Vasoreactivity, Inflammation and vascular effects of Thiazolidinediones in Insulin resistance

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Vasoreactiviteit, Inflammatie en vasculaire effecten van Thiazolidinedionen in Insuline resistentie

(met een samenvatting in het Nederlands)

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“Twee halve waarheden maken nog geen hele waarheid”
(Multatuli)

Aan mijn ouders
Voor Marieke en Valerie

Content

INTRODUCTION

Atherosclerosis
Vasoreactivity
Insulin resistance and the Metabolic syndrome
PPAR-γ agonism and Thiazolidinediones
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Atherosclerosis

General introduction

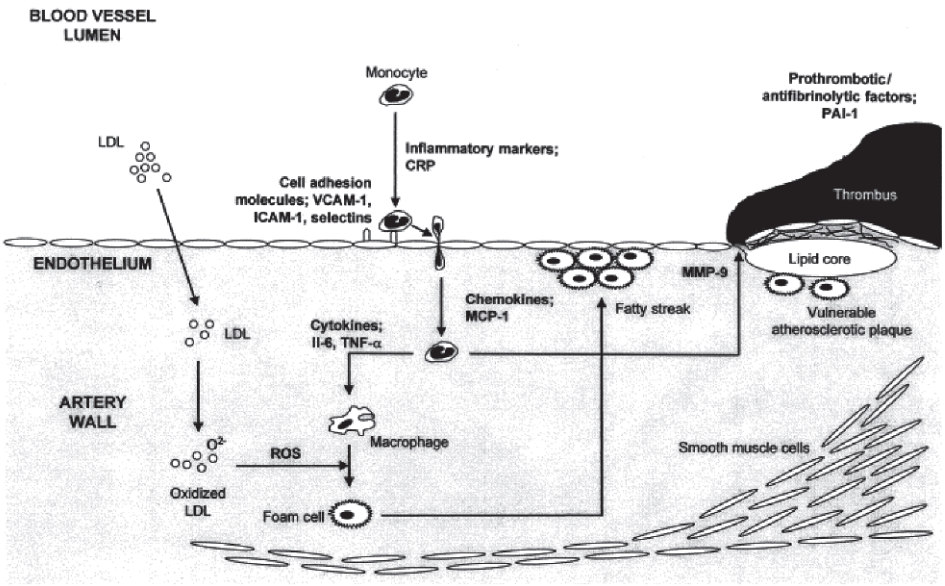
Cardiovascular disease is the most common cause of morbidity and mortality in the Western world.[1] In developing countries, a transition is taking place towards urbanization, industrialization, and more Western life styles which will inevitably lead to a further increase in cardiovascular disease.[2,3] The term cardiovascular disease includes clinical manifestations of arterial atherosclerosis, such as peripheral artery disease, cerebrovascular disease, and coronary artery disease. Well-established risk factors are dyslipidemia, smoking, diabetes mellitus, hypertension, and abdominal obesity.[4] The pathogenesis of atherosclerosis is considered to be multifactorial, i.e. caused by a combination of genetic predisposition, lifestyle, and environmental influences.

Pathogenesis of atherosclerosis

For decades, atherosclerosis has been considered a disease of the arteries caused by excessive cholesterol storage. In recent years, advances have been made in our understanding of the pathophysiological mechanisms that lead to atherosclerosis. Accumulating evidence suggests that the atherosclerotic disease process in the arterial wall is the consequence of an intricate interplay between numerous related and interacting processes and pathways. The endothelial cells that line the luminal vascular surface play a pivotal role in maintaining the hemostatic balance. Ross[5,6] proposed a model for the pathogenesis of atherosclerosis that was based on the response to injury. Injury to the endothelium is an initiating event in atherogenesis. It triggers the immune system and the coagulation cascade. Endothelial cells (EC) produce adhesion molecules (VCAM-1 and ICAM-1) and monocyte-chemotactic-protein-1 (MCP-1), but also prostaglandin-2, nitric oxide (NO), endothelium-derived relaxing factor (EDRF) and thromboxane A2, all influencing vascular tone, vascular structure and hemostatic properties of the vessel wall.[7] The main determinant of the anti-atherosclerotic properties of the endothelium, endothelium-derived NO, is intimately involved in the regulation of vascular tone, vascular growth and coagulation.[8-11]

The immunological and inflammatory response is generated by the adhesion of leukocytes to the endothelium.[8] Adhered monocytes synthesize and express tissue

Figure. Pathophysiology of atherosclerosis



factor, factor VII, high-affinity binding sites for fibrinogen and also produce many cytokines including IL-6 and TNF-α. The adhesion of platelets to leukocytes is mediated by P-selectin expressed on the platelet plasma membrane.[12] Adhesion is followed by migration to the sub-endothelium and differentiation, in appearance of oxidized low-density lipoprotein (ox-LDL), into macrophages. Monocyte derived macrophages and T-lymphocytes are observed in all stages of atherosclerosis.[13,14] Early lesions, the so-called fatty streaks, consist of isolated groups of macrophages containing lipid droplets (macrophage foam cells).[13] The genesis of fatty streaks consists of transport of cholesterol into the arterial wall, reverse cholesterol transport from peripheral tissues back to the liver, oxidation of constituents of these lipoproteins which may trigger an inflammatory response in the vessel wall, pro-inflammatory mediators which may enhance lipoprotein uptake by macrophages and enhance their pro-inflammatory response, protection against oxidation and inflammation by enzymes on some lipoproteins, and pro-oxidative enzymes which may interfere with the reverse cholesterol transport. The arterial inflammatory reaction stimulates proliferation and migration of vascular smooth muscle cells (VSMC)

that become intermixed with the area of inflammation. A continuous inflammation and the formation of fibrous tissue lead to further progression and enlargement of the lesion. In advanced atherosclerotic lesions a fibrous cap overlies an atheromatous core of lipid, necrotic and sometimes calcified material. It is now generally accepted that rupture of a vulnerable plaque due to infiltration of inflammatory cells rather than gradual occlusion of an artery, often leads to an acute clinical manifestation of atherosclerosis.[15] In the presence of one or more risk factors for atherogenesis (e.g. dyslipidemia, diabetes mellitus, hypertension), early atherosclerotic lesions may progress and become mature atherosclerotic plaques. The dangerous and potentially lethal consequences of atherosclerosis are the destabilization of the advanced plaque, which may culminate in sudden disruption of the plaque surface, triggering luminal thrombosis. Plaque disruption with superimposed thrombosis is the most frequent pathoanatomic substrate underlying the acute coronary syndromes (unstable angina, acute myocardial infarction, and sudden coronary death) and ischemic stroke.[16]

References

1. Braunwald E. Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N Engl J Med. 1997;337:1360-1369.
2. Yusuf S, Reddy S, Ounpuu S et al. Global burden of cardiovascular diseases: Part II: variations in cardiovascular disease by specific ethnic groups and geographic regions and prevention strategies. Circulation. 2001;104:2855-2864.
3. Yusuf S, Reddy S, Ounpuu S et al. Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. Circulation. 2001;104:2746-2753.
4. Yusuf S, Hawken S, Ounpuu S et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet. 2004;364:937-952.
5. Ross R. The pathogenesis of atherosclerosis--an update. N Engl J Med. 1986;314:488-500.
6. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature. 1993;362:801-809.
7. Luscher TF. 1993 Mack Forster Award Lecture. Review. The endothelium as a target and mediator of cardiovascular disease. Eur J Clin Invest. 1993;23:670-685.
8. Patarroyo M, Makgoba MW. Leucocyte adhesion to cells. Molecular basis, physiological relevance, and abnormalities. Scand J Immunol. 1989;30:129-164.
9. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993;329:2002-2012.
10. Gimbrone MA, Jr. Vascular endothelium: an integrator of pathophysiologic stimuli in atherosclerosis. Am J Cardiol. 1995;75:67B-70B.
11. Busse R, Fleming I. Regulation and functional consequences of endothelial nitric oxide formation. Ann Med. 1995;27:331-340.
12. Rinder HM, Bonan JL, Rinder CS et al. Dynamics of leukocyte-platelet adhesion in whole blood. Blood. 1991;78:1730-1737.
13. Stary HC, Chandler AB, Glagov S et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Arterioscler Thromb. 1994;14:840-856.
14. Stary HC, Chandler AB, Dinsmore RE et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation. 1995;92:1355-1374.
15. Libby P. What have we learned about the biology of atherosclerosis? The role of inflammation. Am J Cardiol. 2001;88:3J-6J.
16. Hangartner JR, Charleston AJ, Davies MJ et al. Morphological characteristics of clinically significant coronary artery stenosis in stable angina. Br Heart J. 1986;56:501-508.

Vasoreactivity

Endothelium

Cardiovascular risk factors such as hypercholesterolemia, hypertension, diabetes and smoking may impair the normal protective function of the vascular endothelium. The endothelium is a selective barrier located between the circulating blood and the VSMC. It is a larg organ, which covers an area of approximately 1 to 7m^2 and weighs about 1kg in an adult human. The endothelium is metabolically active and functional integrity of the endothelium is important for the maintenance of blood-flow and the prevention of atherothrombosis. The endothelium releases factors that are involved in both pro-atherogenic actions such as vasoconstriction, platelet aggregation, leukocyte adhesion, thrombogenesis, and VSMC proliferation and anti-atherogenic actions such as vasorelaxation, fibrinolysis, inhibition of platelet aggregation, and inhibition of leukocyte adhesion, in a balanced way.[1-3] In endothelial dysfunction, a disturbance of this balance between vasoconstricting, prothrombotic, and proliferative factors (endothelin-1 (ET-1), reactive oxygen species (ROS), angiotensin-II (Ang-II), thromboxane A2) and vasodilating, anti-thrombotic, and anti-proliferative factors (NO, endothelium-derived hyperpolarizing factor, natriuretic peptides, and prostacyclin) occurs, which results in a pro-atherogenic state.[4-9] Endothelial function in humans can be measured in several ways and serves as a tool for early assessment of cardiovascular risk.

Old fashioned measurements of endothelial function

Several endothelial factors have been put forward as tools for assessing endothelial function. Endothelial function can be estimated by measuring endothelium-derived proteins such as von Willebrand factor (vWF), plasma ET-1, modulators of coagulation such as plasma thrombomodulin, plasma tissue plasminogen activator (tPA), and plasminogen activator inhibitor-1 (PAI-1), as well as adhesion molecules such as VCAM-1 and ICAM-1.[10,11] It should be recognized that these endothelium-derived proteins can be affected by a variety of stimuli. Non-specific confounders limit the use of vWF and P-selectin as specific markers of endothelial function in subjects with acute-phase reactions and platelet activation.[12] Although some of these markers are elevated in cardiovascular diseases and correlations with cardiovascular diseases or other risk factors have been shown, they lack sensitivity and/or specificity for assessment of endothelial function in individual patients.[13]

Nitric oxide

NO is an important endothelium-derived vasodilating factor.[14,15] Besides vasodilation, NO also inhibits adhesion of leukocytes to the endothelium,[16] inhibits platelet-vessel wall interaction,[17,18] decreases endothelial permeability,(19) inhibits VSMC proliferation and migration,[20,21] and reduces vessel tone.[22] NO was originally described as endothelium-derived relaxing factor (EDRF).[14] NO is synthesized by NO synthase from L-arginine.[23] The conversion from L-arginine to NO can be inhibited by false substrates for the NO synthase, e.g. by NG-monomethyl-L-arginine (L-NMMA) or NG-nitro-L-arginine methyl ester (L-NAME).[24] Two subtypes of NO-synthases may be distinguished: a constitutive NO is produced by endothelial cells in response to shear stress by the enzyme endothelial nitric oxide synthase (eNOS), and an inducible isoform (iNOS) as an important inflammatory mediator released by macrophages in response to immunological stimuli.[25] Different co-factors are necessary, such as calcium, calmodulin, heme, tetrahydrobiopterin (BH_4), and reduced nicotamine adenine dinucleotide phosphate (NADPH).[26,27] NO rapidly diffuses to either the blood or abluminally to the underlying VSMC, where it activates the enzyme guanylate cyclase, inducing an accumulation of cyclic guanosine monophosphate (cGMP) in the VSMC.[28] NO not only stimulates cGMP but is also rapidly inactivated by binding to heme and by reacting with oxygen radicals, yielding methemoglobin, nitrate and peroxynitrite.[29] Therefore, NO-bioavailability is the result of the balance between NO-synthesis and breakdown. Under normal, healthy conditions, low concentrations of NO and nanomolar concentrations of peroxynitrite are produced, both resulting in vasodilative and/or anti-atherosclerotic actions. However, in pathophysiological situations like diabetes,[29-33] hypercholesterolemia,[34-38] and smoking,[39-42] NO-availability *in vivo* is impaired as a result of reduced NO-formation, enhanced NO-breakdown or both. Reduced NO-formation can be the result of decreased availability of L-arginine,[34] or co-factors for eNOS.[34,40,42] Nevertheless, a major factor of impaired NO-availability may be enhanced degradation of NO by reactions with oxygen radicals produced by enzymes such as xanthine oxidase,[44] NADH- and NADPH oxidase.[45,46] Moreover, under certain pathophysiological conditions, eNOS itself can synthesize mainly superoxide instead of NO.[47-49] This NO-uncoupling phenomenon is characterized by reduced availability of NO and increased production of highly cytotoxic oxidant peroxynitrite. Because of its reactivity, direct measurement of NO itself *in vivo* is virtually impossible. Alternatively, NO-dependent vasodilation is probably one of the most reliable and practically useful estimations of endothelial function. There are different ways of stimulating or inhibiting NO-release and different ways to measure this effect in order to assess NO-dependent vasodilation.

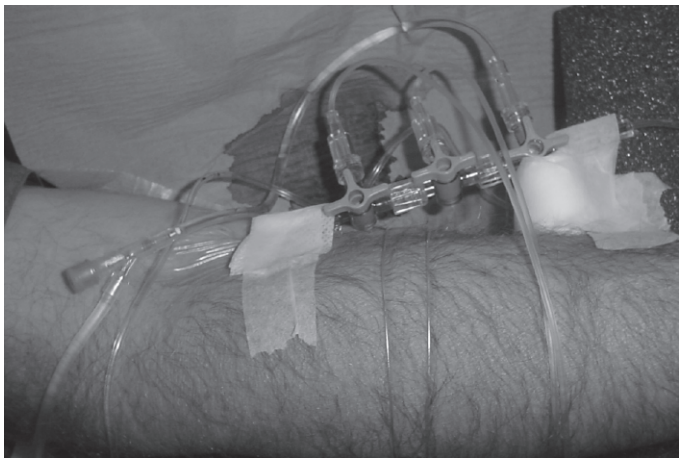
Coronary endothelial assessment

Coronary angiography is routinely performed to detect stenotic coronary artery lesions in symptomatic patients. In addition to morphological details of the coronary arteries, endothelial function of resistance and epicardial conduit vessels can be simultaneously investigated. The resistance vessels regulate coronary blood flow. Endothelial function of these resistance vessels can be demonstrated using an intracoronary Doppler flow velocity transducer. Endothelial function of coronary conduit vessels can be demonstrated by measuring the changes in diameter. Intracoronary infusion of acetylcholine at a dose between 10^{-8} to 10^{-4} mmol/l is generally used as a distinctive agonistic provocation.[50-54] In healthy arteries this will result in increased coronary dilation and flow due to specific stimulation of the muscarinic 1 receptor on the luminal side of the endothelial cell. Coronary flow decreases and epicardial vessels constrict if endothelial function is impaired due to lack of NO availability, thus allowing a direct effect of acetylcholine on the subendothelial muscarinic 2 receptor on the VSMC, establishing a paradoxal vasoconstriction.

Determination of forearm blood flow (FBF) using venous occlusion plethysmography

Plethysmography has been used to measure blood flow in resistance vasculature beds for more than a century. For endothelial function measurements venous occlusion plethysmography of the forearm is most widely used.[30-32,35,55] The mean blood flow of

Figure. Determination of FBF using occlusion plethysmography



FBF: volume changes measured using mercury in elastic strain gauges and the intra-arterial canula for local infusion of vasoactive compounds.

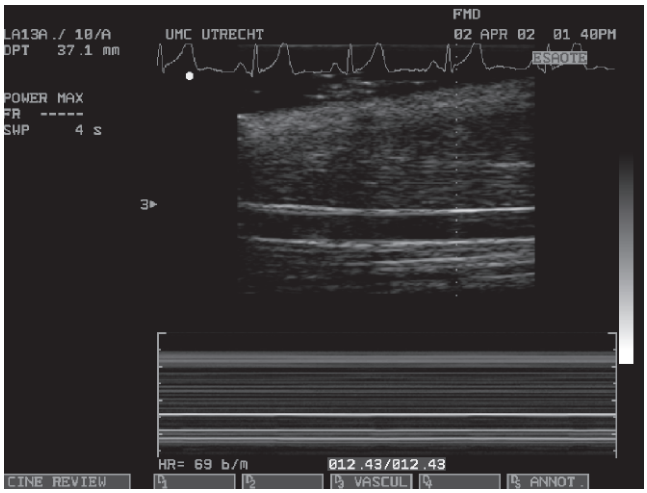
the forearm is measured by recording an increase in forearm volume that occurs during temporary interruption of venous outflow by inflating a pneumatic cuff to 40 mmHg at the upper arm leaving arterial inflow unimpeded. A wrist cuff, inflated 40 mmHg above systolic blood pressure, excludes flow from the hand, since the latter reflects thermoregulation rather than a resistance vessel bed. The initial increase in volume is due to arterial inflow. The volume change can be measured using mercury in elastic strain gauges wrapped around the forearm at 1/3 of the forearm length distal from the olecranon. At this part the forearm contains the largest proportion of muscle. An intra-arterial canula is placed to allow for local infusion of vasoactive compounds. The basal blood flow is measured during infusion of 0.9% saline.

Endothelium-dependent vasodilation can be induced using compounds such as serotonin, acetylcholine and bradykinin, which all more or less stimulate NO-formation. Serotonin, physiologically released by platelets, acts via the luminal serotonin receptor and specifically stimulates eNOS. Acetylcholine binds to the muscarinic 1 receptor on the endothelial cell and activates eNOS as well as endothelium-dependent VSMC-hyperpolarization. In physiologic situations acetylcholine is not a natural NO-stimulator. Acetylcholine is rapidly degraded by cholinesterase. In many human studies acetylcholine was used as agonist of NO-release. However, firstly acetylcholine has been shown to induce multiple vascular effects such as NO-release,[14] release of vasodilative and vasoconstrictive prostaglandins[56] and release of EDHF.[57] Secondly, since muscarinic receptors on VSMC directly mediate vasoconstriction, blunting of the acetylcholine-induced vasodilation may be unrelated to a change in the NO-pathway. Serotonin induces a large initial rise in bloodflow, followed by a vasodilatation that sustains less. In contrast to the partial inhibition of acetylcholine-induced vasodilation by the NOS-inhibitor L-NMMA, the sustained vasodilation induced by serotonin is completely abolished by co-infusion of L-NMMA.[58] Bradykinin acts via the luminal bradykinin receptor on endothelial cells and stimulates besides NO also EDHF. Bradykinin causes vasodilation independent of NO, predominantly through hyperpolarization.[59] Therefore, we prefer serotonin to investigate specific NO-mediated vasodilation. Nitroprusside, serving as exogenous NO-donor, induces endothelium-independent vasodilation by directly acting on the VSMC. Inhibition of basal NO-activity is possible with specific NO-blockers such as L-NMMA and L-NAME. Changes in FBF in the measured arm are the result of vaso-active compounds. Besides this local reaction, systemic factors such as sympathetic arousal and blood pressure also can influence FBF. Comparison of flow between the measurement arm and the contralateral arm (M/C ratio) excludes these systemic factors.

Non-invasive measurement of flow-mediated dilation (FMD) of the conduit brachial artery

A non-invasive technique to measure endothelial function is the assessment of FMD using an ultrasound device.[60,61] Assessment of endothelial function is based on changes in vessel diameter. Endothelium-dependent vasodilation is non-invasively induced by blood flow increase or shear stress. To induce an increase in blood-flow in the forearm, a cuff is inflated to 50 mmHg above systolic pressure distal to the transducer. Deflating this cuff after 4-5 minutes results in hyperemia lasting for approximately 2 minutes while causing vasodilation for up to 20 minutes.[62] In healthy subjects, a brachial artery normally dilates approximately 5-10%.[60,61] L-NMMA abolishes the vasodilative effect of hyperemia in the brachial artery, demonstrating that NO is the main mediator of FMD.[63] The non-endothelium-dependent vasodilative response can be measured using sublingually administrated nitroglycerine.
It is important to be aware of influencing factors like baseline diameter. The vasodilator responses are inversely related to the vessel size. The accuracy and reproducibility are good. An interobserver variability of 2.9% and a respons variability of 1.4% are reported.[64] The number of patients required in an intervention trial depends on the hypothesized improvement in FMD and the type of study; thus,

Figure. FMD of the conduit brachial artery



FMD: measuring endothelial function using an ultrasound device; the ultrasonic view of the brachial artery.

larger groups are needed for a parallel compared to a cross-over design.[65,66] There is a close relationship of vascular function between the coronary and forearm conduit vessels. Patients with coronary artery endothelial dysfunction, manifested as vasoconstriction in response to intracoronary acetylcholine, had significantly impaired FMD in the brachial artery compared to patients with normal coronary endothelial function (FMD 4.8% vs. 10.8%, p<0.01). Also patients with coronary artery disease had an attenuated FMD response compared to patients with angiographically smooth coronary arteries (FMD 4.5% vs. 9.7%, p,0.02). Both coronary endothelial dysfunction and presence of coronary artery disease were strong predictors of reduced FMD responses. The positive predictive value of reduced FMD response (FMD < 3%) in predicting coronary endothelial dysfunction was 95%.[67]

Conditions for proper assessment of endothelial function

Noteworthy, different factors affect measurements of endothelial function, which demand rigid standardization to avoid modulating effects.

The sympathetic nervous system is an important regulator of coronary and peripheral vascular tone.[68-75] In this respect, FMD was practically abolished during sympathetic stimulation by baroreceptor unloading which was prevented during local alpha adrenergic blockade with phentolamine.[76] Thus symptomatic treatment modalities which affect sympathetic outflow (e.g. diuretics, β-blockers) may also improve FMD secondary to a decreased sympathetic activity.

Circadian rhythm is important in coronary conduit vessels. In coronary segments with endothelial dysfunction, the constrictor response to acetylcholine or the dilator response to nitroglycerine was significantly greater in the morning than in the afternoon. This suggests a potentially protective role for the endothelium in modulating variations in coronary tone that may contribute to increased incidence of cardiovascular events in the early morning.[77] According to this phenomenon, FBF and FMD have found to be markedly reduced in the morning compared to the afternoon.[78,79] Hypercholesterolemia is associated with endothelial dysfunctionand can be improved by treatment with cholesterol lowering drugs.[35-38,80-82] Also high-fat meals impair endothelial function while several compounds such as vitamin C and E, folic acid, ACE-inhibition and AT-I receptor blockade, prevent this impairment.[83-88] In addition, elevated glucose levels can impair endothelial function as well.[89-92] FMD response of the brachial artery is impaired in postmenopausal women and related to the estradiol level in premenopausal women. This suggests that estrogens have a protective effect on the endothelium.[93-98] Endothelium-mediated vasodilation of coronary and peripheral arteries is inversely correlated to age.[99-103]

Several studies revealed impaired endothelial function of the coronary and brachial conduit vessels and forearm resistance vessels in smokers compared to non-smokers.[39-42] This is probably due to decreased NO-availability by radical stress or decreased eNOS-activity since the L-NMMA-induced vasoconstrictive effect was diminished. BH4, an important co-factor for eNOS, improves the endothelial function in both forearm resistance- and conduit vessels.[40-42]

As discussed above, some factors improve endothelium-mediated vasodilation, whereas others deteriorate endothelial relaxation. Thus, for greatest accuracy, it is important that the patient is investigated in a quiet room at constant room temperature (20-24°C) in a comfortable position. The patient should have been fasting for 6-12 hours.

Relation between endothelial dysfunction and cardiovascular morbidity

Endothelial dysfunction plays an important role in the pathogenesis of coronary artery disease and is considered to be an early manifestation of atherosclerosis. Multiple investigators have clearly demonstrated impaired endothelial function of coronary conduit and resistance arteries in atherosclerotic patients.[53,65,66] In addition, impairment of peripheral endothelium-dependent vasodilation has been demonstrated in patients with coronary artery disease.[104-106] Moreover, the progressive impairment of the endothelium correlates with the progression of coronary atherosclerosis.[107] Even more important, endothelial dysfunction is not only associated with atherosclerosis and its risk factors but also with cardiovascular outcome. To identify cardiac events using FMD in the brachial artery in patients with non-specific chest pain a specificity of 51%, a sensitivity of 86% and a negative predicting value of 93% was found.[108-109]

References

1. Cines DB, Pollak ES, Buck CA et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood. 1998;91:3527-3561.
2. Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. FASEB J. 1989;3:2007-2018.
3. Vane JR, Anggard EE, Botting RM. Regulatory functions of the vascular endothelium. N Engl J Med. 1990;323:27-36.
4. Burnett JC, Jr. Coronary endothelial function in health and disease. Drugs. 1997;53 Suppl 1:20-29.
5. Honing ML, Morrison PJ, Banga JD et al. Nitric oxide availability in diabetes mellitus. Diabetes Metab Rev. 1998;14:241-249.
6. Luscher TF. 1993 Mack Forster Award Lecture. Review. The endothelium as a target and mediator of cardiovascular disease. Eur J Clin Invest. 1993;23:670-685.
7. Luscher TF, Barton M. Biology of the endothelium. Clin Cardiol. 1997;20:II-10.
8. Oemar BS, Tschudi MR, Godoy N et al. Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. Circulation. 1998;97:2494-2498.
9. Verhaar MC, Rabelink TJ. Endothelial function: strategies for early intervention. Cardiovasc Drugs Ther. 1998;12 Suppl 1:125-134.
10. Blann AD, Dobrotova M, Kubisz P et al. von Willebrand factor, soluble P-selectin, tissue plasminogen activator and plasminogen activator inhibitor in atherosclerosis. Thromb Haemost. 1995;74:626-630.
11. Stehouwer CD. Is measurement of endothelial dysfunction clinically useful? Eur J Clin Invest. 1999;29:459-461.
12. Schrama YC, van Dam T, Fijnheer R et al. Cyclosporine is associated with endothelial dysfunction but not with platelet activation in renal transplantation. Neth J Med. 2001;59:6-15.
13. Blann AD, Taberner DA. A reliable marker of endothelial cell dysfunction: does it exist? Br J Haematol. 1995;90:244-248.
14. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980;288:373-376.
15. Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. Prog Cardiovasc Dis. 1995;38:87-104.
16. Lefer AM. Nitric oxide: nature's naturally occurring leukocyte inhibitor. Circulation. 1997;95:553-554.
17. de Graaf JC, Banga JD, Moncada S et al. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. Circulation. 1992;85:2284-2290.
18. Azuma H, Ishikawa M, Sekizaki S. Endothelium-dependent inhibition of platelet aggregation. Br J Pharmacol. 1986;88:411-415.
19. Kubes P. Nitric oxide modulates epithelial permeability in the feline small intestine. Am J Physiol. 1992;262:G1138-G1142.

20. Sarkar R, Meinberg EG, Stanley JC et al. Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. Circ Res. 1996;78:225-230.
21. Cornwell TL, Arnold E, Boerth NJ et al. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. Am J Physiol. 1994;267:C1405-C1413.
22. Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. Lancet. 1989;2:997-1000.
23. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature. 1988;333:664-666.
24. Palmer RM, Rees DD, Ashton DS et al. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem Biophys Res Commun. 1988;153:1251-1256.
25. Palmer RM, Bridge L, Foxwell NA et al. The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. Br J Pharmacol. 1992;105:11-12.
26. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. Cell. 1994;78:915-918.
27. Marletta MA. Nitric oxide synthase: aspects concerning structure and catalysis. Cell. 1994;78:927-930.
28. Cohen RA, Vanhoutte PM. Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. Circulation. 1995;92:3337-3349.
29. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996;271:C1424-C1437.
30. Calver A, Collier J, Vallance P. Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. J Clin Invest. 1992;90:2548-2554.
31. O'Driscoll G, Green D, Maiorana A et al. Improvement in endothelial function by angiotensin-converting enzyme inhibition in non-insulin-dependent diabetes mellitus. J Am Coll Cardiol. 1999;33:1506-1511.
32. O'Driscoll G, Green D, Rankin J et al. Improvement in endothelial function by angiotensin converting enzyme inhibition in insulin-dependent diabetes mellitus. J Clin Invest. 1997;100:678-684.
33. Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. Diabetes Care. 1996;19:257-267.
34. Stroes E, Kastelein J, Cosentino F et al. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest. 1997;99:41-46.
35. Stroes ES, Koomans HA, de Bruin TW et al. Vascular function in the forearm of hypercholesterolaemic patients off and on lipid-lowering medication. Lancet. 1995;346:467-471.
36. Gilligan DM, Guetta V, Panza JA et al. Selective loss of microvascular endothelial function in human hypercholesterolemia. Circulation. 1994;90:35-41.
37. Chowienczyk PJ, Watts GF, Cockcroft JR et al. Sex differences in endothelial function in normal and hypercholesterolaemic subjects. Lancet. 1994;344:305-306.
38. Chowienczyk PJ, Watts GF, Cockcroft JR et al. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. Lancet. 1992;340:1430-1432.

39. Celermajer DS, Sorensen KE, Georgakopoulos D et al. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. Circulation. 1993;88:2149-2155.
40. Ueda S, Matsuoka H, Miyazaki H et al. Tetrahydrobiopterin restores endothelial function in long-term smokers. J Am Coll Cardiol. 2000;35:71-7.
41. Zeiher AM, Schachinger V, Minners J. Long-term cigarette smoking impairs endothelium-dependent coronary arterial vasodilator function. Circulation. 1995;92:1094-1100.
42. Heitzer T, Brockhoff C, Mayer B et al. Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers : evidence for a dysfunctional nitric oxide synthase. Circ Res. 2000;86:E36-E41.
43. Drexler H, Zeiher AM, Meinzer K et al. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. Lancet. 1991;338:1546-1550.
44. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest. 1993;91:2546-2551.
45. Mohazzab KM, Kaminski PM, Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. Am J Physiol. 1994;266:H2568-H2572.
46. Pagano PJ, Ito Y, Tornheim K et al. An NADPH oxidase superoxide-generating system in the rabbit aorta. Am J Physiol. 1995;268:H2274-H2280.
47. Wever RM, Luscher TF, Cosentino F et al. Atherosclerosis and the two faces of endothelial nitric oxide synthase. Circulation. 1998;97:108-112.
48. Stroes E, Hijmering M, van Zandvoort M et al. Origin of superoxide production by endothelial nitric oxide synthase. FEBS Lett. 1998;438:161-164.
49. Stroes ES, van Faassen EE, van Londen GJ et al. Oxygen radical stress in vascular disease: the role of endothelial nitric oxide synthase. J Cardiovasc Pharmacol. 1998;32 Suppl :S14-S21.
50. Ludmer PL, Selwyn AP, Shook TL et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med. 1986;315:1046-1051.
51. Zeiher AM, Drexler H, Wollschlaeger H et al. Coronary vasomotion in response to sympathetic stimulation in humans: importance of the functional integrity of the endothelium. J Am Coll Cardiol. 1989;14:1181-1190.
52. Zeiher AM, Drexler H. Coronary hemodynamic determinants of epicardial artery vasomotor responses during sympathetic stimulation in humans. Basic Res Cardiol. 1991;86 Suppl 2:203-213.
53. Zeiher AM, Drexler H, Wollschlager H et al. Endothelial dysfunction of the coronary microvasculature is associated with coronary blood flow regulation in patients with early atherosclerosis. Circulation. 1991;84:1984-1992.
54. Vita JA, Treasure CB, Nabel EG et al. Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. Circulation. 1990;81:491-497.
55. Panza JA, Casino PR, Kilcoyne CM et al. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. Circulation. 1993;87:1468-1474.

56. Diederich D, Yang Z, Luscher TF. Chronic cyclosporine therapy impairs endothelium-dependent relaxation in the renal artery of the rat. J Am Soc Nephrol. 1992;2:1291-1297.
57. Nagao T, Illiano S, Vanhoutte PM. Heterogeneous distribution of endothelium-dependent relaxations resistant to NG-nitro-L-arginine in rats. Am J Physiol. 1992;263:H1090-H1094.
58. Bruning TA, van Zwieten PA, Blauw GJ et al. No functional involvement of 5-hydroxytryptamine1 A receptors in nitric oxide-dependent dilatation caused by serotonin in the human forearm vascular bed. J Cardiovasc Pharmacol. 1994;24:454-461.
59. Honing ML, Smits P, Morrison PJ et al. Bradykinin-induced vasodilation of human forearm resistance vessels is primarily mediated by endothelium-dependent hyperpolarization. Hypertension. 2000;35:1314-1318.
60. Celermajer DS, Sorensen KE, Gooch VM et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet. 1992;340:1111-1115.
61. Sorensen KE, Celermajer DS, Spiegelhalter DJ et al. Non-invasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. Br Heart J. 1995;74:247-253.
62. Leeson P, Thorne S, Donald A et al. Non-invasive measurement of endothelial function: effect on brachial artery dilatation of graded endothelial dependent and independent stimuli. Heart. 1997;78:22-27.
63. Joannides R, Haefeli WE, Linder L et al. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation. 1995;91:1314-1319.
64. Uehata A, Lieberman EH, Gerhard MD et al. Noninvasive assessment of endothelium-dependent flow-mediated dilation of the brachial artery. Vasc Med. 1997;2:87-92.
65. Zeiher AM, Krause T, Schachinger V et al. Impaired endothelium-dependent vasodilation of coronary resistance vessels is associated with exercise-induced myocardial ischemia. Circulation. 1995;91:2345-2352.
66. Egashira K, Inou T, Hirooka Y et al. Impaired coronary blood flow response to acetylcholine in patients with coronary risk factors and proximal atherosclerotic lesions. J Clin Invest. 1993;91:29-37.
67. Takase B, Uehata A, Akima T et al. Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease. Am J Cardiol. 1998;82:1535-1538.
68. Antony I, Aptecar E, Lerebours G et al. Coronary artery constriction caused by the cold pressor test in human hypertension. Hypertension. 1994;24:212-219.
69. Cardillo C, Kilcoyne CM, Cannon RO, III et al. Impairment of the nitric oxide-mediated vasodilator response to mental stress in hypertensive but not in hypercholesterolemic patients. J Am Coll Cardiol. 1998;32:1207-1213.
70. Cardillo C, Kilcoyne CM, Quyyumi AA et al. Role of nitric oxide in the vasodilator response to mental stress in normal subjects. Am J Cardiol. 1997;80:1070-1074.
71. Jeserich M, Schindler T, Olschewski M et al. Vitamin C improves endothelial function of epicardial coronary arteries in patients with hypercholesterolaemia or essential hypertension--assessed by cold pressor testing. Eur Heart J. 1999;20:1676-1680.

72. Schoder H, Silverman DH, Campisi R et al. Effect of mental stress on myocardial blood flow and vasomotion in patients with coronary artery disease. J Nucl Med. 2000;41:11-16.
73. Dietz NM, Rivera JM, Eggener SE et al. Nitric oxide contributes to the rise in forearm blood flow during mental stress in humans. J Physiol. 1994;480 (Pt 2):361-368.
74. Harris CW, Edwards JL, Baruch A et al. Effects of mental stress on brachial artery flow-mediated vasodilation in healthy normal individuals. Am Heart J. 2000;139:405-411.
75. Lindqvist M, Melcher A, Hjemdahl P. Flow dependence of forearm noradrenaline overflow, as assessed during mental stress and sodium nitroprusside infusion. J Hypertens. 1999;17:91-97.
76. Hijmering ML, Stroes ES, Olijhoek J et al. Sympathetic activation markedly reduces endothelium-dependent, flow-mediated vasodilation. J Am Coll Cardiol. 2002;39:683-688.
77. el Tamimi H, Mansour M, Pepine CJ et al. Circadian variation in coronary tone in patients with stable angina. Protective role of the endothelium. Circulation. 1995;92:3201-3205.
78. Etsuda H, Takase B, Uehata A et al. Morning attenuation of endothelium-dependent, flow-mediated dilation in healthy young men: possible connection to morning peak of cardiac events? Clin Cardiol. 1999;22:417-421.
79. Houben AJ, Slaaf DW, Huvers FC et al. Diurnal variations in total forearm and skin microcirculatory blood flow in man. Scand J Clin Lab Invest. 1994;54:161-168.
80. Egashira K, Hirooka Y, Kai H et al. Reduction in serum cholesterol with pravastatin improves endothelium-dependent coronary vasomotion in patients with hypercholesterolemia. Circulation. 1994;89:2519-2524.
81. Treasure CB, Klein JL, Weintraub WS et al. Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. N Engl J Med. 1995;332:481-487.
82. Anderson TJ, Meredith IT, Yeung AC et al. The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion. N Engl J Med. 1995;332:488-493.
83. Plotnick GD, Corretti MC, Vogel RA. Effect of antioxidant vitamins on the transient impairment of endothelium-dependent brachial artery vasoactivity following a single high-fat meal. JAMA. 1997;278:1682-1686.
84. Vogel RA, Corretti MC, Plotnick GD. Effect of a single high-fat meal on endothelial function in healthy subjects. Am J Cardiol. 1997;79:350-354.
85. Ong PJ, Dean TS, Hayward CS et al. Effect of fat and carbohydrate consumption on endothelial function. Lancet. 1999;354:2134.
86. Williams MJ, Sutherland WH, McCormick MP et al. Impaired endothelial function following a meal rich in used cooking fat. J Am Coll Cardiol. 1999;33:1050-1055.
87. Wilmink HW, Banga JD, Hijmering M et al. Effect of angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor antagonism on postprandial endothelial function. J Am Coll Cardiol. 1999;34:140-145.

88. Wilmink HW, Stroes ES, Erkelens WD et al. Influence of folic acid on postprandial endothelial dysfunction. Arterioscler Thromb Vasc Biol. 2000;20:185-188.
89. Akbari CM, Saouaf R, Barnhill DF et al. Endothelium-dependent vasodilatation is impaired in both microcirculation and macrocirculation during acute hyperglycemia. J Vasc Surg. 1998;28:687-694.
90. Kawano H, Motoyama T, Hirashima O et al. Hyperglycemia rapidly suppresses flow-mediated endothelium-dependent vasodilation of brachial artery. J Am Coll Cardiol. 1999;34:146-154.
91. Shige H, Ishikawa T, Suzukawa M et al. Endothelium-dependent flow-mediated vasodilation in the postprandial state in type 2 diabetes mellitus. Am J Cardiol. 1999;84:1272-4, A9.
92. Williams SB, Cusco JA, Roddy MA et al. Impaired nitric oxide-mediated vasodilation in patients with non-insulin-dependent diabetes mellitus. J Am Coll Cardiol. 1996;27:567-574.
93. English JL, Jacobs LO, Green G et al. Effect of the menstrual cycle on endothelium-dependent vasodilation of the brachial artery in normal young women. Am J Cardiol. 1998;82:256-258.
94. Hashimoto M, Akishita M, Eto M et al. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. Circulation. 1995;92:3431-3435.
95. Kawano H, Motoyama T, Kugiyama K et al. Gender difference in improvement of endothelium-dependent vasodilation after estrogen supplementation. J Am Coll Cardiol. 1997;30:914-919.
96. Taddei S, Virdis A, Ghiadoni L et al. Menopause is associated with endothelial dysfunction in women. Hypertension. 1996;28:576-582.
97. McCrohon JA, Woo KS, Celermajer DS. A comparison of endothelial function in Caucasian and Chinese women before and after the menopause. Maturitas. 2000;35:31-37.
98. Lim SC, Caballero AE, Arora S et al. The effect of hormonal replacement therapy on the vascular reactivity and endothelial function of healthy individuals and individuals with type 2 diabetes. J Clin Endocrinol Metab. 1999;84:4159-4164.
99. Egashira K, Inou T, Hirooka Y et al. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. Circulation. 1993;88:77-81.
100. Gerhard M, Roddy MA, Creager SJ et al. Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. Hypertension. 1996;27:849-853.
101. Taddei S, Virdis A, Mattei P et al. Aging and endothelial function in normotensive subjects and patients with essential hypertension. Circulation. 1995;91:1981-1987.
102. Sarabi M, Millgard J, Lind L. Effects of age, gender and metabolic factors on endothelium-dependent vasodilation: a population-based study. J Intern Med. 1999;246:265-274.
103. Celermajer DS, Sorensen KE, Spiegelhalter DJ et al. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. J Am Coll Cardiol. 1994;24:471-476.
104. Lieberman EH, Gerhard MD, Uehata A et al. Flow-induced vasodilation of the human brachial artery is impaired in patients <40 years of age with coronary artery disease. Am J Cardiol. 1996;78:1210-1214.
105. Zhang X, Zhao SP, Li XP et al. Endothelium-dependent and -independent functions are impaired in patients with coronary heart disease. Atherosclerosis. 2000;149:19-24.

106. Neunteufl T, Katzenschlager R, Hassan A et al. Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease. Atherosclerosis. 1997;129:111-118.
107. Zeiher AM, Drexler H, Wollschlager H et al. Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. Circulation. 1991;83:391-401.
108. Neunteufl T, Heher S, Katzenschlager R et al. Late prognostic value of flow-mediated dilation in the brachial artery of patients with chest pain. Am J Cardiol. 2000;86:207-210.
109. Suwaidi JA, Hamasaki S, Higano ST et al. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation. 2000;101:948-954.

Insulin resistance

General introduction

The prevalence of type 2 diabetes (DM2) has soared in the past decades because of changing lifestyles and eating habits. Obesity associated with insulin resistance is one of the main determinants of the increased incidence of DM2. The major long-term complications of DM2 are an increased risk of myocardial infarction, stroke and peripheral vascular disease. Although microvascular complications cause considerable morbidity in patients with DM2, up to 80% of patients die from macrovascular pathology.[1] Treatment of individual risk factors has been shown to reduce cardiovascular events in DM2. Dysglycemia does not appear to be the major determinant of cardiovascular disease in DM2, a concept supported by observations in the UK Prospective Diabetes Study (UKPDS).[2] Patients with impaired glucose tolerance (IGT) and/or impaired fasting glucose are so called pre-diabetic and are at risk for developing DM2.[3] Although in DM2 elevated plasma glucose concentrations may induce vascular damage, in pre-diabetic patients the increased cardiovascular risk is most likely the result of the pathophysiological phenomenon of insulin resistance which leads to the occurrence of risk factors (e.g. elevated bloodpressure, low HDL-cholesterol, elevated triglycerides) and causes impaired vasoreactivity.[4]

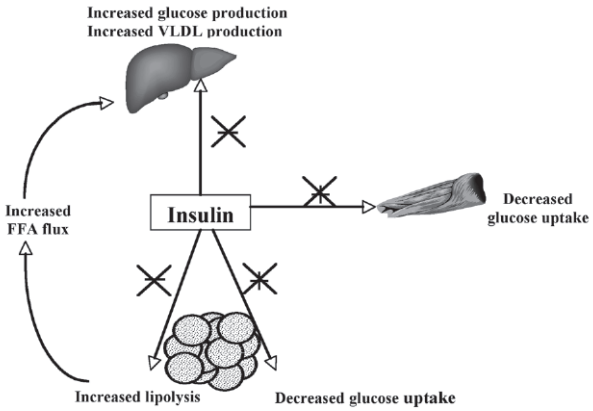
Pathophysiology of insulin resistance

Insulin has several physiological functions. This hormone is the most important regulator for the plasma glucose level by stimulating the disposal of glucose in skeletal muscle- and adipose tissue and decreasing the hepatic glucose production. Furthermore insulin stimulates the lipogenesis and glycogen- and protein-synthase activity in adipocytes, liver and skeletal muscle, and inhibits glycogenolysis, lipolysis and protein breakdown. In addition, insulin plays a role in the regulation of cell growth and differentiation.

Insulin resistance means that when insulin binds to the insulin receptor on the cell surface, this stimulation is not strong enough to induce the normal intracellular signal transduction with the consequence of insufficient reaction of target organs on plasma levels of insulin. In case of a sufficient pancreatic β-cell-function, insulin resistance will lead to a compensatory hyperinsulinemia to maintain normoglycemia. Hyperglycemia, follows by further increase in insulin resistance and after failing of the pancreatic β-cell-function. Adipose tissue, especially intra-abdominal obesity, plays an important role in insulin resistance. Adipose tissue is no longer only a depot, but can be seen as an endocrine organ.[5] Several products of adipose tissue may influence

development of insulin resistance. Important adipocyte-derived factors are FFA, but the adipocytokine TNF-α, adiponectin, and leptin. High levels of free fatty acids (FFA) have been linked to (the induction of) insulin resistance because increased FFA production in the liver leads to increased gluconeogenesis and decreased glucose metabolism in skeletal muscle.[6] Furthermore high levels of FFA will lead to an increase of very low-density lipoprotein (VLDL) together with an increase of plasma triglycerides.[7] In an insulin resistant state there is an attenuated lipoprotein lipase (LPL) acitivity. Lipoprotein lipase is involved in the lipolysis of VLDL. Decreased breakdown of VLDL particles leads to reduced availability of small VLDL fragments and an increase in triglyceride-rich high-density lipoprotein (HDL) particles via cholesterolestertransfer-protein (CETP). Triglyceride-rich HDL particles are faster cleared by the liver resulting in a decreased HDL plasma concentration and an increased triglyceride concentration.[8] The expression of TNF-α by adipose tissue is upregulated in obesity and TNF-α levels are increased in patients with features of the insulin resistance syndrome (such as endothelial dysfunction) inducing a higher risk of recurrent coronary events.[9,10] Adiponectin is an adipocyte-derived hormone that decreases insulin resistance. A low adiponectin plasma concentration precedes a decrease in whole-body insulin sensitivity in humans.[11-13] The role of leptin in insulin resistance is controversial, but leptin might interfere with insulin signalling in certain cell types. Hyperinsulinemia also causes hypertension. Normally insulin has a vasodilating effect, however with insulin resistance, insulin can cause sympathetic activation, production of the vasoconstrictor ET-1, and increase of renal salt retention. Together with, or leading to, endothelial dysfunction these mechanisms can result in hypertension.[14]

Figure. Pathophysiology of insulin resistance



Insulin resistance syndrome

Taken together, insulin resistance is generally regarded as an important feature of a cluster of risk factors of cardiovascular disease. These risk factors (dyslipidemia, hypertension, glucose intolerance, hyperinsulinemia, obesity, low-grade inflammation, endothelial dysfunction and hypercoagulability) often precede clinically manifest DM2. However, the interaction between different cardiovascular risk factors and defects in insulin signalling is very complex. Most persons with multiple metabolic risk factors are insulin resistant which leads to the concept that insulin resistance is the major cause of the cluster metabolic syndrome.[15]

Metabolic syndrome

The metabolic syndrome, which involves a cluster of cardiovascular risk factors including hypertension, obesity, glucose intolerance, endothelial dysfunction, dyslipidemia and a proinflammatory state, is generally considered to be of major importance in the pathophysiology of DM2 and is associated with an increased risk for cardiovascular complications.[16-18] According to the Adult Treatment Panel III (ATP III) the metabolic syndrome is diagnosed when 3 or more criteria are present. The ATP III definition of the metabolic syndrome is the most commonly used definition and most practical for clinical use:[19]

1) Abdominal obesity (waist circumference > 102cm in men and >88cm in women).
2) High blood pressure (≥ 130mmHg systolic or ≥ 85mmHg diastolic).
3) Hypertriglyceridemia (serum triglycerides ≥ 1.70mmol/l).
4) Low serum HDL cholesterol (<1.04mmol/l in men and < 1.29mmol/l in women).
5) High fasting serum glucose (≥ 6.1mmol/l).

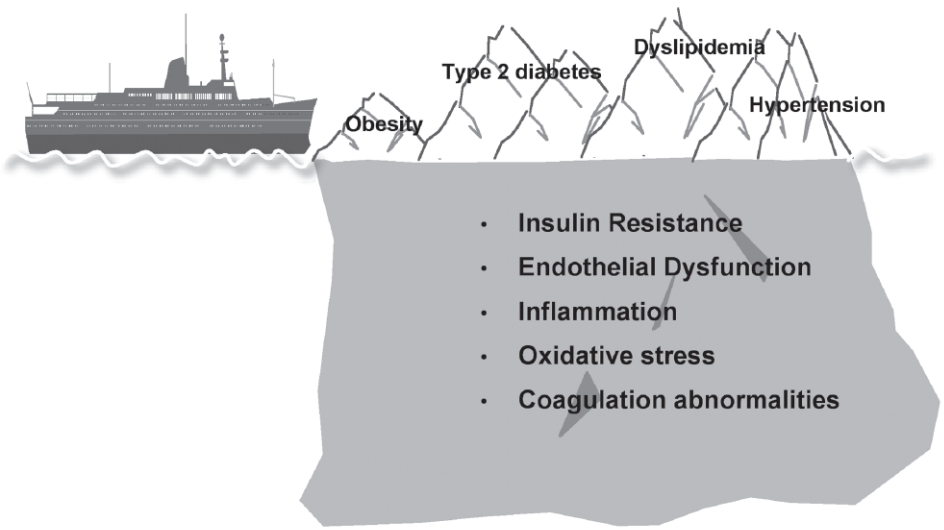
The increase in prevalence of the metabolic syndrome is of great concern worldwide. The for age adjusted prevalence of the MS among assumable healthy persons is around 24% worldwide.[20,21] In a French study, using a modified WHO-definition for the metabolic syndrome, the prevalence for women was 12 % and for men 23%.[22] For patients with manifest arterial disease the prevalence is 45%[23] and for patients with DM2 80%.[17] In a population based cohort study the odds ratio (adjusted for age, sex, and follow-up duration) for the development of diabetes in patients with impaired fasting glucose was 10.0 (95% CI 6.1-16.5).[24] The presence of the metabolic syndrome at baseline increased the risk for the development of diabetes mellitus almost

2-fold in American Indians[25] and in Finnish men a 4-fold increase was shown.[26] Presence of the metabolic syndrome gives a nearly 3-fold increase in cardiovascular related mortality compared to subjects without the metabolic syndrome.[27] In addition, the metabolic syndrome is associated with advanced vascular damage in patients with coronary heart disease, stroke, peripheral arterial disease or abdominal aortic aneurysm[28] and untreated essential hypertension.[29]

It is assumable that the cardiovascular risk for patients with the metabolic syndrome can just be explained by the sum of the separate cardiovascular risk factors. However, Scuteri et al. showed that the age-associated increases in vascular thickness and stiffness was more than expected from the separate cardiovascular risk factors in relation to the metabolic syndrome.[30] Moreover, the increased cardiovascular risk for patients belonging to the metabolic syndrome can not be completely explained by the traditional models for risk-scoring like the Framingham-score.[31] This increased cardiovascular risk may be due to the combination of nontraditional markers, which all have a relation with insulin resistance, together with the separate traditional components of the metabolic syndrome (hyperglycemia, hypertension, low plasma HDL cholesterol, high plasma triglyceride levels, and obesity). The nontraditional markers are endothelial dysfunction, inflammation, hyperinsulinemia, oxidative stress hypercoagulability together with decreased fibrinolysis, and increased small-dense-LDL.[32-34] Several studies showed a relation between hyperinsulinemia, increased plasma FFA levels, and more oxidized small-dense LDL with endothelial dysfunction.[35-37] Also enhanced low-grade inflammation induces endothelial dysfunction by increased production of cytokines like C-reactive protein (CRP), TNF-α, and IL-6.[38-40] In addition, increased hs-CRP is associated with obesity, insulin resistance, and endothelial dysfunction.[41] The more components of the metabolic syndrome are present in the same person, the higher the plasma CRP concentration.[42] Noteworthy is that CRP not only seems to function as an indicator for cardiovascular disease, but CRP may also play a role as a risk factor in atherogenesis.[43]

Endothelial dysfunction, an early step in atherogenesis, is also present in the metabolic syndrome and may be a cause or a consequence of insulin resistance. The Framingham study already showed that abdominal obesity is the main hypertensinogenic factor.[44] Like already mentioned earlier, obesity is also the cause of insulin resistance, diabetes mellitus type 2, left ventricular hypertrophy, hyperlipidemia, and thus related to an increased risk for atherosclerotic diseases. A direct association between hypertension and BMI has been observed in cross-sectional and longitudinal population studies from early childhood to old age.[45] The mechanism

by which obesity raises blood pressure is not fully understood, but increased BMI is associated with an increase in plasma volume and cardiac output. These alterations and blood pressure can be decreased by weight loss in both normotensive and hypertensive subjects. Furthermore, blood pressure in obese adolescents is sodium-sensitive, and fasting insulin is the best predictor of this sensitivity; after weight loss the blood pressure decreases and the salt sensitivity is reduced.[46] The variables that best predict sodium sensitivity are fasting plasma insulin, plasma aldosterone, and plasma norepinephrine, supporting the hypothesis that bloodpressure is sensitive to dietary sodium and that this sensitivity may be due to the combined effect of hyperinsulinemia, hyperaldosteronism, and increased activity of the sympathetic nervous system.[47] Hyperinsulinemia increases both sympathetic nerve activity and sodium and water retention and the expected vasodilation, upon binding of insulin to the endothelial insulin receptor and causing activation of eNOS, is impaired in insulin resistant states.[48] It is also the heterogeneous effect of endogenous NO on proliferation along the vascular tree that relates to the two different phenomenons of insulin resistant vascular dysfunction. Endothelial dysfunction exists in conduit arteries, while elevated vascular resistance of resistance arteries is observed in essential hypertension.[49,50]



Although a pathophysiological construct seems plausible, future research must unrevel pathophysiology and clinical use before the metabolic syndrome can be designated as a 'syndrome'.[51] The individual components that make up the syndrome should be treated coherently. These are the visible mountains of the floating iceberg above the water. However there are other risk factors underneath the water surface. Awareness of the underlying disorders is important for understanding the pathophyiology and thus coherent treatment: be aware for insulin resistance and its associated (non-) traditional risk factors.

References

1. Stevens RJ, Coleman RL, Adler AI et al. Risk factors for myocardial infarction case fatality and stroke case fatality in type 2 diabetes: UKPDS 66. Diabetes Care. 2004;27:201-207.
2. Turner RC, Millns H, Neil HA et al. Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23). BMJ. 1998;316:823-828.
3. Caballero AE. Metabolic and vascular abnormalities in subjects at risk for type 2 diabetes: the early start of a dangerous situation. Arch Med Res. 2005;36:241-249.
4. Haffner SM, Stern MP, Hazuda HP et al. Cardiovascular risk factors in confirmed prediabetic individuals. Does the clock for coronary heart disease start ticking before the onset of clinical diabetes? JAMA. 1990;263:2893-2898.
5. Jazet IM, Pijl H, Meinders AE. Adipose tissue as an endocrine organ: impact on insulin resistance. Neth J Med. 2003;61:194-212.
6. Rebrin K, Steil GM, Getty L et al. Free fatty acid as a link in the regulation of hepatic glucose output by peripheral insulin. Diabetes. 1995;44:1038-1045.
7. Boden G, Shulman GI. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. Eur J Clin Invest. 2002;32 Suppl 3:14-23.
8. Ginsberg HN. Diabetic dyslipidemia: basic mechanisms underlying the common hypertriglyceridemia and low HDL cholesterol levels. Diabetes. 1996;45 Suppl 3:S27-S30.
9. Feinstein R, Kanety H, Papa MZ et al. Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. J Biol Chem. 1993;268:26055-26058.
10. Ridker PM, Rifai N, Pfeffer M et al. Elevation of tumor necrosis factor-alpha and increased risk of recurrent coronary events after myocardial infarction. Circulation. 2000;101:2149-2153.
11. Stefan N, Vozarova B, Funahashi T et al. Plasma adiponectin concentration is associated with skeletal muscle insulin receptor tyrosine phosphorylation, and low plasma concentration precedes a decrease in whole-body insulin sensitivity in humans. Diabetes. 2002;51:1884-1888.
12. Tschritter O, Fritsche A, Thamer C et al. Plasma adiponectin concentrations predict insulin sensitivity of both glucose and lipid metabolism. Diabetes. 2003;52:239-243.
13. Weyer C, Funahashi T, Tanaka S et al. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab. 2001;86:1930-1935.
14. Reaven GM, Lithell H, Landsberg L. Hypertension and associated metabolic abnormalities--the role of insulin resistance and the sympathoadrenal system. N Engl J Med. 1996;334:374-381.
15. Einhorn D, Reaven GM, Cobin RH et al. American College of Endocrinology position statement on the insulin resistance syndrome. Endocr Pract. 2003;9:237-252.
16. Hu G, Qiao Q, Tuomilehto J et al. Prevalence of the metabolic syndrome and its relation to all-cause and cardiovascular mortality in nondiabetic European men and women. Arch Intern Med. 2004;164:1066-1076.

17. Isomaa B, Almgren P, Tuomi T et al. Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care. 2001;24:683-689.
18. Watson KE, Peters Harmel AL, Matson G. Atherosclerosis in type 2 diabetes mellitus: the role of insulin resistance. J Cardiovasc Pharmacol Ther. 2003;8:253-260.
19. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA. 2001;285:2486-2497.
20. Ford ES, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA. 2002;287:356-359.
21. Panagiotakos DB, Pitsavos C, Chrysohoou C et al. Impact of lifestyle habits on the prevalence of the metabolic syndrome among Greek adults from the ATTICA study. Am Heart J. 2004;147:106-112.
22. Marques-Vidal P, Mazoyer E, Bongard V et al. Prevalence of insulin resistance syndrome in south-western France and its relationship with inflammatory and hemostatic markers. Diabetes Care. 2002;25:1371-1377.
23. Gorter PM, Olijhoek JK, van der GY et al. Prevalence of the metabolic syndrome in patients with coronary heart disease, cerebrovascular disease, peripheral arterial disease or abdominal aortic aneurysm. Atherosclerosis. 2004;173:363-369.
24. de Vegt F, Dekker JM, Jager A et al. Relation of impaired fasting and postload glucose with incident type 2 diabetes in a Dutch population: The Hoorn Study. JAMA. 2001;285:2109-2113.
25. Resnick HE, Jones K, Ruotolo G et al. Insulin resistance, the metabolic syndrome, and risk of incident cardiovascular disease in nondiabetic american indians: the Strong Heart Study. Diabetes Care. 2003;26:861-867.
26. Laaksonen DE, Lakka HM, Niskanen LK et al. Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. Am J Epidemiol. 2002;156:1070-1077.
27. Lakka HM, Laaksonen DE, Lakka TA et al. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA. 2002;288:2709-2716.
28. Olijhoek JK, van der GY, Banga JD et al. The metabolic syndrome is associated with advanced vascular damage in patients with coronary heart disease, stroke, peripheral arterial disease or abdominal aortic aneurysm. Eur Heart J. 2004;25:342-348.
29. Schillaci G, Pirro M, Vaudo G et al. Metabolic syndrome is associated with aortic stiffness in untreated essential hypertension. Hypertension. 2005;45:1078-1082.
30. Scuteri A, Najjar SS, Muller DC et al. Metabolic syndrome amplifies the age-associated increases in vascular thickness and stiffness. J Am Coll Cardiol. 2004;43:1388-1395.
31. Girman CJ, Rhodes T, Mercuri M et al. The metabolic syndrome and risk of major coronary events in the Scandinavian Simvastatin Survival Study (4S) and the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS). Am J Cardiol. 2004;93:136-141.

32. Reaven G. The metabolic syndrome or the insulin resistance syndrome? Different names, different concepts, and different goals. Endocrinol Metab Clin North Am. 2004;33:283-303.
33. Sakkinen PA, Wahl P, Cushman M et al. Clustering of procoagulation, inflammation, and fibrinolysis variables with metabolic factors in insulin resistance syndrome. Am J Epidemiol. 2000;152:897-907.
34. Hulthe J, Bokemark L, Wikstrand J et al. The metabolic syndrome, LDL particle size, and atherosclerosis: the Atherosclerosis and Insulin Resistance (AIR) study. Arterioscler Thromb Vasc Biol. 2000;20:2140-2147.
35. Wheatcroft SB, Williams IL, Shah AM et al. Pathophysiological implications of insulin resistance on vascular endothelial function. Diabet Med. 2003;20:255-268.
36. Steinberg HO, Baron AD. Vascular function, insulin resistance and fatty acids. Diabetologia. 2002;45:623-634.
37. Chait A, Brazg RL, Tribble DL et al. Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. Am J Med. 1993;94:350-356.
38. Fichtlscherer S, Rosenberger G, Walter DH et al. Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. Circulation. 2000;102:1000-1006.
39. Yudkin JS, Kumari M, Humphries SE et al. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis. 2000;148:209-214.
40. Chia S, Qadan M, Newton R et al. Intra-Arterial Tumor Necrosis Factor-{alpha} Impairs Endothelium-Dependent Vasodilatation and Stimulates Local Tissue Plasminogen Activator Release in Humans. Arterioscler Thromb Vasc Biol. 2003;23:695-701.
41. Yudkin JS, Stehouwer CD, Emeis JJ et al. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? Arterioscler Thromb Vasc Biol. 1999;19:972-978.
42. Ridker PM, Buring JE, Cook NR et al. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. Circulation. 2003;107:391-397.
43. Blake GJ, Ridker PM. C-reactive protein, subclinical atherosclerosis, and risk of cardiovascular events. Arterioscler Thromb Vasc Biol. 2002;22:1512-1513.
44. Ashley FW, Jr., Kannel WB. Relation of weight change to changes in atherogenic traits: the Framingham Study. J Chronic Dis. 1974;27:103-114.
45. Haffner SM, Ferrannini E, Hazuda HP et al. Clustering of cardiovascular risk factors in confirmed prehypertensive individuals. Hypertension. 1992;20:38-45.
46. Rocchini AP, Key J, Bondie D et al. The effect of weight loss on the sensitivity of blood pressure to sodium in obese adolescents. N Engl J Med. 1989;321:580-585.
47. Carretero OA, Oparil S. Essential hypertension. Part I: definition and etiology. Circulation. 2000;101:329-335.

48. Modan M, Halkin H, Almog S et al. Hyperinsulinemia. A link between hypertension obesity and glucose intolerance. J Clin Invest. 1985;75:809-817.
49. Falloon BJ, Heagerty AM. In vitro perfusion studies of human resistance artery function in essential hypertension. Hypertension. 1994;24:16-23.
50. Rizzoni D, Porteri E, Castellano M et al. Vascular hypertrophy and remodeling in secondary hypertension. Hypertension. 1996;28:785-790.
51. Grundy SM, Cleeman JI, Daniels SR et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation. 2005;112:2735-2752.

PPAR-γ agonism and Thiazolidinediones

Our understanding of the so-called cardiovascular dysmetabolic syndrome has been improved by the discovery of nuclear peroxisome proliferator-activated receptors (PPARs). PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily. As transcription factors, PPARs regulate the expression of numerous genes and affect glycaemic control, lipid metabolism, vascular tone and inflammation. Activation of the subtype PPAR-γ improves insulin sensitivity. Expression of PPAR-γ is present in several cell types involved in the process of atherosclerosis. Thus, modulation of PPAR-γ activity is an interesting therapeutic approach to reduce cardiovascular events. Thiazolidinediones are PPAR-γ agonists and constitute a new class of pharmacological agents for the treatment of type 2 (non-insulin–dependent) diabetes mellitus. Two such compounds are currently available for clinical use: rosiglitazone and pioglitazone. Thiazolidinediones improve insulin sensitivity and glycaemic control in patients with type 2 diabetes. In addition, improvement in endothelial function, a decrease in inflammatory conditions, a decrease in plasma levels of free fatty acids and lower blood pressure have been observed, which may have important beneficial effects on the vasculature.

Several questions remain to be answered about PPAR-γ agonists, particularly with respect to the role of PPAR-γ in vascular pathophysiology. More needs to be known about the adverse effects of Thiazolidinediones, such as hepatotoxicity, increased low-density lipoprotein cholesterol levels and increased oedema. The paradox of adipocyte differentiation with weight gain concurring with the insulin-sensitising effect of Thiazolidinediones is not completely understood. The decrease in blood pressure induced by Thiazolidinedione treatment seems incompatible with an increase in the plasma volume, and the discrepancy between the stimulation of the expression of CD36 and the antiatherogenic effects of the Thiazolidinediones also needs further explanation. Long-term clinical trials of Thiazolidinediones with cardiovascular endpoints are currently in progress.

In conclusion, studying the effects of Thiazolidinediones may shed more light on the mechanisms involved in the insulin resistance syndrome. Furthermore, Thiazolidinediones could have specific, direct effects on processes involved in the development of vascular abnormalities.

Outline of the thesis

Cardiovascular disease is the leading cause of morbidity and mortality in Western countries. The term cardiovascular disease comprises clinical manifestations of arterial atherosclerosis, such as peripheral artery disease, cerebrovascular disease, and coronary artery disease. Well-established risk factors are dyslipidemia, smoking, diabetes mellitus, hypertension, and clustered in association with abdominal obesity. In the pathophysiology of atherosclerosis, based on the respons to injury mechanism, the pathophysiological phenomenons **endothelial dysfunction** and **inflammation** are playing a pivitol role.

The endothelium has been identified as the central transducer through which risk factors can cause atherosclerosis and its clinical complications. The central pathway in this event is the activation of the endothelium. Basically this involves the switch from a healthy condition with low concentrations of NO resulting in vasodilative and/or anti-atherosclerotic actions. However, in pathophysiological situations NO-availability *in vivo* is impaired because of redox signalling in the activated endothelium, as a result of reduced NO-formation, enhanced NO-breakdown or both. There are two major consequences of this endothelial activation:

First, it will lead to the production of chemokines and expression of adhesion molecules. This will support the recruitment of inflammatory cells into the vessel wall. Although this system is physiological in the context of host-defense it may become inappropriate with prolonged periods of endothelial cell activation secondary to cardiovascular risk factors. As a result, an inflammatory phenotype evolves in the vessel wall which leads to atherogenesis and plaque rupture.

Second, the loss of NO-bioavailability by the endothelium, due to risk factors, affects the vessel wall structure. There are some preliminary indications that NO inhibits VSMC-proliferation in the vascular wall. As a result one could hypothesize that loss of NO-activity would result in increased VSMC-proliferation. This may accelerate the development of the atherosclerotic plaque, but may also lead to thicker, remodelled vessels. As a result these vessels become stiffer and start to produce pressure load on the heart.

In **Chapter 1** we first would like to explore the hypothesis that endothelial function (in the sense of NO-bioavailability) is an important determinant of vessel wall structure. In conduit arteries, endothelial dysfunction is initiating atherogenesis. The other phenomenon of endothelial dysfunction is the elevated vascular resistance of resistance arteries (microcirculation) as observed in essential hypertension.

We addressed this heterogeneity of vascular remodeling along the vascular tree and postulated that this regional difference may be related to a heterogeneous effect of eNOS on proliferation in conduit arteries vs. resistance vessels.
In the current thesis we would like to explore several aspects of the described model of vascular injury in the setting of type 2 diabetes. Type 2 diabetes is emerging as a worldwide epidemic and currently about 200 million people are affected worldwide. An important driver for this increased incidence is the associated increase in patients with insulin resistance (approximately 400 million worldwide right now). This insulin resistance is driven by obesity and secondary to obesity, free fatty acid fluxes towards other tissues than adipocytes (like the muscle and liver). However, genetic factors, particularly in Asian people, seem to play a role as well.
Clinically it would be useful to correlate the severity of insulin resistance to the severity of alterations in vessel wall structure due to loss of NO-activity. In **chapter 2** we investigated in a cross-sectional survey (2105 patients) to determine whether carotid artery stiffness was increased in ('pre-diabetic') patients with the metabolic syndrome and in patients who developed overt type 2 diabetes.
Because our group extensively studied all kind of other markers of endothelial dysfunction in diabetes and obesity (and thus insulin resistance), we did not further explore these concepts and took the experience already gained, to address possible treatment. Thinking about a treatment of the underlying disorders, the discovery of nuclear peroxisome proliferator-activated receptors (PPARs) and subsequent insight into their role in several metabolic pathways was a major breakthrough in our understanding of pathophysiological mechanisms underlying the insulin resistance syndrome.
Thiazolidinediones (like pioglitazone) are clinically available agonists of the PPAR-γ subtype and constitute a new class of antihyperglycaemic agents. Activation of PPAR-γ not only improves insulin sensitivity but may also have additional beneficial vascular effects.
The aim of a main part of this thesis is to focus on the potential role of Thiazolidinediones in the pathophysiological mechanisms involved in vascular disease.
Chapter 3 is an overview of the metabolic and additional vascular effects of Thiazolidinediones.
In **chapter 4** we investigated the direct vascular effects of pioglitazone on the capacity of the vasculature to maintain its NO-release, in type 2 diabetic patients, in a double blind and crossover design. As measurement of NO-activity in the vessel wall, flow-mediated dilation in the conduit brachial artery was used. We focused on relatively short-term application of pioglitazone to tease out direct vascular effects from its indirect metabolic effects.

We subsequently investigated whether a beneficial effect of TZDs on NO-bio-availability in type 2 diabetes also translates into better protection of the vessel wall from inflammatory stimuli.
To this end we investigated in **chapter 5**, whether diabetic subjects could maintain NO dominated endothelial function in the presence of increased concentrations of TNF-α. Therefore we first investigated the effect of TNF-α on endothelium-dependent vasodilation and secondly the effects of short-term pioglitazone treatment on TNF-α induced endothelial dysfunction in patients with type 2 diabetes mellitus.
In addition, we investigated *ex vivo* whether TZDs changed the properties of monocytes to adhere to the endothelium, or to produce cytokines.
Therefore, we examined in **chapter 6**, in a parallel controlled, *ex vivo* study, the effects of incubation with TZDs, on monocyte-endothelium-adherence under flow conditions.
In **chapter 7** we studied the cytokine production in whole blood from type 2 diabetes patients after short-term pioglitazone treatment, in a double blind and crossover design.

CHAPTER 1

Vessel-specific stimulation of protein synthesis by nitric oxide synthase inhibition -role of extracellular signal–regulated kinases 1/2-

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Abstract

Introduction Although conduit arteries develop hypertrophy after chronic NO synthesis blockade, resistance arteries remodel without hypertrophy under the same conditions. Similar findings have been described in essential hypertension. We postulated that this regional difference may be related to a heterogeneous effect of endogenous NO on proliferation along the vascular tree.

Methods and Results Newly synthesized proteins were radiolabeled in rats with [3H]L-leucine in basal conditions and during NO synthase inhibition, with or without PD98059 (inhibitor of the extracellular signal–regulated kinases [ERK] 1/2). Blocking the generation of NO by 3 different L-arginine analogues increased protein synthesis by an average of 75% in the aorta, in association with enhanced ERK 1/2 phosphorylation. PD98059 significantly reduced L-arginine analogue–induced protein synthesis and ERK 1/2 phosphorylation, confirming the involvement of ERK 1/2 as an important signaling element. In small arteries, L-arginine analogues did not influence the extent of protein synthesis, although phosphorylation of ERK 1/2 was also enhanced. To determine the role of NO in a condition of enhanced protein synthesis, angiotensin-II was infused for 24 hours. Angiotensin-II augmented protein synthesis in mesenteric arteries and the aorta, and was additive to NO synthase blockade in the aorta.

Conclusion Endogenous NO exerts a tonic inhibitory influence on aortic growth, with limited impact on small arteries in basal and hypertrophic conditions. This heterogeneous role of NO on vascular growth may explain the heterogeneity of vascular remodeling observed in essential hypertension, a condition associated with endothelial dysfunction.

Keywords aorta, arteries, hypertrophy, kinase, nitric oxide, nitric oxide synthase

Introduction

The endothelium is an important modulator of vascular reactivity and structure, and NO is one of the main products synthesized and released by endothelial cells.[1] Most of the biological effects of NO are mediated by cGMP and include regulation of vascular tone and endothelial permeability, inhibition of platelet adhesion and aggregation, and inhibition of leukocyte–endothelial cell interactions.[2] In addition, early studies in vascular smooth muscle cells (VSMCs) in culture have shown that NO donors inhibit cellular proliferation[3-6], which is an important event in the pathogenesis of atherosclerosis, restenosis, and possibly hypertension.[7] Among the signaling pathways related to NO inhibition of cell proliferation, inhibition of extracellular signal–regulated kinase (ERK) 1/2 phosphorylation has been proposed to be important, considering the pivotal role of this signaling event in VSMC growth.[6,8]

In hypertension, arteries adapt to the pressure-induced elevation in wall stress by changing their geometry.[9,10] Indeed, the elevated vascular resistance observed in hypertension is associated with an increased media thickness–lumen diameter ratio (remodeling) of resistance arteries.[11,12]

In essential hypertension, the amount of material in the vessel wall is not augmented but appears to be rearranged around a smaller lumen, and the process has been called eutrophic remodeling.[13] In contrast, large arteries undergo mainly hypertrophic remodeling, because their lumen size is generally not reduced, and wall thickness increases in an effort to compensate for the increased wall stress.[10,14]

Chronic inhibition of NO synthesis with NG-nitro-L-arginine methyl ester (L-NAME) leads to hypertension. Interestingly, in this model, small arteries undergo eutrophic remodeling in proportion to the elevation of arterial pressure.[15,16] In addition, under conditions of stimulated growth with exogenous angiotensin-II (Ang-II) administration, chronic NO synthase inhibition does not worsen growth development but blunts it in cerebral arteries.[17] These observations argue against a prominent inhibitory role of NO on VSMC growth in small arteries *in vivo*. In the aorta, however, chronic NO inhibition with L-NAME promotes medial hypertrophy and fibronectin deposition, suggesting a growth-inhibitory role for NO in large arteries.[18] Our postulate is that endogenous NO exerts growth regulation in large arteries but that there is no such regulation in small arteries, possibly because of the different influence of signaling along the vascular tree. To address this issue, the vascular growth response to endogenous NO inhibition was determined in small and large arteries simultaneously under basal and Ang-II–stimulated conditions *in vivo*. Furthermore, we tested the potential of ERK 1/2 to represent an important determinant of the heterogeneity.

Methods

Under anesthesia, the left femoral vein and artery of Sprague-Dawley rats (300 to 400 g) were catheterized with a polyethylene tubing that was tunneled subcutaneously, exteriorized at the back of the neck, and protected by a tethering system. In rats treated with Ang-II, an osmotic pump delivering 400 ng/kg per minute was inserted subcutaneously at the same time.

Twenty hours after surgery, arterial pressure and heart rate were recorded. In the first series of experiments, a bolus of L-arginine analogues was administered through the venous catheter to control and Ang II-treated rats. Three different L-arginine analogues were used: L-NAME, N^G-methyl-L-arginine (L-NMMA), and N^G-nitro-L-arginine (L-NA), all at a dose of 3 mg/kg (n=8 per group). One hour after this bolus, a 4-hour intravenous infusion of L-(4,5-^{3}H)leucine was started.[19] A second dose of L-arginine analogues was administered intra-arterially 90 minutes after the start of the infusion. In the second series of experiments, rats received vehicle, L-NA (as above), PD98059 (10 mg/kg IP, at the time of the first L-NA administration, n=8), or the combination of L-NA and PD98059 (n=8). L-(4,5-^{3}H)leucine infusion was started 1 hour after injection of the drugs in half of the animals. The protocols were approved by the Animal Care Committee of Université de Montréal.

Protein synthesis measurement and autoradiography

Frozen aortas and mesenteric arteries were powdered in liquid nitrogen, and proteins were precipitated overnight in trichloroacetic acid (TCA), washed once with TCA and twice with water, and solubilized in KOH to which the scintillation liquid was added. The other half of the tissue was also left overnight in TCA, and the precipitate was solubilized in NaOH to measure protein concentration by the method of Lowry et al.[20] The final results are expressed as counts per minute per milligram protein. Slides of paraffin-embedded aortic sections were soaked in an autoradiography emulsion, dried, and kept at 4°C for 8 weeks in complete darkness. Slides were then developed, fixed, and counter-stained with hematoxylin to reveal the nuclei. Images were digitalized at a final magnification of x 400.

Phosphospecific immunoblot of ERK 1/2, cGMP, and plasma renin activity measurements

ERK 1/2 activity was estimated by Western blot, with the use of a phosphospecific antibody, as previously described by Touyz et al.[21] Equal amounts of proteins (20 μg) were loaded on a 10% SDS–polyacrylamide gel. Proteins were then transferred to a polyvinylidene difluoride membrane and incubated with a phospho-specific ERK 1/2

antibody. The membrane was then washed and incubated with a second antibody. The membrane was incubated with enhanced chemiluminescence (ECL) Western blotting reagents and exposed on Hyperfilm ECL. Results were normalized to control values on each gel to account for methodological variation.
cGMP measurements were performed by using the acetylation procedure, as suggested by the Biotrak cGMP enzyme immunoassay system. Plasma renin activity (PRA) was assessed by a commercial radioimmunoassay kit on plasma samples taken from 6 control rats and 6 rats treated with L-NA (2 injections of 3 mg/kg). Samples were taken before and 5 hours after the first drug injection. An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Results

Hemodynamic measurements
In the first series of experiments, administration of L-arginine analogues in 2 boluses over a 5-hour period increased mean arterial pressure (MAP) only slightly compared with preadministration values, with only L-NA having a significant effect (Table). The dose of Ang II selected did not produce a significant elevation of MAP in 24 hours. Administered in combination with Ang II, L-NA and L-NAME produced a significant pressor effect (Table). In the second series of experiments, PD98059 administered either alone or with L-NA did not modify arterial pressure significantly (Table).

Protein Synthesis and autoradiography
Basal protein synthesis was 406±42 and 276±26 cpm/mg protein in mesenteric arteries and in the aorta, respectively (Figure 1). In mesenteric arteries, the administration of L-arginine analogues 1 hour before labeled L-leucine infusion did not influence the extent of protein synthesis (Figure 1A). However, in the aorta, the analogues enhanced protein synthesis by 70% to 86% (Figure 1B). Ang II significantly increased protein synthesis by 70% in small (mesenteric) and by 66% in large (aortic) arteries (Figure 1). When endogenous NO production was blocked by L-arginine analogues in addition to Ang II, we observed no modification of protein synthesis in mesenteric arteries (Figure 1C), but we did observe enhanced protein synthesis ranging from 58% to 72% in the aorta (Figure 1D). The effects of Ang II and L-arginine analogues were clearly additive in the aorta. The augmented protein synthesis found in the aorta did not correlate with mean, systolic, or diastolic arterial pressure (data not shown).

Table. MAP values in awake freely moving rats taken before and averaged during the 5h of drug administration

Group	Predrug MAP (mmHg)	Postdrug MAP (mmHg)
Control	109 ± 4	
L-NAME	108 ± 3	113 ± 3
L-NMMA	123 ± 8	122 ± 5
L-NA	112 ± 5	130 ± 4*
Ang II	116 ± 7	
Ang II + L-NAME	109 ± 5	140 ± 6*
Ang II + L-NMMA	125 ± 8	121 ± 6
Ang II + L-NA	120 ± 5	138± 4*
Control	98 ± 3	
L-NA	101 ± 4	105 ± 2
L-NA + PD98059	102 ± 10	108 ± 7
PD98059	98 ± 6	95 ± 7

Values are mean ± SEM;
In control and Ang II-treated rats, there was no acute drug administration;
* $p<0.05$

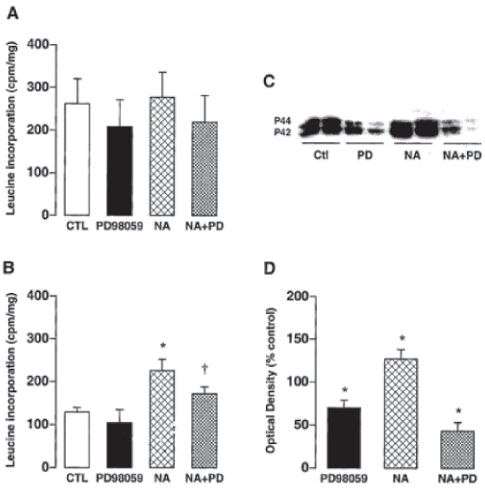
In our second series of experiments, the effect of L-NA on protein synthesis was reproduced. Indeed, it amplified protein synthesis in the aorta but not in small arteries (Figure 2A and 2B). PD98059, which had no significant effect on its own, blocked L-NA–induced augmentation of protein synthesis in the aorta by 56%.

Autoradiography of aortic cross sections was performed to localize the enhanced protein synthesis in the aortic wall. It revealed that most of protein synthesis occurred in the media and intima in an evenly distributed fashion, both in control conditions and after L-NA treatment (Figure 3). Indeed, the number of silver grains in the adventitia appears reduced compared with those remaining in the aortic wall.

ERK 1/2 activity

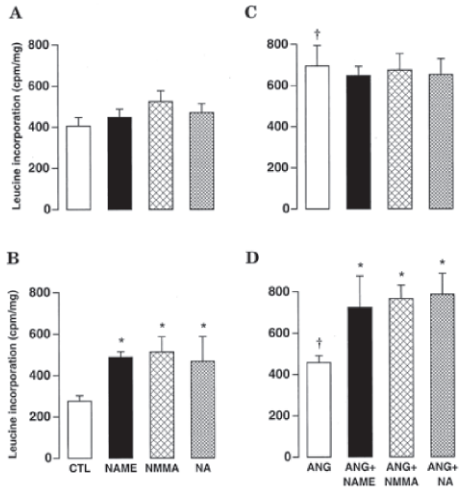
In the aorta, ERK 1/2 phosphorylation increased after L-NA administration (Figure 2C and 2D). As expected, this effect was abrogated by PD98059, which even decreased ERK 1/2 phosphorylation below control levels. ERK 1/2 activity was also significantly enhanced in small mesenteric arteries during L-NA administration (165±7% from control).

Figure 1.



Effect of NOS inhibition on [3H]L-leucine incorporation in proteins from rat mesenteric arteries (A and C) and rat thoracic aortic segments (B and D) at the basal level (A and B) and after Ang II (ANG) treatment (C and D). CTL indicates control; NAME=L-NAME; NMMA=L-NMMA; and NA=L-NA. Results are expressed as the mean ± SEM in counts per minute per milligram protein (n=8 per group). *p<0.05 vs respective CTL, and †p<0.05 vs true CTLs (ANOVA+Bonferroni correction for multiple comparisons).

Figure 2.

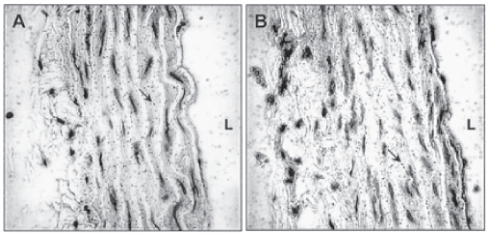


A and B: Effect of PD98059 on basal and NOS inhibition–induced [3H]L-leucine incorporation in proteins from small mesenteric arteries (A) and thoracic aortic segments (B). CTL indicates control; NA=L-NA; NA+PD=L-NA+PD98059. * p<0.05 vs control (CTL), and † = p<0.05 vs L-NA (ANOVA+Bonferroni correction for multiple comparisons).

C: Representative Western blot for ERK 1/2 (p44/p42), with use of a phospho-specific antibody in aortic protein extracts from 2 control (Ctl) rats, 2 PD-treated rats, 2 NA-treated rats, and 2 NA+PD–treated rats.

D: Mean corrected optical density of Western blots for 6 to 8 animals per group, performed on 3 separated experiments. * p<0.05 vs 100 (Ctl value, performed by 1-sample analysis).

Figure 3.



Representative autoradiography of aortic cross sections in a control rat (A) and a rat treated with L-NA for 5 hours (B). The arrows point to one of the numerous silver grains scattered throughout the aortic wall. It is noteworthy that the adventitia is poorly labeled. Quantification was performed by use of a more sensitive method (see Methods and Figure 1). L indicates lumen.

cGMP and plasma renin activity measurements

To confirm that the L-arginine analogues reduced NO production and that Ang II exerted its effect independently from NO, we measured aortic cGMP levels in control and in L-NMMA– and Ang II–treated rats. Two and a half hours after the second bolus injections of L-NMMA, cGMP levels were halved (2.2±0.3 fmol/mg compared with 4.3±0.4 fmol/mg in the control group). In contrast, Ang-II had no significant effect on vascular cGMP levels (4.0±0.5 fmol/mg).

To rule out any global effect of Ang II on protein synthesis during NOS inhibition, PRA was measured. In control rats, repeated measurements of PRA were similar at the beginning and 5 hours later (6.2±1.2 and 7.1±1.1 ng angiotensin I/mL per hour, respectively). In L-NA–treated animals, however, PRA decreased from 4.3±0.4 to 1.7±0.5 ng angiotensin I/mL per hour after the 2 bolus injections of the drug.

Discussion

Using a model enabling us to measure pharmacological modulation of protein synthesis, we report that endogenous NO exerts tonic inhibition of vascular wall growth in a vessel-specific manner. In large arteries, NOS blockade stimulated protein synthesis by enhancing ERK 1/2 phosphorylation, whereas in small arteries, NOS blockade did not induce protein synthesis despite elevation of ERK 1/2 phosphorylation. These results suggest heterogeneity in vascular growth responses to NOS blockade and to ERK 1/2 activation.

Although vascular heterogeneity to the relaxing properties of NO has been reported,[22] to the best of our knowledge the concept of vascular heterogeneity to the antiproliferative action of NO has never been put forward and has never been examined directly in the same animals. NOS inhibition did not augment protein synthesis in small arteries, consistent with previous reports, including ours, showing that chronic NO deficiency leads to eutrophic remodeling but not to hypertrophy (no change in cross-sectional area despite an enhanced media/lumen ratio) of small mesenteric and cerebral arteries.[15,16,22,23] During chronic NO deficiency, these arteries normally undergo eutrophic remodeling in proportion to the elevation of arterial pressure.[16] One exception may be the coronary circulation, which seems to have pressure-independent remodeling, possibly related to local Ang II formation.[24,25] Indeed, Ang II leads to a pressure-independent hypertrophic remodeling of small arteries when it is administered for long periods[26-28], and we could measure a significant increase of protein synthesis after 24 hours of administration in the present model.

In large arteries, NOS blockade, confirmed by cGMP measurements, enhanced protein synthesis and ERK 1/2 phosphorylation, demonstrating that endogenous NO exerts a tonic inhibition of vascular growth. The involvement of ERK 1/2 in the growth response was confirmed by the efficacy of PD98059 to reduce it. The partial effect of PD98059 suggests that other signaling events could also contribute to the response. Alternatively, higher doses of PD98059 may be required, but 10 mg/kg proved to block 100% of Ang II–induced vascular protein synthesis (C. Daigle, P. Moreau, unpublished data, 2001). The goal of using Ang II was to determine the modulation, by NOS inhibition, of enhanced vascular protein synthesis (compared with physiological protein synthesis) and not to specifically look for interactions. In fact, measurement of PRA shows that acute NOS inhibition decreases the circulating renin-angiotensin system. It appears quite clear that the 2 treatments had additive effects in large arteries, suggesting that NO also exerts a modulation of protein synthesis in conditions of enhanced growth. One may argue that pressure elevation secondary to NOS blockade could promote arterial remodeling, thus explaining part of the enhanced protein synthesis. However, we could not find a correlation between mean, systolic, and diastolic pressure values and protein synthesis, arguing against a secondary effect of NOS blockade. In addition, it is quite clear that increased protein synthesis can occur without elevation of arterial pressure, as we have shown with the administration of a subpressor dose of Ang II.

The enhanced protein synthesis during NOS inhibition could result directly from the reduction of NO production, inasmuch as NO has been shown to modulate growth

in vitro.[5,6] However, the *in vivo* situation is more complex, and numerous molecules could be recruited and contribute to the enhancement of protein synthesis and the remodeling process. In that respect, it has been demonstrated that chronic NOS inhibition leads to ACE activation and to the formation of a proinflammatory milieu in the vasculature that could influence VSMC protein synthesis and the development of hypertrophy.[25,29,30] Thus, the *in vivo* measurement of protein synthesis provides an assessment of the final integration of the tissue to the different circulating and local influences and clearly demonstrates that under NOS inhibition, large arteries react with a trophic response partly involving ERK 1/2.

The heterogeneous growth response to NOS inhibition between large and small arteries is not unlikely, because many other vascular functions, including growth responses, differ between distant segments of the arterial tree.[31] In an effort to characterize the heterogeneity at the molecular level, ERK 1/2 phosphorylation was measured, because it represents a central element of growth signaling. Furthermore, NO donors have been shown to interact with this element by reducing its phosphorylation.[6] Our *in vivo* study extends these *in vitro* findings by showing that NOS inhibition amplifies ERK 1/2 phosphorylation. However, activation of ERK 1/2 was observed in both small and large arteries, suggesting that it is not its activation but its vascular effect that is heterogeneous. We are currently testing the role of ERK 1/2 in small-artery vasoconstriction *in vivo* under NOS inhibition, because this pathway has been shown to be involved in resistance artery VSMC contraction[32,33], a condition associated with the attenuation of growth responses.[34]

The method used in the present study does not allow for the determination of the nature of the proteins newly synthesized by NOS inhibition or Ang II, although most of the activity appears to be located in the media and the intima with NOS inhibition. Kato et al[18] have reported that both hypertensive stimuli, administered alone or in combination, lead to medial thickening of the aorta by cellular hypertrophy and enhanced matrix deposition in the form of fibronectin. It is also noteworthy that intimal and adventitial cell division occurred only during the second day of Ang II administration and not with NOS inhibition. Thus, although not confirmed in the present study, previous results suggest that both intracellular and extracellular proteins account for the elevation of protein synthesis during NOS inhibition and Ang II administration, at least in large arteries.

It has been suggested that L-NAME exerts NO-independent negative metabolic effects on protein synthesis that could account for the lack of hypertrophy, at least in the heart.[35] In vessels, we found no evidence that L-NAME is less specific than

other L-arginine analogues in terms of growth regulation at doses comparable to that used to induce hypertension (6 mg/kg over 5 hours versus 50 mg/kg per day in chronic studies). Although we solely measured protein synthesis, it represents a prerequisite to the development of vascular hypertrophy, and it appears unlikely that L-NAME has a nonspecific effect. This does not exclude the possibility that L-NAME, being a muscarinic antagonist *in vitro*[36], could affect the proliferation of specific tissues, such as astrocytes and prostate cancer cells, which have been shown to be modulated by muscarinic interventions.[37,38]

In conclusion, we report that NO is an endogenous inhibitor of vascular protein synthesis in conduit arteries under physiological and Ang II–stimulated conditions, in part by modulating ERK 1/2 phosphorylation. In contrast, in small arteries from the mesenteric circulation, endogenous NO does not exert any growth inhibition, although it also modulates ERK 1/2 activity. Our findings may help to explain the heterogeneous remodeling of large and small arteries in conditions of endothelial dysfunction, such as essential hypertension.

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References

1. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature. 1988;333:664-666.
2. Moreau P, Nava E, Takase H, Lüscher TF. Local regulation of vascular function: focus on endothelium-dependent mechanisms in normotension, hypertension and atherosclerosis. In: Zanchetti A, Mancia G, ed. Pathophysiology of Hypertension. Amsteram, the Netherlands: Elsevier;1997:975-1006. Birkenhäger WH, Reid JL, ed. Handbook of Hypertension; Vol 17.
3. Kariya K, Kawahara Y, Araki S et al. Antiproliferative action of cyclic GMP-elevating vasodilators in cultured rabbit aortic smooth muscle cells. Atherosclerosis. 1989;80:143-147.
4. Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest. 1989;83:1774-1777.
5. Sarkar R, Webb RC. Does nitric oxide regulate smooth muscle cell proliferation? A critical appraisal. J Vasc Res. 1998;35:135-142.
6. Jeremy JY, Rowe D, Emsley AM et al. Nitric oxide and the proliferation of vascular smooth muscle cells. Cardiovasc Res. 1999;43:580-594.
7. Lüscher T, Dubey RK. Endothelium and platel-derived vasoactive substances: role in the regulation of vascular tone and growth. In: Laragh JH, Brenner BM, eds. Hypertension: Pathophysiology, Diagnosis, and Management. 2nd ed. New York, NY: Raven Press, Ltd; 1995:609-630.
8. Servant MJ, Giasson E, Meloche S. Inhibition of growth factor-induced protein synthesis by a selective MEK inhibitor in aortic smooth muscle cells. J Biol Chem. 1996;271:16047-16052.
9. Heagerty AM, Aalkjaer C, Bund SJ et al. Small artery structure in hypertension. Dual processes of remodeling and growth. Hypertension. 1993;21:391-397.
10. Laurent S, Vanhoutte P, Cavero I et al. The arterial wall: a new pharmacological and therapeutic target. Fundam Clin Pharmacol. 1996;10:243-257.
11. Falloon BJ, Heagerty AM. In vitro perfusion studies of human resistance artery function in essential hypertension. Hypertension. 1994;24:16-23.
12. Rizzoni D, Porteri E, Castellano M et al. Vascular hypertrophy and remodeling in secondary hypertension. Hypertension. 1996;28:785-790.
13. Mulvany MJ, Baumbach GL, Aalkjaer C et al. Vascular remodeling. Hypertension. 1996;28:505-506.
14. Safar ME. Arteries in Clinical Hypertension. Philadelphia, Pa: Lippincott-Raven Publishers; 1996:97.
15. Li JS, Schiffrin EL. Resistance artery structure and neuroeffector mechanisms in hypertension induced by inhibition of nitric oxide synthase. Am J Hypertens. 1994;7:996-1004.
16. Moreau P, Takase H, Kung CF et al. Structure and function of the rat basilar artery during chronic nitric oxide synthase inhibition. Stroke. 1995;26:1922-1928.

17. Moreau P, Takase H, d'Uscio LV et al. Effect of chronic nitric oxide deficiency on angiotensin II-induced hypertrophy of rat basilar artery. Stroke. 1998;29:1031-1035.
18. Kato H, Hou J, Chobanian AV et al. Effects of angiotensin II infusion and inhibition of nitric oxide synthase on the rat aorta. Hypertension. 1996;28:153-158.
19. McNulty PH, Young LH, Barrett EJ. Response of rat heart and skeletal muscle protein in vivo to insulin and amino acid infusion. Am J Physiol. 1993;264:E958-E965.
20. Lowry OH, Rosebrough NJ, Farr AL et al. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265-275.
21. Touyz RM, Deng LY, He G et al. Angiotensin II stimulates DNA and protein synthesis in vascular smooth muscle cells from human arteries: role of extracellular signal-regulated kinases. J Hypertens. 1999;17:907-916.
22. Ferrer M, Encabo A, Conde MV et al. Heterogeneity of endothelium-dependent mechanisms in different rabbit arteries. J Vasc Res. 1995;32:339-346.
23. Deng LY, Thibault G, Schiffrin EL. Effect of hypertension induced by nitric oxide synthase inhibition on structure and function of resistance arteries in the rat. Clin Exp Hypertens. 1993;15:527-537.
24. Numaguchi K, Egashira K, Takemoto M et al. Chronic inhibition of nitric oxide synthesis causes coronary microvascular remodeling in rats. Hypertension. 1995;26:957-962.
25. Takemoto M, Egashira K, Usui M et al. Important role of tissue angiotensin-converting enzyme activity in the pathogenesis of coronary vascular and myocardial structural changes induced by long-term blockade of nitric oxide synthesis in rats. J Clin Invest. 1997;99:278-287.
26. Griffin SA, Brown WC, MacPherson F et al. Angiotensin II causes vascular hypertrophy in part by a non-pressor mechanism. Hypertension. 1991;17:626-635.
27. Moreau P, d'Uscio LV, Shaw S et al. Angiotensin II increases tissue endothelin and induces vascular hypertrophy: reversal by ET(A)-receptor antagonist. Circulation. 1997;96:1593-1597.
28. Simon G, Illyes G, Csiky B. Structural vascular changes in hypertension: role of angiotensin II, dietary sodium supplementation, blood pressure, and time. Hypertension. 1998;32:654-660.
29. Gonzalez W, Fontaine V, Pueyo ME et al. Molecular plasticity of vascular wall during N(G)-nitro-L-arginine methyl ester-induced hypertension: modulation of proinflammatory signals. Hypertension. 2000;36:103-109.
30. Tomita H, Egashira K, Kubo-Inoue M et al. Inhibition of NO synthesis induces inflammatory changes and monocyte chemoattractant protein-1 expression in rat hearts and vessels. Arterioscler Thromb Vasc Biol. 1998;18:1456-1464.
31. Daemen MJ, De Mey JG. Regional heterogeneity of arterial structural changes. Hypertension. 1995;25:464-473.
32. Matrougui K, Eskildsen-Helmond YE, Fiebeler A et al. Angiotensin II stimulates extracellular signal-regulated kinase activity in intact pressurized rat mesenteric resistance arteries. Hypertension. 2000;36:617-621.

33. Touyz RM, He G, Deng LY et al. Role of extracellular signal-regulated kinases in angiotensin II-stimulated contraction of smooth muscle cells from human resistance arteries. Circulation. 1999;99:392-399.
34. Allen SP, Wade SS, Prewitt RL. Myogenic tone attenuates pressure-induced gene expression in isolated small arteries. Hypertension. 1997;30:203-208.
35. Bartunek J, Weinberg EO, Tajima M et al. Chronic N(G)-nitro-L-arginine methyl ester-induced hypertension: novel molecular adaptation to systolic load in absence of hypertrophy. Circulation. 2000;101:423-429.
36. Buxton IL, Cheek DJ, Eckman D et al. NG-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. Circ Res. 1993;72:387-395.
37. Rayford W, Noble MJ, Austenfeld MA et al. Muscarinic cholinergic receptors promote growth of human prostate cancer cells. Prostate. 1997;30:160-166.
38. Guizzetti M, Costa P, Peters J et al. Acetylcholine as a mitogen: muscarinic receptor-mediated proliferation of rat astrocytes and human astrocytoma cells. Eur J Pharmacol. 1996;297:265-273.

CHAPTER 2

Carotid arterial stiffness is marginally increased in the metabolic syndrome and markedly increased in type 2 diabetes mellitus in patients with manifestations of arterial disease

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Submitted

Condensed abstract

We performed a cross-sectional study to determine whether the metabolic syndrome and type 2 diabetes mellitus are associated with carotid artery stiffness in 2105 patients with manifestations of arterial disease.
We conclude that (increasing number of components of) the metabolic syndrome is associated with marginally increased carotid artery stiffness, while type 2 diabetes is associated with a marked increase in carotid artery stiffness, in patients with already manifestations of arterial disease.

Abstract

Introduction Insulin resistance is generally considered to be of major importance in the pathophysiology of the metabolic syndrome and type 2 diabetes mellitus, both high-risk conditions for cardiovascular complications. Carotid artery stiffness is increasingly recognized as an important predictor of cardiovascular morbidity and mortality. Therefore in the present study we determined whether the metabolic syndrome (MetSyn) and type 2 diabetes mellitus (DM2) are associated with carotid artery stiffness in patients with already clinical manifestations of arterial disease.

Methods and Results A cross-sectional study in 2105 patients with manifest arterial disease (cerebral, coronary or peripheral artery disease, renal artery stenosis or an aneurysm of the abdominal aorta) was performed. The difference in carotid stiffness between patients with and without MetSyn and with and without DM2 was studied with linear regression analysis.
Compared to patients without DM2 (N=1112), patients with DM2 (N=301) had increased arterial stiffness. Generally, carotid stiffness did not differ as much between patients with (N=922) or without (N=1112) MetSyn. Excluding the patients with also DM2 (N=230) from the MetSyn-group diminished this relation even more. Furthermore, in the population as a whole, carotid artery stiffness increases with increasing number of components of the metabolic syndrome. In addition, this association is not as clear after exclusion of the patients with DM2 from the MetSyn-group. From all the components of the MetSyn only high blood pressure and high fasting glucose influenced carotid artery stiffness.

Conclusions Increasing number of components of the metabolic syndrome is associated with marginally increased carotid artery stiffness, while type 2 diabetes is associated with largely increased carotid artery stiffness, in patients with already manifestations of arterial diseases.

Keywords carotid artery stiffness, diabetes mellitus, metabolic syndrome.

Introduction

The metabolic syndrome (MetSyn), which involves a cluster of cardiovascular risk factors including hypertension, obesity, glucose intolerance, endothelial dysfunction, dyslipidemia and a proinflammatory state, is associated with an increased risk for cardiovascular complications.[1-3] The MetSyn is associated with advanced vascular damage in patients with coronary heart disease, cardiovascular events after a myocardial infarction,[4] stroke, peripheral arterial disease or abdominal aortic aneurysm[5] and untreated essential hypertension.[6] As insulin resistance is the major underlying pathophysiological mechanism, the MetSyn often precedes the development of DM2. The chance of developing DM2 is 10-fold in patients with impaired fasting glucose.[7] The presence of the metabolic syndrome increased the risk for the development of diabetes mellitus almost 2-fold in American Indians[8] and in Finnish men a 4-fold increase was shown.[9]

Carotid artery stiffness is increasingly recognized as an important predictor of cardiovascular morbidity and mortality. While progress in non-invasive ultrasonography has enabled reliable measurement of carotid intima media-thickness as a strong surrogate of coronary atherosclerosis,[10] additional functional measurements of carotid arteries are providing information on viscoelastic properties, such as arterial stiffness.[11] Increased arterial stiffness is considered an independent risk factor for cardiovascular disease[12,13] and stroke[14,15]. Although elasticity of central arteries is influenced by the aging process per se,[16] hemodynamic forces and other cardiovascular risk factors associated with insulin resistance are also important.[17,18] Previous studies already showed increasing arterial stiffness with deteriorating glucose tolerance status[19,20] and with increasing number of risk factors related to the MetSyn in healthy volunteers.[21,22] Studying the presence of carotid artery stiffness may provide further insight in cardiovascular risk in patients with MetSyn compared to DM2 patients, in a group of patients already at high risk for developing cardiovascular diseases.

Therefore in the present study we determined whether the MetSyn and DM2 are associated with carotid artery stiffness in patients with already clinical manifestations of arterial disease.

Subjects and methods

Study population

We used data from patients enrolled in the SMART study (Second Manifestations of ARTerial disease). The SMART study is an ongoing prospective single-center cohort study in patients with manifest arterial disease or cardiovascular risk factors (cerebral, coronary or peripheral artery disease, renal artery stenosis or an aneurysm of the abdominal aorta). Starting in September 1996, consecutive patients aged 18 to 80 years, referred to the University Medical Center Utrecht (UMCU) with manifest arterial disease or a cardiovascular risk factor underwent a vascular screening including a questionnaire, blood chemistry and ultrasonography. Written informed consent was obtained from all participants. The study was approved by the medical ethics committee of the University Medical Center Utrecht. The rationale and design of the SMART-study have been described in detail elsewhere.[23]

Carotid stiffness assessment was part of the vascular screening until august 2003 resulting in a study population of 2105 patients. In 2055 of these patients, all 5 elements of the metabolic syndrome were known.

Vascular screening

Vascular screening was conducted on a single day at the University Medical Center Utrecht (UMCU). Blood samples were collected after an overnight fast. Glucose, total cholesterol, triglycerides and HDL-cholesterol were measured by standard enzymatical laboratory methods (Vitros 250; Johnson/Johnson). LDL-cholesterol was calculated by use of Friedewald's formula. Height and weight were measured without shoes and heavy clothing. Blood pressure was measured in supine position at the right brachial artery every 4 minutes during the arterial stiffness measurement with a semiautomatic oscillometric device (Omega 1400, Invivo Research Laboratories Inc., Broken Arrow, OK, USA). Use of current medication was available.[23] Common carotid intima-media thickness (CIMT) was measured at the left and right common carotid arteries with an ATL Ultramark 9 (Advanced Technology Laboratories, Bethel, WA, USA) equipped with a 10-MHz linear array transducer as previously described elsewhere.[23] The mean CIMT was calculated in each patient.

Carotid artery stiffness

Stiffness was assessed by measurement of distension of the left and right common carotid arteries. The distension of an artery is the change in diameter in systole relative to the diastolic diameter during the cardiac cycle. The displacement of the walls of

the left and right common carotid artery was measured with a Wall Track System (Scanner 200, Pie Medical, Maastricht, The Netherlands) equipped with a 7.5 MHz linear array transducer and vessel wall moving detector system. After a rest of at least 5 minutes in supine position, patients were examined in supine position with the head turned approximately 45 degrees away from the side examined. The left and right carotid arteries were examined separately. Measurements were performed in the distal common carotid artery 2 cm proximal to the origin of the carotid bulb as described elsewhere.[24] In short, at the right carotid artery 5 measurements were performed. Each assessment lasted 4 seconds and comprised several cardiac cycles. First, the distension of the cardiac cycles within a single measurement was averaged. Next, the results of the five assessments were averaged. A similar procedure was used for the left carotid artery. The mean of the left and right carotid artery measurements was taken as distension measurement for one individual. The same procedure was followed for lumen diameter measurements. An intra-observer variability study on distension and end-diastolic lumen diameter measurements showed a coefficient of variation of 6.2% and 2.1% respectively. Between observers, the coefficient was 7.3% and 3.5%, respectively.[24]

Adjusted carotid distension was the primary stiffness measure,[11,25] using blood pressure simultaneously measured at the brachial artery at 4-minute intervals and adjusting all models for the end-diastolic diameter of the common carotid artery. In addition, traditional indexes of arterial stiffness were used for comparison. β stiffness index was determined as ln(SBP/DBP)/(ΔD/Dd) with SBP indicating systolic blood pressure, DPB indicating diastolic blood pressure, ΔD indicating the carotid distension and Dd the carotid end-diastolic diameter. Distensibility coefficient (DC) in 10^{-3}*kPa^{-1} was (2*ΔD/Dd)/PP. Cross-sectional compliance coefficient (CC) in mm^2*kPa-1 was given as (π*Dd*ΔD)/(2*PP) with PP indicating pulse pressure (SBP – DBP). Petersons modulus (E_p) in kPa *10^2 was defined as (PP*Dd)/ΔD. Young's elastic modulus (YEM) in kPa *10^3 was (PP*Dd2)/(ΔD*2*IMT). Increasing distension, DC and CC imply decreasing stiffness. Distensibility is the relative change in diameter and compliance is the absolute change in diameter with pressure. Peterson's (elastic) modulus is the pressure change required for (theoretic) 100% increase in diameter, and Young's modulus is pressure per square millimeter required for (theoretic) 100% extension.[26] If more pressure change is required it implies increasing stiffness.

Metabolic syndrome

The MetSyn was diagnosed according to the Adult Treatment Panel III criteria, including three or more of the following metabolic abnormalities[27]:

1) Abdominal obesity (waist circumference > 102 cm in men and > 88 cm in women).
2) High blood pressure (≥ 130 mmHg systolic or ≥ 85 mmHg diastolic); patients on anti-hypertensive medication were regarded as having high blood pressure.
3) Hypertriglyceridemia (serum triglycerides ≥ 1.70 mmol/l).
4) Low serum HDL cholesterol (< 1.04 mmol/l in men and < 1.29 mmol/l in women).
5) High fasting serum glucose (≥ 6.1 mmol/l).

Diabetes mellitus

Patients having a fasting glucose ≥ 7.0 mmol/l and/or use of glucose-lowering medication or insulin were defined as having DM2.

Data analysis

Baseline characteristics were determined separately for patients with diabetes mellitus (DM2), patients with the metabolic syndrome (MetSyn), patients with both DM2 and MetSyn and patients with neither DM2 nor MetSyn. The relation of MetSyn and DM2 with the separate parameters of arterial stiffness was studied with linear regression analysis, using separate models. First, adjustments were made for age and sex. Additional adjustments were performed for mean arterial pressure (MAP: (2*DBP + SBP)/3). MetSyn and DM2 were entered separately and patients without, respectively, MetSyn and DM2 served as reference groups. Because the categories of DM2 and MetSyn are not mutually exclusive, both DM2 and MetSyn were adjusted for age, sex and mean arterial pressure. Patients without DM2 and MetSyn served as reference group. Our primary stiffness parameter was carotid distension, adjusted for blood pressure and end-diastolic diameter in the model. Accordingly, adjustments were made for carotid end-diastolic diameter in all models with carotid distension as dependent variable. To additionally study the relation of MetSyn and carotid stiffness independent of DM2, linear regression models were performed, excluding patients with DM2.

Carotid stiffness in relation to the number of components of the metabolic syndrome was studied by calculating the mean adjusted carotid stiffness, using analysis of covariance (ANCOVA, general linear model procedure). Adjustments were made for age, sex and mean arterial pressure. This was repeated after exclusion of patients with DM2.

Finally, the relation of the separate components of the metabolic syndrome with carotid distension and the different carotid stiffness indexes was studied using linear regression analysis. The relation was studied univariately and was additionally adjusted for age, sex and mean arterial pressure. The relation between high blood pressure and carotid stiffness was studied without adjustment for mean arterial pressure.

Results

Table 1. Baseline characteristics (N=2105)

	MetSyn (N=922) (230 with DM2)	DM2 (N=301) (230 with MetSyn)	DM2 and MetSyn (N=230)	No DM2 or MetSyn (N=1112)
Male gender (%)	71	69	67	79
Age (years)	60 ± 10	61 ± 10	61 ± 10	60 ± 11
BMI (kg/m^{-2})	28 ± 4	27 ± 4	28 ± 10	25 ± 3
Ever smoking (%)	82	77	76	84
Total cholesterol (mmol/l)	5.6 ± 1.3	5.4 ± 1.4	5.2 ± 1.2	5.4 ± 1.1
LDL cholesterol (mmol/l)	3.5 ± 1.0	3.2 ± 1.0	3.2 ± 1.1	3.4 ± 1.0
Creatinin (μmol/l)	77 ± 24	77 ± 25	78 ± 28	74 ± 20
Cockroft creatinin clearance (μmol/l)	77 ± 24	77 ± 25	78 ± 28	74 ± 20
Albuminuria (%)	18	24	25	11
Homocysteine (Ìmol/l)	15 ± 7	15± 8	15 ± 8	15 ± 8
Cerebral vascular disease (%)	29	37	37	28
Coronary disease (%)	53	53	53	55
Abdominal aneurysm (%)	12	7	7	11
Periferal vascular disease (%)	32	33	33	24
Ras-inhibitors (%)	25	35	40	15
Thrombocyte-aggregation inhibitors (%)	60	62	63	64
Calcium-antagonists	25	24	27	20
Diuretics (%)	18	22	24	9
β-blockers (%)	47	41	46	38
Statins (%)	41	47	47	40
Nitrates (%)	18	17	18	20
CCA characteristics:				
Distension (Ìm)	423 ± 151	408 ± 145	409 ± 142	429 ± 143
End diastolic diameter (Ìm)	8192 ± 1114	8248 ± 987	8272 ± 1004	7888 ± 1097
β index	12.9 ± 6.3	14.2 ± 6.9	14.1 ± 6.8	11.7 ± 6.4
DC (10^{-3}*kPa^{-1})	12.9 ± 5.7	11.8 ± 5.5	11.6 ± 5.2	15.1 ± 6.7
CC (mm^2*kPa^{-1})	0.66 ± 0.29	0.62 ± 0.27	0.61 ± 0.26	0.71 ± 0.29
Peterson's modulus (kPa *10^2)	1.90 ± 0.99	2.09 ± 1.08	2.08 ± 1.02	1.67 ± 1.02
YEM (kPa *10^3)	0.83 ± 0.44	0.88 ± 0.48	0.90 ± 0.49	0.74 ± 0.46
Components of the MetSyn:				
Waist circumference (cm)	101 ± 10	99 ± 11	101 ± 11	92 ± 9
Systolic blood pressure (mmHg)	145 ± 19	147 ± 21	148 ± 20	138 ± 20
Diastolic blood pressure (mmHg)	80 ± 10	80 ± 10	80 ± 10	79 ± 10
HDL cholesterol (mmol/l)	1.02 ± 0.27	1.11 ± 0.33	1.02 ± 0.28	1.30 ± 0.34
Triglycerides (mmol/l)	2.6 ± 2.2	2.1 ± 1.2	2.4 ± 1.2	1.5 ± 0.9
Fasting serum glucose (mmol/l)	7.2 ± 2.6	9.6 ± 3.4	9.7 ± 3.5	5.6 ± 0.7

Values are mean ± SD;
CCA: common carotid artery; β index: β stiffness index; DC: distensibility coefficient; CC: compliance coefficient; YEM: Young's elastic modulus; The groups are not 'mutually exclusive'

Anthropometric, hemodynamic and metabolic baseline parameters

The anthropometric, hemodynamic, and metabolic baseline parameters of the study population (N=2105) are shown in Table 1. All patients had a recent manifestation of arterial disease and were classified according to have the MetSyn (N=922 of whom 230 also with DM2), DM2 (N=301 of whom 230 also have the MetSyn), or patients with both DM2 and MetSyn (N=230), and patients with neither DM2 nor MetSyn (N=1112).

Differences in mean carotid artery stiffness in patients with and without MetSyn and DM2

In Table 2 the difference in carotid artery stiffness between patients with and without MetSyn, and patients with and without DM2 is given. A statistical significant positive relation was observed between carotid artery stiffness and the MetSyn.

Table 2. Differences in mean carotid artery stiffness in patients with and without MetSyn and DM2

MetSyn (N=922):	**MetSyn vs. without MetSyn** adjusted for age and sex	**MetSyn vs. without MetSyn** additionally adjusted for mean arterial pressure	**both MetSynand DM2 vs. no MetSyn or DM2** adjusted for age, sex and mean arterial pressure
Distension (μm)	-9.6 (-21.5;2.3)	-6.2 (-18.0;5.7)	-3.2 (-15.4;9.0)
β index	0.9 (0.4;1.4)	0.6 (0.1;1.1)	0.3 (-0.2;0.8)
DC (10^{-3}*kPa^{-1})	-1.8 (-2.2;-1.4)*	-1.1 (-1.5;-0.7)*	-0.8 (-1.2;-0.4)*
CC (mm^2*kPa^{-1})	-0.02 (-0.05;-0.001)*	0.001 (-0.020;0.023)	0.01 (-0.01;0.03)
Peterson's (kPa *10^2)	0.2 (0.1;0.3)*	0.06 (-0.01;0.13)	0.02 (-0.05;0.1)
YEM (kPa *10^3)	0.08 (0.04;0.12)*	0.03 (-0.004;0.07)	0.02 (-0.02;0.06)
DM2 (N=301):	**DM2 vs. without DM2** adjusted for age and sex	**DM2 vs. without DM2** additionally adjusted for mean arterial pressure	**both MetSyn and DM2 vs. no MetSyn or DM2** adjusted for age, sex and mean arterial pressure
Distension (μm)	-18.5 (-35.1;-1.9)*	-17.3 (-33.8;-0.9)*	-16.2 (-33.2;0.8)
β index	1.7 (1.0;2.4)*	1.6 (0.9;2.2)*	1.4 (0.7;2.1)*
DC (10^{-3}*kPa^{-1})	-2.0 (-2.6;-1.4)*	-1.6 (-2.2;-1.1)*	-1.3 (-1.9;-0.8)*
CC (mm^2*kPa^{-1})	-0.05 (-0.09;-0.02)*	-0.04 (-0.07;-0.01)*	-0.05 (-0.08;-0.01)*
Peterson's (kPa *10^2)	0.3 (0.2;0.4)*	0.2 (0.1;0.3)*	0.2 (0.1;0.3)*
YEM (kPa *10^3)	0.09 (0.04;0.15)*	0.07 (0,02;0.12)*	0.06 (0.01;0.11)*

Values are mean differences with 95% CI

* $p < 0.05$

However this association generally disappeared after adjustment for mean arterial pressure. Patients with DM2 had stiffer arteries than non-diabetic patients, irrespective of the measured stiffness parameter. Adjustment for mean arterial pressure did not materially affect this association. Furthermore, excluding DM2 patients from the MetSyn-group, weakened the observed positive relation of carotid artery stiffness (Table 3).

Table 3. Differences in mean carotid artery stiffness in patients with and without MetSyn, after excluding DM2

MetSyn (N=692):	**MetSyn vs. without MetSyn** adjusted for age and sex	**MetSyn vs. without MetSyn** additionally adjusted for mean arterial pressure
Distension (μm)	-5.7 (-18.8;7.4)	-2.7 (-15.6;10.3)
β index	0.3 (-0.3;0.8)	0.1 (0.4;0.6)*
DC (10^{-3}*kPa^{-1})	-1.1 (-1.6;-0.6)*	-0.6 (-1.1;-0.2)*
CC (mm^2*kPa^{-1})	-0.04 (-0.07;-0.02)*	-0.02 (-0.04;-0.001)
Peterson's (kPa *10^2)	0.06 (-0.02;0.14)	0.00 (-0.08;0.08)
YEM (kPa *10^3)	0.02 (-0.02;0.06)	-0.01 (-0.04;0.03)

Values are mean differences with 95% CI

* p <0.05

Association between carotid artery stiffness and the number of components of the MetSyn

In Table 4 is shown that increase in the number of components of the metabolic syndrome did increase carotid artery stiffness. Patients who had all five criteria constituting the MetSyn had significantly the highest β index (range 429-408), and Peterson's index (range 1.71-1.84). The DC decreased significantly with increasing number of components of the MetSyn (range 15.8-13.0). In addition, this association is not as clear after excluding patients with DM2 from the MetSyn-group. (Table 5).

Table 4. Relation between carotid artery stiffness and the number of MetSyn components

MetSyn	Adjusted mean (range)					
components (N)	Distension (μm)	β index	DC (10^{-3}*kPa^{-1})	CC (mm^2*kPa^{-1})	Peterson's (kPa *10^2)	YEM (kPa *10^3)
0	429 (404-454)	11.4 (10.4-12.4)	15.8 (15.0-16.6)	0.68 (0.64-0.73)	1.71 (1.56-1.86)	0.76 (0.68-0.83)
1	434 (422-447)	12.0 (11.5-12.5)	14.7 (14.3-15.2)	0.70 (0.67-0.72)	1.74 (1.67-1.82)	0.79 (0.73-0.80)
2	423 (412-434)	12.3 (11.9-12.8)	14.0 (13.6-14.4)	0.68 (0.66-0.70)	1.79 (1.73-1.86)	0.77 (0.74-0.81)
3	428 (416-440)	12.4 (11.9-12.9)	13.7 (13.3-14.1)	0.69 (0.67-0.71)	1.78 (1.71-1.86)	0.78 (0.75-0.82)
4	418 (403-433)	12.9 (12.3-13.5)	13.1 (12.6-13.6)	0.68 (0.65-0.71)	1.87 (1.78-1.96)	0.83 (0.78-0.87)
5	408 (383-433)	12.8 (11.7-13.8)	13.0 (12.1-13.8)	0.69 (0.64-0.74)	1.84 (1.68-1.98)	0.77 (0.69-0.84)
p-trend	0.08	0.005	<0.001	0.77	0.04	0.11

Values are means (range)

Mean values are adjusted for age, sex, and mean arterial pressure

Table 5. Relation between carotid artery stiffness and the number of MetSyn components, after excluding DM2 patients

MetSyn	Adjusted mean (range)					
components (N)	Distension (μm)	β index	DC (10^{-3}*kPa^{-1})	CC (mm^2*kPa^{-1})	Peterson's (kPa *10^2)	YEM (kPa *10^3)
0	430 (405-455)	11.3 (10.3-12.3)	15.9 (15.1-16.7)	0.69 (0.64-0.74)	1.69 (1.54-1.84)	0.75 (0.67-0.82)
1	435 (423-448)	11.9 (11.4-12.4)	14.9 (14.4-15.3)	0.70 (0.68-0.73)	1.72 (1.65-1.80)	0.76 (0.72-0.80)
2	425 (413-437)	12.0 (11.5-12.5)	14.3 (13.9-14.7)	0.69 (0.66-0.71)	1.73 (1.66-1.80)	0.76 (0.73-0.79)
3	431 (418-444)	12.2 (11.6-12.7)	14.0 (13.6-14.4)	0.70 (0.67-0.72)	1.75 (1.67-1.83)	0.76 (0.73-0.80)
4	425 (407-443)	12.4 (11.7-13.1)	13.7 (13.2-14.3)	0.70 (0.67-0.74)	1.79 (1.68-1.90)	0.80 (0.75-0.85)
5	405 (372-438)	12.5 (11.2-13.8)	13.3 (12.2-14.4)	0.71 (0.65-0.78)	1.79 (1.59-1.99)	0.77 (0.67-0.86)
p-trend	0.24	0.06	<0.001	0.64	0.20	0.29

Values are means (range)

Mean values are adjusted for age, sex, and mean arterial pressure

Difference in mean carotid artery stiffness between absence and presence of separate components of the metabolic syndrome

Studying the difference in mean carotid artery stiffness between absence and presence of separate components of the MetSyn showed that only high blood pressure and high fasting glucose were associated with increased carotid artery stiffness. Abdominal obesity, hypertriglyceridemia, and low serum HDL cholesterol did not influence the carotid artery stiffness significantly.

Table 6. Difference in mean carotid artery stiffness between absence and presence of separate components of the metabolic syndrome

MetSyn components:	Abdominal obesity	High blood pressure	Hyper-triglyceridemia	Low serum HDL cholesterol	High fasting glucose
Distension (μm)	-6.0(-20.1;8.1)	-21.1(-36.0;-6.1)*	-1.8(-14.5;10.9)	3.7(-9.0;16.4)	-15.8(-28.7;-2.9)*
adjusted for age, sex and mean arterial pressure	-0.3(-13.7;13.2)	7.2(-7.3;21.7)	-11.2(-23.1;0.8)	-4.6(-16.5;7.4)	-2.8(-15.0;9.4)
β index	0.25(-0.37;0.87)	3.43(2.80;4.06)*	-0.10(-0.65;0.45)	0.05(-0.50;0.61)	1.65(1.09;2.21)*
adjusted for age, sex and mean arterial pressure	-0.08(-0.62;0.47)	1.83(1.25;2.40)*	0.42(-0.06;0.90)	0.57(0.10;1.05)*	0.78(0.29;1.26)*
DC (10^{-3}*kPa^{-1})	-0.71(-1.32;-0.10)*	-6.35(-6.94;-5.77)*	-0.34(-0.89;0.21)	-0.10(-0.45;0.65)	-2.25(-2.80;-1.70)*
adjusted for age, sex and mean arterial pressure	-0.22(-0.65;0.21)	-4.52(-5.01;-4.02)*	-0.83(-1.21;-0.45)*	-0.61(-0.99;-0.23)*	-0.99(-1.38;-0.61)*
CC (mm^2*kPa^{-1})	0.01(-0.03;0.10)	-0.17(-0.20;-0.14)*	0.00(-0.02;0.02)	0.01(-0.02;0.04)	-0.05(-0.07;-0.02)*
adjusted for age, sex and mean arterial pressure	0.02(0.0;0.05)	-0.11(-0.14;-0.09)*	-0.01(-0.03;0.01)	-0.01(-0.03;0.02)	-0.01(-0.03;0.01)
Peterson's (kPa *10^2)	0.05(-0.05;0.15)	0.74(0.64;0.84)*	-0.01(-0.10;0.08)	-0.02(-0.10;0.07)	0.27(0.18;0.36)*
adjusted for age, sex and mean arterial pressure	-0.02(-0.1;0.06)	0.50(0.41;0.59)*	0.05(-0.02;0.12)	0.0890.01;0.15)*	0.10(0.03;0.17)*
YEM (kPa *10^3)	0.00(-0.04;0.05)	0.28(0.24;0.33)*	0.01(-0.03;0.05)	0.01(-0.03;0.05)	0.09(0.05;0.13)*
adjusted for age, sex and mean arterial pressure	-0.02(-0.06;0.02)	0.21(0.16;0.25)*	0.03(-0.01;0.06)	0.04(0.08;0.10)*	0.03(-0.01;0.06)

Values are mean differences with 95% CI

* p<0.05

The relation was studied univariately in a first model and was additionally adjusted for age, sex and mean arterial pressure; the relation of high blood pressure and carotid stiffness was studied without adjustment for mean arterial pressure

Abdominal obesity (waist circumference > 102 cm in men and > 88 cm in women); High blood pressure (≥ 130 mmHg systolic or ≥ 85 mmHg diastolic); Hypertriglyceridemia (serum triglycerides ≥ 1.70 mmol/l); Low serum HDL cholesterol (< 1.04 mmol/l in men and < 1.29 mmol/l in women); High fasting serum glucose (≥ 6.1 mmol/l)

Discussion

The main result of the present study is that (increasing number of components of) the metabolic syndrome is associated with marginally increased carotid artery stiffness, while type 2 diabetes is associated with a marked increase in carotid artery stiffness, in patients with already manifestations of arterial disease. High blood pressure and high fasting glucose are the only components of the metabolic syndrome that were associated with increased carotid artery stiffness.

The increasing prevalence of clustered risk factors in association with central obesity is gaining increased attention worldwide. In line with several other studies in different high-risk populations,[2;28;29] the prevalence of the MetSyn in our study population is nearly 50%. In healthy populations, the presence of the MetSyn is associated with a nearly 3-fold increase in cardiovascular related mortality compared to subjects without the MetSyn,[30] this syndrome calls for a systemic approach to identification and treatment. The individual components that make up the syndrome could be treated separately or with awareness of insulin resistance as the underlying disorder. Patients with MetSyn are also at risk for the development of vascular abnormalities that range from endothelial dysfunction, followed by artery stiffness, to evident atherosclerosis.[31] All separate traditional components of the MetSyn (hyperglycemia, hypertension, low plasma HDL cholesterol, hypertriglyceridemia, and central obesity) are cardiovascular risk factors and are known to cause endothelial dysfunction.[32-34] In DM2 the elevated glucose-levels may add to the development of vascular damage.[35] In MetSyn the glucose-levels are lower and therefore the increased cardiovascular risk is most likely due to metabolic changes associated with insulin resistance causing impaired vasoreactivity.[36] Endothelial dysfunction is a preliminary state causing remodeling of the vessel wall resulting in increased vascular stiffness and decreased compliance/elasticity/distensibility. Decreased NO-bioavailability by reactive oxygen species (ROS) might be a major driving force for instability of atherosclerotic plaques in patients with the MetSyn.[37] Also low-grade inflammation induces endothelial dysfunction by elevated plasma levels of C-reactive protein (CRP), TNF-α, and IL-6.[38-40] Carotid artery stiffness is an indicator for the risk of cardiovascular disease[12,13] and stroke.[14,15] The results of the present study may conclude that the longer the exposure of insulin resistance to the vessel wall, the stronger the positive association with carotid artery stiffness. Compared to patients without DM2, patients with DM2 had increased arterial stiffness, while carotid stiffness did not differ as much between

patients with or without MetSyn. Furthermore, although in the population as a whole, carotid artery stiffness increases with increasing number of components of the metabolic syndrome, this association is not as clear after exclusion of the patients with DM2 from the MetSyn-group. High glucose plasma levels, seen in DM2 but not in MetSyn, and longer exposure of the vessel wall to other metabolic changes associated with insulin resistance may have contributed to increased arterial stiffness.[19,20] MetSyn patients are at high risk for developing type 2 diabetes. The clinical implication of this finding may be that progression of MetSyn to type 2 diabetes may lead to an increase in cardiovascular risk. Therefore, preventing MetSyn patients to develop type 2 diabetes can be an effective strategy to reduce cardiovascular risk.
Increased number of risk factors, as clustered in the MetSyn have been associated with arterial stiffness in healthy volunteers.[21,22] The present study confirms these results in patients with clinical manifestations of vascular disease. This adds to the concept of increased vascular risk in MetSyn patients, indicated by advanced vascular damage as measured by intima media thickness and ankle brachial pressure index, in a previous study in patients with manifest vascular disease.[5] However, supposed to be an independent predictor for cardiovascular disease[12,13] and stroke,[14,15], carotid artery stiffness was no independent risk factor for vascular events in the studied population of this present study.[41] This could explain the minimal correlation between carotid artery stiffness and MetSyn.

We acknowledge some limitations of our study. Firstly, there is not a single clinical definition for the clustering of risk factors associated with abdominal obesity. The MetSyn can be described by several definitions, which implies that it may be difficult to compare outcomes of different studies. Most commonly used, because more easy to measure in daily clinical practice, is the working definition suggested by the ATP III. Recently also the International Diabetes Federation (IDF) based their MetSyn definition on modified ATP III criteria.[42] Although insulin resistance seems to be a major underlying disorder, the pathophysiology of MetSyn is not completely clear yet. Secondly, this survey is a cross-sectional study so only assumptions about possible etiological relationships can be made without the possibility to draw conclusions in terms of causality. Furthermore, adjusted carotid distension, together with other traditional indexes of arterial stiffness (β index, DC, CC, Peterson's, and YEM), was used as stiffness measurements. Although the several different measures can make the interpretation difficult, it may also give an overview of carotid artery stiffness.

We conclude that (increasing number of components of) the metabolic syndrome is associated with marginally increased carotid artery stiffness, while type 2 diabetes is associated with a marked increase in carotid artery stiffness, in patients with already manifestations of arterial disease. Preventing MetSyn-patients to progress to DM2 may reduce the cardiovascular risk.

References

1. Hu G, Qiao Q, Tuomilehto J et al. Prevalence of the metabolic syndrome and its relation to all-cause and cardiovascular mortality in nondiabetic European men and women. Arch Intern Med. 2004;164:1066-1076.
2. Isomaa B, Almgren P, Tuomi T et al. Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care. 2001;24:683-689.
3. Watson KE, Peters Harmel AL, Matson G. Atherosclerosis in type 2 diabetes mellitus: the role of insulin resistance. J Cardiovasc Pharmacol Ther. 2003;8:253-260.
4. Levantesi G, Macchia A, Marfisi R et al. Metabolic syndrome and risk of cardiovascular events after myocardial infarction. J Am Coll Cardiol. 2005;46:277-283.
5. Olijhoek JK, van der Graaf Y, Banga JD et al. The metabolic syndrome is associated with advanced vascular damage in patients with coronary heart disease, stroke, peripheral arterial disease or abdominal aortic aneurysm. Eur Heart J. 2004;25:342-348.
6. Schillaci G, Pirro M, Vaudo G et al. Metabolic syndrome is associated with aortic stiffness in untreated essential hypertension. Hypertension. 2005;45:1078-1082.
7. de Vegt F, Dekker JM, Jager A et al. Relation of impaired fasting and postload glucose with incident type 2 diabetes in a Dutch population: The Hoorn Study. JAMA. 2001;285:2109-2113.
8. Resnick HE, Jones K, Ruotolo G et al. Insulin resistance, the metabolic syndrome, and risk of incident cardiovascular disease in nondiabetic american indians: the Strong Heart Study. Diabetes Care. 2003;26:861-867.
9. Laaksonen DE, Lakka HM, Niskanen LK et al. Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. Am J Epidemiol. 2002;156:1070-1077.
10. O'Leary DH, Polak JF. Intima-media thickness: a tool for atherosclerosis imaging and event prediction. Am J Cardiol. 2002;90:18L-21L.
11. Liao D, Arnett DK, Tyroler HA et al. Arterial stiffness and the development of hypertension. The ARIC study. Hypertension. 1999;34:201-206.
12. Arnett DK, Evans GW, Riley WA. Arterial stiffness: a new cardiovascular risk factor? Am J Epidemiol. 1994;140:669-682.
13. Gatzka CD, Cameron JD, Kingwell BA et al. Relation between coronary artery disease, aortic stiffness, and left ventricular structure in a population sample. Hypertension. 1998;32:575-578.
14. Dijk JM, van der Graaf Y, Grobbee DE et al. Increased arterial stiffness is independently related to cerebrovascular disease and aneurysms of the abdominal aorta: the Second Manifestations of Arterial Disease (SMART) Study. Stroke. 2004;35:1642-1646.
15. Dijk JM, van der Graaf Y, Grobbee DE et al. Carotid stiffness indicates risk of ischemic stroke and TIA in patients with internal carotid artery stenosis: the SMART study. Stroke. 2004;35:2258-2262.

16. Avolio AP, Deng FQ, Li WQ et al. Effects of aging on arterial distensibility in populations with high and low prevalence of hypertension: comparison between urban and rural communities in China. Circulation. 1985;71:202-210.
17. DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care. 1991;14:173-194.
18. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes. 1988;37:1595-1607.
19. Henry RM, Kostense PJ, Spijkerman AM et al. Arterial stiffness increases with deteriorating glucose tolerance status: the Hoorn Study. Circulation. 2003;107:2089-2095.
20. Salomaa V, Riley W, Kark JD et al. Non-insulin-dependent diabetes mellitus and fasting glucose and insulin concentrations are associated with arterial stiffness indexes. The ARIC Study. Atherosclerosis Risk in Communities Study. Circulation. 1995;91:1432-1443.
21. Scuteri A, Najjar SS, Muller DC et al. Metabolic syndrome amplifies the age-associated increases in vascular thickness and stiffness. J Am Coll Cardiol. 2004;43:1388-1395.
22. Urbina EM, Srinivasan SR, Kieltyka RL et al. Correlates of carotid artery stiffness in young adults: The Bogalusa Heart Study. Atherosclerosis. 2004;176:157-164.
23. Simons PC, Algra A, van de Laak MF et al. Second manifestations of ARTerial disease (SMART) study: rationale and design. Eur J Epidemiol. 1999;15:773-781.
24. Simons PC, Algra A, Bots ML et al. Common carotid intima-media thickness and arterial stiffness: indicators of cardiovascular risk in high-risk patients. The SMART Study (Second Manifestations of ARTerial disease). Circulation. 1999;100:951-957.
25. Stork S, van den Beld AW, von Schacky C et al. Carotid artery plaque burden, stiffness, and mortality risk in elderly men: a prospective, population-based cohort study. Circulation. 2004;110:344-348.
26. O'Rourke MF, Staessen JA, Vlachopoulos C et al. Clinical applications of arterial stiffness; definitions and reference values. Am J Hypertens. 2002;15:426-444.
27. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA. 2001;285:2486-2497.
28. Meigs JB. Epidemiology of the metabolic syndrome, 2002. Am J Manag Care. 2002;8:S283-S292.
29. Rantala AO, Kauma H, Lilja M et al. Prevalence of the metabolic syndrome in drug-treated hypertensive patients and control subjects. J Intern Med. 1999;245:163-174.
30. Lakka HM, Laaksonen DE, Lakka TA et al. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA. 2002;288:2709-2716.
31. Caballero AE. Metabolic and vascular abnormalities in subjects at risk for type 2 diabetes: the early start of a dangerous situation. Arch Med Res. 2005;36:241-249.
32. Baron AD. Vascular reactivity. Am J Cardiol. 1999;84:25J-27J.

33. Steinberg HO, Baron AD. Vascular function, insulin resistance and fatty acids. Diabetologia. 2002;45:623-634.
34. Wheatcroft SB, Williams IL, Shah AM et al. Pathophysiological implications of insulin resistance on vascular endothelial function. Diabet Med. 2003;20:255-268.
35. McVeigh GE, Brennan GM, Johnston GD et al. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia. 1992;35:771-776.
36. Haffner SM, Stern MP, Hazuda HP et al. Cardiovascular risk factors in confirmed prediabetic individuals. Does the clock for coronary heart disease start ticking before the onset of clinical diabetes? JAMA. 1990;263:2893-2898.
37. Standl E, Schnell O. A new look at the heart in diabetes mellitus: from ailing to failing. Diabetologia. 2000;43:1455-1469.
38. Fichtlscherer S, Rosenberger G, Walter DH et al. Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. Circulation. 2000;102:1000-1006.
39. Yudkin JS, Kumari M, Humphries SE et al. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis. 2000;148:209-214.
40. Chia S, Qadan M, Newton R et al. Intra-Arterial Tumor Necrosis Factor-{alpha} Impairs Endothelium-Dependent Vasodilatation and Stimulates Local Tissue Plasminogen Activator Release in Humans. Arterioscler Thromb Vasc Biol. 2003;23:695-701.
41. Dijk JM, Algra A, van der Graaf Y et al. Carotid stiffness and the risk of new vascular events in patients with manifest cardiovascular disease. The SMART study. Eur Heart J. 2005;26:1213-1220.
42. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet. 2005;365:1415-1428.

CHAPTER 3

Metabolic and additional vascular effects of Thiazolidinediones

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Introduction

Cardiovascular disease is the number one cause of morbidity and mortality in the Western world. Several risk factors for the development of cardiovascular disease have been identified. Some of these risk factors (dyslipidaemia, hypertension, glucose intolerance, hyperinsulinaemia, obesity, low-grade inflammation, endothelial dysfunction and hypercoagulability) have been found to cluster and often precede clinically manifest type 2 (noninsulin–dependent) diabetes mellitus. Insulin resistance is generally regarded as an important feature of this cluster of risk factors and therefore the term 'insulin resistance syndrome' has been coined.[1] 'Cardiovascular dysmetabolic syndrome',[2] or 'syndrome X'[3] are other terms which have been used to describe this metabolic state.

The prevalence of type 2 diabetes has soared in the past decades because of changing lifestyles and eating habits. Obesity associated with insulin resistance is one of the main determinants of the increase in occurrence of type 2 diabetes. Not surprisingly, the major long-term complications of type 2 diabetes are an increased risk of myocardial infarction, stroke and peripheral vascular disease. Although microvascular complications cause considerable morbidity in patients with type 2 diabetes, up to 80% of patients die from macrovascular pathology.[4]

Treatment of individual risk factors has been shown to reduce cardiovascular events in type 2 diabetes. Dysglycaemia does not appear to be the major determinant of cardiovascular disease in type 2 diabetes, a concept supported by observations in the UK Prospective Diabetes Study.[5] Therefore, targeting the underlying pathophysiological mechanisms of the insulin resistance syndrome may be a more logical and beneficial strategy for reduction of cardiovascular morbidity and mortality. Pharmacological modulation of the insulin resistance syndrome will not only improve glycaemic control, but may also have beneficial effects on inflammation, dyslipidaemia and possibly other components of the syndrome independently from improvements in glucose metabolism.

The discovery of nuclear peroxisome proliferator-activated receptors (PPARs) and subsequent insight into their role in several metabolic pathways was a major breakthrough in our understanding of pathophysiological mechanisms underlying the insulin resistance syndrome.[6]

Thiazolidinediones are clinically available agonists of the PPAR-γ subtype and constitute a new class of antihyperglycaemic agents. Activation of PPAR-γ not only improves insulin sensitivity but may also have additional beneficial vascular effects. The aim of this review is to focus on the potential role of thiazolidinediones in the pathophysiological mechanisms involved in vascular disease.

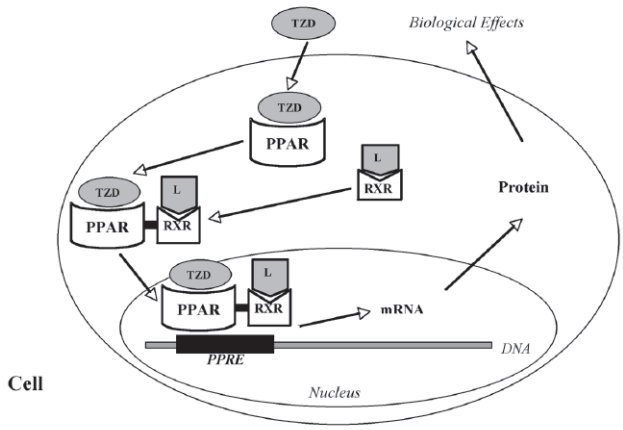
1. Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily, which include receptors for steroids, retinoid and thyroid hormones.[7-9] Once PPARs are activated by ligand binding, they form heterodimers with the ligand-activated retinoic acid receptor (RXR). Through its DNA binding domain, this heterodimer binds to specific DNA sequences, called PPAR-responsive elements (PPREs), and induces transcriptional activation of specific genes (figure 1).[10] PPARs function as regulators of glucose, lipid and protein metabolism, and influence cellular proliferation, differentiation and apoptosis. They also play a role in neoplastic proliferation and inflammatory diseases.[11]

Three subtypes of PPARs are known: PPAR-α, PPAR-γ and PPAR-δ. The tissue distribution of these subtypes varies considerably.[10-12] Whereas PPAR-δ is ubiquitously distributed, its function remains to be elucidated. PPAR-α is found in liver, intestine, kidney, heart, adipose tissue, skeletal muscle and recently in vascular cells.[13] PPAR-α has an important role in lipid metabolism. Its molecular targets include genes for enzymes that are important for the β-oxidation of fatty acids.[14] Synthetic ligands for this receptor subtype are fibric acid derivatives, which are used in clinical practice as lipid-lowering agents. PPAR-γ is found in adipose tissue, pancreas, skeletal muscle and vasculature.[10,12,13,15] High levels of expression are found in adipocytes. In addition, PPAR-γ is also expressed in macrophages, T cells, neutrophils, epithelial cells and smooth muscle cells.[16]

Figure 1. Mechanism of action of the peroxisome proliferator-activated receptors (PPARs)

L = ligand; PPRE = PPAR-responsive elements; RXR = retinoic acid receptor; TZD = thiazolidinediones

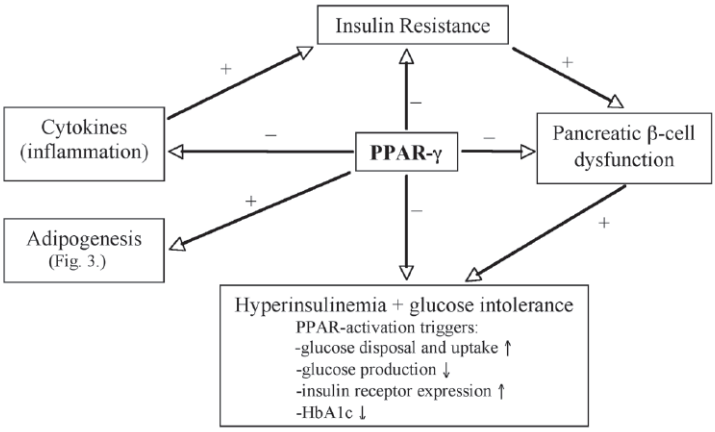


The most potent natural ligands are 13-hydroxyoctadecadienoic acid (HODE) and 15-deoxyΔ-prostaglandin J_2 (15d-PGJ2).[17] Thiazolidinediones are potent synthetic ligands for PPAR-γ activation.

2. Thiazolidinediones

Thiazolidinediones are a new class of drugs that act primarily by improving insulin sensitivity in different target tissues such as liver, skeletal muscle and adipose tissue. They have been shown to improve glycaemic control in patients with type 2 diabetes and appear to have favourable direct effects on other components of the insulin resistance syndrome because of the role of PPAR-γ in vascular physiology (figure 2).[18,19] Thiazolidinediones are chemically and functionally unrelated to other classes of oral antihyperglycaemic agents. Two compounds in this class are currently available for clinical use, namely, rosiglitazone, which was approved by the US Food and Drug Administration (FDA) in May 1999, and pioglitazone, which was approved in July 1999. Troglitazone, the first drug of this class, was marketed in the US from March 1997 until it was withdrawn in March 2000, when the FDA decided that the risk of hepatotoxicity associated with troglitazone therapy outweighed its potential benefits. The mode of action and magnitude of effects of different thiazolidinediones show some variation.[20-23] Pioglitazone may perhaps also have PPAR-α agonistic effects, which is of interest with regard to lipid-lowering effects similar to fibric acid derivatives.

Figure. 2. The central role of peroxisome proliferator-activated receptor (PPAR)-γ in vascular physiology
HbA1c = glycosylated haemoglobin



3. Thiazolidinediones and glycaemic control

Several thiazolidinediones have been shown to improve insulin sensitivity by increasing glucose disposal in skeletal muscle and decreasing hepatic glucose production. Thiazolidinediones increase glycogen synthase activity and glucose metabolism in skeletal muscle but also in adipocytes. They also decrease gluconeogenesis in cultured hepatocytes.[23-25] Stimulation of PPAR-γ normalises glucose uptake associated with glucose transporter 4 (GLUT4) expression and stimulates insulin receptor expression and activation.[26-32]
Improvement of glycaemic control by thiazolidinediones has been shown in different animal models of diabetes[33-36] and in patients with type 2 diabetes. Although troglitazone has been withdrawn from the market, it was very effective in lowering plasma glucose, insulin and glycosylated haemoglobin (HbA1c) levels in patients with type 2 diabetes.[37-40] Rosiglitazone resulted in significant reductions in fasting plasma glucose, HbA1c and insulin levels, and was more effective than troglitazone in maintaining low fasting plasma glucose levels in the long term.[41] Rosiglitazone also improved glycaemic control in patients with type 2 diabetes when administered in combination with metformin, sulphonylurea derivatives or insulin.[42,43] Similar significant decreases in fasting plasma glucose and HbA1c levels are achieved with pioglitazone in monotherapy;[44,45] furthermore, combination therapy with metformin, sulphonylurea derivatives or insulin, improves glycaemic control more than pioglitazone monotherapy does.[45] Unfortunately, studies comparing the individual effects of thiazolidinediones with metformin in glycaemic control have not been published yet. In addition, thiazolidinediones improve insulin sensitivity in nondiabetic insulin-resistant states, such as obese individuals and individuals with impaired glucose tolerance.[46,47]

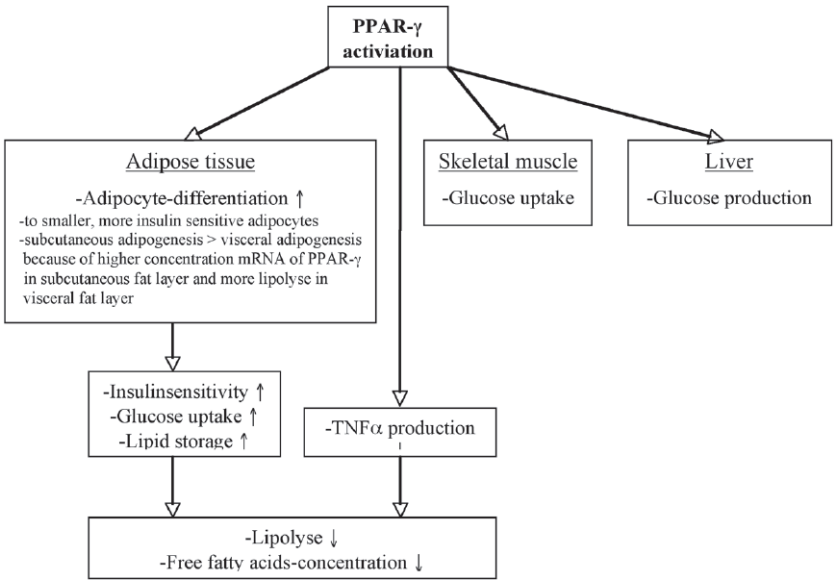
What mechanisms could be involved in the beneficial effects of thiazolidinediones on glycaemic control and insulin resistance? Since obesity, causing insulin resistance, is a main determinant for the development of type 2 diabetes, mechanisms related to adipocyte function are likely to be involved.

3.1 Differentiation of adipocytes

PPAR-γ is expressed mainly in adipose tissue and is a key factor in the differentiation of adipocytes and adipogenesis (figure 3).[48-49] PPAR stimulation alters adipocyte metabolism by increasing the expression of specific adipocyte genes involved in glucose regulation (e.g. GLUT4, lipoprotein lipase (LPL), fatty acid transporter

protein, Acyl CoA synthase and malic enzymes).[50] Recent observations in PPAR-γ knockout mice show that homozygous PPAR-γ-null mice are completely devoid of adipose tissue and that mice heterozygous for the mutation (PPAR-γ +/- mice) are characterised by a decreased adipose tissue mass.[51] These *in vivo* results are further supported by *in vitro* data showing that embryonic stem cells lacking both copies of PPAR-γ fail to differentiate into adipocytes after appropriate treatment, whereas embryonic stem cells expressing PPAR-γ readily differentiate into adipocytes.[52] Moreover, forced expression of PPAR-γ in fibroblasts makes them differentiate into adipocytes.[53] Pioglitazone affects the early stage of adipocyte differentiation and enhances growth arrest, protein synthesis and hypertrophy of 3T3-L1 adipocytes.[54] Exposure of 3T3-L1 adipocytes to tumour necrosis factor (TNF)-α, a potent inhibitor of adipocyte differentiation, results in lipid depletion and a complete reversal of adipocyte differentiation.[55] Consistent with the opposing effects of PPAR-γ and TNF-α in adipose tissue, treatment of obese animals with PPAR-γ agonists reduces the adipose tissue expression of TNF-α, which contributes to the weight gain.[56]

Figure 3. The peroxisome proliferator-activated receptor (PPAR)-γ paradox of increased adipogenesis and beneficial diabetic treatment TNF-α= tumour necrosis factor-α



An interesting concept is the 'lipid-steal hypothesis'.[53,57] This hypothesis states that stimulation of adipose differentiation leads to increased numbers of small adipocytes, which are thought to be more sensitive to insulin than large adipocytes. These smaller adipocytes take up free fatty acids more easily and thus reduce free fatty acid flux to the muscles or liver. Pioglitazone strongly induces adipocyte differentiation and increases adipocyte glucose utilisation at post-absorptive insulin levels *in vivo*.[27] However, thiazolidinediones also exert beneficial effects on glucose and lipid metabolism in the absence of adipose tissue,[28] suggesting that an alteration in adipocyte differentiation cannot be a sole explanation for the improvement in insulin sensitivity.

How can the paradox of beneficial effects of PPAR-γ activation with improvement of insulin resistance on one hand and stimulation of adipogenesis on the other hand be explained?

Thiazolidinedione-induced adipogenesis occurs mainly in subcutaneous fat and not in visceral fat. An increase in visceral fat is associated with a higher cardiovascular risk. In line with this, levels of mRNA for PPAR-γ and leptin are higher in subcutaneous fat than in visceral fat.[58] Furthermore, 3T3-L1 pre-adipocytes in subcutaneous fat become resistant to apoptosis after differentiation into mature adipocytes, a process stimulated by PPAR-γ activation and resulting in decreased apoptosis in subcutaneous fat.[59] Finally, the recently identified protein, resistin, a protein that causes insulin resistance in mice and which is inhibited by PPAR-γ activation, may also be involved.[60] Unfortunately, these results are controversial because resistin is not found in humans, once again showing the discrepancy between experimental animal studies and human physiology.

3.2 Modulation of tissue triglyceride content

Improvement in insulin resistance is associated with a decrease in the triglyceride content of liver and skeletal muscle. Treatment with thiazolidinediones reduces the triglyceride content in liver and skeletal muscle, which may be an important factor in the observed improvement in peripheral glucose disposal and decreased hepatic glucose output.[61] In addition, thiazolidinediones also lower the triglyceride content of β cells, which is associated with an improvement of β cell function.[62] Supporting these data is the clinical observation that the high ratio of proinsulin to insulin typically found in patients with type 2 diabetes mellitus is normalised upon thiazolidinedione treatment, suggesting an effect of these drugs on β cell function.[39,63]

3.3 Effect on adipocyte-derived factors

Other mechanisms of the thiazolidinediones include regulation of storage and release of adipocyte-derived signaling factors that affect insulin sensitivity of muscle. These factors include free fatty acids, adiponectin, TNF-α and leptin.

3.3.1 Free fatty acids

Fatty acids are key mediators of the storage or release of adipocyte-derived signaling factors affecting insulin sensitivity. High levels of free fatty acids have been linked to the induction of insulin resistance, because increased free fatty acid metabolism in the liver leads to increased gluconeogenesis.[64] There is evidence for a direct regulatory effect of fatty acids on the production of macrophage lipoprotein lipase (involved in the pathogenesis of atherosclerosis) in the vascular wall.[65] It is well established that increased fatty acid levels decrease glucose metabolism in muscle. Because fatty acids are ligands for PPAR-γ, activation of PPARs by thiazolidinediones increases fatty acid clearance in adipose tissue with a concomitant decrease in the uptake of fatty acids in muscle, which potentially improves insulin sensitivity.[66-67]

The mechanism underlying these effects may be related to the regulation by PPAR-γ of the expression of the fatty acid transporter CD36, which is also implicated in the control of insulin sensitivity.[68-70] There is also a correlation between PPAR-γ transactivation and intracellular levels of liver fatty acid-binding protein, which could explain the decrease in plasma levels of free fatty acids.[71] Furthermore, PPAR-γ is required for the expression of adipocyte phosphoenolpyruvate carboxykinase (PEPCK). PEPCK is the key enzyme in glyceroneogenesis, an important metabolic pathway that limits the release of non-esterified fatty acids from adipocytes.[72] Therefore, PEPCK could be a major target gene for the antidiabetic actions of thiazolidinediones. Thus, lowering the elevated plasma levels of non-esterified fatty acids is likely to be an important mechanism to explain the beneficial metabolic effects induced by thiazolidinediones.

3.3.2 Adiponectin

Adiponectin is an adipocyte-derived hormone that decreases insulin resistance by lowering the triglyceride content of muscle and liver in obese mice. This effect results from increased expression of molecules involved in fatty-acid combustion in muscle. Moreover, insulin resistance in lipoatrophic mice can be completely reversed by physiological doses of adiponectin and leptin.[73]) In addition, adiponectin suppresses adhesion molecule expression in vascular endothelial cells and inhibits

cytokine production by macrophages. Recent publications show that thiazolidinediones can markedly enhance the expression and secretion of adiponectin *in vitro* and *in vivo*, possibly (partly) mediated by antagonising the suppressive effect of TNF-α on the production of adiponectin.[74] However, the exact role of adiponectin in insulin resistance in humans has not been elucidated.

3.3.3 Tumour necrosis factor-α

The expression of TNF-α by adipose tissue is upregulated in obesity and TNF-α levels are increased in patients with features of the insulin resistance syndrome. This cytokine decreases PPAR-γ expression, insulin receptor synthesis and activation, and glucose uptake in adipose tissue, skeletal muscle and liver by attenuating the expression of the glucose transporter GLUT4.[31,75-77] Chronic hyperglycaemia is associated with increased TNF-α production, which may be derived from adipose tissue.[78] Thiazolidinediones restore sensitivity to insulin by down-regulating adipose cytokines such as TNF-α.[6,79,80] Furthermore, it has been shown that pioglitazone improves TNF-α-induced insulin resistance by improving insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate.[81]

3.3.4 Leptin

Thiazolidinediones have also been implicated in the regulation of leptin expression. Administration of thiazolidinediones reduces the expression of leptin mRNA and protein in adipocytes *in vivo* and *in vitro*.[82] The role of leptin in insulin resistance is controversial, but some reports indicate that leptin might interfere with insulin signalling in certain cell types.

In conclusion, glycaemic control and insulinsensitising properties of thiazolidinediones may involve a wide range of inter-related mechanisms in different target tissues involved in insulin activity and glucose production and uptake.

4. Thiazolidinediones and additional vascular effects

4.1 Improvement of endothelial (dys-)function

Atherosclerotic disease is characterised by endothelial dysfunction. Endothelial dysfunction is characterised by decreased availability of endothelium-derived nitric oxide (NO) and can be assessed clinically by impaired vasoreactivity of the brachial artery after an ischaemic or other stimulus. Impaired endothelial function has

prognostic significance for future development of cardiovascular events. All known cardiovascular risk factors are associated with endothelial dysfunction.[83,84] Endothelial dysfunction appears to be an important feature of the insulin resistance syndrome. Upon binding to the endothelial insulin receptor, insulin activates endothelial NO synthase (eNOS), thereby stimulating NO production, resulting in vasodilation. This vasodilation is impaired in insulin-resistant states, which has been termed vascular insulin resistance. Patients with insulin-resistant states such as obesity, hypertension and type 2 diabetes exhibit blunted insulin-mediated vasodilation and impaired endothelium-dependent vasodilation.[85] Quenching of NO by decreased NO or an increased inactivation of NO by reactive oxygen species (ROS) might be a major driving force for instability of atherosclerotic plaques in patients with diabetes.[86]

In both obese people and healthy volunteers, it was shown that a single oral dose of troglitazone improved the ischaemia-induced flow-mediated vasodilatation in the forearm.[87-88] Normalisation of impaired brachial artery vasoreactivity also occurred during troglitazone therapy in individuals with peripheral vascular disease and impaired glucose tolerance.[89]

How can thiazolidinediones improve endothelial function? Improved metabolic control will most likely contribute to the effects observed. High levels of glucose and free fatty acids stimulate ROS production, for example through protein kinase C-dependent activation of nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] oxidase.[90] Reduction of the glucose and free fatty acid concentrations by thiazolidinediones will therefore have beneficial effects. A reduction in formation of ROS by both polymorphonuclear leukocytes and mononuclear cells after administration of troglitazone may also contribute to improvement in endothelial function.[88] Incubation with insulin plus pioglitazone improves vasodilation induced by acetylcholine, suggesting that pioglitazone augments the endothelium-dependent vasodilation mediated by insulin.[91]

Direct effects of thiazolidinediones on vascular smooth muscle cells have also been observed. Thiazolidinediones attenuate vasoconstriction as well as inhibit L-type Ca^{2+} currents in vascular smooth muscle cells (VSMC) *in vitro*.[92] The vasodilative action of pioglitazone after removal of the endothelium[93] is not yet completely understood. Pioglitazone appears to act mainly on VSMC rather than the vascular endothelium. However, expression of PPAR-γ mRNA is very low in VSMC.[94]

In patient groups with a high incidence of cardiovascular diseases and endothelial dysfunction (congestive heart failure, diabetes, atherosclerosis) TNF-α levels are increased. There may be an interesting link between TNF-α and endothelial function

because of the direct association between TNF-α and NO bioavailability. TNF-α downregulates mRNA for eNOS by shortening its half-life in human umbilical vein endothelial cells.[96] In a rat model, recombinant TNF-α infusion *in vivo* depresses endothelium-dependent relaxation without decreasing mean arterial pressure.[96] In addition, brief exposure of the human forearm resistance artery to TNF-α may increase the basal bioavailability of the vasoconstrictor prostaglandin and reduce the basal bioavailability of NO. However, in acetylcholine-stimulated endothelium-dependent vasodilatation, TNF-α did not impair the vascular function, maybe because of an overwhelming NO bioavailability in healthy humans.[97] It is noteworthy to mention that interpretation of these results are difficult in the light of the effects of TNF-α on the inducible form of NOS (iNOS).

4.2 Decreased inflammatory conditions

Low-grade inflammation plays an important role in the initiation and progression of cardiovascular diseases.[98] Accumulation of monocyte-derived lipid-loaded macrophages or foam cells, smooth muscle cell proliferation and de novo formation of extracellular matrix results in the formation of the atherosclerotic plaque.[99] Markers of inflammation, such as the acute-phase protein C-reactive protein (CRP), TNF-α and interleukin (IL)-6, are increased in patients with the insulin resistance syndrome.[100,101] Elevated serum levels of CRP, which is indicative of a low-grade inflammatory state, are associated with a diminished systemic endothelial vasodilator function.[102]

PPARs are mainly expressed in adipocytes and could have an important role in downregulation of the inflammatory cytokine TNF-α as discussed in sections 3.3.3 and 4.1. Expression of PPARs in macrophages, T cells and neutrophils suggests that they may have an important role in modulating the function of inflammatory cells.[16]

4.2.1 Modulation of PPAR activity in inflammatory cells

Several studies have reported that PPAR agonists dampen inflammatory responses in macrophages. PPAR-γ agonists inhibit tissue factor expression in human monocytes and macrophages,[103,104] and PPAR-γ agonists reduce macrophage homing to atherosclerotic plaques.[106] PPAR-γ is a negative regulator of macrophage activation and may limit chronic inflammation by inhibiting the induced expression of circulating vascular cell adhesion molecule-1 (VCAM-1) and monocytes without affecting the acute inflammation mediated by endothelial-leucocyte adhesion molecule (E-selectin).[106,107] *In vitro*, PPAR-γ agonists suppress the release of inflammatory cytokines, such as TNF-α, IL-1 and IL-6, from monocytes at agonist

concentrations similar to those effective in promoting adipogenesis.[108] An increased expression of PPAR-γ during the differentiation of monocytes and macrophages initially suggested that PPAR-γ may regulate macrophage differentiation.[10,109] However, there are also studies, performed with PPAR-γ–deficient stem cells, suggesting that PPAR-γ is not essential for either myeloid development, or for certain functions of mature macrophages such as phagocytosis and inflammatory cytokine production.[16,110] Several reports indicate that PPAR-γ agonists dampen macrophage inflammatory responses by reducing the expression of matrix-degrading metalloproteinases, cytokines, NO and modified lipoprotein receptors,[107,108,110,11] whereas others do not.[16,112] Part of this contradiction can be explained by the widely varying doses of PPAR-γ agonists used. At high concentrations these agents appear to have PPAR-γ-independent actions, which are as yet poorly understood.[113]

4.2.2 CD36 expression in mononuclear cells

PPAR-γ stimulation induces CD36 gene expression.[70,110,114] CD36 is a transporter of longchain fatty acids and is a high-affinity receptor for oxidised low-density lipoproteins (oxLDL).[115] CD36-deficient mice have a 6-fold reduction in atheroma compared with controls,[116] probably because of a reduced uptake of oxLDL, which results in diminished foam cell formation.[109] Since PPAR-γ increases the expression of CD36, there is concern about the overall antiatherogenic effect of thiazolidinediones. Some investigators have reported that PPAR activation leads to an induction of foam cell formation from macrophages,[70,109,110] whereas others have reported suppression of inflammatory cytokines and induction of cholesterol efflux from macrophages as antiatherogenic effects of PPAR activation.[114,117] However, overall results indicate that foam cell development can occur in the absence of PPAR-γ and that PPAR-γ agonists decrease atherosclerosis in animal models of LDL receptor and apolipoprotein E deficiency.[118] The induction by PPAR of cholesterol efflux through the adenosine triphosphate (ATP)-binding cassette transporter 1 (ABCA1) may be a counterbalancing mechanism. The liver X receptor-α (LXRα) and the scavenger receptor A (SRA) may have a central role in this concept of cholesterol efflux induced by PPAR activation.[60,110,117]

Recently, CD36-deficient humans were found to have an increased insulin resistance, including higher plasma triglyceride and glucose levels, lower plasma high-density lipoprotein (HDL) cholesterol levels and much higher blood pressure than controls.[69] So far, most studies show a net antiatherogenic effect of thiazolidinediones, but the major mechanisms for this still have to be clarified.[80;119] Agonists of PPAR-α

and PPAR-γ inhibit the cardiac expression of TNF-α, in part by antagonising nuclear factor-κB activity.[120]
Taken together, these complex observations suggest that thiazolidinediones may have beneficial effects in modulating the inflammatory state and thus atherogenesis.

4.3 Effects on the lipid profile
Dyslipidaemia is a well-established risk factor for the formation of atherosclerotic plaques. Insulin resistance and type 2 diabetes are associated with a characteristic pattern of lipid abnormalities, including an increased number of small dense LDL particles, elevated plasma triglyceride levels, and low plasma high density lipoprotein (HDL) levels.[121] The disturbance of lipid metabolism may not be the result of insulin resistance alone, but may also be directly involved in the metabolic abnormalities observed. Evidence obtained from obese animal models (eg. rats fed high-fat diets) shows excess accumulation of muscle triglyceride together with the development of insulin resistance.[122] Several studies demonstrate an increased muscle triglyceride content in insulin-resistant states in humans as well.[123-125] The factors leading to this accumulation are not clear yet, but it could well be a result of elevated circulating free fatty acids associated with impaired triglyceride clearance, or reduced muscle free fatty acid oxidation.

4.3.1 Reduction of plasma triglycerides
In humans, troglitazone[4,22] and pioglitazone[126,127] lower triglyceride levels by approximately 9 to 20%. In contrast, mean triglyceride levels were increased after rosiglitazone treatment by 38.4%.[128] The exact mechanism by which thiazolidinediones affect triglyceride levels is currently not known. Pioglitazone has been reported to increase the expression of lipoprotein lipase and to decrease the expression of apolipoprotein C-III (key players in plasma triglyceride metabolism), indicating that pioglitazone has PPAR-α agonistic activity.[129] The triglyceride-lowering action of PPAR-γ activation may be the result of a reduction in fatty acid and triglyceride synthesis, and consequently a decrease in the production of very-lowdensity lipoprotein (VLDL).

4.3.2 Effects on lipoprotein metabolism
Several trials have been conducted to study the effects of thiazolidinediones on plasma lipoproteins. In general there appears to be an increase in HDL (up to ≈20%).[130] The increase in HDL levels is likely to be explained by the decrease in triglyceride levels.

LDL levels tend to increase (≈10%),[130] are unaffected or are lowered (≈15%) by thiazolidinedione treatment.[128,131,132] However, the concomitant changes in plasma HDL and LDL levels resulted in unaltered LDL to HDL ratios.[4,130] The increase in total cholesterol and LDL cholesterol levels observed in several studies is cause for concern. This increase may be predominantly caused by larger, buoyant LDL particles. Larger LDL particles are less prone to oxidative modification and are therefore thought to be less atherogenic.[133,134] Support for this hypothesis comes from a study showing that troglitazone increases the resistance of LDL cholesterol to oxidation.[135;136] Recent studies reported a decrease in LDL levels after pioglitazone treatment.[128,131,132] There appears to be a differential effect of thiazolidinediones: LDL levels apparently increase more during rosiglitazone than pioglitazone treatment.[127,137] In a head to head comparison, treatment with pioglitazone was associated with overall greater beneficial effects on blood lipid levels (total cholesterol, HDL, LDL and triglycerides) than treatment with rosiglitazone, while similar effects were demonstrated in respect to weight gain and glycaemic control.[131,132] A possible reason is that pioglitazone may perhaps also have PPAR-α agonistic effects, which is in line with lipid-lowering effects of fibric acid derivatives. The different effects of the various thiazolidinediones on lipid metabolism need further investigation, but considering its central role in lipid metabolism, pharmacological modulation of PPAR-γ activity by thiazolidinediones may result in an overall improvement of the dyslipidaemic phenotype.[127,138,139]

4.4 Lowering blood pressure

Troglitazone and rosiglitazone decrease blood pressure by ≈10%. This effect has been observed in patients with hypertension and type 2 diabetes,[140,141] individuals with normal blood pressure and type 2 diabetes,[141,142] and obese individuals without diabetes.[47,143] Pioglitazone therapy decreased arterial pressure in rat models of hypertension[92,93] and prevented the development of hypertension.[144] Other animal and human studies have shown that thiazolidinediones decrease blood pressure associated with decreased insulin levels and improvement of endothelial function.[91,145,146] However, the exact role of decreased insulin levels on the thiazolidinedione-mediated regulation of blood pressure is debated because some reports show insulin- and glucose-independent blood pressure–lowering mechanisms.[87,147] It has also been suggested that thiazolidinediones may lower blood pressure by a direct vascular effect involving decreased calcium uptake into vascular cells.[93,148,149] Alternatively, a thiazolidinedione-induced decrease in the activity of the renin-angiotensin system and of the sympathetic system may also play an important role in the modulation of blood pressure.[91]

In conclusion, thiazolidinediones lower blood pressure by multiple mechanisms, including a decrease in plasma insulin levels.

4.5 Additional antiatherogenic effects

4.5.1 Intimal hyperplasia

The proliferation and migration of vascular smooth muscle cells play a role in the pathogenesis and progression of atherosclerosis. Troglitazone has been shown to inhibit VSMC growth and intimal hyperplasia.[150] In clinical trials, troglitazone reduced intimal hyperplasia in patients with type 2 diabetes, with and without coronary stent implants.[151,152] Pioglitazone shows similar effects; a significant decrease in the intima-media thickness of the carotid arteries was observed as early as 3 and 6 months after its administration in patients with type 2 diabetes.[153] Pioglitazone also reduced the VSMC density of rat carotid arterial intima induced by balloon catheterisation and had vasculo-protective effects against neointimal thickening and hypertensive vascular hypertrophy.[154,155] New insights show that pioglitazone is a potent inducer of apoptosis in vascular lesions. Furthermore, thiazolidinediones inhibit VSMC migration mediated by multiple chemoattractants and attenuate the development of intimal hyperplasia in animal models of balloon catheter vascular injury.[156] The underlying mechanism of a reduction in intimal hyperplasia by thiazolidinediones is not known but improved insulin sensitivity may play an important role.[157]

4.5.2 Effects on the prothrombotic state

Increased levels of the inhibitor of fibrinolysis, plasminogen activator inhibitor-1 (PAI-1), create a prothrombotic state. Levels of PAI-1 are increased in patients with type 2 diabetes and are strongly correlated with body mass index, insulin resistance and fasting levels of insulin, triglycerides and HDL cholesterol. So far, only troglitazone has been shown to reduce PAI-1 to near-normal levels in patients with diabetes.[158] Pioglitazone decreases PAI-1 production in cultured human umbilical vein endothelial cells *in vitro*.[159] Thus, thiazolidinediones may have favourable effects on cardiovascular events by improvement of the prothrombotic state.

5. Special considerations

Thiazolidinedione treatment is associated with some undesirable effects. Some of these adverse effects need further consideration.

5.1 Increase in bodyweight

Gain of bodyweight is a dose-dependent adverse effect of thiazolidinediones, whether administered alone or in combination with other antihyperglycaemic agents, especially sulphonylureas.[160] The weight gain, 4kg on average, plateaus

after 6 months. Despite the weight gain, thiazolidinediones clearly decrease insulin resistance as discussed in section 3. An increased fat mass consisting of small adipocytes and increased plasma volume have been proposed to explain these observations.

5.1.1 Increased fat mass

It is believed that the thiazolidinedione-induced differentiation of adipocytes and adipogenesis, as discussed in section 3.1, may be partly responsible for the increase in bodyweight seen in humans and animals.[161,162]

In humans, long-term troglitazone treatment results in increased accumulation of subcutaneous fat without a change in the total amount of visceral fat, probably because of the activation of PPAR-γ subcutaneously.[163] Thus, troglitazone appears to promote fat accumulation in subcutaneous adipose tissue rather than in visceral adipose tissue, which may have little impact on atherogenesis.[164] One study even shows that troglitazone treatment of patients with type 2 diabetes decreases the intra-abdominal fat mass but does not affect the total body fat mass or bodyweight.[165] Thus, increased adipocyte differentiation associated with increased bodyweight may not be as harmful as first thought, but the clinical significance of this modest weight change will require further evaluation in long-term studies.

5.1.2 Increase in plasma volume

The weight gain caused by thiazolidinedione treatment is also associated with an increase in the plasma volume, which occurs whether thiazolidinediones are administered alone or in combination with metformin or sulphonylureas. Again, there is a paradox because thiazolidinediones cause a substantial decrease in blood pressure while increasing plasma volume. An explanation could be the effects of thiazolidinediones on downregulation of endothelin-1, a potent vasoconstrictor.[166]

As a consequence of an increased plasma volume, haemoglobin and haematocrit levels are decreased. These haematological alterations are observed during the first weeks of therapy but do not change further thereafter.

Because of this plasma volume expansion, thiazolidinediones are not recommended for patients with heart failure (New York Heart Association class III or IV).

5.2 Hepatotoxicity

As mentioned in section 2, troglitazone was withdrawn from the market because of an increased risk of idiosyncratic hepatic toxicity. Three cases of severe hepatotoxicity have recently been reported with rosiglitazone.[167,168] It is uncertain whether the drug directly induces these hepatic disturbances. All patients recovered fully after

discontinuing treatment. Currently, no cases of pioglitazone-induced severe hepatotoxicity have been reported but it must be realised that there is considerably less clinical experience with rosiglitazone and pioglitazone than with troglitazone. The largest study conducted so far shows no evidence of hepatotoxic effects observed in studies that involved 5006 patients taking rosiglitazone as monotherapy or combination therapy for 5508 person-years.[169] These findings suggest that the idiosyncratic liver toxicity observed with troglitazone is unlikely to be a thiazolidinedione class effect.
Patients with poorly controlled type 2 diabetes may have moderate elevations of serum alanine transferase (ALT) that will decrease with improved glycaemic control during treatment with rosiglitazone or other antihyperglycaemic agents. Thiazolidinediones should not be given to patients with signs of serious hepatic dysfunction. However, in patients with non-alcoholic steatohepatitis and small increases in plasma transaminases, thiazolidinediones may be particularly useful because of their beneficial effects on visceral fat accumulation; regular monitoring of plasma transaminases is recommended in these patients.

5.3 Effects on gonadal function in women
Since the increased insulin sensitivity induced by thiazolidinediones is associated with an improvement of ovulation and fertility in woman with polycystic ovary syndrome (PCOS) and with an increased estrogen clearance in pre- and postmenopausal women, caution should be taken in women receiving oral contraceptives or hormone replacement therapy. Medications with a higher estrogen content may be beneficial to avoid the possibility of reduced effectiveness. Thiazolidinediones should not be administered to women during pregnancy and breast-feeding since it is not known whether these drugs have teratogenic effects or are secreted in human breast milk.

5.4 Drug interactions
According to the 1B-text, pioglitazone is metabolised in the liver by hydroxylation of aliphatic methyl groups, mainly through the cytochrome P450 (CYP) isoenzymes CYP3A4 and CYP2C9. *In vitro* studies have suggested that xenobiotic oxidations by CYP enzymes are more substantially affected by troglitazone and its metabolites than pioglitazone or rosiglitazone.[170] Troglitazone directly and competitively inhibits the steroidogenic enzyme P450c17, whereas rosiglitazone and pioglitazone exert a direct but weaker inhibitory effect on P450c17.[171] Indeed the results of further *in vitro* studies indicate that pioglitazone has a low potential for drug interactions and

there is no evidence to date that pioglitazone induces the hepatic CYP isoform CYP3A4 system. However, to our knowledge no in vivo studies have been published to further establish the effects of pioglitazone on drug metabolism regarding interference with the CYP3A4 isoform. An article by Prueksaritanont et al. studied the interactions between simvastatin and troglitazone or pioglitazone in healthy individuals and concluded that the modest effect of troglitazone on simvastatin pharmacokinetics was in agreement with the suggestion that troglitazone is an inducer of CYP3A4, while the lack of pharmacokinetic effect of pioglitazone on simvastatin supported the expectation that this combination may be used safely.[172] To date there are more published clinical trials investigating pioglitazone and, even though it is circumstantial evidence, there is indeed no evidence that pioglitazone induces the hepatic CYP isoform CYP3A4 system.[132,169] However, until more long-term data are available regular monitoring of liver enzymes is still recommended.

6. Conclusion

The co-occurrence of metabolic disorders such as type 2 diabetes, dyslipidaemia, hypertension, hypercoagulability, vasculopathy, obesity and atherosclerotic disease, and the central role of insulin resistance in this cluster, provide a target to potentially reduce vascular incidents. Until now these vascular risk factors have been treated separately and thus patients often need polypharmacy. Obviously, insulin resistance plays a key role and pharmacological intervention aimed at the insulin resistance syndrome may therefore have beneficial effects on several cardiovascular risk factors, resulting in a decreased risk of future cardiovascular disease.

Thiazolidinediones are uniquely able to exert direct beneficial effects on insulin resistance by binding to PPAR-γ and probably to PPAR-α. As transcription factors, PPARs regulate the expression of numerous genes with key roles in glucose and lipid metabolism. In addition, activation of PPARs could improve vascular function and inflammatory processes resulting in additional vascular effects.

Several issues are yet to be resolved. The apparent paradox of adipocyte differentiation with weight gain concurring with the insulin-sensitising effects of thiazolidinediones is not completely understood. The thiazolidinedione-induced decrease in blood pressure accompanied by an increase in the plasma volume has not been fully explained. The discrepancy of the stimulation of expression of CD36 and the anti-atherogenic effect of the thiazolidinediones also needs to be further explained. It would be interesting to know whether thiazolidinediones act directly by activating

PPAR-γ or stimulate PPAR-α activity at the same time, which could also explain the broad metabolic and additional vascular effects of thiazolidinediones. An important issue that needs to be resolved is the importance of raised cholesterol levels, in particular raised LDL levels, caused by some thiazolidinediones. Future research may provide answers to these questions, particularly with respect to the role of PPAR-γ in vascular pathophysiology. Although the concept of thiazolidinediones is very promising, long-term clinical trials concerning cardiovascular end points are needed.

References

1. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes. 1988;37:1595-1607.
2. Fagan TC, Deedwania PC. The cardiovascular dysmetabolic syndrome. Am J Med. 1998;105:77S-82S.
3. Meigs JB. Invited commentary: insulin resistance syndrome? Syndrome X? Multiple metabolic syndrome? A syndrome at all? Factor analysis reveals patterns in the fabric of correlated metabolic risk factors. Am J Epidemiol. 2000;152:908-911.
4. Mudaliar S, Henry R. NEW ORAL THERAPIES FOR TYPE 2 DIABETES MELLITUS: The Glitazones or Insulin Sensitizers. Annu Rev Med. 2001;52:239-257.
5. Turner RC, Millns H, Neil HA et al. Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23). BMJ. 1998;316:823-828.
6. Vamecq J, Latruffe N. Medical significance of peroxisome proliferator-activated receptors. Lancet. 1999;354:141-148.
7. Issemann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature. 1990;347:645-650.
8. Lemberger T, Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. Annu Rev Cell Dev Biol. 1996;12:335-363.
9. Mangelsdorf DJ, Thummel C, Beato M et al. The nuclear receptor superfamily: the second decade. Cell. 1995;83:835-839.
10. Willson TM, Brown PJ, Sternbach DD et al. The PPARs: from orphan receptors to drug discovery. J Med Chem. 2000;43:527-550.
11. Chinetti G, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. Inflamm Res. 2000;49:497-505.
12. Loviscach M, Rehman N, Carter L et al. Distribution of peroxisome proliferator-activated receptors (PPARs) in human skeletal muscle and adipose tissue: relation to insulin action. Diabetologia. 2000;43:304-311.
13. Marx N, Libby P, Plutzky J. Peroxisome proliferator-activated receptors (PPARs) and their role in the vessel wall: possible mediators of cardiovascular risk? J Cardiovasc Risk. 2001;8:203-210.
14. Elangbam CS, Tyler RD, Lightfoot RM. Peroxisome proliferator-activated receptors in atherosclerosis and inflammation--an update. Toxicol Pathol. 2001;29:224-231.
15. Dubois M, Pattou F, Kerr-Conte J et al. Expression of peroxisome proliferator-activated receptor gamma (PPARgamma) in normal human pancreatic islet cells. Diabetologia. 2000;43:1165-1169.
16. Moore KJ, Rosen ED, Fitzgerald ML et al. The role of PPAR-gamma in macrophage differentiation and cholesterol uptake. Nat Med. 2001;7:41-47.
17. Forman BM, Tontonoz P, Chen J et al. 15-Deoxy-delta 12, 14-prostaglandin J_2 is a ligand for the adipocyte determination factor PPAR gamma. Cell. 1995;83:803-812.

18. Chaiken RL, Eckert-Norton M, Pasmantier R et al. Metabolic effects of darglitazone, an insulin sensitizer, in NIDDM subjects. Diabetologia. 1995;38:1307-1312.
19. Spiegelman BM. PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. Diabetes. 1998;47:507-514.
20. Maggs DG, Buchanan TA, Burant CF et al. Metabolic effects of troglitazone monotherapy in type 2 diabetes mellitus. A randomized, double-blind, placebo-controlled trial. Ann Intern Med. 1998;128:176-185.
21. Sironi AM, Vichi S, Gastaldelli A et al. Effects of troglitazone on insulin action and cardiovascular risk factors in patients with non-insulin-dependent diabetes. Clin Pharmacol Ther. 1997;62:194-202.
22. Suter SL, Nolan JJ, Wallace P et al. Metabolic effects of new oral hypoglycemic agent CS-045 in NIDDM subjects. Diabetes Care. 1992;15:193-203.
23. Preininger K, Stingl H, Englisch R et al. Acute troglitazone action in isolated perfused rat liver. Br J Pharmacol. 1999;126:372-378.
24. Raman P, Judd RL. Role of glucose and insulin in thiazolidinedione-induced alterations in hepatic gluconeogenesis. Eur J Pharmacol. 2000;409:19-29.
25. Zierath JR, Ryder JW, Doebber T et al. Role of skeletal muscle in thiazolidinedione insulin sensitizer (PPARgamma agonist) action. Endocrinology. 1998;139:5034-5041.
26. Arakawa K, Ishihara T, Aoto M et al. Actions of novel antidiabetic thiazolidinedione, T-174, in animal models of non-insulin-dependent diabetes mellitus (NIDDM) and in cultured muscle cells. Br J Pharmacol. 1998;125:429-436.
27. Hallakou S, Doare L, Foufelle F et al. Pioglitazone induces in vivo adipocyte differentiation in the obese Zucker fa/fa rat. Diabetes. 1997;46:1393-1399.
28. Hallakou S, Foufelle F, Doare L et al. Pioglitazone-induced increase of insulin sensitivity in the muscles of the obese Zucker fa/fa rat cannot be explained by local adipocyte differentiation. Diabetologia. 1998;41:963-968.
29. Matsuhisa M, Shi ZQ, Wan C et al. The effect of pioglitazone on hepatic glucose uptake measured with indirect and direct methods in alloxan-induced diabetic dogs. Diabetes. 1997;46:224-231.
30. Shimaya A, Kurosaki E, Shioduka K et al. YM268 increases the glucose uptake, cell differentiation, and mRNA expression of glucose transporter in 3T3-L1 adipocytes. Horm Metab Res. 1998;30:543-548.
31. Szalkowski D, White-Carrington S, Berger J et al. Antidiabetic thiazolidinediones block the inhibitory effect of tumor necrosis factor-alpha on differentiation, insulin-stimulated glucose uptake, and gene expression in 3T3-L1 cells. Endocrinology. 1995;136:1474-1481.
32. Tanaka T, Itoh H, Doi K et al. Down regulation of peroxisome proliferator-activated receptorgamma expression by inflammatory cytokines and its reversal by thiazolidinediones. Diabetologia. 1999;42:702-710.
33. Kraegen EW, James DE, Jenkins AB et al. A potent in vivo effect of ciglitazone on muscle insulin resistance induced by high fat feeding of rats. Metabolism. 1989;38:1089-1093.

34. Lee MK, Miles PD, Khoursheed M et al. Metabolic effects of troglitazone on fructose-induced insulin resistance in the rat. Diabetes. 1994;43:1435-1439.
35. Miles PD, Romeo OM, Higo K et al. TNF-alpha-induced insulin resistance in vivo and its prevention by troglitazone. Diabetes. 1997;46:1678-1683.
36. Miles PD, Higo K, Romeo OM et al. Troglitazone prevents hyperglycemia-induced but not glucosamine-induced insulin resistance. Diabetes. 1998;47:395-400.
37. Fonseca VA, Valiquett TR, Huang SM et al. Troglitazone monotherapy improves glycemic control in patients with type 2 diabetes mellitus: a randomized, controlled study. The Troglitazone Study Group. J Clin Endocrinol Metab. 1998;83:3169-3176.
38. Inzucchi SE, Maggs DG, Spollett GR et al. Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. N Engl J Med. 1998;338:867-872.
39. Prigeon RL, Kahn SE, Porte D, Jr. Effect of troglitazone on B cell function, insulin sensitivity, and glycemic control in subjects with type 2 diabetes mellitus. J Clin Endocrinol Metab. 1998;83:819-823.
40. Spencer CM, Markham A. Troglitazone. Drugs. 1997;54:89-101.
41. Nolan JJ, Jones NP, Patwardhan R et al. Rosiglitazone taken once daily provides effective glycaemic control in patients with Type 2 diabetes mellitus. Diabet Med. 2000;17:287-294.
42. Wolffenbuttel BH, Gomis R, Squatrito S et al. Addition of low-dose rosiglitazone to sulphonylurea therapy improves glycaemic control in Type 2 diabetic patients. Diabet Med. 2000;17:40-47.
43. Wolffenbuttel BH, Sels JP, Huijberts MS. Rosiglitazone. Expert Opin Pharmacother. 2001;2:467-478.
44. Aronoff S, Rosenblatt S, Braithwaite S et al. Pioglitazone hydrochloride monotherapy improves glycemic control in the treatment of patients with type 2 diabetes: a 6-month randomized placebo-controlled dose-response study. The Pioglitazone 001 Study Group. Diabetes Care. 2000;23:1605-1611.
45. Einhorn D, Rendell M, Rosenzweig J et al. Pioglitazone hydrochloride in combination with metformin in the treatment of type 2 diabetes mellitus: a randomized, placebo-controlled study. The Pioglitazone 027 Study Group. Clin Ther. 2000;22:1395-1409.
46. Berkowitz K, Peters R, Kjos SL et al. Effect of troglitazone on insulin sensitivity and pancreatic beta-cell function in women at high risk for NIDDM. Diabetes. 1996;45:1572-1579.
47. Nolan JJ, Ludvik B, Beerdsen P et al. Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. N Engl J Med. 1994;331:1188-1193.
48. Chawla A, Schwarz EJ, Dimaculangan DD et al. Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology. 1994;135:798-800.
49. Sandouk T, Reda D, Hofmann C. The antidiabetic agent pioglitazone increases expression of glucose transporters in 3T3-F442A cells by increasing messenger ribonucleic acid transcript stability. Endocrinology. 1993;133:352-359.
50. Vidal-Puig AJ, Considine RV, Jimenez-Linan M et al. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. J Clin Invest. 1997;99:2416-2422.

51. Miles PD, Barak Y, He W et al. Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency. J Clin Invest. 2000;105:287-292.
52. Fajas L, Debril MB, Auwerx J. Peroxisome proliferator-activated receptor-gamma: from adipogenesis to carcinogenesis. J Mol Endocrinol. 2001;27:1-9.
53. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell. 1994;79:1147-1156.
54. Takamura T, Nohara E, Nagai Y et al. Stage-specific effects of a thiazolidinedione on proliferation, differentiation and PPARgamma mRNA expression in 3T3-L1 adipocytes. Eur J Pharmacol. 2001;422:23-29.
55. Torti FM, Torti SV, Larrick JW et al. Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor beta. J Cell Biol. 1989;108:1105-1113.
56. Hofmann C, Lorenz K, Braithwaite SS et al. Altered gene expression for tumor necrosis factor-alpha and its receptors during drug and dietary modulation of insulin resistance. Endocrinology. 1994;134:264-270.
57. Okuno A, Tamemoto H, Tobe K et al. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. J Clin Invest. 1998;101:1354-1361.
58. Montague CT, O'Rahilly S. The perils of portliness: causes and consequences of visceral adiposity. Diabetes. 2000;49:883-888.
59. Niesler CU, Urso B, Prins JB et al. IGF-I inhibits apoptosis induced by serum withdrawal, but potentiates TNF-alpha-induced apoptosis, in 3T3-L1 preadipocytes. J Endocrinol. 2000;167:165-174.
60. Steppan CM, Bailey ST, Bhat S et al. The hormone resistin links obesity to diabetes. Nature. 2001;409:307-312.
61. Sreenan S, Keck S, Fuller T et al. Effects of troglitazone on substrate storage and utilization in insulin-resistant rats. Am J Physiol. 1999;276:E1119-E1129.
62. Shimabukuro M, Zhou YT, Lee Y et al. Troglitazone lowers islet fat and restores beta cell function of Zucker diabetic fatty rats. J Biol Chem. 1998;273:3547-3550.
63. Fonseca V, Rosenstock J, Patwardhan R et al. Effect of metformin and rosiglitazone combination therapy in patients with type 2 diabetes mellitus: a randomized controlled trial. JAMA. 2000;283:1695-1702.
64. Rebrin K, Steil GM, Getty L et al. Free fatty acid as a link in the regulation of hepatic glucose output by peripheral insulin. Diabetes. 1995;44:1038-1045.
65. Michaud SE, Renier G. Direct regulatory effect of fatty acids on macrophage lipoprotein lipase: potential role of PPARs. Diabetes. 2001;50:660-666.
66. Martin G, Schoonjans K, Staels B et al. PPARgamma activators improve glucose homeostasis by stimulating fatty acid uptake in the adipocytes. Atherosclerosis. 1998;137 Suppl:S75-S80.
67. Randle PJ. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. Diabetes Metab Rev. 1998;14:263-283.
68. Aitman TJ, Glazier AM, Wallace CA et al. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. Nat Genet. 1999;21:76-83.

69. Miyaoka K, Kuwasako T, Hirano K et al. CD36 deficiency associated with insulin resistance. Lancet. 2001;357:686-687.
70. Tontonoz P, Nagy L, Alvarez JG et al. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell. 1998;93:241-252.
71. Wolfrum C, Borrmann CM, Borchers T et al. Fatty acids and hypolipidemic drugs regulate peroxisome proliferator-activated receptors al. Proc Natl Acad Sci U S A. 2001;98:2323-2328.
72. Glorian M, Duplus E, Beale EG et al. A single element in the phosphoenolpyruvate carboxykinase gene mediates thiazolidinedione action specifically in adipocytes. Biochimie. 2001;83:933-943.
73. Yamauchi T, Kamon J, Waki H et al. The mechanisms by which both heterozygous PPARgamma deficiency and PPARgamma agonist improve insulin resistance. J Biol Chem. 2001.
74. Maeda N, Takahashi M, Funahashi T et al. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes. 2001;50:2094-2099.
75. Stephens JM, Lee J, Pilch PF. Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem. 1997;272:971-976.
76. Uysal KT, Wiesbrock SM, Marino MW et al. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature. 1997;389:610-614.
77. Zhang B, Berger J, Hu E et al. Negative regulation of peroxisome proliferator-activated receptor-gamma gene expression contributes to the antiadipogenic effects of tumor necrosis factor-alpha. Mol Endocrinol. 1996;10:1457-1466.
78. Feinstein R, Kanety H, Papa MZ et al. Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. J Biol Chem. 1993;268:26055-26058.
79. Fukuzawa M, Satoh J, Qiang X et al. Inhibition of tumor necrosis factor-alpha with anti-diabetic agents. Diabetes Res Clin Pract. 1999;43:147-154.
80. Peraldi P, Xu M, Spiegelman BM. Thiazolidinediones block tumor necrosis factor-alpha-induced inhibition of insulin signaling. J Clin Invest. 1997;100:1863-1869.
81. Iwata M, Haruta T, Usui I et al. Pioglitazone ameliorates tumor necrosis factor-alpha-induced insulin resistance by a mechanism independent of adipogenic activity of peroxisome proliferator--activated receptor-gamma. Diabetes. 2001;50:1083-1092.
82. De Vos P, Lefebvre AM, Miller SG et al. Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma. J Clin Invest. 1996;98:1004-1009.
83. Brown AA, Hu FB. Dietary modulation of endothelial function: implications for cardiovascular disease. Am J Clin Nutr. 2001;73:673-686.
84. Clarkson P, Celermajer DS, Donald AE et al. Impaired vascular reactivity in insulin-dependent diabetes mellitus is related to disease duration and low density lipoprotein cholesterol levels. J Am Coll Cardiol. 1996;28:573-579.
85. Baron AD. Vascular reactivity. Am J Cardiol. 1999;84:25J-27J.

86. Standl E, Schnell O. A new look at the heart in diabetes mellitus: from ailing to failing. Diabetologia. 2000;43:1455-1469.
87. Fujishima S, Ohya Y, Nakamura Y et al. Troglitazone, an insulin sensitizer, increases forearm blood flow in humans. Am J Hypertens. 1998;11:1134-1137.
88. Garg R, Kumbkarni Y, Aljada A et al. Troglitazone reduces reactive oxygen species generation by leukocytes and lipid peroxidation and improves flow-mediated vasodilatation in obese subjects. Hypertension. 2000;36:430-435.
89. Avena R, Mitchell ME, Nylen ES et al. Insulin action enhancement normalizes brachial artery vasoactivity in patients with peripheral vascular disease and occult diabetes. J Vasc Surg. 1998;28:1024-1031.
90. Inoguchi T, Li P, Umeda F et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes. 2000;49:1939-1945.
91. Kotchen TA, Zhang HY, Reddy S et al. Effect of pioglitazone on vascular reactivity in vivo and in vitro. Am J Physiol. 1996;270:R660-R666.
92. Zhang F, Sowers JR, Ram JL et al. Effects of pioglitazone on calcium channels in vascular smooth muscle. Hypertension. 1994;24:170-175.
93. Buchanan TA, Meehan WP, Jeng YY et al. Blood pressure lowering by pioglitazone. Evidence for a direct vascular effect. J Clin Invest. 1995;96:354-360.
94. Hattori Y, Hattori S, Kasai K. Troglitazone upregulates nitric oxide synthesis in vascular smooth muscle cells. Hypertension. 1999;33:943-948.
95. Yoshizumi M, Perrella MA, Burnett JC, Jr. et al. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res. 1993;73:205-209.
96. Wang P, Ba ZF, Chaudry IH. Administration of tumor necrosis factor-alpha in vivo depresses endothelium-dependent relaxation. Am J Physiol. 1994;266:H2535-H2541.
97. Nakamura M, Yoshida H, Arakawa N et al. Effects of tumor necrosis factor-alpha on basal and stimulated endothelium-dependent vasomotion in human resistance vessel. J Cardiovasc Pharmacol. 2000;36:487-492.
98. Ridker PM. High-sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease. Circulation. 2001;103:1813-1818.
99. Libby P. Changing concepts of atherogenesis. J Intern Med. 2000;247:349-358.
100. Margaglione M, Cappucci G, Colaizzo D et al. C-reactive protein in offspring is associated with the occurrence of myocardial infarction in first-degree relatives. Arterioscler Thromb Vasc Biol. 2000;20:198-203.
101. Pickup JC, Mattock MB, Chusney GD et al. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia. 1997;40:1286-1292.

102. Fichtlscherer S, Rosenberger G, Walter DH et al. Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. Circulation. 2000;102:1000-1006.
103. Marx N, Mackman N, Schonbeck U et al. PPARalpha Activators Inhibit Tissue Factor Expression and Activity in Human Monocytes. Circulation. 2001;103:213-219.
104. Neve BP, Corseaux D, Chinetti G et al. PPARalpha Agonists Inhibit Tissue Factor Expression in Human Monocytes and Macrophages. Circulation. 2001;103:207-212.
105. Pasceri V, Wu HD, Willerson JT et al. Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators. Circulation. 2000;101:235-238.
106. Jackson SM, Parhami F, Xi XP et al. Peroxisome proliferator-activated receptor activators target human endothelial cells to inhibit leukocyte-endothelial cell interaction. Arterioscler Thromb Vasc Biol. 1999;19:2094-2104.
107. Ricote M, Li AC, Willson TM et al. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature. 1998;391:79-82.
108. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature. 1998;391:82-86.
109. Nagy L, Tontonoz P, Alvarez JG et al. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell. 1998;93:229-240.
110. Chawla A, Barak Y, Nagy L et al. PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med. 2001;7:48-52.
111. Marx N, Sukhova G, Murphy C et al. Macrophages in human atheroma contain PPARgamma: differentiation-dependent peroxisomal proliferator-activated receptor gamma(PPARgamma) expression and reduction of MMP-9 activity through PPARgamma activation in mononuclear phagocytes in vitro. Am J Pathol. 1998;153:17-23.
112. Thieringer R, Fenyk-Melody JE, Le Grand CB et al. Activation of peroxisome proliferator-activated receptor gamma does not inhibit IL-6 or TNF-alpha responses of macrophages to lipopolysaccharide in vitro or in vivo. J Immunol. 2000;164:1046-1054.
113. Moore KJ, Fitzgerald ML, Freeman MW. Peroxisome proliferator-activated receptors in macrophage biology: friend or foe? Curr Opin Lipidol. 2001;12:519-527.
114. Li AC, Brown KK, Silvestre MJ et al. Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. J Clin Invest. 2000;106:523-531.
115. Abumrad N, Harmon C, Ibrahimi A. Membrane transport of long-chain fatty acids: evidence for a facilitated process. J Lipid Res. 1998;39:2309-2318.
116. Febbraio M, Podrez EA, Smith JD et al. Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. J Clin Invest. 2000;105:1049-1056.
117. Chinetti G, Lestavel S, Bocher V et al. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. Nat Med. 2001;7:53-58.

118. Plutzky J. Peroxisome proliferator-activated receptors in endothelial cell biology. Curr Opin Lipidol. 2001;12:511-518.

119. Aitman TJ. CD36, insulin resistance, and coronary heart disease. Lancet. 2001;357:651-652.

120. Takano H, Nagai T, Asakawa M et al. Peroxisome proliferator-activated receptor activators inhibit lipopolysaccharide-induced tumor necrosis factor-alpha expression in neonatal rat cardiac myocytes. Circ Res. 2000;87:596-602.

121. Ginsberg HN, Huang LS. The insulin resistance syndrome: impact on lipoprotein metabolism and atherothrombosis. J Cardiovasc Risk. 2000;7:325-331.

122. Kraegen EW, Cooney GJ, Ye J et al. Triglycerides, fatty acids and insulin resistance--hyperinsulinemia. Exp Clin Endocrinol Diabetes. 2001;109:S516-S526.

123. Fontbonne A, Eschwege E, Cambien F et al. Hypertriglyceridaemia as a risk factor of coronary heart disease mortality in subjects with impaired glucose tolerance or diabetes. Results from the 11-year follow-up of the Paris Prospective Study. Diabetologia. 1989;32:300-304.

124. Laakso M, Lehto S, Penttila I et al. Lipids and lipoproteins predicting coronary heart disease mortality and morbidity in patients with non-insulin-dependent diabetes. Circulation. 1993;88:1421-1430.

125. Syvanne M, Taskinen MR. Lipids and lipoproteins as coronary risk factors in non-insulin-dependent diabetes mellitus. Lancet. 1997;350 Suppl 1:SI20-SI23.

126. Rosenblatt S, Miskin B, Glazer NB et al. The impact of pioglitazone on glycemic control and atherogenic dyslipidemia in patients with type 2 diabetes mellitus. Coron Artery Dis. 2001;12:413-423.

127. Yamasaki Y, Kawamori R, Wasada T et al. Pioglitazone (AD-4833) ameliorates insulin resistance in patients with NIDDM. AD-4833 Glucose Clamp Study Group, Japan. Tohoku J Exp Med. 1997;183:173-183.

128. Gegick CG, Altheimer MD. Comparison of effects of thiazolidinediones on cardiovascular risk factors: observations from a clinical practice. Endocr Pract. 2001;7:162-169.

129. Auwerx J, Schoonjans K, Fruchart JC et al. Regulation of triglyceride metabolism by PPARs: fibrates and thiazolidinediones have distinct effects. J Atheroscler Thromb. 1996;3:81-89.

130. Raskin P, Rendell M, Riddle MC et al. A randomized trial of rosiglitazone therapy in patients with inadequately controlled insulin-treated type 2 diabetes. Diabetes Care. 2001;24:1226-1232.

131. Boyle PJ, King AB, Olansky L et al. Effects of pioglitazone and rosiglitazone on blood lipid levels and glycemic control in patients with type 2 diabetes mellitus: a retrospective review of randomly selected medical records. Clin Ther. 2002;24:378-396.

132. Khan MA, St Peter JV, Xue JL. A prospective, randomized comparison of the metabolic effects of pioglitazone or rosiglitazone in patients with type 2 diabetes who were previously treated with troglitazone. Diabetes Care. 2002;25:708-711.

133. Hirano T, Yoshino G, Kazumi T. Troglitazone and small low-density lipoprotein in type 2 diabetes. Ann Intern Med. 1998;129:162-163.

134. Tack CJ, Smits P, Demacker PN et al. Troglitazone decreases the proportion of small, dense LDL and increases the resistance of LDL to oxidation in obese subjects. Diabetes Care. 1998;21:796-799.

135. Cominacini L, Young MM, Capriati A et al. Troglitazone increases the resistance of low density lipoprotein to oxidation in healthy volunteers. Diabetologia. 1997;40:1211-1218.
136. Cominacini L, Garbin U, Fratta PA et al. Troglitazone reduces LDL oxidation and lowers plasma E-selectin concentration in NIDDM patients. Diabetes. 1998;47:130-133.
137. Chen Z, Ishibashi S, Perrey S et al. Troglitazone inhibits atherosclerosis in apolipoprotein E-knockout mice: pleiotropic effects on CD36 expression and HDL. Arterioscler Thromb Vasc Biol. 2001;21:372-377.
138. Colca JR, Dailey CF, Palazuk BJ et al. Pioglitazone hydrochloride inhibits cholesterol absorption and lowers plasma cholesterol concentrations in cholesterol-fed rats. Diabetes. 1991;40:1669-1674.
139. Mimura K, Umeda F, Hiramatsu S et al. Effects of a new oral hypoglycaemic agent (CS-045) on metabolic abnormalities and insulin resistance in type 2 diabetes. Diabet Med. 1994;11:685-691.
140. Ogihara T, Rakugi H, Ikegami H et al. Enhancement of insulin sensitivity by troglitazone lowers blood pressure in diabetic hypertensives. Am J Hypertens. 1995;8:316-320.
141. Sung BH, Izzo JL, Dandona P et al. Vasodilatory effects of troglitazone improve blood pressure at rest and during mental stress in type 2 diabetes mellitus. Hypertension. 1999;34:83-88.
142. Ghazzi MN, Perez JE, Antonucci TK et al. Cardiac and glycemic benefits of troglitazone treatment in NIDDM. The Troglitazone Study Group. Diabetes. 1997;46:433-439.
143. Tack CJ, Ong MK, Lutterman JA et al. Insulin-induced vasodilatation and endothelial function in obesity/insulin resistance. Effects of troglitazone. Diabetologia. 1998;41:569-576.
144. Kaufman LN, Peterson MM, DeGrange LM. Pioglitazone attenuates diet-induced hypertension in rats. Metabolism. 1995;44:1105-1109.
145. Grinsell JW, Lardinois CK, Swislocki A et al. Pioglitazone attenuates basal and postprandial insulin concentrations and blood pressure in the spontaneously hypertensive rat. Am J Hypertens. 2000;13:370-375.
146. Walker AB, Chattington PD, Buckingham RE et al. The thiazolidinedione rosiglitazone (BRL-49653) lowers blood pressure and protects against impairment of endothelial function in Zucker fatty rats. Diabetes. 1999;48:1448-1453.
147. Zhang HY, Reddy SR, Kotchen TA. Antihypertensive effect of pioglitazone is not invariably associated with increased insulin sensitivity. Hypertension. 1994;24:106-110.
148. Knock GA, Mishra SK, Aaronson PI. Differential effects of insulin-sensitizers troglitazone and rosiglitazone on ion currents in rat vascular myocytes. Eur J Pharmacol. 1999;368:103-109.
149. Pershadsingh HA, Szollosi J, Benson S et al. Effects of ciglitazone on blood pressure and intracellular calcium metabolism. Hypertension. 1993;21:1020-1023.
150. Law RE, Meehan WP, Xi XP et al. Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia. J Clin Invest. 1996;98:1897-1905.
151. Minamikawa J, Tanaka S, Yamauchi M et al. Potent inhibitory effect of troglitazone on carotid arterial wall thickness in type 2 diabetes. J Clin Endocrinol Metab. 1998;83:1818-1820.

152. Takagi T, Akasaka T, Yamamuro A et al. Troglitazone reduces neointimal tissue proliferation after coronary stent implantation in patients with non-insulin dependent diabetes mellitus: a serial intravascular ultrasound study. J Am Coll Cardiol. 2000;36:1529-1535.
153. Koshiyama H, Shimono D, Kuwamura N et al. Rapid communication: inhibitory effect of pioglita zone on carotid arterial wall thickness in type 2 diabetes. J Clin Endocrinol Metab. 2001;86:3452-3456.
154. Igarashi M, Takeda Y, Ishibashi N et al. Pioglitazone reduces smooth muscle cell density of rat carotid arterial intima induced by balloon catheterization. Horm Metab Res. 1997;29:444-449.
155. Yoshimoto T, Naruse M, Shizume H et al. Vasculo-protective effects of insulin sensitizing agent pioglitazone in neointimal thickening and hypertensive vascular hypertrophy. Atherosclerosis. 1999;145:333-340.
156. Goetze S, Xi XP, Kawano H et al. PPAR gamma-ligands inhibit migration mediated by multiple chemoattractants in vascular smooth muscle cells. J Cardiovasc Pharmacol. 1999;33:798-806.
157. Howard G, O'Leary DH, Zaccaro D et al. Insulin sensitivity and atherosclerosis. The Insulin Resistance Atherosclerosis Study (IRAS) Investigators. Circulation. 1996;93:1809-1817.
158. Fonseca VA, Reynolds T, Hemphill D et al. Effect of troglitazone on fibrinolysis and activated coagulation in patients with non-insulin-dependent diabetes mellitus. J Diabetes Complications. 1998;12:181-186.
159. Kato K, Satoh H, Endo Y et al. Thiazolidinediones down-regulate plasminogen activator inhibitor type 1 expression in human vascular endothelial cells: A possible role for PPARgamma in endothelial function. Biochem Biophys Res Commun. 1999;258:431-435.
160. Schwartz S, Raskin P, Fonseca V et al. Effect of troglitazone in insulin-treated patients with type II diabetes mellitus. Troglitazone and Exogenous Insulin Study Group. N Engl J Med. 1998;338:861-866.
161. Pickavance L, Widdowson PS, King P et al. The development of overt diabetes in young Zucker Diabetic Fatty (ZDF) rats and the effects of chronic MCC-555 treatment. Br J Pharmacol. 1998;125:767-770.
162. Tafuri SR. Troglitazone enhances differentiation, basal glucose uptake, and Glut1 protein levels in 3T3-L1 adipocytes. Endocrinology. 1996;137:4706-4712.
163. Akazawa S, Sun F, Ito M et al. Efficacy of troglitazone on body fat distribution in type 2 diabetes. Diabetes Care. 2000;23:1067-1071.
164. Mori Y, Murakawa Y, Okada K et al. Effect of troglitazone on body fat distribution in type 2 diabetic patients. Diabetes Care. 1999;22:908-912.
165. Kelly IE, Han TS, Walsh K et al. Effects of a thiazolidinedione compound on body fat and fat distribution of patients with type 2 diabetes. Diabetes Care. 1999;22:288-293.
166. Fukunaga Y, Itoh H, Doi K et al. Thiazolidinediones, peroxisome proliferator-activated receptor gamma agonists, regulate endothelial cell growth and secretion of vasoactive peptides. Atherosclerosis. 2001;158:113-119.
167. Al Salman J, Arjomand H, Kemp DG et al. Hepatocellular injury in a patient receiving rosiglitazone. A case report. Ann Intern Med. 2000;132:121-124.

168. Forman LM, Simmons DA, Diamond RH. Hepatic failure in a patient taking rosiglitazone. Ann Intern Med. 2000;132:118-121.

169. Lebovitz HE, Kreider M, Freed MI. Evaluation of Liver Function in Type 2 Diabetic Patients During Clinical Trials: Evidence that rosiglitazone does not cause hepatic dysfunction. Diabetes Care. 2002;25:815-821.

170. Yamazaki H, Suzuki M, Tane K et al. In vitro inhibitory effects of troglitazone and its metabolites on drug oxidation activities of human cytochrome P450 enzymes: comparison with pioglitazone and rosiglitazone. Xenobiotica. 2000;30:61-70.

171. Arlt W, Auchus RJ, Miller WL. Thiazolidinediones but not metformin directly inhibit the steroidogenic enzymes P450c17 and 3beta -hydroxysteroid dehydrogenase. J Biol Chem. 2001;276:16767-16771.

172. Prueksaritanont T, Vega JM, Zhao J et al. Interactions between simvastatin and troglitazone or pioglitazone in healthy subjects. J Clin Pharmacol. 2001;41:573-581.

CHAPTER 4

Short-term pioglitazone treatment improves vascular function irrespective of metabolic changes in patients with type 2 diabetes

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Condensed abstract

To determine whether pioglitazone influences endothelial function directly we examined in a randomised, cross-over, placebo-controlled, double blind trial, the effects of 4 weeks pioglitazone treatment in 20 male type 2 diabetic patients.
We conclude that short-term pioglitazone treatment ameliorates endothelial dysfunction in conduit arteries irrespective of significant beneficial changes in plasma levels of insulin, FFA, adiponectin or CRP in type 2 diabetics.

Abstract

Objective Pioglitazone, a PPAR-γ agonist, does not only improve insulin resistance and glycemic control but may also have additional beneficial vascular effects in patients with type 2 diabetes. Low-grade inflammation, free fatty acids and adiponectin may play a role in modulation of vascular function. We studied the effect of 4 week pioglitazone treatment on endothelial function, metabolic changes and C-reactive protein in patients with type 2 diabetes.

Methods and Results A randomised, cross-over, placebo-controlled, double blind trial was performed in which pioglitazone 30 mg once daily was administered in 20 patients with type 2 diabetes on oral anti-hyperglycemic agents for 4 weeks. Shear stress-induced flow mediated dilation (FMD) of the brachial artery was used as outcome parameter for vascular function.
Brachial artery endothelial function was significantly increased by pioglitazone treatment compared to placebo (FMD 5.4±0.5 vs. 3.1±0.5 %, p=0.001). Endothelium-independent vasodilation was not different between the two study periods. Pioglitazone treatment reduced insulin, FFA and C-reactive protein concentrations compared to placebo (18.3±2.4 vs. 14.8±2.1 mU/l; p=0.03, 641±46 vs. 542±33 μmol/l; p=0.04 and 3.5±0.6 mg/l vs. 2.6±0.5; p= 0.01, respectively). A significant increase in plasma adiponectin concentration (3.95±0.57μg/ml vs. 7.59±0.95; p=0.002) was also observed. No correlations were found between these metabolic changes and the improvement of conduit artery endothelial function.

Conclusions Short-term pioglitazone treatment ameliorates endothelial dysfunction in conduit arteries irrespective of changes in insulin, FFA, adiponectin or CRP in type 2 diabetics.

Keywords adiponectin, C-reactive protein, diabetes mellitus, endothelial function, nitric oxide, pioglitazone.

Introduction

Patients with type 2 diabetes have a high risk to develop micro- and macrovascular complications.[1] Insulin resistance, which drives hypertension, glucose intolerance, endothelial dysfunction, dyslipidemia and a proinflammatory state, is generally considered to be of major importance in the pathophysiology of type 2 diabetes and cardiovascular complications. A therapeutic strategy that targets insulin resistance in type 2 diabetes may have beneficial effects on the cardiovascular risk.

Thiazolidinediones (TZDs) are a class of oral anti-hyperglycemic agents that can improve insulin resistance. TZDs such as pioglitazone and rosiglitazone are ligands for peroxisome proliferator-activated receptors (PPARs), in particular PPAR-γ. These nuclear transcription factors are involved in glucose homeostasis, lipid and lipoprotein metabolism and adipogenesis. TZDs improve glycemic control by increasing insulin sensitivity and have also been shown to favorably modulate other components of the metabolic syndrome.[2] In addition, pparγ-agonists may have direct effects on vessel wall structure, inflammation and endothelial function.

Endothelial dysfunction, metabolic abnormalities related to insulin resistance and a proinflammatory state, are characteristic of patients with type 2 diabetes. Endothelial dysfunction is characterised by decreased availability of endothelium-derived nitric oxide (NO) and can be assessed clinically by impaired vasoreactivity of the brachial artery after an ischemic stimulus with flow-mediated dilation.[3] Previous studies showed already improvement of endothelial vasoreactivity in insulin resistant patients (obesity, polycystic ovary syndrome and type 2 diabetes) with TZD treatment. However many studies were done with troglitazone, now withdrawn from the market, and/or with long treatment periods.[4-7] In a non-randomised, open-label study, pioglitazone treatment for 3 months resulted in improvement of endothelial function in type 2 diabetic patients.[8] In this study pioglitazone treatment also significantly reduced the metabolic abnormalities associated with type 2 diabetes. To tease out a direct vascular effect on vascular function of pioglitazone from its indirect metabolic effects we studied, double blind and crossover, the direct vascular effect of short-term (4 weeks) treatment with pioglitazone on endothelial function in patients with type 2 diabetes.

Subjects and methods

Subjects

Twenty male, non-smoking patients with type 2 diabetes were recruited. All patients were treated with oral anti-hyperglycemic agents (9 patients with sulphonylurea derivates, 4 patients with metformin and 7 patients with a combination of metformin and sulphonylurea derivates) which continued during the study. Subjects with poor glycemic control (HbA1c> 9%) were not included. Other relevant exclusion criteria were presence of macro- or microvascular disease and use of vasoactive medication (e.g. beta-blockers, calcium entry blockers, ACE-inhibitors, angiotensin type 1 receptor blockers, statins, aspirin, non-steroidal inflammatory drugs).
The protocol was approved by the ethical review board of the University Medical Center Utrecht (UMCU). All subjects gave written informed consent. Measurements were carried out in accordance with local institutional guidelines in a Good Clinical Practice-certified unit.

Study design

The study was designed as a prospective, randomised, crossover, placebo-controlled, double blind trial. Patients eligible to take part in the study were randomised to receive pioglitazone 30 mg once daily (Eli Lilly, Indianapolis, U.S.A.) or placebo for 4 weeks in addition to their oral anti-hyperglycemic agents. Forearm vascular function was performed after four weeks followed by a washout period of six weeks. Crossover of therapy occurred at the end of the washout period with reanalysis of vascular function at 4 weeks. At the beginning and at the end of each 4-week treatment period laboratory parameters were determined. Patients were instructed to fast for at least 10 hours prior to the tests. No study medication or other medication was used on the morning of the study days.

Methods

Assessment of vascular function

Experiments were performed in a temperature-controlled room (22-24°C) in the morning. Vascular function tests after pioglitazone and placebo treatment were performed at the same time for each individual in order to exclude daytime variability. Upon arrival after an overnight fast, patients were asked to rest ina supine position for 20 minutes before ultrasonographic assessment of post-ischemic flow-mediated vasodilation (FMD) of the brachial artery. This procedure was followed by blood withdrawal for laboratory measurements.

For assessment of post-ischemic NO-dependent FMD of the brachial artery, ultrasound measurements were performed at the elbow of the right arm using a vessel wall-movement system (Wall Track System, Pie Medical) that consists of a 10 MHz linear array transducer connected to a data acquisition system and a personal computer.[9] In order to optimize quality of the ultrasound images of the arterial wall, ultrasound gel, a 200 ml water bag (as conductive medium), and a fixed probe holder were used. An optimal two-dimensional B-mode image of the brachial artery was obtained. An M-line perpendicular to the vessel was selected and the ultrasound system was switched to M-mode. The vessel-movement detector system registers the end-diastolic vessel diameter repeatedly during a period of 12 seconds. The first three measurements were averaged to provide a baseline arterial diameter. By inflation of a blood pressure cuff for 5 minutes at a pressure of 200 mmHg, ischemia was applied to the forearm distal to the location of the transducer. Ultrasonographic measurements continued for 4 minutes after cuff release at 30-second intervals. The widest lumen diameter was taken as a measure for maximal post-ischemic vasodilation. In eight patients sublingual nitroglycerine spray (400μg) was administered after a 15-minute rest in order to allow the arterial diameter to return to its baseline value. After 2, 3, 4 and 5 minutes the vasodilatory respons to nitroglycerine was assessed as previously described. FMD and nitroglycerine-induced vasodilation were calculated as the percentage change relative to the baseline diameter.

Laboratory assessment

Fasting blood was drawn and plasma was frozen at –20°C until further analysis. Glucose, creatinine, serum alanine transferase (ALT), serum aspartate transferase (AST), total cholesterol, triglycerides and high-density lipoprotein cholesterol (HDL-C) were measured by standard enzymatical laboratory methods (Vitros 250; Johnson/Johnson). Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula. HbA1c and free fatty acids (FFA) were photometrically performed (Hitachi 911; Roche). Insulin levels were determined with an immunological method (Immulite 2000; Diagnostic Prod Corp.). Measurements of plasma adiponectin, interleukin-6 (IL-6) and high-sensitive C-reactive protein (hs-CRP) were performed with a commercially available kit (ELISA; R&D Systems Inc.).

Statistical analysis

Results from vascular function tests and laboratory analysis are expressed as mean + standard error. Differences in FMD and metabolic parameters between pioglitazone and placebo treatment were analysed with a paired t-test (95% confidence interval). In case of non-normal distribution the Wilcoxon Signed Rank test was used. Statistical

significance was taken at the 5% level. Correlations between the change in FMD and the change in metabolic or inflammatory parameters were analysed by linear regresion.

Results

All subjects completed the study. The demographic, clinical and laboratory characteristics are shown in Table 1. No carry-over effects between the two treatment periods were observed for any parameter.

Table 1. Clinical characteristics at baseline and cross-over

	Baseline values at study start with placebo (N=10)	**Baseline values at crossover to pioglitazone** (N=10)	**Baseline values at study start with pioglitazone** (N=10)	**Baseline values at crossover to placebo** (N=10)
Age (yrs)	58±2	58±2	55±3	55±3
Weight (kg)	86.6±5.2	89.0±5.2	98.2±5.7	99.0±5.7
BMI (kg/m2)	28.8±1.6	29.3±1.6	29.9±1.6	30.2±1.6
Waist (cm)	104.6±2.8	104.6±2.8	111.8±4.2	111.8±4.2
SBP (mmHg)	159±4	148±5	138±7	142±7
DBP (mmHg)	92±2	90±2	85±3	90±3
Fasting glucose (mmol/l)	7.9±0.8	6.3±0.5	9.1±0.5	8.1±0.6
Insulin (mU/l)	18.3±5.0	14.1±2.4	23.5±4.	20.6±3.3
HOMA-R	7.5±2.7	7.7±2.7	10.2±2.2	7.6±1.4
HbA1c (%)	7.0±0.4	6.6±0.4	7.1±0.3	6.9±0.3
Total cholesterol (mmol/l)	5.7±0.3	5.2±0.2	5.8±0.3	5.6±0.3
HDL cholesterol (mmol/l)	1.21±0.08	1.20±0.08	1.14±0.06	1.10±0.09
LDL cholesterol (mmol/l)	3.4±0.28	3.2±0.2	3.8±0.19	3.7±0.19
Triglycerides (mmol/l)	2.24±0.43	1.75±0.25	1.84±0.32	1.80±0.27
Free fatty acids (μmol/l)	656±80	615±62	699±101	654±77
Adiponectin (ng/ml)	5073±954	4143±648	4759±926	3497±657
ASAT (U/l)	35±3	32±3	27±3	27±5
ALAT (U/l)	40±7	34±4	37±8	39±10
Creatinine (μmol/l)	79±4	78±4	74±1	76±2
Hematocriet (l/l)	0.45±0.01	0.44±0.01	0.46±0.01	0.46±0.01
hs-CRP (mg/l)	3.3±0.5	4.3±0.8	4.2±1.1	4.2±0.9
Interleukin-6 (pg/ml)	2.18±0.60	2.43±0.41	1.99±0.39	2.17±0.33

Values are mean ±SEM

Influence of pioglitazone on anthropometric, hemodynamic and metabolic parameters
Table 2 reports the outcome of pioglitazone- or placebo treatment on anthropometric, hemodynamic and metabolic parameters.
Short-term pioglitazone treatment for 4 weeks already reduced insulin and FFA concentrations compared to placebo (18.3±2.4 vs. 14.8±2.1 mU/l; p=0.03 and 641±46 vs. 542±33 μmol/l; p=0.04, respectively).

A significant increase in adiponectin plasma levels was observed during pioglitazone treatment compared to placebo after only 4 weeks (3948±573 vs. 7591±953 ng/ml, p=0.002).

Table 2. Clinical characteristics after treatment

	Placebo (N=20)	**Pioglitazone** (N=20)	**p-value**
Weight (kg)	94.4±4.1	94.6±3.9	0.68
SBP (mmHg)	136±4	135±3	0.94
DBP (mmHg)	91±2	87±2	0.89
Fasting plasma glucose (mmol/l)	7.1±0.4	6.7±0.4	0.19
Insulin (mU/l)	18.3±2.4	14.8±2.1	0.03*
HOMA-R	6.1±1.1	4.9±0.9	0.06
HbA1c (%)	6.8±0.2	6.7±0.2	0.50
Total cholesterol (mmol/l)	4.9±0.2	5.0±0.2	0.38
HDL cholesterol (mmol/l)	1.02±0.04	1.04±0.05	0.36
LDL cholesterol (mmol/l)	3.1±0.14	3.2±0.16	0.69
Triglycerides (mmol/l)	1.60±0.12	1.59±0.17	0.60
Free fatty acids (μmol/l)	641±46	542±33	0.04*
Adiponectin (ng/ml)	3948±573	7591±953	0.002*
ASAT (U/l)	27±2	28±3	0.88
ALAT (U/l)	34±5	35±5	0.65
Creatinine(μmol/l)	72±2	69±2	0.09
Hematocriet (l/l)	0.43±0.01	0.41±0.01	0.08
CRP (mg/l)	3.5±0.6	2.6±0.5	0.01*
Interleukin-6 (pg/ml)	1.71±0.21	1.62±0.22	0.17

Values are mean ±SEM;
p-values are reported for comparison between placebo and pioglitazone
* p-values < 0.05

Furthermore a significant decrease in CRP concentration was observed during pioglitazone treatment compared to placebo after only 4 weeks (3.5±0.6 vs. 2.6±0.5 mg/l, p=0.01) while IL-6 plasma concentrations did not change significantly.

We did not observe a difference in the effects of pioglitazone between patients using metformin or a combination of metformin and sulphonylurea derivates and the group of patients only treated with sulphonylurea derivates (data not shown). No change in weight or blood pressure was observed during pioglitazone treatment. Fasting glucose concentrations and lipids were not different between the pioglitazone and placebo treatment periods. Edema did not occur in any patient on pioglitazone treatment. Pioglitazone treatment was not associated with liver enzyme abnormalities.

Effects of pioglitazone on brachial artery vasoreactivity

The baseline arterial diameter was similar during pioglitazone and placebo treatment (Table 3).

Table 3. Ultrasound assessment of brachial artery vasoreactivity

	Placebo	Pioglitazone	p-value
Baseline diameter (mm)	4.45±0.10	4.44±0.10	0.39
Post-ischemic maximum diameter (mm)	4.59±0.11	4.68±0.11	0.01*
FMD after ischemia (%)	3.08±0.53	5.38±0.46	0.001*
Nitroglycerin-induced maximum diameter (mm)	5.14±0.10	5.24±0.10	0.17
Nitroglycerin-induced vasodilation (%)	12.04±1.09	14.49±1.36	0.09

Values are mean ±SEM;

p-values are reported for difference between placebo and pioglitazone after 4 weeks of treatment.

* p-values < 0.05

Flow-mediated vasodilation (FMD) was significantly increased by pioglitazone (3.08±0.53% vs. 5.38±0.46, p=0.001) (Table 3). As ultrasound recordings of the post-ischemic arterial diameter were performed every 30 seconds during 5 minutes in our protocol, a difference between post-ischemic vasoreactivity at all time points between pioglitazone and placebo treatment could be observed (Figure 1).

Figure 1. Post-ischemic flow-mediated vasodilation

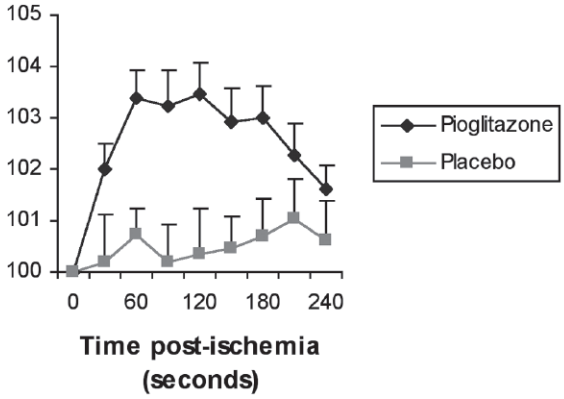
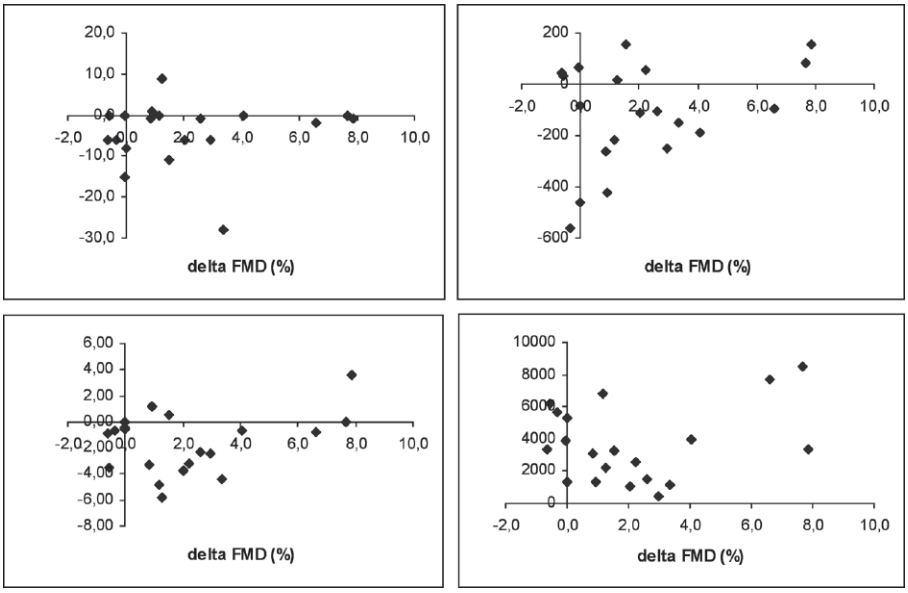


Figure 2. Relationship between the change in FMD and the change in insulin, free fatty acids, CRP and adiponectin after short-term pioglitazone treatment



There were no significant differences in the effects of pioglitazone between the group of patients using metformin or a combination of metformin and sulphonylurea derivates and the group of patients only treated with sulphonylurea derivates. Nitroglycerin-induced endothelium-independent vasodilation of the brachial artery was not significantly different between both treatments (12.04±1.09% vs. 14.49±1.36, p=0.09) indicating that there seemed to be no alteration of large vessel vascular smooth muscle cell (VSMC) responsiveness to NO (Table 3).

Correlation between endothelial function, metabolic and inflammatory changes
We used a study-design with short-term pioglitazone treatment to avoid known long-term metabolic improvement of insulin sensitivity to investigate direct vascular effects of pioglitazone. Although already after treatment for 4 weeks, significant beneficial changes in insulin, free fatty acids, adiponectin and CRP were observed in our study no correlation was present between the beneficial changes in insulin, FFA, adiponectin or CRP, and the improvement of FMD (Figure 2).

Discussion

In the current study we demonstrate that short-term pioglitazone treatment ameliorates endothelial dysfunction in conduit arteries irrespective of significant beneficial changes in plasma levels of insulin, FFA, adiponectin or CRP in type 2 diabetics.

To tease out metabolic effects from direct vascular effects we aimed to study relatively short-term actions of pioglitazone rather than long-term effects. Long-term TZD treatment has been shown to significantly change body composition and to have profound effects on the glycemic and lipid profile. The first metabolic effects were found as early as the 2-4th week of therapy but maximal decreases occurred after 10-14 weeks[10-12]. Most clinical studies observed substantial improvements in metabolic parameters by TZDs after 8- or more weeks of treatment.[13] These long-term anthropometric and metabolic alterations are likely to significantly modulate vascular function and inflammatory parameters. Using a 4-week treatment period we did not observe significant differences in fasting lipids and glucose between the pioglitazone and placebo treatment period. Nevertheless, insulin, FFA, adiponectin

and CRP concentrations were beneficially changed already. No relation was found between the changes in insulin, FFA, adiponectin or CRP, and the improvement of FMD. However, in view of the limited number of patients studied, such a relation can also not be entirely excluded.
Our results are in line with previous studies showing antiatherogenic effects of pioglitazone in type 2 diabetic patients independent of its antidiabetic effect.[8,14] An explanation could be the direct effects on the vascular wall of TZDs. PPAR-γ mRNA is expressed in endothelial and vascular smooth muscle cells.[15] TZDs increase NO release from endothelial cells without altering expression of endothelial nitric oxide synthase.[16] A direct effect on NO availability by pioglitazone may have contributed to the increase in FMD.
Other direct effects of pioglitazone on vascular smooth muscle cells *in vitro* have also been observed. These actions include attenuation of vasoconstriction as well as inhibition of L-type Ca^{2+} currents in VSMC.[17,18] In order to study vascular smooth muscle responsiveness to NO we administered nitroglycerin, a nitric oxide donor, before ultrasound assessment of brachial artery diameter. No significant difference was observed in endothelium-independent vasodilation between placebo and pioglitazone treatment indicating that pioglitazone does not seem to alter large vessel VSMC responsiveness to NO.

Interestingly, pioglitazone also significantly decreased C-reactive protein (CRP) after only four weeks of therapy. Concentrations of CRP are increased in patients with type 2 diabetes. This is considered to reflect a low-grade inflammatory state that is likely to contribute to the increased cardiovascular morbidity and mortality in patients with type 2 diabetes. CRP concentrations correlate with several components of the metabolic syndrome[19] and CRP is considered a strong predictor of cardiovascular events. Our results are in accordance with a study by Haffner et al. who showed a reduction in CRP after 26 weeks of treatment with another TZD, rosiglitazone.[20]

Although our data suggest no major role for adiponectin, FFA or insulin in improvement of conduit artery endothelial function during pioglitazone, the sample size is a limiting factor to more reliably assess the possible existence of a relationship between these parameters and endothelial function. An increase in adiponectin by pioglitazone has previously been shown[8] and adiponectin can directly stimulate NO production by phosphorylating eNOS by AMP-activated protein kinase in

endothelial cells *in vitro*.[21] High levels of FFA impair NO production by generation of reactive oxygen species (ROS).[22] In addition, FFA decrease insulin sensitivity in peripheral tissues. We included subjects who were only using oral anti-hyperglycemic agents. Since insulin could have profound effects on the vasculature, caution should be taken to extrapolate our results to patients with type 2 diabetes who use combination treatment consisting of pioglitazone and insulin. In addition, our patients did not use vasoactive medication. Many patients with type 2 diabetes use pharmacologic agents such as angiotensin-converting enzym blockers, angiotensin-II antagonists and statins improve endothelial function. It remains to be determined whether pioglitazone has additional benefits on endothelial function in these patients.

In conclusion, 4-weeks pioglitazone treatment improves conduit artery endothelial function, independent of metabolic changes in patients with type 2 diabetes. This may contribute to decreased cardiovascular risk in these patients.

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References

1. Watson KE, Peters Harmel AL, Matson G. Atherosclerosis in type 2 diabetes mellitus: the role of insulin resistance. J Cardiovasc Pharmacol Ther. 2003;8:253-260.
2. Martens FM, Visseren FL, Lemay J et al. Metabolic and additional vascular effects of thiazolidinediones. Drugs. 2002;62:1463-1480.
3. Raitakari OT, Celermajer DS. Flow-mediated dilatation. Br J Clin Pharmacol. 2000;50:397-404.
4. Caballero AE, Saouaf R, Lim SC et al. The effects of troglitazone, an insulin-sensitizing agent, on the endothelial function in early and late type 2 diabetes: a placebo-controlled randomized clinical trial. Metabolism. 2003;52:173-180.
5. Paradisi G, Steinberg HO, Shepard MK et al. Troglitazone therapy improves endothelial function to near normal levels in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2003;88:576-580.
6. Tack CJ, Ong MK, Lutterman JA et al. Insulin-induced vasodilatation and endothelial function in obesity/insulin resistance. Effects of troglitazone. Diabetologia. 1998;41:569-576.
7. Wang TD, Chen WJ, Lin JW et al. Effects of rosiglitazone on endothelial function, C-reactive protein, and components of the metabolic syndrome in nondiabetic patients with the metabolic syndrome. Am J Cardiol. 2004;93:362-365.
8. Satoh N, Ogawa Y, Usui T et al. Antiatherogenic effect of pioglitazone in type 2 diabetic patients irrespective of the responsiveness to its antidiabetic effect. Diabetes Care. 2003;26:2493-2499.
9. Joannides R, Haefeli WE, Linder L et al. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation. 1995;91:1314-1319.
10. Aronoff S, Rosenblatt S, Braithwaite S et al. Pioglitazone hydrochloride monotherapy improves glycemic control in the treatment of patients with type 2 diabetes: a 6-month randomized placebo-controlled dose-response study. The Pioglitazone 001 Study Group. Diabetes Care. 2000;23:1605-1611.
11. Einhorn D, Rendell M, Rosenzweig J et al. Pioglitazone hydrochloride in combination with metformin in the treatment of type 2 diabetes mellitus: a randomized, placebo-controlled study. The Pioglitazone 027 Study Group. Clin Ther. 2000;22:1395-1409.
12. Rosenblatt S, Miskin B, Glazer NB et al. The impact of pioglitazone on glycemic control and atherogenic dyslipidemia in patients with type 2 diabetes mellitus. Coron Artery Dis. 2001;12:413-423.
13. Yki-Jarvinen H. Thiazolidinediones. N Engl J Med. 2004;351:1106-1118.
14. Langenfeld MR, Forst T, Hohberg C et al. Pioglitazone Decreases Carotid Intima-Media Thickness Independently of Glycemic Control in Patients With Type 2 Diabetes Mellitus. Results From a Controlled Randomized Study. Circulation. 2005.
15. Hattori Y, Hattori S, Kasai K. Troglitazone upregulates nitric oxide synthesis in vascular smooth muscle cells. Hypertension. 1999;33:943-948.
16. Calnek DS, Mazzella L, Roser S et al. Peroxisome proliferator-activated receptor gamma ligands increase release of nitric oxide from endothelial cells. Arterioscler Thromb Vasc Biol. 2003;23:52-57.

17. Song J, Walsh MF, Igwe R et al. Troglitazone reduces contraction by inhibition of vascular smooth muscle cell Ca^{2+} currents and not endothelial nitric oxide production. Diabetes. 1997;46:659-664.
18. Zhang F, Sowers JR, Ram JL et al. Effects of pioglitazone on calcium channels in vascular smooth muscle. Hypertension. 1994;24:170-175.
19. Ridker PM, Buring JE, Cook NR et al. C-Reactive Protein, the Metabolic Syndrome, and Risk of Incident Cardiovascular Events: An 8-Year Follow-Up of 14 719 Initially Healthy American Women. Circulation. 2003;107:391-397.
20. Haffner SM, Greenberg AS, Weston WM et al. Effect of rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. Circulation. 2002;106:679-684.
21. Chen H, Montagnani M, Funahashi T et al. Adiponectin stimulates production of nitric oxide in vascular endothelial cells. J Biol Chem. 2003;278:45021-45026.
22. Steinberg HO, Paradisi G, Hook G et al. Free fatty acid elevation impairs insulin-mediated vasodilation and nitric oxide production. Diabetes. 2000;49:1231-1238.

CHAPTER 5

TNF-α induces endothelial dysfunction in diabetic adults, an effect reversible by the PPAR-γ agonist pioglitazone

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Condensed abstract

To determine whether pioglitazone influences TNF-α induced endothelial dysfunction we examined in a randomised, parallel, placebo-controlled, double blind trial, the effects of 4 weeks pioglitazone treatment in 16 male type 2 diabetic patients. We conclude that intra-arterial TNF-α induces impairment of endothelial function in type 2 diabetes. Short-term pioglitazone treatment blocks this impairment.

Abstract

Introduction Inflammation contributes to the pathogenesis of cardiovascular disease. Tumor necrosis factor (TNF)-α in particular, is a key mediator of inflammation and vascular dysfunction and progression of atherosclerotic disease. Pioglitazone, a PPAR-γ agonist, not only improves insulin sensitivity, but may also have anti-inflammatory effects. The aims of this study were to investigate the acute effects of local intra-arterial infusion with low-dose TNF-α on resistance vessel endothelial function in type 2 diabetes and to determine whether short-term pioglitazone treatment protects against vascular dysfunction induced by this inflammatory stimulus.

Methods and Results A randomised, parallel, placebo-controlled, double blind trial with 30 mg pioglitazone once daily for 4 weeks was performed in 16 male patients with type 2 diabetes. Forearm plethysmography (FBF) was used to evaluate the effect on resistance vessel responses of intra-arterial administration of seroton in (NO-dependent vasodilation) and nitroprusside (endothelium-independent vasodilation) followed by another FBF-measurement during the second hour of intra-arterial infusion with TNF-α (10ng/100 ml FAV/min for 2h). Endothelial-dependent FBF of type 2 diabetic patients was significantly impaired (25.4%) by intra-arterial TNF-α infusion ($p < 0.02$), while nitroprusside-induced vasodilation did not change. Treatment with pioglitazone for 4 weeks completely blocked TNF-α-induced impairment of endothelial-dependent FBF compared to placebo. No significant changes in plasma concentrations of TNF-α, IL-6, soluble TNF-α-receptors or CD40L were observed.

Conclusions Pioglitazone treatment can convey direct protection against cytokine (TNF-α)-induced endothelial dysfunction in humans with an increased cardiovascular risk due to type 2 diabetes.

Keywords diabetes mellitus, endothelial function, inflammation, pioglitazone, tumour necrosis factor-α.

Introduction

The metabolic syndrome, which involves a cluster of cardiovascular risk factors including hypertension, obesity, glucose intolerance, endothelial dysfunction, dyslipidemia and a proinflammatory state, is generally considered to be of major importance in the pathophysiology of type 2 diabetes and cardiovascular complications.[1] Endothelial activation is considered the transducer by which risk factors lead to progression of atherosclerosis. It is present in patients with increased cardiovascular risk including the metabolic syndrome[2] and is a predictor for development of cardiovascular events.[3] Endothelial activation is characterised by decreased availability of endothelium-derived nitric oxide (NO) and can be assessed clinically by impaired forearm blood flow (FBF) to NO-agonists.

Low-grade chronic vascular inflammation contributes to the pathogenesis of cardiovascular disease. Elevated levels of proinflammatory proteins are predictive for both cardiovascular disease and type 2 diabetes.[4] The proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in particular, are elevated in patients with endothelial dysfunction and ischemic heart disease.[5,6] TNF-α downregulates mRNA for endothelial nitric oxide synthase (eNOS) by shortening its half-life in endothelial cells.[7] In addition *in vivo* intra-arterial TNF-α causes an acute local vascular inflammation that is associated with impaired endothelium-dependent vasomotion in young healthy non smoking men because of an acute local vascular-wall inflammation confirmed by the local rise of IL-6.[8] Another recent study in healthy lean male volunteers showed impairment of endothelium-dependent vasodilation upon intra-arterial TNF-α infusion.[9]

Thiazolidinediones (TZDs), such as pioglitazone and troglitazone, are a class of oral anti-hyperglycemic agents and ligands for peroxisome proliferator-activated receptors (PPARs), in particular PPAR-γ. These nuclear transcription factors are involved in glucose homeostasis, lipid and lipoprotein metabolism and adipogenesis. TZDs improve glycemic control by increasing insulin sensitivity, but may also have potential anti-inflammatory effects.[10]

Therefore in the present study we first investigated the effect of TNF-α on endothelium-dependent vasodilation and secondly the effects of short-term pioglitazone treatment on TNF-α induced endothelial dysfunction in patients with type 2 diabetes mellitus.

Subjects and methods

Subjects

Sixteen male, non-smoking patients with type 2 diabetes were recruited. All patients were treated with oral anti-hyperglycemic agents (7 patients with sulphonylurea derivates, 3 patients with metformin only and 6 patients with a combination of metformin and sulphonylurea derivates) which was continued during the study. Subjects with very poor glycemic control (HbA1c >9%) were excluded. Other exclusion criteria were presence of macro- or microvascular disease and the use of vasoactive medication (e.g. beta-blockers, calcium entry blockers, ACE-inhibitors, angiotensin type 1 receptor blockers, statins, aspirin, non-steroidal inflammatory drugs). The protocol was approved by the ethical review board of the University Medical Center Utrecht (UMCU). All subjects gave written informed consent. Measurements were carried out in accordance with local institutional guidelines in a Good Clinical Practice-certified unit.

Study design

The study was a prospective, randomised, parallel, placebo-controlled, double blind trial. Patients eligible to take part in the study were randomised in two patient-groups: 8 patients received placebo in addition to their current oral anti-hyperglycemic agents for 4 weeks first followed by measurement of forearm vascular function without intra-arterial TNF-α infusion and secondly directly followed by measurement of forearm vascular function during the second hour of intra-arterial TNF-α infusion (10ng/100 ml forearm volume (FAV)/min for 2h); 8 patients received pioglitazone 30mg once daily (Eli Lilly, Indianapolis, U.S.A.) in addition to their current oral anti-hyperglycemic agents for 4 weeks first followed by measurement of forearm vascular function without intra-arterial TNF-α infusion and secondly directly followed by measurement of forearm vascular function during the second hour of intra-arterial TNF-α infusion (10ng/100 ml FAV/min for 2h). At the end of each 4-week treatment period laboratory parameters were determined. Patients were instructed to fast for at least 10 hours prior to the tests. No study medication or other medication was used on the morning of the study day.

Methods

Assessment of vascular function

Experiments were performed in a temperature-controlled room (22-24°C) in the morning. All vascular function tests after pioglitazone and placebo treatment were performed at the same time for each individual in order to exclude daytime variabi-

lity. Upon arrival, patients were asked to rest in a supine position for 20 minutes before insertion of intra-arterial and intravenous cannula, blood withdrawal for laboratory measurements and determination of forearm blood flow (FBF) using venous occlusion plethysmography.

For assessment of FBF by venous occlusion plethymography (Hokanson EC-4, Bellevue, Wash., USA), both forearms were supported above heart level. The brachial artery of the non-dominant arm was cannulated with a 20-gauge catheter (Arrow International, USA) after local anaesthesia. Bilateral forearm blood flow was determined using mercury-in-silastic strain gauges and a microcomputer based, R-wave triggered system for online monitoring according to established methods.[11] Intra-arterial blood pressure was continuously monitored. Baseline measurements were performed at least 45 minutes after cannulation of the brachial artery in order to allow stabilisation of baseline blood flow. Vasoactive agents were dissolved in NaCl 0.9% and infused at a constant rate of 90 ml/h. All infusates were prepared in our hospitals pharmacy department in accordance to GMP guidelines.

Sequential infusions of vasoactive agents were performed. Serotonin (5-HT, Sigma Chemicals, St. Louis, Mo., USA) was infused into the brachial artery at increasing doses of 0.6, 1.8, 6.0 ng/100 ml FAV/min. This protocol has previously been shown to cause a dose-dependent increase in endothelium-dependent, NO-mediated vasodilation.[12] Sodium nitroprusside (SNP, Merck, Germany) was infused at increasing doses of 20, 60, 180 and 600 ng/100 ml FAV/min to assess endothelium-independent vasodilation. Serotonin- and SNP infusions were performed in random order in order to avoid any bias related to the order of drug infusion. Each dose was infused for 5-7 minutes and only during the last 2 to 3 min FBF was recorded. Five-minute intervals were applied between each dose. In order to allow recovery of forearm blood flow after administration of a vasoactive agent, a 20-minute rest between infusions of different vasoactive agents was applied. Average values of FBF of the cannulated and control arm were obtained from the last four to six consecutive recordings of each infusion period. The ratio of flow in the cannulated measurement (M) and non-cannulated control (C) arm was calculated for each recording (M:C ratio). The FBF for each dose is expressed as the percentage change of M:C ratio from baseline M:C ratio (M:C%).

TNF-α infusion

Human recombinant TNF-α (10ng/100 ml FAV/min Tasonermin, Boehringer-Ingelheim, Germany; 1/8 of dosis used in healthy young volunteers[13]) was infused in the cannulated brachial artery for 2 hours. During the second hour of TNF-α infusion

serotonin- and SNP infusions were performed as well as FBF measurements. Blood was drawn from the infused arm, 60- and 120 minutes after start of intra-arterial TNF-α infusion, for measurements of TNF-α and IL-6 concentrations. Blinded sham control with an intra-arterial salt infusion was not performed due to the invasive nature of the procedure.

Laboratory assessment

At the end of each 4-week treatment period fasting blood was drawn before the start of FBF measurements and plasma was frozen at –20°C until further analysis. Glucose, creatinine, serum alanine transferase (ALT), serum aspartate transferase (AST), total cholesterol, triglycerides and high-density lipoprotein cholesterol (HDL-C) were measured by standard enzymatical laboratory methods (Vitros 250; Johnson/Johnson). Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula. HbA1c and free fatty acids (FFA) were photometrically performed (Hitachi 911; Roche). Insulin levels were determined with an immunological method (Immulite 2000; Diagnostic Prod Corp.). HOMA-R was calculated (fasting glucose (mmol/L) x fasting insulin (mU/mL)/22.5). Measurements of plasma TNF-α, soluble TNF-α receptors 60kD and 80kD and IL-6 were performed with commercially available high-sensitive kits (ELISA; R&D Systems Inc./ minimum detectable doses resp.: 0.06 pg/ml, 0.2 pg/ml, 0.43 pg/ml, and 0.016 pg/ml). Measurement of circulating sCD40L was also performed with a commercially available high-sensitive kit (ELISA; BenderMed Systems/ minimum detectable dose: 0.005 ng/ml).

Statistical analysis

Results from vascular function tests and laboratory analysis are expressed as mean + standard error. Differences in metabolic parameters as well as in FBF (for each vasoactive agent on different concentration/time-points) between both treatments were analysed with (un-)paired t-tests (95% confidence interval). In case of non-normal distribution the Wilcoxon Signed Rank test was used. Statistical significance was taken at the 5% level.

Results

Anthropometric, hemodynamic and metabolic baseline parameters

Table 1 shows the anthropometric, hemodynamic, inflammatory and metabolic baseline parameters of both groups.

Table 1. Baseline characteristics

	Placebo (N=8)	**Pioglitazone** (N=8)
Age	55 ± 3	57 ± 2
Weight (kg)	89 ± 9	95 ± 4
BMI	28 ± 2	30 ± 1
RRsyst (mmHg)	151 ± 8	144 ± 6
RRdiast (mmHg)	90 ± 3	86 ± 5
Fasting plasma glucose (mmol/l)	7.8 ± 0.8	7.6 ± 0.6
Insulin (mU/l)	19.2 ± 6.4	18.8 ± 4.0
HOMA-R	8.3 ± 3.9	6.7 ± 1.6
HbA1c (%)	7.4 ± 0.4	7.0 ± 0.5
Total cholesterol (mmol/l)	5.9± 0.3	5.6± 0.3
HDL cholesterol (mmol/l)	1.10 ± 0.06	1.20 ± 0.09
LDL cholesterol (mmol/l)	3.7 ± 0.2	3.7 ± 0.3
Triglycerides (mmol/l)	2.1 ± 0.5	1.6 ± 0.2
Free fatty acids (μmol/l)	591 ± 85	553 ± 41
Adiponectin (mg/l)	3.7 ± 0.1	2.1 ± 0.2
ASAT (U/l)	32 ± 4	26 ± 2
ALAT (U/l)	35 ± 8	32 ± 4
Creatinin (μmol/l)	74 ± 2	78 ± 5
Hematocrite (l/l)	0.45 ± 0.01	0.44 ± 0.01
CRP (mg/l)	3.4 ± 0.4	4.5 ± 1.2
Interleukin-6 (ng/l)	1.8 ± 0.3	1.8 ± 0.3
TNF-α (ng/l)	2.2 ± 0.2	2.7 ± 0.3

Values are mean ± SEM

During TNF-α-infusion the intra-arterial TNF-α-concentration increased. No significant changes were observed in anthropometric, hemodynamic, metabolic and inflammatory parameters after 4 weeks of pioglitazone- or placebo treatment (Table 2). In addition we did not observe a difference in the effects of pioglitazone

between the group of patients using metformin alone or a combination of metformin and sulphonylurea derivates and the group of patients only treated with sulphonylurea derivates. Edema did not occur in any patient on pioglitazone treatment. Pioglitazone treatment was not associated with liver enzyme abnormalities.

Table 2. Clinical characteristics after treatment

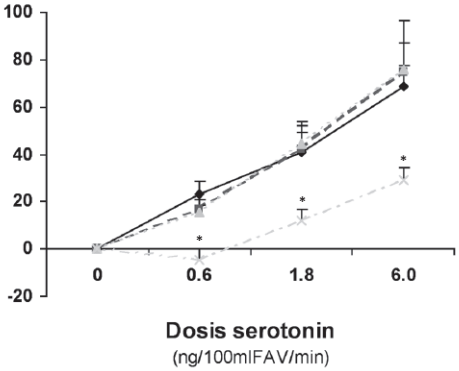
	Placebo (N=8)	**Pioglitazone** (N=8)	**p-values**
Weight (kg)	90 ± 8	96 ± 5	0.53
BMI	28 ± 2	30 ± 1	0.33
RRsyst (mmHg)	138 ± 5	139 ± 4	0.77
RRdiast (mmHg)	80 ± 4	82 ± 5	0.74
Fasting plasma glucose (mmol/l)	6.7 ± 0.4	6.3 ± 0.5	0.55
Insulin (mU/l)	16.0 ± 2.6	12.0 ± 2.9	0.44
HOMA-R	5.4 ± 1.0	3.8 ± 1.3	0.50
HbA1c (%)	6.9 ± 0.2	6.9 ± 0.4	0.97
Total cholesterol (mmol/l)	5.2 ± 0.3	5.1 ± 0.3	0.69
HDL cholesterol (mmol/l)	1.04 ± 0.04	1.06 ± 0.09	0.83
LDL cholesterol (mmol/l)	3.2 ± 0.2	3.3 ± 0.2	0.75
Triglycerides (mmol/l)	1.7 ± 0.2	1.4 ± 0.1	0.33
Free fatty acids (İmol/l)	536 ± 90	487 ± 32	0.64
Adiponectin (mg/l)	2.9 ± 0.7	5.5 ± 0.1	0.10
ASAT (U/l)	30 ± 4	24 ± 3	0.24
ALAT (U/l)	30 ± 6	32 ± 4	0.75
Creatinin (μmol/l)	69 ± 2	71 ± 5	0.67
Hematocrite (l/l)	0.41 ± 0.01	0.40 ± 0.01	0.37
CRP (mg/l)	2.7 ± 0.6	3.3 ± 0.8	0.57
Interleukin-6 (ng/l)	1.7 ± 0.3	1.5 ± 0.2	0.53
TNF-α (ng/l)	2.3 ± 0.2	2.7 ± 0.3	0.25
sTNFr 60kD (μg/l)	2.1 ± 0.1	2.5 ± 0.3	0.23
sTNFr 80kD (μg/l)	5.7 ± 0.3	9.5 ± 2.7	0.18
CD40L (μg/l)	8.5 ± 1.3	7.3 ± 0.8	0.45
IL-6 after 60min infusion (ng/l)	2.0 ± 0.6	1.6 ± 0.2	0.57+
IL-6 after 120min infusion (ng/l)	3.4 ± 1.2	2.9 ± 0.9	0.77+
TNF-α after 60min infusion (ng/l)	9.4 ± 1.5	8.8 ± 1.6	0.81+
TNF-α after 120min infusion (ng/l)	22.1 ± 5.7	14.2 ± 1.6	0.21+

Values are mean ± SEM;

p-values are reported for comparison between placebo and pioglitazone;

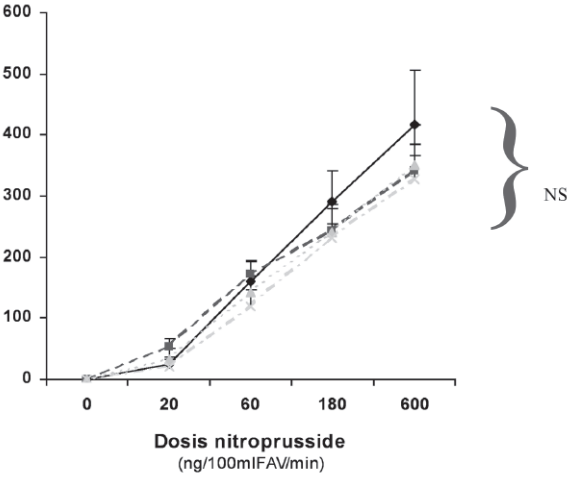
+: p-values are reported for comparison between with- or without TNF-α-infusion

Figure 1a. Endothelial-dependent serotonin-induced vasodilation of the forearm



Values are mean±SEM;
-◆-: placebo, N=8;
-■-: pioglitazone, N=8;
-X-: placebo + TNF-α infusion (10ng/min for 2h), N=8;
-▲-: pioglitazone + TNF-α infusion (10ng/min for 2h), N=8;
*: p-value 0.01 is reported for comparison of intra-arterial TNF-α infusion vs. no TNF-α infusion in the placebo group; p-value 0.02 is reported for comparison of pioglitazone + intra-arterial TNF-α infusion vs. placebo + intra-arterial TNF-α infusion; Analyses by (un-)paired t-tests on different concentration/time-points.

Figure 1b. Endothelial-independent nitroprusside-induced vasodilation of the forearm



Values are mean±SEM;
-◆-: placebo, N=8;
-■-: pioglitazone, N=8;
-X-: placebo + TNF-α infusion (10ng/min for 2h), N=8;
-▲-: pioglitazone + TNF-α infusion (10ng/min for 2h), N=8;
NS: no comparisons are significant; Analyses by (un-)paired t-tests on different concentration/time-points.

Influence of intra-arterial TNF-α infusion on forearm blood flow
Intra-arterial TNF-α infusion had no effects on heart rate or blood pressure. Basal forearm blood flow was similar in all groups.
The serotonin-induced endothelial-dependent FBF of patients treated with placebo for 4 weeks was impaired by intra-arterial TNF-α infusion ($p<0.02$) (Figure 1a).

Effects of pioglitazone and intra-arterial TNF-α infusion on forearm blood flow
Basal forearm blood flow was similar in the placebo- and pioglitazone-treated patients. After treatment with pioglitazone for 4 weeks followed by an intra-arterial TNF-α infusion the serotonin-induced endothelium-dependent vasodilation in the forearm vascular bed stayed at the level of the serotonin-induced endothelium-dependent vasodilation of the placebo group- or the pioglitazone group- without intra-arterial TNF-α infusion. Compared to the serotonin-induced endothelium-dependent vasodilation in the forearm vascular bed of the placebo treated patients with intra-arterial TNF-α infusion, the serotonin-induced endothelium-dependent vasodilation in the forearm vascular bed of the pioglitazone treated patients improved significantly ($p<0.02$) (Figure 1a). Administration of the endothelium-independent vasodilator nitroprusside caused an increase in FBF that was at the same level in the pioglitazone group as it was in the placebo group, indicating that there was no alteration in large vessel VSMC responsiveness to NO (Figure 1b).

Furthermore the endothelial-independent nitroprusside-induced vasodilation did not change indicating that there was no alteration of large vessel vascular smooth muscle cell (VSMC) responsiveness to NO (Figure 1b).

Discussion

Inflammation contributes to the pathogenesis of cardiovascular disease and elevated levels of pro-inflammatory proteins are predictive of both cardiovascular disease and type 2 diabetes.[14] Especially in obese individuals TNF-α is elevated and is associated with ischemic heart disease and endothelial dysfunction.[15,16] The main outcome of the present study is that low dose intra-arterial TNF-α infusion induces an acute impairment of endothelial function in type 2 diabetes and that (short-term) pioglitazone treatment blocks this impairment completely independently of metabolic changes.

In vitro TNF-α plays a role in acute coronary syndromes by increasing expression of adhesion molecules on the endothelial cell surface and the ensuing recruitment of inflammatory cells, and by decreasing vascular smooth muscle cells viability. In agreement with such mechanisms TNF-α levels have been associated with plaque instability by decreasing VSMC viability.[13] *In vivo* previous studies showed impairment of endothelium-dependent vasodilation in healthy subjects by low dose intra-arterial TNF-α infusion.[8] Type 2 diabetic patients already have endothelial dysfunction and it could therefore be hypothesized that inflammatory stimuli like TNF-α do not further affect endothelial function. However, in our study we now show that raising the intra-arterial TNF-α concentration in type 2 diabetes locally, to levels that are towards the range of the levels found in conditions such as congestive heart failure[14] and acute coronary syndromes[15], further impairs endothelium-dependent vasodilation. This effect appeared to be endothelium specific as vasodilation to nitroprusside was unaltered. Treatment with pioglitazone for 4 weeks completely protected against TNF-α induced depression of endothelium-dependent vasodilation. It should be noticed that baseline levels of cytokines and TNF-α were not affected by pioglitazone treatment nor was there any change in metabolic indices in this short term study. This is not unexpected as usually the metabolic effects of TZD treatment only become apparent after 8 weeks of treatment.[16,17] Nevertheless, basal endothelial function was also restored to normal values (Figure 1a).

These data indicate that pioglitazone specifically and directly changed endothelial function, and in particular the endothelial responsiveness to TNF-α. Indeed, the PPAR-γ isoform is expressed in endothelial cells and its activation has been associated with enhanced NO availability[18-20] and a reduced potential of endothelial cells to switch to an inflammatory phenotype.[21-23] There appears to be a generalized repression of NF-κB, CCAAT/enhancer-binding protein, and activator protein-1– mediated transcription of inflammatory genes.[24-25] The exact mechanism is still unknown, but probably involves increased levels of co-repressor molecules or transcriptional superregu¬lation, for example by chromatin remodeling, as has been described for activation of other nuclear hormone receptors.[30] In addition endothelial release of the vasoconstrictor peptide endothelin-1 is suppressed by TZD's.[27]

The direct vascular anti-inflammatory properties of TZD's have previously been demonstrated in animal models. For example Angiotensin-II (Ang-II) infusion in rats resulted in endothelial dysfunction, increased medio/lumen ration and vascular

inflammation. All these changes where abrogated by TZD treatment independently of metabolic effects suggesting direct interference of TZD's with signaling cascades that lead to these events.[28] Our current study extends these properties of TZD's to humans with increased cardiovascular risk. These observations may provide an explanation for some of the recent findings where TZD treatment could reduce the progression of intima-media thickness[29], a proxy for atherosclerotic burden and cardiovascular risk[30], in patients with coronary artery disease but without diabetes. In these studies only minimal changes in metabolic parameters were observed.

We only included subjects using oral anti-hyperglycemic agents. Since insulin has profound effects on the vasculature, caution should be taken to extrapolate our results to patients with type 2 diabetes using pioglitazone in combination with insulin. In addition, patients using vasoactive medication were excluded from our study while it would also be interesting to study the additional benefits of pioglitazone in these patients.

In conclusion, pioglitazone treatment can convey direct protection against cytokine (TNF-α)-induced endothelial dysfunction in humans with an increased cardiovascular risk due to type 2 diabetes.

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References

1. Watson KE, Peters Harmel AL, Matson G. Atherosclerosis in type 2 diabetes mellitus: the role of insulin resistance. J Cardiovasc Pharmacol Ther 2003;8:253-260.
2. McVeigh GE, Brennan GM, Johnston GD, McDermott BJ, McGrath LT, Henry WR, Andrews JW, Hayes JR. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 1992;35:771-776.
3. Zeiher AM. Endothelial vasodilator dysfunction: pathogenetic link to myocardial ischaemia or epiphenomenon? Lancet 1996;348 Suppl 1:s10-s12.
4. Haddy N, Sass C, Droesch S, Zaiou M, Siest G, Ponthieux A, Lambert D, Visvikis S. IL-6, TNF-alpha and atherosclerosis risk indicators in a healthy family population: the STANISLAS cohort. Atherosclerosis 2003;170:277-283.
5. Ridker PM, Rifai N, Pfeffer M, Sacks F, Lepage S, Braunwald E. Elevation of tumor necrosis factor-alpha and increased risk of recurrent coronary events after myocardial infarction. Circulation 2000;101:2149-2153.
6. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation 2000;101:1767-1772.
7. Yoshizumi M, Perrella MA, Burnett JC, Jr., Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res 1993;73:205-209.
8. Chia S, Qadan M, Newton R, Ludlam CA, Fox KA, Newby DE. Intra-Arterial Tumor Necrosis Factor-{alpha} Impairs Endothelium- Dependent Vasodilatation and Stimulates Local Tissue Plasminogen Activator Release in Humans. Arterioscler Thromb Vasc Biol 2003;23:695-701.
9. Rask-Madsen C, Dominguez H, Ihlemann N, Hermann T, Kober L, Torp-Pedersen C. Tumor necrosis factor-alpha inhibits insulin's stimulating effect on glucose uptake and endothelium-dependent vasodilation in humans. Circulation 2003;108:1815-1821.
10. Martens FM, Visseren FL, Lemay J, de Koning EJ, Rabelink TJ. Metabolic and additional vascular effects of thiazolidinediones. Drugs 2002;62:1463-1480.
11. Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. Hypertension 1995;25:918-923.
12. Bruning TA, Chang PC, Blauw GJ, Vermeij P, van Zwieten PA. Serotonin-induced vasodilatation in the human forearm is mediated by the "nitric oxide-pathway": no evidence for involvement of the 5-HT3-receptor. J Cardiovasc Pharmacol 1993;22:44-51.
13. Anwar A, Zahid AA, Scheidegger KJ, Brink M, Delafontaine P. Tumor necrosis factor-alpha regulates insulin-like growth factor-1 and insulin-like growth factor binding protein-3 expression in vascular smooth muscle. Circulation 2002;105:1220-1225.
14. Dutka DP, Elborn JS, Delamere F, Shale DJ, Morris GK. Tumour necrosis factor alpha in severe congestive cardiac failure. Br Heart J 1993;70:141-143.

15. Mizia-Stec K, Gasior Z, Zahorska-Markiewicz B, Janowska J, Szulc A, Jastrzebska-Maj E, Kobielusz-Gembala I. Serum tumour necrosis factor-alpha, interleukin-2 and interleukin-10 activation in stable angina and acute coronary syndromes. Coron Artery Dis 2003;14:431-438.
16. Khan MA, St Peter JV, Xue JL. A prospective, randomized comparison of the metabolic effects of pioglitazone or rosiglitazone in patients with type 2 diabetes who were previously treated with troglitazone. Diabetes Care 2002;25:708-711.
17. Yki-Jarvinen H. Thiazolidinediones. N Engl J Med 2004;351:1106-1118.
18. Calnek DS, Mazzella L, Roser S, Roman J, Hart CM. Peroxisome proliferator-activated receptor gamma ligands increase release of nitric oxide from endothelial cells. Arterioscler Thromb Vasc Biol 2003;23:52-57.
19. Pistrosch F, Passauer J, Fischer S, Fuecker K, Hanefeld M, Gross P. In Type 2 Diabetes, Rosiglitazone Therapy for Insulin Resistance Ameliorates Endothelial Dysfunction Independent of Glucose Control. Diabetes Care 2004;27:484-490.
20. Satoh N, Ogawa Y, Usui T, Tagami T, Kono S, Uesugi H, Sugiyama H, Sugawara A, Yamada K, Shimatsu A, Kuzuya H, Nakao K. Antiatherogenic effect of pioglitazone in type 2 diabetic patients irrespective of the responsiveness to its antidiabetic effect. Diabetes Care 2003;26:2493-2499.
21. Fukuzawa M, Satoh J, Qiang X, Miyaguchi S, Sakata Y, Nakazawa T, Ikehata F, Ohta S, Toyota T. Inhibition of tumor necrosis factor-alpha with anti-diabetic agents. Diabetes Res Clin Pract 1999;43:147-154.
22. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 1998;391:82-86.
23. Peraldi P, Xu M, Spiegelman BM. Thiazolidinediones block tumor necrosis factor-alpha-induced inhibition of insulin signaling. J Clin Invest 1997;100:1863-1869.
24. Ruan H, Pownall HJ, Lodish HF. Troglitazone antagonizes TNF-alpha -induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NF-kB. J Biol Chem 2003.
25. Takata Y, Kitami Y, Yang ZH, Nakamura M, Okura T, Hiwada K. Vascular inflammation is negatively autoregulated by interaction between CCAAT/enhancer-binding protein-delta and peroxisome proliferator-activated receptor-gamma. Circ Res 2002;91:427-433.
26. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 2002;108:465-474.
27. Delerive P, Martin-Nizard F, Chinetti G, Trottein F, Fruchart JC, Najib J, Duriez P, Staels B. Peroxisome proliferator-activated receptor activators inhibit thrombin-induced endothelin-1 production in human vascular endothelial cells by inhibiting the activator protein-1 signaling pathway. Circ Res 1999;85:394-402.

28. Diep QN, El Mabrouk M, Cohn JS, Endemann D, Amiri F, Virdis A, Neves MF, Schiffrin EL. Structure, endothelial function, cell growth, and inflammation in blood vessels of angiotensin II-infused rats: role of peroxisome proliferator-activated receptor-gamma. Circulation 2002;105:2296-2302.
29. Sidhu JS, Kaposzta Z, Markus HS, Kaski JC. Effect of rosiglitazone on common carotid intima-media thickness progression in coronary artery disease patients without diabetes mellitus. Arterioscler Thromb Vasc Biol 2004;24:930-934.
30. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK, Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med 1999;340:14-22.

CHAPTER 6

Monocyte-endothelium adhesion (under flow conditions) is increased in type 2 diabetes and can be reduced by PPAR-γ agonists pioglitazone and troglitazone

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Submitted

Condensed abstract

To determine whether PPAR-γ agonists pioglitazone and troglitazone modify monocyte-endothelium- adhesion we examined in an ex vivo study the effects of incubated monocytes and HUVEC in 6 male type 2 diabetic patients and 6 matched male healthy volunteers. Monocyte-endothelium-adhesion was studied under flow conditions. We conclude that monocytes from patients with type 2 diabetes adhere more to endothelium compared to monocytes from healthy volunteers. Pioglitazone- or troglitazone pre-treatment of monocytes reduce monocyt adhesion.

Abstract

Introduction Monocytes are involved in atherosclerotic lesion development, destabilization and plaque rupture. PPAR-γ agonists, like pioglitazone and troglitazone, regulate glucose homeostasis, lipid metabolism and may have anti-inflammatory effects, which may influence atherogenesis. In this study we determined the effect of PPAR-γ agonism on adhesion of monocytes from patients with type 2 diabetes vs healthy volunteers to endothelial cells under flow conditions ex vivo.

Methods and Results Monocytes were isolated from 6 male patients with type 2 diabetes and 6 age-matched male healthy volunteers. The isolated monocytes were incubated with pioglitazone, troglitazone, DMSO and RPMI and added to confluent HUVEC. The adherence capacity of the monocytes in a small volume perfusion chamber was measured by realtime video microscopy.
Monocyte-adhesion under flow conditions showed significant more monocyte adhesion in type 2 diabetic patients compared to healthy controls: 212 counts per mm^2±17 vs 158 counts per mm^2±9 (p=0.018). Incubation with pioglitazone (1μM) lowered monocyte-adhesion in type 2 diabetic patients: 162 counts per mm^2±16 (p=0.07). Troglitazone (1μM) did lower adhesion of monocytes from type 2 diabetic patients significantly: 147 counts per mm^2±11 (p=0.008). Any differences in expression of (adhesion-)surface-antigens CD14, CD11b/CD18, CD62L and CD36 could not be observed.

Conclusions Monocyte-endothelium-adhesion is increased in type 2 diabetes and can be reduced by PPAR-γ agonists pioglitazone and troglitazone without changes in CD14, CD11b/18, CD62L and CD36 expression on monocytes.

Keywords diabetes mellitus, monocyt-endothelium adhesion, inflammation, pioglitazone, troglitazone.

Introduction

Atherosclerotic disease in large vessels is the major cause of morbidity and mortality in patients with type 2 diabetes (DM2).[1] The early phase of atherosclerosis involves the recruitment of inflammatory cells from the circulation, adhesion to the endothelium and transendothelial migration. This process is predominantly mediated by cellular adhesion molecules which are expressed on the vascular endothelium in response to several inflammatory stimuli.[2,3] The first step in adhesion, the rolling of monocytes along the endothelial surface is mediated by selectines. For firm adhesion of monocytes to the endothelium, integrins, vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1, all expressed on the surface of vascular endothelial cells, are involved.[4] The progression of the atherosclerotic plaque eventually leading to plaque rupture is to view upon as an chronic inflammatory process.[5] Elevated plasma levels of endothelial adhesion molecules[6] and enhanced monocyte-adhesion to endothelium under static conditions[7,8] have already been documented in patients with type 2 diabetes. It would be important to know whether these results are similar under, physiologically more relevant, flow conditions and whether this process can be pharmacologically influenced.

Thiazolidinediones (TZDs), such as pioglitazone and troglitazone, are a class of oral anti-diabetic agents and are ligands for peroxisome proliferator-activated receptors (PPARs), in particular PPAR-γ. These nuclear transcription factors are involved in glucose homeostasis, lipid and lipoprotein metabolism and adipogenesis. TZDs improve glycemic control by increasing insulin sensitivity, but may also have potential anti-inflammatory effects.[9] Pioglitazone treatment leads to a 16% reduction in cardiovascular events.[10] PPAR-γ agonists may limit chronic inflammation by inhibiting VCAM-1 induction[11-13] and reduce macrophage homing to atherosclerotic plaques.[14] Because of conflicting findings the consequences of PPAR-γ activation on monocyt/macrophage inflammatory responses have led to much debate regarding the role of PPAR in atherosclerosis.

In the present study we investigated the effects of PPAR-γ agonists on the adhesion to endothelium and the expression of surface proteins of monocytes from healthy volunteers compared to monocytes derived from patients with type 2 diabetes.

Subjects and methods

Subjects

Six male, non-smoking patients with type 2 diabetes were recruited. All patients were treated with oral anti-hyperglycemic agents (3 patients with sulphonylurea derivates, 1 patient with metformin and 2 patients with a combination of metformin and sulphonylurea derivates). Subjects with very poor glycemic control (HbA1c> 9%) were excluded. Other relevant exclusion criteria were presence of macro- or micro-vascular disease and use of vasoactive medication (e.g. beta-blockers, calcium entry blockers, ACE-inhibitors, angiotensin type 1 receptor blockers, statins, aspirin, non-steroidal inflammatory drugs). Six healthy male volunteers were age-matched .

Methods

Reagents

Trypsine; Gelatine 2% solution type B from bovine skin G1393; EDTA: $C_{10}H_{14}N_2O_8Na_2.2H_2O$, molmassa : 372,2 (E-4884); Histopaque-1077 (Sigma, St. Louis MO); RPMI-1640 containing 25mM hepes buffer-glutamine and L-glutamine (Gibco, Grand Island NY); Dimethyl sulfoxide (DMSO); M199 medium with Earle's Salt, 2,2g/L $NaHCO_3$, L-glutamine, without L-amino-acids (Gibco-BRL/Invitrogen 31150-022); Fetal Calf Serum (FCS); Penicillin/Streptomycine; Heparin 5000E/ml (Leo 5 ml, RVG 01372); Bovine Pituitary Extract (BPE) (Gibco-BRL/Invitrogen 13028-014); PBS (our own hospital pharmacy); Ficoll Paque Tm Plus (Amersham Biosciences 17-1440-03); CD14, CD11b/CD18, CD62L and CD36 microbeats (Miltenyi Biotec); Carboxylfluorescentmarker CFDA-SE. IL-1, (Peprotech Inc); Propidium Iodide/ Annexin V-FITC staining solution (BD Biosiences); Pioglitazone powder (Takeda Pharmaceuticals); Troglitazone powder (Sankyo Pharma GmbH).

HUVEC preparation

HUVEC (±10.000) were seeded in each well of gelatin-coated 96-well plates 3-4 days before experiments. Only confluent monolayers, as confirmed by microscopic inspection the day before the assay, were used for experiments. Confluent cultures of HUVEC were incubated with pioglitazone (1μM), troglitazone (1μM), DMSO 10^{-5} and RPMI for 20h, all with medium and stimulated with TNF-α for 9h at 37°C.

$CD14^+$ MNC preparation

The mononuclear cell (MNC) fraction was prepared by dilution with an equal volume of PBS with 2 % EDTA at room temperature and layered over a solution containing 3ml

Ficoll-Histopaque per 10ml and centrifuged at 2000rpm for 20 min. The mononuclear cell layer was harvested and transferred to a fresh tube, mixed with 3 volumes of PBS, and centrifuged at 2000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in buffer. Magnetically labelled CD14 antibodies (20μl) per $1x10^7$ cells were added and incubated for 15 min. Monocytes were separated using Magnetic Cell Sorting (MACS). After purification, monocytes were labelled with the fluorescent marker CFDA-SE by incubating the monocytes in PBS containing 0.2 μl/ml of the CFDA-SE at 37°C for 20 min, and centrifuged at 1500 rpm for 5 min. Fluorescent cells were pelleted and resuspended ($1x10^6$/ml) in RPMI 1640, and incubated at 37°C for 15 min to wash out excess of CFDA-SE. Cells were pelleted and resuspended in RPMI and divided in fresh tubes. Tubes were centrifuged at 3000rpm, 3 min and supernatant was removed. Incubation at 37°C with pioglitazone (1μM), troglitazone (1μM), DMSO 10^{-5} and RPMI, all with 10% autologe serum, was performed for 20h.
Cells were washed once with RPMI and resuspended at $1x10^6$ cells/ml. The cells expressed CD14 as determined by FACS analysis (>95%).

Monocyte-adhesion assay under flow conditions
For measurement of monocyte-endothelium-adhesion under flow conditions the isolated monocytes ($1x10^5$ in 50μl/well) were incubated with pioglitazone (1μM), troglitazone (1μM), DMSO 10^{-5} and RPMI, all with 10% autologe serum for 20h and were added to confluent and TNF-α pre-stimulated HUVEC. HUVEC was also incubated with pioglitazone (1μM), troglitazone (1μM), DMSO 10-5 and RPMI, all with medium for 20h.
Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber as previously described by van Zanten et al.[15] This micro-chamber has a slit height of 0.2 mm and width of 2 mm. The chamber contains a circular plug on which a coverslip (18 mm x 18 mm) with HUVEC was mounted.
Monocytes ($1x10^6$ cells/ml) were suspended in perfusion buffer (20 mM Hepes, 132 mM NaCl, 6 mM KCL, 1 mM $MgSO_4$, 1.2 mM KH_4PO_4, 5 mM glucose, 1 mM $CaCl_2$, 0.5 % human serum albumin, pH 7.4) and were aspirated from a reservoir through the perfusion chamber. Monocyte perfusions were performed as individual runs at 37°C for 5 minutes at shear stress 1.0 dyn/cm² to obtain firmly adhering cells on the endothelial surface.[16] Shear stress was increased to 2 dyn/cm² and recording of the images on video was started. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE, Leica, Weitzlar, Germany) equipped with a B/W CCD-video-camera (Sanyo, Osaka, Japan), coupled to a VHS video recorder. The monocytes

in contact with the surface, appeared as bright white-centered cells after proper adjustment of the microscope during recording. Twentyfive video images were evaluated for the number of adhered cells per mm2 of endothelial cell surface using dedicated routines made in the image-analysis software Optimas 6.1 (Media Cybernetics systems, Silverspring, MD).

Laboratory assessment
Fasting blood was drawn to obtain peripheral blood mononuclear cells and plasma was frozen at –20°C until further analysis. Glucose was measured by standard enzymatical laboratory methods (Vitros 250; Johnson/Johnson). HbA1c was photometrically performed (Hitachi 911; Roche). Expression of CD14, CD11b/CD18, CD62L and CD36 (Miltenyi Biotec) on mononuclear cells were determined by FACS analysis. Viability of the mononuclear cells were quantitatively determined by the percentage of cells undergoing apoptosis by Annexin V-FITC and Propidium Iodide (BD Biosciences).

Statistical analysis
Results are expressed as mean + standard error. Differences in parameters between the different groups were analysed with one-way ANOVA analysis followed by an unpaired t-test (95% confidence interval). In case of non-normal distribution the Wilcoxon Signed Rank test was used. Statistical significance was taken at the 5% level.

Results

Monocyte-endothelium adhesion in DM2 patients compared to healthy controls
In table 1. the differences between the 6 male healthy volunteers and the 6 age-and sex-matched DM2 patients are shown. The body mass index (BMI), fasting plasma glucose concentrations and HbA1c levels are elevated in the DM2 patients as expected. The total white bloodcell count and the plasma concentration of monocytes were similar in both groups.
Monocyte-adhesion under flow conditions was significant higher in monocytes derived from DM2 patients compared to healthy controls: 212±17 vs 158±9 counts per mm2 (p=0.018) (see also figure 2). Differences in expression of the (adhesion-) surface-antigens CD14, CD11b/CD18, CD62L and CD36 on monocytes could not be observed (see also Table 3).

Table 1. Healthy volunteers- or type 2 diabetes patients-characteristics

	Control (N=6)	**DM2** (N=6)	**p-value**
Age (y)	52±3	54±3	0.55
BMI	24.5±0.4	28.0±1.9	0.11
Fasting plasma glucose (mmol/l)	4.7±0.2	8.6±1.3	0.03*
HbA1c (%)	5.5±0.1	6.9±0.4	0.04*
White Bloodcell Count (exp9)	5.5±0.6	5.9±0.8	0.72
Monocytes in WBC (%)	8.8±0.4	8.5±0.8	0.81
Adhesion (counts per mm2)	158±9	212±17	0.018*
CD14 (mean effluoresence)	263±46	372±107	0.37
CD11b/CD18 (mean effluoresence)	2746±508	2820±629	0.93
CD62L (mean effluoresence)	23±25	80±43	0.31
CD36 (mean effluoresence)	1417±294	1478±200	0.87

Values are mean ±SEM;
p-values are reported for comparison between healthy volunteers vs patients with type 2 diabetes.
* p-values < 0.05

Table 2. Effects of pioglitazone- or troglitazone treatment in type 2 diabetes

	Control=DMSO (N=6)	Pioglitazone 1μM (N=6)	Troglitazone 1μM (N=6)	p-value
Pio/Tro				
Viability (% cell death)	6.6±1.7	4.3±0.9	5.0±0.9	0.27/0.43
Adhesion (counts per mm²)	206±14	162±16	147±11	0.07/0.008*
CD14 (mean effluoresence)	339±115	319±85	312±87	0.89/0.86
CD11b/CD18 (mean effluoresence)	2633±572	2561±653	2430±663	0.94/0.83
CD62L (mean effluoresence)	20±67	96±60	5±26	0.42/0.85
CD36 (mean effluoresence)	1451±245	1467±336	1439±341	0.97/0.98

Values are mean ±SEM;
p-values are reported for comparison between pioglitazone treatment vs vehicle DMSO and troglitazone treatment vs vehicle DMSO, in patients with type 2 diabetes.
* p-values < 0.05

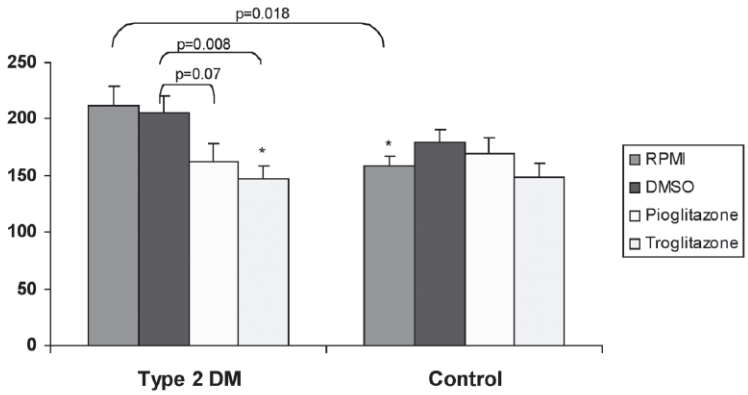
Effect of pioglitazone and troglitazone on monocyte-endothelium adhesion

In table 2. the effects of monocyte incubation with pioglitazone or troglitazone are shown. Because both PPAR-γ agonists needed to be dissolved in DMSO, the effects of pioglitazone- or troglitazone treatment are compared with the effects caused by DMSO alone. While DMSO can be harmful for the cells together with viability-checks also RPMI-controls were performed in all experiments. The percentage of cell death as measured by the propidium iodide/ annexin V-FITC staining-method did not exceed the 9.5% in any experiment and there were no differences in cell death observed between all groups.

Adhesion of monocytes from DM2 patients was lowered by incubation with pioglitazone (1μM) compared to the vehicle DMSO: 206±14 vs 162±16 counts per mm² (p=0.07) and incubation with troglitazone (1μM) did lower adhesion in type 2 diabetic patients significantly compared to the vehicle DMSO: 206±14 vs 147 ±11 counts per mm² (p=0.008) (see figure 1).

Differences in expression of the (adhesion-) surface-antigens CD14, CD11b/CD18, CD62L and CD36 on monocytes could not be observed (see also Table 3).

Figure 1. Monocyte-endothelium-adhesion under flow conditions



Values are mean±SEM;

p-value 0.018 is reported for comparison between patients with type 2 diabetes vs healthy volunteers;

p-value 0.07 is reported for comparison between pioglitazone treatment 1ÌM vs vehicle DMSO in patients with type 2 diabetes;

p-value 0.008 is reported for comparison between troglitazone treatment 1μM vs vehicle DMSO in patients with type 2 diabetes.

Table 3. Expression of surface-antigens on monocytes (mean effluoresence)

	Control (N=6)	**DM2** (N=6)	**p-value**
CD14 (RPMI)	263±46	372±107	0.37
CD14 (DMSO 10^{-5})	260±73	339±115	0.97/0.84
CD14 (Pioglitazone 1μM)	291±85	319±85	0.60/0.89
CD14 (Troglitazone 1μM)	209±40	312±87	0.58/0.86
CD11b/CD18 (RPMI)	2746±508	2820±629	0.93
CD11b/CD18 (DMSO 10^{-5})	2490±326	2633±572	0.68/0.83
CD11b/CD18 (Pioglitazone 1μM)	2199±433	2561±653	0.60/0.94
CD11b/CD18 (Troglitazone 1μM)	2525±520	2430±663	0.96/0.83
CD62L (RPMI)	23±25	80±43	0.31
CD62L (DMSO 10^{-5})	4±20	20±67	0.61/0.47
CD62L (Pioglitazone 1μM)	47±45	96±60	0.44/0.42
CD62L (Troglitazone 1μM)	0±45	5±26	0.83/0.85
CD36 (RPMI)	1417±294	1478±200	0.87
CD36 (DMSO 10^{-5})	1213±238	1451±245	0.60/0.93
CD36 (Pioglitazone 1μM)	1400±312	1467±336	0.64/0.97
CD36 (Troglitazone 1μM)	1294±322	1439±341	0.85/0.98

Values are mean ± SEM;
p-values are reported for:
In case of RPMI: comparison between healthy volunteers vs patients with type 2 diabetes;
In case of DMSO: comparison between RPMI incubation vs incubation with vehicle DMSO in healthy volunteers/patients with type 2 diabetes;
In case of pioglitazone and troglitazone: comparison between pioglitazone-or troglitazone incubation vs their vehicle DMSO in healthy volunteers/patients with type 2 diabetes.

Discussion

The main outcome of our study is that monocyte-endothelium-adhesion is increased in monocytes derived from DM2 patients. Furthermore this monocyte-endothelium-adhesion can be reduced by PPAR-γ agonists pioglitazone and troglitazone without changes in CD14, CD11b/18, CD62L and CD36 expression on monocytes.

Enhanced monocyte-endothelium adhesion in type 2 diabetic patients

Increased adherence of mononuclear cells from patients with type 2 diabetes mellitus to cultured endothelium *in vitro* may explain why accelerated atherosclerosis occurs in

patients with DM2 since the adherence of mononuclear cells to the endothelium represents the earliest step in atherosclerosis.7 Increased adhesiveness of diabetic monocytes to endothelial cells, but under static conditions and with cultured bovine aortic endothelial cells (BAEC) has been shown.[8] However, in the present study we showed under, physiologically more relevant, flow conditions significantly more adhesion to endothelium of monocytes derived from DM2 patients compared to healthy volunteers.
Potential determinants of increased monocyte-endothelial cell adhesion in type 2 diabetes include oxidative stress and advanced glycation end products (AGEs). Glycoxidation products accumulate in tissue collagen with age and at an accelerated rate in diabetes. Possible sources of oxidative stress and damage to proteins in diabetes include free radicals generated by autoxidation reactions of sugars, sugar adducts to protein, and by autoxidation of unsaturated lipids in plasma and membrane proteins.[17] Furthermore elevated plasma concentrations of the adhesion molecules ICAM-1 and VCAM-1 are seen in patients with DM1[6] or DM2.[18] In the present study we did not use diabetic endothelium but focussed on differences in adhesiveness of monocytes from type 2 diabetics compared to monocytes form healthy volunteers. Activated monocytes can activate endothelial cells resulting in production of CAMs and cytokines like interleukin-6 and interleukin-8, and monocyte chemoattractant protein-1 (MCP-1). These effects combined will lead to recruitment and activation of monocytes, as expressed by increased selectins and integrins on the outer membrane, causing eventually firm adhesion to the vessel wall.[16] From *in vitro* studies it is known that acute stimulation of monocytes results in rapid shedding of CD62L[19,20] or translocation of stored receptors like CD11b.[25] However, in this study no differences in the adhesion-surface antigens CD14, CD11b/18, CD62L and CD36 were observed. An alternative activator route for monocytes may be elevated levels of glucose, lipoproteins, and free fatty acids (FFA). Furthermore, these directly activated monocytes can activate the endothelium or destabilize atherosclerotic lesions by the production of ROS, tumor necrosis factor-α (TNF-α), and degradative enzymes like collagenase and gelatinase.[22]

Incubation with pioglitazone or troglitazone reduced adhesion in type 2 diabetics
The exact mechanism of a PPAR-γ induced decrease in monocyte-adhesion is likely to be a combination of anti-inflammatory effects upon the endothelium as well as an effect upon the monocytes. It is assumable that the inhibitory effect of PPAR-γ activation on NF-κB, STAT1 and AP-1[23,24] leads to decreased expression of adhesion molecule expression on monocytes and endothelium (like VCAM-1 and ICAM-1), eventually leading to decreased adhesion[11-13] and thus reducing the formation

of atherosclerotic plaques.[14] PPAR-γ agonism has an important anti-atherogenic influence on the endothelium and will therefore also reduce monocyte-endothelium-adhesion. Unfortunately we were not able to perform a unilateral incubation of monocytes or HUVEC alone to differentiate between the effects of PPAR-γ agonism directly on the endothelium, and the effects of PPAR-γ agonism directly on the monocytes. But, to exclude at least different effects of PPAR-γ agonism on endothelium, only HUVEC of non-diabetic subjects was used in all experiments to get proof of principle in the present study.

The observed reduction of monocyte-endothelium-adhesion by the PPAR-γ agonists pioglitazone and troglitazone under physiological flow-conditions means an inhibition in the early step of atherogenesis. In another study pioglitazone inhibited the expression of adhesion molecules on the endothelium but also decreased CD11b/CD18 expression on stimulated leukocytes.[11] However in the present study we did not find any difference in CD11b/CD18 expression on monocytes in DM2 patients compared to healthy volunteers. Incubation with pioglitazone or troglitazone did not affect CD11b/CD18 and other (adhesion-) surface-antigens like CD14, CD62L and CD36 expression on monocytes. These results are in line with a *in vitro* study by Toriumi et al. showing a reduction of monocyte-adhesion from a U937 cell line to HUVEC under flow by pre-incubation with pioglitazone. Also in that study the expression levels of CD11a, CD18, and CD49d were not affected after treatment but they found a reduction of actin filament and a decrease in RhoA GTPase activity.[25] Probably diabetic monocytes are in a more inflammatory activated state and thus more sensitive for PPAR-γ agonism. In the present set of experiments it is possible that monocytes already become activated. However this should implicate an underestimation of the results. Staels et al. found that whereas PPAR-α is already present in undifferentiated monocytes, PPAR-γ expression is yet induced upon differentiation into macrophages.[26]

In conclusion, monocyte-endothelium-adhesion is increased in monocytes derived from DM2 patients and this increased monocyte-endothelium-adhesion can be reduced by PPAR-γ agonists pioglitazone and troglitazone without changes in CD14, CD11b/18, CD62L and CD36 expression on monocytes. PPAR-γ agonists may be beneficial in the prevention of atherosclerosis in type 2 diabetes patients.

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References

1. Turner RC, Millns H, Neil HA, Stratton IM, Manley SE, Matthews DR, Holman RR. Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23). BMJ 1998;316:823-828.
2. Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. Atherosclerosis 2003;170:191-203.
3. Libby P. Changing concepts of atherogenesis. J Intern Med 2000;247:349-358.
4. Plutzky J. Atherosclerotic plaque rupture: emerging insights and opportunities. Am J Cardiol 1999;84:15J-20J.
5. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-126.
6. Fasching P, Veitl M, Rohac M, Streli C, Schneider B, Waldhausl W, Wagner OF. Elevated concentrations of circulating adhesion molecules and their association with microvascular complications in insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 1996;81:4313-4317.
7. Carantoni M, Abbasi F, Chu L, Chen YD, Reaven GM, Tsao PS, Varasteh B, Cooke JP. Adherence of mononuclear cells to endothelium in vitro is increased in patients with NIDDM. Diabetes Care 1997;20:1462-1465.
8. Desfaits AC, Serri O, Renier G. Normalization of plasma lipid peroxides, monocyte adhesion, and tumor necrosis factor-alpha production in NIDDM patients after gliclazide treatment. Diabetes Care 1998;21:487-493.
9. Martens FM, Visseren FL, Lemay J, de Koning EJ, Rabelink TJ. Metabolic and additional vascular effects of thiazolidinediones. Drugs 2002;62:1463-1480.
10. Dormandy JA, Charbonnel B, Eckland DJ, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefebvre PJ, Murray GD, Standl E, Wilcox RG, Wilhelmsen L, Betteridge J, Birkeland K, Golay A, Heine RJ, Koranyi L, Laakso M, Mokan M, Norkus A, Pirags V, Podar T, Scheen A, Scherbaum W, Schernthaner G, Schmitz O, Skrha J, Smith U, Taton J. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. Lancet 2005;366:1279-1289.
11. Imamoto E, Yoshida N, Uchiyama K, Kuroda M, Kokura S, Ichikawa H, Naito Y, Tanigawa T, Yoshikawa T. Inhibitory effect of pioglitazone on expression of adhesion molecules on neutrophils and endothelial cells. Biofactors 2004;20:37-47.
12. Jackson SM, Parhami F, Xi XP, Berliner JA, Hsueh WA, Law RE, Demer LL. Peroxisome proliferator-activated receptor activators target human endothelial cells to inhibit leukocyte-endothelial cell interaction. Arterioscler Thromb Vasc Biol 1999;19:2094-2104.
13. Wang N, Verna L, Chen NG, Chen J, Li H, Forman BM, Stemerman MB. Constitutive activation of peroxisome proliferator-activated receptor-gamma suppresses pro-inflammatory adhesion molecules in human vascular endothelial cells. J Biol Chem 2002;277:34176-34181.

14. Pasceri V, Wu HD, Willerson JT, Yeh ET. Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators. Circulation 2000;101:235-238.
15. Henrita vZ, Saelman EU, Schut-Hese KM, Wu YP, Slootweg PJ, Nieuwenhuis HK, de Groot PG, Sixma JJ. Platelet adhesion to collagen type IV under flow conditions. Blood 1996;88:3862-3871.
16. Ulfman LH, Kuijper PH, van der Linden JA, Lammers JW, Zwaginga JJ, Koenderman L. Characterization of eosinophil adhesion to TNF-alpha-activated endothelium under flow conditions: alpha 4 integrins mediate initial attachment, and E-selectin mediates rolling. J Immunol 1999;163:343-350.
17. Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes 1991;40:405-412.
18. Devaraj S, Jialal I. Low-density lipoprotein postsecretory modification, monocyte function, and circulating adhesion molecules in type 2 diabetic patients with and without macrovascular complications: the effect of alpha-tocopherol supplementation. Circulation 2000;102:191-196.
19. Kuijper PH, Gallardo Torres HI, van der Linden JA, Lammers JW, Sixma JJ, Zwaginga JJ, Koenderman L. Neutrophil adhesion to fibrinogen and fibrin under flow conditions is diminished by activation and L-selectin shedding. Blood 1997;89:2131-2138.
20. Kansas GS. Selectins and their ligands: current concepts and controversies. Blood 1996;88:3259-3287.
21. Miller LJ, Bainton DF, Borregaard N, Springer TA. Stimulated mobilization of monocyte Mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. J Clin Invest 1987;80:535-544.
22. Jordan JE, Zhao ZQ, Vinten-Johansen J. The role of neutrophils in myocardial ischemia-reperfusion injury. Cardiovasc Res 1999;43:860-878.
23. Cuzzocrea S, Pisano B, Dugo L, Ianaro A, Maffia P, Patel NS, Di Paola R, Ialenti A, Genovese T, Chatterjee PK, Di Rosa M, Caputi AP, Thiemermann C. Rosiglitazone, a ligand of the peroxisome proliferator-activated receptor-gamma, reduces acute inflammation. Eur J Pharmacol 2004;483:79-93.
24. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 1998;391:79-82.
25. Toriumi Y, Hiraoka M, Watanabe M, Yoshida M. Pioglitazone reduces monocyte adhesion to vascular endothelium under flow by modulating RhoA GTPase and focal adhesion kinase. FEBS Lett 2003;553:419-422.
26. Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, Staels B. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. J Biol Chem 1998;273:25573-25580.

CHAPTER 7

Short-term pioglitazone treatment does not affect cytokine production of monocytes in contrast with the natural PPAR-γ ligand prostaglandin J_2

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Condensed abstract

To determine whether PPAR-γ agonism influences cytokine production directly we examined, in a randomised, cross-over, placebo-controlled, double blind trial, the effects of 4 weeks pioglitazone treatment on TNF-α, IL-6, and IL-10 production in with LPS-stimulated whole blood from 20 male type 2 diabetic patients ex vivo. The effects of pioglitazone on isolated monocytes are compared with the natural PPAR-γ ligand prostaglandin J_2 (PGJ_2) *in vitro*.
We conclude that short-term pioglitazone treatment does not affect TNF-α, IL-6, and IL-10 production of monocytes in contrast with the natural PPAR-γ ligand PGJ_2.

Abstract

Introduction Monocyte derived lipid-loaded macrophages are involved in atherosclerotic lesion development, destabilization and plaque-rupture. PPAR-γ agonists, like pioglitazone, are regulators of glucose homeostasis, lipid metabolism and appear to have anti-inflammatory effects, which may influence atherogenesis. In this study we determined the effect of PPAR-γ agonism on cytokine production in whole blood from type 2 diabetics with pioglitazone ex vivo. The effects of pioglitazone on isolated monocytes are compared with the natural PPAR-γ ligand prostaglandin J_2 (PGJ_2) *in vitro*.

Methods and Results A randomised, cross-over, placebo-controlled, double blind trial was performed in which pioglitazone 30 mg once daily was administered in 20 patients with type 2 diabetes for 4 weeks. Whole blood was stimulated ex vivo with different concentrations LPS (0, 0.6, and 6 ng/ml stimulated for 15min, and 4h). No differences were observed between the pioglitazone- vs. the placebo-group in LPS-stimulated TNF-α, IL-6, and IL-10 production.
Peripheral blood mononuclear cells and isolated monocytes (1x10^4 in 200 μl/well) from 4 healthy volunteers, stimulated for 20h with lipopolysaccharides (LPS) (100 pg/ml), phorbol myristate acetate (PMA) (10 ng/ml) and okadaic acid (40 ng/ml) have been incubated with various concentrations of pioglitazone (0, 11, 33 and 100 μM) *in vitro*. Pioglitazone did not affect TNF-α, and IL-6 production. The natural PPAR-γ ligand PGJ_2 (0, 5.6, 16.7 and 50 μM) inhibited the cytokine production.

Conclusions Short-term pioglitazone treatment does not affect TNF-α, IL-6, and IL-10 production of monocytes in contrast with the natural PPAR-γ ligand PGJ_2.

Keywords cytokine production, diabetes mellitus, IL-6, pioglitazone, TNF-α.

Introduction

Low-grade chronic vascular inflammation contributes to the pathogenesis of cardiovascular disease. Elevated levels of proinflammatory proteins are predictive for both cardiovascular disease and type 2 diabetes.[1] The proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in particular, are elevated in patients with endothelial dysfunction and ischemic heart disease.[2,3] In contrast, serum levels of the potent anti-inflammatory cytokine IL-10 have been shown to be decreased in patients with acute coronary syndromes,[4] thus suggesting that reduced levels of IL-10 may favour plaque instability and accelerate the development of acute coronary syndromes. The progression of the atherosclerotic plaque eventually leading to plaque rupture is to view upon as an chronic inflammatory process.[5] Atherosclerotic disease in large vessels is the major cause of morbidity and mortality in patients with type 2 diabetes (DM2).[6]
Thiazolidinediones (TZDs), such as pioglitazone, are a class of oral anti-diabetic agents and are ligands for peroxisome proliferator-activated receptors (PPARs), in particular PPAR-γ. These nuclear transcription factors are involved in glucose homeostasis, lipid and lipoprotein metabolism and adipogenesis. TZDs improve glycemic control by increasing insulin sensitivity, but may also have potential anti-inflammatory effects.[7] Several lines of evidence suggest that PPAR-γ ligands may influence the development of atherosclerosis due to anti-inflammatory effects of monocytes by potent inhibition of nuclear factor-κB (NF-κB)-dependent transcription.[8-11] In contrast to these data, others report that IL-6 and TNF-α levels were not affected by TZD treatment.[12,13] Because of these conflicting results we investigated the direct effects of pioglitazone on cytokine production of human-monocytes ex vivo and in comparison with the natural PPAR-γ ligand PGJ_2 *in vitro*.

Subjects and methods

Subjects

Twenty male, non-smoking patients with type 2 diabetes were recruited. All patients were treated with oral anti-hyperglycemic agents (9 patients with sulphonylurea derivates, 4 patients with metformin and 7 patients with a combination of metformin

and sulphonylurea derivates) which continued during the study. Subjects with poor glycemic control (HbA1c >9%) were not included. Other relevant exclusion criteria were presence of macro- or microvascular disease and use of vasoactive medication (e.g. beta-blockers, calcium entry blockers, ACE-inhibitors, angiotensin type 1 receptor blockers, statins, aspirin, non-steroidal inflammatory drugs).
The protocol was approved by the ethical review board of the University Medical Center Utrecht (UMCU). All subjects gave written informed consent. Measurements were carried out in accordance with local institutional guidelines in a Good Clinical Practice-certified unit.

Study design
The original study was designed as a prospective, randomised, crossover, placebo-controlled, double blind trial.[14] Patients eligible to take part in the study were randomised to receive pioglitazone 30 mg once daily (Eli Lilly, Indianapolis, U.S.A.) or placebo for 4 weeks in addition to their oral anti-hyperglycemic agents. Blood sampling for cytokine-production measurements was performed after four weeks followed by a washout period of six weeks. Crossover of therapy occurred at the end of the washout period with reanalysis of the cytokine-production at 4 weeks. At the beginning and at the end of each 4-week treatment period laboratory parameters were determined. Patients were instructed to fast for at least 10 hours prior to the tests. No study medication or other medication was used on the morning of the study days.

Methods
LPS stimulation of whole blood ex vivo
Whole blood samples of 4.5 ml were collected using sterile endotoxin-free blood collection tubes containing heparin. Venapuncture and transportation were performed uniformly in all experiments without agitating the samples. Samples were directly divided into 0.3 ml aliquots to be stimulated with different concentrations LPS (Boivin method, Difco Laboratories, Detroit). Obtained LPS final concentrations were 0, 0.6, and 6 ng/ml (RPMI-diluted: Gibco, Grand Island, NY). Stimulation was directly performed for 15min and 4h at 20°C. After stimulation the aliquots were centrifuged twice at 600xg and the supernatants were directly stored at -80°C.
Measurements of plasma TNF-α, IL-6, and IL-10 were performed with commercially available kits (ELISA; R&D Systems Inc./ minimum detectable doses resp.: 0.5, 0.7 and 3.9 pg/ml).

Other laboratory assessments
Glucose, creatinine, serum alanine transferase (ALT), serum aspartate transferase (AST), total cholesterol, triglycerides and high-density lipoprotein cholesterol (HDL-C) were measured by standard enzymatical laboratory methods (Vitros 250; Johnson/Johnson). Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula. HbA1c and free fatty acids (FFA) were photometrically performed (Hitachi 911; Roche). Insulin levels were determined with an immunological method (Immulite 2000; Diagnostic Prod Corp.).

LPS stimulation of peripheral blood mononuclear cells and isolated monocytes in vitro
Confirmation was provided by pilot-measurements of TNF-α, and IL-6 production from peripheral blood mononuclear cells (MNC) or isolated monocytes ($1x10^4$ in 200 μl/well) from 4 healthy volunteers, stimulated for 20h with LPS (100 pg/ml), PMA (10 ng/ml; Sigma-Aldrich) and okadaic acid (40 ng/ml; Sigma-Aldrich), in the presence of various concentrations of pioglitazone (0, 11, 33 and 100 μM) and the natural PPAR-γ ligand PGJ_2 (0, 5.6, 16.7 and 50 μM; Sigma-Aldrich).
The MNC fraction was prepared by dilution with an equal volume of PBS with 2 % EDTA at 20°C and layered over a solution containing 3ml Ficoll-Histopaque per 10ml and centrifuged at 2000rpm for 20 min.
Isolated monocytes were obtained by harvesting the mononuclear cell layer, transfered to a fresh tube, mixed with 3 times the volume of PBS, and centrifuged at 2000 rpm for 5 min. The supernatant was removed and the pellet was resuspended. Metal beats coated with CD14 antibodies (20μl; 0.01M; Sigma-Aldrich) per $1x10^7$ cells were added and incubated for 15 min. Monocytes were separated using Magnetic Cell Sorting (MACS).

Statistical analysis
Results from laboratory analyses are expressed as mean + standard error. Differences in metabolic parameters between pioglitazone and placebo treatment were analysed with a paired t-test (95% confidence interval). In case of non-normal distribution the Wilcoxon Signed Rank test was used. Statistical significance was taken at the 5% level.

Results

All subjects completed the study. The demographic, clinical and laboratory characteristics are shown in Table 1. No carry-over effects between the two treatment periods were observed for any parameter.

Table 1. Clinical characteristics at baseline and cross-over

	Baseline values at study start with placebo (N=10)	**Baseline values at crossover to pioglitazone** (N=10)	**Baseline values at study start with pioglitazone** (N=10)	**Baseline values at crossover to placebo** (N=10)
Age (yrs)	58±2	58±2	55±3	55±3
Weight (kg)	86.6±5.2	89.0±5.2	98.2±5.7	99.0±5.7
BMI	28.8±1.6	29.3±1.6	29.9±1.6	30.2±1.6
Waist (cm)	104.6±2.8	104.6±2.8	111.8±4.2	111.8±4.2
SBP (mmHg)	159±4	148±5	138±7	142±7
DBP (mmHg)	92±2	90±2	85±3	90±3
Fasting glucose (mmol/l)	7.9±0.8	6.3±0.5	9.1±0.5	8.1±0.6
Insulin (mU/l)	18.3±5.0	14.1±2.4	23.5±4.	20.6±3.3
HOMA-R	7.5±2.7	7.7±2.7	10.2±2.2	7.6±1.4
HbA1c (%)	7.0±0.4	6.6±0.4	7.1±0.3	6.9±0.3
Total cholesterol (mmol/l)	5.7±0.3	5.2±0.2	5.8±0.3	5.6±0.3
HDL cholesterol (mmol/l)	1.21±0.08	1.20±0.08	1.14±0.06	1.10±0.09
LDL cholesterol (mmol/l)	3.4±0.28	3.2±0.2	3.8±0.19	3.7±0.19
Triglycerides (mmol/l)	2.24±0.43	1.75±0.25	1.84±0.32	1.80±0.27
Free fatty acids (μmol/l)	656±80	615±62	699±101	654±77
ASAT (U/l)	35±3	32±3	27±3	27±5
ALAT (U/l)	40±7	34±4	37±8	39±10
Creatinine (μmol/l)	79±4	78±4	74±1	76±2
Hematocriet (l/l)	0.45±0.01	0.44±0.01	0.46±0.01	0.46±0.01
hs-CRP (mg/l)	3.3±0.5	4.3±0.8	4.2±1.1	4.2±0.9

Values are mean ±SEM

Influence of pioglitazone on anthropometric, hemodynamic and metabolic parameters
Table 2. reports the outcome of pioglitazone- or placebo treatment on anthropometric, hemodynamic and metabolic parameters. Short-term pioglitazone treatment for 4 weeks already reduced insulin and FFA concentrations compared to placebo (18.3±2.4 vs. 14.8±2.1 mU/l; p=0.03 and 641±46 vs. 542±33 μmol/l; p=0.04, respectively). Furthermore a significant decrease in CRP concentration was observed during pioglitazone treatment compared to placebo after only 4 weeks (3.5±0.6 vs. 2.6±0.5 mg/l, p=0.01). We did not observe a difference in the effects of pioglitazone between patients using metformin or a combination of metformin and sulphonylurea derivates and the group of patients only treated with sulphonylurea derivates (data not shown). No change in weight or blood pressure was observed during pioglitazone treatment. Fasting glucose

Table 2. Clinical characteristics after treatment

	Placebo (N=20)	**Pioglitazone** (N=20)	**p-value**
Weight (kg)	94.4±4.1	94.6±3.9	0.68
SBP (mmHg)	136±4	135±3	0.94
DBP (mmHg)	91±2	87±2	0.89
Fasting plasma glucose (mmol/l)	7.1±0.4	6.7±0.4	0.19
Insulin (mU/l)	18.3±2.4	14.8±2.1	0.03*
HOMA-R	6.1±1.1	4.9±0.9	0.06
HbA1c (%)	6.8±0.2	6.7±0.2	0.50
Total cholesterol (mmol/l)	4.9±0.2	5.0±0.2	0.38
HDL cholesterol (mmol/l)	1.02±0.04	1.04±0.05	0.36
LDL cholesterol (mmol/l)	3.1±0.14	3.2±0.16	0.69
Triglycerides (mmol/l)	1.60±0.12	1.59±0.17	0.60
Free fatty acids (μmol/l)	641±46	542±33	0.04*
ASAT (U/l)	27±2	28±3	0.88
ALAT (U/l)	34±5	35±5	0.65
Creatinine(μmol/l)	72±2	69±2	0.09
Hematocriet (l/l)	0.43±0.01	0.41±0.01	0.08
CRP (mg/l)	3.5±0.6	2.6±0.5	0.01*

Values are mean ±SEM;
p-values are reported for comparison between placebo and pioglitazone
* p-values < 0.05

concentrations and lipids were not different between the pioglitazone and placebo treatment periods. Edema did not occur in any patient on pioglitazone treatment. Pioglitazone treatment was not associated with liver enzyme abnormalities.

Effects of pioglitazone on TNF-α, IL-6, and IL-10 production in LPS-stimulated whole blood from type 2 diabetic patients *ex vivo*

In table 3. the production of TNF-α, IL-6, and IL-10 in whole blood from type 2 diabetic patients after treatment with pioglitazone or placebo for 4 weeks is shown. The effects of different concentrations and different incubation times of LPS-stimulation of whole blood are also shown. No significant differences were observed between the pioglitazone- vs. the placebo-group in TNF-α, IL-6, and IL-10 production.

Table 3. Cytokine production in LPS-stimulated whole blood system after treatment

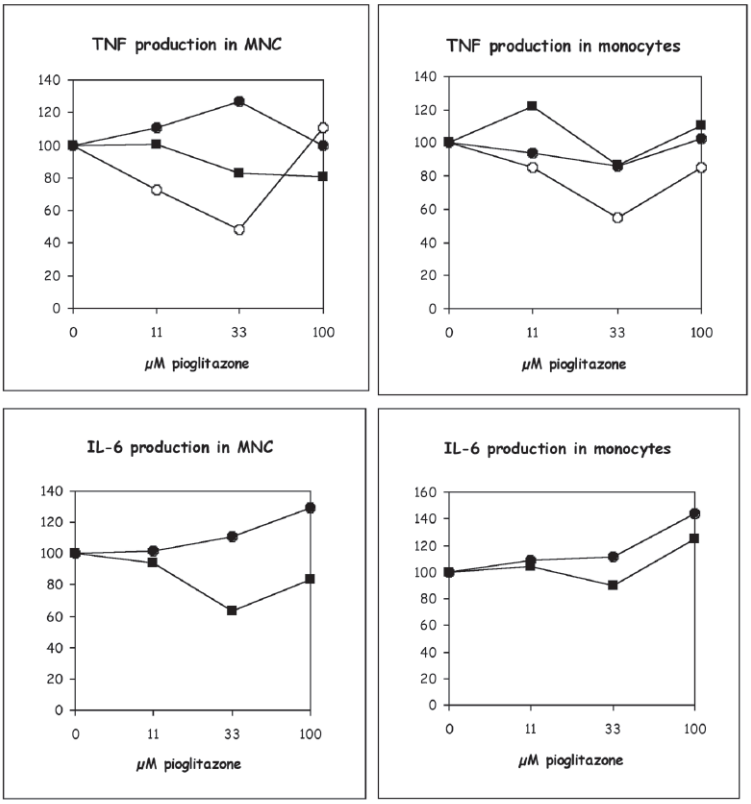
		Placebo (N=20)	**Pioglitazone** (N=20)	**p-value**
TNF-α (pg/ml)	Plasma	4.7±0.9	3.9±0.7	0.24
	LPS 0.6 ng/ml for 15min	4.8±0.8	4.1±0.8	0.37
	LPS 0.6 ng/ml for 4h	356±78	354±57	0.97
	LPS 6 ng/ml for 15 min	5.0±0.9	5.1±1.3	0.92
	LPS 6 ng/ml for 4h	724±153	648±128	0.55
IL-6 (pg/ml)	Plasma	13.6±1.4	11.4±2.7	0.43
	LPS 0.6 ng/ml for 15 min	12.5±3.6	10.4±1.6	0.55
	LPS 0.6 ng/ml for 4h	1336±296	1169±276	0.46
	LPS 6 ng/ml for 15 min	12.1±2.1	14.4±2.5	0.34
	LPS 6 ng/ml for 4h	2830±396	3184±410	0.44
IL-10 (pg/ml)	Plasma	6.0±2.2	3.7±1.0	0.25
	LPS 0.6 ng/ml for 15 min	14.3±8.1	7.8±4.5	0.11
	LPS 0.6 ng/ml for 4h	14.5±7.5	11.5±5.9	0.17
	LPS 6 ng/ml for 15 min	11.8±7.3	6.1±3.5	0.16
	LPS 6 ng/ml for 4h	12.8±6.1	9.8±3.8	0.32

Values are mean ±SEM;
p-values are reported for comparison between placebo and pioglitazone

Effects of pioglitazone on TNF-α and IL-6 production by MNC and isolated monocytes from healthy volunteers *in vitro*

Incubation with various concentrations of pioglitazone (0, 11, 33 and 100 μM) for 20h did not affect TNF-α (Figure 1a + 1b) or IL-6 (Figure 1c + 1d) production in peripheral blood mononuclear cells (Figure 1a + 1c) or isolated monocytes (Figure 1b + 1d) from healthy volunteers stimulated with LPS (100 pg/ml) or PMA (10 ng/ml) or okadaic acid (40 ng/ml) for 20h.

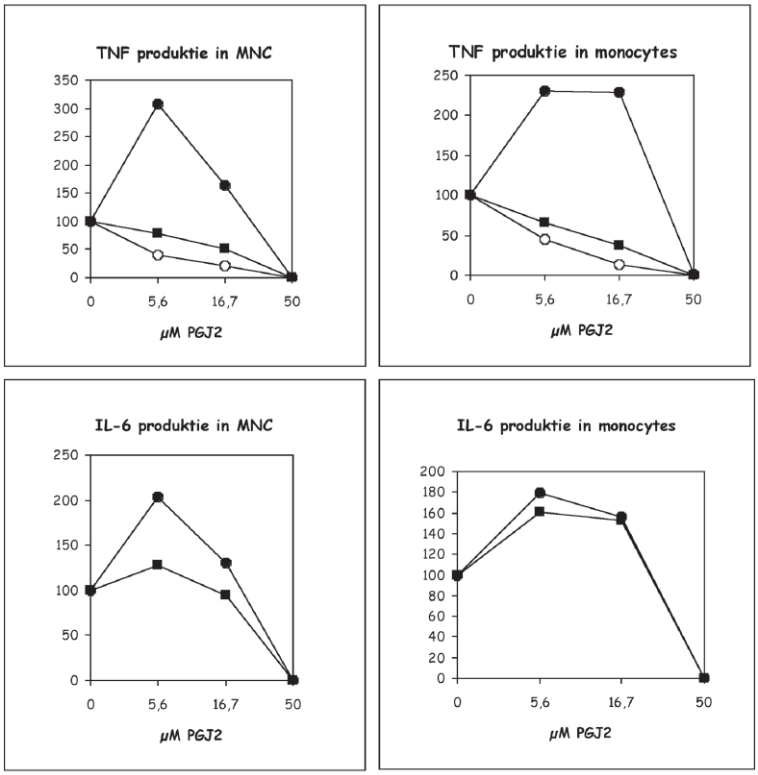
Figure 1. Effects of pioglitazone on TNF-α and IL-6 production by MNC and isolated monocytes from healthy volunteers *in vitro*



Effects of PGJ_2 on TNF-α and IL-6 production by MNC and isolated monocytes from healthy volunteers *in vitro*

Incubation with various concentrations of the natural PPAR-γ ligand PGJ_2 (0, 5.6, 16.7 and 50 μM) for 20h resulted in a decrease in TNF-α (Figure 2a + 2b) and IL-6 (Figure 2c + 2d) production by peripheral blood mononuclear cells (Figure 2a + 2c) and isolated monocytes (Figure 2b + 2d) from healthy volunteers stimulated with LPS (100 pg/ml) and okadeic acid (40 ng/ml).

Figure 2. Effects of PGJ_2 on TNF-α and IL-6 production by MNC and isolated monocytes from healthy volunteers *in vitro*



Discussion

In the current study we demonstrated that pioglitazone did not affect cytokine production directly. Neither in LPS-stimulated whole blood ex vivo after short-term pioglitazone treatment for 4 weeks in type 2 diabetics, nor after incubation for 20h with various concentrations of pioglitazone in peripheral blood MNC or isolated monocytes from healthy volunteers *in vitro*. In contrast, the natural PPAR-γ ligand PGJ_2 did decrease TNF-α, and IL-6 production in peripheral blood MNC and isolated monocytes from healthy volunteers *in vitro*.

In the present study we focused on the potential direct effects of the PPAR-γ agonist pioglitazone on monocytes. The hypothesis is that PPAR-γ agonism can bring monocytes in a less active state resulting in reduced cytokine production after stimulation. Several lines of evidence suggest that PPAR-γ ligands may beneficially influence the development of atherosclerosis due to anti-inflammatory effects of monocytes by potent inhibition of NF-κB-dependent transcription.[8,15] Incubation of human monocytes with synthetic PPAR-γ ligands inhibits the production of inflammatory cytokines.[9,14] The original report of this effect by Jiang et al suggested that troglitazone and the natural PPAR-γ ligand PGJ_2 selectively abrogate cytokine production (TNF-α and IL-6) by PMA stimulation of human monocytes, but not by LPS.[10] In a mouse model of atherosclerosis, Li et al. demonstrated that TZDs reduce TNF-α expression in the aortic root.[11] We and others previously demonstrated anti-inflammatory effects of pioglitazone in diabetic patients as measured by attenuation of the postprandial increase of neutrophils, IL-6 and IL-8[17], and a decrease in systemic low-grade plasma levels of C-reactive protein (CRP) after only 4 weeks of pioglitazone therapy.[14] Also after 26 weeks of treatment with another TZD, rosiglitazone, a reduction in CRP has been shown.[18] In contrast to these data, other reports indicated that IL-6 and TNF-α levels were not affected by TZD treatment in db/db mice in response to LPS challenge.[12] Another study, performed in rat peritoneal macrophages, also reported an increase in LPS-stimulated TNF-α production after rosiglitazone treatment.[13] The IL-6 plasma level, a strong inducer of CRP, was expected to decrease too after 4 weeks of pioglitazone treatment, but this was not the case.[14] In line with the results of the present study, Staels et al. found that whereas PPAR-α is already present in undifferentiated monocytes, PPAR-γ expression is yet induced upon differentiation into macrophages.[19] This may also explain the observed inhibition of cytokine production by the putative endogenous PPAR-γ ligand PGJ_2.

That PGJ_2 is stronger in its inhibition may be explained by differences in PPAR-α agonistic activities by PPAR-γ-agonists.[20] Furthermore PGJ_2 has shown pronounced inhibition of inflammatory cytokines production by inhibition of nuclear factor-κB (NF-κB)-dependent transcription although this is not very selective for PPAR-γ and also acts via PPAR-independent mechanisms.[21-23]
Another reason for the variety in anti-inflammatory effects of PPAR-γ agonism on monocytes may be the difficulty to study monocytes *in vitro*. We determined the cytokine production in a whole blood stimulation system to reduce the risk of activation of monocytes to a minimum. It is known that monocytes can already be activated when removed from their environment. Although cytokine production induced by LPS in whole blood samples ex vivo varies in healthy subjects by 60 to 75% on the basis of heritability alone.[24] In combination with a small laboratory error and small intra-individual variation makes whole blood stimulation a reliable system for cytokine production measurements.[25] It is clear that there are many conflicting findings on the effects of PPAR-γ activation on monocyte/macrophage inflammatory responses and many studies are being revisited using different PPAR-ligands at various concentrations. It appears likely that the anti-inflammatory effects of several PPAR-γ agonists may vary depending on the source (e.g. primary vs. transformed cells, mouse vs. human), the state of differentiation/activation of monocytes/macrophages studied, and the selectivity of the PPAR-γ agonists used.

We conclude that short-term pioglitazone treatment does not affect TNF-α, IL-6, and IL-10 production of monocytes directly in contrast to the natural PPAR-γ ligand PGJ_2. The present study provides evidence that systemic anti-inflammatory effects of pioglitazone are not due to direct effects on monocytes but probably because of general inhibition of the NF-κB pathway, improvement of adipocyte function, and a decrease of endothelial cell activation. These changes may in turn affect monocyte function.

References

1. Haddy N, Sass C, Droesch S et al. IL-6, TNF-alpha and atherosclerosis risk indicators in a healthy family population: the STANISLAS cohort. Atherosclerosis. 2003;170:277-283.
2. Ridker PM, Rifai N, Pfeffer M et al. Elevation of tumor necrosis factor-alpha and increased risk of recurrent coronary events after myocardial infarction. Circulation. 2000;101:2149-2153.
3. Ridker PM, Rifai N, Stampfer MJ et al. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation. 2000;101:1767-1772.
4. Smith DA, Irving SD, Sheldon J et al. Serum levels of the antiinflammatory cytokine interleukin-10 are decreased in patients with unstable angina. Circulation. 2001;104:746-749.
5. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340:115-126.
6. Turner RC, Millns H, Neil HA et al. Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23). BMJ. 1998;316:823-828.
7. Martens FM, Visseren FL, Lemay J et al. Metabolic and additional vascular effects of thiazolidinediones. Drugs. 2002;62:1463-1480.
8. Moore KJ, Fitzgerald ML, Freeman MW. Peroxisome proliferator-activated receptors in macrophage biology: friend or foe? Curr Opin Lipidol. 2001;12:519-527.
9. Ricote M, Li AC, Willson TM et al. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature. 1998;391:79-82.
10. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature. 1998;391:82-86.
11. Li AC, Brown KK, Silvestre MJ et al. Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. J Clin Invest. 2000;106:523-531.
12. Thieringer R, Fenyk-Melody JE, Le Grand CB et al. Activation of peroxisome proliferator-activated receptor gamma does not inhibit IL-6 or TNF-alpha responses of macrophages to lipopolysaccharide in vitro or in vivo. J Immunol. 2000;164:1046-1054.
13. Guyton K, Bond R, Reilly C et al. Differential effects of 15-deoxy-delta(12,14)-prostaglandin J_2 and a peroxisome proliferator-activated receptor gamma agonist on macrophage activation. J Leukoc Biol. 2001;69:631-638.
14. Martens FM, Visseren FL, de Koning EJ et al. Short-Term Pioglitazone Treatment Improves Vascular Function Irrespective of Metabolic Changes in Patients With Type 2 Diabetes. J Cardiovasc Pharmacol. 2005;46:773-778.
15. Mohanty P, Aljada A, Ghanim H et al. Evidence for a potent antiinflammatory effect of rosiglitazone. J Clin Endocrinol Metab. 2004;89:2728-2735.
16. Ricote M, Welch JS, Glass CK. Regulation of macrophage gene expression by the peroxisome proliferator-activated receptor-gamma. Horm Res. 2000;54:275-280.

17. van Wijk JP, Cabezas MC, Coll B et al. Effects of rosiglitazone on postprandial leukocytes and cytokines in type 2 diabetes. Atherosclerosis. 2005.
18. Haffner SM, Greenberg AS, Weston WM et al. Effect of rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. Circulation. 2002;106:679-684.
19. Chinetti G, Griglio S, Antonucci M et al. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. J Biol Chem. 1998;273:25573-25580.
20. Hirakata M, Tozawa R, Imura Y et al. Comparison of the effects of pioglitazone and rosiglitazone on macrophage foam cell formation. Biochem Biophys Res Commun. 2004;323:782-788.
21. Straus DS, Pascual G, Li M et al. 15-deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. Proc Natl Acad Sci U S A. 2000;97:4844-4849.
22. Chawla A, Barak Y, Nagy L et al. PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med. 2001;7:48-52.
23. Delerive P, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors in inflammation control. J Endocrinol. 2001;169:453-459.
24. Westendorp RG, Langermans JA, Huizinga TW et al. Genetic influence on cytokine production and fatal meningococcal disease. Lancet. 1997;349:170-173.
25. van der Linden MW, Huizinga TW, Stoeken DJ et al. Determination of tumour necrosis factor-alpha and interleukin-10 production in a whole blood stimulation system: assessment of laboratory error and individual variation. J Immunol Methods. 1998;218:63-71.

CHAPTER 8

General discussion, summary and conclusions

Cardiovascular disease is the leading cause of morbidity and mortality in Western countries. The term cardiovascular disease comprises clinical manifestations of arterial atherosclerosis, such as peripheral artery disease, cerebrovascular disease, and coronary artery disease. Well-established risk factors are dyslipidemia, smoking, diabetes mellitus, hypertension, and clustered in association with abdominal obesity. In the pathophysiology of atherosclerosis, based on the respons to injury mechanism, the pathophysiological phenomenons **endothelial dysfunction** and **inflammation** are playing a pivitol role.

The endothelium has been identified as the central transducer through which risk factors can cause atherosclerosis and its complications. The central pathway is the activation of the endothelium causing endothelial dysfunction. Endothelial dysfunction is characterized by a shift towards reduced vasodilation, a pro-inflammatory state, and pro-thrombic properties. Mechanisms that participate in the reduced vasodilatory responses include reduced NO generation, oxidative stress, and reduced production of hyperpolarizing factor. Vasoactive peptides (such as Ang-II and ET-1), hypercholesterolemia, altered insulin signalling, and hyperglycemia, together with upregulation of adhesion molecules, generation of chemokines (such as MCP-1), and production of PAI-1, participate in these reduced vasodilatory responses, the inflammatory response, and contribute to a pro-thrombic state. Basically this involves the switch from a healthy condition, with concentrations of NO resulting in vasodilative and/or anti-atherosclerotic actions, to a situation of vasoactive dysfunction. Besides vasodilation, NO inhibits adhesion of leukocytes to the endothelium, inhibits platelet-vessel wall interaction, decreases endothelial permeability, inhibits VSMC proliferation and migration, and reduces vessel tone. However, in pathophysiological situations NO-availability *in vivo* is impaired because of redox signalling in the activated endothelium, as a result of reduced NO-formation, enhanced NO-breakdown or both. Summarized there are two major consequences of this endothelial activation:

First it will lead to the production of chemokines and expression of adhesion molecules. This will support the recruitment of inflammatory cells into the vessel wall. Although this system is physiological in the context of host-defense it may become inappropriate with prolonged periods of endothelial cell activation secondary to cardiovascular risk factors. As result, an inflammatory phenotype evolves in the vessel wall which leads to atherogenesis and plaque rupture. Evidence that atherosclerosis is a chronic inflammatory disease emerged when pathologic studies showed that T-lymphocytes and macrophages are present in the shoulder regions of atherosclerotic plaques in individuals who died from acute myocardial events.

Subsequent studies implicated a multitude of pro-inflammatory targets that appear to be involved in atherogenesis, atherosclerosis and plaque rupture. Atherosclerosis starts with the entry of monocytes into the vessel wall. This occurs as a result of monocytes following a chemical gradient of chemoattractant cytokines, produced by an injured endothelium. However the pathogenic mechanism accounting for this relation is yet unclear. It has been shown that inflammation is a contributor to atherosclerosis and its complications implicating that inflammation itself may represent a therapeutic target.

Second, the loss of NO-bioavailability by the endothelium, due to risk factors, affects the vessel wall structure. There are some preliminary data showing that NO inhibits VSMC proliferation in the vascular wall. As a result one could hypothesize that loss of NO-activity would result in increased VSMC-proliferation. This may accelerate the development of the atherosclerotic plaque, but may also lead to thicker, remodelled vessels. As a result these vessels become stiffer and start to produce pressure load on the heart.

Endothelial dysfunction is associated with most forms of cardiovascular disease such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure. Furthermore the severity of endothelial dysfunction has been shown to have prognostic value for cardiovascular events. Endothelial dysfunction is not only associated with cardiovascular disease but may also precede its development. In contrast, correction of endothelial dysfunction may be associated with reduced cardiovascular risk. Treatment of the causes of endothelial activation may restore endothelial function.

In **Chapter 1** we explored the hypothesis that endothelial function (in the sense of NO-bioavailability) is an important determinant of vessel wall structure. In conduit arteries, endothelial dysfunction is initiating atherogenesis. The other phenomenon of endothelial dysfunction is the elevated vascular resistance of resistance arteries (microcirculation) as observed in essential hypertension. We addressed this heterogeneity of vascular remodeling along the vascular tree and postulated that this regional difference may be related to a heterogeneous effect of eNOS on proliferation in conduit arteries vs. resistance vessels. We found that eNOS exerts a tonic inhibitory influence on aortic growth, with limited impact on small arteries, in basal and hyperthrophic conditions. This heterogeneous role of NO on vascular growth may explain the heterogeneity of vascular remodeling due to endothelial activation. These results may also explain the various effects of a stimulus on endothelial function, measured in the conduit brachial artery with FMD vs. measurements in the resistance vascular bed with FBF (chapter 4 and 5).

In the current thesis we explored several aspects of the described model of vascular injury in the setting of type 2 diabetes. Type 2 diabetes is emerging as a worldwide epidemic and currently about 200 million people are affected worldwide. An important cause for this increased incidence is the associated increase in patients with **insulin resistance** (approximately 400 million worldwide right now). Insulin resistance is driven by central obesity and is secondary to free fatty acid fluxes towards muscle and liver. However, genetic factors, particularly in Asian people, seem to play a role as well. Several risk factors such as glucose intolerance, hyperinsulinemia, obesity, dyslipidemia, hypertension, but also endothelial dysfunction and inflammation, have been found to cluster and often precede type 2 diabetes mellitus. Seeing the importance for primary prevention of early identification, the National Cholesterol Education Program (NCEP) of the US created in 2001 a readily applicable definition for daily clinical practice for this cluster of metabolic abnormalities, often referred to as **the metabolic syndrome**. It is assumable that the cardiovascular risk for patients belonging to the metabolic syndrome can just be calculated out of the sum of the separate cardiovascular risk factors. However, there are also data pointing towards a higher risk than expected from the separate cardiovascular risk factors (high triglycerides, low HDL, hyperglycemia, and high blood pressure) in relation to the metabolic syndrome. Treatment of these risk factors decreases the risk for cardiovascular events. Although it is difficult to distinguish between the relative effects of insulin resistance, it induces clearly a significantly increased cardiovascular and cerebrovascular risk. This effect is consistent across the spectrum of worsening glycemic control, from the onset of impaired glucose tolerance to the development of clinical diabetes.

Although a pathophysiological construct seems plausible, future research must unrevel pathophysiology and clinical use before the metabolic syndrome can be designated as a 'syndrome'. The individual components that make up the syndrome should be treated coherently. These are the end-sites of the vascular tree. However other risk factors act like trunk of this vascular tree. Awareness of the underlying disorders is important for understanding the pathophysiology and thus coherent treatment: be aware for insulin resistance and its associated (non-) traditional risk factors like endothelial dysfunction and inflammation.

Clinically it would be useful to correlate the severity of insulin resistance to the severity of alterations in vessel wall structure due to loss of NO-activity. In **chapter 2** we investigated a cross-sectional survey (2105 patients) and showed that the metabolic syndrome (and increasing number of components of the metabolic syndrome)

is associated with marginally increased carotid artery stiffness, while type 2 diabetes is associated with a marked increase in carotid artery stiffness. The increase in artery stiffness seemed consistent across the spectrum of worsening glycemic control.

Despite the extensive data analyzing the connections between diabetes mellitus and atherosclerosis, it remains unclear whether diabetic atherosclerosis is merely a similar but accelerated process recapitulating non-diabetic atherosclerosis. Certainly hyperglycemia itself may be a unique aspect of diabetes. Accelerated atherosclerosis may be due to hyperglycemia, resulting in elevated formation of advanced glycation endproducts (AGEs). In addition, insulin resistance leads to hypertension, hypertriglyceridemia, low HDL, small dense LDL, diminished fibrinolysis, and increased thrombogenicity. Since dysglycemia does not appear to be the major determinant of cardiovascular disease in type 2 diabetes, targeting the underlying pathophysiological mechanisms of the insulin resistance syndrome may be a more logical and beneficial strategy for reduction of cardiovascular morbidity and mortality. Pharmacological modulation of insulin resistance will not only improve glycemic control, but may also have beneficial effects on inflammation, endothelial dysfunction, dyslipidaemia and possibly other components of the syndrome independently from improvements in glucose metabolism.
The discovery of nuclear peroxisome proliferator-activated receptors (PPARs) and subsequent insight into their role in several metabolic pathways was a major breakthrough in the understanding of pathophysiological mechanisms underlying the insulin resistance syndrome. The **Thiazolidinediones (TZDs)** as a drug subclass are PPAR-γ agonists that have been exhaustively shown to improve peripheral (predominantly skeletal muscle) insulin sensitivity in both animals and humans. Two agents from this class, rosiglitazone and pioglitazone, are currently approved for use in patients with type 2 diabetes mellitus. An earlier TZD, troglitazone, was found to have similar effects on insulin sensitivity but was withdrawn from commercial use because of hepatotoxicitiy. Available TZDs improve insulin sensitivity predomin antly at the adipose level, with smaller hepatic effects. The increase in adipocyte differentiation and the redistribution of fat deposits from the abdominal to the subcutaneous space may also indirectly improve insulin sensitivity. Improvement of insulin sensitivity with TZDs results in a decreased demand for insulin secretion, which may prolong the viability of β-cells. Although the mechanisms by which TZDs may improve insulin sensitivity are not fully elucidated, observed reductions in FFA concentrations may mediated at least part of the effect. There are also preliminary

reports indicating that TZDs upregulate the production of adiponectin, an adipocyte-derived protein associated with improved insulin sensitivity.
In addition to their effects on insulin sensitivity, TZDs have demonstrated both direct and indirect vascular effects, including improved endothelial function, decreased vascular inflammation, lowered plasma FFA levels, improved LDL phenotype, and inhibition of VSMC proliferation. The TZDs have been demonstrated to reduce elevations in blood pressure and microalbuminuria and to improve fibrinolysis with consequent reversal of the procoagulant state. More recently attention has also been drawn to PPAR-γ independent mechanisms in the vascular wall. There are some indications that TZDs may reduce atherosclerotic progression in non-diabetic subjects as well. Data evaluating the effects of TZDs on surrogate markers of cardiovascular diseases are more and more available and appear quite promising. The aim of a main part of this thesis is to focus on the potential role of Thiazolidinediones in the pathophysiological mechanisms involved in vascular disease.
Chapter 3 is an overview of the metabolic and additional vascular effects of Thiazolidinediones.
In **chapter 4** we investigated the direct vascular effects of pioglitazone on the capacity of the vasculature to maintain its NO-release, in type 2 diabetic patients, in a double blind and crossover design. As measurement of NO-activity in the vessel wall, flow-mediated dilation in the conduit brachial artery was used. We focused on relatively short-term application of pioglitazone to tease out direct vascular effects from its indirect metabolic effects. Short-term pioglitazone treatment did ameliorate endothelial dysfunction in conduit arteries irrespective of significant beneficial changes in plasma levels of insulin, FFA, adiponectin, or CRP in type 2 diabetics, indicating direct vascular effects of pioglitazone.
We subsequently investigated whether a beneficial effect of TZDs on NO-bioavailability in type 2 diabetes also translates into better protection of the vessel wall from inflammatory stimuli.
To this end we investigated in **chapter 5**, whether diabetic subjects could maintain NO dominated endothelial function in the presence of increased concentrations of TNF-α. Therefore we first investigated the effect of TNF-α on endothelium-dependent vasodilation and secondly the effects of short-term pioglitazone treatment on TNF-α induced endothelial dysfunction in patients with type 2 diabetes mellitus. Intra-arterial TNF-α did induce impairment of endothelial function in type 2 diabetes, indicating an inflammatory endothelial injury while short-term pioglitazone treatment blocked this impairment, indicating direct anti-inflammatory effects.

However, in this study the endothelial function did not improve in the resistance vascular bed (measured with FBF), in contrast to the improvement in the conduit brachial artery (chapter 4). The heterogeneous role of NO on vascular growth along the vascular tree (as discussed in chapter 2), may one of the reasons for this difference in effects on endothelial function.
In addition, we investigated *ex vivo* whether TZDs changed the properties of monocytes to adhere to the endothelium, or to produce cytokines.
Therefore, we examined in **chapter 6**, in an *ex vivo* study, the effects of incubation of monocytes with TZDs on monocyte-endothelium-adherence under flow conditions. Monocytes from patients with type 2 diabetes adhered more to endothelium compared to monocytes from healthy volunteers. Pioglitazone- or troglitazone pre-treatment of monocytes reduced this monocyte adhesion.
In **chapter 7** we studied the cytokine production in whole blood from type 2 diabetes patients after short-term pioglitazone treatment, in a double blind and crossover design. However, short-term pioglitazone treatment did not affect TNF-α, IL-6, and IL-10 production of monocytes, confirming the results presented in chapter 6, that pioglitazone does not have direct anti-inflammatory effects on monocytes.

Although the mechanistic studies presented in this thesis add to the current understanding of both atherosclerosis and the pharmacologic effects of TZDs, results from large-scale clinical studies are needed to evaluated the effects on clinical endpoints. TZDs significantly reduced carotid arterial intima media thickness compared with placebo. Furthermore a study performed with intravascular ultrasound scanning showed that pioglitazone reduced neointimal tissue proliferation after coronary stent implantation in patients with type 2 diabetes mellitus.
Recently the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROACTIVE) pioglitazone treatment reduced the relative risk for the combined endpoint death, myocardial infarction, and stroke with 16%. Compared to placebo, pioglitazone reduced HbA1c by 0.5%, the plasma triglyceride concentration by 13.2%, the LDL/HDL ratio by 5.3%, and the blood pressure by 3 mmHg, while the HDL level increased by 8.9%. Other trials, like Rosiglitazone Evaluated for Cardiac outcomes and Regulation of Glycemia in Diabetes (RECORD), Carotid Intima Media Thickness in Atherosclerosis Using Pioglitazone (CHICAGO), and Pioglitazone Effect on Regression of Intravascular Sonographic Coronary obstruction Prospective Evaluation (PERISCOPE), are planned to report in 2007. The Study of Atherosclerosis with Ramipril and Rosiglitazone (STARR) is being conducted as a sub-study of the Diabetes Reduction

Approaches with Ramipril and Rosiglitazone Medications (DREAM) trial and is planned to have a follow up from 5 years.
Several issues concerning TZD treatment are yet to be resolved. The apparent paradox of adipocyte differentiation with weight gain concurring with the insulin-sensitising effects of TZDs is not completely understood. The TZD-induced decrease in blood pressure accompanied by an increase in the plasma volume has not been fully explained but make the TZDs contra-indicated for patients with heart failure. An important issue that needs to be resolved is the importance of raised cholesterol levels, in particular raised LDL levels, caused by rosiglitazone. Future research may provide answers to these questions, particularly with respect to the role of PPAR-γ, but also PPAR-α, in vascular pathophysiology. Although the concept of TZDs is promising, further research and additional long-term clinical trials concerning cardiovascular endpoints, are needed.

This thesis particularly puts forward the interplay between endothelial dysfunction and inflammation as biomarkers for cardiovascular disease.
Endothelial function (in the sense of NO-bioavailability) is an important determinant of vessel wall structure. eNOS exerts a tonic inhibitory influence on aortic growth, with limited impact on small arteries, in basal and hyperthrophic conditions. This heterogeneous role of NO on vascular growth may explain the heterogeneity of vascular remodeling (eutrophic in the resistance vascular bed and hypertrophic in the conduit arteries) due to endothelial activation. It may also explain the various effects of a stimulus on endothelial function, measured in the conduit brachial artery with FMD vs. measurements in the resistance vascular bed with FBF.
Furthermore it helps to understand the complicated pathophysiology behind the clustered risk factors in association with central obesity by linking endothelial dysfunction to insulin resistance. Increase in artery stiffness seems consistent across the spectrum of worsening glycemic control. Targeting the underlying pathophysiological mechanism of the clustered risk factors in association with central obesity may be a more logical and beneficial strategy for reduction of cardiovascular morbidity and mortality. Using the gained information of the beneficial metabolic effects of TZDs as insulin-sensitizers, this thesis reveals also potential direct vascular effects of TZDs improving insights in the pathophysiology of atherogenesis in insulin resistance. Focussing on endothelial dysfunction, short-term pioglitazone treatment induced maintenance of NO-release, irrespective of changes in plasma levels of insulin, FFA, adiponectin, or CRP, indicating a direct vascular effect of TZDs. Intra-arterial TNF-α induced impairment of endothelial function in type 2 diabetes while

short-term pioglitazone treatment blocked this impairment, indicating direct anti-inflammatory effects on the endothelium. TZD-pre-treatment reduced adhesion to the endothelium of diabetic monocytes. Furthermore, short-term pioglitazone treatment did not affect cytokine-production of monocytes. These studies indicate direct anti-inflammatory effects of pioglitazone on endothelium but not on monocytes.

Studying the effects of TZDs on endothelial function and inflammation may shed more light on the mechanisms involved in the clustered cardiovascular risk factors.

NEDERLANDSE SAMENVATTING

Hart- en vaatziekten zijn doodsoorzaak nummer 1 in de Westerse wereld. De term hart- en vaatziekten omvat alle klinische manifestaties van atherosclerose zoals perifeer vaatlijden, cerebrovasculaire ziekten en coronair vaatlijden. Bekende risico factoren zijn dyslipidemie, roken, diabetes mellitus, hypertensie en obesitas. De pathofysiologie van atherosclerose is gebaseerd op een 'respons to injury' principe, waardoor minder traditionele factoren, zoals **endotheeldisfunctie** en **ontsteking**, een noodzakelijke rol spelen.

Het endotheel is geïdentificeerd als de centrale regulator die ontregelt wordt door verscheidene risicofactoren waardoor atherosclerose en de daarbij horende complicaties kunnen ontstaan. Voornamelijk activatie van het endotheel door risicofactoren kan endotheeldisfunctie geven. Endotheeldisfunctie is gekarakteriseerd door een verschuiving van de processen die zich afspelen in het endotheel richting minder vasodilaterend, meer ontsteking en meer trombogeen. Mechanismen die betrokken zijn bij een verminderd vasodilaterend effect zijn, minder NO produktie, oxidatieve stress en minder produktie van hyperpolarizing factor. Vasoactieve peptiden zoals Ang-II and ET-1, hypercholesterolemie, onevenwichtige insuline signalling en hyperglycemie, samen met opregulatie van adhesie molekulen, produktie van chemokines zoals MCP-1, en productie van PAI-1, zorgen allemaal samen voor een verminderde vasodilatatie, de ontsteking en een trombogene toestand. Basaal ontstaat endotheeldisfunctie door een verschuiving van een gezonde conditie met lage concentraties NO welke vaatverwijding en/of anti-inflammatoire effecten geeft. Naast vaatverwijding, remt NO ook de adhesie van leukocyten aan het endotheel, remt NO de gladde spiercel-proliferatie en migratie, en vermindert het de vaattonus. In pathofysiologische situaties is de NO-beschikbaarheid *in vivo* echter, door redox signalling in het geactiveerde endotheel, verminderd door minder NO-productie, toegenomen NO-afbraak of beiden. Samengevat heeft zulk een pathofysiologische activatie van het endotheel twee gevolgen:

Allereerst zal dit leiden tot een productie van chemokines en een tot expressie komen van adhesie-moleculen. Dat heeft weer tot gevolg dat ontstekingscellen door de vaatwand zullen worden aangetrokken. In fysiologische omstandigheden is dit systeem er voor de bescherming tegen indringers, maar het kan echter ook doorslaan bij langdurige endotheel activatie door cardiovasculaire risicofactoren. In de vaatwand ontstaat nu een inflammatoir fenotype dat weer kan leiden tot atherogenesis en plaque-rupturen. Het besef dat atherosclerose gebaseerd is op chronische ontsteking begint bij pathologisch onderzoek waarbij T-lymfocyten en macrophagen worden aangetoond in de schouder van een atherosclerotische plaque in patiënten die zijn gestorven door een acute hartaanval. Hierop volgende studies geven steeds

meer aanwijzingen dat inflammatoire processen betrokken zijn bij atherogenese, atherosclerose en plaque ruptuur. Het begin van atherosclerose is het migreren van monocyten van het lumen de vaatwand in, waarbij de monocyten gedreven worden door een chemische aantrekking welke is ontstaan door beschadiging van het endotheel. Ondanks het feit dat het precieze pathofysiologische mechanisme hierachter nog niet geheel ontrafeld is, is het overduidelijk dat door de centrale rol die inflammatie speelt bij het ontstaan van atherosclerose, het remmen van deze ontstekings-procesen daadwerkelijk als behandeling van atherosclerose een veelbelovend aspect kan zijn.
Het andere effect, van verminderde NO-beschikbaarheid in het endotheel door cardiovasculaire risicofactoren, blijkt de verandering van de vaatwand-structuur. NO lijkt de gladde spiercel-proliferatie in de vaatwand te remmen. Verminderde NO-beschikbaarheid zou dan dus een toename van deze gladde spiercel-proliferatie ten gevolgen hebben. Dit zal dan weer de ontwikkeling tot een atherosclerotische plaque versnellen, maar ook leiden tot dikkere, van structuur veranderde vaten. Hierdoor worden de desbetreffende vaten stijver en geven dus onwillekeurig verhoogde druk op het hart- en vaatstelsel.
Endotheeldisfunctie is geassocieerd met alle vormen van hart- en vaatziekten zoals hypertensie, coronair vaatlijden, chronisch hartfalen, perifeer vaatlijden, diabetes en chronisch nierfalen. De mate van endotheeldisfunctie blijkt een prognostische maat voor cardiovasculaire events. Endotheeldisfunctie is niet alleen geassocieerd met hart- en vaatziekten maar blijkt ook, als een soort voorbode, een voorspeller voor cardiovasculaire events. Het behandelen en/of tegengaan van endotheeldisfunctie lijkt daarmee logischerwijs het risico op hart- en vaatziekten terug te dringen. Het gaat hier dan om de behandeling van de onderliggende aandoening die de endotheeldisfunctie veroorzaakt. Maar ook het behandelen van de losse componenten, die ieder op zich endotheeldisfunctie veroorzaken, zal helpen.
In **hoofdstuk 1** hebben we gekeken naar de hypothese dat endotheelfunctie (obv NO-beschikbaarheid) een belangrijke rol speelt in veranderingen van de vaatwand-structuur. Endotheeldisfunctie, als begin van atherogenese, speelt zich af in de grote vaten, terwijl er toegenomen vasculaire weerstand ontstaat in de weerstandsvaten bij essentiële hypertensie. Gepostuleerd kan worden dat deze heterogeniteit best wel eens te maken zou kunnen hebben met een heterogeen effect van eNOS op de proliferatie in grote vaten versus de kleine weerstandsvaten. Inderdaad blijkt eNOS een tonisch remmende werking te hebben op de groei in de aorta, terwijl er nauwelijks effect bereikt wordt door eNOS in de kleine weerstandsvaten, zowel in basale- als al in hypertrofische toestand. Dit verschil in effect van NO op de

vasculaire groei op verschillende plekken in het vasculaire netwerk kan de heterogeniteit van vasculaire remodeling, een adaptatie welke geassocieerd is met endotheeldisfunctie,verklaren. Ook zou het een verklaring kunnen zijn voor de soms verschillende resultaten van endotheelfunctie metingen bij dezelfde stimuli tussen een FMD-meting van de grote a. brachialis versus een FBF-meting van het onderarms-weerstandsvaatbed (zie hoofdstuk 4 en 5).

In dit proefschrift hebben we verschillende aspecten van het zojuist beschreven model van vaatschade onderzocht bij type 2 diabetes mellitus. Grote bezorgdheid bestaat bij het wereldwijd enorm toenemen van de prevalentie van diabetes (op dit moment zo'n 200 miljoen mensen wereldwijd). Een belangrijke rol speelt de enorme toename van patiënten met **insulineresistentie** (op dit moment zo'n 400 miljoen mensen wereldwijd). Deze insulineresistentie is vooral geassocieerd met obesitas, en daaraan gekoppeld de vrije vetzuur-flux naar andere weefsels dan de adipocyten (zoal het spierweefsel en de lever). Daarnaast spelen genetische veranderingen, met name bij Aziatische mensen, een belangrijke rol. Verschillende risico factoren, zoals glucoseintolerantie, hyperinsulinemie, obesitas, dyslipidemie, hypertensie, maar ook endotheeldisfunctie en inflammatie, komen vaak samen voor in één patiënt en resulteren vaak in het ontstaan van klinische diabetes mellitus type 2. Door in 2001 een voor de kliniek goed hanteerbare definitie te formuleren voor dit cluster van metabole abnormaliteiten, **het metabool syndroom** genaamd, voorziet de National Cholesterol Education Program (NCEP) van de VS het grote belang van primaire preventie en dus vroege opsporing hiervan. Het klinkt logisch wanneer wordt aangenomen dat het totale cardiovasculaire risico voor patiënten die tot het metabool syndroom behoren gewoon berekend kan worden uit de som van de verschillende betrokken cardiovasculaire risico factoren samen. Er bestaan echter ook data die suggereren dat het cardiovasculaire risico voor patiënten behorende tot het metabool syndroom zelfs hoger ligt dan de som der delen. Behandeling van alle tot het metabool syndroom behorende afzonderlijke pathofysiologische abnormaliteiten zal zeer zeker leiden tot risicoreductie voor cardiovasculaire events. Het is noodzakelijk om populatie-studies welke focussen op behandeling van de bekende cardiovasculaire risico factoren zoals obesitas, hoog plasma LDL cholesterol, hoog trygliceriden gehalte, laag plasma HDL, hyperglycemie en hoge bloeddruk, te continueren. Deze bekende risico factoren zijn namelijk, om het in een metafoor uit te drukken, de uiteinden van de vasculaire boom. Echter, we moeten ons wel bedenken dat de takken van de vasculaire boom gedragen worden door de stam. Deze metafoor is bedoeld om aan te geven dat er een drager bestaat voor alle tot nog toe bekende risico factoren,

bestaande uit niet-traditionele onderliggende pathofysiologische fenomenen. Deze pathofysiologische fenomenen zoals endotheeldisfunctie, inflammatie, oxidatieve stress, stollings-abnormaliteiten en ectopische vetophoping, zijn allen geassocieerd met insulineresistentie. Het is moeilijk precies te achterhalen wat nu werkelijk de effecten zijn van insulineresistentie, maar er bestaat duidelijk een significante relatie met verhoogd cardiovasculair- en cerebrovasculair risico. Het interessante is dat deze relatie stand houdt, zelfs versterkt, naarmate de glycemische controle verslechtert: van het begin van glucose intolerantie tot de ontwikkeling van klinische type 2 diabetes mellitus.
Ondanks het feit dat een pathofysiologische constructie logisch lijkt moet toekomstig onderzoek uitwijzen of we inderdaad pathofysiologisch en klinisch kunnen spreken van een 'metabool syndroom'.
Klinisch zou het zinvol zijn wanneer de ernst van insulineresistentie gekoppeld zou kunnen worden aan de ernst van vaatstijfheid ontstaan door verminderde NO-beschikbaarheid. In **hoofdstuk 2** hebben we een cross-sectioneel onderzoek gedaan om te kijken of er een relatie bestaat tussen vaatstijfheid van de carotis van 2105 patiënten met manifestaties van arterieel vaatlijden en het metabool syndroom en hoe deze zich verhoudt tot de relatie van vaatstijfheid van de carotis en type 2 diabetes mellitus. We kunnen concluderen in lijn met de hypothese dat naarmate de glycemische control verslechtert, en er dus sprake is van meer insulineresistentie, er ook een sterkere relatie bestaat met vaatstijfheid van de carotis in patiënten met manifestaties van arterieel vaatlijden. De vaatstijfheid is iets vergroot bij patiënten met het metabool syndroom en neemt toe naarmate een patiënt meer componenten van het metabool syndroom heeft. De meeste vaatstijfheid blijkt echter aanwezig bij patiënten met type 2 diabetes mellitus.

Ondanks uitgebreid onderzoek naar het verband tussen diabetes en de onvermijdelijke cardiovasculaire complicaties is het helaas nog niet helemaal duidelijk of de aggresiever lijkende atherosclerose, ontstaan in een diabetische situatie, ook anders is dan de atherosclerose die ontstaat in niet-diabetische situatie. Hyperglycemie is inderdaad een uniek aspect van diabetes. Glucose zelf heeft al zo zijn invloed, maar ook de alternatieve pathofysiologische consequenties van hyperglycemie spelen hierin een rol. Advanced glycation endproducts (AGEs), veroorzakers van meer plaque instabiliteit, zijn hiervan een voorbeeld. Bij insulineresistentie is er echter ook sprake van enkele metabole abnormaliteiten zoals hypertensie, hypertriglyceridemie, laag HDL gehalte, small dense LDL en meer trombogeniteit, die ook allen betrokken zijn bij atherosclerose bij niet-diabeten. Zo lijkt het logisch(er) om bij het

proberen cardiovasculaire complicaties te reduceren, ons te richten op de onderliggende pathofysiologische mechanismen van insulineresistentie. De stam van de vasculaire boom weer in gedachte, zal farmacologische modulatie van het insulineresistentie syndroom niet alleen de glycemische controle verbeteren, maar zal ook gunstige effecten hebben op ontsteking, endotheeldisfunctie, dyslipidemie en mogelijk nog op andere componenten van het syndroom, zelfs onafhankelijk van het glucose metabolisme.

De ontdekking van nuclear peroxisome proliferator-activated receptors (PPARs) en het inzicht in hun werking en daarmee hun rol in verschillende metabole pathways heeft voor veel meer begrip gezorgd van de onderliggende pathofysiologische mechanismen van het insulineresistentie syndroom. **Thiazolidinedionen (TZDs)** behoren tot de PPAR-γ agonisten, een klasse medicijnen dat uitgebreid heeft aangetoond in mens en dier de insuline gevoeligheid (met name in skeletspieren) te verbeteren. Twee van deze klasse medicijnen, rosiglitazon en pioglitazon, zijn op dit moment geregisteerd voor patienten met type 2 diabetes mellitus. Een eerdere TZD, troglitazon, had dezelfde insulinegevoeligheid verbeterende werking, maar is van de markt gehaald wegens onacceptabele hepatotoxiciteit. Beschikbare TZDs verbeteren de insulinegevoeligheid met name in het vetweefsel, en in mindere mate via het levermetabolisme. Indirecte verbetering van insulinegevoeligheid wordt ook bewerkstelligd door meer adipocyt differentiatie en de redistributie van vetopslag van abdominal naar subcutaan. Verbetering van de insulinegevoeligheid door TZDs resulteert in een verminderde vraag naar insulinsecretie, hetgeen weer de levensvatbaarheid van de β-cellen ten goede komt. De precieze mechanismen waarmee TZDs de insulinegevoeligheid verbeteren is nog niet geheel opgehelderd, maar daling in de vrije vetzuur concentraties draagt daar zeker aan bij. Sommige studies beweren ook dat TZDs de produktie van adiponectine, een uit vetweefsel komend eiwit dat geassocieerd is met verbetering van insulinegevoeligheid, opreguleren.

Naast de effecten op insulinegevoeligheid hebben TZDs ook gedemonstreerd directe en indirecte vasculaire effecten te hebben. TZDs verbeteren endotheelfunctie, remmen ontsteking (in de vaatwand), reduceren de vrije vetzuur concentraties, verbeteren het LDL-profiel en remmen gladde spiercelproliferatie. Ze verlagen bloeddruk, verminderen de microalbuminemie en verbeteren de fibrinolyse. Tegenwoordig wordt ook gekeken naar PPAR-γ onafhankelijke mechanismen in de vaatwand en lijken TZDs ook atherosclerose te verminderen in niet-diabeten. Er worden steeds meer veelbelovende data gepubliceerd over de effecten van TZDs op surrogaatmarkers van hart- en vaatziekten. Het doel van een overgroot deel van dit proefschrift is

na te gaan wat de potenties zijn van het mechanisme van Thiazolidinedionen in de pathofysiologie van vasculaire ziekte.
Hoofdstuk 3 geeft een overzicht van de metabole en additionele vasculaire effecten van Thiazolidinedionen.
In **hoofdstuk 4** hebben we onderzocht of pioglitazon, direct de endotheelfunctie kan beïnvloeden door behoud van voldoende NO-productie in type 2 diabetes patïenten. In een gerandomiseerde, cross-over, placebo-gecontroleerde en dubbel-blinde studie, is de NO-activiteit in de vaatwand gemeten middels FMD van de a.brachialis. Om directe vasculaire effecten te kunnen onderscheiden van de indirecte metabole effecten hebben we naar een relatief korte behandelingsperiode gekeken. Het blijkt, dat door het behandelen van patiënten met type 2 diabetes mellitus met pioglitazon voor een korte tijd, de endotheelfunctie op directe wijze verbetert zonder indirecte hulp van significante gunstige metabole veranderingen in insuline, vrije vetzuren, adiponectine of CRP.
Om de gedachte door te trekken, hebben we verder gekeken of dit gunstige effect van TZDs op de NO-beschikbaarheid in type 2 diabetes, ook te vertalen valt in een betere bescherming van de vaatwand voor inflammatoire stimuli.
Daarom hebben we in **hoofdstuk 5** de twee biomarkers, endotheeldisfunctie en inflammatie, gekoppeld en gekeken of diabetici hun NO-gemedieerde endotheelfunctie op peil kunnen houden ondanks een verhoogde concentratie TNF-α. In een gerandomiseerde, placebo-gecontroleerde en dubbel-blinde studie is onderzocht of pioglitazon TNF-α geïnduceerde endotheeldisfunctie kan beïnvloeden. Intra-arteriele infusie van TNF-α geeft een inflammatoire beschadiging van de vaatwand waardoor endotheeldisfunctie optreedt in type 2 diabetes. Korte-termijn behandeling met pioglitazon kan deze geïnduceerde endotheeldisfunctie anti-inflammatoir blokkeren. Echter, in hoofdstuk 4 hebben we een duidelijke directe endotheelfunctie-verbetering gevonden middels FMD-metingen in de a. brachialis, maar vinden we die (nog) niet bij de FBF-meting van het onderarms-weerstandsvaatbed. De in hoofdstuk 2 beschreven heterogeniteit van NO kan een mogelijke verklaring zijn voor dit verschil.
Om de mogelijke anti-inflammatoire effecten van TZDs verder te bestuderen hebben we *ex vivo* onderzocht of TZDs de eigenschappen van adhesie van monocyten aan het endotheel, of de productie van cytokinen, kunnen beïvloeden.
Daarom is in **hoofdstuk 6** ex vivo gekeken naar het adhesie-gedrag van monocyten van type 2 diabeten op HUVEC onder flow. Het blijkt dat monocyten van patiënten met type 2 diabetes meer adheren aan endotheel, in vergelijking met monocyten

van de gezonder vrijwilligers. Pioglitazon- of troglitazon-behandeling van de monocyten verminderen echter deze adhesie aan het endotheel. **Hoofdstuk 7** is een *ex vivo*, gerandomiseerde, cross-over, placebo-gecontroleerde, dubbel-blinde studie waarbij het effect van kortdurende pioglitazon behandeling op de cytokine-produktie in volbloed bestudeerd wordt. De conclusie is, dat korte-termijn behandeling met pioglitazon geen effect heeft op de TNF-α-, IL-6-, of IL-10-produktie van monocyten en daarmee misschien het idee van hoofdstuk 6 bevestigt dat TZDs geen directe anti-inflammatoire effecten op monocyten genereren.

Natuurlijk zijn dit soort fundamentele interventiestudies van belang om meer te kunnen begrijpen van atherosclerose en de farmacologische effecten van TZDs en meer te verwachten resultaten van andere studies zullen aan dit inzicht bijdragen. Op dit moment zijn voornamelijk de effecten van TZDs op surrogaat cardiovasculaire eindpunten bij patienten bekend. TZDs reduceren significant de arteriële intima dikte van de carotis, in vergelijking met placebo. Daarnaast blijkt pioglitazon de neo-intima proliferatie na coronaire stentplaatsing, gevisualiseerd middels intravasculaire echografie, te verminderen in patiënten met type 2 diabetes mellitus.
In de recent gepubliceerde Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROACTIVE) vermindert pioglitazon het relatieve risico op het gecombineerde eindpunt sterfte, een hartinfarct, of een hersenbloeding, met 16%. Metabole parameters veranderen ook ten gunste: HbA1c concentraties werden 0.5% minder, de plasma triglyceride concentratie daalde met 13.2%, de LDL/HDL ratio met 5.3%, en de bloeddruk daalde met 3 mmHg, terwijl het plasma HDL-gehalte steeg met 8.9%. Andere trials, zoals de Rosiglitazone Evaluated for Cardiac outcomes and Regulation of Glycemia in Diabetes (RECORD), Carotid Intima Media Thickness in Atherosclerosis Using Pioglitazone (CHICAGO), en Pioglitazone Effect on Regression of Intravascular Sonographic Coronary obstruction Prospective Evaluation (PERISCOPE), zullen allen hun resultaten prijsgeven in 2007. De Study of Atherosclerosis with Ramipril and Rosiglitazone (STARR) is opgezet als een onderdeel van de Diabetes Reduction Approaches with Ramipril and Rosiglitazone Medications (DREAM) trial en duurt 5 jaar.
Toch blijven er nog de nodige vraagtekens bestaan voor TZD-behandeling die in de toekomst opgelost zullen (moeten) worden. De paradox van adipocyt-differentiatie en de waargenomen gemiddelde gewichtstoename van maximaal enkele kilo's bij gebruik van de insuline gevoeligheid verbeterende TZDs is nog niet geheel duidelijk. Verder is het nog onduidelijk dat TZDs de bloeddruk verlagen terwijl er een expansie

van het plasmavolume optreedt, waardoor gebruik van TZDs terecht gecontraïndiceerd is bij patiënten met hartfalen. Verder onderzoek zal nog moeten plaatsvinden naar soms geobserveerde stijging van cholesterolconcentraties, met name het atherogene LDL. Toekomstig onderzoek zal vele antwoorden vinden op deze en meer vragen. Zeker zal het belang van PPAR-γ, maar ook PPAR-α in de vasculaire pathofysiologie ontrafeld worden. Het concept van de TZDs is zeer veelbelovend, maar laten we nog enkele jaren van onderzoek, -misschien leidend tot nog betere farmacologische behandelingsstrategieën-, en de resultaten van meer lange-termijn klinische trials op cardiovasculaire eindpunten afwachten.

In dit proefschrift zien we met name het samenspel tussen de belangrijke biomarkers voor hart- en vaatziekten: endotheeldisfunctie en inflammatie.
Endotheelfunctie (de mate van NO-beschikbaarheid) is een belangrijke factor voor de vaatwandstructuur. Inderdaad blijkt eNOS een tonisch remmende werking te hebben op de groei in de aorta, terwijl er nauwelijks effect bereikt wordt door eNOS in de kleine weerstandsvaten, zowel in basale- als al in hypertrofische toestand. Dit verschil in effect van NO op de vasculaire groei op verschillende plekken in het vasculaire netwerk kan de heterogeniteit van vasculaire remodeling (eutrofisch in het weerstandvaatbed en hypertrofisch in de geleidingsvaten), een adaptatie welke geassocieerd is met endotheeldisfunctie, verklaren. Ook zou het een verklaring kunnen zijn voor de soms verschillende resultaten van endotheelfunctie metingen bij dezelfde stimuli tussen een FMD-meting van de grote a. brachialis versus een FBF-meting van het onderarms-weerstandsvaatbed.
Verder kan het helpen de gecompliceerde pathofysiologie achter het geclusterd voorkomen van met obesitas geassocieerde risicofactoren beter te begrijpen, door endotheeldisfunctie te koppelen aan insulineresistentie: naarmate de glycemische control verslechtert, en er dus sprake is van meer insulineresistentie, bestaat er ook een sterkere relatie met vaatstijfheid van de carotis in patiënten met manifestaties van arterieel vaatlijden. De vaatstijfheid is iets vergroot bij patiënten met het metabool syndroom en neemt toe naarmate een patiënt meer componenten van het metabool syndroom heeft. De meeste vaatstijfheid blijkt echter aanwezig bij patiënten met type 2 diabetes mellitus.
Het lijkt logisch(er) om bij het proberen cardiovasculaire complicaties te reduceren, ons te richten op de onderliggende pathofysiologische mechanismen van de geclusterde met obesitas geassocieerde risicofactoren. Gebruik makend van de kennis dat TZDs, als insuline-sensitizers, gunstige metabole effecten hebben, geeft dit

proefschrift ook een idee van directe vasculaire effecten van TZDs. Dit vergroot niet alleen de therapeutische mogelijkheden, maar zorgt ook voor meer inzicht in de pathofysiologie van de atherogenese in insulineresistentie. Het blijkt, dat door het behandelen van patiënten met type 2 diabetes mellitus met pioglitazon voor een korte tijd, de endotheelfunctie op directe wijze verbetert zonder indirecte hulp van significante gunstige metabole veranderingen in insuline, vrije vetzuren, adiponectine of CRP.

De gunstige effecten van TZDs op de NO-beschikbaarheid in type 2 diabetes, zijn ook te vertalen in een betere bescherming van de vaatwand voor inflammatoire stimuli: intra-arteriele infusie van TNF-α geeft een inflammatoire beschadiging van de vaatwand waardoor endotheeldisfunctie optreedt in type 2 diabetes. Korte-termijn behandeling met pioglitazon kan deze geïnduceerde endotheeldisfunctie anti-inflammatoir blokkeren. De suggestie wordt gewekt dat dit anti-inflammatoire effect voornamelijk op het endotheel gericht is, aangezien de in type 2 diabeten verhoogde monocyt-endotheel-adhesie geremd kan worden met TZDs zonder een direct effect op de monocyt. Ook de cytokinen-productie wordt niet beïnvloed door TZDs.

Het bestuderen van de vasculaire effecten van TZDs op endotheelfunctie en inflammatie zorgt voor meer inzicht in de pathofysiologie achter de geclusterd voorkomende cardiovasculaire risicofactoren.

ABBREVIATIONS

ACE	Angiotensin converting enzyme
Ang-II	Angiotensin-II
AT	Angiotensin
BH4	Tetrahydrobiopterin
BMI	Body mass index (weight in kilograms divided by the square height in meters)
CETP	Cholesterolestertransferprotein
cGMP	cyclic Guanosine monophosphate
CRP	C-reactive protein
DM2	Diabetes mellitus type 2
EC	Endothelial cells
EDHF	Endothelium derived hyperpolarizing factor
EDRF	Endothelium derived relaxing factor = NO
eNOS	endothelial Nitric oxide synthase
E-selectin	Endothelial-leucocyte adhesion molecule
ET-1	Endothelin-1
FBF	Forearm blood flow
FFA	Free fatty acids
FMD	Flow-mediated dilation
GLUT 4	Glucose transporter 4
HDL	High-density lipoprotein
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular adhesion molecule-1
IL-6	Interleukin-6
L-NAME	NG-nitro-L-arginine methyl ester
L-NMMA	NG-monomethyl-L-arginine
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide/ endotoxin
MCP-1	Monocyte chemotactic protein-1
MNC	Mononuclear cells
MetSyn	Metabolic syndrome
NADPH	Nicotamine adenine dinucleotide phosphate
NF-kB	Nuclear factor-kB
NO	Nitric oxide
ox-LDL	Oxidized low density lipoprotein
PAI-1	Plasminogen activator inhibitor
PPAR	Peroxisome proliferator-activated receptors
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor-α
tPA	tissue Plasminogen activator
TZDs	Thiazolidinediones
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cells
vWF	von Willebrand factor

CURRICULUM VITAE

Curriculum Vitae

Fabrice Marcel Anne Clément Martens was born on the 1th of January 1974 in Delft, the Netherlands. After he graduated from the Gymnasium at the Trichter College in Maastricht in 1992, he started Medical School at the University of Leuven (Belgium). A year later he switched to the University of Utrecht. During his medical training he went abroad for a fellowship of Internal Medicine at the Rikshospitalet of Oslo (Norway), and a fellowship of Dermatology at the Royal Perth Hospital, Sir Charles Gairdner Hospital and the Princess Margeret Children Hospital, all in Perth (Australia). His first experience with research was his participation in a project about HLA-matching by heart transplantation at the cardiology department of the University Medical Center Utrecht, under supervision of dr. J.H. Kirkels. He became really enthusiastic for research in the period of 1998-1999, while investigating vessel-wall-remodelling at the Université de Montréal (Canada), under supervision of prof. P. Moreau. In this period his first performance at an international meeting was at the Ninth European Meeting on Hypertension (European Society of Hypertension), Milan.

Fabrice Martens obtained his Medical Degree in January 2001 and started the work described in this thesis at the department of Vascular Medicine of the University Medical Center Utrecht, under supervision of dr. F.L.J. Visseren and prof. dr. T.J. Rabelink. During this period he presented on several international meetings: the Second International Symposium on PPARs, Florence; the 18th Congress of the International Diabetes Federation, Paris; the XIIIth International Symposium on Atherosclerosis (International Atherosclerosis Society), Kyoto; the Scientific Sessions (American Heart Association), Orlando.

In September 2003 he started his 2 year residency Internal Medicine at the University Medical Center Utrecht under supervision of prof. dr. E.E. van der Wall as part of his specialisation to cardiologist. In September 2005 he continued his training in cardiology at the department of Cardiology at the St. Antonius Hospital Nieuwegein, under supervision of dr. W. Jaarsma, where he will become cardiologist in the year 2009.

Fabrice Martens is married to Marieke Volmer and they have an almost 3 month-old daughter Valerie.

PUBLICATIONS

Dao HH, Martens FMAC, Larivière R, Yamaguchi N, Cernacek P, de Champlain J, Moreau P.
Transient involvement of endothelin in hypertrophic remodeling of small arteries.
Journal of Hypertension 2001;19:1-12

Martens FMAC, Demeiliers B, Girardot D, Daigle C, Dao HH, deBlois D, Moreau P.
Vessel specific stimulation of protein synthesis by nitric oxide synthase inhibition: role of extracellular signal-regulated kinases 1/2.
Hypertension 2002;39:16-21

Martens FMAC, Visseren FLJ, Lemay J, de Koning EJP, Rabelink TJ.
Metabolic and additional vascular effects of thiazolidinediones.
Drugs 2002;62(10):1-17

Martens FMAC, Visseren FLJ.
Inflammatie en insulineresistentie; is er een rol voor thiazolidinedionen?
Insuline Resistentie; van pathophysiologie tot behandeling 2003:2(3):52-57

Daigle C, Martens FMAC, Girardot D, Dao HH, Touyz RM, Moreau P.
Signaling of angiotensin II-induced vascular protein synthesis in conduit and resistance arteries *in vivo*.
BMC Cardiovascular Disorders 2004;4(1):6

Olijhoek JK, Martens FMAC, Banga JD, Visseren FLJ.
Het metabool syndroom: een cluster van vasculaire risicofactoren.
Nederlands Tijdschrift voor Geneeskunde 2005;149:859-865

Martens FMAC, Visseren FLJ, de Koning EJP, Rabelink TJ.
Short-term pioglitazone treatment improves vascular function irrespective of metabolic changes in patients with type 2 diabetes.
J Cardiovasc Pharmacol 2005; 46(6):773-778

Martens FMAC, Rabelink TJ, op 't Roodt J, Visseren FLJ.
TNF-α induces endothelial dysfunction in diabetic adults, an effect reversible by the PPAR-γ agonist pioglitazone.
In revision Eur Heart J

Olijhoek JK, van der Graaf Y, Broeyer FJF, Martens FMAC, Visseren FLJ.
In patients with coronary artery disease the NCEP-defined metabolic syndrome, insulin resistance and obesity are all associated with decreased adiponectin levels.
Submitted

Martens FMAC, Rabelink TJ, vd Hilst JCH, Lutgendorf F, de Boer H, Loomans CJM, Zwaginga JJ, Visseren FLJ.
Monocyte-endothelium adhesion (under flow conditions) is increased in type 2 diabetes and can be reduced by PPAR-γ agonists pioglitazone and troglitazone.
Submitted

Martens FMAC, van der Graaf Y, Dijk JM, Olijhoek JK, Visseren FLJ.
Carotid arterial stiffness is marginally increased in the metabolic syndrome and largely increased in type 2 diabetes mellitus in patients with manifestations of arterial disease.
Submitted

Martens FMAC, Rabelink TJ, Westerink J, de Groot ER, Aarden LA, Visseren FLJ.
Short-term pioglitazone treatment does not affect cytokine production of monocytes in contrast with the natural PPAR-γ ligand prostaglandin J_2.

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THE VASCULAR TREE **"treatment from trunk-to-end-sites"**