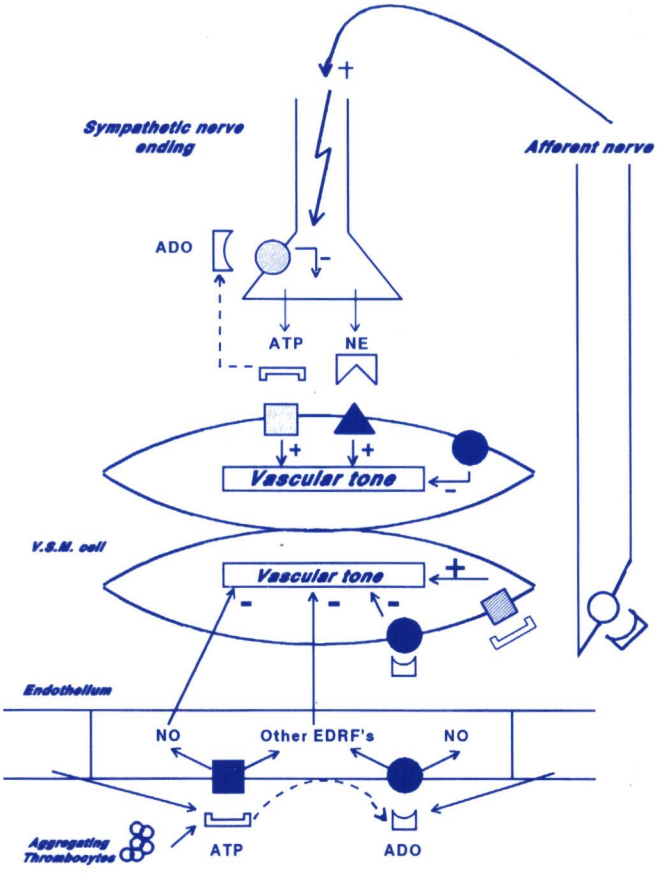
Vascular Pharmacology of Adenosine and Adenosine-5'-triphosphate in Humans



Gerard Rongen

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Vascular Pharmacology of Adenosine and Adenosine-5′-triphosphate in Humans

Vasculaire farmacologie van adenosine en adenosine-5′-trifosfaat bij de mens

Een wetenschappelijke proeve op het gebied van
de Medische Wetenschappen

PROEFSCHRIFT

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aan de Katholieke Universiteit Nijmegen,
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Aan mijn ouders
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CONTENTS

CHAPTER 1

Cardiovascular pharmacology of purines: a general introduction

Background

Since the beginning of this century, the cardiovascular actions of extracellular nucleosides and nucleotides have been recognized [1-4]. Only recently, understanding of their interstitial formation, degradation and mode of action has been increased and their significance in (patho)physiology has been identified [5]. Apart from their cardiovascular actions, they appear to be involved in modulation of the central nervous system, immune function, thrombocyte aggregation and regulation of renal and gastro-intestinal functions [6-14]. Via their interactions with the autonomic nervous system [15,16], they probably interfere with almost any body function. This thesis will focus on two purines: adenosine and adenosine-5'-triphospate (ATP). In this introduction section, we will primarily discuss in-vitro and animal data on the role of purines in the modulation of the cardiovascular system. For a discussion of human in-vivo studies, we refer to the other chapters in this thesis. Interaction with other systems will only be considered as far as it interferes with the cardiovascular system. Since endothelium is thought to play an important role in the vascular effects of purines, some general properties of the endothelium with regard to the regulation of vascular tone will be discussed first.

The role of the endothelium in the regulation of vascular tone

In vitro experiments on the effect of endothelium removal have indicated that a variety of classical pharmacological vasodilators induce their effect in an endothelium-dependent way [17-19]. Among these vasodilators are acetylcholine, adenine nucleotides, histamine, serotonin, thrombin, bradykinin and substance P. These substances stimulate the endothelium to release a labile substance called 'endothelium-derived relaxing factor' (EDRF) [20]. Both nitrovasodilators, with nitric oxide (NO) as active principle, and EDRF stimulate the soluble guanylate cyclase in the smooth muscle cell to form cyclic guanosine monophosphate (cGMP), a second messenger inducing relaxation in smooth muscle cells [21]. Based on this and other similarities Furchgott assumed that EDRF was identical to NO. This has been experimentally confirmed by Moncada's group [22,23] and Ignarro et al. in 1987 [24]. NO is formed from L-arginine [25,26]. This stereospecific reaction is catalyzed by NO-synthase and can be competitively antagonized by L-arginine analogues like N^G-monomethyl-L-arginine (L-NMMA) [27,28]. Two types of NO-synthase are known: a constitutive Ca^{2+}-dependent NO-synthase and an inducible Ca^{2+}-independent NO-synthase [29]. The first type is present in the endothelial cells and in adventitial nerve endings where it may play a role in non-adrenergic and non-cholinergic nerve stimulated vasodilation [30,31]. The second type is expressed in endothelial cells, smooth muscle cells, granulocytes, Kupffer cells and hepatocytes after induction by lipo-

polysaccharides (LPS), interferon gamma or interleukin-1 [32] Under physiological circumstances the Ca^{2+}-dependent NO-synthase is the most important one in regulation of vascular tone [33] The second type may play an important role in sepsis mediating the vasodilation and shock [34]

In vitro the basal vascular tone is increased by removal of the endothelium or by incubation with L-NMMA, indicating the basal release of endothelium-derived NO [27,28,35] Vallance et al have shown that this L-NMMA-induced forearm vasoconstriction is reversed by infusion of L-arginine but not by D-arginine, indicating that NO is also released in basal conditions in humans in vivo [36] Intravenous infusion of L-NMMA in rats induces an increase in blood pressure suggesting that endothelial basal NO-release has an important function in blood pressure regulation [37] However, a central mechanism due to decreased NO mediated inhibition of the sympathetic nervous system may play a role in the pressor response to systemic infusion of L-NMMA in rats [30,38,39]

Hypoxia and shear stress stimulate the NO release providing a mechanism to adapt the circulation to local demands [40,41] Besides direct effects of these factors on the endothelial NO release, local stimulation of the release of endothelium-dependent vasodilator substances from passing erythrocytes (adenosine triphosphate (ATP)) or from endothelial cells (ATP, acetylcholine, substance P, serotonin) may account for the increased NO-release during hypoxia or shear stress [42,43]

Both adrenergic nerve stimulation- and phenylephrine-induced vasoconstriction are inhibited in the presence of endothelium [44,45] indicating an interaction between the sympathetic nervous system and endothelium This interaction is accomplished by an alpha-receptor-mediated norepinephrine induced release of EDRF [46] Besides this interaction, an endothelium-derived factor may have a presynaptic inhibiting influence on norepinephrine release [47] However, the literature is not unequivocal on this issue [48] The majority of endothelium-dependent vasodilators, like serotonin, acetylcholine and ATP, are able to elicit vasoconstriction in the absence of endothelium, probably by interacting with specific smooth muscle cell receptors [49-51] Like norepinephrine, these compounds may play a role as a neurotransmitter in the sympathetic nervous system [49].

Endothelium-derived nitric oxide is not only a potent vasodilator Like smooth muscle cells, thrombocytes possess a soluble guanylate cyclase [52] Acetylcholine-induced EDRF release stimulates the soluble guanylate cyclase of passing thrombocytes [53] Endothelium-derived NO inhibits thrombocyte adhesion and aggregation by stimulation of thrombocytic soluble guanylate cyclase [54-56] Thrombocytic NO-synthase is stimulated during thrombocyte aggregation providing a negative feedback control [52,57] Prostacycline potentiates the effect of NO on thrombocyte aggregation [58] During aggregation thrombocytes release vasoactive substances which induce an endothe-

lium-dependent vasodilation (ATP, ADP, possibly serotonin), or vasoconstriction in the absence of endothelium (thromboxane A_2, serotonin) [59-61]. These thrombocyte-derived vasoactive substances may also be involved in an EDRF-mediated inhibition of thrombocyte aggregation.

Many questions still remain to be answered in research on endothelial physiology. For instance, other endothelium-derived substances like prostacycline and 'endothelium derived hyperpolarizing factor' may be involved in the endothelium-dependent vasodilation [62-67]. Their relative importance may vary in different vascular beds, different species and different physiological and pathophysiological circumstances [68,69]

Besides EDRF the endothelium is able to release endothelium-derived contracting factors (EDCF's) like endothelin-1, thromboxane A_2 and prostaglandin H_2 [70] Many stimuli that induce release of EDRF also induce the release of endothelin-1 [71-73] Furthermore, endothelin-1 itself is able to stimulate the release of endothelium-derived NO and prostacycline [74-77] The physiological importance of these interactions is speculative, but it is suggested that the endothelin-1-induced release of NO and prostacycline exerts a negative feedback on endothelin production via cGMP- and cAMP-dependent mechanisms [70] The physiological relevance of the inhibition of mitogenesis and proliferation of smooth muscle cells by NO and the opposite effect of endothelin-1 is unknown [78,79].

Formation, release and degradation of adenosine and ATP

Within the cell, *adenosine* is primarily formed by two metabolic routes [80]. First, it is formed from ATP by degradation via adenosine-5'-diphosphate and adenosine-5'-monophosphate (AMP) to adenosine This pathway is accelerated when there is a mismatch between energy supply and demand and probably represents the main route of adenosine formation during ischemia The hydrolysation of AMP is catalysed by 5'-nucleotidase, which is present both in the intracellular (cytosolic form) as well as the extracellular space (membrane bound form) Second, adenosine is formed by hydrolysation of ATP via S-adenosylmethionine to S-adenosylhomocysteine which in turn is hydrolysed to adenosine This reaction is catalysed by S-adenosylhomocysteine-hydrolase This route is oxygen insensitive and is probably of minor importance during ischemic conditions Adenosine diffuses through the cellular membrane between the extracellular and intracellular space This bidirectional process [81] is concentration gradient dependent and is facilitated by a nucleoside transporter that is located in the outer membrane of many cells among which the endothelial cells, erythrocytes, cardiomyocytes and vascular smooth muscle cells.

Apart from a cellular source of adenosine, adenosine formation may occur from extracellular ATP that can rapidly be degraded to adenosine by widespread ecto-nucleotidases which are present at the extracellular surface of vascular smooth muscle cells and endothelium [82-86]. The endothelium appears to play a key role in uptake and metabolism of luminally applied adenosine [87-89], but it is also a major source of endogenous adenine nucleosides and nucleotides. The endothelial release of adenine nucleosides and nucleotides is increased by beta-adrenergic stimulation [85]. Norepinephrine-induced activation of 5'-nucleotidase may play a role in this interaction between endothelial adenosine formation and the sympathetic nervous system [90].

Adenosine-5'-triphosphate is released by exocytosis from aggregating thrombocytes and from sympathetic nerve endings where it is coreleased with norepinephrine [15]. In most cells, cytoplasmatic ATP concentrations are high and simple diffusion of ATP through the cell membrane may partly account for extracellular ATP. During increased blood flow [42,72,91], sympathetic nerve stimulation [92], or application of well known vasodilators like bradykinin and acetylcholine [93], endothelial cells appear to release ATP. Cardiac ischemia evokes an increase in plasma nucleotide concentration in the venous effluent which is underestimated by vascular ecto-nucleotidase activity [85]. Under hypoxic conditions, erythrocytes release ATP [94].

As mentioned above, extracellular ATP is rapidly degraded to adenosine by ecto-nucleotidases. During normal oxygenation, intracellular adenosine and AMP concentrations are low. In this situation, extracellular adenosine will rapidly be transported to the intracellular compartment where it is further inactivated by phosphorylation to AMP or deamination to inosine. An extracellular form of adenosine deaminase exists. The relative importance of extracellular versus intracellular adenosine deaminase differs between species [95]. In man, intracellular deamination after uptake is probably of most importance [95].

Purinergic receptors

Cardiovascular responses to purines are thought to be mediated by membrane bound receptors that interact with their extracellular ligand to evoke an intracellular signal (increase or decrease in concentrations of Ca^{2+}, cAMP or cGMP for example) that ultimately changes cellular function (i.e. smooth muscle cell relaxation or contraction, reduction or increase in neuronal firing rate or neurotransmitter release) [96]. Several lines of evidence support this view and point towards a subdivision in P_1- and P_2-purinergic receptors. This subdivision is based on the potency order of agonists (ATP > ADP > AMP > adenosine and adenosine > AMP > ADP > ATP for the P_2- and P_1-

purinergic receptor respectively) and the P_1- but not P_2-purinergic receptor antagonistic properties of xanthine derivatives [6]

The existence of P_1-purinergic receptors has been demonstrated using adenosine analogues that are less or not affected by degradation, specific antagonists derived from the methylxanthines caffeine and theophylline, (intra)cellular measurement of second messenger systems (cAMP, intracellular Ca^{2+} concentrations, ion currents, membrane potentials), receptor ligand labelling studies, receptor purification and DNA cloning studies [97] Based on ligand specificity and effect on intracellular cAMP levels, P_1-purinergic receptors are subdivided in A_1- and A_2-adenosine-receptors. This differentiation has been verified by molecular techniques [98,99] Both receptors are linked to so called G proteins A_1-receptor stimulation evokes a G_i-mediated decrease in adenylate cyclase activity with subsequent reduction in intracellular cAMP levels The A_2-receptor is associated with a G_s binding protein and stimulation of this receptor increases adenylate cyclase activity with a subsequent increase in intracellular cAMP levels Apart from this adenylate cyclase mediated response to P_1-receptor stimulation, both receptor subtypes have been associated with a variety of other second messenger systems [5] like for instance an ATP-dependent potassium channel (K_{ATP}-channel). A_1-receptor-mediated opening of K_{ATP}-channels may play a role in the phenomenon of ischemic preconditioning (see below) Recently, the existence of a distinct A_3-adenosine receptor has been suggested [100] Advances in molecular biology will probably reveal many other subclasses of the adenosine receptor Whether stimulation of these receptors give rise to a specific pharmacological or physiological effect, distinct from A_1- or A_2-receptor stimulation remains to be elucidated

Based on rank order differences in agonist potencies and different vascular effects, the vascular P_2-purinergic receptor has been subdivided in the P_{2x}- and P_{2y}-purinergic receptors [101,102] P_{2x}-purinergic receptors are located on vascular smooth muscle cells and mediate vasoconstriction The P_{2y}-purinergic receptor mediates a vasodilator response either via stimulation of the endothelium to produce endothelium-derived relaxing factors (nitric oxide, prostacycline) or via a direct effect on the smooth muscle cell [62,69,103-106] Apart from these two receptor subtypes, the existence of separate vascular 'pyrimidine' or 'nucleotide' receptors has been suggested [107-111]. A more definite characterization of cardiovascular P_2-receptors continues to be hindered by the lack of selective, competitive receptor antagonists [108,109] The P_{2x}-purinergic receptor agonist alpha,beta-methylene ATP, has been successfully applied to perform radioligand studies on P_2-purinergic receptors [112] and to desensitize P_{2x}-purinergic receptors [113]. However, repeated application of alpha,beta-methylene ATP not only inhibits the vasoconstrictor effect of ATP, but may also attenuate the vasoconstrictor effect of norepinephrine, angiotensin II and vasopressin [114] In addition, the potency of alpha,beta-

methylene ATP to inhibit ATP-induced effects varies enormously [115]. To date, P_2-purinergic receptors have not been isolated with molecular techniques. The second messenger systems that are involved in mediating its effects are not exactly known but phospholipase C activation, inositol phosphate production and subsequent increases in intracellular Ca^{2+} concentrations appear to be involved [104].
Apart from vascular P_2-purinergic receptors, specific P_{2T}-purinergic receptors have been demonstrated on thrombocytes [116]. Adenosine-5'-diphosphate is the most potent endogenous agonist for this receptor and receptor stimulation results in thrombocyte activation and subsequent thrombus formation. Adenosine-5'-triphosphate is an endogenous antagonist for this receptor.

Effects of cardiovascular purinergic receptor stimulation

Vascular wall

Adenosine-5'-triphosphate may exert two opposite vascular actions that are mediated by two distinct P_2-purinergic receptors located on endothelium and vascular smooth muscle cells (see figure 1). The endothelial receptor evokes an endothelium-mediated vasodilator response. Apart from nitric oxide, prostacycline and/or 'EDHF' (endothelium-derived hyperpolarizing factor) may be involved as endothelium-derived relaxing factor [63,103,104]. Since the endothelium plays an important role in degradation and uptake of purines, only a minor part of luminally applied ATP will probably reach the vascular smooth muscle cells [82,83]. Indeed, if endothelial function is intact, ATP as released during thrombocyte aggregation induces a vasodilator response [59].

On vascular smooth muscle cells, P_{2x}-purinergic receptor stimulation results in a vasoconstrictor response [117]. For reasons as mentioned above, this receptor will preferentially be stimulated by interstitially released ATP as occurs from sympathetic nerve endings.

In most vessels, *adenosine* induces a vasodilator response via A_2-adenosine receptor activation (see figure 1) [118]. The A_1 receptor can mediate vascular relaxation as well [119] although this receptor subtype is generally believed to be involved in a vasoconstrictor action of adenosine in some vascular beds (see below). In the absence of an intact endothelium, the vasodilator response is still present indicating that an intact endothelium is not obligate for adenosine to establish its effect [120]. However, some studies show a reduced vasodilator response to adenosine when the endothelium is removed, indicating that adenosine receptors may be present on the endothelial cells [106]. Furthermore, inhibition of NO-synthase has been shown to inhibit adenosine-mediated vasodilation [121]. Recently, A_2-adenosine receptors have been

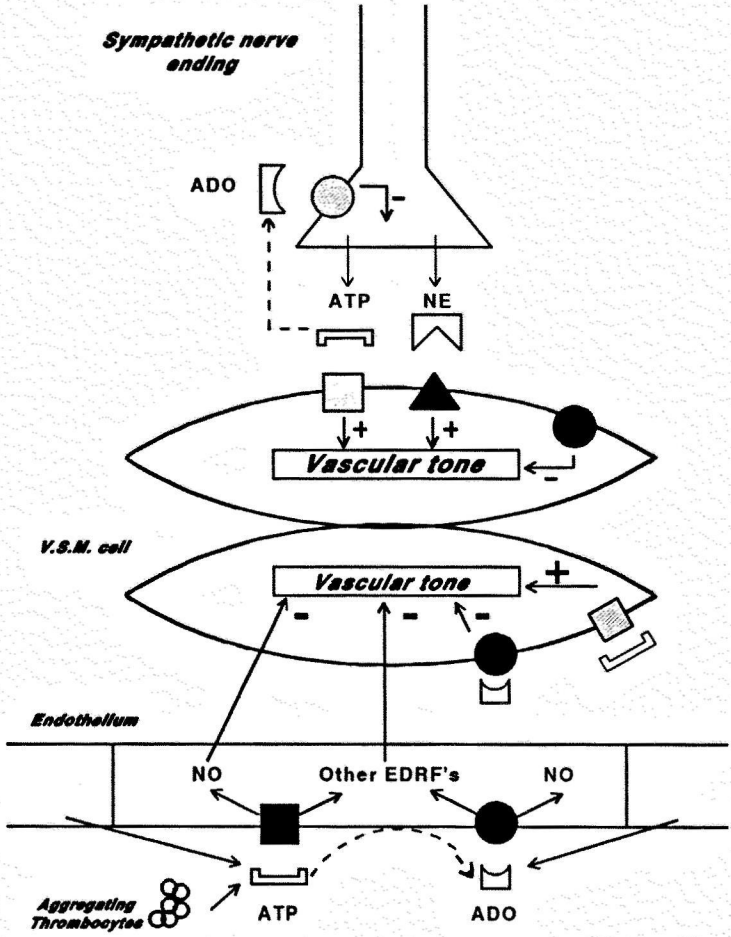

Figure 1. A simplified presentation of the proposed interactions (based on in vitro studies and animal in vivo studies) between the purines adenosine (ADO) and adenosine-5'-triphosphate (ATP) and the sympathetic nervous system and endothelium with respect to the regulation of vascular tone. Squares represent P_2-purinergic receptors (P_{2x}-purinergic receptor: shaded boxes, P_{2y}-purinergic receptors: black boxes); Circles represent P_1-purinergic receptors (A_1-purinergic receptor: shaded circle, A_2-purinergic receptor: black circle); Triangle represents the alpha adrenoceptor; V.S.M. cell: vascular smooth muscle cell; NO: nitric oxide; EDRF: endothelium derived relaxing factor; NE: norepinephrine.

demonstrated on human endothelial cells [122]. There is some debate with regard to the functional significance of these endothelial adenosine receptors [123]. Facing the key role of the endothelium in purine degradation and uptake, the site of adenosine application or formation may determine whether the vasodilator response is mediated by a direct effect on the vascular smooth muscle cells (interstitial adenosine) or whether the endothelium is involved (luminal adenosine) [124].

Although adenosine induces a vasodilator response in most vessels, two important exceptions are known: the pulmonary vascular bed [125] and the preglomerular afferent arterioles in the kidney (see below). In these vessels adenosine induces a vasoconstrictor response that is thought to be mediated by A_1-purinergic receptors. In the lung, this effect might be implicated in hypoxia-induced vasoconstriction, and in the kidney adenosine-induced afferent vasoconstriction may be involved in the autoregulation of the glomerular filtration rate (see below).

Apart from its direct influence on vascular tone, purines have been shown to affect norepinephrine release from sympathetic nerve endings by presynaptic modulation (see figure 1). Most studies on this matter point towards an adenosine-induced A_1-purinergic receptor-mediated response. ATP, via a yet unclassified purinergic receptor subtype, may be involved as well [126,127].

Based on the above mentioned experimental data, Burnstock hypothesized that ATP is co-released with norepinephrine by sympathetic nerve endings, induces a vasoconstrictor response in concert with norepinephrine and is rapidly degraded to adenosine which presynaptically inhibits sympathetic neurotransmitter release thus providing a negative feedback loop [15,128]. The activity of this feedback loop may be controlled by ecto-nucleotidase activity [129].

Heart

Apart from coronary vasodilation and presynaptic inhibition of norepinephrine release (see above), purines have negative chronotropic, dromotropic and inotropic effects in isolated heart preparations. ATP is thought to induce its negative chronotropic and dromotropic effects after degradation to adenosine [130,131]. However, under certain experimental conditions, atropine or vagotomy can attenuate the negative chrono/dromotropic actions of ATP, suggesting that the ATP effect is at least partially vagally mediated [132,133].

Negative chronotropic actions. Adenosine depresses sinoatrial node activity and shifts the earliest site of atrial activation from the SA node towards the crista terminalis region. At high concentrations, adenosine may even cause a SA exit block. Adenosine may also depress atrioventricular and ventricular escape rhythms. In patients with a ventricular escape rhythm, heart rate is reduced in response to adenosine, especially when

the escape rhythm is accelerated with isoproterenol. These effects are probably mediated by A_1-adenosine receptor stimulation [134]. In the intact organism, the adenosine-induced stimulation of reflex mechanisms may result in an increase in heart rate, which strongly contrasts with its effect on the SA-node (see chapter 2)

Negative dromotropic actions. From the beginning of purine research, adenosine is known to induce heart block [1]. Adenosine prolongs atrioventricular conduction time dose-dependently. Its site of action is probably the proximal portion of the AV junction. Based on the rank order of potency of different adenosine analogs, A_1-adenosine receptors are thought to be involved [135]. This action of adenosine is used clinically to terminate supraventricular tachycardias, especially in situations where calcium antagonists are contra-indicated [136,137].

(Anti)arrhythmic properties. Adenosine has been shown to attenuate ischemia- and catecholamine-induced arrhythmias [138-140]. This action is mediated by A_1 adenosine receptors, probably by preventing catecholamine-induced calcium influx and subsequent afterdepolarizations [134]. Adenosine-5'-triphosphate also inhibits isoproterenol-induced calcium influx, probably by stimulating A_1-adenosine receptors after degradation to adenosine [134]. When ATP alone (i.e. without isoproterenol pretreatment) is applied to isolated myocardial cells, calcium influx is increased. This effect is supposed to be related to P_2-purinergic receptor stimulation [141] and may contribute to ATP-induced automaticity. Alternatively, in isolated myocardial cells ATP hydrolysis may activate the chloride/bicarbonate exchanger. The subsequent intracellular acidification may induce arrhythmic activity, providing a P_2-purinergic receptor-independent mechanism [142].

Negative inotropic actions. Adenosine has a negative inotropic effect on both atrial and ventricular myocardium when contractility is increased with isoproterenol probably by antagonizing the isoproterenol-induced increase in intracellular cAMP [134,143]. In the atrium, adenosine also has a direct, i.e without isoproterenol pretreatment, negative inotropic action probably by increasing potassium efflux and subsequent hyperpolarization. These effects are mediated by A_1-adenosine receptor stimulation [134]. In contrast to adenosine, ATP has a positive inotropic action in both atrial and ventricular myocardial cells [144] by increasing intracellular calcium concentration.

Kidney

In contrast to most other vascular beds, *adenosine* induces a vasoconstrictor response in the outer renal cortex which appears to be confined to the glomerular afferent arterioles and which is mediated by A_1-adenosine receptors. Simultaneously, adenosine dilates the efferent glomerular arterioles via A_2-adenosine receptor stimulation [145]. Acting in concert, these effects of adenosine result in a reduction of glomerular filtration rate. The vasoconstrictor effect occurs immediately upon adenosine infusion, while the

vasodilator response develops more slowly. These opposing effects of adenosine may explain the biphasic response of renal blood flow but a constant depression of glomerular filtration rate during infusion of adenosine in the renal artery [145,146]. Adenosine-induced release of EDRF probably contributes to the efferent arteriolar vasodilation [145,146]. The adenosine-mediated reduction in glomerular filtration rate has been proposed to be involved in tubuloglomerular feedback. This term has been ascribed to the phenomenon that an increase in the perfusion rate of fluid in the distal nephron results in a vasoconstrictive response of the afferent arteriole such that the glomerular filtration rate of that nephron is diminished. An increased tubular volume load would be sensed by the macula densa evoking a paracrine signal to the glomeruli and a subsequent reduction in glomerular filtration rate. Inhibition of the tubuloglomerular feedback by selective adenosine receptor antagonists and adenosine deaminase as well as stimulation of the phenomenon by adenosine deaminase inhibition and nucleoside transport inhibition serve as important evidence in favour of adenosine as a mediator of this phenomenon [5]
Adenosine also affects tubular function resulting in an increased water and sodium excretion (although an increased sodium reabsorption has also been observed). In the nanomolar range, adenosine and its analogs stimulate A_1-adenosine receptors resulting in reduced intracellular cAMP concentrations, providing a mechanism to attenuate the effects of vasopressin and parathyroid hormone, which are both acting via stimulation of adenylate cyclase [14]. In addition, adenosine inhibits renal renin release via A_1-adenosine receptor stimulation [147-154], resulting in reduced angiotensin II production. Renal erythropoietin production is increased by A_2-adenosine receptor stimulation [155].

The renal effects of *ATP* are less well assessed. In the rat, ATP induces a vasoconstrictor response that appears to be most pronounced in the afferent (preglomerular) arterioles. The efferent arterioles are not affected by ATP [156].

Adenosine: a natural defense against the sequelae of ischemia

Extracellular formation of adenosine is increased during hypoxia and ischemia [157-160]. The sympathetic nervous system may play a role in this hypoxia or ischemia-induced adenosine release [161-163]. Several effects of this locally formed adenosine are assumed to be beneficial in situations of reduced oxygen supply [164]. In the heart, the negative inotropic and chronotropic effects will reduce oxygen demand. The electrophysiological effects of adenosine will reduce the risk of ischemia-induced arrhythmias. Presynaptic inhibition of norepinephrine release will attenuate the possible deleterious effects of norepinephrine like arrhythmogenesis, vasoconstriction, thrombocyte aggregation and direct cytotoxicity [165] However, the relation between adenosine, cardioprotection and sympathetic nervous system is complex since norepinephrine is able to

augment ischemia-induced adenosine formation by activation of 5'-nucleotidase [90] Furthermore, norepinephrine (like adenosine) has been shown to induce ischemic preconditioning (see below) [90] Adenosine-induced vasodilation will increase oxygen supply In addition, adenosine inhibits thrombocyte aggregation [166] and attenuates neutrophil function [13,167] possibly reducing the formation of leucocyte-derived free radicals during ischemia and reperfusion In several animal models, adenosine has been shown to reduce myocardial infarct size and to prevent ischemia-induced cardiac arrhythmias [168-174] Adenosine appears to be involved in preconditioning defined as the increased tolerance of myocardium to a prolonged ischemic insult achieved by an initial brief exposure to ischemia and reperfusion [175] In humans, preconditioning has also been suggested [176-179] In animals, adenosine-induced preconditioning is mediated by A_1-adenosine receptor stimulation An A_3-adenosine receptor may also be involved [180,181] Protein kinase C and ATP-dependent potassium channels appear to play a key role in the postreceptor mechanism of preconditioning [182,183]

In the kidney, adenosine only marginally affects renal blood flow (due to a combination of afferent arteriolar contraction and efferent vasodilation) but reduces tubular oxygen demand by attenuating glomerular filtration rate and tubular function In contrast to this theoretical beneficial effects of adenosine in renal ischemia, the adenosine receptor antagonist theophylline prevented renal ischemic damage in rabbits [184]

In the brain, adenosine can attenuate the synaptic release of the exocytotic aminoacids glutamate and aspartate by stimulation of presynaptic A_1-adenosine receptors Postsynaptically, adenosine depresses membrane Ca^{2+} permeability and increases K^+ and Cl permeability resulting in hyperpolarisation, and a reduced oxygen demand [185] Endogenous adenosine prevents the occurrence of convulsions [186] Dilation of cerebral arterioles will increase oxygen supply Adenosine receptor stimulation and elevation of endogenous adenosine levels appear to reduce cerebral infarct size in animals [187]

Pharmacological interventions

To date, pharmacological interventions are primarily directed towards adenosine-mediated effects An increasing number of more or less selective P_1- and P_2-purinergic receptor agonists and antagonists have been described For a detailed description, the reader is referred to some excellent reviews on this matter [6,97,116]

Apart from compounds that directly interact with the adenosine receptor, some drugs have been developed with an indirect action More specifically, these drugs enhance adenosine release (AICA riboside [188-190]) or reduce adenosine degradation by inhibiting cellular uptake of adenosine (nucleoside transport inhibitors nitrobenzylthioinosine, dipyridamole, dilazep and mioflazine derivatives like draflazine [191,192]) or

by inhibiting adenosine deaminase activity (deoxicoformycin). In addition, some compounds have been described that facilitate the binding of adenosine to its receptor through an allosteric mechanism These allosteric enhancers may have the theoretical advantage of a possible adenosine receptor subtype specificity [193] These indirectly acting drugs have in common that they affect adenosine-mediated effects only at sites of endogenous adenosine release, providing a tool to prevent systemic side effects that are associated with systemic infusion of adenosine or its analogues

Outline of this thesis

In most studies that are presented in this thesis, the 'perfused forearm technique' is used With this technique, it is possible to quantify the effect of vasoactive substances on the forearm circulation without interference by possible systemic effects of the used compounds like baroreflex activation

After cannulation of the brachial artery (an anatomical end artery), vasoactive substances can be infused using highly accurate perfusion pumps The resulting changes in forearm blood flow can be quantified by strain gauge plethysmography [194] To confine the measurements to the muscle vascular bed as much as possible, all measurements are performed during occlusion of the hand circulation which is largely determined by the skin vasculature [195] Simultaneous measurements of forearm blood flow in the non-infused arm, permit us to detect possible changes in forearm blood flow that do not reflect local effects of the intra arterial infusions

In the first part of this thesis (chapters 2-4), the effects of endogenous adenosine are investigated In particular, its interaction with the sympathetic nervous system is explored First, the effects of various grades of systemic nucleoside transport inhibition, as induced with draflazine, on plasma catecholamines, some hemodynamic parameters and ventilation are investigated (chapter 2 and 3) The hypothesis is tested that systemic nucleoside transport inhibition at a grade without any effect on catecholamines, blood pressure or heart rate still potentiates the forearm vasodilator response to exogenous adenosine. Next, the role of endogenous adenosine in modulation of forearm norepinephrine release is investigated in baseline conditions and during stimulation of the sympathetic nervous system (chapter 4) This study tests the hypothesis that increased extracellular concentrations of endogenous adenosine results in a reduced norepinephrine release in response to sympathetic nerve stimulation

The second part of this thesis focuses on the interaction between two purines (adenosine and adenosine-5'-triphosphate) on the one hand and the endothelium of the forearm vascular bed on the other In a pilot study, the ability of N^G-monomethyl-L-

arginine-acetate (L-NMMA) is investigated to reduce the formation of nitric oxide in our experimental setting. Acetylcholine is used as an endothelium-dependent vasodilator since this compound has been shown to induce vasodilation via stimulation of nitric oxide in the human forearm vascular bed [36]. The interaction between methacholine and L-NMMA is also studied because this compound has frequently been used as a substitute for acetylcholine in pharmacological studies of the human forearm vascular bed [196-199] without comparing the two substances with respect to their ability to stimulate NO-synthase (chapter 5). Searching for possible P_{2x}-purinergic receptors in the human forearm vasculature, the forearm vascular response to adenosine-5'-triphosphate (ATP) is further characterized (chapter 6). To this end, theophylline and L-NMMA are used to reduce P_1-purinergic receptor activation and NO-formation as possible mechanisms of ATP-induced vasodilation. In a final attempt to demonstrate ATP-induced forearm vasoconstriction as a possible marker of P_{2x}-purinergic receptor activation, the effect of extremely high ATP dosages are studied.

As outlined in the introduction section, nitric oxide may play a role in the vasodilator response to luminally applied adenosine. To assess this possibility in the human forearm vascular bed, the interaction between L-NMMA and adenosine is studied (chapter 7). Additionally, the role of potassium channels in signal transduction after adenosine receptor stimulation by luminally applied adenosine is investigated using tolbutamide and quinidine as K-channel blockers.

Impaired adenosine responsiveness, as suggested in animal studies [200,201], could contribute to the devastating sequelae of ischemic disease in patients with diabetes mellitus. To explore this possible mechanism in humans, the forearm vasodilator response to adenosine is studied in patients with uncomplicated insulin-dependent diabetes mellitus and compared with healthy age-matched control subjects (chapter 8).

CHAPTER 2

Hemodynamic and neurohumoral effects of various grades of selective adenosine transport inhibition in humans: Implications for its future role in cardioprotection

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ABSTRACT

In 12 healthy male volunteers (27-53 years), a placebo-controlled randomized double blind cross-over trial was performed to study the effect of the intravenous injection of 0.25, 0.5, 1, 2, 4 and 6 mg draflazine (a selective nucleoside transport inhibitor) on hemodynamic and neurohumoral parameters and ex vivo nucleoside transport inhibition (NTI). We hypothesized that an intravenous draflazine dosage without effect on hemodynamic and neurohumoral parameters would still be able to augment the forearm vasodilator response to intra-arterially infused adenosine. Heart rate (electrocardiography), systolic blood pressure (Dinamap 1846 SX; Critikon, Portanje Electronica BV, Utrecht, The Netherlands), plasma norepinephrine and epinephrine increased dose-dependently and could almost totally be abolished by caffeine pretreatment indicating the involvement of adenosine receptors. Draflazine did not affect forearm blood flow (venous occlusion plethysmography). Intravenous injection of 0.5 mg draflazine did not affect any of the measured hemodynamic parameters but still induced a significant ex vivo nucleoside-transport inhibition of 31.5 ± 4.1 % ($P < 0.05$ versus placebo). In a subgroup of ten subjects the brachial artery was cannulated to infuse adenosine (0.15, 0.5, 1.5, 5, 15 and 50 μg/100 ml forearm per min) before and after intravenous injection of 0.5 mg draflazine. Forearm blood flow amounted 1.9 ± 0.3 ml/100 ml forearm per min for placebo and 1.8 ± 0.2, 2.0 ± 0.3, 3.8 ± 0.9, 6.3 ± 1.2, 11.3 ± 2.2 and 19.3 ± 3.9 ml/100 ml forearm per min for the six incremental adenosine dosages, respectively. After the intravenous draflazine infusion, these values were 1.6 ± 0.2 ml/100 ml forearm per min for placebo and 2.1 ± 0.3, 3.3 ± 0.6, 5.8 ± 1.1, 6.9 ± 1.4, 14.4 ± 2.9 and 23.5 ± 4.0 ml/100 ml forearm per min, respectively (Friedman ANOVA: $P < 0.05$ before versus after draflazine infusion). In conclusion, a 30-50 % inhibition of adenosine transport significantly augments the forearm vasodilator response to adenosine without significant systemic effects. These results suggest that draflazine is a feasible tool to potentiate adenosine-mediated cardioprotection in man.

INTRODUCTION

Adenosine has important cardioprotective properties that are mediated by stimulation of adenosine receptors, located on the outer cell membrane. In animals, infarct size is reduced when adenosine is infused either before ischemia or during the reperfusion period [168,169,202]. Infusion of a selective adenosine receptor antagonist increases infarct size, indicating a role for endogenous adenosine as a cardioprotective autacoid [169]. Adenosine is a mediator of ischemic preconditioning [168,169,171], defined as the increased tolerance of myocardium to a prolonged ischemic insult achieved by an initial brief

exposure to ischemia and reperfusion [203]. This phenomenon has originally been described in animals [175]. Also, in humans a preconditioning effect has been suggested [176,177]. At present, many potentially cardioprotective effects of adenosine are known, like inhibition of neutrophil activation with subsequent reduced free radical formation, inhibition of thrombocyte aggregation, vasodilation, presynaptic inhibition of norepinephrine release, opening of potassium channels, and repletion of purine stores [204]. From a pharmacological point of view it seems of interest to develop agents with a comparable local effect on the myocardium [205]. Within this concept, long-acting adenosine receptor agonists are not useful because these drugs elicit pharmacological effects, not only during ischemia but continuously, and not only in the myocardium but in nearly all organ systems, resulting in a large list of side effects [206,207]. Inhibition of the cellular uptake of extracellular adenosine might be an alternative approach to circumvent the disadvantages of intravenous adenosine infusions [208]. In animals, this concept has been evaluated by the use of adenosine transport inhibitors like, for instance, dipyridamole. Dipyridamole appears to potentiate myocardial preconditioning [209]. In humans, dipyridamole is not a suitable tool to potentiate the cardioprotective effect of endogenous adenosine [210,211] and this might be due to its nonspecific actions like stimulation of prostacycline release and inhibition of phosphodiesterase [212,213].

Recently, a new adenosine transport inhibitor, called draflazine, has become available for human investigation. This active (-)-enantiomer of the piperazine derivate R 75231 is a highly specific adenosine transport inhibitor with a tighter binding to the transporter and looser binding to plasma proteins when compared with dipyridamole [192]. In rabbits, R 75231 has shown to prevent death from catecholamine-induced cardiac toxicity [214] and to improve functional recovery after cardiac ischemia [215]. In pigs, R 75231 reduces ischaemia-induced arrhythmias [216]. The present study explores the hemodynamic and neurohumoral effects of various grades of adenosine transport inhibition in man. Our results convincingly show that inhibition of adenosine transport by 70% or more increases blood pressure, heart rate and plasma catecholamines. In contrast, low grade inhibition to 30-50 % does not evoke any systemic hemodynamic changes but still potentiates the vasodilator effects of adenosine at a site of increased supply. These observations should be taken into account when draflazine is used as an 'on demand' drug to potentiate the beneficial effects of adenosine as released during ischemia.

SUBJECTS AND METHODS

Subjects

After approval of the local ethics committee, twelve normotensive nonsmoking healthy Caucasian male volunteers signed written informed consent before participation in the study. Demographic data are shown in table 1. They had no history of hypertension, diabetes mellitus or drug allergy, and did not use concomitant medication. In all volunteers a physical examination, routine laboratory investigation, and a twelve-electrocardiography lead were performed to exclude cardiovascular, pulmonary, renal, liver, or neurologic disease.

TABLE 1: Demographic characteristics

N = 12	mean (range)
age (years)	41 (27-53)
weight (kg)	73.3 (62.3-88.5)
length (cm)	179.5 (172.0-189.0)
systolic blood pressure (mmHg)*	129 (112-140)
diastolic blood pressure (mmHg)*	75 (62-88)
heart rate (bpm)‡	63 (48-72)

* Auscultatory measurements with sphygmomanometer after 5 minutes of supine rest (systolic blood pressure: Korotkoff I; diastolic blood pressure: Korotkoff V)
‡ Measured by pulse frequency counting after 5 minutes of supine rest

Methods

'Critical dose' finding (part 1)

In this study, the circulatory effects of six dosages of draflazine were investigated (0.25, 0.5, 1, 2, 4 and 6 mg).

First, in a double-blind randomized cross-over design, the hemodynamic and neurohumoral effects of placebo and 1, 2 or 4 mg draflazine were studied during four sessions per volunteer, that were separated by at least one week. At least one week thereafter, and after proven tolerance to the previous dosages, the effect of 6 mg draflazine was studied in a single blind fashion. This 6-mg dose was not included in the randomized double blind approach for safety reasons, on request of the ethics committee. After the randomization code had been broken, data analysis showed that all dosages, including the lowest, induced a statistically significant increase in heart rate. Therefore, the trial was

prolonged with two visits per volunteer, separated by at least one week, to study the hemodynamic effects of 0.25 and 0.5 mg draflazine in a double-blind randomized fashion.

Each experiment was performed in the morning after a 24-hour abstinence from caffeine-containing products and an overnight fast of at least 10 hours. Both arms were intravenously cannulated to infuse drug (left arm) and to collect blood (right arm). After an equilibration period of at least 45 minutes in supine position, placebo or drug was intravenously infused for 15 minutes using an automatic syringe infusion pump (type STC-521, Terumo Corporation, Tokyo, Japan). From five minutes before until 60 minutes after the start of the infusion, heart rate was measured by standard electrocardiography and continuously recorded on tape (Oxford 9000 with ADgi preamplifier, band pass 0.05-40 Hz, (-3 dB); Oxford Medical, Gorinchem, the Netherlands). Starting immediately before and continuing until 60 minutes after the start of the drug infusion, blood pressure (Dinamap 1846 SX, Critikon, Portanje electronica BV, Utrecht, The Netherlands) and forearm blood flow (FBF) were measured at the left arm at five- and fifteen minute intervals, respectively. FBF was registered by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (EC4, D.E. Hokanson, Inc., Washington DC, USA). The upper arm collecting cuff was inflated using a rapid cuff inflator (E-20, D.E. Hokanson, Inc., Washington DC, USA). At least one minute before the FBF measurements, the circulation of the left hand was occluded by inflation of a wrist cuff to 200 mmHg. FBF was recorded three times per minute during three minutes.

Before the start of each intravenous injection, blood was collected for the measurement of the plasma caffeine concentration.

Immediately before and 15, 30 and 60 minutes after the start of the intravenous infusion of placebo, 1, 2, 4 or 6 mg draflazine, blood was collected to determine plasma epinephrine (EPI), norepinephrine (NE), and adenosine concentration and to measure ex vivo adenosine transport inhibition (see Analytical Methods). Additionally, 15, 30 and 60 minutes after the start of the infusion, blood was collected to measure whole blood draflazine concentrations (see Analytical Methods). After administration of 0.25 and 0.5 mg draflazine, only adenosine transport inhibition and draflazine concentrations were determined.

Effect of the adenosine receptor antagonist caffeine on hemodynamic and neurohumoral responses to draflazine (part 2)

To investigate the contribution of adenosine-receptor stimulation in the hemodynamic and neurohumoral responses to draflazine, in a subgroup of ten subjects, informed consent was obtained to repeat the intravenous injection of 4 mg draflazine during adenosine receptor blockade with caffeine, an adenosine receptor antagonist in humans

[206]. Forty minutes before the start of the draflazine injection, a ten minute intravenous caffeine infusion was started (4 mg/kg), as described previously [217]. Blood pressure, heart rate, catecholamines, plasma caffeine concentration and adenosine transport inhibition were measured immediately before the start of the caffeine infusion, immediately before the start of the draflazine infusion and at regular time intervals thereafter as mentioned above. Draflazine-induced changes in hemodynamic and neurohumoral parameters were compared with those as obtained in part 1 of this study.

Effect of 0.5 mg draflazine on the forearm vasodilator response to adenosine (part 3)

In ten of the twelve subjects informed consent was obtained for a third study part to investigate the effect of 0.5 mg draflazine ('critical dose') on the forearm vasodilator response to intra-arterially infused adenosine. This dose was chosen because it was the highest dose of draflazine which did not affect baseline hemodynamic or neurohumoral parameters. Before the start of the study, the subjects were asked to abstain from caffeine-containing products for at least 24 hours. All tests were performed in the supine position after an overnight fast, starting at 8.00 AM. After administration of a 2% local anaesthesia (xylocaïne; Astra Pharmaceutical Products Inc., Worcester, MA), the left brachial artery was cannulated with a 20-gauge catheter (Angiocath, Deseret Medical, Inc., Becton Dickinson and Co., Sandy, UT) for both intra-arterial adenosine infusion (automatic syringe infusion pump, [type STC-521, Terumo Corp.]) and blood pressure recording (Hewlett Packard GmbH, Böblingen, Germany). Forearm blood flow was registered simultaneously on both forearms by electrocardiography-triggered venous occlusion plethysmography as stated above.

The experiment started with the measurement of baseline FBF during placebo infusion (NaCl, 0.9%). The effect of six increasing dosages of adenosine (0.15, 0.5, 1.5, 5, 15 and 50 μg/100 ml forearm per min) were compared with placebo (NaCl 0.9%). Prolonged occlusion of the hand circulation can cause discomfort with subsequent effects on blood pressure and heart rate. Therefore, a 5-minute-rest was allowed between the placebo infusion and the first adenosine infusion and between the third and fourth adenosine infusion. Forty-five minutes after the last adenosine infusion, the intra-arterial placebo infusion was repeated and followed by a fifteen minute intravenous injection of 0.5 mg draflazine in the right arm. Subsequently, the vasodilator response to adenosine was studied again. Additionally, the ex vivo adenosine transport inhibition was measured at regular time intervals. During all procedures, total infusion was adjusted to forearm volume as measured by water displacement and kept at a constant rate of 100 μl/100 ml forearm per min. Placebo and each adenosine dosage were infused for 4 minutes.

In a separate group of six healthy nonsmoking male volunteers the same protocol was performed except for the intravenous infusion of draflazine (time control study).

Analytical methods

Samples for determination of plasma caffeine concentration were analyzed with a reversed-phase HPLC method (limit of detection: 0.2 μg/ml) [218].

Plasma levels of EPI and NE were determined simultaneously by a HPLC method with fluorometric detection, in which catecholamines are concentrated from plasma by liquid-liquid extraction and derivated with the selective fluorescent agent 1,2-diphenyl-ethylenediamine prior to chromatography as has been described in more detail elsewhere [219]. The interassay coefficients of variation in our laboratory are 7.2% and 8.5% at a mean concentration of 0.15 and 1.02 nmol/liter for EPI and NE respectively (N=52).

To measure plasma adenosine concentration, 2 ml blood was collected and directly mixed during collection with 2 ml blocker solution using a specially designed device. The blocker solution contained the adenosine deaminase inhibitor erythro-9(2-hydroxy-3-nonyl)-adenosine (EHNA, 10 μM), the adenosine transport inhibitor dipyridamole (20 μM) and the thrombocyte aggregation inhibitor indomethacin (2 mg/liter). Immediately after blood collection the blood/blocker mixture was centrifuged (model 3200; Eppendorf North America, Inc., Madison, WI) at 3000 rpm for 2 minutes and the plasma was deproteinated with perchloric acid as described before [220]. The extract was kept frozen at -20 °C until the adenosine concentration was determined in duplicate by reversed-phase, high performance liquid chromatography using a nonlinear gradient.

In five subjects 12 ml blood/blocker mixture was collected before administration of 0.25 or 0.5 mg draflazine and divided into 6 equal portions. Adenosine standards were added to five separate blocker/mixture portions. The six portions were handled as stated above. The expected increases in adenosine concentration, as compared with the control blood/blocker mixture were 0.029, 0.052, 0.110, 0.210, and 0.405 μM. The averaged recoveries were 115.9 ± 31.3, 144.7 ± 24.6, 109.6 ± 11.6, 114.5 ± 15.6 and 126.7 ± 12.2 % for the five increasing standards, respectively. The detected increase in plasma adenosine concentration tended to be overestimated, although this was not statistically significant (P=0.3, n=5; Friedman ANOVA). In each individual a maximal correlation of 1.0 was observed between expected and measured adenosine concentration. The individual regression coefficients ranged from 1.0 to 1.7 (mean±SE: 1.3±0.1). The intra-assay variability, calculated as the coefficient of variation in the duplicate determinations at baseline (five duplicate-determinations in 11 subjects) was 16.2±2.2 %. The short-term intra-individual variability in measured adenosine concentration, calculated as the coefficient of variation of the four measurements during placebo infusion, was 20.7±7.2 %.

Ex vivo adenosine transport inhibition was measured by standardized incubation of erythrocytes with adenosine. Four ml blood was drawn into a vial containing 1 ml acid, citrate, and dextrose (85 mM trisodium citrate, 65 mM citric acid and 20 g/liter glucose)

and further handled as described before [216]. The percentage inhibition of adenosine transport (ATI %) was calculated as:

$$ATI\% = (A_x - A_0) x 100/(1 - A_0)$$

in which A_0 represents the adenosine concentration as proportion of the sum of the concentration of adenosine, inosine and hypoxanthine as determined in the sample collected just before the drug infusion and A_x represents this proportion as determined in the sample collected after the start of the drug infusion.

Whole-blood draflazine concentration was detected by HPLC (limit of detection: 5.0 ng/ml).

Drugs and solutions

Sterile solutions of draflazine or placebo in a formulation with 5 % hydroxypropyl-β-cyclodextrine (Janssen Pharmaceutica Inc., Beerse, Belgium), were prepared with NaCl 0.9% on the morning of the study day by a specially trained research nurse who was not otherwise involved in the practical performance of the trial. The randomization code was broken at the end of the trial, after all calculations on forearm blood flow were performed. Sterile solutions of caffeine (OPG Pharma, Utrecht, The Netherlands) and adenosine (Sigma Chemical Co., St Louis, MO) were freshly prepared by the investigator with NaCl 0.9% as solvent.

Statistics

Heart rate, as derived from continuously recorded R-R intervals, was averaged for each consecutive five-minute interval. The five-minute interval just before draflazine or placebo infusion was taken as baseline. For the other hemodynamic and neurohumoral parameters, the values obtained just before the intravenous infusion were taken as baseline. For each experiment, the effect of drug administration was calculated at each time point as change from baseline. Since hemodynamic parameters appeared to be normally distributed ($P > 0.1$; Shapiro-Wilk test for normality) differences in changes from baseline between placebo and draflazine administration were assessed by an ANOVA for repeated measurements with the drug dosage and time as within-subject factors. The neurohumoral parameters were not normally distributed ($P < 0.1$; Shapiro-Wilk test for normality). If an overall analysis by Friedman two-way nonparametric ANOVA showed significant differences in responses ($P < 0.05$, by means of Chi-square approximation), the paired Wilcoxon signed rank test was used to detect which draflazine dosages were different from placebo. During the intra-arterial study (part 3), mean arterial pressure was measured continuously during each recording of FBF and averaged per FBF registration. Forearm vascular resistance (FVR) was calculated from simultane-

ously measured mean arterial pressure and FBF and expressed as arbitrary units (AU). Additionally, the ratio of each simultaneously measured FBF (FBF infused/FBF control arm) was calculated. FBFs, the calculated flow ratios and FVRs obtained during each four minutes of placebo infusion or during the last two minutes of each drug infusion were averaged to one value. Adenosine-induced effects were expressed both as absolute and percentage change from preceding placebo infusion. The overall effect of draflazine on the adenosine dose response curve was analyzed by Friedman two-way nonparametric ANOVA. All results are expressed as mean$\pm$SE unless indicated otherwise; $P<0.05$ (two sided) was considered to indicate statistical significance.

RESULTS

Plasma caffeine levels were determined to check the compliance with respect to the caffeine abstinence. For part 1 of this study, in four subjects, the plasma caffeine concentration was below the limit of detection for all visits. In six subjects the plasma caffeine concentration remained below 1 mg/liter. In one subject caffeine was only detectable before the infusion of 1 mg draflazine (1.4 mg/liter) and in another subject, caffeine was detectable during 5 of the 7 visits ranging from 0.5 to 1.6 mg/liter. For part 2 of this study, plasma caffeine was detectable in two subjects being 0.2 mg/liter for both. In part 3 of this study, plasma caffeine was detectable in 3 subjects being 0.8, 0.3 and 0.25 mg/liter. These plasma caffeine concentrations indicate a good compliance with regard to the caffeine abstinence. In the second part of the study, caffeine was administered in a dose of 4 mg/kg. Thirty minutes after the caffeine infusion and immediately before the start of the draflazine infusion, plasma caffeine concentration was on average 5.7$\pm$0.2 mg/liter.

Subjective side effects to draflazine

Up to 2 mg draflazine, no subjective side effects occurred. In one subject, the 4-mg dose induced a slight headache and a feeling of dyspnoea. Previous intravenous injection of caffeine completely prevented these complaints. In 8 of the 12 subjects, the 6-mg dose induced temporary subjective side effects ranging from a feeling of excitement (n=4) and/or headache (n=4) to nausea (n=3) that was accompanied by vomiting and chest pain (without electrocardiographic changes) in one subject. These subjective side effects might have affected the recordings, resulting in less accurate measurements during and after the administration of 6 mg draflazine as compared with the lower dosages.

Critical dose finding

Table 2 shows mean baseline values for all measured parameters. Overall, no statistically significant differences in baseline values were observed.

Figure 1 shows the time course of changes in blood pressure and heart rate during the varying draflazine infusions as compared with placebo. Up to 0.5 mg, draflazine did not induce a significant increase in heart rate. Heart rate increased by maximally 2.0 ± 1.1 for placebo and 1.8 ± 0.5, 3.3 ± 0.8, 6.1 ± 0.7, 15.2 ± 2.3, 29.2 ± 2.6, and 32.5 ± 2.8 bpm for 0.25, 0.5, 1, 2, 4 and 6 mg draflazine respectively ($P<0.01$ versus placebo for 1, 2, 4 and 6 mg draflazine, $n=12$). Up to 1 mg draflazine, systolic blood pressure was not significantly affected. At higher dosages, the maximal mean systolic blood pressure response increased dose-dependently by 9.1 ± 1.4, 12.5 ± 3.2 and 18.0 ± 3.1 mmHg for 2, 4 and 6 mg draflazine respectively ($P<0.05$ versus placebo, $n=12$). The most important increase in systolic blood pressure occurred during the first 15 minutes after starting the draflazine infusion. Neither diastolic blood pressure, mean arterial pressure nor forearm blood flow were significantly affected by draflazine. When forearm vascular tone was expressed as vascular resistance (ratio of mean arterial pressure and forearm blood flow), still no effect of draflazine on forearm vasculature could be observed.

The effect of draflazine on plasma NE and EPI concentration are shown in figure 2. Starting with 2 mg, draflazine induced a dose-dependent increase in plasma NE concentration that reached a maximum at 30 minutes after starting the infusion. At this time point, the increase in NE concentration was 0.7 ± 0.1, 1.0 ± 0.2 and 1.7 ± 0.3 nmol/liter for 2, 4 and 6 mg draflazine respectively ($P<0.05$ versus placebo, $n=12$).

After infusion of 1 mg draflazine, the EPI concentration was not significantly affected. Infusion of 2 mg draflazine induced a small increase in EPI concentration of 0.02 ± 0.01 nmol/liter at 15 minutes after starting the draflazine infusion ($P<0.05$ versus placebo, $n=12$). Up to 60 minutes after the start of the infusion of 4 or 6 mg draflazine, the EPI concentration continuously increased maximally by 0.15 ± 0.03 and 0.31 ± 0.06 nmol/liter respectively ($P<0.05$ versus placebo, $n=12$).

The course of ex vivo adenosine transport inhibition is shown in figure 3. Fifteen minutes after the start of each draflazine infusion, maximal adenosine transport inhibition was achieved being 0.2 ± 0.7 for placebo and 10.2 ± 2.3, 31.5 ± 4.1, 70.4 ± 1.5, 80.6 ± 0.9, 89.6 ± 1.1, and 92.6 ± 0.6 % for 0.25, 0.5, 1, 2, 4 and 6 mg draflazine respectively ($P<0.05$ for each dosage versus placebo).

TABLE 2: Baseline characteristics before each drug infusion (mean±SE)

	Draflazine dosage						
	placebo	0.25 mg	0.5 mg	1 mg	2 mg	4 mg	6 mg
Systolic blood pressure (mmHg)	108.2±3.0	108.5±2.5	108.5±2.9	107.5±3.0	105.4±2.8	107.9±2.7	104.8±3.7
Diastolic blood pressure (mmHg)	68.6±1.6	69.3±1.6	69.5±1.5	66.4±1.4	66.1±1.9	69.0±1.9	66.1±2.2
Mean arterial blood pressure (mmHg)	83.4±2.0	82.9±1.8	83.7±1.8	81.3±1.5	80.4±2.3	83.2±2.2	80.3±2.8
Heart rate (bpm)	60.6±2.0	61.7±1.5	60.8±1.9	60.6±2.3	61.5±1.9	59.8±1.8	61.4±2.5
Forearm blood flow (ml/100 ml/min)	1.8±0.3	2.1±0.5	1.6±0.3	1.4±0.2	1.5±0.2	1.3±0.2	1.8±0.2
Noradrenaline (nmol/L)	1.2±0.1	-	-	1.1±0.1	1.2±0.1	1.3±0.2	1.3±0.2
Adrenaline (nmol/L)	0.10±0.02	-	-	0.11±0.02	0.09±0.01	0.09±0.01	0.08±0.02
Adenosine (μmol/L)	0.17±0.07	-	-	0.22±0.08	0.39±0.13	0.39±0.18	0.35±0.17

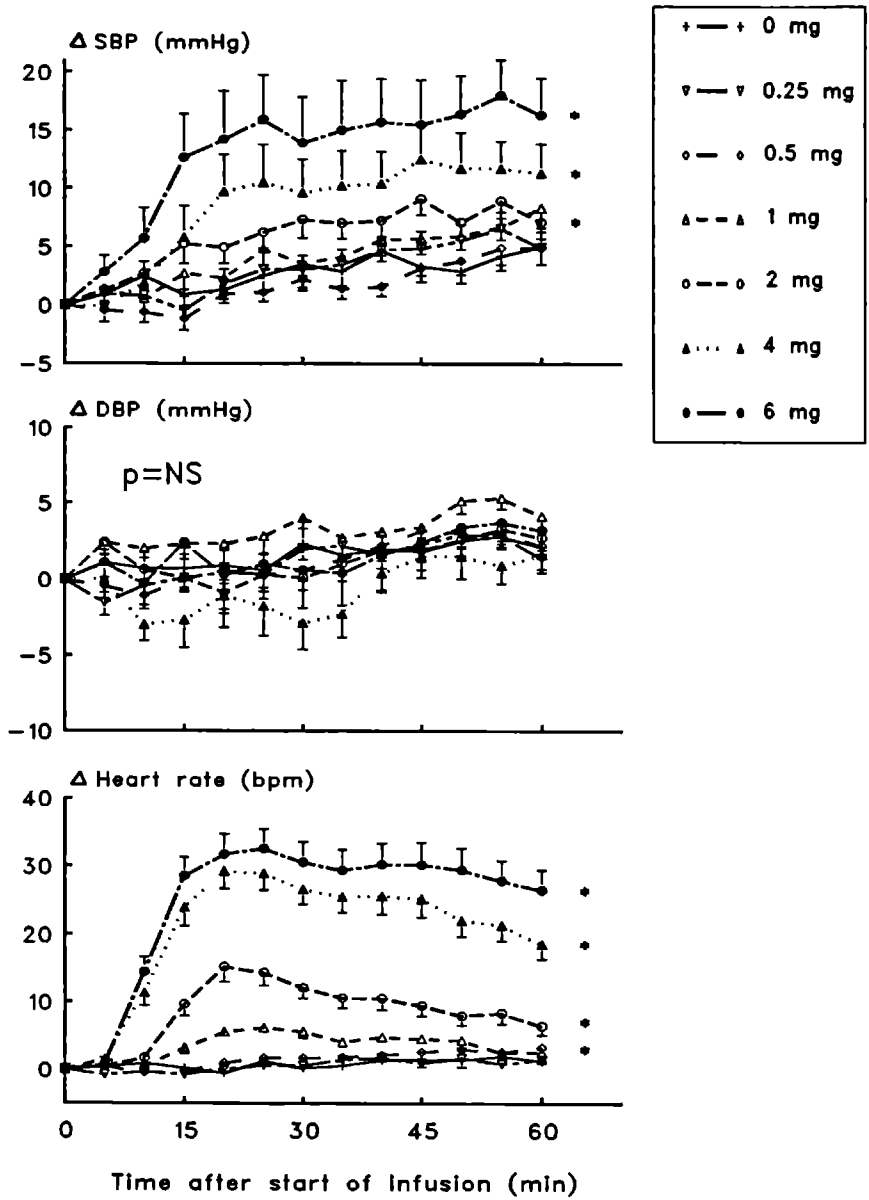

Figure 1. Effect of draflazine on systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate. *, Significant difference with placebo (n = 12)

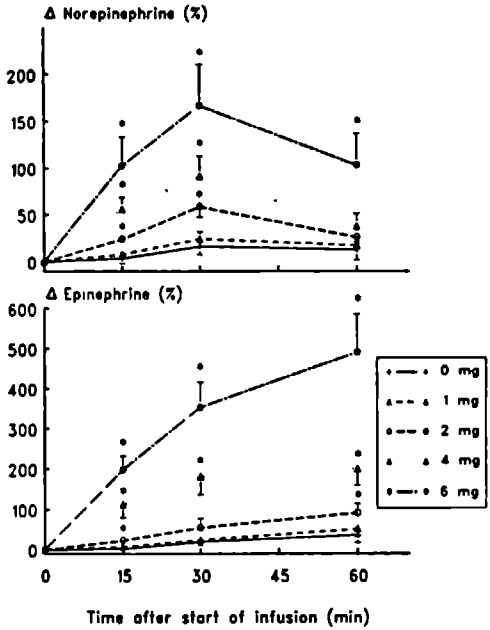

Figure 2. Effect of draflazine on plasma catecholamines. *, Significant difference with placebo (n=12)

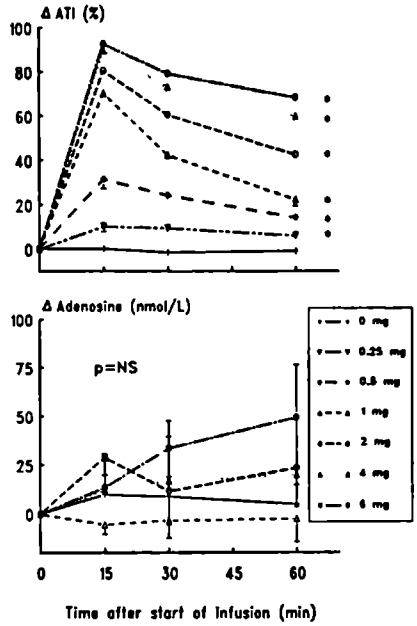

Figure 3. Effect of draflazine on ex vivo nucleoside transport inhibition (top) and plasma-adenosine concentration (bottom), respectively *, Curves that were significantly different from placebo at all time points (n=12)

Sixty minutes after starting the infusion, there still remained a significant adenosine transport inhibition being -0.9±0.8 for placebo and 6.0±0.8, 14.2±1.1, 22.3±3.0, 42.5±1.7, 59.8±1.9 and 68.3±2.0 % for the six draflazine doses, respectively ($P<0.05$ for each dosage versus placebo). For each subject, the relation between drug concentration and ex vivo adenosine transport inhibition was assessed by computer assisted curve fitting using the Hill equation (figure 4). For this analysis, minimal and maximal ex vivo adenosine transport inhibition was set constant at 0 and 100 % respectively. The best fitting drug concentration with 50 % ex vivo adenosine transport inhibition and Hill-coefficient were 74.1±2.4 ng/ml (range: 55.5-88.1 ng/ml; n=12) and 3.2±0.1 (range: 2.5-4.0; n=12), respectively. According to this model, 95±1 % of the variation in ex vivo nucleoside transport inhibition could on average be explained by the blood draflazine concentration.

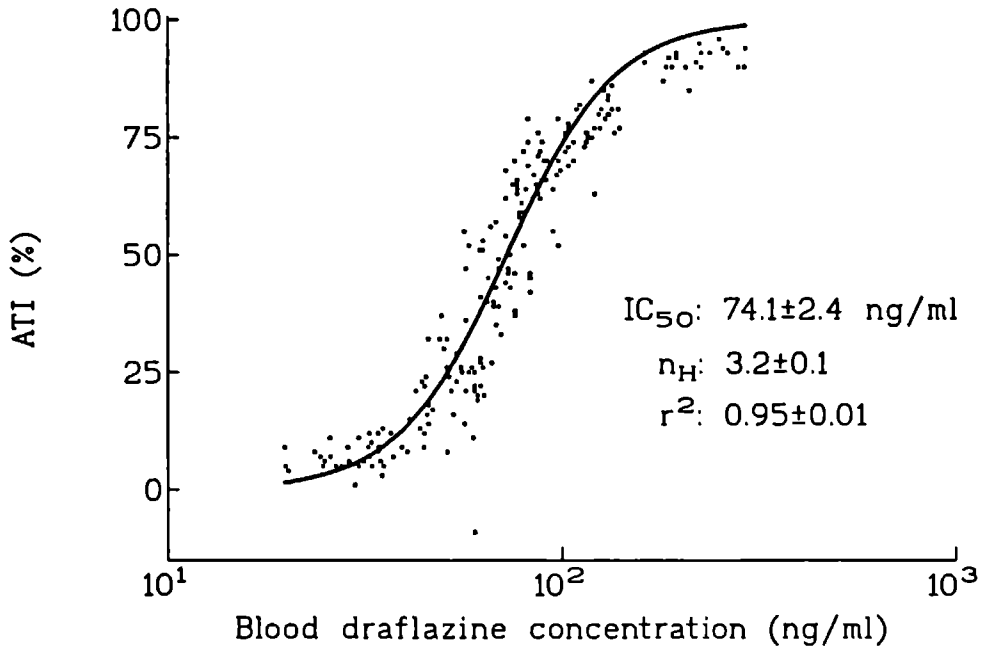

Figure 4. Relation between percent inhibition in adenosine transport and whole-blood draflazine concentration. This relation was evaluated for each subject using the Hill equation. For each individual, the best fitting Hill coefficient and IC_{50} (draflazine concentration that exhibits 50% of the maximal effect) were calculated by computer-assisted curve fitting (n=12).

For each subject, the relation between draflazine-induced heart rate response (difference with response after placebo infusion) and ex vivo adenosine transport inhibition was assessed at 15, 30 and 60 minutes after starting the draflazine infusion (figure 5). Up to approximately 50 % inhibition of adenosine transport, heart rate was unaffected. An equation in the form of $Y=AX^3$ appeared to describe the relation between heart rate response and adenosine transport inhibition most accurately. Nonlinear regression analysis revealed a mean value for A of $(3.0\pm0.3)\,10^{-5}$, $(6.1\pm0.4)\,10^{-5}$ and $(7.7\pm0.8)\,10^{-5}$ at 15, 30 and 60 minutes respectively. With this model, 77±2, 90±2 and 81±3 % of the intra-individual variation in heart rate response could be explained by ex vivo nucleoside transport inhibition at 15, 30 and 60 minutes after starting the draflazine infusion, respectively.

Apart from ex vivo adenosine transport inhibition, figure 3 shows the effect of draflazine on plasma adenosine concentration. Draflazine did not induce significant changes in plasma adenosine concentration ($P>0.5$, $n=11$).

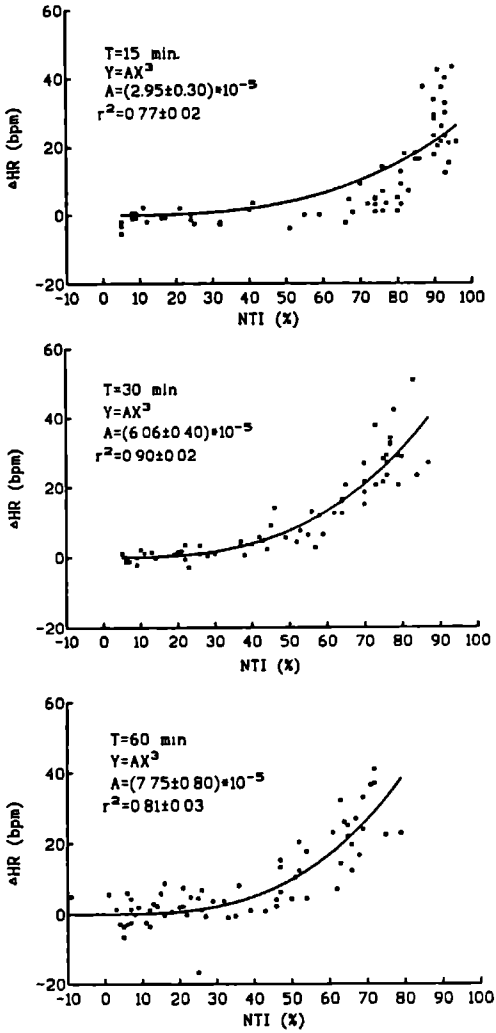

Figure 5. Relation between heart rate response and adenosine transport inhibition. At each time point and for each individual, this relation was evaluated by computer assisted curve fitting. An equation in the form of $Y=AX^3$ appeared to result in the best fitting curves. The individual values for A and r^2 (squared correlation coefficient) were averaged and are depicted in the figure as mean ± SE (n = 12).

Effect of the adenosine receptor antagonist caffeine on hemodynamic and neurohumoral responses to draflazine

Baseline values after caffeine infusion were significantly increased for systolic, diastolic and mean arterial pressure and plasma EPI concentration and reduced for heart rate when compared with baseline values without caffeine pretreatment (see table 3).

Figure 6 shows the effect of intravenous caffeine infusion (4 mg/kg) on the course of systolic blood pressure, heart rate, EPI and NE after draflazine infusion. Caffeine pretreatment reduced the heart rate response to the 4-mg dose of draflazine from

TABLE 3: Effect of caffeine on baseline parameters

N = 10	Before placebo (without caffeine pretreatment)	Before 4 mg draflazine (without caffeine pretreatment)	Before 4 mg draflazine (with caffeine pretreatment)
Systolic blood pressure (mmHg)	108.8±3.4	108.5±3.1	113.0±2.1*‡
Diastolic blood pressure (mmHg)	68.9±1.9	68.9±2.3	73.1±1.6*‡
Mean arterial pressure (mmHg)	83.8±2.3	83.5±2.5	87.3±1.7‡
Heart rate (bpm)	59.0±2.2	58.4±1.4	53.9±1.4*‡
Norepinephrine (nmol/L)	1.2±0.1	1.3±0.2	1.0±0.1
Epinephrine (nmol/L)	0.11±0.02	0.09±0.02	0.14±0.02¶

* and ‡ Significantly different from baseline values before placebo and 4 mg (without caffeine treatment), respectively, ¶ Friedman Two-way ANOVA: p=0.08; Wilcoxon matched-pairs, signed-ranks test: P=0.03 versus 4 mg draflazine (without caffeine pretreatment).

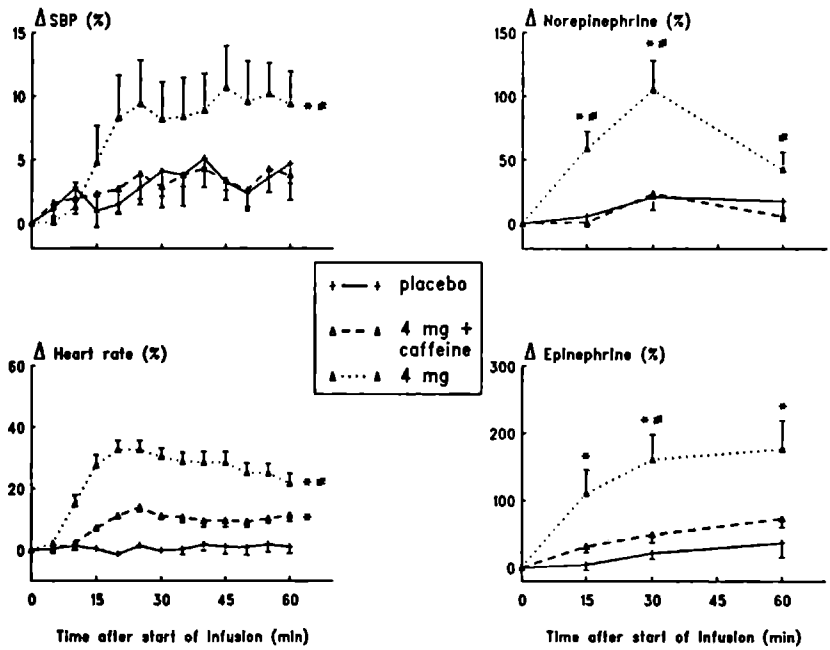

Figure 6. Effect of caffeine pretreatment (4 mg/kg) on the draflazine (4 mg)-induced increases in systolic blood pressure (SBP), heart rate, norepinephrine and epinephrine. * And #, significant differences with placebo or draflazine after caffeine pretreatment, respectively (n=10).

29.3±3.8 bpm (32.6±3.0 %) to 8.8±1.0 (13.9±1.4 %) ($P<0.01$ versus placebo and versus draflazine without caffeine pretreatment). The systolic blood pressure response to draflazine was reduced from 11.7±3.7 mmHg (10.7±3.3 %) without caffeine pretreatment to 4.9±1.7 mmHg (4.3± 1.5 %) after caffeine pretreatment (P=NS versus placebo, $P<0.05$ versus draflazine without caffeine pretreatment).

Caffeine pretreatment reduced the increase in NE concentration after the 4-mg dose of draflazine from 1.1±0.23 nmol/liter (105.6±22.6 %) to 0.2±0.1 nmol/liter (23.7±4.1 %) (P=NS versus placebo and $P<0.05$ versus draflazine infusion without caffeine pretreatment). The increase in plasma EPI concentration after 4 mg draflazine was reduced from 0.14±0.04 nmol/liter (177.0±42.2 %) without caffeine pretreatment to 0.09±0.02 nmol/liter (73.1±12.9 %) after caffeine pretreatment (P=NS versus placebo and $P<0.05$ versus draflazine without caffeine pretreatment).

Draflazine induced a significant inhibition of nucleoside transport of 89.3±1.1, 71.5±1.3 and 59.4±1.8 % at 15, 30 and 60 minutes after the start of the draflazine infusion respectively and these figures did not significantly differ from adenosine transport inhibition as observed after infusion of 4 mg draflazine without caffeine pretreatment.

Effect of 0.5 mg draflazine on forearm vasodilator response to adenosine

Intra-arterial infusion of adenosine increased forearm blood flow dose dependently. The baseline forearm blood flow was 1.9±0.3 and 1.6±0.2 ml/100 ml forearm per min in the infused and control arm, respectively. During the subsequent six incremental adenosine infusions, the forearm blood flow in the infused arm amounted to 1.8±0.2, 2.0±0.3, 3.8±0.9, 6.3±1.2, 11.3±2.2 and 19.3±3.9 ml/100 ml/min, respectively. In the control arm, forearm blood flow remained unchanged. Draflazine did not affect forearm blood flow: in the infused arm, forearm blood flow was 1.8±0.2 and 1.6±0.2 ml/100 ml per min immediately before and after the draflazine infusion, respectively (control arm: 1.3±0.1 and 1.2±0.2 ml/100 ml per min). However, draflazine significantly augmented the adenosine-induced changes in forearm blood flow. After the draflazine infusion, the forearm blood flow in the infused arm amounted 2.1±0.3, 3.3±0.6, 5.8±1.1, 6.9±1.4, 14.4±2.9 and 23.5±4.0 ml/100 ml forearm per min during the six incremental adenosine infusions, respectively ($P<0.05$ versus adenosine infusions before draflazine treatment). In the control arm, forearm blood flow remained unchanged. Figure 7 shows the effect of draflazine on the forearm vasodilator response to adenosine, expressed as percent change in FVR. The same effect of draflazine was observed when results were expressed as forearm vascular resistance or flow ratio both expressed as absolute as well as percent changes from baseline. The time control study did not reveal a

significant change in adenosine-induced forearm vasodilation, excluding significant carry-over effects (see lower panel of figure 7).

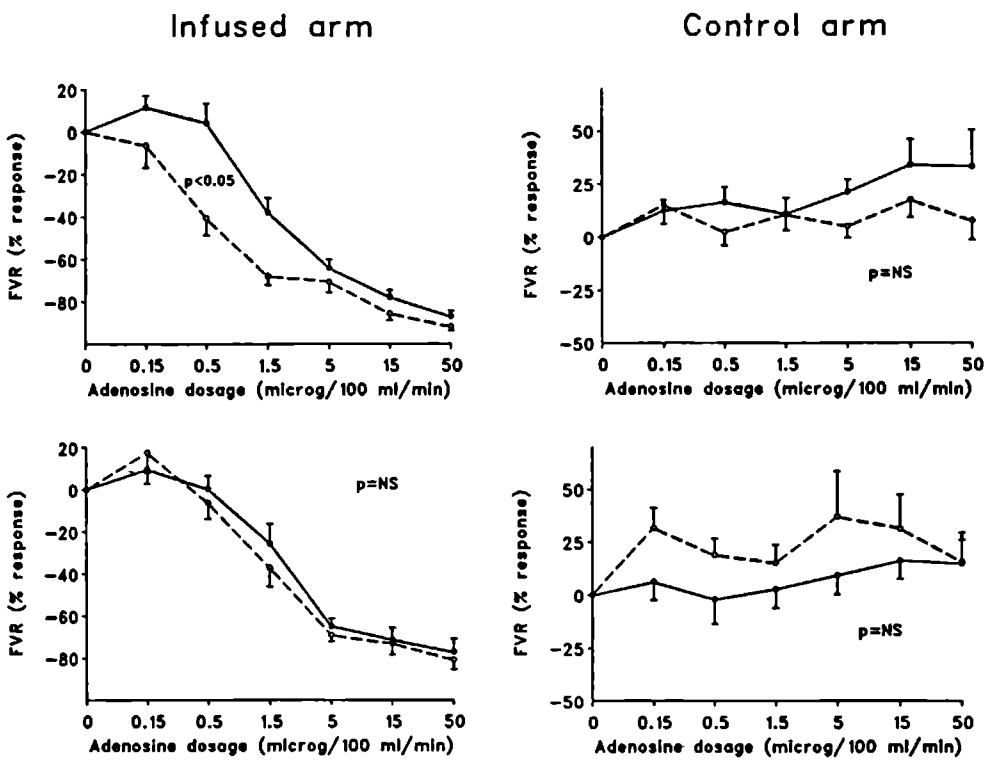

Figure 7. Adenosine-induced forearm vasodilation expressed as forearm vascular resistance (quotient of mean arterial pressure and forearm blood flow) in infused- and control arm. Upper panel: effect of intravenous draflazine treatment (N=10); —: before draflazine infusion; ------: after draflazine infusion. Lower panel. time control study (N=6), —: first dose response curve; ------: second dose response curve. P-values indicate level of significance for the difference between both curves.

Heart rate was 57.5±1.7 and 58.4±1.4 bpm immediately before and after draflazine infusion, respectively (P>0.2), confirming that the used draflazine dosage did not induce systemic effects.

Ex vivo nucleoside transport inhibition was 47.9±3.2, 21.6±1.6 and 15.0±1.5 % at the end of the draflazine infusion, and at the end of the third and sixth intra-arterial adenosine dose, respectively.

DISCUSSION

This study was aimed at finding a draflazine dosage without unwanted hemodynamic and neurohumoral effects that still potentiates the beneficial effects of adenosine at sites of increased formation. Therefore, a dose-response trial was performed that revealed a dose-dependent increase in systolic blood pressure, heart rate, and plasma catecholamines in subjects that refrained from caffeine-containing products for at least 24 hours. Preceding caffeine infusion abolished all draflazine-mediated effects, confirming that they were mediated by adenosine receptor stimulation Heart rate appeared to be the most sensitive parameter to detect unwanted effects. An intravenous dosage of 0.5 mg draflazine did not affect heart rate, but still induced an ex vivo transport inhibition by approximately 30 % To test the hypothesis that this 'critical dose' is able to potentiate the vasodilatory effects of adenosine in humans at sites of increased formation, the perfused forearm technique was used as a model of local adenosine formation Intravenous infusion of 0.5 mg draflazine appeared to augment the forearm vasodilator response to adenosine threefold, suggesting that a low grade adenosine transport inhibition is a feasible approach to exploit the beneficial effects of endogenous adenosine in humans.

Hemodynamic and neurohumoral effects of draflazine

With regard to the cardiovascular system, adenosine can induce direct and indirect effects. The direct effects of adenosine include negative inotropic, chronotropic and dromotropic effects [134], relaxation of vascular smooth muscle (except in the pulmonary and renal vascular bed where vasoconstriction is observed) [5,221], pre- and postsynaptic inhibition of adrenergic neurotransmission [222,223], reduction of renal renin release [148,151,153], stimulation of vascular angiotensin II production [224] and inhibitory effects on cardiovascular centres in the brain stem except for the nucleus tractus solitarii that is excitated by adenosine, resulting in an increased sensitivity of the baroreflex [225]. These effects are thought to be involved in the hypotensive response to intravenously administered adenosine as observed in animals and anesthetized humans The indirect effects are mediated by adenosine-induced stimulation of afferent nerves, including renal [226,227] and myocardial afferent nerves [385], carotid and aortic chemoreceptors [207,228] and forearm (muscle) afferent nerves [229] Stimulation of these afferents results in activation of the sympathetic nervous system and respiratory system [206,230] and a subsequent increase in systolic blood pressure, and plasma renin activity [206,207,230-234]. The increase in heart rate is probably mediated by concomitant deactivation of the parasympathetic nervous system since it can be antagonized by atropine but not by propranolol [235] These indirect effects of intravenous adenosine infusion are dependent on an intact autonomic reflex arc [207], which probably explains why these

effects are blunted in anesthetized humans and animals [236-238]. The increase in systolic blood pressure is not always observed during intravenous infusion of adenosine in healthy volunteers [235,239-241]. This apparent discrepancy in the literature can be explained by differences in caffeine abstinence which is always relatively short or totally absent in the studies in which no increase in systolic blood pressure is observed. After ingestion of two cups of regular coffee, plasma caffeine concentrations are in the range of 4-5 mg/liter [242], which is sufficiently high to antagonize the hemodynamic effects of intravenous adenosine infusion [206]. The plasma half life time of caffeine after ingestion of two cups of coffee ranges from 2 to 8.5 hours [242]. Therefore, a 24 hour period of caffeine abstinence is important to prevent underestimation of adenosine-induced hemodynamic and neurohumoral effects. An increase in systolic blood pressure, heart rate and plasma catecholamines has also been observed in resting man after administration of the adenosine transport inhibitor dipyridamole [217,243] and could be inhibited by previous administration of caffeine or theophylline [217,244], suggesting that adenosine is formed under baseline conditions. In addition to the variable oral bioavailability of dipyridamole and its lack of specificity [212,213,245,246], these excitatory effects of dipyridamole may be an explanation for its disappointing effect on cardiovascular mortality in large trials [210,211].

It was expected from the previous experiments with adenosine and dipyridamole in healthy, conscious human volunteers, that draflazine, too, could induce hemodynamic and neurohumoral effects that would be unfavourable in patients with ischemic heart disease. The present study indeed confirms most of these observations and substantiates them by showing their dependency on the degree of ex vivo adenosine transport inhibition. Moreover, we observed an increase in both EPI and NE during high degrees of selective adenosine transport inhibition, supporting the involvement of the sympathetic nervous system in this excitatory response. The increase in systolic blood pressure without a change in mean arterial pressure excludes baroreflex activation as a possible mechanism for the increased plasma catecholamine concentrations. Activation of sympathetic tone by draflazine-induced myocardial ischemia is very unlikely because the study was performed in healthy subjects without a history of cardiovascular disease. Additionally, continuous electrocardiography did not demonstrate myocardial ischemia. However, a yet unknown direct effect of draflazine on the kinetics of catecholamines can not be excluded.

Effect of caffeine pretreatment on draflazine-induced hemodynamic and neurohumoral responses

Caffeine pretreatment almost completely abolished the effect of draflazine on systolic blood pressure, heart rate, and plasma catecholamines. This antagonism occurred at a plasma caffeine concentration of 5.7 mg/liter, a concentration that can occur after

drinking one cup of coffee [242] The property of caffeine as a competitive adenosine receptor antagonist has been well documented in human in vivo studies [206,221,247,248]. Therefore, the observed antagonism of draflazine-induced hemodynamic and neurohumoral responses by caffeine indicates the involvement of adenosine receptors in draflazine-induced effects.

Thirty minutes after caffeine pretreatment, baseline values of systolic, diastolic, and mean arterial pressure and plasma EPI concentration were increased and baseline heart rate was reduced. These findings are in agreement with previous reports about the effect of caffeine [218]. The changes in baseline blood pressure, heart rate and EPI that were associated with caffeine pretreatment could have interfered with the subsequent effects of draflazine infusion. However, the magnitude of the increased baseline levels after caffeine are almost neglectable when compared with the effects of draflazine.

Draflazine-induced adenosine transport inhibition was not significantly affected by caffeine pretreatment Therefore, the effect of caffeine on draflazine-induced hemodynamic and neurohumoral responses can not be explained by differences in the degree of adenosine transport inhibition

In theory, inhibition of 5'-nucleotidase by caffeine could have diminished the formation of extracellular adenosine [249] However, inhibition of the ecto-5'-nucleotidase, as obtained from rat brain, occurs at a caffeine concentration of 1 0 mM which is considerably higher than the plasma caffeine concentration of 5.7 mg/liter (equivalent to 0 03 mM) as observed in this study. Therefore, caffeine-induced inhibition of extracellular adenosine formation is not likely in the present study.

Draflazine and plasma adenosine concentration

Draflazine failed to increase plasma adenosine concentrations. Two possible explanations will be discussed (*a*) the lack of an observed plasma adenosine increase is due to a measurement error and (*b*) adenosine concentrations are increased in the interstitium only.

(*a*) The precision of the adenosine determinations in the present study does not essentially differ from that reported by others [250]. As stated in methods, the recovery of adenosine added to plasma was high and appeared to be linear within the physiological range. Therefore, a systematic measurement error is unlikely Both German et al. and Sollevi et al. observed a doubling in plasma adenosine concentration after administration of the adenosine transport inhibitor dipyridamole [244,251]. However, in both studies, adenosine formation, uptake and breakdown after blood sampling were not optimally antagonized. German et al. did not use a blocker solution [251,252] while Sollevi et al. used a blocker solution that was mixed with blood immediately after, in stead of during, blood sampling [244]. Therefore, in these studies, baseline plasma adenosine concentra-

tions may have been underestimated. The administered dipyridamole may have affected adenosine uptake by erythrocytes after blood sampling resulting in an artificial increase in measured plasma adenosine concentration. The present study does not rule out a small draflazine-induced increase in plasma adenosine levels since intra-subject variability of the adenosine detection method is relatively high, especially when compared with detection methods of other endogenous compounds like for instance (nor)epinephrine. Plasma adenosine concentrations were measured up to 60 minutes after the start of the draflazine infusion. Therefore, our data do not exclude a detectable increase in plasma adenosine levels after this time point or during more sustained nucleoside transport inhibition.

(*b*) The most likely explanation for adenosine receptor-mediated hemodynamic and neurohumoral effects without any change in plasma adenosine concentrations is that selective adenosine transport inhibition results in accumulation of endogenous adenosine within the interstitium. As discussed in detail elsewhere, the vascular endothelium may act as a strong barrier against passage of metabolites [88,253,254]. In normal tissue, the nucleoside transporter bypasses this barrier but nucleoside transport inhibition may transform the endothelium to a functional barrier preventing adenosine to leave the interstitial space [254]. Interstitial adenosine may originate from ATP that is released from sympathetic nerve endings [102] and subsequently dephosphorylated to adenosine by ecto-phosphatases and ecto-5'-nucleotidase located on the membranes of vascular smooth muscle cells and endothelium. Since ATP is released from sympathetic nerve endings by exocytosis, this source of adenosine is not blocked by adenosine transport inhibition.

Ex vivo nucleoside transport inhibition in relation to draflazine concentration and heart rate response

The relation between blood drug concentration and ex vivo adenosine transport inhibition could well be described by the Hill-equation. Since draflazine concentration was measured in whole blood, this is not necessarily a reflection of draflazine concentration at the level of the nucleoside transporter in the erythrocyte membrane. Therefore, the accuracy and pharmacological importance of the Hill-coefficient and the 50 % inhibiting drug concentration, as determined in this study should not be overvalued. Nevertheless, the high correlation between ex vivo nucleoside transport inhibition and whole-blood draflazine concentration provides an experimental base to use this functional parameter as a tool to monitor draflazine treatment. This is further supported by the high correlation between ex vivo adenosine transport inhibition and heart rate response. The relation between heart rate response and ex vivo nucleoside transport inhibition became steeper over time, reflecting a time delay between draflazine-induced ex vivo nucleoside transport inhibition and its effect on heart rate. Two processes may account for this delay. First, intravenous infusion of draflazine rapidly inhibits nucleoside transport at the erythrocyte

membrane while diffusion of draflazine into the interstitial space and inhibition of adenosine transport in the vascular wall may take more time. Second, it probably takes some time for endogenous adenosine to accumulate and to reach concentrations that are sufficiently high to evoke its full effect on heart rate.

Effect of 0.5 mg draflazine on forearm vasodilator response to adenosine

The forearm vasodilator response to adenosine was significantly augmented by a draflazine dosage that did not induce unwanted hemodynamic or neurohumoral side effects. These results indicate that the cellular uptake of luminal adenosine is inhibited, and that the increased adenosine concentrations are available for stimulation of adenosine receptors that mediate vascular relaxation. The reduced augmentation of adenosine-mediated vasodilation at the three highest adenosine concentrations, could be the result of the decreased nucleoside transport inhibition at the time that the highest adenosine concentration was infused. This is supported by the ex vivo nucleoside transport inhibition which amounted to only 15 % at the end of the sixth adenosine infusion, compared with 22 % at the end of the third adenosine infusion.

Limitations of the study and conclusions

Three limitations of the study should be mentioned. First, the studies were performed in healthy volunteers. We are not informed about the influence of age and varying disease states on the systemic effects of draflazine. Therefore, it cannot be excluded that in the elderly or in patients with conditions like hypertension and cardiovascular disease, a different dose-response pattern to draflazine exists. Second, the hemodynamic- and neurohumoral responses to the adenosine transport inhibitor draflazine were studied after a 24 hour abstinence from caffeine containing products. This abstinence period was included to prevent adenosine antagonism by caffeine. In clinical practice, such a situation will almost never occur. Adenosine receptor upregulation during caffeine use may have occurred and could have exaggerated the effects of adenosine uptake inhibition after short term abstinence from caffeine [255-257]. Third, we studied the acute effects of short-term nucleoside transport inhibition. Our data do not exclude the development of tolerance to increased levels of endogenous adenosine that may arise during long-term nucleoside transport inhibition.

Before it can definitely be concluded that draflazine is a feasible tool to potentiate the cardioprotective effects of adenosine in humans, our findings should be confirmed in the coronary vasculature. Furthermore, the present observation of in vivo adenosine transport inhibition holds for intra-arterially applied adenosine and should be verified for interstitially formed adenosine as during ischemia.

In conclusion, in healthy male volunteers, draflazine is able to inhibit adenosine transport significantly both ex vivo as well as in-vivo in man. Heart rate appeared to be the most sensitive parameter to detect systemic hemodynamic or neurohumoral effects. When a draflazine dosage is injected that does not affect heart rate, the forearm vasodilator response to adenosine is still potentiated. Clinical trials are needed to investigate the possible beneficial effect of adenosine uptake inhibition in the prevention of ischemic injury. In these trials, caffeine intake should be monitored, the optimal draflazine dosage can be determined using heart rate as a marker of unwanted systemic effects and ex vivo adenosine transport inhibition can be used as a tool to monitor efficacy and compliance.

CHAPTER 3

High-grade nucleoside transport inhibition stimulates ventilation in humans.

Rongen GA, Smits P, Bootsma G, Ver Donck K, de Vries A, Thien Th

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ABSTRACT

In six healthy male volunteers a placebo controlled, double-blind, randomized, crossover trial was done to assess the effect of 1, 2, 4 and 6 mg draflazine, a specific nucleoside transport inhibitor, on ventilation. Draflazine increased thoracic excursions dose-dependently by maximally (median with 95% confidence interval) 114.0% (38.3 to 184.8%) without affecting breathing rate. Ex vivo adenosine transport was inhibited by 0% (0 to 1%) after placebo, and 70% (59 to 74%), 81% (76 to 85%), 90% (86 to 93%), and 93% (90 to 96%) after the four increasing draflazine dosages, respectively ($P < 0.05$ for each draflazine dosage versus placebo). These results indicate that endogenously released adenosine may play a role in the regulation of ventilation.

INTRODUCTION

Intravenous injection of adenosine has been reported to stimulate ventilation in humans [207,228,234,248]. The adenosine receptor antagonist theophylline does not reduce ventilation in healthy volunteers [258], suggesting that endogenous adenosine does not play an important role in the regulation of the ventilation. However, theophylline crosses the blood-brain barrier, and the contrasting central and peripheral effects of adenosine may offer an explanation for the lack of effect of theophylline on ventilation [259]. Alternatively, the physiological importance of adenosine can be evaluated by inhibiting its cellular uptake. Although dipyridamole augmented the ventilatory effects of intravenously injected adenosine [260,261], it did not affect ventilation during isocapnic hypoxia when given orally [258]. In contrast, it has recently been reported that intravenously injected dipyridamole stimulates ventilation in humans [262]. Besides the variable oral availability of dipyridamole [245], nonspecific actions such as phosphodiesterase inhibition and stimulation of prostacycline release [212,213] may have interfered, possibly accounting for these contrasting results. Based on these previous observations, we hypothesized that endogenously released adenosine is able to stimulate ventilation in man. To test this hypothesis, we decided to study the effect of intravenously injected draflazine, a specific nucleoside transport inhibitor, on ventilation. Draflazine, the active (-)-enantiomer of R 75 231, has a tight binding to the nucleoside transporter [192]. Phase I studies in man reveal a long duration of action: 50% ex vivo adenosine transport inhibition at four hours after a total dose of 10 mg R 75 231 (resembling 5 mg draflazine) intravenously [254]. The advantage of using draflazine in stead of dipyridamole is that draflazine does not possess nonspecific confounding effects as does dipyridamole. Drug efficacy was monitored by measuring ex vivo nucleoside transport inhibition.

SUBJECTS AND METHODS

Subjects

After approval of the local ethics committee, six normotensive, nonsmoking, healthy, Caucasian, nonobese, male volunteers [age: 43.8 (29-53) years; height: 179.9 (172-189) cm] signed written informed consent before participation. They had no history of pulmonary disease and did not use concomitant medication.

Study design

The ventilatory effects of placebo and 1, 2, 4 or 6 mg of draflazine were studied during five sessions per volunteer, that were separated by at least one week. The administration of placebo or draflazine was randomized and double-blind, except for the highest dose (6 mg). The 6-mg dose was always given in the final session in a single-blind way for safety reasons, because at the time of these experiments this dosage had not been given before. Each experiment was done in the morning after a 24-hour abstinence from caffeine-containing products and an overnight fast of at least 10 hours. Both arms were intravenously cannulated to infuse the drug (left arm) and to collect blood (right arm). Immediately before and after the 15-minute drug infusion, blood was collected for measurement of plasma adenosine [220] and ex vivo nucleoside transport [216]. Ventilation was measured by registration of each thoracic excursion that occurred during three minutes before the start of draflazine infusion (baseline period) and immediately after the draflazine administration [207].

Measurement of ventilation

Thoracic excursions were measured using mercury-in-Silastic strain gauge plethysmography (Hokanson EC4, D.E. Hokanson, Inc.; Washington, DC). A 26-cm mercury-in-Silastic strain gauge was wrapped around the thorax at the mid sternal level. To bridge the remaining gap, both ends of the Silastic tube were attached to an unextensible band at the back of the volunteer. As the thorax expands, the length of the gauge is changed by a corresponding amount. The resulting variations in the voltage drop across the gauge will reflect changes in thoracic circumference. In a separate group of 6 healthy nonobese male volunteers [age: 28.5 (22-36) years; height: 179.9 (172-190) cm], this method was validated against simultaneous spirometric recordings (Figure 1). Although confidence intervals were considerably higher for the plethysmographic technique, an increase in tidal volume as recorded by spirometry almost always accompanied an increase in thoracic excursion for each individual.

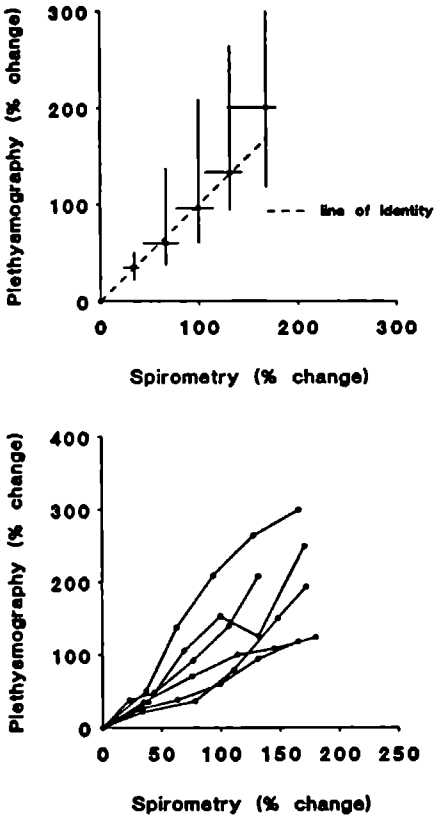

Figure 1. Six subjects were asked to breath with a tidal volume of approximately 600 (baseline), 800, 1000, 1200, 1400 and 1600 ml. They adjust their ventilation by watching a continuous registration of their tidal volume by spirometry. Changes in real tidal volume (spirometry) and thoracic excursions (strain gauge plethysmography) were expressed as percentage change from baseline. Upper panel shows the group averaged changes in thoracic excursion (plethysmography) in comparison with changes in tidal volume (spirometry) expressed as median with 95% confidence interval The lower panel shows individual changes in these two parameters

Analytical methods

Plasma adenosine concentration and ex vivo adenosine transport inhibition were measured as described in chapter 2.

Drugs and solutions

Draflazine or placebo solutions in a formulation with 5 % hydroxypropyl-β-cyclodextrine (Janssen Pharmaceutica, Beerse, Belgium) were prepared with sodium chloride (NaCl 0.9%) by a specially trained research nurse who was not otherwise involved in the practical performance of the trial.

Statistics

For each three-minute registration period, the thoracic excursions were averaged to one value. Effects of placebo or drug infusion were expressed as percentage change from baseline. When an overall analysis by Friedman two-way nonparametric analysis of variance (ANOVA) showed significant differences in responses ($P<0.05$, Chi-square test), the paired Wilcoxon signed rank test was used to find out which draflazine dosages were different from placebo. All results are expressed as median (95% confidence interval).

RESULTS

Figure 2 shows the effect of draflazine on breathing rate and tidal volume. Draflazine did not significantly affect breathing rate. Tidal volume changed by -7.2% (-36.5 to 49.1%) after placebo, and -8.7% (-35.4 to 47.8%), 38.4% (0.0 to 121.3%), 35.0% (-10.5 to 70.4%) and 114.0% (38.3 to 184.8%) after 1, 2, 4 and 6 mg of draflazine, respectively ($P<0.05$ versus placebo for 2, 4 and 6 mg of draflazine).

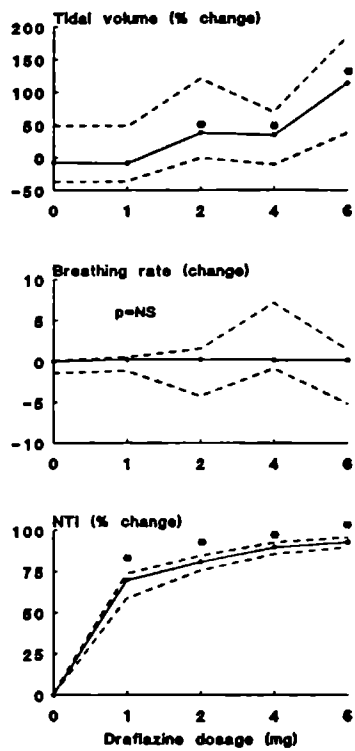

Figure 2. Effect of placebo (0 mg), 1, 2, 4 and 6 mg draflazine on plethysmographically determined tidal volume (upper panel), breathing rate (middle panel) and ex-vivo nucleoside transport inhibition (NTI, lower panel) as determined by standardized incubation of erythrocytes with adenosine. Results are expressed as median with 95% confidence interval. *, Statistically significant differences from placebo (n=6).

Nucleoside transport was inhibited by 0% (0-1%) after placebo, and 70% (59 to 74%), 81% (76 to 85%), 90% (86 to 93%) and 93% (90 to 96%) after the four increasing draflazine dosages, respectively ($P<0.05$ for each draflazine dosage versus placebo). Plasma adenosine concentrations did not significantly change: 0.0 μmol/L (-0.06 to 0.03μmol/L) after placebo and 0.0 μmol/L (-0.02 to 0.0 μmol/L), 0.02 μmol/L (0.0 to 0.11 μmol/L), 0.02 μmol/L (0.0 to 0.11 μmol/L) and 0.0 μmol/L (-0.06 to 0.06 μmol/L) after 1, 2, 4 and 6 mg of draflazine (Friedman: $P=0.12$).

DISCUSSION

These results show that ventilation is increased by high grade nucleoside transport inhibition with draflazine. Until now, nucleoside transport inhibition has been the only known action of this drug [192,214,263,264].

Recently, we have shown that the hemodynamic and neurohumoral effects of high grade nucleoside transport inhibition with draflazine in humans can be antagonized with the adenosine receptor antagonist caffeine [265]. This supports the assumption that the effects of draflazine are mediated by endogenous adenosine. Additionally, draflazine is able to potentiate the vasodilator effect of intra-arterially-infused adenosine in humans [266] confirming the in vivo action of draflazine as a nucleoside transport inhibitor. Therefore, the present results suggest that endogenously released adenosine can play a role in the regulation of the ventilation. Plasma adenosine concentration was not significantly affected by draflazine. This finding contrasts with the observations of others [244,251] who reported a doubling of adenosine concentrations after dipyridamole administration [244,251]. However, in these dipyridamole studies adenosine formation, uptake, and breakdown after blood sampling were not optimally antagonized. Therefore, in these studies baseline plasma adenosine concentrations may have been underestimated, and the administered dipyridamole may have affected adenosine uptake by erythrocytes after blood sampling. The present study does not rule out a small draflazine-induced increase in plasma adenosine levels, because intra-subject variability of the adenosine detection method is high. Additionally, we can not exclude a detectable increase in adenosine concentration during more sustained nucleoside transport inhibition. Alternatively, nucleoside transport inhibition may transform the endothelium to a functional barrier preventing adenosine from leaving the interstitial space and resulting in an increase of adenosine in the interstitial compartment only.

In conclusion, draflazine-induced high grade nucleoside transport inhibition stimulates ventilation in humans. We suggest that this effect is mediated by an increased extracellular adenosine concentration in the interstitial compartment only.

CHAPTER 4

Presynaptic inhibition of norepinephrine release from sympathetic nerve endings by endogenous adenosine

G.A. Rongen, J.W.M. Lenders, G. Lambrou, J.J. Willemsen, H. Van Belle, Th. Thien and P. Smits

Hypertension, provisionally accepted

ABSTRACT

Experimental data suggest that ATP (adenosine-5'-triphosphate) is coreleased with norepinephrine from sympathetic nerve endings. Breakdown of ATP to adenosine in the synaptic cleft may give rise to inhibition of norepinephrine release by stimulation of presynaptic adenosine receptors. We tested this mechanism of action in humans by using a specific nucleoside transport inhibitor (draflazine) as a pharmacological tool to allow accumulation of endogenous adenosine in the synaptic cleft. In a dose-finding study ($n=10$), we first assessed the effect of local draflazine infusion into the brachial artery for five increasing dose steps: 100 to 2000 ng/100 ml forearm per min. Forearm blood flow and vascular resistance were registered at both sides by venous occlusion plethysmography. Ex vivo levels of local nucleoside transport inhibition as determined from sequential blood samples showed a dose-dependent increase which resulted in a decrease in forearm vascular resistance in the infused arm only for the three highest draflazine dosages ($P<0.05$). Since the second dose of draflazine (250 ng/100 ml forearm per min) induced a considerable local nucleoside transport inhibition ($\approx 40\%$), without affecting vascular tone and without a relevant effect on the level of nucleoside transport inhibition at the contralateral side ($\approx 7\%$), this particular dose was used for the main study.

In the main study, the effect of intra-arterial draflazine on sympathetic-mediated norepinephrine spillover during lower body negative pressure (LBNP, -25 mmHg, 15 minutes) was studied by use of the ^{3}H-norepinephrine (NE) isotope dilution technique ($n=25$). During placebo, LBNP induced a significant increase in the total body NE spillover ($29\pm9\%$) as well as in the forearm NE appearance rate ($45\pm13\%$), accompanied by an increase in forearm vascular resistance ($101\pm16\%$) and heart rate ($5\pm2\%$). Draflazine infusion itself did not affect total body NE spillover or hemodynamics, but appeared to increase basal forearm NE appearance rate by $27\pm7\%$, possibly as result of afferent nerve stimulation. During draflazine, LBNP-induced increase in forearm NE appearance rate was significantly reduced as compared with placebo (16 ± 11 versus $45\pm13\%$, $P<0.05$), whereas the total body NE spillover and the systemic hemodynamic parameters responded similarly as during placebo. These observations suggest that during sympathetic stimulation, accumulation of endogenous adenosine in the synaptic cleft can inhibit the release of norepinephrine from sympathetic nerve endings.

INTRODUCTION

In vitro observations have indicated that adenosine-5'-triphosphate (ATP) is coreleased with norepinephrine from sympathetic nerve endings [15]. Extracellular ATP is rapidly degraded by ectophosphatases to adenosine-5'-monophosphate which is subse-

quently dephosphorylated by 5'-nucleotidase to adenosine [82-84]. Then, endogenous adenosine is rapidly cleared from the interstitium, at least in part by cellular uptake through specific nucleoside transporters [254]. In a variety of in-vitro models, adenosine has been shown to inhibit norepinephrine (NE) release from sympathetic nerve endings [222,267,268]. Adenosine A_1-receptors are thought to be involved in this inhibitory effect [267]. In an elegant in vivo study in humans, Taddei et al. have provided indirect evidence for an adenosine-mediated reduction in NE release from sympathetic nerve endings in humans, by demonstrating an augmented forearm vasoconstrictor response to sympathetic stimulation during administration of the adenosine receptor antagonist theophylline [269]. However, NE release itself was not measured in these experiments and, in theory, their observations could have been explained by alterations or interactions at the postsynaptic level [50,223]. Apart from the putative inhibitory action of adenosine at the level of sympathetic nerve endings, it has to be emphasized that adenosine can increase sympathetic nervous system activity in humans by stimulating afferent nerve endings in the vessel wall as well as in the carotid body [207,270,271].

This study was performed to investigate the effect of endogenous adenosine on NE release from sympathetic nerve endings during stimulation of the sympathetic nervous system. To this end, a specific nucleoside transport inhibitor (draflazine) was used as a pharmacological tool to allow accumulation of endogenous adenosine in the synaptic cleft during sympathetic stimulation [254,271]. Appropriate pilot studies were performed to determine the dose of draflazine at which an optimal grade of nucleoside transport inhibition was induced. The current observations support the hypothesis that endogenous adenosine inhibits NE release at the sympathetic nerve ending.

SUBJECTS AND METHODS

After approval by the local ethics committee, a total number of 35 normotensive nonsmoking healthy male volunteers signed written informed consent statements before participation in the study. Their demographic characteristics are summarized in table 1. Ten of these subjects participated in the draflazine dose-finding study, and 25 participated in the main study. They had no history of hypertension, diabetes mellitus or drug allergy and did not use concomitant medication. In all volunteers, a physical examination and a twelve lead electrocardiography was performed to exclude cardiovascular, pulmonary or neurologic disease. The subjects were asked to abstain from caffeine-containing products for at least 24 hours since low concentrations of caffeine have shown to attenuate the effects of adenosine in the forearm vascular bed [221]. All experiments were performed in the morning hours after a ten-hour fasting period, in a temperature controlled room (24-25 °C) and with the subjects in supine position. Since all drug- and volume infusion

rates were calculated per deciliter of forearm tissue, the forearm volume was measured for each individual by water displacement.

TABLE 1: Baseline characteristics of the study groups (mean ± SD)

	Dose-finding study	Main study
N	10	25
Age (years)	29.4 ± 11.9	34.6 ± 16.8
Weight (kg)	73.3 ± 11.5	76.0 ± 10.4
Height (cm)	180.5 ± 9.4	181.4 ± 6.6
Body mass index (kg/m^2)	22.4 ± 2.0	23.0 ± 2.5
Systolic blood pressure (mmHg)#	128 ± 8.0	126 ± 8.9
Diastolic blood pressure (mmHg)#	74.0 ± 9.6	72.5 ± 8.9
Heart rate (bpm)#	70.0 ± 12.1	66.4 ± 9.7
Forearm vascular resistance (AU)	55 ± 11	42 ± 15.6
Forearm volume (L)	1.01 ± 0.16	1.11 ± 0.13

Blood pressure was measured in supine position with the Riva-Rocci technique using Korotkoff I and V for systolic and diastolic blood pressure respectively. Heart rate was measured by pulse frequency counting (radial artery).

After local anesthesia (xylocaine 2%), the left brachial artery was cannulated with a 20-gauge catheter (Angiocath, Deseret Medical, Becton Dickinson Sandy, UT, USA) for both intra-arterial drug infusion (automatic syringe infusion pump, type STC-521, Terumo, Tokyo, Japan) and blood pressure recording (Hewlett Packard GmbH, Böblingen, Germany). In the same arm, a deep antecubital vein was cannulated retrogradely for venous blood sampling. In the opposite arm an antecubital vein was cannulated for blood sampling (dose-finding study) or infusion of tritiated norepinephrine (main study). Forearm blood flow (FBF) was recorded in both forearms by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, D.E. Hokanson, Washington DC, USA). The upper arm collecting cuffs were simultaneously inflated using a rapid cuff inflator (Hokanson E-20). At least one minute before FBF measurements were made, the hand circulation was occluded by inflation of wrist cuffs to 200 mmHg. Forearm blood flow was recorded three times a minute. All experiments were started at least 30 minutes after intra-arterial cannulation. In all experiments total intra-arterial infusion rate was kept constant at 50 μl/100 ml

forearm per min. Before the start of each experiment, venous blood was collected for the measurement of plasma caffeine concentration.

Draflazine dose-finding study

The aim of this dose-finding study was to determine the intra-arterial dose of draflazine which resulted in a sufficient level of nucleoside transport inhibition in the forearm vascular bed without causing systemic effects and without inducing regional vasodilation. We reasoned that prevention of draflazine-induced vasodilation was important because baseline vascular tone is an important determinant of the magnitude of the response to vasomotor stimuli [272].

In 10 subjects, the effect of five increasing dosages of draflazine, infused into the brachial artery, on forearm vascular tone and on local and systemic ex vivo nucleoside transport inhibition was studied. The experiment started with the measurement of baseline FBF, mean arterial pressure and forearm vascular resistance (FVR) during the last four minutes of a five minute saline infusion. The effect of five increasing dosages of draflazine (100, 250, 500, 1000 and 2000 ng/100 ml forearm per min) on FBF and FVR were compared with saline. Each draflazine dosage was infused for 20 minutes. At the end of saline infusion and each draflazine infusion, venous blood was sampled from both arms for ex vivo measurements of nucleoside transport inhibition (see 'analytical methods'). Prolonged occlusion of the hand circulation can cause discomfort. Therefore, wrist cuffs were only inflated during the last 10 minutes of draflazine infusion, and FBF was measured during the last 8 minutes of each draflazine dose.

Main study

In 25 subjects, the lower body was sealed in an airtight Plexiglas™ box. The applied lower body negative pressure (LBNP) was recorded by a manometer connected to the inside of the box. In each subject, a 15-minute LBNP at -25 mmHg was applied twice. The second LBNP was performed 50 minutes after the first one. In a pilot study we have shown that the mean hemodynamic response to LBNP was identical when repeated within one day, with a fall in FBF of 1.3 ± 1.0 and 1.1 ± 0.8 ml/100 ml forearm per min for the first and second LBNP respectively ($n=15$, unpublished data).

Intra-arterial infusion of placebo (NaCl 0.9%) started 10 minutes before each LBNP. The first LBNP was performed during ongoing intra-arterial placebo infusion. In contrast, five minutes before the second LBNP, intra-arterial placebo was switched to draflazine (250 ng/100 ml forearm per min) which was infused until the end of the LBNP. This draflazine dose was based on the results of the dose-finding study. The wrist cuffs were inflated during the ten minutes prior to each LBNP and during the last 10 minutes of LBNP. Forearm blood flow was measured during the placebo infusions,

during the draflazine infusion and during the last 8 minutes of each LBNP. Venous and arterial blood samples were obtained from the infused arm immediately before and at the end of each LBNP for determination of NE kinetics. Additionally, arterial and venous blood was sampled at the end of the second placebo infusion to detect a possible effect of draflazine on baseline NE kinetics.

Levo-[ring-2,5,6-^{3}H]-norepinephrine (specific activity 30-60 Ci/mmol) was infused intravenously in order to assess plasma NE kinetics. The radiotracer was administered intravenously at a constant rate of 1.0 μCi/min, and this infusion was started 20 minutes before the onset of each LBNP. Samples of the infusate were taken at the end of the infusion in order to determine the exact infusion rate and the original activity of ^{3}H-NE in 1.0 ml plasma sample.

Analytical methods

Samples for determination of plasma caffeine concentration were analyzed with a reversed-phase HPLC method (limit of detection: 0.2 μg/ml), as described previously [218].

Blood samples for determination of plasma norepinephrine were collected in prechilled tubes containing glutathione (0.2 M) and EGTA (0.25 M). The tubes were centrifuged at 4 °C and the plasma was separated and stored at -20 °C. Measurements for concentrations of NE and ^{3}H-NE of all plasma samples and infusates occurred within two months after sampling, using high performance liquid chromatography (HPLC) with fluorometric detection after precolumn derivatization with the fluorescent agent 1,2-diphenylethylenediamine. A Gilson fraction collector (Model 201-202), which was connected to an automatic sample injector (Wisp 710B), was used for collecting [^{3}H]-NE into scintillation vials, according to the retention time of the NE standard solution.

Ex vivo nucleoside transport inhibition was measured by standardized incubation of erythrocytes with adenosine. Four ml of blood was drawn into a vial containing 1.0 ml acid/citrate/dextrose (85 mM tri-sodium citrate, 65 mM citric acid and 20 g/L glucose) and further handled as described before [271]. After incubation, the concentration of adenosine, inosine and hypoxanthine was determined in the supernatant with a chemoluminiscence technique. The percentage inhibition of nucleoside transport was calculated as: $(A_x-A_0) \times 100 / (1-A_0)$ in which A_0 represents the adenosine concentration as proportion of the sum of the concentration of adenosine, inosine and hypoxanthine as determined in the sample collected just before the drug infusion and A_x represents this proportion as determined in the sample collected after the start of the drug infusion.

Drugs and solutions

Tritiated norepinephrine was obtained from Du Pont New England Nuclear ('s-Hertogenbosch, The Netherlands). The radionuclide was sterilized using a 0.22 μm filter and diluted in 0.9% NaCl containing acetic (0.2 M) and ascorbic (1 mg/ml) acid. Aliquots of approximately 70 μCi/ml of [^{3}H]-NE were stored at -80 °C until use. Sterilization, dilution, and aliquoting were carried out under nitrogen. Just before use, an aliquot was diluted in 0.9% NaCl.

Sterile draflazine solutions were freshly prepared from 10 ml vials containing 5 mg draflazine diluted in NaCl 0.9% (Janssen Pharmaceutica Inc., Beerse, Belgium). The specificity of this drug as a nucleoside transport inhibitor, and the ability of this drug to increase endogenous adenosine levels in humans has extensively been described before [254,271].

Data analysis

Mean arterial blood pressure was measured continuously during each recording of FBF and averaged per FBF measurement. Forearm vascular resistance (FVR) was calculated as the quotient of the simultaneously registered mean arterial blood pressure and the FBF, and was expressed as arbitrary units (AU). For the draflazine dose-finding, the hemodynamic registrations obtained during placebo infusion and the last 6 minutes of each draflazine infusion were each averaged to one value. Draflazine-induced effects were expressed both as absolute and percentage changes from the preceding placebo infusion. For the main study, the hemodynamic parameters were averaged to one value for the following consecutive periods: Placebo-1 / Placebo+LBNP / Placebo-2 / Draflazine / Draflazine+LBNP. The data obtained during the last 6 minutes of each LBNP were used.

Arterial and venous concentrations of [^{3}H]-NE and NE were used for calculations of the various parameters of norepinephrine kinetics, as previously described [273-275]. Total body NE spillover, the estimated rate of appearance of endogenous NE in arterial plasma, was calculated from arterial plasma NE concentration (NE_a), the arterial steady-state plasma concentration of [^{3}H]-NE ([^{3}H]-NE_a) and the infusion rate of [^{3}H]-NE, according to the equation:

Total body NE spillover (ng/min) = NE_a (ng/L) x {Infusion rate (dpm·min^{-1})} / [^{3}H]-NE_a (dpm/L)

The local forearm NE appearance rate was estimated from:

Forearm NE appearance rate (pg/100 ml forearm per min) = {(FPF x NE_a x f_{NE}) + [FPF x (NE_v - NE_a)]} / (1-f_{NE})

where f_{NE} is the fractional extraction of the tracer across the forearm, calculated as ([^{3}H]NE_a - [^{3}H]NE_v) / [^{3}H]NE_a, and [^{3}H]NE_v and [^{3}H]NE_a is respectively the venous and arterial plasma concentration of [^{3}H]-NE, and FPF is the forearm plasma flow (ml/100 ml forearm per min), calculated from the forearm blood flow and the hematocrit as FBF x (1-Ht).

The changes in the hemodynamic and kinetic parameters induced by LBNP during placebo were compared to those induced by LBNP during draflazine.

All results are presented as mean±SE unless indicated otherwise. To avoid multiple comparison, within subject effects were assessed by ANOVA for repeated measurements first and further analyzed with the paired Student t-test if appropriate (dose-finding study). The results of the main study were analyzed by paired Student t-tests. To minimize multiple comparisons, LBNP-induced changes from baseline were not tested statistically. For the same reason, only FVR, forearm norepinephrine appearance rate and total body spillover were included in the statistical analysis. Differences were considered to be statistically significant at a P-value < 0.05 (two-sided). NS means not significant.

RESULTS

Plasma caffeine levels were determined to check the compliance with respect to the caffeine abstinence. In all volunteers, plasma caffeine concentration was below the limit of detection indicating an excellent compliance to the caffeine free diet. No subjective side effects occurred during the intra-arterial infusion of draflazine.

Dose finding study

Baseline FBF was 2.1±0.3 ml/100 ml forearm per min in the cannulated arm and increased to 2.2±0.4, 2.4±0.3, 3.0±0.4, 3.7±0.5 and 4.2±0.5 ml/100 ml forearm per min at the end of the five increasing draflazine dosages respectively. Expressed as percent change from baseline, FBF was increased by 4.9±7.4, 18.0±6.1, 51.1±13.6, 94.1±20.8, and 123.2±24.5 % at the end of the five increasing draflazine dosages respectively ($n=10$; $P<0.05$ for the four highest dosages). Forearm blood flow in the contralateral arm was not significantly affected during any of the infusions. Baseline FVR was 54.6±10.7 AU in the cannulated arm. Draflazine decreased FVR by 5.3±10.1, 5.7±5.7, 20.4±6.7, 34.8±7.1 and 41.7±6.7 % for 100, 250, 500, 1000 and 2000 ng

draflazine/100 ml forearm per min respectively (n=10; P<0.05 for the three highest dosages). The FVR in the contralateral arm was not significantly affected (figure 1).
In the infused arm ex vivo nucleoside transport inhibition was 7.0±2.4, 42.4±8.8, 69.7±7.7, 80.8±4.8 and 87.1±2.5 % during the five increasing dosages respectively (n=10, P<0.05 for each dosage versus baseline; see figure 1). Likewise, in the non-experimental arm ex vivo NTI increased dose-dependently by 3.9±1.6, 6.8±3.4, 11.9±3.7, 17.8±3.5 and 51.2±3.0 % respectively (n=10, P<0.05 for each dosage versus baseline; see figure 1).

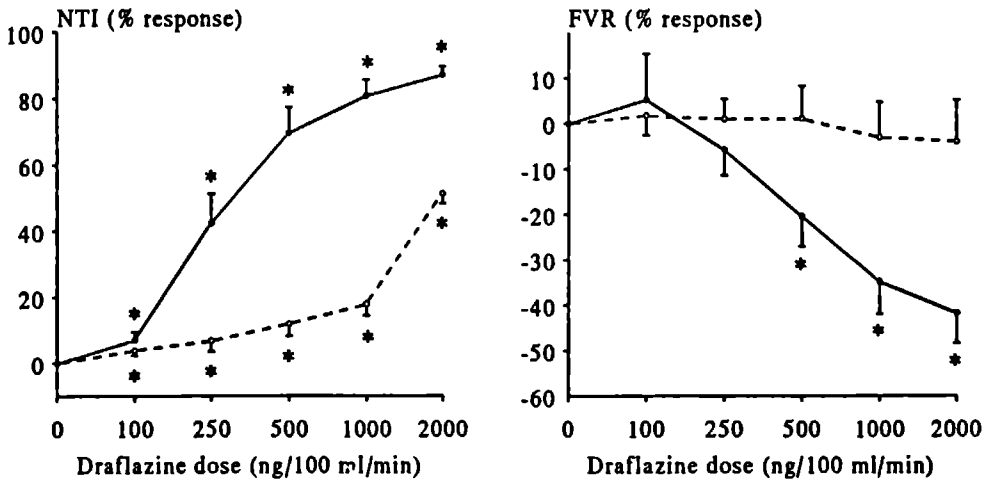

Figure 1. Percentage changes (mean±SE) in nucleoside transport inhibition (left panel), and in the forearm vascular resistance (right panel) as induced by incremental dosages of draflazine, infused into the brachial artery. The solid line represents the data at the side of draflazine infusion, whereas the dashed line shows the data from the contralateral side.
*, P<0.05 versus baseline.

Based on these results, the draflazine dose of 250 ng/100 ml forearm per min was chosen for the main study because this dose induced a sufficient nucleoside transport inhibition in the forearm vascular bed (≈40%) without a relevant increase in this parameter at the contralateral site (≈7%), and without causing a significant vasodilator response at the side of infusion.

Main study

Effect of sympathetic stimulation by LBNP

Table 2 shows the hemodynamic and neurohumoral effects of LBNP with and without concomitant intra-arterial draflazine infusion. During placebo, LBNP induced a fall in FBF from 2.6±0.3 to 1.5±0.2 ml/100 ml forearm per min at the experimental side, and from 2.5±0.3 to 1.5±0.2 ml/100 ml forearm per min at the contralateral side. Mean arterial blood pressure did not change, but heart rate increased by 5.2±1.6 % as a result of the first and second LBNP respectively. Total body NE spillover increased by 28.9±8.6%, whereas the forearm NE appearance rate increased by 45.3±13.3%. Fifty minutes after stopping the first LBNP, all parameters had returned towards baseline level (placebo-1).

Effects of draflazine infusion

In accordance with the results of the dose-finding study, intra-arterial infusion of draflazine at a rate of 250 ng/100 ml forearm per min did hardly affect vascular tone (table 2). Although the total body NE spillover was not altered, draflazine caused an increase in forearm NE appearance rate of 26.6±7.2 % ($P<0.01$ versus baseline).

Effects of draflazine on the response to sympathetic stimulation by LBNP

Application of LBNP during draflazine exerted a hemodynamic response which did not differ from that to the first LBNP, with no change in mean arterial blood pressure and a significant increase in heart rate of 7.3±2.0 %. During draflazine infusion, LBNP induced a fall in forearm blood flow from 3.2±0.4 to 2.1±0.3 ml/100 ml forearm per min at the experimental side and from 2.8±0.3 to 1.9±0.2 ml/100 ml forearm per min at the contralateral side. Forearm vascular resistance showed a bilateral fall without difference between the experimental and contralateral arm, although response was lower as compared with the first LBNP, both on the experimental as well as on the contralateral side.

Figure 2 shows the LBNP-induced percent changes from baseline for the data on norepinephrine. Again, total body NE spillover increased, on average by 45.6±9.3% as opposed to 28.9±8.6% during the first LBNP (first versus second LBNP: $P=0.2$). In contrast, the response of the forearm NE appearance rate to LBNP was almost abolished during draflazine (15.9±10.5%), this response being significantly lower as compared with that to the first LBNP (15.9±10.5 versus 45.3±13.3%, $P<0.05$). In 16 subjects, the response to LBNP was reduced during draflazine infusion as compared with that to the first LBNP.

TABLE 2: Hemodynamic and neurohumoral effects of LBNP with and without concomitant intra-arterial draflazine infusion; mean±SE

	Placebo-1	LBNP-1	Placebo-2	Draflazine	LBNP-2
FVR infused arm (AU)	42±3	87±13 (101±16 %)	40±3	38±3 (-3±2 %)	65±8 (66±11 %)@
FVR control arm (AU)	44±4	92±15 (103±19 %)	49±7	46±5 (-4±3 %)	78±11 (69±12 %)@
Total body NE spillover (ng/min)	373±32	449±34 (29±9 %)	387±42	384±42 (1±3 %)	513±49 (46±9 %)
Forearm norepinephrine Appearance Rate (pg/100 ml/min)	570±75	846±157 (45±13 %)	685±104	815±115 (27±7 %)#	893±133 (16±11 %)@

Percentage changes from baseline are shown between brackets. #, Statistically significant percentage changes from baseline (only tested for the effect of draflazine); @, Statistically significant differences in percentage response between first and second LBNP.

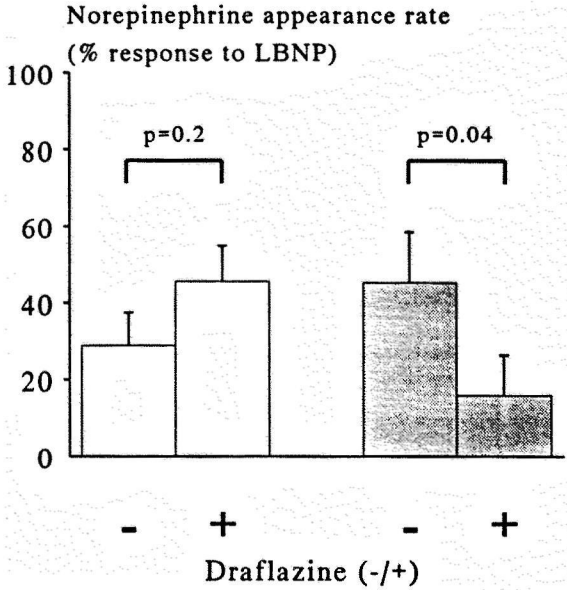

Figure 2. The percentage increase (mean ±SE) in the total body norepinephrine (NE) spillover (left panel) and the forearm NE appearance rate resulting from sympathetic stimulation by application of lower body negative pressure (LBNP), both during intra-arterial infusion of placebo or draflazine (250 ng/-min/dl).

DISCUSSION

The main observation of this study is that infusion of the specific nucleoside transport inhibitor draflazine into the forearm skeletal muscle vascular bed resulted in a blunted response of the local NE appearance rate during sympathetic stimulation. This attenuation only occurred at the side of draflazine infusion, as simultaneous measurements of the total body NE spillover convincingly showed that the overall response of the sympathetic nervous system to LBNP during draflazine (≈45%) was certainly not lower than that to LBNP during placebo (≈30%). The absence of an effect on total body NE spillover completely agrees with the almost negligible level of systemic nucleoside transport inhibition, which was far below the threshold for interference with the sympathetic nervous system [271]. The specificity of draflazine has been tested in various in vitro systems [214]}. Intravenous infusion of draflazine in conscious humans elicits a hemodynamic, neurohumoral and ventilatory response that closely resembles the effects of intravenous infusion of adenosine [206,207,230,248,271,276]. This occurred at higher systemic draflazine blood concentrations than currently used locally, as assessed by ex-vivo nucleoside transport inhibition. By use of caffeine as an adenosine receptor antagonist, we were able to show in a previous study that the circulatory effects of nucleoside transport inhibition by draflazine completely resulted from adenosine receptor stimulation [271]. Consequently, our current data provide evidence for an adenosine receptor-mediated reduction in norepinephrine release from efferent nerve endings during sympa-

thetic stimulation. This reduction in the observed LBNP-induced norepinephrine release from the forearm vascular bed was expected to be accompanied by a parallel reduction in the local vasoconstrictor response to sympathetic stimulation. However, we did not find any evidence for an impaired forearm vasoconstrictor response at the side of draflazine infusion (table 2). This does not necessarily argue against our interpretation of the results, because the forearm vasoconstrictor response also depends on a variety of other factors, including the postsynaptic interaction between adenosine and norepinephrine [50,223], eventual alterations in receptor populations, and changes in the release of cotransmitters like ATP and/or neuropeptide Y [15,277,278].

Our interpretations are mainly based on assessment of the spillover of norepinephrine according to the isotope dilution technique as described by Esler et al. [274]. The tracer ^{3}H-NE is used for determining the amount of norepinephrine that is extracted during passage across a vascular bed, in this case that of the forearm skeletal muscles. However, forearm NE spillover as calculated from these parameters strongly depends on the forearm blood flow [273]. Recently, Chang et al. proposed a correction for this calculated forearm NE spillover by taking into account the amount of released norepinephrine that is also extracted from the forearm vascular bed [275]. This so-called forearm NE appearance rate should be a more accurate measurement of the regional release from norepinephrine, especially for our study because of the induced changes in forearm blood flow as a result of sympathetic stimulation.

In our dose-finding study, intra-arterial infusion of draflazine resulted in a significant dose-dependent inhibition of ex vivo nucleoside transport that was considerably higher in the infused arm as compared with the non-infused contralateral arm. Draflazine dosages of 500 ng/100 ml forearm per min or more elicited vasodilation in the infused arm that was not observed in the control arm indicating a local vasodilator action of draflazine in humans. As demonstrated previously, the effects of draflazine are mediated by adenosine accumulation in the extracellular space and subsequent adenosine receptor stimulation [271]. Therefore, the vasodilator action of draflazine indicates extracellular adenosine accumulation in the non-ischemic human forearm vascular bed. Possible sources of this endogenous adenosine include endothelium, vascular smooth muscle cells and sympathetic nerve endings where adenosine is formed extracellularly by enzymatic degradation of the neurotransmitter adenosine-5'-triphosphate (ATP) [85,279-281].

Unexpectedly, the main study showed that the baseline forearm NE appearance rate increased significantly during draflazine infusion without changes in total body NE spillover. Actually, the dose of 250 ng/100 ml forearm per min was chosen because it was expected to inhibit nucleoside transport inhibition to a sufficient extent without affecting baseline hemodynamic or humoral parameters. Of course, we realized that increasing endogenous adenosine levels may stimulate sympathetic nervous system activity

by stimulation of afferent nerves [207,270,271]. Recently, this has been shown in particular for the forearm skeletal muscle vascular bed [229]. However, dose response data on draflazine have suggested that this afferent stimulation should not occur at the level of nucleoside transport inhibition as reached in our main study [271]. Nonetheless, the present finding indicates that the baseline norepinephrine release from sympathetic nerve endings in the forearm can be slightly stimulated by low dosages of intra-arterially infused draflazine. We have no clear explanation for this observation. If stimulation of adenosine sensitive afferent nerve endings in the forearm vascular bed plays a role in this observation, we must assume that the response of the efferent sympathetic nervous system was a differential side selective response because total body norepinephrine spillover and systemic hemodynamics were unaffected. Along these lines, the contrasting effects of draflazine on baseline versus stimulated forearm NE appearance rate may be mediated by interaction of endogenous adenosine with the sympathetic nervous system at two distinct levels. In figure 3 this hypothesis is summarized in a schedule.

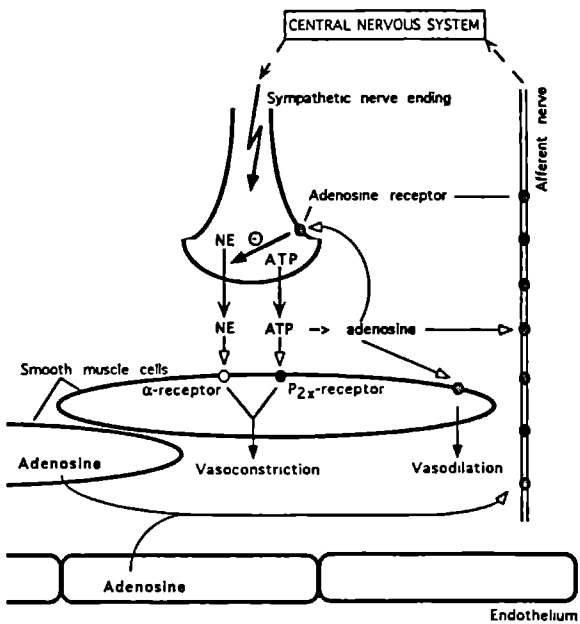

Figure 3. Outline of the potential interactions between endogenous adenosine and the sympathetic nervous system.

First, accumulation of continuously formed interstitial adenosine may increase sympathetic nervous system activity as a result of stimulation of afferent nerve endings in

the forearm vascular bed. Second, during sympathetic stimulation draflazine allows accumulation of endogenous adenosine, as a breakdown product of ATP coreleased in the synaptic cleft, that is able to reduce norepinephrine release from sympathetic nerve endings locally by presynaptic inhibition of neurotransmitter release. Apparently, the first effect of adenosine dominates during baseline conditions, when baseline sympathetic nervous system activity is low. However, the importance of the second interaction increases during sympathetic stimulation by LBNP as adenosine builds up in the synaptic cleft. It has to be emphasized that this dual action of adenosine has already extensively been described for exogenous adenosine [225]. The current results extrapolate this concept to the effects of endogenous adenosine.

CHAPTER 5

N^G-Monomethyl-L-Arginine reduces the forearm vasodilator response to acetylcholine but not to methacholine in humans

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ABSTRACT

We compared the contribution of nitric oxide (NO) in methacholine (MCh)- and acetylcholine (ACh)-induced vasodilation using the NO-synthase inhibitor N^G-monomethyl-L-arginine acetate (L-NMMA-acetate) in two groups (A and B) of 6 healthy male volunteers. The left brachial artery was cannulated for drug infusion and recording of mean arterial pressure (MAP). Forearm blood flow (FBF) was measured on both sides by venous occlusion mercury-in-silastic strain gauge plethysmography. All measurements were performed with occluded hand circulation. Forearm vasodilator response to three increasing dosages of MCh (0.03, 0.3 and 1 μg/100 ml forearm/min; group A) or ACh (0.5, 2 and 8 μg/100 ml forearm/min; group B) was studied first. Forty-five minutes later, these infusions were repeated (after and during local administration of L-NMMA). L-NMMA-acetate infusion alone increased basal forearm vascular resistance (FVR, mean$\pm$SE) by 86.2$\pm$14.5 % and 99.5$\pm$27.4 % in group A and B, respectively ($P<0.05$) without significant FVR changes in the control arm. MCh-induced vasodilation was not attenuated by concomitant L-NMMA-acetate infusion. In contrast, L-NMMA-acetate significantly reduced the averaged percentage decrease in FVR during infusion of ACh from 55.7$\pm$9.1 % to 35.4$\pm$11.8 % ($P<0.05$).

L-NMMA-acetate increased basal vascular tone and reduced the vasodilator response to ACh. MCh induced vasodilation to a similar degree as compared to that obtained with ACh. Nevertheless, MCh-induced vasodilation could not be attenuated by L-NMMA suggesting that NO contributes differentially to MCh- and ACh-induced vasodilation in humans.

INTRODUCTION

The endothelium has important vascular tone-regulating properties mediated by endothelium-derived relaxing and contracting factors (EDRF, EDCF) [73,282-284]. One of these is nitric oxide (NO), which is synthesized from L-arginine by nitric oxide (NO)-synthase [32,285]. Nitric oxide has important vasorelaxing properties mediated by a soluble guanylate cyclase in vascular smooth muscle cell [32]. Both in vitro and in vivo studies have demonstrated that the effect of many endothelium-dependent vasodilators is mediated by NO [36,103,146,286]. Vallance and colleagues were first to show that infusion of N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO-synthase, reduced basal blood flow and attenuated the response to the endothelium-dependent vasodilator acetylcholine (ACh) in the human forearm vascular bed [36]. These findings implicated that L-NMMA could be an important tool to investigate the role of NO in the human forearm in vivo. Methacholine (MCh), a relatively stable ACh analogue

[287,288], is often used as a substitute for ACh in human studies [196,197,289] but no data are available concerning the ability to reduce MCh-induced vasodilation by L-NMMA in vivo Recently, Chowienczyk and co-workers have proposed that MCh- and ACh-induced vasodilation in the human forearm is mediated by different mechanisms [290]. This hypothesis was based on the observed divergent effect of hypercholesterolemia on ACh- and MCh-induced vasodilation, but some methodological problems may have biased the observations [291]. Therefore, we compared the contribution of NO in MCh- and ACh-induced vasodilation, using the commercially available L-NMMA-acetate.

SUBJECTS AND METHODS

After approval was obtained from the local ethics committee, two groups of six normotensive healthy male volunteers (group A and B) signed written informed consent statements before participating in the study Both groups were comparable for age (18-43 years), weight (62 8-90 1 kg), height (172-202 cm), systolic blood pressure (106-130 mmHg), and diastolic blood pressure (50-79 mmHg). Before the start of the study, the subjects were asked to abstain from caffeine-containing products for at least 36 hours and to refrain from smoking for 12 hours. Furthermore, subjects were asked to discontinue any food intake two hours before the experiment All tests were performed with the subject in supine position, starting at 2 00 p m After local anaesthesia (xylocaine, 2%) was induced, the left brachial artery was cannulated with a 20-gauge catheter (Angiocath, Deseret Medical, Inc., Becton Dickinson, Sandy, UT, U S A) for both intra-arterial drug infusion (automatic syringe infusion pump, type STC-521, Terumo, Tokyo, Japan) and blood pressure recording (Hewlett Packard GmbH, Boblingen, Germany). Forearm blood flow (FBF) was registered on both forearms by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, D E. Hokanson, Washington, D.C., U S A). The upper arm collecting cuffs were simultaneously inflated using a rapid cuff inflator (Hokanson E-20) At least one minute before FBF measurements were made, hand circulation was occluded by inflation of the wrist cuffs to 200 mmHg. Forearm blood flow was recorded three times a minute All experiments were started at least 30 minutes after intra-arterial cannulation.

The experiment began with the measurements of baseline FBF during placebo infusion. Figure 1 shows the course of FBF and the schedule of the several drug infusions in group A. The effect of three increasing dosages MCh (0.03-0.3-1.0 μg/100 ml forearm per min) were compared with placebo (NaCl 0.9%) Because prolonged occlusion of the hand circulation can cause discomfort with subsequent effects on blood pressure and heart rate, a 10-minute rest was allowed between the first and second MCh infusion. Forty-five minutes after the last MCh infusion the vasoactive effect of L-NMMA-acetate infusion

(0.1 mg/100 ml forearm per min) was compared with placebo infusion. Subsequently, the lowest MCh dose was infused (0.03 mg/100 ml forearm per min) together with infusion of L-NMMA-acetate (0.05 mg/100 ml forearm per min). Ten minutes thereafter, L-NMMA-acetate was infused again (0.1 mg/100 ml forearm per min), immediately followed by the middle and the highest MCh dosages (0.3-1.0 μg/100 ml forearm per min) together with infusion of L-NMMA-acetate (0.05 mg/100 ml forearm per min). During all procedures, total infusion rate was kept constant at a rate of 100 μl/100 ml forearm per min. Placebo and each drug dosage were infused during 5 minutes. In group B, ACh (0.5, 2 and 8 μg/100 ml forearm per min) was substituted for MCh. Otherwise the same protocol was performed.

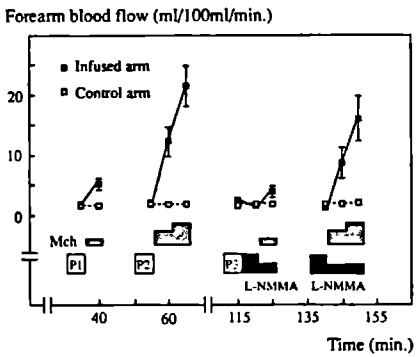

Figure 1. Forearm blood flow (ml/100 ml/min) during increasing dosages of methacholine (0.03-0.3-1 μg/100 ml forearm/min) with and without L-NMMA-acetate (mean±SE). P1, P2 and P3 represent first, second and third placebo infusion respectively.
Mch: methacholine

Drugs and solutions

L-NMMA-acetate, acetylcholine chloride (Sigma Chemical Company, St. Louis, USA) and methacholine chloride (Aldrich Chemical Company, Inc., Bornem, The Netherlands) were reconstituted on the morning of the study day from a sterile lyophilized powder, passed through a 0.2 μm millipore filter, and diluted in 0.9% NaCl.

Statistical analysis

Mean arterial pressure was measured continuously during each recording of FBF and averaged per FBF measurement. Forearm vascular resistance (FVR) was calculated from simultaneously measured MAP and FBF (MAP/FBF) and expressed as arbitrary units (AU). The calculated FVRs obtained during each 5 minutes of placebo infusion or during the last 2 minutes of each drug infusion were averaged to one value. Drug-induced effects were expressed as the percentage of change from preceding placebo infusion (effect of L-NMMA-acetate and MCh or ACh without L-NMMA-acetate) or from preceding L-NMMA-acetate infusion (effect of MCh or ACh with L-NMMA-acetate). To quantify the overall response to ACh or MCh, the percentage responses in FVR to the three dosages of ACh or MCh were averaged to one value, that was assumed to represent

the area under the curve (AUC) [292]. AUCs with and without concomitant L-NMMA infusion were compared to assess the effect of L-NMMA on the vasodilator response to MCh or ACh. All results are expressed as mean±SE unless indicated otherwise and compared by the Wilcoxon's paired signed rank test; $P<0.05$ was taken as statistically significant.

RESULTS

Figure 1 shows the course of the mean FBF throughout the several infusions in group A. In the infused arm in both groups, FVR did not significantly differ between the three consecutive placebo infusions: 50.1±11.6, 56.7±19.2 and 50.8±10.2 AU for group A and 46.8±8.3, 48.8±9.8 and 52.1±14.7 AU for group B. Figure 2 shows L-NMMA-induced percentage changes in FVR for both control and infused arm. In the infused arm of group A, L-NMMA-acetate induced an increase in FVR of 36.7±15.7% ($P=0.07$) and 86.2±14.5% ($P<0.05$) during the first and second infusion respectively. In theory, the effect of the second L-NMMA-acetate infusion could have been influenced by the preceding MCh infusion (0.03 μg/100ml forearm per min). To assess this possible carry over effect, we calculated the percentage difference in FVR between the first and second placebo infusion. The percentage increase in FVR during the second L-NMMA acetate infusion differed significantly from the percentage difference in FVR between the first and second placebo infusion, indicating that a possible carry over effect has not played an important role (86.2±14.5% versus 3.1±9.5% for L-NMMA and placebo respectively; $P<0.05$). Comparable results were obtained in group B: L-NMMA-acetate induced an increase in FVR of 54.6±16.6% ($P<0.05$) and 99.5±27.4% ($P<0.05$ versus baseline and versus placebo) during the first and second infusion, respectively. In the control arm, FVR did not change significantly as a result of L-NMMA-acetate infusion.

Figure 3 shows the effect of L-NMMA on the vasodilator response to MCh and ACh. In the infused arm of group A, FVR decreased with 55.8±9.4%, 69.0±14.6% and 86.9±3.7% during the three increasing MCh dosages. Concomitant L-NMMA-acetate infusion did not affect the vasodilator response to MCh significantly: The AUCs before and during L-NMMA infusion were -70.6±8.8 and -68.6±9.3%, respectively (P=NS). In the infused arm of group B, FVR decreased with 18.9±18.0%, 67.1±7.2% and 81.0±5.3% during the three increasing ACh dosages. The AUCs before and during L-NMMA infusion were -55.7±9.1% and -35.4±11.8% respectively ($P<0.05$). Again, no significant changes in FVR were observed in the control arm.

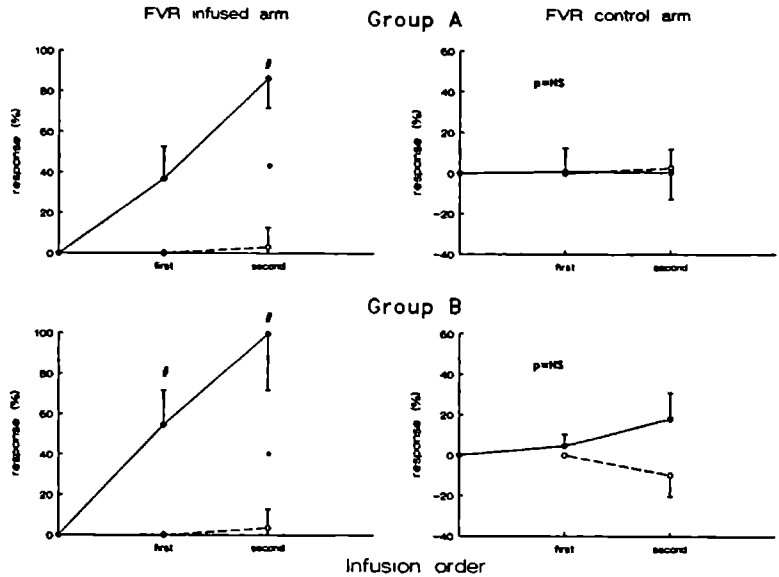

Figure 2. Effect of two consecutive L-NMMA-acetate infusions (0.1 mg/100 ml/min) on forearm vascular resistance (FVR) expressed as percentage change from third placebo infusion (mean±SE). The course in FVR changes during the two L-NMMA-acetate infusions is compared with the course of these parameters during the first two consecutive placebo infusions preceding the methacholine (Group A) or acetylcholine (group B) infusions without L-NMMA-acetate. Solid line: L-NMMA; dashed line: placebo. *, Significant differences between placebo and L-NMMA-acetate infusion. #, Significant differences from baseline.

DISCUSSION

We compared the contribution of NO in MCh- and ACh-induced vasodilation. At present, in vivo measurements of NO release are not technically feasible. It is fortunate that production of NO can be reduced by L-arginine derivatives that competitively antagonize NO-synthase [27,28]. An example is N^G-monomethyl-L-arginine (L-NMMA). In vitro data suggest that L-NMMA also inhibits transmembrane transport of L-arginine [293]. Thus, a reduced intracellular concentration of the substrate of NO-synthase may further contribute to the inhibitory action of L-NMMA on NO production.

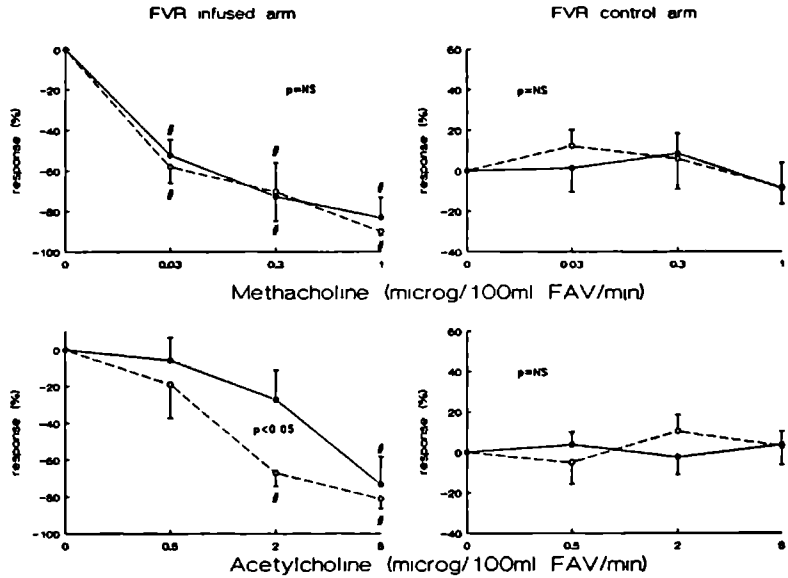

Figure 3. Effect of methacholine (top) and acetylcholine (bottom) infusions on forearm vascular resistance (FVR), expressed as percentage change (mean±SE) from preceding placebo (methacholine or acetylcholine with placebo) or from preceding L-NMMA-acetate infusion (methacholine or acetylcholine with L-NMMA-acetate). Dashed line: with concomitant placebo infusion, Solid line: with concomitant L-NMMA infusion. P-values indicate level of significance for the difference between the AUC's before and during L-NMMA infusion. #, Significant differences from baseline

The L-NMMA-acetate infusion of 0.1 mg/100ml forearm per min induced a significant increase in FVR, indicating that basal NO release was effectively inhibited [36].

The most striking result of the current study is the failure of L-NMMA-acetate to reduce the MCh-induced percentage reduction in FVR, although it caused a degree of vasodilation similar to that caused by ACh. This finding suggests that NO contributes differentially to MCh- and ACh induced vasodilation and supports the hypothesis of Chowienczyk and co-workers [290]. Our results may have been confounded by differences between the two study groups other than the vasodilator used. The existence of a confounding factor is not likely since both study groups consisted of healthy male volunteers very alike with respect to age, blood pressure, baseline FVR and effect of L-NMMA on basal vascular tone.

Infusion of 0.1 mg L-NMMA-acetate/100ml per min was equimolar to the highest dosage of L-NMMA used by Vallance and colleagues (4 μmol/min) [36], who infused this

dosage for 5 minutes just before giving ACh infusions. However, in the present study, L-NMMA was also infused simultaneously with MCh and ACh to ensure that NO-synthase inhibition was effectuated. A reduced dosage of 0.05 mg L-NMMA-acetate was used to prevent possible serious side effects. MCh-induced vasodilation might be reduced by concomitant infusion of higher dosages of L-NMMA.

The lowest ACh dosage did not affect FVR significantly. In contrast, the second ACh infusion induced a vasodilator response that was almost completely abolished by concomitant infusion of L-NMMA, confirming the important role of NO as mediator of the ACh-induced vasodilation. The vasodilator response to the highest ACh dose was not affected by L-NMMA, which can easily be explained by the competitive nature of the NO-synthase inhibition. Other mechanisms than endothelial release of NO may account for the vasodilator response to ACh in the presence of L-NMMA. According to the literature, ACh-induced vasodilation is mediated by at least three different mechanisms: presynaptic inhibition of norepinephrine release, release of NO from endothelial cells and stimulation of prostacyclin production from the blood vessel wall [65,282,294,295]. Furthermore, increasing evidence suggests that ACh is able to induce the release of an endothelium-derived hyperpolarizing factor that can induce vasodilation and is distinct from NO [64].

The divergent effect of L-NMMA on ACh- and MCh-induced vasodilation suggests that NO contributes differentially to MCh- and ACh-induced vasodilation. Studies performed with MCh to assess the influence of various disease states on NO-mediated endothelium-dependent vasodilation, are not conclusive and should be repeated with ACh.

CHAPTER 6

Characterization of ATP-induced vasodilation in the human forearm vascular bed

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ABSTRACT

Animal data indicate that adenosine-5'-triphosphate (ATP), derived from aggregating thrombocytes or endothelium, induces an endothelium-dependent vasodilator response that is mediated by P_{2y}-purinergic receptors and is reduced when high dosages are administered. This reduced vasodilator response to high ATP doses has been associated with the concomitant release of endothelium-derived contracting factors. In contrast to the endothelium-dependent vasodilator response, ATP as released from sympathetic nerve endings induces a P_{2x}-purinergic receptor-mediated vasoconstrictor response that may contribute to the attenuated vasodilator response to high dosages of luminally applied ATP. The dual action of ATP might be important in the pathophysiology of disease states characterized by an impaired endothelial function and increased thrombocyte aggregation. This study was performed to characterize the vascular response to ATP in humans.

The brachial artery was cannulated in 50 healthy male volunteers (age, 18-44 years) for drug infusion and measurement of mean arterial pressure. Forearm blood flow was recorded by venous occlusion strain-gauge plethysmography. ATP induced a dose-dependent vasodilator response that was significantly higher than the effect of equimolar adenosine infusion and that was not reduced by concomitant infusion of the P_1 purinergic receptor antagonist theophylline. The infusion of the NO synthase antagonist N^G-monomethyl-L-arginine (L-NMMA) reduced the averaged fall in forearm vascular resistance (FVR) to acetylcholine (-59±6% [mean±SE] versus -42±8%; $P<0.05$; $n=10$) but did not affect the vasodilator response to ATP (-68±3% versus -64±6%; $P>0.1$; $n=10$) or sodium nitroprusside (SNP; -53±3% versus -49±4%; $P>0.1$; $n=6$). The L-NMMA-induced increase in FVR appeared to be related to the type of vasodilator pretreatment being 94.7±16.7%, 44.9±8.7% and 40.8±7.3% for acetylcholine, ATP and SNP pretreatment, respectively ($P<0.01$ for acetylcholine versus ATP and SNP; $P>0.1$ for ATP versus SNP). In contrast to animal data, high dosages of intra-arterially infused ATP (up to 1000 μg/100 ml forearm per min) did not reveal a reduction in the forearm vasodilator response but appeared to be similar to the maximal forearm vasodilation as observed during postocclusive reactive hyperemia.

These observations indicate that ATP induces a potent dose-dependent vasodilator response that is not mediated by P_1 purinergic receptor stimulation or by the release of nitric oxide. Moreover, in healthy volunteers the vasodilator response to high intra-arterial dosages of ATP is not reduced by the release of endothelium-derived contracting factors or by the stimulation of P_{2x}-purinergic receptors on the smooth muscle cells.

INTRODUCTION

At the end of the 19th century, Heidenhain was probably the first to show that endogenous compounds, as extracted from freshly prepared organs, can elicit circulatory effects such as vasodilation and a fall in blood pressure (BP) when infused intravenously in animals [2]. In 1929, Drury and Szent-Gyorgyi recognized the importance of endogenous nucleotides in this response [1] Their impressive early report on the pharmacology of nucleotides in rats is considered the beginning of modern research on purine pharmacology [102] After a detailed review of the existing literature and own observations, Burnstock et al. [6] proposed a subdivision of purinergic receptors The major subdivision is between P_1-purinergic receptors, with an endogenous agonist potency order of adenosine > adenosine-5'-monophosphate (AMP) >adenosine-5' diphosphate (ADP) >adenosine-5'-triphosphate (ATP) and P_2-purinergic receptors, with an endogenous agonist potency order of ATP > ADP > AMP > adenosine Based on a different potency order of synthesized adenosine analogues and divergent effects on intracellular cyclic adenosine-3'-5'-monophosphate (cAMP) levels, an important second messenger of P_1-purinergic receptor stimulation, P_1-purinergic receptors are subdivided in A_1- and A_2-adenosine receptors [6] In the vascular wall, A_2-adenosine receptors, located on endothelial and smooth muscle cells, are involved in the vasodilator response to adenosine [118] On sympathetic nerve endings, stimulation of the A_1 adenosine receptor results in a reduced release of norepinephrine [268] Methylxanthines like theophylline and caffeine are competitive antagonists on both P_1-purinergic receptor subtypes [206,221,247] Recently, this subdivision has been confirmed by molecular techniques [97]

Largely on the basis of the rank order of agonist potency of structural analogues of ATP, two subtypes of the P_2-purinergic receptor are generally accepted. the P_{2x}- and the P_{2y}-purinergic receptors [101,296,297]

Under physiological circumstances, ATP is coreleased with norepinephrine from sympathetic nerve endings [15] The subsequent stimulation of P_{2x}-purinergic receptors on vascular smooth muscle cells is thought to contribute to the sympathetic nervous system-mediated vasoconstriction [15] Additionally, ATP is released from endothelial cells and aggregating thrombocytes, resulting in a P_{2y}-purinergic receptor-mediated endothelium-dependent vasodilator response that opposes the effect of thrombocyte-derived vasoconstricting agents like thromboxane A_2 and serotonin [59,72,91] Thus, endogenously released ATP might have an important role in physiology and pathophysiology, and it is of interest to characterize the effects of ATP in vivo in humans Up to now, our pharmacological knowledge on ATP is largely based on in vitro data from animal studies Human in vivo data about the direct vascular effects of ATP are scarce Although the vasodilator effect of ATP in humans has been described before [298,299], the mechanism

of action is largely unknown. Of course, endothelial P_{2y}-purinergic receptors are expected to be involved in the vasodilator response to ATP, but this response may also be mediated by adenosine receptors [300], because ATP is rapidly degraded to adenosine [96,301] and because ATP has weak adenosine receptor agonist properties [301,302]. In this report, a series of experiments is presented to elucidate the mechanism of action of the vasodilator property of ATP in humans. This study focuses on three questions in particular:

1) What is the involvement of P_1-purinergic receptor stimulation in the ATP-induced forearm vasodilation?
2) Is nitric oxide (NO) involved in the forearm vasodilator response to ATP?
3) Is there evidence for an ATP-mediated release of endothelium-derived contracting factors (EDCF) or P_{2x}-purinergic receptor stimulation?

SUBJECTS AND METHODS

After approval from the local ethics committee, a total of 50 healthy male volunteers signed written informed consent statements before participation in the study. Their demographic characteristics are summarized in table 1. Before the start of the study, the subjects were asked to abstain from caffeine-containing products for at least 36 hours and to refrain from smoking for at least 12 hours. Furthermore, subjects were asked to discontinue any food intake 2 hours before the start of the experiment. All experiments were performed in the afternoon with the subject in the supine position. After local anesthesia (xylocaine 2%), the left brachial artery was cannulated with a 20-gauge catheter (Angiocath, Deseret Medical, Becton Dickinson, Sandy, UT, U.S.A.) for both intra-arterial drug infusion (automatic syringe infusion pump, type STC-521, Terumo, Tokyo, Japan) and BP recording (Hewlett Packard GmbH, Böblingen, Germany). Forearm blood flow (FBF) was registered in both forearms by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, D.E. Hokanson, Washington DC, U.S.A.). The upper arm collecting cuffs were simultaneously inflated using a rapid cuff inflator (Hokanson E-20). At least one minute before FBF measurements were made, hand circulation was occluded by inflation of wrist cuffs to 200 mmHg. FBF was recorded three times per minute. All experiments were started at least 30 minutes after intra-arterial cannulation. In all experiments, total infusion rate was kept constant at 100 μl/100 ml forearm per min. Saline and all drug dosages were infused for 5 minutes.

TABLE 1: Baseline characteristics of the study groups (mean±SD)

	Total group	time control study	ATP + theophylline	ATP + L-NMMA	ACh + L-NMMA	SNP + L-NMMA	Highly dosed ATP
N	50	6	6	10	10	6	12
Age (years)	27.7±8.0	26.0±7.9	25.0±8.7	31.1±7.6	28.7±8.2	29.2±9 8	25.5±7.2
Weight (kg)	75 8±8.3	74.4±8.8	72.4±11.0	75.8±7.0	77.4±9.1	78.1±4.4	75.8±9.5
Height (cm)	182.6±6.6	178.9±4.7	184.9±6.6	181.4±5.5	183.9±5.8	182.2±9.6	183.4±7.4
Body mass index (kg/m^2)	22.8±2.3	23.3±3.1	21.1±1.9	23.1±2.02	22.9±2.9	23.6±1.6	22.5±2.1
Systolic blood pressure (mmHg)#	127 0±8.0	124.2±11.0	128.0±12.2	126.8±7.7	126.5±5.0	131.3±5.9	126.2±8.0
Diastolic blood pressure (mmHg)#	68 2±9.7	69.0±9.5	69.5±13.5	69.0±8.0	67.7±10.1	68.0±10 4	66.8±10.3
Heart rate (bpm)#	65.2±10.0	63.3±10.6	67.3±8.6	56.4±9.1*	69.6±8.7	71.0±12.4	65.7±7.1
Forearm vascular resistance (AU)	58±34	48±16	47±28	43±18	62±44	49±7	73±33
Forearm volume (L)	1.10±0 14	1.03±0 15	1 08±0 17	1 08±0 14	1.11±0.15	1.18±0.16	1.10±0.12

* Indicates $p<0.05$ for comparison with the other L-NMMA groups, the ATP + theophylline group and the highly dosed ATP group.
# Blood pressure was measured in supine position with the Riva-Rocci technique using Korotkoff I and V for systolic and diastolic blood pressure respectively. Heart rate was measured by pulse frequency counting (radial artery).

Involvement of P_1-purine (adenosine) receptor stimulation in the ATP-induced forearm vasodilator response

In 12 subjects, the vasodilator effect of equimolar dosages of intra-arterially-infused adenosine and ATP were compared. Additionally, the effect of intra-arterially-infused theophylline, a competitive P_1-purinergic receptor antagonist, on the vasodilator response to ATP was studied.

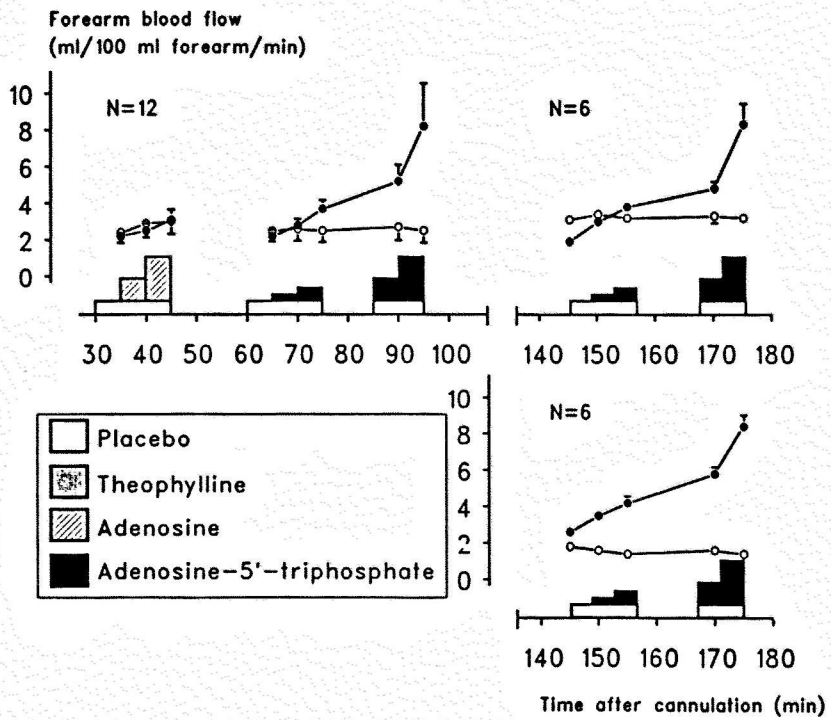

Figure 1. A schematic presentation of the protocol aimed to study the involvement of P_1 purinergic receptors in the ATP-mediated vasodilator response. Adenosine was infused at a rate of 2 and 6 nmol/100 ml forearm/ min and ATP was infused at a rate of 0.2, 0.6, 2 and 6 nmol/100 ml forearm/min. Additionally, the course of the forearm blood flow is shown.
Closed symbols indicate infused arm; open symbols, control arm.

The experiment started with the measurement of baseline FBF during saline infusion (NaCL 0.9%). Figure 1 shows the course of FBF and the schedule of the several drug infusions. The effect of two increasing dosages of adenosine (0.5 and 1.5 μg/100 ml forearm per min, equivalent to 2 and 6 nmol/100 ml per min) was compared with that of

saline infusion. Fifteen minutes after the last adenosine infusion, saline was infused again. Now, the effect of four increasing dosages of ATP (0.1, 0.3, 1.0, and 3.0 μg/100 ml forearm per min, equivalent to 0.2, 0.6, 2 and 6 nmol/100 ml forearm per min) were compared with saline infusion. Because prolonged occlusion of hand circulation can cause discomfort, with subsequent effects on BP and heart rate (HR), a 10-minute rest was allowed between the second and third ATP infusion. Forty-five minutes after the last ATP infusion, saline infusion and the four increasing ATP dosages were repeated in six subjects ("time control study"). In the other six subjects, saline infusion and the four increasing ATP dosages were repeated with concomitant infusion of theophylline (100 μg/100 ml forearm per min). In previous reports, we and others have shown that this dosage of theophylline antagonizes the forearm vasodilator response to intra-arterially-infused adenosine [221,303].

Involvement of nitric oxide (NO) in the ATP-induced forearm vasodilator response

We studied the effect of intra-arterially-infused N^G-monomethyl-L-arginine (L-NMMA), a competitive NO-synthase antagonist, on the vasodilator response to ATP (n=10), to the NO-dependent vasodilator acetylcholine (n=10), and to sodium nitroprusside (SNP, n=6), an NO donor that does not stimulate NO-synthase activity [304]. Acetylcholine, a proven NO-dependent vasodilator in the human forearm [36,305], was used to ascertain the effectiveness of our experimental setup to demonstrate antagonistic properties of L-NMMA (positive control experiment). L-NMMA reduced FBF which might nonspecifically affect the subsequent response to a vasodilator stimulus. Therefore, the interaction between SNP and L-NMMA was also studied (negative-control experiment). Apart from the effect of L-NMMA on the aforementioned vasodilator response to acetylcholine, ATP, and SNP, the forearm vasoconstrictor response to L-NMMA itself was analyzed to find out whether this was related to the effect of the previously infused vasodilator substances on NO synthase.

The experiment started with the measurements of baseline FBF during saline infusion. The effect of three increasing dosages of ATP (0.3, 3 and 10 μg/100 ml forearm per min) were compared with that of saline infusion. Because prolonged occlusion of hand circulation can cause discomfort, with subsequent effects on BP and HR, a 10-minute rest was allowed between the first and second ATP infusions. Forty-five minutes after the last ATP infusion, the vasoactive effect of L-NMMA infusion (0.1 mg/100 ml forearm per min) was compared with that of saline infusion. Subsequently, the lowest ATP dose was infused (0.3 μg/100 ml per min) together with infusion of L-NMMA (0.05 mg/100 ml forearm per min). Ten minutes thereafter, L-NMMA was infused again (0.1 mg/100 ml forearm per min), immediately followed by the middle and the highest ATP dosages (3 and 10 μg/100 ml forearm per min), again together with the lower L-NMMA

dose (0.05 mg/100 ml forearm per min). In the acetylcholine group, acetylcholine (0.5, 2 and 8 μg/100 ml forearm per min) was substituted for ATP. In the SNP group, SNP (0.02, 0.2 and 0.6 μg/100 ml forearm per min) was substituted for ATP. Since SNP is diluted in glucose 5%, in the SNP group glucose 5% was substituted for NaCl 0.9%. Otherwise, the same protocol was performed in the three groups.

Effect of high dosages of intra-arterially infused ATP on forearm vascular tone

In vitro data suggest that high dosages of ATP reveal a vasoconstrictor component compared with the effect of lower ATP dosages [306,307]. Both P_{2x}-purinergic receptor stimulation as well as release of EDCFs may be involved in this response. To study these possible mechanisms in humans, the effects of high dosages of intra-arterially-infused ATP were studied in 12 subjects. The effect of four increasing intra-arterially-infused ATP dosages (10, 30, 100 and 300 μg/100 ml forearm per min) were compared with that of saline infusion. To avoid discomfort, a 10-minute rest was allowed between the second and third ATP infusions. After the first 6 experiments, an interim analysis was performed, revealing no decreased vasodilator response at the highest dosage. In the subsequent 6 experiments, 1000 μg ATP/100 ml forearm per min was infused immediately after the fourth ATP infusion. To be sure that maximal vasodilation occurred in response to the ATP infusions, maximal vasodilation was measured during postocclusive reactive hyperemia according to the well-established method of Pedrinelli et al [308,309]. A cuff applied to the left upper arm was inflated to 300 mmHg for 13 minutes. During the last minute of ischemia, the subjects were asked to perform repeated hand contractions. Immediately after desufflation of the upperarm cuff, FBF measurements were started for at least 2 minutes with occluded hand circulation. The lowest forearm vascular resistance (MAP/FBF) was considered to represent maximal vasodilation.

Drugs and solutions

ATP solutions were freshly prepared from 2-ml ampoules containing 20 mg ATP as disodium salt (Striadyne, Wyeth Laboratories), and were diluted in NaCl 0.9%. L-NMMA-acetate, and acetylcholine chloride (Sigma Chemical Co, St. Louis, MO, U.S.A.), were reconsituted on the morning of the study day from a sterile lyophilized powder, passed through a 0.2 μm Millipore filter, and diluted in NaCl 0.9%. Adenosine (Sigma Chemical Co., St. Louis, MO, U.S.A.) was freshly prepared from 10-ml ampoules containing 20 mg adenosine with NaCl 0.9% as solvent. Theophylline solutions were freshly prepared from 10-ml ampoules containing 24 mg/ml aminophyllinum-hydricum (Euphyllin^{R}, BYK Nederland, Zwanenburg, The Netherlands), and diluted in NaCl 0.9%. SNP was reconstituted immediately before the start of the experiment from a

sterile lyophilized powder, diluted in glucose 5 % (Nipride, Roche Nederland, Mijdrecht, The Netherlands) and protected against light.

Statistical analysis

Mean arterial BP (MAP) was measured continuously during each recording of FBF and averaged per FBF measurement. Forearm vascular resistance (FVR) was calculated from simultaneously measured MAP and FBF (MAP/FBF) and expressed as arbitrary units (AU). The calculated FVRs obtained during each 5 min of saline infusion or during the last 2 min of each drug infusion were averaged to one value. Drug-induced effects were expressed as percentage of change from preceding saline infusion or antagonist infusion. The percentage changes in FVR to each dosage of a vasodilator substance were averaged to one value both before and during antagonist infusion. These two values were compared to assess the effect of an antagonist. All results are mean$\pm$SE unless indicated otherwise. To avoid multiple comparison, within-subject effects were assessed by Friedman two-way nonparametric ANOVA first and further analyzed with the Wilcoxon's paired signed rank test if appropriate. Likewise, differences between groups were assessed with the Kruskall-Wallis nonparametric one-way ANOVA and further analyzed with the Mann-Whitney-*U* test if appropriate; $P<0.05$ (two sided) was considered statistically significant.

RESULTS

Involvement of P_1-purine (adenosine) receptor stimulation in the ATP-induced forearm vasodilator response

Apart from the dosage schedule, figure 1 shows the course of FBF during infusion of adenosine and ATP. FBF in the control arm was not significantly affected during any of the infusions.

Baseline FVR was 47$\pm$6 AU in the cannulated arm. In twelve subjects, the effect of two adenosine dosages and four ATP dosages were studied first. FVR of the infused arm during the first and second saline infusion did not significantly differ (47$\pm$6 and 53$\pm$9 AU, respectively, P=NS; n=12). ATP decreased FVR by 17$\pm$12%, 37$\pm$11%, 52$\pm$7% and 60$\pm$7% for 0.1, 0.3, 1 and 3 μg ATP/100 ml forearm per min respectively (n=12; $P<0.05$ for the three highest dosages). The FVR in the control arm was not significantly affected. In figure 2, the forearm vascular effects of two equimolar adenosine and ATP dosages (2 and 6 nmol/100 ml per min) are compared. Both ATP dosages induced significantly more forearm vasodilation than their equimolar adenosine counterparts (37$\pm$11% versus 9$\pm$6% for 2 nmol/100 ml forearm per min, and 60$\pm$7% versus 21$\pm$7% for 6 nmol/100 ml forearm per min; n=12; $P<0.05$ for both comparisons).

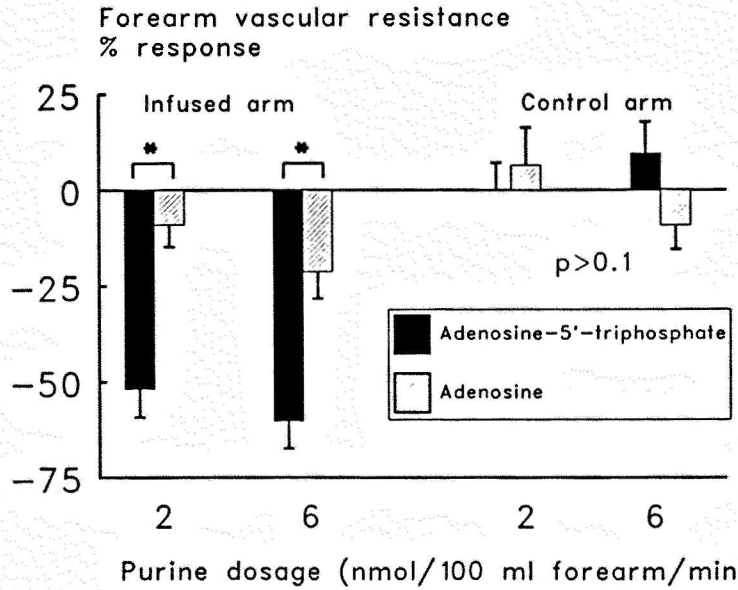

Figure 2. Bar graph comparing forearm vascular responses to two equimolar adenosine and ATP infusions. *, Statistically significant differences between adenosine and ATP. P value indicates the level of significance for the vascular effects in the control arm.

In 6 subjects, the ATP infusions were repeated 45 minutes later. The vasodilator response to ATP did not significantly differ between the two infusion periods: the averaged responses in FVR were -46±8% and -45±10 % in the infused arm for the first and second ATP infusions, respectively (P=NS). In the control arm, vascular resistance remained unchanged (averaged response in FVR, 2±5% and 8±14 % during the first and second infusion period, respectively; n=6, P>0.1). In the other 6 subjects, the ATP infusions were repeated with concomitant infusion of theophylline. FVRs during the second saline infusion and the theophylline infusion were 54±16 and 54±17 AU, respectively (P=NS, n=6). FVR during theophylline infusion did not differ significantly from FVR during the third saline infusion of the time-control study group (54±17 versus 62±8 AU; P=NS, n=6 for both groups). Thus, theophylline did not significantly affect FVR. Likewise, theophylline did not significantly affect the ATP-induced forearm vasodilator response: the averaged responses in FVR were -38±12% and -38±10% for ATP infusions with saline and theophylline, respectively (P=NS, n=6). In the control arm, no changes in FVR were observed.

Involvement of NO in the ATP-induced forearm vasodilator response

FBF in the control arm was not affected by the various drug infusions.

Figure 3 depicts the effect of each dosage of the three vasodilator substances on FVR in the infused arm before and during L-NMMA infusion.

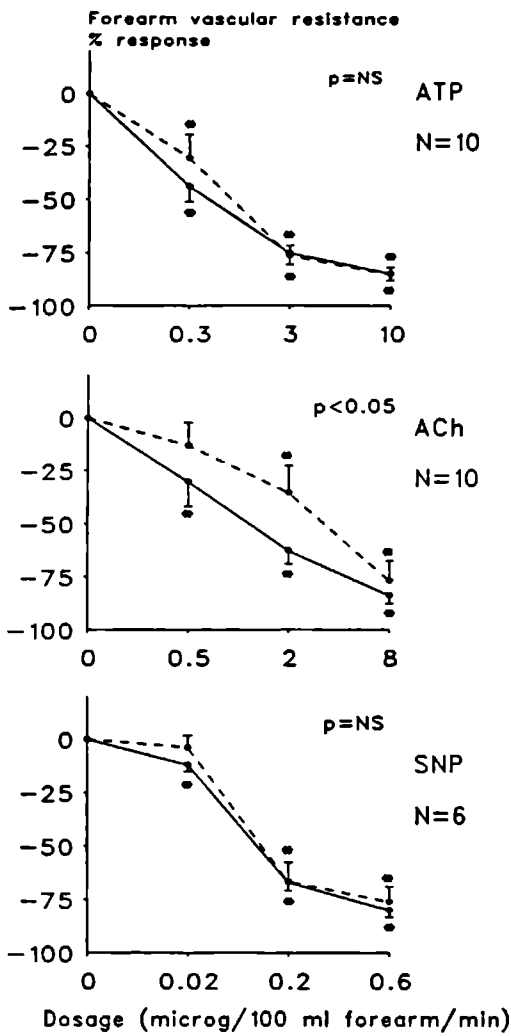

Figure 3. Graphs showing course of forearm vascular resistance in infused arm during the infusion of three increasing dosages of ATP, acetylcholine (ACh) or sodium nitroprusside (SNP). *, P<0.05 versus baseline. P values indicate the level of significance for the effect of L-NMMA on the vasodilator response. NS indicates not significant (P>0.1); solid line, before L-NMMA infusion; dashed line, during L-NMMA infusion.

In the infused arm, for each vasodilator substance, a dose-dependent vasodilation was observed. Besides a smaller effect of the lowest SNP dosage, the three vasodilator substances induced a comparable vasodilator response. Only the acetylcholine-induced

vasodilator response could be antagonized significantly by L-NMMA. The effect was most pronounced for the lower dosages. Table 2 tabulates the effect of L-NMMA on forearm vascular tone and the averaged responses to vasodilator substances before and during L-NMMA infusion. The averaged percentage responses in FVR to the three vasodilator substances were -59±6%, -68±3%, and -53±3% for acetylcholine, ATP, and SNP, respectively. During concomitant infusion of L-NMMA, the averaged responses in FVR were -42±8%, -64±6%, and -49±4% for the acetylcholine, ATP, and SNP group, respectively ($P<0.05$ for effect of L-NMMA on acetylcholine; P=NS for the effect of L-NMMA on ATP and SNP). Besides a small increase during the second SNP infusion with L-NMMA (35.7±14.8%; $P<0.05$ versus baseline), FVR in the control arm was not significantly affected for any of the vasodilators.

TABLE 2: Effect of L-NMMA on baseline forearm vascular resistance and vasodilator response to ATP, ACh and SNP (mean±SE)

	Vasodilator	% Rise in FVR to first L-NMMA infusion	% Rise in FVR to second L-NMMA infusion	Averaged % response in FVR to vasodilator	Averaged % response in FVR to vasodilator (+ L-NMMA)
Infused arm	ACh	64±13*#	95±17*#	-59±6*	-42±8*§
	ATP	37±10*	45±9*	-68±3*	-64±6*
	SNP	33±11*	41±7*	-53±3*@	-49±4*
Control arm	ACh	5±4	16±9	1±5	5±4
	ATP	-3±5	6±5	3±4	1±3
	SNP	5±2	-8±11	6±5	19±10

L-NMMA indicates N^G-monomethyl-L-arginine, ACh, acetylcholine, SNP, sodium nitroprusside, and FVR, forearm vascular resistance. Values are mean ±SE *, Statistically significant difference from baseline ($P<0.05$); §, Statistically significant effect of L-NMMA on vasodilator response ($P<0.05$); #, Statistically significant difference from ATP and SNP group ($P<0.05$);
@, Statistically significant difference with ATP group ($P<0.05$).

Within each group, the vascular resistances during the three saline infusions were not significantly different, indicating that vascular tone had returned toward baseline levels before the second infusion of a vasodilator substance and before the first L-NMMA infusion. Additionally, baseline FVR did not differ significantly between the three groups. During the first L-NMMA infusion, FVR increased in all three groups ($P<0.05$ for acetylcholine [n=10], ATP [n=10] and SNP [n=6] groups). In the acetylcholine group, this vasoconstrictor response differed significantly from the other two groups, being

64.3±12.7% versus 37.2±9.7%, and 33.3±10.7% for the ATP and SNP groups, respectively ($P<0.05$ for the acetylcholine group versus each other group; $P>0.1$ for the ATP group versus the SNP group). These group differences were more pronounced for the second L-NMMA infusion. In the ATP group, the increase in FVR did not differ significantly from the SNP group, being 44.9±8.7% and 40.8±7.3% for the ATP and SNP groups, respectively (ATP versus SNP group, $P=1.0$; first versus second L-NMMA infusion, $P>0.1$ for both groups). However, in the acetylcholine group, L-NMMA now tended to induce a more pronounced increase in FVR of 94.7±16.7% ($P<0.01$ versus ATP and SNP groups; $P=0.09$ versus first L-NMMA infusion).

Group differences in vasoconstrictor response to L-NMMA may have confounded the effect of L-NMMA on the response to vasodilator substances. To investigate this possibility, a correlation between the vasoconstrictor response to L-NMMA and the L-NMMA-induced reduction in averaged percentage response in FVR to acetylcholine and ATP was analyzed for each agonist separately. This analysis revealed that with an increasing vasoconstrictor response to L-NMMA, the inhibition of agonist-induced vasodilation was reduced ($r=-0.56$; $P=0.09$ for acetylcholine and $r=-0.66$; $P<0.05$ for ATP). Therefore, it is unlikely that the reduced vasoconstrictor response to L-NMMA in the ATP-group masked a possible inhibitory effect of L-NMMA on ATP-induced vasodilation.

Effect of high dosages of intra-arterially infused ATP on forearm vascular tone

Baseline FVR was 73±10 AU. Figure 4 shows the percentage response in FVR during the four increasing ATP dosages. In the control arm, FVR was not significantly affected (Friedman ANOVA, $P>0.1$; $n=12$). In the infused-arm, FVR was 9±2, 8±3, 6±2 and 5±2 AU during infusion of 10, 30, 100 and 300 μg ATP/100 ml per min, respectively ($P<0.01$ versus baseline for all dosages, $n=12$). These values are distorted because of one outlayer with a FVR in the infused arm of 77 AU during saline and 26, 35, 32 and 28 AU during the four increasing ATP dosages, respectively. After exclusion of this outlayer, FVR was 8±2, 5±1, 4±1, and 2±0.2 AU for the four increasing ATP dosages, repectively ($P<0.01$ versus baseline for all dosages, $n=11$). Thus, up to 300 μg ATP/100 ml forearm per min, FVR was continuously reduced. In six subjects this dose-response curve was extended to 1000 μg ATP/ 100 ml forearm per min, which was infused immediately after 300 μg ATP/100 ml forearm per min. On average, FVR was reduced to 3±1 AU during infusions of 1000 μg ATP/100 ml forearm per min, respectively (P=NS for 300 versus 1000 μg ATP/100 ml forearm per min, $n=6$). In four of the 6 subjects, FVR in the control arm increased slightly during the highest ATP dosage ($P>0.1$). The minimal FVR during postocclusive reactive hyperemia was 3±0.2 AU and

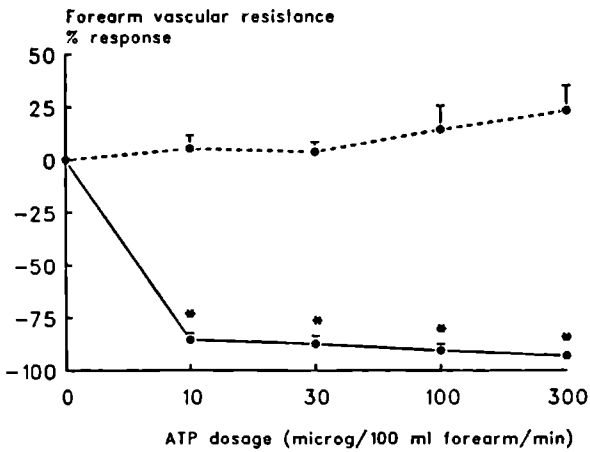

Figure 4. Graph showing effect of high dose of ATP on forearm vascular resistance. Solid line indicates infused arm; dashed line, control arm.
*, $P<0.05$ versus baseline; $n=11$.

did not differ significantly from FVR during infusion of 300 ($n=12$) or 1000 μg ATP/100 ml forearm per min ($n=6$).

DISCUSSION

In vitro studies have demonstrated pharmacological effects of ATP, that might have important physiological and pathophysiological implications. ATP released from sympathetic nerve endings at the adventitial side of a blood vessel, results in a vasoconstrictor response, mediated by so-called P_{2x}-purinergic receptors on smooth muscle cells. On the other hand, luminally released ATP from aggregating thrombocytes or the endothelium itself stimulate endothelial P_{2y}-purinergic receptors, resulting in an NO-mediated vasodilator response [50]. To assess the relevance of these in vitro observations, we now characterized the vascular in vivo effects of ATP in men. The perfused forearm technique was used because this model has been validated previously as a method to study direct vascular effects in humans [194,310]. In this model, the possibility of measuring FBF in the noninfused arm has methodological advantages because it enables us to detect systemic actions of the infused drugs, such as baroreflex activation, that could potentially counteract the local effects.

Our results demonstrate that ATP induces vasodilation even at low concentrations in the nanomolar range, which are thought to occur under physiological conditions [311]. This vasodilator response appeared to be unrelated to P_1-purinergic receptor stimulation and NO release. The vasodilator response was not reduced at high ATP infusion rates.

Involvement of P_1-purine (adenosine) receptor stimulation in the ATP-induced forearm vasodilator response

The vascular effects of equimolar dosages of ATP and adenosine, the degradation product of ATP with the highest P_1-purinergic receptor agonist activity, demonstrate that P_1-purinergic receptor stimulation does hardly contribute to the ATP-induced vasodilation. This view is further supported by the fact that theophylline did not affect the vasodilator response to ATP. Since theophylline has been shown to antagonize the forearm vasodilator response to adenosine [221,303], the present observation rules out that P_1-purinergic receptor stimulation is involved in the ATP-induced forearm vasodilator response. This is in agreement with most in vitro studies [62,105,117,297,312]. In theory, theophylline could have inhibited intracellular phosphodiesterase activity. Since cAMP is thought to be an important second messenger of A_2 purinergic receptor stimulation, this effect could have counteracted the P_1-purinergic receptor antagonizing action of theophylline. This problem has been addressed by others using the forearm vasodilator response to theophylline as a marker of phosphodiesterase inhibition [269,303]. They found that theophylline infusions up to 100 μg/100 ml per min did not affect forearm vascular tone but significantly antagonized the adenosine-induced vasodilation. In our study, the same theophylline dosage was used without any effect on vascular tone. Therefore, it is very unlikely that inhibition of phosphodiesterase has contributed to the results.

In view of the extensive literature on in vitro vascular effects of ATP, the exclusion of P_1-purinergic receptor involvement strongly suggests the existence of the P_{2y}-purinergic receptor in the human forearm vascular bed [102]. However, the involvement of a recently suggested "pyrimidine receptor" cannot be excluded [307]. Specific P_2-purinergic receptor antagonists are needed to resolve these problems in P_2-purinergic receptor classification.

Involvement of NO in the ATP-induced forearm vasodilator response

L-NMMA, a competitive inhibitor of NO synthase without affinity for the muscarine receptor, was used in this study to inhibit the formation of vascular NO [28,313]. Acetylcholine is known to stimulate NO synthase both in vitro and in vivo in the human forearm [36,305]. Therefore, this agonist was used as a positive control. SNP is regarded as an NO donor that directly stimulates soluble guanylate cyclase without involvement of NO synthase [304], and therefore was a suitable negative control in this study part.

The vasodilator response to acetylcholine was reduced significantly by L-NMMA, indicating that the L-NMMA dosage used in this study is able to inhibit NO synthase significantly. In contrast, the ATP-induced vasodilator response could not be inhibited by concomitant L-NMMA infusion, despite a similar degree of vasodilation induced by ATP and acetylcholine. This finding supports the argument that NO does not significantly

contribute to ATP-induced vasodilation. The SNP-induced forearm vasodilation was not affected by concomitant L-NMMA infusion, ruling out the possibility that agonist-induced vasodilation was affected by L-NMMA-evoked precontraction. During the second series of SNP infusions, FVR in the control arm increased temporarily. A possible systemic effect of the concomitant L-NMMA infusion is not likely, because it was not observed in the ATP and acetylcholine groups and because FVR in the control arm returned to baseline levels during the last administration of SNP with concomitant L-NMMA infusion. Whatever the cause might be, it did not seriously affect the results obtained from the infused arm, since it was only a small change in FVR that did not significantly differ from FVR fluctuations in the control arm during SNP administration with concomitant saline infusion.

In all three groups, L-NMMA induced a significant increase in FVR, confirming that L-NMMA in the dosages used was able to inhibit baseline NO production [36]. This effect of L-NMMA differed between the three groups, indicating an influence of agonist pretreatment on NO synthase activity. During the second L-NMMA infusion, ten minutes after the lowest agonist infusion, these between-group differences were more pronounced, supporting this view (see table 2). Since SNP does not stimulate NO synthase, the effect of L-NMMA in the SNP group can be regarded as unaffected by the previous infusions of SNP. In the acetylcholine group, the vasoconstrictor response to L-NMMA was significantly higher than in the SNP group, indicating that NO synthase was still activated 50 minutes after the last acetylcholine infusion, although FVR had returned to baseline levels. In the ATP group, L-NMMA-induced vasoconstriction was similar to that observed in the SNP group during both the first and second L-NMMA infusion. In combination with the lack of effect of L-NMMA on ATP-induced vasodilation these results indicate that ATP does not stimulate NO-synthase activity in the human forearm vascular bed. In theory, kinetic differences between acetylcholine and ATP could explain the between-group differences for the effect of L-NMMA on FVR. However, both acetylcholine and ATP have a short half-life of a few seconds because of rapid degradation by choline esterase and ectonucleotidases, respectively. Furthermore, both substances induced a similar amount of vasodilation and in both the ATP and acetylcholine group, FVR had returned to baseline levels before L-NMMA was infused. Therefore, kinetic differences between the two agonists are not able to explain the divergent effect of L-NMMA in the acetylcholine and ATP group. Probably, NO synthase remained activated after acetylcholine infusion without continuous muscarine receptor stimulation.

One might argue that the group differences in L-NMMA-evoked contractions reflect group differences in NO synthase sensitivity to L-NMMA. However, this would assume a positive correlation between L-NMMA-induced vasoconstriction and the inhibitory effect of L-NMMA on the forearm vasodilator response to acetylcholine and

ATP. In contrast, a negative correlation was observed. Therefore, we believe that our results are not relevantly confounded by group differences in NO synthase sensitivity to L-NMMA. In addition, the existence of a confounding factor is not likely, because the study groups consisted of healthy male volunteers very alike with respect to age, BP, and baseline FVR.

The present observation that ATP does not activate NO synthase in the human forearm contrasts with most in vitro studies [103,307,314]. An important question remains: How is the ATP-induced vasodilation mediated in the human forearm? With regard to the endothelium, in vitro studies indicate that besides NO, prostacyclin is also released after endothelial stimulation with ATP and may be involved in a vasodilator response [69,104]. Other, yet unidentified, endothelium-derived relaxing factors like the "endothelium-derived hyperpolarizing factor" may be involved as well [63,64]. Therefore, the present finding does not exclude the possibility that ATP induces its vasodilator response in the human forearm by an endothelium-dependent mechanism. On the other hand, some in vitro studies have demonstrated an endothelium-independent component in the ATP-induced vasodilator response [62,105,106]. The ATP-dependent potassium channel may be involved in ATP-induced vasodilation [315]. Further research is needed to determine the precise role of the endothelium in the ATP-induced forearm vasodilator response.

Effect of high dosages of intra-arterially infused ATP on forearm vascular tone

In vitro studies have shown a reduced vasodilator response to high dosages of ATP [297,306,307]. Two possible mechanisms have been proposed: simultaneous stimulation of P_{2x}-purinergic receptors [307] and concomitant release of EDCFs [306]. In the present study, these in vitro observations could not be reproduced in the human forearm. Maximal ATP-induced vasodilation did not differ from post occlusive reactive hyperemia, confirming a previous observation [316]. Postocclusive reactive hyperemia can be regarded as a condition of maximal and total vascular relaxation [308,309]. The vasodilator response to ATP was not reduced even at the very high dosage of 1000 μg ATP/100 ml forearm per min, indicating that the vasodilator effect of high intra-arterial dosages of ATP is not reduced by the release of EDCFs. The presence of an intact endothelium and the existence of a highly active vascular ectophosphatase system could have prevented ATP from reaching the vascular smooth muscle cells [82,317]. Therefore, the present observations do not exclude the existence of P_{2x}-purinergic receptors in the human forearm vasculature.

In conclusion, intra-arterial infusion of ATP induces a forearm vasodilator response that is not mediated by P_1-purinergic receptor stimulation or by NO release. In healthy volunteers, the effect of high doses of ATP is not reduced by the potential release

of EDCFs or stimulation of P_{2x}-purinergic receptors. The exact mechanism of the vasodilator response to ATP in the human forearm remains to be elucidated. However, our results convincingly indicate that the intravascular release of ATP or ADP from thrombocytes is not expected to induce vasoconstriction in a healthy vascular system. This, of course, does not exclude a vasoconstrictor response to interstitially released ATP.

CHAPTER 7

Endothelial release of nitric oxide contributes to the vasodilator effect of adenosine in humans

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ABSTRACT

The endogenous nucleoside adenosine plays an important role in the regulation of vascular tone, especially during ischemia. Experimental data derived from animal models suggest that nitric oxide (NO) contributes to the vasodilator effect of adenosine. The primary purpose of this investigation was to determine whether the endothelial release of NO contributes to adenosine-induced vasodilation in humans.

Venous occlusion plethysmography was used to assess the forearm blood flow (FBF) responses to graded intra-arterial infusions of adenosine (1.5 - 500 μg/min). Dose response curves were constructed before and during intra-arterial infusion of the NO-synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA, 2 mg/min, n=6) or vehicle (n=6). Prior to L-NMMA, adenosine caused a dose-dependent increase in FBF from 2.3 to 15.9 ml/min/dl. During concurrent infusion of L-NMMA, adenosine increased FBF from 1.7 to 10.0 ml/min/dl, and this change from baseline was significantly reduced as compared with that before L-NMMA ($P<0.05$). L-NMMA also attenuated the FBF-response to adenosine when the basal constrictor effect of L-NMMA was prevented by co-infusion of the NO-donor nitroprusside (n=6, $P<0.01$). In contrast, L-NMMA did not affect the FBF-response to intra-arterial infusion of the endothelium-independent vasodilator verapamil (from 2.0 to 13.9 before L-NMMA and from 1.3 to 13.6 ml/min/dl during L-NMMA, n=6, P=NS). The second objective of this study was to determine whether the adenosine-induced release of nitric oxide is mediated by activation of endothelial potassium channels, putatively coupled to adenosine receptors. Thus, the FBF-response to adenosine was measured before and during infusion of the ATP-dependent K^+-channel blocker tolbutamide (1 mg/min, n=6), or the K^+-channel blocker quinidine (0.5 mg/min, n=6). The adenosine-mediated increments in FBF were not attenuated by either K^+-channel blocker.

Adenosine-induced vasodilation in humans is mediated, at least in part, by endothelial release of nitric oxide. The transducing mechanism of this phenomenon is not known, but does not appear to involve the activation of either ATP-dependent or quinidine-sensitive K^+-channels.

INTRODUCTION

Apart from its role as a constituent of the intracellular energy source adenosine-'5-triphosphate (ATP), the endogenous nucleoside adenosine also has important effects in the extracellular compartment. In the cardiovascular system, extracellular adenosine is a regulatory substance, matching blood flow to alterations in tissue oxygen supply and/or demand [318]. Until recently, the vasodilator effect of adenosine was thought to be based

on direct stimulation of A_2-adenosine receptors on vascular smooth muscle cells, which mediate an increase in the second messenger, cyclic adenosine-3,5'-monophosphate (cAMP), via stimulation of adenylate cyclase. Therefore, this agent has been frequently used in animal as well as human studies to evaluate endothelium-independent vasodilation [319,320]. However, recent investigations have disputed the endothelium-independent character of adenosine-mediated vasodilation. Several vascular preparations have shown attenuated responses to adenosine or adenosine analogues after previous inhibition of endothelial nitric oxide synthesis [321,322], or after rubbing of the endothelium [122,323,324]. Also, infusion of large sized adenosine agonists (N^6-octylamine-adenosine coupled to carboxylated latex microspheres), which were assumed to be confined to the intravascular space, induced an obvious decrease in coronary vascular resistance in the saline-perfused guinea pig heart, presumably via an endothelium-derived mechanism [123]. Moreover, functional adenosine receptors have been identified on the endothelium, even in human aortic endothelial cells [122].

Several potential mechanisms could mediate the endothelial release of nitric oxide during adenosine administration. Stimulation of endothelial adenosine receptors may mobilize calcium and thereby activate endothelial NO-synthase [32]. Adenosine A_1-receptors have been demonstrated to be coupled to ATP-dependent potassium channels by guanine nucleotide binding proteins, in particular by the $G\alpha_i$-protein [325]. The $G\alpha_i$-proteins can mobilize calcium by stimulating phosphoinositide-specific phospholipase C activity causing hydrolysis of phosphatidyl-inositol-4,5-biphosphate [326]. Also, adenosine can stimulate NO-release by the hyperpolarization of endothelial cells resulting from activation of ATP-dependent potassium channels [315,327]. In addition, potassium channel activation and NO-release can be triggered non-specifically by increments in shear stress related to the raised flow [328].

In the present study we evaluated whether the endothelial release of nitric oxide plays an important role in the vasodilating effects of adenosine in humans and, if so, whether the aforementioned potential mechanisms are involved in that NO-release. Human data on this subject are especially important because of the large inter-species differences in endothelial pharmacology [329]. To address these questions, the effects of adenosine were assessed in vivo by the perfused forearm technique, both before and during blockade of the production of nitric oxide by the NO-synthase inhibitor, N^G-monomethyl-L-arginine, and before and during blockade of potassium channels by the sulphonylurea derivative, tolbutamide, and by the antiarrhythmic drug, quinidine. These studies as well as appropriate control experiments allow us to conclude that the vascular effects of adenosine in humans are based, at least partly, on the endothelial release of nitric oxide. This adenosine-induced release of nitric oxide is not mediated by activation of ATP-dependent potassium channels or by quinidine-sensitive potassium channels.

SUBJECTS AND METHODS

Thirty nine healthy volunteers participated in the study, and all signed a written informed consent before participation. The health status of each volunteer was assured by medical history and a physical examination as well as by laboratory investigations to assess lipid levels. Demographic data of the participants are shown in table 1. Subjects with high blood pressure (> 140/90 mmHg), high concentrations of serum cholesterol (low density lipoproteins above the 75th percentile of their age group) and/or those who smoked cigarettes were excluded from participation because these features may interfere with the nitric oxide pathway [198,330-332].

TABLE 1: Demographic data for all participants

	Mean ± SD	Range
Age (years)	29.9 ± 7.3	18-44
Gender (male / female)	17 /22	---
Height (m)	1.71 ± 0.09	1.50-1.83
Weight (kg)	65.8 ± 9.7	47.7-81.8
Body Mass Index (kg/m^2)	22.5 ± 3.0	17.0-30.3
Systolic Blood Pressure (mmHg)	120 ± 11	101-144
Diastolic Blood Pressure	62 ± 8	47-86
Heart Rate (bpm)	58 ± 2	46-78
Total Cholesterol (mg/dl)	4.3 ± 0.6	2.9-5.5
HDL-Cholesterol (mg/dl)	1.3 ± 0.3	0.9-1.9
LDL-Cholesterol (mg/dl)	2.6 ± 0.6	1.6-3.6

Each subject participated in one experiment of approximately 4 hours duration. The experiments were performed in a quiet and temperature-controlled room (22 °C). The participants were all asked to abstain from food, alcohol and caffeine for at least 12 hours before the test. Since caffeine acts as a potent adenosine receptor antagonist in the human cardiovascular system [221], we measured plasma caffeine levels at the time of the test to be sure of the subjects compliance with respect to the abstinence recommendations. The subjects remained in the supine position throughout the test. After arrival in the laboratory, the left brachial artery was cannulated under local anesthesia with a 20-gauge catheter which was used for intra-arterial drug infusion as well as for blood pressure monitoring (Gould Inc, Cleveland, Ohio). Drugs were delivered with an automatic syringe infusion pump (Harvard Apparatus, South Natick, Massachusetts).

Forearm blood flow (FBF) recordings were started after an equilibration period of at least 40 minutes. Forearm blood flow was measured in each arm by venous occlusion mercury-in-silastic strain gauge plethysmography (Hokanson EC4, D.E.Hokanson, Washington, U.S.A.) as previously described [198]. Both arms rested in slings on heart level, with the forearms slightly elevated to ensure a sufficient venous return. To be sure that FBF recordings referred predominantly to the forearm skeletal muscle circulation, the hand circulation was occluded during all FBF-recordings by a wrist cuff inflated 100 mmHg above the systolic blood pressure [195].

Assessment of the role of nitric oxide in the vasodilator response to adenosine and verapamil

In the first subgroup of 6 subjects, the vasodilator response to increasing dosages of adenosine was investigated. Baseline measurements were taken during infusion of placebo into the brachial artery (glucose 5 %). Then, adenosine was infused intra-arterially in six increasing dosages for 4 minutes per dose (adenosine dosages: 1.5 - 5 - 15 - 50 - 150- 500 μg/min). Measurements were performed during the last two minutes of each infusion. After an equilibration interval of 60 minutes in which the FBF was allowed to return towards baseline levels, new baseline recordings were obtained during placebo-infusion. Subsequently, infusion of N^G-monomethyl-L-arginine (L-NMMA) into the brachial artery was started in a dose of 2 mg/min. After 15 minutes of the L-NMMA-infusion alone, hemodynamic measurements were taken to evaluate the vaso-active effects of L-NMMA itself. Thereafter, the six increasing adenosine dosages were again administered and co-infused with L-NMMA. The total duration of L-NMMA infusion was approximately 40 minutes. In an additional series of three subjects, we ensured that the single infusion of L-NMMA of 2 mg/min for 40 minutes induced a steady state vasoconstrictor response, and did not progress over time.

In the next group of 6 subjects similar experiments were performed, with a similar time schedule and the same dosages of adenosine and L-NMMA, but now the L-NMMA-infusion was accompanied by a low intra-arterial dose of the NO-donor sodium nitroprusside (SNP). Previous studies have shown that L-NMMA infusion induces a vasoconstrictor response in the forearm vascular bed by inhibiting the baseline release of nitric oxide from the endothelium [36]. From a methodological point of view, this change in baseline may complicate the interpretation of the results because the baseline vascular resistance is an important determinant of the response to a vasodilator stimulus [272], and also because the achieved concentrations of adenosine will be proportionally higher. We determined from pilot studies that an intra-arterial dose of sodium nitroprusside of 0.2 μg/min was appropriate to counteract the vasoconstrictor response to L-NMMA. Thus, this dose of nitroprusside was co-infused with L-NMMA. The "clamping" of the

NO-mediated vascular tone by the concomitant infusion of L-NMMA and nitroprusside has recently been shown to be a useful approach in animal experiments on this subject [333].

An additional six subjects were studied for a time control purpose. The same time schedule was used as in the other adenosine experiments, but instead the second dose response curve with adenosine was performed in the presence of placebo instead of active drug. This study was done to be sure that within one experimental session the forearm vasodilator response to adenosine did not change over time.

In theory, nitric oxide release from the endothelium may have been triggered non-specifically by the adenosine-induced increase of shear stress resulting from the high forearm blood flows. In order to evaluate this non-specific stimulus of nitric oxide release, we measured FBF-responses to the endothelium-independent calcium entry blocker verapamil before and after L-NMMA (2 mg/min) in another six subjects. Verapamil was used in four dose steps of 5 minutes each (10 - 30 - 100 - 300 μg/min). Since verapamil has a longer half life than adenosine, we waited for 90 minutes to wash out the drug after the first series of verapamil infusion. Apart from these details the time schedule was similar to the previous experiments.

Assessment of the role of potassium channel activation in the vasodilator response to adenosine

Recently, it has been shown that adenosine receptors are coupled to ATP-dependent potassium channels [325]. These channels can be blocked specifically by sulphonylurea derivatives, not only in the pancreatic beta cells, but also at the level of the vessel wall [334]. Therefore, adenosine dose response studies were performed in a fourth group of 6 volunteers before and during the intrabrachial infusion of tolbutamide (1 mg/min). The infusion of tolbutamide started 15 minutes before the first adenosine dose. Since systemic dosages of tolbutamide may induce hypoglycemia by stimulating insulin release, and because insulin increases skeletal muscle blood flow and alters vascular responsiveness to drugs in the forearm model [335,336], arterial blood samples for glucose and insulin were taken just before and after tolbutamide to demonstrate that no systemic effects occurred at this low dose.

In an additional 6 healthy volunteers, the vascular response to adenosine was assessed before and during the intra-arterial infusion of quinidine 0.5 mg/min. By use of the patch clamp technique, studies have shown that quinidine blocks several types of potassium channels [337-341], including those activated by adenosine. To limit the cumulative dose of quinidine in healthy volunteers, we only used the four highest instead of all six adenosine dosages in these subjects. Measurements of FBF, and subsequent administration of the four adenosine dosages were started after 10 minutes of

quinidine-infusion, and the total infusion time of quinidine was restricted to 30 minutes (cumulative dose 15 mg).

Drugs and solutions

Adenosine was prepared for each experiment by diluting Adenocard vials (6 mg / 2ml, Fujisawa Pharmaceutical Co, Deerfield, Illinois) in saline. N^G-monomethyl-L-arginine acetate was purchased from Calbiochem, and diluted in glucose 5% just before the experiments. Sodium nitroprusside was purchased from Elkins-Sinn, Inc. (Cherry Hill, New Hampshire). Protected from light it was dissolved in glucose 5% just before administration. Verapamil HCl (American Regent Laboratories Inc., Shirley, New York) was diluted with glucose 5%. For the tolbutamide experiments, sterile tolbutamide sodium (Orinase Diagnostic, The Upjohn Company, Kalamazoo, Minnesota) was dissolved in saline (NaCl 0.9%). For the quinidine studies, we used quinidine gluconate vials (Eli Lilly, Indianapolis, Indiana) of 800 mg/10 ml diluted in glucose 5%. Depending on the experiment, glucose 5% or saline was used as placebo infusion. All drugs and placebo infusions were administered at the same infusion rate of 0.4 ml/min.

Statistics and calculations

The effects of adenosine itself were analysed by comparing the hemodynamic variables at baseline and at the six dose levels by one-way analysis of variance (ANOVA) with repeated measures. Post-hoc comparisons between the different dosages were made by Scheffé-F-tests. The paired t-test was used for the assessment of the effects of L-NMMA, tolbutamide or quinidine on baseline parameters. In order to evaluate the effect of the intervention (NO-synthase blockade, K^+-channel blockade) on the adenosine or verapamil responses, two-way repeated measures ANOVA was performed on the changes from baseline. Since the mean arterial blood pressure was not affected by either drug infusion (see results), FBF-changes were assumed to represent changes in foream vascular tone. Differences were considered to be statistically significant at $P<0.05$ (two-tailed). All results are presented as mean values$\pm$SE, unless indicated otherwise.

RESULTS

Vasodilator response to adenosine

To assess the vascular response to graded adenosine infusion, the data of the first dose response curve for all experiments with the six adenosine dosages were pooled ($n=24$). Table 2 summarizes the results of this analysis. Adenosine induced a dose-dependent increase in FBF, which was significantly different from baseline for the third and higher dosages. The dose-dependency was supported by significant differences

between dosages (Scheffé-F-tests: $P<0.05$). One hour after cessation of adenosine, the FBF was identical to the baseline level. As shown in table 2, there were no changes in

TABLE 2: The results of graded intra-arterial adenosine infusion on hemodynamic parameters

ADENOSINE DOSE	FBF-ex# (ml/min/dl)	FBF-con^{NS} (ml/min/dl)	FVR# (arbitrary units)	MAP^{NS} (mmHg)	HR^{NS} (bpm)
Baseline (μg/min)	2.2±0.2	1.8±0.2	40.2±2.7	79.2±2.0	58.3±1.8
1.5	2.3±0.2	1.8±0.1	40.3±2.9	78.8±1.7	57.8±1.7
5	2.6±0.2	1.8±0.2	34.0±2.5	78.5±1.9	58.3±1.6
15	4.4±0.5	1.8±0.1	23.3±2.3*	78.7±1.9	58.4±1.8
50	7.8±0.9*	1.7±0.2	13.1±1.5*	77.7±1.9	58.6±1.7
150	12.2±1.3*	1.8±0.1	8.8±1.2*	78.6±2.0	57.9±1.9
500	16.7±2.1*	1.8±0.1	7.2±1.2*	79.0±1.9	59.2±1.8
After wash out (60 minutes)	2.1±0.2	1.8±0.1	44.4±3.9	81.1±1.9	57.9±1.8

Values are presented as mean data ± SE (n=24).
FBF: forearm blood flow at the experimental (-ex) as well as at the control side (-con)
FVR: forearm vascular resistance at the experimental side (calculated as the quotient of mean arterial pressure and FBF)
MAP: mean arterial pressure
HR: heart rate.
#, $P<0.001$ by repeated measures ANOVA. ^{NS} Not significant by repeated measures ANOVA.
*, $P<0.05$ by Post-hoc Scheffé F-tests (significantly different versus baseline, versus the wash-out value as well as versus previous adenosine dose).

flow in the non-infused forearm, or changes in blood pressure or heart rate during adenosine infusion, arguing against any systemic effect of the dosages used. In the subset of experiments where the adenosine infusions were repeated after one hour without any intervention (time controls), adenosine increased FBF the first time from 2.3±0.4 to 15.2±4.7, and the second time from 1.9±0.7 to 16.0±7.2 ml/min/dl. There was no significant difference between the FBF-changes from baseline between this first and second series of measurements.

Plasma caffeine levels could not be detected in 20 of 23 subjects in whome it was measured (lower limit of assay: 0.5 mg/l). In the remaining three subjects, caffeine concentration ranged from 1.8 to 4.0 mg/l. Despite detectable caffeine levels in these three subjects, each showed a vasodilator response to adenosine that was comparable to that observed in the others, justifying inclusion of their data in the results. Moreover, statistical analysis after exclusion of these particular individuals did not affect the outcome of the findings.

Vasoconstrictor response to N^G-monomethyl-L-arginine

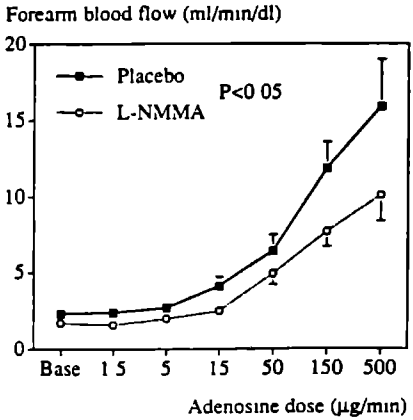
To assess the effect of L-NMMA on basal forearm blood flow, the data of six L-NMMA-adenosine-, three single L-NMMA- and six L-NMMA-verapamil-experiments were pooled. After 15 minutes of intra-arterial L-NMMA infusion, FBF decreased significantly from 2.1±0.2 to 1.5±0.1 ml/min/dl (n=15, paired t-test: $P<0.01$). In contrast, the FBF of the contralateral non-infused arm remained constant during this infusion (1.8±0.1 before versus 1.8±0.1 during L-NMMA). Moreover, L-NMMA-infusion did not change the mean arterial blood pressure (81.3±2.3 vs 79.1±2.7 mmHg, P=NS) or heart rate (56.1±2.2 vs 54.9±2.1 beats/min, P=NS), indicating that local L-NMMA-infusion did not affect systemic hemodynamics. In the three subjects, in whome L-NMMA was the only drug given, the FBF fell from 1.8±0.3 at baseline to 1.3±0.2 and 1.6±0.1 ml/min/dl after 15 and 40 minutes respectively, arguing against a progressive vasoconstrictor effect after 15 minutes of infusion.

Effects of NO-synthase blockade on vasodilator responses

Figure 1 illustrates the FBF response to the six increasing adenosine dosages during the placebo and L-NMMA-infusions. Adenosine in the presence of placebo induced a dose-dependent increase in FBF from 2.3±0.2 to 15.9±3.1 ml/min/dl. During co-infusion with L-NMMA, adenosine increased FBF from 1.7 to 10.0 ml/min/dl, this response being significantly reduced when compared with the first dose response curve. The concomitant infusion of L-NMMA and adenosine elicited no significant changes in contralateral FBF (from 1.8±0.2 at baseline to 2.3± 0.3 ml/min/dl at the highest adenosine dose), mean arterial pressure (from 80±4 to 83±4 mmHg) or heart rate (from 55±2 to 54±2 beats/min)

In the second series of experiments, the vasoconstrictor effect of L-NMMA was counteracted by concomitant infusion of sodium nitroprusside. Again, in the presence of placebo, adenosine caused a dose-dependent forearm vasodilator effect with an increase of FBF from 1.9±0.4 to 14.9±2.0 ml/min/dl (figure 2). After returning to baseline conditions, the combined infusion of L-NMMA and nitroprusside did not significantly change FBF (2.0±0.3 versus 2.2±0.3 ml/min/dl, n=6, P=NS). The subsequent administration of the six adenosine dosages elicited dose-dependent increments in FBF from 2.2±0.3 to 9.8±2.4 ml/min/dl; the changes from baseline were significantly reduced as compared with before adminstration of L-NMMA and nitroprusside ($P<0.01$) (figure 2). No significant changes occurred in contralateral FBF (from 1.7±0.3 at baseline to 1.8± 0.3 ml/min/dl at the highest adenosine dose), mean arterial pressure (from 79±4 to 81±3 mmHg) or heart rate (from 53±2 to 53±3 beats/min)

The intra-arterial infusion of L-NMMA did not change the forearm vasodilator response to verapamil.



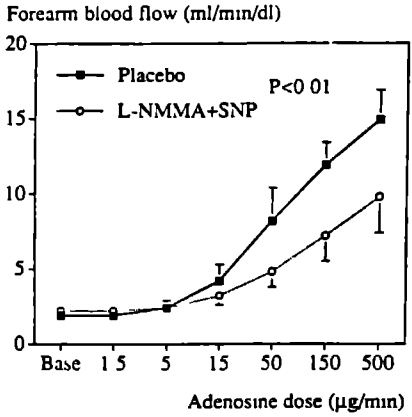
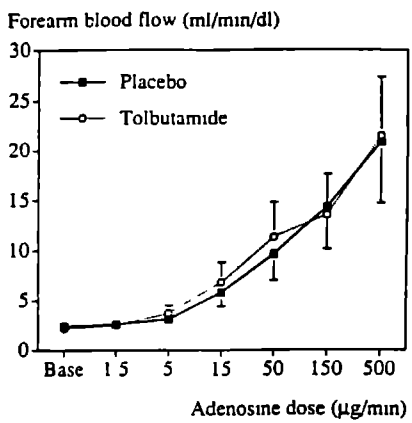


Figure 1 (left) and figure 2 (right): The forearm blood flow (FBF) during graded intra-arterial adenosine infusion, both in the presence of placebo (squares/solid line) as well as during concomitant infusion of N^G-monomethyl-L-arginine (L-NMMA, circles/dashed line, figure 1) or during the combined infusion of N^G-monomethyl-L-arginine and sodium nitroprusside (L-NMMA+SNP, circles/dashed line, figure 2) is presented as mean±SE The p-value refers to the statistical difference between conditions for these dose-responses as analyzed by repeated measures ANOVA



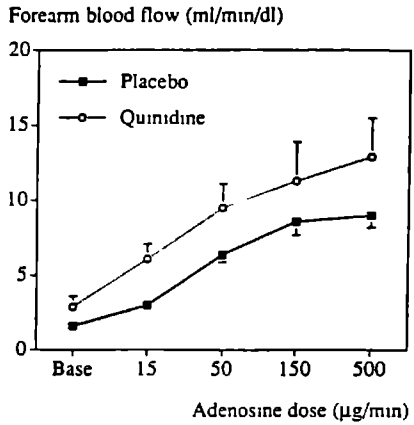


Figure 3 (left) and figure 4 (right): The forearm blood flow (FBF) during graded intra-arterial adenosine infusion, both in the presence of placebo (squares/solid line) as well as during concomitant infusion of the potassium channel blocker, tolbutamide (figure 1) or quinidine (figure 4) Values are presented as mean±SE There were no significant differences in the dose-response relationship between placebo and tolbutamide or quinidine as analyzed by repeated measures ANOVA

Effects of potassium channel blockade on adenosine responses

During placebo, the FBF-changes from baseline for the four increasing verapamil dosages averaged 2.3±0.5, 4.2±0.7, 8.6±1.9 and 11.9±2.8 ml/min/dl, whereas these numbers were 1.7±0.2, 3.5±0.9, 6.7±1.4 and 12.2±2.4 ml/min/dl during concomitant L-NMMA-administration. No significant effects on systemic hemodynamics were observed during the combined infusion of L-NMMA and verapamil. The contralateral FBF was 1.9±0.2 before and 1.6±0.3 ml/min/dl during the highest verapamil dose. Respective values for mean arterial blood pressure were 79±2 and 79±2 mmHg, and for heart rate were 56±4 and 58±4 beats/min.

Figure 3 demonstrates the effects of tolbutamide on the vasodilator response to adenosine. Tolbutamide infusion into the brachial artery did not change the baseline FBF (2.3±0.3 versus 2.2±0.3 ml/min/dl). Moreover, the adenosine-induced increase in FBF was not reduced by tolbutamide.

Figure 4 illustrates the effects of quinidine on the forearm vasodilator response to adenosine infusion. In this series, adenosine increased FBF from 1.6±0.3 to 9.0±0.8 ml/min/dl. After equilibration, the FBF returned to a baseline value of 1.5 ml/min/dl. Ten minutes of quinidine-infusion increased the baseline FBF significantly to 2.9±0.7 ml/min/dl ($P<0.05$). However, regional quinidine infusion did not significantly affect the adenosine-induced increase in FBF from baseline.

Throughout these two series of experiments, there were no changes in contralateral FBF, blood pressure or heart rate. The plasma insulin concentrations averaged 4.4±1.2 before and 4.5±1.2 μU/ml after tolbutamide administration (P=NS), while the glucose levels were 84±3 and 81±2 mg/dl, respectively.

DISCUSSION

The results of this study enable us to conclude that the vasodilator response to adenosine in humans is mediated, at least in part, by the endothelial release of nitric oxide. The following evidence supports this conclusion: 1) blockade of NO-synthase by L-NMMA significantly reduced the forearm blood flow response to adenosine, 2) the vehicle control experiments ensured that this reduced response could not be attributed to time, 3) the observation that L-NMMA attenuated adenosine responses could not be attributed to a change in basal vascular resistance, because restoration of baseline conditions by the addition of a low dose of the nitroprusside did not change the results. We reasoned that the co-infusion of nitroprusside and L-NMMA was the most ideal approach to correct for the L-NMMA-associated change in baseline vascular resistance. Addition of exogenous NO by infusion of nitroprusside was thought to restore the baseline biology of the vascular wall, and to recondition its responsiveness to vaso-active

stimuli [333]. Also, the vasoconstrictor effect of L-NMMA (when infused alone) did not progress over time, and thus could not account for the L-NMMA-mediated reduction in adenosine responses. Apart from the pathophysiological consequences of our conclusion, it is obvious that the use of adenosine for the assessment of endothelium-independent vasodilation in peripheral resistance vessels is not a valid approach in human research.

Several recent studies in animals support our observations [321,322,324,342], but others do not [319,343]. Species differences and different experimental conditions may contribute to this discrepancy. In the perfused forearm technique used in this study, the drug is administered into the vascular lumen, thus the exposure to adenosine is much higher for the vascular endothelium than for the underlying smooth muscle cell layer, especially because of the efficient uptake of adenosine by the endothelium [124,344]. In several in vitro vascular preparations the exposure is more balanced, and as such direct relaxant effects on smooth muscle cells mediated by stimulation of A_2-adenosine receptors may have been more pronounced [124].

Shear stress as a mechanism of action

A number of transducing mechanisms should be considered as mediators for adenosine's ability to release endothelium-derived nitric oxide. For example, the adenosine-induced release of NO might have been triggered non-specifically by the increase in flow (shear stress) rather than by the specific stimulation of endothelial adenosine receptors. However, in previous studies with the same technique, the intra-arterial infusion of L-NMMA reduced the relaxant effects of acetylcholine, but not those of nitroprusside, arguing against a contribution of flow-related NO-release during drug-induced elevations of forearm blood flow [345]. Furthermore, in the current experiment, L-NMMA did not attenuate the vasodilator response to the calcium entry blocker verapamil, an endothelium-independent vasodilator, despite achieving blood flow responses comparable to that observed in the mid dose range of adenosine. Our data therefore point towards a more specific interaction between L-NMMA and adenosine. We and others have shown previously that low intrabrachial dosages of the adenosine receptor antagonists caffeine and theophylline are able to block the forearm vasodilator response to adenosine [221,303], enabling us to postulate that the adenosine-mediated release of NO results from stimulation of endothelial adenosine receptors. Unfortunately, selective agonists and antagonists for the different subtypes of adenosine receptors are not available for human use, therefore it is not possible at this time to determine whether the adenosine-induced NO-release in humans is mediated by stimulation of endothelial A_1- or A_2-adenosine receptors.

Role of potassium channels

We postulated that activation of potassium channels may serve as an intermediate step, transducing stimulation of adenosine receptors to the synthesis and/or release of nitric oxide from the endothelium. Since equipotent dosages of the related sulphonylurea derivative glibenclamide have been shown to attenuate the vasodilator response to pharmacological opening of K_{ATP}-channels in the forearm vascular bed [346,347], we think that our negative observation of tolbutamide can not be attributed to ineffective dosing. Consequently, our data support the conclusion that K_{ATP}-channel-opening does not contribute to the vasodilator response to intra-arterial adenosine infusion in humans. In theory, K_{ATP}-channel-opening might have contributed to the vaso-active effects of adenosine at two different levels. At the level of the endothelium, K_{ATP}-channel-opening may hyperpolarize endothelial cells [348], and as such may activate endothelial NO-release by increasing the influx of calcium [348]. In contrast to increases of calcium influx in endothelial cells after hyperpolarization, calcium influx decreases after membrane hyperpolarization in vascular smooth muscle cells [348]. Therefore, opening of potassium channels also results in vasodilation at the level of vascular smooth muscle cells. It must be emphasized that because of our intraluminal administration of adenosine, its effects may have been predominantly endothelium-dependent, and therefore we think that our tolbutamide data do not exclude a role of K_{ATP}-channel activation in the smooth muscle relaxing effects of adenosine.

Although we realize that quinidine has several pharmacological properties, including sodium channel blockade and anti-adrenergic effects, its recently established potassium channel blocking properties made this drug an additional tool for our study, especially because quinidine was able to attenuate the vasodilator response to adenosine in animal studies [349]. Quinidine increased basal forearm blood flow, but did not attenuate the vasodilator response to adenosine. The effect of quinidine on the baseline flow may well be explained by its alpha-adrenergic blocking properties. In line with the reasoning on tolbutamide, our data argue against a role of quinidine-sensitive potassium channels in the adenosine-mediated release of NO, but do not exclude an interaction between quinidine and adenosine at the level of vascular smooth muscle cells.

Pathophysiologic Implications

Apart from effects on vascular tone, adenosine has other important properties, including inhibition of platelet aggregation [350], inhibition of leukocyte activation [167], and presynaptic inhibition of norepinephrine release [268]. Furthermore, adenosine appears to mediate ischemic preconditioning in the myocardium [169]. The release of endogenous adenosine may also contribute to the reactive hyperemic response after ischemia [315,351], as well as to exercise-induced vasodilation [352]. In recent years,

nitric oxide has been demonstrated to affect several of these pathophysiological phenomena in a similar way [315,351,353-356]. Since adenosine is released from tissues, including the endothelium, during anoxia or ischemia [357], the currently observed relation between adenosine and endothelial nitric oxide release makes endogenous adenosine a likely candidate for triggering NO-release during ischemia. We think that our results create the foundation for future studies on the interrelationship between the release of endogenous adenosine and of nitric oxide during several pathophysiological conditions in humans.

CHAPTER 8

Preserved vasodilator response to adenosine in insulin-dependent diabetes mellitus

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ABSTRACT

Experimental data derived from animal models suggest that the endogenous nucleoside adenosine has important cardioprotective properties. The potent vasodilator effects of adenosine may contribute to this cardioprotection as ischemia-induced release of endogenous adenosine has been suggested to adjust local blood flow to the metabolic demands of the tissue. Interestingly, the vascular effects of adenosine appeared to be impaired in animal models for diabetes mellitus. This observation may be of importance with respect to the increased cardiovascular mortality in diabetes. Therefore, we investigated the in vivo vasodilator effects of adenosine in insulin-dependent diabetic patients.

In twelve uncomplicated insulin-dependent male diabetic patients and twelve healthy male age-matched subjects, the brachial artery was cannulated for infusion of adenosine (0.15, 0.5, 1.5, 5, 15 and 50 μg/100 ml/min) and for measurement of mean arterial pressure (MAP). Forearm blood flow (FBF) was measured by venous occlusion mercury-in-silastic strain gauge plethysmography. Maximal vasodilation was assessed by standardized post occlusive reactive hyperaemia (PORH). Baseline forearm blood flow was 2.7 ± 0.4 and 1.8 ± 0.2 ml/100 ml/min for the diabetic patients and control group respectively. In the diabetic patients, adenosine infusion raised forearm blood flow to 2.4 ± 0.4, 2.6 ± 0.4, 4.4 ± 0.7, 6.3 ± 1.0, 9.8 ± 1.5 and 14.2 ± 2.1 ml/100 ml/min for the respective dosages. In the control group these values were 1.7 ± 0.2, 1.9 ± 0.3, 3.2 ± 0.8, 6.0 ± 1.2, 10.9 ± 2.1 and 17.1 ± 3.4 ml/100 ml/min respectively ($p>0.1$ for between group comparison). Forearm blood flow at the contralateral side was not significantly affected by the placebo and adenosine infusions. Comparable results were obtained when results were expressed as changes in forearm vascular resistance or forearm blood flow ratio (FBF infused arm/FBF control arm). Maximal vasodilation did not differ between the two groups.

We conclude that the forearm vasodilator response to adenosine is preserved in uncomplicated insulin-dependent diabetic patients. This observation argues against a primary role of a reduced adenosine responsiveness in the cardiovascular sequelae of diabetes.

INTRODUCTION

Adenosine has potentially important cardioprotective properties like inhibition of neutrophil activation with subsequent reduced free radical formation, inhibition of thrombocyte aggregation, vasodilation, presynaptic inhibition of norepinephrine release, and opening of potassium channels [204]. These effects are mediated by adenosine receptors, located on the outer cell membrane. In animals, myocardial infarct size is

reduced when adenosine is infused either before ischaemia or during the reperfusion period [168,169,202]. In addition, adenosine reduces the incidence of ischemia-induced arrhythmias [358]. Infusion of a selective adenosine receptor antagonist increases infarct size, indicating a role for endogenous adenosine as a cardioprotective autacoid [171]. Since the vasodilator action of adenosine is thought to play a role in the local adjustment of oxygen demand to oxygen supply [359,360], this may contribute to the cardioprotective properties of adenosine.

Interestingly, an impaired responsiveness to the vasodilator effect of adenosine has been observed in animal models for diabetes mellitus [200,201]. Several mechanisms may be responsible for this reduced responsiveness to adenosine. Animal and human data have indicated both fascilitating as well as inhibiting interactions between adenosine and the sympathetic nervous system [206,222,223,229,269,271]. Furthermore, human and animal studies show that adenosine-induced vasodilation is at least partially mediated by the endothelium [123,361,362]. Since insulin alters sympathetic nervous system activity [363,364] and diabetes mellitus has been associated with reduced endothelium-dependent vasodilation [199,289,292,365], both neural and endothelial mechanisms may be involved in the reduced reactivity to adenosine in these descriptive animal studies. Additionally, direct actions of adenosine on cardiac and vascular muscle cells may be reduced in patients with diabetes mellitus.

Although vascular reactivity in human diabetes has been studied extensively over the past few years [199,289,292,365], no human data are available on the responsiveness to the endogenous nucleoside adenosine. Since diabetes is an independent risk factor for developing cardiovascular disease [366], and is often associated with concomitant hypercholesterolaemia and hypertension which further attributes to an increased risk of ischaemic heart disease [367,368], an impairment in adenosine responsiveness may be of clinical interest. Pharmacological compounds are currently being developed to potentiate the action of endogenous adenosine at sites of ischemia [271]. In this context it is valuable to know if diabetic patients exhibit decreased responses to adenosine. Furthermore, a reduced vascular responsiveness to adenosine may also be of importance in the metabolic control of patients with diabetes mellitus since adenosine enhances glucose uptake in some animal models [369,370]. This metabolic effect of adenosine may in part be due to its effect on blood flow [371]. Therefore, we evaluated the vasodilator response to adenosine in patients with uncomplicated insulin dependent diabetes mellitus and compared these observations with a carefully matched control group.

PATIENTS AND METHODS

Patients

After approval of the local ethics committee, twelve normotensive nonsmoking caucasian male patients with insulin-dependent diabetes mellitus were selected from our outpatient population. Diabetes mellitus was diagnosed at least 5 years before participation to this study. Patients with evidence of macro- or microvascular disease were excluded from the study because these vascular complications would result in a nonspecific impairment of the reactivity to any vasodilator substance. Macrovascular disease was assessed by taking their history (no coronary artery disease, heart failure, cerebrovascular disease, peripheral vascular disease or foot ulcers), physical examination and a twelve lead electrocardiogram. Microvascular disease was excluded by demonstrating the absence of orthostatic hypotension and peripheral loss of sensibility, by a normal fundoscopy, and by an albumin excretion ratio less than 20 μg/min. None of the patients used medication other than subcutaneous insulin injection. Only patients with a glycosylated haemoglobin concentration (HbA1c) between 7 and 10 % as measured during insulin treatment were included.

Table 1: Demographic characteristics of the study groups (mean ± SD)

	Diabetic subjects	Healthy subjects
N	12	12
Male/female	12/0	12/0
Age (years)	34.6 ± 5.9	34.8 ± 6.0
Body Mass Index (kg/m^2)	23.6 ± 2.4	23.3 ± 2.4
Systolic blood pressure (mmHg)*	123 ± 10.4	118 ± 8 2
Diastolic blood pressure (mmHg)*	63.2 ± 8.6	62.4 ± 4.9
Mean arterial pressure (mmHg)*	85.2 ± 9.8	82.4 ± 6.8
Heart rate (beats per minute)#	67.3 ± 16.8	57.4 ± 9.6
HbA_{1c} (%)	8.5 ± 0.9	
Time after diagnosis (years)	16.2 ± 8.3	
Glucose (mmol/L)@	13.0 ± 5.1	

*, Intra-arterially measured during placebo infusion; #, Measured by electrocardiographic recordings during placebo infusion; @, Determined during the experiment; for each subject the 6 determinations were averaged to one value.

The control group consisted of twelve male non-smoking healthy caucasian volunteers. These subjects were carefully matched for age, blood pressure and body weight. They had no history of diabetes mellitus and did not use concomitant medication. Physical examination and twelve lead electrocardiography did not reveal any abnormalities. Demographic data of the study groups are shown in table 1.

Methods

Before the start of the study, the subjects were asked to abstain from caffeine-containing products for at least 24 hours, because caffeine is a potent adenosine receptor antagonist [221]. In all participating subjects, the plasma caffeine concentration was below the limit of detection as measured in a sample that was collected immediately before starting the experiments (reversed phase HPLC; minimal level of detection: 0.2 μg/ml [218]). All tests were performed in a temperature controlled laboratory (22-23 °C), with participants in the supine position, after an overnight fast, starting at 8.00 am.

From a methodological point of view, the level of plasma insulin and glucose concentrations throughout the study is a very important issue. Recent studies have convincingly shown that baseline skeletal muscle flow in humans is not affected by hyperglycemia [372], whereas hyperinsulinemia induces an obvious increase in baseline skeletal muscle flow as well as in forearm vascular reactivity [335,373-375]. To avoid confounding of our results by insulin-mediated vasodilation, we instructed the diabetic subjects to skip their morning dose of insulin and not to use a breakfast. This was done in order to achieve low and steady state plasma insulin levels during the time of experiments. Although even lower insulin levels would have been reached by also skipping the long-acting insulin injection of the evening before the experiment, this would have introduced the risk of the development of ketosis or keto-acidosis, a factor which would certainly have affected the results. Since no insulin was administered in the morning hours, we had to accept the varying fasting glucose levels throughout the experiments, since correcting the glucose levels would inevitably have increased insulin levels. As stated above, recent data have convincingly shown that plasma glucose levels up to 15 mmol/l do neither affect baseline forearm blood flow nor vascular reactivity of the forearm vascular bed [372].

After local anaesthesia (Xylocaïne, 2%), the left brachial artery was cannulated with a 20-gauge catheter (Angiocath, Deseret Medical, Inc., Becton Dickinson and Co, Sandy, UT, U.S.A.) for both intra-arterial adenosine infusion (automatic syringe infusion pump, type STC-521, Terumo Corporation, Tokyo, Japan) and blood pressure recording (Hewlett Packard GmbH, Böblingen, Germany). Forearm blood flow was registered simultaneously on both forearms by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, D.E. Hokanson,

Inc., Washington, U.S.A). The upper arm collecting cuff was inflated using a rapid cuff inflator (Hokanson E-20, D.E. Hokanson, Inc., Washington, U.S.A.). At least one minute before the FBF measurements, the circulation of the left hand was occluded by inflation of a wrist cuff to 200 mmHg. Forearm blood flow was recorded three times per minute during the four minute placebo infusion and during the last two minutes of each adenosine infusion.

The experiment started with the measurement of baseline forearm blood flow during placebo infusion (NaCl, 0.9%). Apart from the course in the forearm blood flow, figure 1 shows the schedule of the several drug infusions. The effect of six increasing dosages of adenosine (Sigma Chemical Co., St Louis, MO, U.S.A.; 0.15, 0.5, 1.5, 5, 15 and 50 μg/100 ml forearm/min) were compared with placebo (NaCl 0.9%). Prolonged occlusion of the hand circulation can cause discomfort with subsequent effects on blood pressure and heart rate.

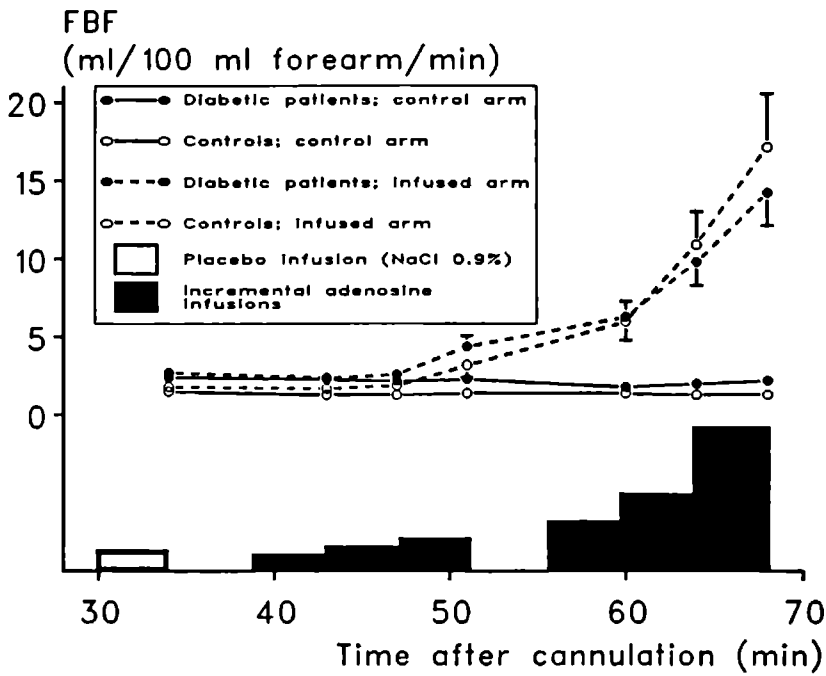

Figure 1. The course in forearm blood flow (FBF) before and during the intra-arterial infusion of adenosine is presented for the diabetic patients and the control group. There was no statistically significant difference between the groups (ANOVA for repeated measurements: P=1.0).

Therefore, a 5-minute rest period with desufflation of the wrist cuffs was allowed between the placebo infusion and the first adenosine dose and between the third and fourth

adenosine dose. During all procedures, total volume infusion was adjusted to forearm volume as measured by water displacement and kept at a constant rate of 100 μl/100 ml forearm/min. Placebo and each adenosine dosage were infused during 4 minutes.

To exclude structural vascular changes in the diabetic patients, maximal vasodilation was measured during post occlusive reactive hyperaemia (PORH) according to the well-established method of Pedrinelli et al [308,309] twenty minutes after the end of the final adenosine infusion. A cuff applied to the left upper arm was inflated to 300 mm Hg for 13 minutes. During the last minute of ischaemia the subjects were asked to perform repeated hand contractions. Immediately after desufflation of the upperarm cuff, FBF measurements were started for at least 2 minutes with occluded hand circulation. The lowest forearm vascular resistance (MAP/FBF) was considered to represent maximal vasodilation.

In the diabetic group, blood glucose concentrations were determined 6 times: immediately after arterial cannulation, after infusion of placebo, after the third and after the sixth adenosine dose, just before the test of maximal vasodilation and just before decannulation (Accutrend, type 1284851, Boehringer, Mannheim, Germany). Prior to the intra-arterial adenosine infusions, 10 ml arterial blood was collected with Li-heparin as coagulant in 9 diabetic patients and in 4 control subjects for the determination of plasma insulin and detection of insulin antibodies. Plasma insulin was measured by radioimmunoassay using a specific antiserum raised in a guinea pig against human insulin. A second antibody was used to separate the antibody-bound and free fractions. Insulin antibodies were detected by incubation of the samples with ^{125}I-insulin and subsequent precipitation with polyethyleneglycol [376].

Statistics

Mean arterial pressure (MAP) was measured continuously during each recording of forearm blood flow (FBF) and averaged per FBF registration. Forearm vascular resistance (FVR) was calculated from simultaneously measured MAP and FBF (MAP/FBF) and expressed as arbitrary units (AU). Additionally, the ratio of each simultaneously measured FBF (FBF infused arm/FBF control arm) was calculated. Forearm blood flows, the calculated flow ratios and FVR's obtained during each four minutes of placebo infusion or during the last two minutes of each drug infusion were averaged to one value. Adenosine-induced effects were expressed both as absolute and percentage change from preceding placebo infusion. Differences in responses to adenosine between the two study groups were analysed with an ANOVA for repeated measurements with the adenosine dosage as within-subject factor and the presence of diabetes mellitus as between-subject factor. Differences in baseline values were assessed with the unpaired Student t-test. Correlations were performed using the Pearson correlation coefficient.

Since plasma insulin concentrations did not show a Gaussian distribution, group differences in insulin levels were analyzed by the Mann-Whitney U test. All results are expressed as mean±SE unless indicated otherwise; $P<0.05$ (two sided) was considered to indicate statistical significance.

RESULTS

Baseline FBFs in the infused arm were 2.7±0.4 and 1.8±0.2 ml/100 ml forearm/min for the diabetic patients and control group respectively ($P=0.07$). In the control arm these values were 2.4±0.4 and 1.5±0.1 ml/100 ml forearm/min ($P=0.05$). During the adenosine infusions, FBF in the infused arm of the diabetic patients amounted 2.4±0.4, 2.6±0.4, 4.4±0.7, 6.3±1.0, 9.8±1.5 and 14.2±2.1 ml/100 ml forearm/min for the respective adenosine dosages of 0.15, 0.5, 1.5, 5, 15 and 50 μg adenosine/100 ml forearm/min. In the control group these values were 1.7±0.2, 1.9±0.3, 3.2±0.8, 6.0±1.2, 10.9±2.1 and 17.1±3.4 ml/100 ml forearm/min, respectively (see figure 1). In both groups, FBF in the control arm was not significantly affected during the placebo and adenosine infusions. Overall, repeated measures ANOVA did not reveal a significant difference between the two groups (between subject effect: $P=1.0$). Comparable results were obtained when results were expressed as absolute or relative changes in FBF from baseline.

Baseline FVRs in the infused arm were 39.6±5.2 and 57.5±7.6 AU for the diabetic patients and controls, respectively ($P=0.07$). In the control arm these values were 45.7±6.3 and 63.7±5.9 AU, respectively ($P<0.05$). During adenosine infusion, FVRs in the infused arm of the diabetic patients were 44.6±6.6, 41.9±5.4, 29.9±7.1, 20.5±4.6, 16.6±5.7 and 12.2±5.7 AU for the six increasing adenosine dosages, respectively. In the controls, these values were 57.5±7.6, 58.5±7.2, 56.1±7.7, 45.8±9.0, 24.6±5.5, 16.0±4.6 and 13.2±5.2 AU, respectively. Overall, the course of FVR in the infused arm did not significantly differ between the two groups (between-subject effect: $P=0.2$). In both groups, FVR in the control arm was not affected. Comparable results were obtained when adenosine-induced changes in FVR were expressed as absolute or relative changes from baseline (see figure 2).

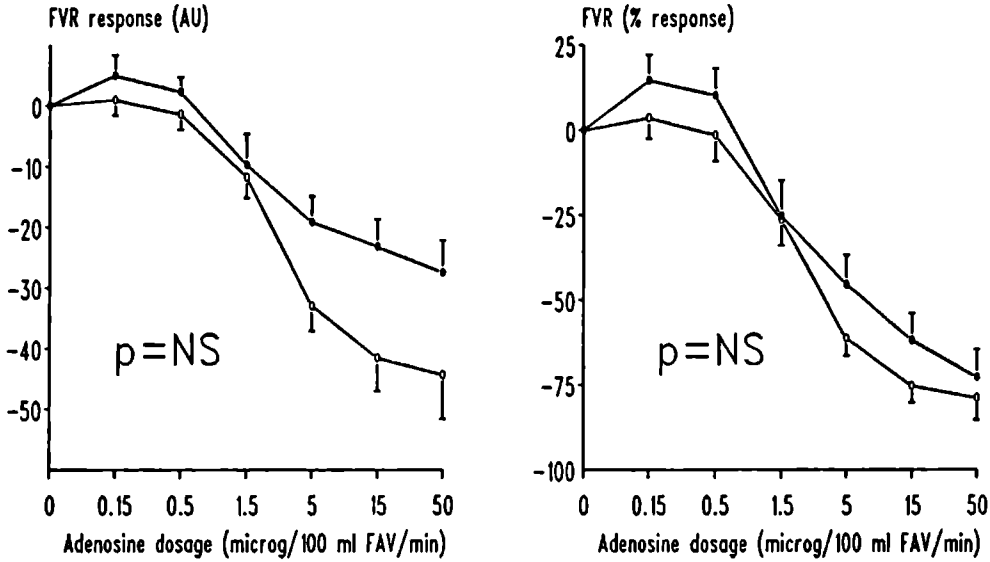

Figure 2. Adenosine-induced forearm vasodilation in controls (open symbols) and diabetic patients (closed symbols) expressed as absolute and percetage change in forearm vascular resistance (FVR) from baseline. P values indicate the level of significance for between group effects (ANOVA for repeated measurements).

Because we observed slight differences in baseline FBF- and FVR-values between the diabetic patients and the control group, we also analyzed the results of the FBF-ratio ($FBF_{infused\ arm}$ divided by $FBF_{contralateral\ arm}$). Assessment of the percent changes of this ratio has been shown to be an appropriate method to analyze dose response curves [292,377]. During placebo infusion, the FBF-ratio was equal in both groups and numbered 1.2 ± 0.1.

In the diabetic patients, the FBF-ratios increased to 1.0 ± 0.1, 1.2 ± 0.1, 2.1 ± 0.4, 4.4 ± 1.0, 5.9 ± 1.2 and 9.2 ± 2.5 during the six increasing adenosine dosages, respectively. In the controls, these values were 1.3 ± 0.1, 1.5 ± 0.2, 2.2 ± 0.4, 4.2 ± 0.8, 8.9 ± 1.5 and 14.7 ± 2.1, respectively. Overall, the course of the ratio did not significantly differ between the two groups (between-subject effect: $P=0.2$). Comparable results were obtained when adenosine-induced changes in the ratio were expressed as absolute or relative changes from baseline.

The minimal FVR that occurred during post-occlusive reactive hyperaemia did not differ between diabetic patients and controls: 3.2 ± 0.2 versus 3.4 ± 0.3 AU ($P=0.6$).

The individual courses of blood glucose concentration are shown in figure 3. Within each individual, glucose levels remained reasonably stable. However, between the patients a high variation in averaged glucose level existed ranging from 3.4 to 20.6 mmol/liter. There was no correlation between the individual plasma glucose concentration and the vascular responsiveness to adenosine in the diabetic patients ($r=-0.2$, $P=0.5$). Plasma insulin concentration was 21.8 ± 2.5 mE/L ($N=9$) in the diabetic patients versus 6.5 ± 1.3 mE/liter ($N=4$) in the controls ($P<0.01$). In four diabetic patients, insulin antibodies could be detected. After exclusion of these patients, plasma insulin concentration was still significantly higher in the diabetic patients as compared with the control subjects (20.8 ± 3.8 versus 6.5 ± 0.9 mE/liter; $P<0.05$).

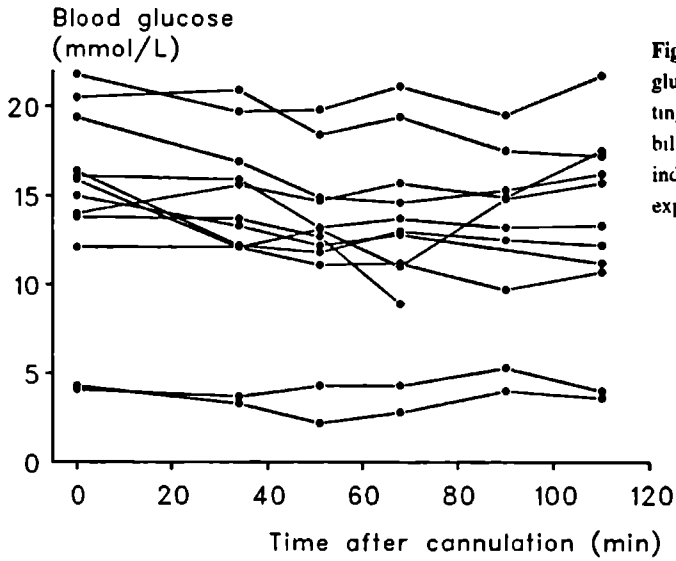

Figure 3. Individual courses of blood glucose concentrations, demonstrating the *large* inter-individual variability as well as the *small* intra-individual variability during the experiments.

DISCUSSION

This study was performed to investigate whether the forearm vasodilator response to adenosine is affected in patients with insulin-dependent diabetes mellitus. Normotensive non-smoking diabetic patients without evidence of macro- or microvascular complications were selected to prevent possible confounding by structural arteriolar or microvascular changes. Maximal forearm vasodilation in the diabetic patients appeared to be equal to that of the age-matched control group confirming the absence of structural abnormalities in the forearm vascular bed of the diabetic subjects. In these carefully selected patients, we observed a preserved vasodilator response to adenosine in the forearm vascular bed.

This observation argues against a primary role of reduced adenosine responsiveness in the cardiovascular sequellae of diabetes.

The current results are in contrast with several observations in animal models for diabetes [200,201] that studied the responsiveness of the heart and coronary vasculature to adenosine. We can not exclude the possibility that diabetes mellitus differentially affects the coronary and forearm vascular bed in humans. However, other possible explanations should be discusses as well.

It has to be emphasized that animal models for diabetes represent a true insulinopenic state. In contrast, as a result of subcutaneous administration of insulin as opposed to the physiological release of insulin into the portal vein, the levels of insulin are elevated in treated patients with insulin-dependent diabetes mellitus. As such, treated patients with insulin-dependent diabetes do not represent an insulinopenic state, not even in the fasting state when plasma insulin concentrations reach their trough level. This may well explain the preserved adenosine responsiveness in our patients, because treatment of diabetic animals with insulin also restored the impaired responses to adenosine [201]. Since we did not study the effects of adenosine in a true insulinopenic state, our data do not exclude an interaction between insulin and adenosine. Nonetheless, our present results do allow the conclusion that the vascular responsiveness to adenosine is preserved in patients with diabetes mellitus who are regularly treated with insulin.

In the diabetic patients, baseline forearm blood flow and forearm vascular resistance were slightly different from that of the control subjects. This interesting phenomenon has been described before [292] and is not only confined to the forearm but has also been shown for the retinal, renal and cutaneous circulation [378-380]. It already exists in the early course of the disease before diabetic complications have developed [292,380]. Although the exact mechanism of this 'hyperdynamic circulation' in diabetic patients is not known, it may be related to the development of complications like diabetic nephropathy and diabetic microangiopathy [380]. Especially because of this difference in baseline forearm blood flow, we also included the results on the FBF-ratio (see method section). For the FBF-ratio, the diabetic patients and the control group had exactly the same baseline values. Using this parameter, statistical analysis of the adenosine responses revealed no difference between the two groups.

The main observation of the present study is that adenosine-mediated forearm vasodilation is not significantly affected in insulin-dependent diabetic patients. The response to the two highest adenosine dosages tended to be slightly reduced in the diabetic patients, but this difference did not reach statistical significance. In contrast to the two highest dosages, the responses to the lower dosages were very similar between the two groups. We regard these lower dosages more representative for the local physiological

increases in adenosine concentration which are probably needed for the small adjustments of local flow in order to constantly balance tissue oxygen demand and supply.

The effect of intra-arterially supplied adenosine is determined by the adenosine concentration, adenosine receptor density and receptor function. Adenosine concentrations depend on the rate of cellular adenosine uptake. In theory, diabetes mellitus may impair this cellular uptake of adenosine [381]. Therefore, a reduced adenosine receptor density or function could have been masked by differences in adenosine kinetics between the two groups. However, the clinical significance of a possible receptor dysfunction is limited when the overall vasodilator effect of adenosine is not reduced in vivo as shown in the present study.

In conclusion, the vasodilator response to adenosine is preserved in patients with insulin-dependent diabetes mellitus who are regularly treated with insulin. This observation argues against a primary role of impaired adenosine responsiveness in the cardiovasculsar sequelae of diabetes.

CHAPTER 9

Summary and conclusions

Based on in vitro studies and in vivo experiments in animals, *adenosine* has been shown to induce vasodilation, to reduce norepinephrine release from sympathetic nerve endings by a presynaptic mechanism and to exhibit negative inotropic, dromotropic and chronotropic actions. These effects of adenosine are mediated by stimulation of adenosine receptors (P_1-purinergic receptors) that are located at the outer cell membrane. In anesthetized animals, these effects occur both during local and systemic administration of adenosine.

Studies in several animal models have demonstrated a cardioprotective effect of adenosine during ischemia by reducing the incidence of ischemia-induced arrhythmias and infarct size. Endogenous adenosine is thought to play an important role in ischemic preconditioning, defined as the increased tolerance of myocardium to a prolonged ischemic insult achieved by an initial brief exposure to ischemia and reperfusion. Exploitation of the cardioprotective effects of adenosine in humans would be of great therapeutic advantage.

Unfortunately, in unanesthetized humans, intravenous infusion of adenosine results in an increase in systolic blood pressure, heart rate, plasma catecholamine levels and ventilation. These systemic effects are potentially deleterious in patients with ischemic heart disease and are thought to be mediated by afferent nerve activation and subsequent stimulation of the sympathetic nervous system. In contrast to these systemic effects of adenosine, local infusion of adenosine into the brachial or coronary artery induces vasodilation and counteracts sympathetic nerve-induced vasoconstriction. In humans, both local and systemic effects of adenosine are mediated by specific adenosine receptors and can be antagonized by adenosine receptor antagonists, such as theophylline and caffeine.

Extracellular adenosine is rapidly taken up by various cells like endothelial cells, smooth muscle cells and erythrocytes. This process is mediated by facilitated diffusion and can be inhibited by nucleoside transport inhibitors like dipyridamole and draflazine. Nucleoside transport inhibition results in increased concentrations of adenosine at sites of adenosine formation only. Nucleoside transport inhibitors might circumvent the problems that are associated with intravenous adenosine to induce adenosine receptor-mediated cardioprotection. Additionally, it can be used to study the possible effects of endogenously formed adenosine.

In vitro studies have shown a dual action of *adenosine-5'triphosphate* (ATP) on the vessel wall: an endothelium-dependent vasodilator response and an endothelium-independent vasoconstrictor response. Based on studies with stable ATP analogues, at least two distinct ATP receptors (P_2-purinergic receptors) are assumed to exist on the outer cell membrane: the P_{2y}-purinergic receptor (on endothelial cells) and the P_{2x}-

purinergic receptor (on vascular smooth muscle cells), mediating vasodilation and vasoconstriction, respectively

An increasing amount of in vitro studies show an ATP-dependent component in the sympathetic nervous system-induced vasoconstriction. Based on these observations, Burnstock hypothesized that ATP is a cotransmitter in the sympathetic nervous system, which induces vasoconstriction and which, after breakdown to adenosine, inhibits norepinephrine release presynaptically (see figure 1 of chapter 1).

Apart from the sympathetic nervous system, extracellular ATP originates from aggregating thrombocytes. Endothelial damage, like in coronary atherosclerosis, promotes thrombocyte aggregation with subsequent ATP release In the absence of a functionally intact endothelium, ATP-evoked vasoconstriction may contribute to vasospasm and to induction of acute myocardial infarction. Therefore, demonstration of this dual action of ATP in humans would be of pathophysiological importance

In this thesis, the role of adenosine and ATP in the regulation of vascular tone is investigated. In most of the described experiments, we used the perfused forearm technique to quantify vascular reactivity to vasoactive substances in vivo The brachial artery is cannulated for infusion of vasoactive substances The forearm vascular response is quantified by strain gauge plethysmography a method that accurately measures changes in blood flow.

RESULTS AND DISCUSSION

In chapter 2 and 3, the hemodynamic, neurohumoral and respiratory effects of the nucleoside transport inhibitor draflazine are characterized A double blind placebo-controlled randomized cross-over trial was performed in twelve healthy male volunteers. Intravenous infusion of draflazine at dosages of 1 mg or higher, elicited the classical response as observed during intravenous adenosine infusion. Heart rate appeared to be the most sensitive marker of systemic effects. Forearm vascular tone was not affected by systemic nucleoside transport inhibition. All systemic effects of draflazine could significantly be antagonized by caffeine, indicating the involvement of P_1-purinergic receptor stimulation After intravenous infusion of 0 5 mg draflazine, a dosage that did not elicit any systemic effect, the forearm vasodilator response to intra-arterially infused adenosine could still be potentiated, suggesting that low grade nucleoside transport inhibition is a feasable tool to potentiate adenosine-mediated cardioprotection in humans.
Ex vivo nucleoside transport inhibition was used to quantify the grade of nucleoside transport inhibition. Before and after the infusion of draflazine, blood is collected to measure the rate of adenosine uptake by erythrocytes. Since erythrocyte membranes contain the draflazine sensitive nucleoside transporter, the ex vivo transport inhibition of

applied adenosine might reflect nucleoside transport inhibition in vivo. This assumption is supported by our observation that ex vivo nucleoside transport inhibition was significantly correlated with both whole blood draflazine concentration and the heart rate response to draflazine. No effect on heart rate was observed at levels of ex vivo nucleoside transport inhibition of 50% and lower.

Thus, high grade nucleoside transport inhibition revealed the continuous formation of extracellular adenosine in healthy volunteers. Where is this endogenous adenosine produced and what is the physiological importance of this endogenous adenosine? Based on in vitro studies, we speculated that there are at least two potential sources of endogenous adenosine: the endothelium and the sympathetic nervous system (see chapter 1, figure 1). Furthermore, we hypothesized that adenosine, as derived from sympathetic nerve endings (after breakdown of the cotransmitter ATP) inhibits norepinephrine release by stimulating presynaptic adenosine receptors.

Two experiments were performed to get some answers to these fundamental questions (chapter 4). First, in ten healthy male volunteers draflazine was infused in the brachial artery at five increasing dosages. The intra-arterial route of administration was chosen to prevent the systemic effects that are associated with intravenous draflazine infusion. Forearm vascular resistance did not significantly change at levels of ex vivo nucleoside transport inhibition of 40% and lower. High graded locally induced nucleoside transport inhibition evoked a small vasodilator response. Although the physiological significance of this small vasodilator response (as compared with the effect of intraarterially infused adenosine) is uncertain, it indicates the local production of extracellular adenosine in the forearm during baseline conditions. Second, in 25 healthy male subjects the effect of 250 ng draflazine/100 ml forearm/min was studied on forearm norepinephrine release at baseline conditions and during stimulation of the sympathetic nervous system using lower body negative pressure (LBNP). Both local (forearm) and systemic norepinephrine release were quantified using a tracer technique. Baseline forearm norepinephrine release *increased* during local draflazine infusion. In contrast, the LBNP-induced stimulation of forearm norepinephrine release was *reduced* during intra-arterial infusion of draflazine. Systemic norepinephrine release was not affected by local draflazine infusion, indicating that the effects on forearm norepinephrine release were probably specifically related to the infusion of draflazine. The findings support the assumption that the formation of endogenous adenosine is increased during activation of the sympathetic nervous system, resulting in presynaptic inhibition of norepinephrine release. The increased baseline norepinephrine appearance rate can be explained by assuming an increased firing rate of sympathetic nerves in the infused forearm in response to local forearm afferent nerve stimulation (see figure 3 of chapter 4).

In the next four chapters, the interaction between endothelium and purines are studied in more detail To study the involvement of nitric oxide in ATP and adenosine-mediated forearm vasodilation we first performed a pilot study to find a dosage of L-NMMA that was able to antagonize the vasodilator response to acetylcholine, a well known NO-dependent vasodilator During this process, we observed that methacholine, a muscarinic receptor agonist that was used frequently in our laboratory as a substitute for the more labile acetylcholine, induced a forearm vasodilation that could not be inhibited by L-NMMA, suggesting a differential contribution of NO to acetylcholine and methacholine-induced vasodilation (chapter 5) This observation is supported by the results of Chowienczyk et al [382] but contrasts with the findings of Bruning et al who were able to antagonize methacholine-induced vasodilation with concomitant L-NMMA infusion [383] These contrasting observations that became available to us after publication of our results will be discussed in perspective of the results of later experiments with L-NMMA (see below)

In chapter 6, experiments are described that were performed to characterize the forearm vasodilator response to ATP The main purpose of these studies was to demonstrate a dual action of ATP on vascular tone (see above) First, the involvement of P_1-purinergic receptor stimulation in ATP-induced forearm vasodilation was investigated ATP appeared to induce much more vasodilation than equimolar dosages of adenosine Furthermore, the P_1-purinergic receptor antagonist theophylline could not antagonize ATP-induced vasodilation indicating that P_1-purinergic receptor stimulation does not occur during intra-arterial infusion of ATP Second, we tried to inhibit the ATP-induced vasodilator response by co-infusion with L-NMMA ATP induced vasodilation was not antagonized by L-NMMA suggesting that NO is not involved in the forearm vasodilator response to ATP The L-NMMA-induced vasoconstriction appeared to depend on the type of vasodilator substance that was infused 50 minutes prior to L-NMMA infusion After acetylcholine pretreatment, a substance that stimulates NO-synthase in the human forearm, L-NMMA-induced forearm vasoconstriction was significantly more pronounced than after sodium nitroprusside (SNP, a NO donor without a stimulating effect on NO synthase) or ATP pretreatment Probably, this pretreatment effect is caused by prolonged NO-synthase activation after acetylcholine infusion Interestingly, forearm blood flow had returned to baseline levels prior to L-NMMA infusion suggesting that other (endothelial?) factors counterbalanced the increased NO formation The similarity between SNP and ATP pretreatment with respect to L-NMMA-induced vasoconstriction further supports our conclusion that ATP-induced forearm vasodilation is not mediated by NO Third, we tried to overrule the vasodilator response to ATP by infusing extremely high dosages of ATP Based on in vitro data, we expected to find a reduced vasodilator response during infusion of the highest ATP dosages because of concomitant P_{2x}-purinergic receptor activation

and/or release of endothelium-derived contracting factors. However, even at very high dosages the ATP-induced vasodilator response was not reduced. Endothelial metabolization of ATP, preventing luminally applied ATP from reaching the putative P_{2x}-purinergic receptor on vascular smooth muscle cells, probably explains our failure to demonstrate the existence of P_{2x}-purinergic receptors in the forearm vascular bed.

Urged by the previously mentioned conflicting results with respect to the interaction between L-NMMA and methacholine, we decided to reanalyse our data by comparing the vascular effects of acetylcholine, methacholine and sodium nitroprusside, focusing on the effect of vasodilator pretreatment on L-NMMA-induced vasoconstriction (Table 1). This analysis revealed a similar behaviour of acetylcholine and methacholine, indicating that both compounds are able to increase NO-synthase activity which is in agreement with the results of Bruning et al. [383]. However, we were not able to show an effect of L-NMMA on methacholine-induced vasodilation while Bruning et al. observed similar effects of L-NMMA on acetylcholine and methacholine-induced vasodilation. These conflicting observations are probably due to differences in the way that results were expressed. Bruning et al. calculated plasma drug concentrations from infusion rate and plasma flow, in order to correct for L-NMMA-induced reduction in baseline blood fow and subsequent effect on plasma drug concentrations by reduced dilution. This method assumes that local degradation of the infused drug is not affected by blood flow. However, this assumption is probably not true for labile substances like acetylcholine, ATP and SNP. For example, for ATP it has been reported that local degradation is augmented when flow is reduced [384]. Assuming the same effect of blood flow on degradation of acetylcholine in the forearm, the effect of a reduced dilution on final plasma acetylcholine concentration during L-NMMA infusion will (partially) be counterbalanced by an increased degradation.

TABLE 1: Effect of L-NMMA on baseline forearm vascular resistance in the infused arm (mean ± SE)

Vasodilator pretreatment	Number of subjects	Baseline FVR (AU)	% Rise in FVR to first L-NMMA infusion	% Rise in FVR to second L-NMMA infusion
ACh	10	59 ± 11	64 ± 13*	95 ± 17*#
MCh	6	51 ± 10	37 ± 16	86 ± 14*#
SNP	6	59 ± 8	33 ± 11*	41 ± 7*

L-NMMA indicates N^G-monomethyl-L-arginine; ACh, acetylcholine; SNP, sodium nitroprusside; MCh, methacholine; and FVR, forearm vascular resistance. *, Statistically significant difference from baseline ($P<0.05$); #, Statistically significant difference from SNP group ($P<0.05$). In the control arm, FVR was not significantly affected by the drug infusions. For description of statistical analysis: see chapter 6.

Methacholine is less affected by degradation Therefore, Bruning et al have probably overestimated the effect of L-NMMA on acetylcholine-induced vasodilation, whereas we have underestimated the effect of L-NMMA on methacholine-induced vasodilation The advantage of the analysis as performed in table 1 is that baseline vascular tone was similar for the three groups Although this analysis indicates that both acetylcholine and methacholine are able to stimulate NO-synthase, it does not exclude a possible difference in vasodilator mechanism between the two compounds Further research is needed to address this question

In chapter 7, the involvement of NO in the adenosine-mediated forearm vasodilator response was investigated L-NMMA significantly inhibited the adenosine-induced vasodilator response indicating that this effect of luminally applied adenosine is (partially) mediated by endothelial NO-synthase activation and subsequent NO formation Furthermore, the role of potassium channels in adenosine-induced forearm vasodilation was explored using tolbutamide and quinidine as potassium channel blockers Tolbutamide did not affect the vasodilator response to adenosine, indicating that ATP-dependent potassium channels are probably not involved in the vasodilator response to luminally applied adenosine Using quinidine, a less selective potassium channel blocker, similar results were obtained

A reduced responsiveness to adenosine would be of disadvantage during ischemia. In animal models of insulin-dependent diabetes mellitus, a reduced adenosine-induced vasodilator response has been observed in the coronary vascular bed Since diabetes mellitus is an independent risk factor for cardiovascular disease, an impaired responsiveness to adenosine may be of clinical interest Therefore, we evaluated the vasodilator response to adenosine in uncomplicated insulin-dependent diabetes mellitus and compared these observations with an age-matched control group (chapter 8) The forearm vasodilator response was not reduced in the patients with diabetes mellitus The difference between the animal data and our results can probably be explained by differences in insulin concentration in animal studies, a reduced responsiveness to adenosine was only observed in insulinopenic animals while in our patient group high insulin concentrations were measured in peripheral blood samples

CONCLUSIONS

1. Systemic high grade nucleoside transport inhibition (ex vivo nucleoside transport inhibition > 50%) induces a dose-dependent increase in heart rate, systolic blood pressure, plasma catecholamine concentration and respiration The P_1-purinergic receptor antagonist caffeine antagonizes the neurohumoral and hemodynamic effects indicating the involvement of adenosine receptor stimulation.
2. Systemic low grade nucleoside transport inhibition augments the forearm vasodilator response to intra-arterially infused adenosine without inducing systemic effects suggesting that low grade nucleoside transport inhibition is a feasable tool to potentiate the cardioprotective effects of endogenous adenosine.
3 In contrast to systemic high grade nucleoside transport inhibition, forearm inhibition of nucleoside transport evokes a local vasodilator response, demonstrating counteracting systemic and local effects of endogenous adenosine
4 Local low grade nucleoside transport inhibition in the forearm increases baseline forearm norepinephrine release probably by activation of forearm afferent nerve endings and subsequent stimulation of forearm sympathetic firing rate. In contrast, low grade nucleoside transport inhibition reduces the increase in forearm norepinephrine release in response to activation of the sympathetic nervous system supporting the hypothesis that endogenous adenosine inhibits norepinephrine release from sympathetic nerve endings in humans
5. In healthy volunteers, local ATP infusion induces a dose-dependent forearm vasodilator response Vasodilation already occurs in the nanomolar range and is not reduced during infusion of extremely high ATP dosages
6 Adenosine- but not ATP-induced forearm vasodilation is mediated by nitric oxide.
7. The forearm vasodilator response to adenosine is preserved in patients with uncomplicated insulin-dependent diabetes mellitus who are treated with insulin.

CHAPTER 10

Samenvatting en conclusies

Door middel van in vitro en in vivo experimenten met dieren, heeft men aangetoond dat *adenosine* vaatverwijding induceert, de afgifte van noradrenaline door sympathische zenuwuiteinden remt via een presynaptisch mechanisme en tevens in staat is om de hartfrequentie te verlagen, de prikkelgeleiding in het hart te vertragen en de contractiekracht van het hart te verminderen Deze effecten van adenosine worden gemedieerd door stimulatie van adenosinereceptoren (P_1-purinerge receptoren), gelokaliseerd op de celmembraan. Bij dieren die onder narcose zijn gebracht, treden deze effecten zowel na locale (arteriele) als systemische (veneuze) toediening van adenosine op

Dierexperimenteeel onderzoek heeft aangetoond dat adenosine een beschermend effect heeft ten aanzien van myocardischemie, zowel door het verlagen van de kans op hartritmestoornissen als door het reduceren van de infarctgrootte. Endogeen adenosine wordt een belangrijke rol toegedacht in 'ischemic preconditioning' Ischemic preconditioning wordt gedefinieerd als een toegenomen tolerantie van het hartspierweefsel voor de gevolgen van langdurige afsluiting van een kransslagader door een eerdere kortdurende blootstelling aan ischemie en reperfusie. Het uitbuiten van de cardioprotectieve eigenschappen van adenosine bij de mens zou van groot therapeuthisch nut kunnen zijn.

Helaas geeft intraveneus toegediend adenosine bij mensen, die niet onder invloed zijn van anesthetica, aanleiding tot een stijging van bloeddruk, hartfrequentie, plasmacatecholamineconcentratie en ademfrequentie. Deze systemische effecten van adenosine zijn potentieel schadelijk voor patienten met een ischemische hartziekte. Men veronderstelt dat deze effecten worden teweeggebracht door stimulatie van afferente zenuwvezels leidend tot stimulatie van het sympathisch zenuwstelsel. In tegenstelling tot deze systemische effecten van adenosine, geeft lokale infusie van deze stof in de armslagader of een kransslagader aanleiding tot lokale vaatverwijding en vermindert het de vasoconstictieve werking van het sympathisch zenuwstelsel. Bij de mens worden zowel de systemische als de lokale effecten van adenosine veroorzaakt door stimulatie van specifieke adenosinereceptoren. Deze effecten kunnen worden geantagoneerd door adenosinereceptorantagonisten zoals theophylline en coffeine

Extracellulair adenosine wordt snel opgenomen door diverse celtypen zoals endotheelcellen, gladde spiercellen en rode bloedcellen. De opname van adenosine wordt versneld door een proces van gefaciliteerde diffusie en kan worden geremd door nucleosidetransportremmers zoals dipyridamol en draflazine. Remming van het nucleosidetransport leidt alleen tot verhoging van de adenosineconcentratie op die plaatsen waar adenosine ook gevormd wordt. Hierdoor is het wellicht mogelijk om het cardioprotectief effect van adenosine te versterken zonder gehinderd te worden door de problemen die verbonden zijn aan het systemisch toedienen van adenosine of adenosine-analogen.

Daarnaast kunnen nucleosidetransportremmers gebruikt worden om de mogelijke effecten van endogeen gevormd adenosine te bestuderen.

In vitro onderzoek heeft aan het licht gebracht dat *adenosine-5'-trifosfaat* (ATP) twee tegengestelde effecten heeft op de vaatwand: een vaatverwijdend effect dat teweeg wordt gebracht met tussenkomst van het endotheel (dit is de één cellaag dikke binnenbekleding van een bloedvat) en een vasoconstrictief effect dat ontstaat wanneer ATP direkt in kan werken op de gladde spiercellen van de vaatwand. Op basis van studies met stabiele ATP-analogen wordt verondersteld dat er tenminste twee verschillende ATP receptoren (P_2-purinerge receptoren) in de vaatwand aanwezig zijn: de P_{2y}-purinerge receptor (op endotheelcellen) en de P_{2x}-purinerge receptor (op de gladde spiercellen van de vaatwand) die na stimulatie respectievelijk vaatverwijding en vaatvernauwing veroorzaken.

Een toenemend aantal studies laat zien dat ATP een rol speelt in de regulatie van de vaattonus door het sympathisch zenuwstelsel. Op basis van deze studies, postuleerde Burnstock dat ATP een rol speelt als cotransmitter in het sympathisch zenuwstelsel. Hieronder wordt verstaan dat naast noradrenaline ook ATP door de zenuwuiteinden van het sympathisch zenuwstelsel wordt afgegeven. Dit ATP induceert in de vaatwand vasoconstrictie en remt, na afbraak tot adenosine, presynaptisch de afgifte van noradrenaline (zie figuur 1 van hoofdstuk 1).

Behalve uit het sympathisch zenuwstelsel, komt ATP ook vrij uit aggregerende thrombocyten. Beschadiging van endotheel, zoals bij atherosclerose in coronair arteriën, bevordert thrombocytenaggregatie resulterend in afgifte van ATP. In afwezigheid van een functioneel intact endotheel, zou dit ATP vasoconstrictie teweeg kunnen brengen die zou kunnen bijdragen aan vasospasme en inductie van een hartinfarct Daarom zou het aantonen van het tweeledig effect van ATP op de vaatwand bij de mens van groot pathofysiologisch belang kunnen zijn

In dit proefschrift wordt de betekenis onderzocht van adenosine en ATP voor de regulatie van de vaattonus. In de meeste experimenten die in dit boek beschreven worden, wordt gebruik gemaakt van het 'onderarmsmodel' om de vasculaire reactiviteit van vasoactieve stoffen in vivo te kunnen kwantificeren. De onderarmsslagader (a. brachialis) wordt bij deze methode gecannuleerd voor infusie van vasoactieve stoffen. De vasculaire respons in de onderarm wordt gekwantificeerd met behulp van kwiktouwtjes plethysmografie: een methode waarmee nauwkeurig veranderingen in bloeddoorstroming kunnen worden vastgelegd.

RESULTATEN EN DISCUSSIE

In hoofdstuk 2 en 3 worden de hemodynamische, neurohumorale en ventilatoire effecten van draflazine gekarakteriseerd. Een dubbelblind, placebo-gecontroleerd en gerandomiseerd cross-over experiment werd uitgevoerd bij twaalf gezonde mannelijke vrijwilligers. Intraveneuze infusie van draflazine in een dosering van 1 mg of hoger, gaf aanleiding tot een klassieke respons zoals die in het verleden ook werd gezien na toediening van adenosine. De hartfrequentie bleek de meest gevoelige parameter om systemische effecten te detecteren. De vaattonus in de onderarm werd door systemische nucleosidetransportremming niet beïnvloed. Voorbehandeling met coffeïne antagoneerde alle systemische effecten van draflazine, wijzend op de betrokkenheid van P_1-purinerge receptorstimulatie. Na de intraveneuze toediening van 0,5 mg draflazine, een dosering die niet leidde tot systemische effecten, kon het vaatverwijdend effect van intra-arteriëel toegediend adenosine in de onderarm nog steeds worden versterkt. Dit suggereert dat geringe remming van het nucleosidetransportsysteem het cardioprotectief effect van adenosine bij de mens zou kunnen potentiëren.

Ex vivo metingen werden gebruikt om de mate van nucleosidetransportremming te kwantificeren. Voor en na draflazine-infusie werd bloed afgenomen om de snelheid te meten waarmee adenosine wordt opgenomen door erythrocyten. Aangezien het membraan van erythrocyten de draflazine-gevoelige nucleosidetransporter bevat, is ex vivo transportremming van toegediend adenosine wellicht een weerspiegeling van de mate van nucleosidetransportremming in vivo. Deze veronderstelling wordt ondersteund door onze observatie dat ex vivo resultaten correleren met zowel de draflazineconcentratie in bloed alsook met de stijging van de hartfrequentie die optreedt na toediening van draflazine. De hartfrequentie werd niet door draflazine beïnvloed zolang de ex vivo nucleosidetransportremming onder de 50% bleef.

Bovenstaande experimenten brachten aan het licht dat bij gezonde vrijwilligers continu extracellulair adenosine wordt gevormd. Waar wordt dit adenosine geproduceerd en wat is de fysiologische betekenis van dit endogeen adenosine? Gebaseerd op in vitro onderzoek, speculeerden we dat er tenminste twee mogelijke bronnen van endogeen adenosine bestaan: het endotheel en het sympathisch zenuwstelsel (zie hoofdstuk 1, figuur 1). Vervolgens veronderstelden we dat adenosine, zoals dat afkomstig is uit sympathische zenuwuiteinden (na afbraak van de cotransmitter ATP) de noradrenaline-afgifte remt door stimulatie van presynaptische adenosinereceptoren.

Twee experimenten werden uitgevoerd om antwoord te vinden op deze fundamentele vragen (hoofdstuk 4). Ten eerste werden bij tien gezonde vrijwilligers vijf opklimmende doseringen draflazine in de a. brachialis geïnfundeerd. De intra-arteriële wijze van

toediening werd gekozen ter voorkoming van systemische effecten die verbonden zijn aan de systemische toediening van draflazine Tot het niveau van 40% lokale ex vivo nucleosidetransportremming (250 ng draflazine/100 ml onderarm/min) werd de vaatweerstand in de onderarm niet significant beinvloed Sterkere remming van het nucleosidetransport in de onderarm gaf aanleiding tot een geringe vaatverwijding In vergelijking met het effect van intra-arterieel toegediend adenosine, is het vaatverwijdend effect van nucleosidetransportremming slechts gering en de fysiologische betekenis ervan is onduidelijk Echter, het optreden van enige vaatverwijding toont wel aan dat er ook in de onderarm extracellulair adenosine wordt gevormd onder normale (= niet ischemische) condities In het tweede experiment, werd bij 25 gezonde mannelijke vrijwilligers het effect van 250 ng draflazine/100 ml onderarm/min bestudeerd op de afgifte van noradrenaline in de onderarm onder basale condities en tijdens stimulatie van het sympathisch zenuwstelsel door het aanbrengen van een onderdruk om het onderlichaam (LBNP) Zowel de lokale (onderarm) als systemische noradrenaline-afgifte werden bepaald met behulp van een tracer-techniek De basale afgifte van noradrenaline in de onderarm *nam toe* tijdens de lokale infusie van draflazine Echter, de LBNP-geinduceerde toename van de noradrenaline afgifte in de onderarm *nam af* tijdens intra-arteriele toediening van draflazine De systemische noradrenaline-afgifte werd niet beinvloed door de lokale'draflazine' toediening, hetgeen aantoont dat de effecten op de afgifte van noradrenaline in de onderarm waarschijnlijk specifiek gerelateerd zijn aan de infusie van draflazine Deze bevindingen ondersteunen de veronderstelling dat de vorming van endogeen adenosine is toegenomen tijdens activatie van het sympathisch zenuwstelsel, resulterend in een presynaptische remming van de noradrenaline afgifte De toegenomen basale afgifte van noradrenaline kan worden verklaard door aan te nemen dat de vuurfrequentie van sympathische zenuwvezels in de geintundeerde onderarm is toegenomen ten gevolge van de stimulatie van lokale afferente zenuwvezels (zie figuur 3 van hoofdstuk 4)

In de volgende vier hoofdstukken wordt de interaktie tussen endotheel en purines bestudeerd. We wilden de betekenis vaststellen van stikstofoxide (NO), een vaatverwijdende substantie die geproduceerd wordt door het endotheel, voor de vaatverwijding die tot stand komt onder invloed van ATP en adenosine Hiertoe werd eerst een pilot studie uitgevoerd om een dosering te vinden van de NO-synthase remmer L-NMMA, die in staat was om het vaatverwijdend effect van acetylcholine te verminderen Acetylcholine is namelijk het prototype NO-afhankelijke vaatverwijder Tijdens het uitvoeren van deze inleidende studie ontdekten we dat methacholine, een muscarinereceptoragonist die regelmatig door onze onderzoeksgroep werd gebruikt als substituut voor het meer labiele acetylcholine, een vaatverwijding in de onderarm veroorzaakt die niet door L-NMMA geremd kon worden Deze bevinding suggereert dat de bijdrage van NO aan het vaatverwijdend effect van methacholine en acetylcholine verschilt voor deze twee stoffen

(hoofdstuk 5) Deze observatie wordt ondersteund door de bevindingen van Chowienczyk et al. [382] maar verschilt van de resultaten van Bruning et al. die in staat waren om ook het vaatverwijdend effect van methacholine te remmen met behulp van L-NMMA-infusie [383]. Deze tegengestelde observaties, die voor ons beschikbaar kwamen na publicatie van onze resultaten, zullen worden bediscussieerd in het licht van de resultaten van onze latere experimenten met L-NMMA (zie onder).

In hoofdstuk 6 worden de experimenten beschreven die werden uitgevoerd om het vaatverwijdend effect van ATP in de onderarm te typeren De belangrijkste doelstelling van deze experimenten was het aantonen van een tweeledig effect van ATP op de vaattonus (zie boven) Ten eerste werd de betekenis van P_1-purinerge receptorstimulatie voor de ATP-geinduceerde vaatverwijding in de onderarm vastgesteld. ATP bleek een veel sterker vaatverwijding te induceren dan equimolaire adenosinedoseringen Verder bleek dat de P_1-purinerge receptorantagonist theophylline de ATP-geinduceerde vaatverwijding niet kon antagoneren Deze resultaten tonen aan dat tijdens intra-arteriele toediening van ATP geen stimulatie optreedt van P_1-purinerge receptoren. Vervolgens probeerden we de ATP-geiduceerde vaatverwijding te remmen met gelijktijdige infusie van L-NMMA. De ATP-geinduceerde vaatverwijding werd niet door L-NMMA geantagoneerd Dit suggereert dat NO niet is betrokken in het vaatverwijdend effect van ATP. De L-NMMA-geinduceerde vasoconstrictie bleek afhankelijk te zijn van het type vaatverwijder dat 50 minuten voor de L-NMMA infusie werd geinfundeerd Na voorbehandeling met acetylcholine, een stof die NO-synthase stimuleert in de onderarm, was de L-NMMA-geinduceerde vasoconstrictie significant sterker dan na voorbehandeling met natrium nitroprusside (SNP, een NO donor zonder stimulerend effect op NO-synthase) of voorbehandeling met ATP. Waarschijnlijk wordt dit effect van voorbehandeling veroorzaakt door aanhoudende activatie van NO-synthase na acetylcholine-infusie. Interessant is dat de onderarmsdoorbloeding was teruggekeerd naar het uitgangsniveau voordat L-NMMA werd geinfundeeerd Dit suggereert dat andere (endotheliale?) factoren het effect van toegenomen NO-produktie tegenwerken De overeenkomst tussen SNP- en ATP-voorbehandeling op de L-NMMA-geinduceerde vasoconstrictie ondersteunt onze conclusie dat NO niet is betrokken in de ATP-geinduceerde vaatverwijding Tenslotte probeerden we het vaatverwijdend effect van ATP te reduceren door infusie van extreem hoge doseringen ATP. Gebaseerd op de resultaten van in vitro experimenten, verwachtten we een gereduceerde vaatverwijding te vinden tijdens infusie van de hoogste ATP-doseringen vanwege gelijktijdige activatie van P_{2x}-purinerge receptoren en/of afgifte van endotheliale vasoconstrictieve factoren Echter, zelfs tijdens infusie van zeer hoge ATP-doseringen was het vaatverwijdend effect niet gereduceerd. De afbraak van ATP door het endotheel voorkomt dat luminaal toegediend ATP de veronderstelde P_{2x}-purinerge receptor op

gladde spiercellen bereikt en dit verklaart waarschijnlijk waarom wij niet in staat waren om het bestaan van P_{2x}-purinerge receptoren in het vaatbed van de onderarm aan te tonen

Aangespoord door de eerder genoemde tegengestelde resultaten met betrekking tot de interaktie tussen L-NMMA en methacholine, besloten we onze gegevens opnieuw te analyseren door de vasculaire effecten van acetylcholine, methacholine en natrium nitroprusside met elkaar te vergelijken waarbij we ons concentreerden op het effect van voorbehandeling met deze vaatverwijdende stoffen op het vasoconstrictief effect van L-NMMA (tabel 1) Deze analyse bracht aan het licht dat het vasoconstrictief effect van L-NMMA na voorbehandeling met methacholine niet significant verschilt met het effect van L-NMMA na voorbehandeling met acetylcholine Deze bevinding wijst erop dat beide stoffen in staat zijn tot activatie van NO-synthase hetgeen overeenstemt met de gegevens van Bruning et al [383] Hoe is dan het verschil tussen beide studies te verklaren voor wat betreft het effect van L-NMMA op de methacholine-geinduceerde vaatverwijding? Waarschijnlijk wordt dit verschil verklaard door een verschil in de wijze waarop de resultaten worden gepresenteerd Bruning et al berekenden plasmaconcentraties van acetylcholine en methacholine op basis van infusiesnelheid en bloeddoorstroming (plasma flow) Zij deden dit om te corrigeren voor het effect van L-NMMA op de uitgangsflow waardoor de concentratie van de vaatverwijdende stof in de bloedbaan wordt beinvloed (er treedt minder verdunning op) Daarbij veronderstelt men dat de afbraak van de geinfundeerde stof in de onderarm niet wordt beinvloed door de stroomsnelheid van het bloed Echter, deze aanname is, met name voor labiele stoffen zoals acetylcholine, ATP en SNP, waarschijnlijk niet juist Bijvoorbeeld, voor ATP is beschreven dat lokaal meer ATP wordt afgebroken wanneer de doorbloeding afneemt [384] Wanneer we een zelfde effect van de doorbloeding veronderstellen op de lokale afbraak van acetylcholine, dan zal, onder invloed van L NMMA, het effect van de verminderde verdunning op de uiteindelijke acetylcholineconcentratie (gedeeltelijk) worden tegengegaan door de toegenomen afbraak Aangezien methacholine stabieler is dan acetylcholine, wordt de uiteindelijke concentratie van methacholine minder door degradatie beinvloed Hierdoor hebben Bruning et al het effect van L-NMMA op de acetylcholine-geinduceerde vaatverwijding waarschijnlijk overschat, terwijl wij het effect van L-NMMA op de methacholine-geinduceerde vaatverwijding hebben onderschat Het voordeel van de analyse zoals beschreven in tabel 1 is dat de uitgangswaarde van de onderarmsdoorbloeding gelijk was voor de drie groepen Hoewel deze analyse aantoont dat zowel methacholine als acetylcholine de produktie van NO stimuleren, kan niet worden uitgesloten dat het vaatverwijdend mechanisme verschillend is voor deze twee stoffen Er is meer onderzoek nodig om deze vraag te kunnen beantwoorden

TABEL 1: Effect van L-NMMA op de vaatweerstand van de geïnfundeerde onderarm (gemiddelde ± SE)

Vaatverwijder waarmee werd voorbehandeld	Aantal proefpersonen	Uitgangs FVR (AU)	% Stijging in FVR tijdens eerste L-NMMA infusie	% Stijging in FVR tijdens tweede L-NMMA infusie
ACh	10	59 ± 11	64 ± 13*	95 ± 17*#
MCh	6	51 ± 10	37 ± 16	86 ± 14*#
SNP	6	59 ± 8	33 ± 11*	41 ± 7*

L-NMMA betekent N^G-monomethyl-L-arginine; ACh, acetylcholine; MCh, methacholine; SNP, natrium nitroprusside; en FVR, onderarms vaatweerstand. *, Statistisch significant verschil met uitgangswaarde ($P<0.05$); #, Statistisch significant verschil met SNP groep ($P<0.05$). In de controle arm werd de FVR niet significant beïnvloed door de infusie van vasoactieve stoffen. Voor een beschrijving van de statistische analyse: zie hoofdstuk 6.

In hoofdstuk 7 werd de rol van NO in de adenosine-geïnduceerde vaatverwijding onderzocht. L-NMMA remde het vaatverwijdend effect van adenosine. Dit betekent dat het vaatverwijdend effect van luminaal aangeboden adenosine (gedeeltelijk) wordt gemediëerd door activatie van NO-synthase waardoor het endotheel meer NO gaat afgeven. Tevens werd de rol van kaliumkanalen in de adenosine-geïnduceerde vaatverwijding bestudeerd met behulp van de kaliumkanaal-blokkerende middelen tolbutamide en kinidine. Tolbutamide had geen effect op de vaatverwijding tijdens adenosineinfusie. ATP-afhankelijke kaliumkanalen spelen dus waarschijnlijk geen rol in het vaatverwijdend effect van luminaal aangeboden adenosine. Met kinidine, een minder selectief kaliumkanaal-blokkerend middel, werden vergelijkbare resultaten verkregen.

Een verminderde respons op adenosine zou nadelig kunnen zijn tijdens ischemie. In diermodellen van insuline-afhankelijke diabetes mellitus is een verminderd vaatverwijdend effect van adenosine in de coronairvaten gevonden. Aangezien diabetes mellitus een onafhankelijke risikofaktor is voor het krijgen van hart- en vaatziekten, zou een verminderde gevoeligheid voor adenosine bij de mens van klinische betekenis kunnen zijn. Daarom evalueerden we het vaatverwijdend effect van adenosine bij patiënten met ongecompliceerde insuline-afhankelijke diabetes mellitus en vergeleken we de resultaten met een leeftijd-gewogen controlegroep (hoofdstuk 8). Het vaatverwijdend effect van adenosine in de onderarm was bij de patiënten met suikerziekte niet verminderd. Het verschil tussen de dierexperimentele gegevens en onze resultaten kan waarschijnlijk worden verklaard door verschillen in insulineconcentratie: in de dierexperimentele studies werd een verminderde gevoeligheid voor adenosine alleen vastgesteld bij insulinopene dieren terwijl in onze patiëntengroep hoge insulineconcentraties werden gemeten in perifere bloedmonsters.

CONCLUSIES

1. Een hoge mate van systemische nucleosidetransportremming (ex vivo nucleosidetransportremming > 50%) induceert een dosis-afhankelijke verhoging in hartfrequentie, systolische bloeddruk, plasmacatecholamineconcentratie en ventilatie. De P_1-purinerge receptorantagonist coffeine antagoneert de neurohumorale en hemodynamische effecten, hetgeen de betrokkenheid van adenosinereceptorstimulatie aantoont.
2. Systemische matige nucleosidetransportremming versterkt het effect van intra-arterieel toegediend adenosine op de onderarmsdoorbloeding.
3. In tegenstelling tot systemische nucleosidetransportremming, induceert lokale remming van het nucleosidetransport in de onderarm een lokale vaatverwijdende respons. Dit toont aan dat de lokale effecten worden tegengewerkt door de systemische effecten van endogeen adenosine.
4. Wanneer een matige nucleosidetransportremming lokaal in de onderarm wordt toegepast, dan blijkt de basale afgifte van noradrenaline in de onderarm toe te nemen. Dit effect ontstaat waarschijnlijk door lokale activatie van afferente zenuwvezels resulterend in een toename van de vuurfrequentie van efferente sympathische zenuwvezels in de onderarm. Hier staat tegenover dat matige nucleosidetransportremming in de onderarm de toename van noradrenaline-afgifte onder invloed van activatie van het sympathisch zenuwstelsel vermindert Deze bevinding ondersteunt de hypothese dat endogeen adenosine de afgifte van noradrenaline door sympathische zenuwuiteinden bij de mens remt
5. Bij gezonde vrijwilligers induceert lokale ATP infusie een vaatverwijdend effect dat al optreedt bij ATP concentraties in het nanomolaire bereik Deze vaatverwijding is niet verminderd tijdens infusie van extreem hoge doseringen ATP
6 Adenosine- maar niet ATP-geïnduceerde vaatverwijding in de onderarm wordt gemedieerd door stikstofoxide (NO).
7. Het effect van adenosine op de doorbloeding van de onderarm blijft in stand bij patienten met ongecompliceerde insuline-afhankelijke diabetes mellitus die behandeld worden met insuline-injecties

REFERENCES

1. Drury AN, Szent-Gyorgyi A: The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J Physiol (Lond)* 1929;68 213-237
2. Zipf K: Uber die physiologische und pharmakologische bedeutung kreislaufwirksamer, intermediarer stoffwechselprodukte. *Klin Wschr* 1931,10:1521-1523
3. Bennet DW, Drury AN: Further observations relating to the physiological activity of adenine compounds. *J Physiol (Lond)* 1931;72:288-320
4. Zipf K: Die chemische Natur der "depressorischen Substanz" des Blutes. *Arch f exp Pathol u Pharmakol* 1931;160:579-598
5. Olsson RA, Pearson JD: Cardiovascular purinoceptors. *Physiol Rev* 1990;70:761-845
6. Burnstock G: Classification and characterization of purinoceptors, in Jacobson KA, Daly JW, Manganiello V (ed):*Purines in cellular signaling - Targets for new drugs*. New York, Springer-Verlag, 1990, pp 241-253
7. Edwards FA, Gibb AJ, Colquhoun D ATP Receptor-mediated synaptic currents in the central nervous system. *Nature* 1992;359:144-147
8. Salmon JE, Cronstein BN Fc gamma receptor-mediated functions in neutrophils are modulated by adenosine receptor occupancy. A_1-receptors are stimulatory and A_2-receptors are inhibitory. *J Immunol* 1990;145:2235-2240
9. Priebe T, Platsoucas CD, Nelson JA Adenosine receptors and modulation of natural killer cell activity by purine nucleosides. *Cancer Res* 1990,50:4328-4331
10. Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams M: The adenosine/neutrophil paradox resolved: human neutrophils possess both A_1- and A_2-receptors that promote chemotaxis and inhibit O_2 generation, respectively. *J Clin Invest* 1990,85 1150-1157
11. Axtell RA, Sandborg RR, Smolen JE, Ward PA, Boxer LA: Exposure of human neutrophils to exogenous nucleotides causes elevation in intracellular calcium, transmembrane calcium fluxes, and an alteration of a cytosolic factor resulting in enhanced superoxide production in response to FMLP and arachidonic acid. *Blood* 1990;75.1324-1332
12. Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G: Neutrophil adherence to endothelium is enhanced via adenosine A_1-receptors and inhibited via adenosine A_2-receptors. *J Immunol* 1992,148:2201-2206
13. Cronstein BN, Rosenstein ED, Kramer SB, Weissmann G, Hirschhorn R. Adenosine a Physiologic modulator of superoxide anion generation by human neutrophils. Adenosine acts via an A_2-receptor on human neutrophils *J Immunol* 1985,135 1366-1371
14. Spielman WS, Arend LJ: Adenosine receptors and signaling in the kidney. *Hypertension* 1991;17:117-130
15. Burnstock G, Sneddon P: Evidence for ATP and noradrenaline as cotransmitters in sympathetic nerves. *Clin Sci* 1985;68 Suppl 10.89s-92s
16. Burnstock G: Sympathetic purinergic transmission in small blood vessels. *Trends Pharmacol Sci* 1988;9:116-117
17. de Mey JG, Claeys M, Vanhoutte PM Endothelium-dependent inhibitory effects of acetylcholine, adenosine triphosphate, thrombin and arachidonic acid in the canine femoral artery. *J Pharmacol Exp Ther* 1982;222:166-173
18. Chand N, Altura BM: Acetylcholine and bradykinin relax intrapulmonary arteries by acting on endothelial cells: role in lung vascular diseases. *Science* 1981,213:1376-1379

19. Cherry PD, Furchgott RF, Zawadzki JV, Jothianandan D: Role of endothelial cells in relaxation of isolated arteries by bradykinin *Proc Natl Acad Sci USA* 1982;79·2106-2110
20. Furchgott RF: Role of endothelium in responses of vascular smooth muscle. *Circ Res* 1983;53:557-573
21. Murad F, Rapaport RM, Fiscus R: Role of cyclic-GMP in relaxations of vascular smooth muscle. *J Cardiovasc Pharmacol* 1985;7 (suppl. 3) S111-S118
22. Palmer RM, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;327:524-526
23. Hutchinson PJ, Palmer RM, Moncada S: Comparative pharmacology of EDRF and nitric oxide on vascular strips. *Eur J Pharmacol* 1987;141 445-451
24. Ignarro LJ, Byrns RE, Buga GM, Wood KS: Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ Res* 1987;61:866-879
25. Palmer RMJ, Ashton DS, Moncada S. Vascular endothelium cells synthesize nitric oxide from L-arginine. *Nature* 1988;333·664-666
26. Schmidt HHHW, Nau H, Wittfoht W, Gerlach J, Prescher K-E, Klein MM, Niroomand F, Bohme E: Arginine is a physiological precursor of endothelium-derived nitric oxide. *Eur J Pharmacol* 1988;154:213-216
27. Rees DD, Palmer RMJ, Hodson HF, Moncada S: A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br J Pharmacol* 1989,96.418-424
28. Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 1990;101:746-752
29. Mulsch A, Bassenge E, Busse R: Nitric oxide synthesis in endothelial cytosol: evidence for a calcium-dependent and a calcium-independent mechanism. *Naunyn Schmiedebergs Arch Pharmacol* 1989;340·767-770
30. Bredt DS, Hwang PM, Snyder SH: Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 1990;347:768-770
31. Kim N, Azadzoi KM, Goldstein I, Saenz de Tejada I: A nitric oxide-like factor mediates noradrenergic-noncholinergic neurogenic relaxation of penile corpus cavernosum smooth muscle. *J Clin Invest* 1991;88:112-118
32. Moncada S, Palmer RM, Higgs EA: Nitric oxide· physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109-142
33. Lopez Jaramillo P, Gonzalez MC, Palmer RM, Moncada S: The crucial role of physiological Ca2+ concentrations in the production of endothelial nitric oxide and the control of vascular tone. *Br J Pharmacol* 1990;101·489-493
34. Petros A, Bennett D, Vallance P: Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* 1991;338·1557-1558
35. Chester AH, O'Neil GS, Moncada S, Tadjkarimi S, Yacoub MH: Low basal and stimulated release of nitric oxide in atherosclerotic epicardial coronary arteries. *Lancet* 1990;336:897-900
36. Vallance P, Collier J, Moncada S· Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* 1989;2:997-1000
37. Rees DD, Palmer RMJ, Moncada S: Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* 1989;86.3375-3378

38. Sakuma I, Togashi H, Yoshioka M, Saito H, Yanagida M, Tamura M, Kobayashi T, Yasuda H, Gross SS, Levi R: NG-Methyl-L-arginine, an inhibitor of L-arginine-derived nitric oxide synthesis, stimulates renal sympathetic nerve activity in vivo: A role for nitric oxide in the central regulation of sympathetic tone? *Circ Res* 1992;70:607-611
39. Lacolley PJ, Lewis SJ, Brody MJ: Role of sympathetic nerve activity in the generation of vascular nitric oxide in urethane-anesthetized rats. *Hypertension* 1991;17:881-887
40. Pohl U, Busse R: Hypoxia stimulates release of endothelium-derived relaxant factor. *Am J Physiol* 1989;256:H1595-H1600
41. Rubanyi GM, Romero JC, Vanhoutte PM: Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol* 1986;250:H1145-H1149
42. Milner P, Kirkpatrick KA, Ralevic V, Toothill V, Pearson J, Burnstock G: Endothelial cells cultured from human umbilical vein release ATP, substance P and acetylcholine in response to increased flow. *Proc R Soc Lond* 1990,241 245-248
43. Burnstock G, Lincoln J, Feher E, Hopwood AM, Kirkpatrick K, Milner P, Ralevic V: Serotonin is localized in endothelial cells of coronary arteries and released during hypoxia: apossible new mechanism for hypoxia-induced vasodilatation of the rat heart. *Experientia* 1988;44:705-707
44. Tesfamariam B, Weisbrod RM, Cohen RA: Endothelium inhibits responses of rabbit carotid artery to adrenergic nerve stimulation. *Am J Physiol* 1987;253:H792-H798
45. Carrier GO, White RE: Enhancement of alpha-1 and alpha-2 adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta. *J Pharmacol Exp Ther* 1985,232:682-687
46. Cocks TM, Angus JA: Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature* 1983;305.627-630
47. Cohen RA, Weisbrod RM. Endothelium inhibits norepinephrine release from adrenergic nerves of rabbit carotid artery. *Am J Physiol* 1988,254 H871-H878
48. Wennmalm A, Benthin G, Karwatowska Prokopczuk E, Lundberg J, Petersson AS: Release of endothelial mediators and sympathetic transmitters at different coronary flow rates in rabbit hearts. *J Physiol (Lond)* 1991;435:163-173
49. Burnstock G: Local mechanisms of blood flow control by perivascular nerves and endothelium. *J Hypertens* 1990;8 (suppl 7):S95-S106
50. Burnstock G, Kennedy C: A dual function for adenosine 5'-triphosphate in the regulation of vascular tone; Excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. *Circ Res* 1985;58:319-330
51. Golino P, Piscione F, Willerson JT, Cappelli-Bigazzi M, Focaccio A, Villari B, Indolfi C, Russolillo E, Condorelli M, Chiariello M. Divergent effects of serotonin on coronary-artery dimensions and blood flow in patients with atherosclerosis and control patients. *N Engl J Med* 1991;324:641-648
52. Radomski MW, Palmer RM, Moncada S: Characterization of the L-arginine:nitric oxide pathway in human platelets. *Br J Pharmacol* 1990;101:325-328
53. Pohl U, Busse R: EDRF increases cyclic GMP in platelets during passage through the coronary vascular bed. *Circ Res* 1989;65:1798-1803
54. Mellion BT, Ignarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, Kadowitz PJ: Evidence for the inhibitory role of guanosine 3',5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 1981;57:946-955

55. Radomski MW, Palmer RM, Moncada S: Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* 1987;ii:1057-1058

56. de Graaf JC, Banga JD, Moncada S, Palmer RMJ, de Groot PG, Sixma JJ: Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation* 1992;85:2284-2290

57. Radomski MW, Palmer RM, Moncada S: Modulation of platelet aggregation by an L-arginine-nitric oxide pathway. *Trends Pharmacol Sci* 1991;12:87-88

58. Radomski MW, Palmer RM, Moncada S: The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol* 1987;92:639-646

59. Houston S, Shepherd JT, Vanhoutte PM: Aggregating human platelets cause direct contraction and endothelium-dependent relaxation of isolated canine coronary arteries; Role of serotonin, thromboxane A_2, and adenine nucleotides. *J Clin Invest* 1986;78:539-544

60. Cohen RA, Shepherd JT, Vanhoutte PM: Inhibitory role of the endothelium in the response of isolated coronary arteries to platelets. *Science* 1983,221:273-274

61. Kaul S, Heistad DD, Mugge A, Armstrong ML, Piegors DJ, Lopez JA: Vascular responses to platelet activation in normal and atherosclerotic primates in vivo. *Arterioscler Thromb* 1991;11:1745-1751

62. Keef KD, Pasco JS, Eckman DM: Purinergic Relaxation and Hyperpolarization in Guinea Pig and Rabbit Coronary Artery - Role of the Endothelium. *J Pharmacol Exp Ther* 1992;260:592-600

63. Taylor SG, Weston AH: Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol Sci* 1988;9:272-274

64. Garland CJ, McPherson GA: Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br J Pharmacol* 1992;105:429-435

65. Forstermann U, Mugge A, Alheid U, Haverich A, Frolich JC: Selective attenuation of endothelium-mediated vasodilation in atherosclerotic human coronary arteries. *Circ Res* 1988;62:185-190

66. Gryglewski RJ, Botting RM, Vane JR: Mediators produced by the endothelial cell. *Hypertension* 1988;12:530-548

67. Myers PR, Guerra R,Jr., Harrison DG: Release of multiple endothelium-derived relaxing factors from porcine coronary arteries. *J Cardiovasc Pharmacol* 1992;20.392-400

68. Rand VE, Garland CJ: Endothelium-dependent relaxation to acetylcholine in the rabbit basilar artery: importance of membrane hyperpolarization. *Br J Pharmacol* 1992;106:143-150

69. Mitchell JA, Denucci G, Warner TD, Vane JR: Different patterns of release of endothelium-derived relaxing factor and prostacyclin. *Br J Pharmacol* 1992;105:485-489

70. Luscher TF, Boulanger CM, Dohi Y, Yang Z: Endothelium-derived contracting factors. *Hypertension* 1992;19:117-130

71. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;332:411-415

72. Milner P, Bodin P, Loesch A, Burnstock G: Rapid release of endothelin and ATP from isolated aortic endothelial cells exposed to increased flow. *Biochem Biophys Res Commun* 1990;170:649-656

73. Vane JR, Anggard EE, Botting RM: Regulatory functions of the vascular endothelium. *N Engl J Med* 1990;323:27-36

74 Kiowski W, Luscher TF, Linder L, Buhler FR. Endothelin-1-induced vasoconstriction in humans; reversal by calcium channel blockade but not by nitrovasodilators or endothelium-derived relaxing factor. *Circulation* 1991,83:469-475

75. Rakugi H, Nakamaru M, Tabuchi Y, Nagano M, Mikami H, Ogihara T. Endothelin stimulates the release of prostacyclin from rat mesenteric arteries. *Biochem Biophys Res Commun* 1989;160:924-928

76. de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, Vane JR: Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor *Proc Natl Acad Sci USA* 1988;85:9797-9800

77. Lerman A, Sandok EK, Hildebrand FL, Burnett JC: Inhibition of endothelium-derived relaxing factor enhances endothelin-mediated vasoconstriction. *Circulation* 1992,85 1894-1898

78 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

79 Badr KF, Murray JJ, Breyer MD, Takahashi K, Inagami T, Harris RC Mesangial cell, glomerular and renal vascular responses to endothelin in the rat kidney, elucidation of signal transduction pathways. *J Clin Invest* 1989;83 336-342

80 Meghji P: Storage, release, uptake, and inactivation of purines *Drug Development Res* 1993;28:214-219

81. Meghji P, Rubio R, Berne RM. Intracellular adenosine formation and its carrier-mediated release in cultured embryonic chick heart cells *Life Sci* 1988,43:1851-1859

82 Pearson J, Carleton J, Gordon J Metabolism of adenine nucleotides by ectoenzymes of vascular endothelial and smooth-muscle cells in culture *Biochem J* 1980,190 421-429

83. Juul B, Aalkjaer C, Plesner L. Ecto-nucleotidase activity of intact isolated small arteries. *Blood Vessels* 1991,28 299

84. Welford LA, Cusack NJ, Hourani SMO: The structure-activity relationships of ectonucleotidases and of excitatory P_2-purinoceptors evidence that dephosphorylation of ATP analogues reduces pharmacological potency. *Eur J Pharmacol* 1987,141:123-130

85. Borst MM, Schrader J. Adenine nucleotide release from isolated perfused guinea pig hearts and extracellular formation of adenosine. *Circ Res* 1991,68:797-806

86 Headrick JP, Matherne GP, Berne RM Myocardial adenosine formation during hypoxia - Effects of ecto-5'-nucleotidase inhibition *J Mol Cell Cardiol* 1992,24 295-303

87. Gorman MW, Bassingthwaighte JB, Olsson RA, Sparks HV. Endothelial cell uptake of adenosine in canine skeletal muscle *Am J Physiol* 1986,250 H482-H489

88 Kroll K, Kelm MK, Burrig KF, Schrader J Transendothelial transport and metabolism of adenosine and inosine in the intact rat aorta *Circ Res* 1989,64:1147-1157

89 Pearson JD, Carleton JS, Hutchings A, Gordon JL: Uptake and metabolism of adenosine by pig aortic endothelial and smooth muscle cells in culture. *Biochem J* 1978;170.265-271

90. Kitakaze M, Hori M, Morioka T, Minamino T, Takashima S, Sato H, Shinozaki Y, Chujo M, Mori H, Inoue M, et al : Alpha 1-adrenoceptor activation mediates the infarct size-limiting effect of ischemic preconditioning through augmentation of 5'-nucleotidase activity *J Clin Invest* 1994,93:2197-2205

91. Ralevic V, Milner P, Kirkpatrick KA, Burnstock G: Flow-induced release of adenosine 5'-triphosphate from endothelial cells of the rat mesenteric arterial bed. *Experientia* 1992;48:31-34

92. Sedaa KO, Bjur RA, Shinozuka K, Westfall DP: Nerve and drug-induced release of adenine nucleosides and nucleotides from rabbit aorta. *J Pharmacol Exp Ther* 1990;252:1060-1067

93. Yang S, Cheek DJ, Westfall DP, Buxton IL: Purinergic axis in cardiac blood vessels. Agonist-mediated release of ATP from cardiac endothelial cells. *Circ Res* 1994;74:401-407

94. Bergfeld GR, Forrester T: Release of ATP from Human Erythrocytes in Response to a Brief Period of Hypoxia and Hypercapnia. *Cardiovasc Res* 1992;26:40-47

95. Van Belle H: Uptake and deamination of adenosine by blood Species differences, effect of pH, ions, temperature and metabolic inhibitors. *Biochim Biophys Acta* 1969;192.124-132

96. Gordon JL: Extracellular ATP: effects, sources and fate *Biochem J* 1986;233.309-319

97. Van Galen PJM, Stiles GL, Michaels G, Jacobson KA: Adenosine A_1- and A_2-receptors: structure-function relationships. *Med Res Rev* 1992;12:423-471

98. Furlong TJ, Pierce KD, Selbie LA, Shine J: Molecular characterization of a human brain adenosine-A_2 Receptor. *Mol Brain Res* 1992;15:62-66

100 Zhou QY, Li CY, Olah ME, Johnson RA, Stiles GL, Civelli O: Molecular cloning and characterization of an adenosine receptor -The A_3-adenosine receptor *Proc Natl Acad Sci USA* 1992;89:7432-7436

101. Burnstock G, Kennedy C: Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen Pharmacol* 1985;16:433-440

102. Ralevic V, Burnstock G: Roles of P_2-purinoceptors in the cardiovascular system. *Circulation* 1991;84:1-14

103. Mathie RT, Ralevic V, Alexander B, Burnstock G: Nitric oxide is the mediator of ATP-induced dilatation of the rabbit hepatic arterial vascular bed. *Br J Pharmacol* 1991;103:1602-1606

104. Boeynaems JM, Pearson JD: P_2-purinoceptors on vascular endothelial cells: physiological significance and transduction mechanisms. *Trends Pharmacol Sci* 1990;11:34-37

105. Mathieson JJ, Burnstock G: Purine-mediated relaxation and constriction of isolated rabbit mesenteric artery are not endothelium-dependent *Eur J Pharmacol* 1985,118 221-229

106. Vuorinen P, Porsti I, Metsaketela T, Manninen V, Vapaatalo H, Laustiola KE: Endothelium-dependent and endothelium-independent effects of exogenous ATP, adenosine, GTP and guanosine on vascular tone and cyclic nucleotide accumulation of rat mesenteric artery. *Br J Pharmacol* 1992,105.279-284

107. Davidson JS, Wakefield IK, Sohnius U, van der Merwe PA, Millar RP: A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology* 1990;126:80-87

108. O'Connor SE, Dainty IA, Leff P: Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol Sci* 1991;12:137-141

109. Vials AJ, Burnstock G: Effects of pyrimidines on the guinea-pig coronary vasculature. *Br J Pharmacol* 1993;110:1091-1097

110. von Kugelgen I, Starke K: Evidence for two separate vasoconstriction-mediating nucleotide receptors, both distinct from the P_2-receptor, in rabbit basilar artery: a receptor for pyrimidine nucleotides and a receptor for purine nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* 1990;341.538-546

111 Mannix RJ, Moatter T, Kelley KA, Gerritsen ME Cellular signaling responses mediated by a novel nucleotide receptor in rabbit microvessel endothelium *Am J Physiol* 1993,265 H675-H680

112 Bo XN, Burnstock G [3H]-alpha, beta-methylene ATP, a radioligand labelling P_2-purinoceptors *J Auton Nerv Syst* 1989,28 85-88

113 Sneddon P, Burnstock G Inhibition of excitatory junction potentials in guinea-pig vas deferens by alpha, beta-methylene-ATP further evidence for ATP and noradrenaline as cotransmitters *Eur J Pharmacol* 1984,100 85-90

114 Dalziel HH, Gray GA, Drummond RM, Furman BL, Sneddon P Investigation of the selectivity of alpha, beta methylene ATP in inhibiting vascular responses of the rat in vivo and in vitro *Br J Pharmacol* 1990,99 820-824

115 Stjarne L, Msghina M, Stjarne E Individual variations in potency of alpha,beta-methylene ATP as inhibitor of the excitatory junction current in mouse vas deferens caused by sympathetic nerve stimulation *Acta Physiol Scand* 1989,137 315-316

116 Cusack NJ, Hourani SM Subtypes of P_2 purinoceptors Studies using analogues of ATP *Ann N Y Acad Sci* 1990,603 172-181

117 Ralevic V, Mathie RT, Alexander B, Burnstock G Characterization of P_{2X}- and P_{2Y}-purinoceptors in the rabbit hepatic arterial vasculature *Br J Pharmacol* 1991,103 1108-1113

118 Mathie RT, Alexander B, Ralevic V, Burnstock G Adenosine-induced dilatation of the rabbit hepatic arterial bed is mediated by A_2-purinoceptors *Br J Pharmacol* 1991,103 1103-1107

119 Merkel LA, Lappe RW, Rivera LM, Cox BF, Perrone MH Demonstration of vasorelaxant activity with an A_1-selective adenosine agonist in porcine coronary artery - Involvement of potassium channels *J Pharmacol Exp Ther* 1992,260 437-443

120 Sabouni MH, Ramagopal MV, Mustafa SJ Relaxation by adenosine and its analogs of potassium-contracted human coronary arteries *Naunyn Schmiedebergs Arch Pharmacol* 1990,341 388-390

121 Parent R, Pare R, Lavallee M Contribution of Nitric Oxide to Dilation of Resistance Coronary Vessels in Conscious Dogs *Am J Physiol* 1992 262 H10-H16

122 Iwamoto T, Umemura S, Toya Y, Uchibori T, Kogi K, Takagi N, Ishii M Identification of adenosine A_2 receptor-cAMP system in human aortic endothelial cells *Biochem Biophys Res Commun* 1994,199 905-910

123 Balcells E, Suarez J, Rubio R Functional role of intravascular coronary endothelial adenosine receptors *Eur J Pharmacol* 1992,210 1-9

124 Headrick JP, Northington FJ, Hynes MR, Matherne GP, Berne RM Relative responses to luminal and adventitial adenosine in perfused arteries *Am J Physiol* 1992,263 H1437-H1446

125 Neely CF, Haile DM, Cahill BE, Kadowitz PJ Adenosine and ATP produce vasoconstriction in the feline pulmonary vascular bed by different mechanisms *J Pharmacol Exp Ther* 1991,258 753-761

126 von Kugelgen I, Schoffel E, Starke K Inhibition by nucleotides acting at presynaptic P_2-receptors of sympathetic neuro-effector transmission in the mouse isolated vas deferens *Naunyn Schmiedebergs Arch Pharmacol* 1989,340 522-532

127 Westfall DP, Shinozuka K, Forsyth KM, Bjur RA Presynaptic purine receptors *Ann N Y Acad Sci* 1990,604 130-135

128 Burnstock G, Crowe R, Kennedy C, Torok J Indirect evidence that purinergic modulation of perivascular adrenergic neurotransmission in the portal vein is a physiological process *Br J Pharmacol* 1984,82 359-368

129 Richardson PJ, Brown SJ, Bailyes EM, Luzio JP Ectoenzymes control adenosine modulation of immunoisolated cholinergic synapses *Nature* 1987,327 232 234

130 Ragazzi E, Wu S-N, Shryock J, Belardinelli L Electrophysiological and receptor binding studies to assess activation of the cardiac adenosine receptor by adenine nucleotides *Circ Res* 1991,68 1035-1044

131 Burnstock G, Meghji P The effect of adenyl compounds on the rat heart *Br J Pharmacol* 1983,79 211-218

132 Belhassen B, Ilia R, Pelleg A, Greenspan A, Horowitz L A comparative study of the electrophysiologic effects of striadyne, adenosine triphosphate and adenosine in the canine heart *Cardiology* 1985,72 113-122

133 Pelleg A, Belhassen B, Ilia R, Laniado S Comparative electrophysiologic effects of adenosine triphosphate and adenosine in the canine heart influence of atropine, propranolol, vagotomy, dipyridamole and aminophylline *Am J Cardiol* 1985,55 571-576

134 Belardinelli L, Berne RM The cardiac effects of adenosine *Prog Cardiovasc Dis* 1989,32 73 97

135 Belardinelli L, Pelleg A Cardiac electrophysiology and pharmacology of adenosine *J Cardiovasc Electrophysiol* 1990,1 327-339

136 DiMarco JP, Sellers TD, Berne RM, West GA, Belardinelli L Adenosine electrophysiologic effects and therapeutic use for terminating paroxysmal supraventricular tachycardia *Circulation* 1983,68 1254-1263

137 Rankin AC, Brooks R, Ruskin JN, Mcgovern BA Adenosine and the treatment of supraventricular tachycardia *Am J Med* 1992,92 655-664

138 Wainwright CL, Parratt JR An antiarrhythmic effect of adenosine during myocardial ischemia and reperfusion *Eur J Pharmacol* 1988,146 183 194

139 Wilber DJ, Bearman J, Olshansky B, Kall J, Kopp D Adenosine-sensitive ventricular tachycardia Clinical characteristics and response to catheter ablation *Circulation* 1993,87 126-134

140 Lerman B Response of nonreentrant catecholamine mediated ventricular tachycardia to endogenous adenosine and acetylcholine Evidence for myocardial receptor-mediated effects *Circulation* 1993,87 382-390

141 Alvarez JL, Mongo K, Scamps F, Vassort G Effects of purinergic stimulation on the Ca current in single frog cardiac cells *Pflugers Arch* 1990,416 189-195

142 Scamps F, Vassort G Mechanism of extracellular ATP-induced depolarization in rat isolated ventricular cardiomyocytes *Pflugers Arch* 1990 417 309-316

143 Sato H, Hori M, Kitakaze M, Takashima S, Inoue M, Kitabatake A, Kamada T Endogenous adenosine blunts beta-adrenoceptor-mediated inotropic response in hypoperfused canine myocardium *Circulation* 1992,85 1594-1603

144 Scamps F, Legssyer A, Mayoux E, Vassort G The mechanism of positive inotropy induced by adenosine triphosphate in rat heart *Circ Res* 1990,67 1007-1016

145 Thompson CI, Spielman WS Renal hemodynamic effects of exogenously administered adenosine and polyadenylic acid *Am J Physiol* 1992,263 F816-F823

146. Okumura M, Miura K, Yamashita Y, Yukimura T, Yamamoto K: Role of endothelium-derived relaxing factor in the in vivo renal vascular action of adenosine in dogs. *J Pharmacol Exp Ther* 1992;260:1262-1267

147. Brown NJ, Porter J, Ryder D, Branch RA: Caffeine potentiates the renin response to diazoxide in man. Evidence for a regulatory role of endogenous adenosine. *J Pharmacol Exp Ther* 1991;256:56-61

148. Taddei S, Arzilli F, Arnghi P, Salvetti A: Dipyridamole decreases circulating renin-angiotensin system activity in hypertensive patients. *Am J Hypertens* 1992;5:29-31

149. Spielman WS, Thompson CI· A proposed role for adenosine in the regulation of renal hemodynamics and renin release. *Am J Physiol* 1982;242:F423-F435

150. Cook CB, Churchill PC: Effects of renal denervation on the renal responses of anesthetized rats to cyclohexyladenosine. *Can J Physiol Pharmacol* 1984;62:934-938

151. Arend LJ, Haramati A, Thompson CI, Spielman WS: Adenosine-induced decrease in renin release: dissociation from hemodynamic effects. *Am J Physiol* 1984,247·F447-F452

152. Spielman WS: Antagonistic effect of theophylline on the adenosine-induced decreased in renin release. *Am J Physiol* 1984,247.F246-F251

153. Kurtz A, Della Bruna R, Pfeilschifter J, Bauer C: Role of cGMP as second messenger of adenosine in the inhibition of renin release. *Kidney Int* 1988,33:798-803

154. Tseng CJ, Kuan CJ, Chu H, Tung CS Effect of caffeine treatment on plasma renin activity and angiotensin I concentrations in rats on a low sodium diet. *Life Sci* 1993;52:883-890

155. Fisher JW, Nakashima J: The role of hypoxia in renal production of erythropoietin. *Cancer* 1992;70.928-939

156. Inscho EW, Ohishi K, Navar LG: Effects of ATP on pre- and postglomerular juxtamedullary microvasculature. *Am J Physiol* 1992,263.F886-F893

157. Edlund A, Fredholm BB, Patrignani P, Patrono C, Wennmalm A, Wennmalm M: Release of two vasodilators, adenosine and prostacyclin, from isolated rabbit hearts during controlled hypoxia. *J Physiol (Lond)* 1983;340.487-501

158. Vanwylen DGL, Williams AG, Downey HF Interstitial purine metabolites and lactate during regional myocardial hypoxia. *Cardiovasc Res* 1993;27:1498-1503

159. Van Belle H, Goossens F, Wynants J: Formation and release of purine catabolites during hypoperfusion, anoxia, and ischemia. *Am J Physiol* 1987;252:H886-H893

160. Van Belle H: Myocardial purines during ischemia, reperfusion and pharmacological protection. *Mol Physiol* 1985;8:615-630

161. Herrmann SC, Feigl EO: Adrenergic blockade blunts adenosine concentration and coronary vasodilation during hypoxia. *Circ Res* 1992,70.1203-1216

162. Toombs CF, Wiltse AL, Shebuski RJ. Ischemic preconditioning fails to limit infarct size in reserpinized rabbit myocardium; implication of norepinephrine release in the preconditioning effect. *Circulation* 1993;88[part 1]:2351-2358

163. Kitakaze M, Hori M, Kamada T: Role of adenosine and its interaction with alpha adrenoceptor activity in ischaemic and reperfusion injury of the myocardium. *Cardiovasc Res* 1993;27:18-27

164. Ely SW, Berne RM: Protective effects of adenosine in myocardial ischemia. *Circulation* 1992;85:893-904

165. Julius S: Sympathetic hyperactivity and coronary risk in hypertension. *Hypertension* 1993;21:886-893

166. Kitakaze M, Hori M, Sato H, Takashima S, Inoue M, Kitabatake A, Kamada T: Endogenous adenosine inhibits platelet aggregation during myocardial ischemia in dogs. *Circ Res* 1991;69:1402-1408

167. Cronstein BN, Levin RI, Belanoff J, Weissmann G, Hirschhorn R: Adenosine: an endogenous inhibitor of neutrophil-mediated injury to endothelial cells. *J Clin Invest* 1986,78:760-770

168. Thornton JD, Liu GS, Olsson RA, Downey JM: Intravenous pretreatment with A_1-selective adenosine analogues protects the heart against infarction. *Circulation* 1992,85 659-665

169. Toombs CF, McGee DS, Johnston WE, Vinten-Johansen J: Myocardial protective effects of adenosine; Infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation* 1992;86:986-994

170. Thornton JD, Thornton CS, Downey JM: Effect of adenosine receptor blockade - Preventing protective preconditioning depends on time of initiation. *Am J Physiol* 1993;265:H504-H508

171. Zhao ZQ, McGee DS, Nakanishi K, Toombs CF, Johnston WE, Ashar MS, Vinten-Johansen J: Receptor-mediated cardioprotective effects of endogenous adenosine are exerted primarily during reperfusion after coronary occlusion in the rabbit. *Circulation* 1993;88:709-719

172. Shizukuda Y, Mallet RT, Lee SC, Downey HF. Hypoxic preconditioning of ischaemic canine myocardium. *Cardiovasc Res* 1992,26:534-542

173. Auchampach JA, Gross GJ: Adenosine A_1-receptors, K_{ATP} channels, and ischemic preconditioning in dogs. *Am J Physiol* 1993,264 H1327-H1336

174. Schjott J, Jynge P, Holten T, Brurok H: Ischaemic episodes of less than 5 minutes produce preconditioning but not stunning in the isolated rat heart. *Acta Physiol Scand* 1994,150.281-291

175. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986,74 1124-1136

176. Deutsch E, Berger M, Kussmaul WG, Hirshfeld JW, Herrmann HC, Laskey WK: Adaptation to ischemia during percutaneous transluminal coronary angioplasty. *Circulation* 1990,82:2044-2051

177. Yellon DM, Alkhulaifi AM, Pugsley WB Preconditioning the human myocardium. *The Lancet* 1993;342:276-277

178. Vaitkus PT, Miller JM, Buxton AE, Josephson ME, Laskey WK: Ischemia-induced changes in human endocardial electrograms during percutaneous transluminal coronary angioplasty. *Am Heart J* 1994;127:1481-1490

179 Ottani F, Galvani M, Ferrini D, Sorbello F, Limonetti P, Pantoli D, Rusticali F: Prodromal angina limits infarct size. A role for ischemic preconditioning *Circulation* 1995,91:291-297

180. Liu GS, Richards SC, Olsson RA, Mullane K, Walsh RS, Downey JM. Evidence that the adenosine A_3-receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovasc Res* 1994;28:1057-1061

181. Armstrong S, Ganote CE: Adenosine receptor specificity in preconditioning of isolated rabbit cardiomyocytes: evidence of A_1-receptor involvement. *Cardiovasc Res* 1994,28:1049-1056

182. Ytrehus K, Liu Y, Downey JM: Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am J Physiol* 1994;266:H1145-H1152

183. Grover GJ, Sleph PG, Dzwonczyk S Role of myocardial ATP-sensitive potassium channels in mediating preconditioning in the dog heart and their possible interaction with adenosine A_1-receptors. *Circulation* 1992;86:1310-1316

184. Gouyon J-B, Guignard J-P: Theophylline prevents the hypoxemia-induced renal hypodynamic changes in rabbits. *Kidney Int* 1988;33:1078-1083

185. Simpson RE, Oregan MH, Perkins LM, Phillis JW: Excitatory transmitter amino acid release from the ischemic rat cerebral cortex - Effects of adenosine receptor agonists and antagonists. *J Neurochem* 1992;58:1683-1690

186. Murray TF, Zhang G, Franklin PH: Manipulation of endogenous adenosine affects seizure susceptibility. *Drug Development Res* 1993,28 410-415

187. Miller LP, Hsu C: Therapeutic potential for adenosine receptor activation in ischemic brain injury. *J Neurotrauma* 1992;9:S563-S577

188. Dixon R, Gourzis J, McDermott D, Fujitaki J, Dewland P, Gruber H: AICA-riboside: safety, tolerance, and pharmacokinetics of a novel adenosine-regulating agent *J Clin Pharmacol* 1991;31:342-347

189. Bolling SF, Groh MA, Mattson AM, Grinage RA, Gallagher KP: Acadesine (AICA-Riboside) improves postischemic cardiac recovery. *Ann Thorac Surg* 1992;54:93-98

190. Dorion M, Rouleau J, Kingma JG: Failure of AICA riboside to limit infarct size during acute myocardial infarction in rabbits. *J Cardiovasc Pharmacol* 1992;19.69-77

191. Williams EF, Barker PH, Clanachan AS: Nucleoside transport in heart. species differences in nitrobenzylthioinosine binding, adenosine accumulation, and drug-induced potentiation of adenosine action. *Can J Physiol Pharmacol* 1984;62:31-37

192. Van Belle H, Janssen PAJ: Comparative pharmacology of nucleoside transport inhibitors. *Nucleosides & Nucleotides* 1991,10 975-982

193. Amoah-Apraku B, Xu J, Lu JY, Pelleg A, Bruns RF, Belardinelli L: Selective potentiation by an A_1-adenosine receptor enhancer of the negative dromotropic action of adenosine in the guinea pig heart. *J Pharmacol Exp Ther* 1993;266.611-617

194. Whitney RJ: The measurement of volume changes in human limbs. *J Physiol (Camb)* 1953;121:1-27

195. Lenders J, Janssen G-J, Smits P, Thien T: Role of the wrist cuff in forearm plethysmography. *Clin Sci* 1991,80:413-417

196. Kubo SH, Rector TS, Bank AJ, Williams RE, Heifetz SM: Endothelium-dependent vasodilation is attenuated in patients with heart failure. *Circulation* 1991,84 1589-1596

197. Creager MA, Cooke JP, Mendelsohn ME, Gallagher SJ, Coleman SM, Loscalzo J, Dzau VJ: Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. *J Clin Invest* 1990;86:228-234

198. Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest* 1992;90:1248-1253

199. Johnstone MT, Creager SJ, Scales KM, Cusco JA, Lee BK, Creager MA: Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *Circulation* 1993;88:2510-2516

200. Durante W, Sunahara FA, Sen AK: Effect of diabetes on metabolic coronary dilatation in the rat. *Cardiovasc Res* 1989;23:40-45

201. Downing SE: Restoration of coronary dilator action of adenosine in experimental diabetes. *Am J Physiol* 1985,249:H102-H107

202. Norton ED, Jackson EK, Turner MB, Virmani R, Forman MB: The effects of intravenous infusions of selective adenosine A_1-receptor and A_2-receptor agonists on myocardial reperfusion injury. *Am Heart J* 1992;123 332-338

203. Mullane K: Myocardial preconditioning - Part of the adenosine revival. *Circulation* 1992;85:845-847

204. Forman MB, Velasco CE, Jackson EK: Adenosine attenuates reperfusion injury following regional myocardial ischemia. *Cardiovasc Res* 1993;27:9-17

205. Cohen MV, Downey JM: Ischaemic preconditioning: can the protection be bottled? ***Lancet*** 1993;342:6

206. Smits P, Boekema P, Thien T, de Abreu R, van 't Laar A: Evidence for an antagonism between caffeine and adenosine in the cardiovascular system in man. *J Cardiovasc Pharmacol* 1987;10:136-143

207. Biaggioni I, Olafsson B, Robertson RM, Hollister AS, Robertson D: Cardiovascular and respiratory effects of adenosine in conscious man. Evidence for chemoreceptor activation. *Circ Res* 1987;61:779-786

208. Van Belle H: Specific metabolically active antiischemic agents: adenosine and nucleoside transport inhibitors, in Singh B, Dzan V, Vanhoutte P, Woosley R (ed):*Cardiovascular Pharmacology and Therapeutics*. New York, Churchill Livingstone, 1993, pp 217-235

209. Miura T, Ogawa T, Iwamoto T, Shimamoto K, Iimura O: Dipyridamole potentiates the myocardial infarct size-limiting effect of ischemic preconditioning. *Circulation* 1992;86:979-985

210. PARIS II Research Group : Persantine-Aspirin reinfarction study. Part II. Secondary coronary prevention with persantine and aspirine. *J Am Coll Cardiol* 1986;7:251-269

211. The Persantine-Aspirin Reinfarction Study Research Group : Persantine and aspirin in coronary heart disease. *Circulation* 1980;62:449-461

212. Blass KE, Block HU, Forster W, Ponicke K: Dipyridamole: a potent stimulator of prostacyclin (PGI_2) biosynthesis. *Br J Pharmacol* 1980;68:71-73

213. McElroy FA, Philp RB: Relative potencies of dipyridamole and related agents as inhibitors of cyclic nucleotide phosphodiesterases: possible explanation of mechanism of inhibition of platelet function. *Life Sci* 1975;17:1479-1493

214. Van Belle H, Verheyen W, Donck KV, Janssen PAJ, Robertson JIS: Prevention of catecholamine-induced cardiac damage and death with a nucleoside transport inhibitor. *J Cardiovasc Pharmacol* 1992;20:173-178

215. Masuda M, Demeulemeester A, Chen CC, Hendrikx M, Van Belle H, Flameng W: Cardioprotective effects of nucleoside transport inhibition in rabbit hearts. *Ann Thorac Surg* 1991;52:1300-1305

216. Wainwright CL, Parratt JR, Van Belle H: The antiarrhythmic effects of the nucleoside transporter inhibitor, R75231, in anaesthetized pigs. *Br J Pharmacol* 1993;109:592-599

217. Smits P, Straatman C, Pijpers E, Thien T: Dose-dependent inhibition of the hemodynamic response to dipyridamole by caffeine. *Clin Pharmacol Ther* 1991;50:529-537

218. Smits P, Hoffmann H, Thien T, Houben H, van 't Laar A: Hemodynamic and humoral effects of coffee after beta-1-selective and nonselective beta-blockade. *Clin Pharmacol Ther* 1983;34:153-158

219. van der Hoorn FAJ, Boomsma F, Man in 't Veld AJ, Schalekamp MADH: Determination of catecholamines in human plasma by high-performance liquid chromatography: comparison between a new method with fluorescence detection and an established method with electrochemical detection. *J Chromatography* 1989;487:17-28

220 de Abreu RA, van Baal JM High-performance liquid chromatographic determination of purine and pyrimidine bases, ribonucleosides, deoxyribonucleosides and cyclic ribonucleotides in biological fluids *J Chromatography* 1982,229 67-75

221 Smits P, Lenders JW, Thien T Caffeine and theophylline attenuate adenosine-induced vasodilation in humans *Clin Pharmacol Ther* 1990,48 410 418

222 Kubo T, Su C Effects of adenosine on [3H]norepinephrine release from perfused mesenteric arteries of SHR and renal hypertensive rats *Eur J Pharmacol* 1983,87 349-352

223 Smits P, Lenders JW, Willemsen JJ, Thien T Adenosine attenuates the response to sympathetic stimuli in humans *Hypertension* 1991,18 216-223

224 Taddei S, Virdis A, Favilla S, Salvetti A Adenosine activates a vascular renin-angiotensin system in hypertensive subjects *Hypertension* 1992,19 672-675

225 Biaggioni I Contrasting excitatory and inhibitory effects of adenosine in blood pressure regulation *Hypertension* 1992,20 457-465

226 Katholi RE, Hageman GR, Whitlow PL, Woods WT Hemodynamic and afferent renal nerve responses to intrarenal adenosine in the dog *Hypertension* 1983,5 1149 1154

227 Katholi RE, Whitlow PL, Hageman GR, Woods WT Intrarenal adenosine produces hypertension by activating the sympathetic nervous system via the renal nerves in the dog *J Hypertens* 1984,2 349-359

228 Watt AH, Reid PG, Stephens MR, Routledge PA Adenosine induced respiratory stimulation in man depends on site of infusion Evidence for an action on the carotid body? *Br J Clin Pharmacol* 1987,23 486-490

229 Costa F, Biaggioni I Role of adenosine in the sympathetic activation produced by isometric exercise in humans *J Clin Invest* 1994,93 1654 1660

230 Biaggioni I, Killian TJ, Mosqueda Garcia R Robertson RM, Robertson D Adenosine increases sympathetic nerve traffic in humans *Circulation* 1991,83 1668-1675

231 Biaggioni I, Onrot J, Hollister AS, Robertson D Cardiovascular effects of adenosine infusion in man and their modulation by dipyridamole *Life Sci* 1986,39 2229-2236

232 Fuller RW, Maxwell DL, Conradson TBG, Dixon CMS, Barnes PJ Circulatory and respiratory effects of infused adenosine in conscious man *Br J Clin Pharmacol* 1987,24 309-317

233 Edlund A, Sollevi A, Linde B Haemodynamic and metabolic effects of infused adenosine in man *Clin Sci* 1990,79 131-138

234 Reid PG, Watt AH, Routledge PA, Smith AP Intravenous infusion of adenosine but not inosine stimulates respiration in man *Br J Clin Pharmacol* 1987,23 331-338

235 Conradson TB, Clarke B, Dixon CM, Dalton RN, Barnes PJ Effects of adenosine on autonomic control of heart rate in man *Acta Physiol Scand* 1987,131 525-531

236 Fukunaga AF, Flacke WE, Bloor BC Hypotensive effects of adenosine and adenosine triphosphate compared with sodium nitroprusside *Anesth Analg* 1982,61 273-278

237 Lappe RW, Sheldon JH, Cox BF Selective adenosine-2 agonist produces both direct and reflex tachycardia in normotensive rats *J Cardiovasc Pharmacol* 1992,19 460-463

238 Sollevi A, Lagerkranser M, Irestedt L, Gordon E, Lindquist C Controlled hypotension with adenosine in cerebral aneurysm surgery *Anesthesiology* 1984,61 400-405

239 Conradson TBG, Dixon CMS, Clarke B, Barnes PJ Cardiovascular effects of infused adenosine in man potentiation by dipyridamole *Acta Physiol Scand* 1987,129 387-391

240 Maxwell DL, Fuller RW, Conradson TB, Dixon CMS, Aber V, Hughes JMB, Barnes PJ Contrasting effects of two xanthines, theophylline and enprofylline, on the cardio-respiratory stimulation of infused adenosine in man *Acta Physiol Scand* 1987,131 459-465

241 Bush A, Busst CM, Clarke B, Barnes PJ Effect of infused adenosine on cardiac output and systemic resistance in normal subjects *Br J Clin Pharmacol* 1989,27 165-171

242 Smits P, Thien T, van 't Laar A Circulatory effects of coffee in relation to the pharmacokinetics of caffeine *Am J Cardiol* 1985,56 958-963

243 Lucarini AR, Picano E, Marini C, Favilla S, Salvetti A, Distante A Activation of sympathetic tone during dipyridamole test *Chest* 1992,102 444-447

244 Sollevi A, Ostergren J, Fagrell B, Hjemdahl P Theophylline antagonizes cardiovascular responses to dipyridamole in man without affecting increases in plasma adenosine *Acta Physiol Scand* 1984,121 165-171

245 Stringer KA, Branconi JM, Abadier R, Wilton JH, Royal SH Disposition of oral dipyridamole in patients undergoing Thalium 201 myocardial imaging *Pharmacotherapy* 1992,12 83-87

246 Rardon DP, Kovacs RJ, Bailey JC Adenosine and prostacyclin independent electrophysiological effects of dipyridamole in guinea-pig papillary muscles and canine cardiac Purkinje fibers *J Pharmacol Exp Ther* 1984,231 206-213

247 Biaggioni I, Paul S, Puckett A, Arzubiaga C Caffeine and theophylline as adenosine receptor antagonists in humans *J Pharmacol Exp Ther* 1991,258 588-593

248 Smits P, Schouten J, Thien T Respiratory stimulant effects of adenosine in man after caffeine and enprofylline *Br J Clin Pharmacol* 1987,24 816-819

249 Fredholm BB, Lindgren E Inhibition of soluble 5'-nucleotidase from rat brain by different xanthine derivatives *Biochem Pharmac* 1983,32 2832 2834

250 Feldman MD, Ayers CR, Lehman MR, Taylor HE, Gordon VL, Sabia PJ, Ras D, Skalak TC, Linden J Improved detection of ischemia-induced increases in coronary sinus adenosine in patients with coronary artery disease *Clin Chem* 1992,38 256-262

251 German DC, Kredich NM, Bjornsson TD Oral dipyridamole increases plasma adenosine levels in human beings *Clin Pharmacol Ther* 1989,45 80-84

252 German DC, Kredich NM A radioenzymatic assay for plasma adenosine *Anal Biochem* 1984,142 536-541

253 Mohrman DE, Heller LJ Transcapillary adenosine transport in isolated guinea pig and rat hearts. *Am J Physiol* 1990,259 H772-H783

254 Van Belle H Nucleoside transport inhibition a therapeutic approach to cardioprotection via adenosine? *Cardiovasc Res* 1993,27 68-76

255 Mosqueda Garcia R, Tseng CJ, Biaggioni I, Robertson RM, Robertson D Effects of caffeine on baroreflex activity in humans *Clin Pharmacol Ther* 1990,48 568-574

256 von Borstel RW, Wurtman RJ, Conlay LA Chronic caffeine consumption potentiates the hypotensive action of circulating adenosine *Life Sci* 1983,32 1151-1158

257 Robertson D, Wade D, Workman R, Woosley RL Tolerance to the humoral and hemodynamic effects of caffeine in man *J Clin Invest* 1981,67 1111-1117

258 Parsons ST, Griffiths TL, Christie JM, Holgate ST Effect of theophylline and dipyridamole on the respiratory response to isocapnic hypoxia in normal human subjects *Clin Sci* 1991,80 107-112

259 Barraco R, Ergene E, El-Ridi M Purinergic receptors in the nucleus tractus solitarius mediate distinct cardiorespiratory response patterns *Drug Development Res* 1993,28 309-314

260. Jonzon B, Sylven C, Beermann B, Brandt R. Adenosine receptor mediated stimulation of ventilation in man. *Eur J Clin Invest* 1989,19:65-71

261. Watt AH, Routledge PA: Dipyridamole modulation of heart rate and ventilatory changes produced by intravenous adenosine boluses in man. *Br J Clin Pharmac* 1987;23:631P-632P

262. Engelstein ED, Somers V, Clary MP, Leman BB, Rea RF: Endogenous adenosine uptake inhibition increases muscle sympathetic nerve activity in conscious humans *Circulation* 1992;86·I-368-I-368 (Abstract)

263. Mian R, Marshall JM: The role of adenosine in dilator responses induced in arterioles and venules of rat skeletal muscle by systemic hypoxia *J Physiol Lond* 1991;443·499-511

264. IJzerman AP, Thedinga KH, Custers AFCM, Hoos B, Van Belle H Inhibition of nucleoside transport by a new series of compounds related to lidoflazine and mioflazine *Eur J Pharmacol - Mol Pharmacol Section* 1989;172.273-281

265. Rongen GA, Smits P, Willemsen J, Ver Donck K, Van Belle H, Thien Th· High grade adenosine uptake inhibition stimulates the sympathetic nervous system in man *J Hypertens* 1994;12 (suppl. 3):S160 (Abstract)

266. Rongen GA, Smits P, Van Belle H, Thien T Low grade nucleoside transport inhibition potentiates the in-vivo effect of adenosine in man at a site of increased supply *J Hypertens* 1994,12 (suppl. 3).S160 (Abstract)

267. von Kugelgen I, Spath L, Starke K Stable adenine nucleotides inhibit <H-3>-noradrenaline release in rabbit brain cortex slices by direct action at presynaptic adenosine A_1-receptors. *Naunyn-Schmied Arch Pharmacol* 1992,346:187-196

268. Wennmalm M, Fredholm BB, Hedqvist P Adenosine as a modulator of sympathetic nerve-stimulation-induced release of noradrenaline from the isolated rabbit heart. *Acta Physiol Scand* 1988;132:487-494

269. Taddei S, Pedrinelli R, Salvetti A: Sympathetic nervous system-dependent vasoconstriction in humans. Evidence for mechanistic role of endogenous purine compounds. *Circulation* 1990;82:2061-2067

270. Engelstein ED, Lerman BB, Somers VK, Rea RF Role of arterial chemoreceptors in mediating the effects of endogenous adenosine on sympathetic nerve activity *Circulation* 1994,90 2919-2926

271. Rongen GA, Smits P, Ver Donck K, Willemsen JJ, de Abreu RA, Van Belle H, Thien T: Hemodynamic and neurohumoral effects of various grades of selective adenosine transport inhibition in humans. Implications for its future role in cardioprotection. *J Clin Invest* 1995;95:658-668

272. Myers HA, Honig CR: Influence of initial resistance on magnitude of response to vasomotor stimuli. *Am J Physiol* 1969,216:1429-1436

273. Grossman E, Chang PC, Hoffman A, Tamiat M, Kopin IJ, Goldstein DS: Tracer norepinephrine kinetics: dependence on regional blood flow and the site of infusion. *Am J Physiol* 1991;260.R946-R952

274. Esler M, Jennings G, Korner P, Willett I, Dudley F, Hasking G, Anderson W, Lambert G: Assessment of human sympathetic nervous system activity from measurements of norepinephrine turnover. *Hypertension* 1988;11:3-20

275. Chang PC, Kriek E, Brummelen van P: Sympathetic activity and presynaptic adrenoceptor function in patients with longstanding essential hypertension. *J Hypertension* 1994,12·179-190

276 Rongen GA, Smits P, Bootsma G, Ver Donck K, de Vries A, Thien T High-grade nucleoside transport inhibition stimulates ventilation in humans *J Clin Pharmacol* 1995,35 357-361

277 Clarke J, Benjamin N, Larkin S, Webb D, Maseri A, Davies G Interaction of neuropeptide Y and the sympathetic nervous system in vascular control in man *Circulation* 1991,83 774-777

278 Taddei S, Salvetti A, Pedrinelli R Persistence of sympathetic-mediated forearm vasoconstriction after alpha-blockade in hypertensive patients *Circulation* 1989,80 485-490

279 Deussen A, Moser G, Schrader J Contribution of coronary endothelial cells to cardiac adenosine production *Pflugers Arch* 1986,406 608-614

280 Schutz W, Schrader J, Gerlach E Different sites of adenosine formation in the heart *Am J Physiol* 1981,240 H963-H970

281 Burnstock G, Griffith SG, Sneddon P Autonomic nerves in the precapillary vessel wall *J Cardiovasc Pharmacol* 1984,6 Suppl 2 S344-S353

282 Linder L, Kiowski W, Buhler FR, Luscher TF Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo, blunted response in essential hypertension *Circulation* 1990,81 1762-1767

283 Mann J, Farrukh IS, Michael JR Mechanisms by which endothelin 1 induces pulmonary vasoconstriction in the rabbit *J Appl Physiol* 1991,71 410-416

284 Furchgott RF, Zawadski JV The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine *Nature* 1980,288 373-376

285 Griffith TM, Edwards DH, Lewis MJ, Newby AC, Henderson AH The nature of endothelium-derived vascular relaxant factor *Nature* 1984,308 645-647

286 Vallance P, Collier J, Moncada S Nitric oxide synthesised from L-arginine mediates endothelium dependent dilatation in human veins in vivo *Cardiovasc Res* 1989,23 1053-1057

287 Taylor P Cholinergic agonists, in Goodman Gilman A, Rall TW, Nies AS, Taylor P (ed) *Goodman and Gilman s The Pharmacological basis of therapeutics* New York, Pergamon press, 1990, pp 122-130

288 Molitor H A comparative study on the effects of five choline compounds used in therapeutics acetylcholine chloride,acetyl beta-methylcholine chloride, carbaminoyl choline, ethyl ether beta-methylcholine chloride, carbaminoylbeta-methylcholine chloride *J Pharmacol Exp Ther* 1936,58 337-360

289 Smits P, Kapma J-A, Jacobs M C, Lutterman J, Thien T Endothelium-dependent vascular relaxation in patients with type I diabetes mellitus *Diabetes* 1992,(In Press)

290 Chowienczyk PJ, Watts GF, Cockcroft JR, Ritter JM Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia *Lancet* 1992,340 1430-1432

291 Rongen GA, Jacobs MC, Smits P, Thien T Endothelium dependent vasodilation in hypercholesterolaemic subjects *Lancet* 1993,341 490-491 (letter)

292 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

293 Bogle RG, Moncada S, Pearson JD, Mann GE Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter *Br J Pharmacol* 1992,105 768-770

294 Duckles SP, Garcia Villalon AL Characterization of vascular muscarinic receptors rabbit ear artery and bovine coronary artery *J Pharmacol Exp Ther* 1990,253 608-613

295 Bungardt E, Vockert E, Feifel R, Moser U, Tacke R, Mutschler E, Lambrecht G, Surprenant A: Characterization of muscarinic receptors mediating vasodilation in guinea-pig ileum submucosal arterioles by the use of computer-assisted videomicroscopy. *Eur J Pharmacol* 1992,213:53-61

296. Houston DA, Burnstock G, Vanhoutte PM: Different P_2-purinergic receptor subtypes of endothelium and smooth muscle in canine blood vessels. *J Pharmacol Exp Ther* 1987,241:501-506

297. Martin GN, Thom SAM, Sever PS: The effects of adenosine triphosphate (ATP) and related purines on human isolated subcutaneous and omental resistance arteries. *Br J Pharmacol* 1991;102:645-650

298. Duff F, Patterson C, Shepherd JT: A quantitative study of the response to adenosine triphosphate of the blood vessels of the human hand and forearm *J Physiol (Lond)* 1954,125 581-589

299. Imaizumi T, Takeshita A, Suzuki S, Yoshida M, Ando S, Nakamura M Age-independent forearm vasodilatation by acetylcholine and adenosine 5'-triphosphate in humans. *Clin Sci* 1990;78:89-93

300. Kennedy C, Burnstock G: ATP produces vasodilation via P_1-purinoceptors and vasoconstriction via P_2-purinoceptors in the isolated rabbit central ear artery. *Blood Vessels* 1985,22 145-155

301. Moody CJ, Meghji P, Burnstock G: Stimulation of P_1-purinoceptors by ATP depends partly on its conversion to AMP and adenosine and partly on direct action. *Eur J Pharmacol* 1984;97:47-54

302. Collis MG, Pettinger SJ· Can ATP stimulate P1-receptors in guinea-pig atrium without conversion to adenosine? *Eur J Pharmacol* 1982;81 521-529

303. Taddei S, Pedrinelli R, Salvetti A· Theophylline is an antagonist of adenosine in human forearm arterioles *Am J Hypertens* 1991;4 256-259

304. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ, Gruetter CA: Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* 1981;218.739-749

305. Rongen GA, Smits P, Thien T: N^G-monomethyl-L-ARG reduces the forearm vasodilator response to acetylcholine but not to methacholine in humans. *J Cardiovasc Pharmacol* 1993,22:884-888

306. Mombouli JV, Vanhoutte PM· Purinergic endothelium-dependent and-independent contractions in rat aorta *Hypertension* 1993;22 577-583

307. Ralevic V, Burnstock G. Effects of Purines and Pyrimidines on the Rat Mesenteric Arterial Bed. *Circ Res* 1991;69:1583-1590

308. Pedrinelli R, Taddei S, Spessot M, Salvetti A: Maximal post-ischaemic forearm vasodilation in human hypertension: a re-assessment of the method. *J Hypertens* 1987,5 (suppl 5).S431-S433

309 Pedrinelli R, Spessot M, Salvetti A: Reactive hyperemia during short-term blood flow and pressure changes in the hypertensive forearm. *J Hypertens* 1990;8:467-471

310. Chang PC, van Brummelen P: Calibration and variability of forearm blood flow, measured by strain-gauge plethysmography. *J Cardiovasc Pharmacol* 1987;10 (Suppl. 5) S123-S125

311. Born GV, Kratzer MA: Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. *J Physiol (Lond)* 1984,354 419-429

312. Kennedy C, Burnstock G· Evidence for two types of P_2-purinoceptor in longitudinal muscle of the rabbit portal vein. *Eur J Pharmacol* 1985;111:49-56

313. Buxton IL, Cheek DJ, Eckman D, Westphall DP, Sanders KM, Keef KD NG-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarine receptor antagonists. *Circ Res* 1993;72:387-395

314. Dominiczak AF, Quilley J, Bohr F: Contraction and relaxation of rat aorta in response to ATP. *Am J Physiol* 1991;261:H243-H251

315. Leipert B, Becker BF, Gerlach E: Different endothelial mechanisms involved in coronary responses to known vasodilators. *Am J Physiol* 1992;262:H1676-H1683

316. Conway J: A vascular abnormality in hypertension; a study of blood flow in the forearm. *Circulation* 1963;27:520-529

317. Juul B, Luscher ME, Aalkjaer C, Plesner L: Nucleotide hydrolytic activity of isolated intact rat mesenteric small arteries. *Biochim Biophys Acta* 1991;1067.201-207

318. Olsson RA, Bunger R: Metabolic control of coronary blood flow. *Progr Cardiovasc Dis* 1987;29:369-387

319. White DG, Drew GM, Gurden JM, Penny DM, Roach AG, Watts IS. The effect of N^G-nitro-L-arginine ester upon basal blood flow and endothelium-dependent vasodilation in the dog hindlimb. *Br J Pharmacol* 1993;108:763-768

320. Treasure CB, Vita JA, Cox DA, Fish D, Gordon JB, Mudge GH, Colucci WS, John Sutton MGS, Selwyn AP, Alexander RW, Ganz P. Endothelium-dependent dilation of the coronary microvasculature is impaired in dilated cardiomyopathy. *Circulation* 1990;81:772-779

321. Vials A, Burnstock G: A_2-purinoceptor-mediated relaxation in the guinea-pig coronary vasculature: a role for nitric oxide. *Br J Pharmacol* 1993;109:424-429

322. Okamura M, Miura K, Yamashita Y, Yukimura T, Yamamoto K: Role of endothelium-derived relaxing factor in the in vivo renal vascular action of adenosine in dogs. *J Exp Pharmacol Ther* 1992;260:1262-1267

323. Headrick JP, Berne RM: Endothelium-dependent and -independent relaxations of adenosine in guinea pig aorta. *Am J Physiol* 1990,259:H62-H67

324. Rose'Meyer RB, Hope W: Evidence that A_2 purinoceptors are involved in endothelium-dependent relaxation of the rat thoracic aorta. *Br J Pharmacol* 1990;100:576-580

325. Kirsch GE, Codina J, Birnbaumer L, Brown AM: Coupling of ATP-sensitive K-channels to A_1-receptors by G proteins in rat ventricular myocytes. *Am J Physiol* 1990;259:H820-H826

326. Liao JK, Homcy JC: The G proteins of the $G\alpha_i$ and $G\alpha_q$ family couple the bradykinin receptor to the release of endothelium-derived relaxing factor. *J Clin Invest* 1993;92:2168-2172

327. Seiss Geuder M, Mehrke G, Daut J: Sustained hyperpolarization of cultured guinea pig coronary endothelial cells induced by adenosine. *J Cardiovasc Pharmacol* 1992;20 Suppl 12.S97-100

328. Cooke JP, Rossitch E,Jr., Andon NA, Loscalzo J, Dzau VJ: Flow activates an endothelial potassium channel to release an endogenous nitrovasodilator. *J Clin Invest* 1991;88:1663-1671

329. Luscher TF, Vanhoutte PM: Endothelium-dependent responses in human blood vessels. Human versus animals. *Trends Pharmacol Sci* 1988;9.181-184

330. Kiowski W, Linder L, Stoschitzky K: Diminished vascular response to inhibition of endothelium-derived nitric oxide and enhanced vasoconstriction to exogenously administered endothelin-1 in clinically healthy smokers. *Circulation* 1994;90:27-34

331. Panza JA, Quyyumi AA, Brush JE, Epstein SE: Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med* 1990;323:22-27

332. Gilligan DM, Guetta V, Panza JA, Garcia CE, Quyyumi AA, Cannon III RO. Selective loss of microvascular endothelial function in human hypercholesterolemia. *Circulation* 1994;90:35-41

333. Koller A, Sun D, Huang A, Kaley G: Corelease of nitric oxide and prostaglandins mediates flow-dependent dilation of rat gracilis arterioles. *Am J Physiol* 1994;267:H326-H332

334. Meisheri KD, Khan SA, Martin JL: Vascular pharmacology of ATP-sensitive K-channels: interactions between glyburide and K-channel-openers. *J Vasc Res* 1993,302·2-12

335. Anderson EA, Mark AL· The vasodilator action of insulin. Implications for the insulin hypothesis of hypertension. *Hypertension* 1993,21·136-141

336. Lembo G, Rendina V, Iaccarino G, Lamenza F, Volpe M, Trimarco B. Insulin reduces reflex forearm sympathetic vasoconstriction in healthy humans. *Hypertension* 1993; 21 1015-1019.

337. Benz I, Kohlhardt M: Blockade of cardiac outward rectifying K-channels by TEA and class III antiarrhythmics-evidence against a single drug-sensitive channel site *Eur Biophys J* 1994;22:437-446

338. Imaizumi Y, Giles WR: Quinidine-induced inhibition of transient outward current in cardiac muscle. *Am J Physiol* 1987,253 H704-H708

339. Richards NW, Dawson DC: Single potassium channels blocked by lidocaine and quinidine in isolated turtle colon epithelial cells *Am J Physiol* 1986,251:C85-C89

340. Salata JJ, Wasserstrom JA Effects of quinidine on action potentials and ionic currents in isolated canine ventricular myocytes. *Circ Res* 1988,62·324-337

341. Roden DM, Bennett PB, Snyders DJ, Balser JR, Hodeghem LM. Quinidine delays Ik-activation in guinea pig ventricular myocytes *Circ Res* 1988,62·1055-1058

342. Read MA, Boura ALA, Walters WAW Vascular actions of purines in the foetal circulation of the human placenta *Br J Pharmacol* 1993,110 454-460

343. Kirkeboen KA, Naess PA, Offstad J, Ilebekk A: Effects of regional inhibition of nitric oxide synthesis in intact porcine hearts *Am J Physiol* 1994,266 H1516-H1527

344. Nees S, Herzog V, Becker BF, Bock M, Des Rosier C: The coronary endothelium· a highly active metabolic barrier for adenosine. *Basic Res Cardiol* 1985,80 515-529

345. Rongen GA, Smits P, Thien T: Characterization of ATP-induced vasodilation in the human forearm vascular bed. *Circulation* 1994;90·1891-1898

346. Ashcroft SJH, Ashcroft FM: Properties and functions of ATP-sensitive potassium channels. *Cellular Signaling* 1990;2.197-214

347. Bijlstra PJ, Smits P, Lutterman JA, Thien T: Glibenclamide inhibits the vasodilator effect of potassium channel activation in man *Diabetologia* 1994,37 (Suppl 1) A162(Abstract)

348 Janigro D, West GA, Gorden EL, Winn HR· ATP-sensitive K^+-channels in rat aorta and brain microvascular endothelial cells. *Am J Physiol* 1993;265:C812-C821

349. Burnstock G: Purinergic nerves. *Pharmacol Rev* 1972,24:509-581

350. Edlund A, Siden A, Sollevi A: Evidence for an anti-aggregatory effect of adenosine at physiological concentrations and for its role in the action of dipyridamole. *Thromb Res* 1987;45:183-190

351. Maekawa K, Saito D, Obayashi N, Uchida S, Haraoka S. Role of endothelium-derived nitric oxide and adenosine in functional myocardial hyperemia. *Am J Physiol* 1994,267:H166-H173

352 Goonewardene IP, Karim F: Attenuation of exercise-induced vasodilation by adenosine deaminase in anaesthetized dogs *J Physiol* 1991,442·65-79

353. Clancy RM, Leszczynska-Piziak J, Abramson SB: Nitric oxide, an endothelial cell relaxation factor, inhibits neutophil superoxide anion production via a direct action on the NADPH oxidase *J Clin Invest* 1992,90.1116-1121

354 Siegfried MR, Erhardt J, Rider T, Ma XL, Lefer AM Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia-reperfusion. *J Pharmacol Exp Ther* 1992,260 668-675

355 Vegh A, Szekeres L, Parratt J Preconditioning of the ischaemic myocardium, involvement of the L-arginine nitric oxide pathway *Br J Pharmacol* 1992,107 648-652

356 Loscalzo J, Vita JA Ischemia, hyperemia, exercise, and nitric oxide Complex physiology and complex molecular adaptations *Circulation* 1994,90 2556-2559

357 Smolenski RT, Kochan Z, McDouall R, Page C, Seymour A-ML, Yacoub MH Endothelial nucleotide catabolism and adenosine production *Cardiovasc Res* 1994,28 100-104

358 Boachie-Ansah G, Kane KA, Parratt JR Is adenosine an endogenous myocardial protective (antiarrhythmic) substance under conditions of ischemia? *Cardiovasc Res* 1993,27 77-83

359 Berne RM Cardiac nucleotides in hypoxia possible role in regulation of coronary blood flow *Am J Physiol* 1963,204 317-322

360 McKenzie JE, Steffen RP, Haddy FJ Relationships between adenosine and coronary resistance in conscious exercising dogs *Am J Physiol* 1982,242 H24

361 Smits P, Williams SB, Lipson DE, Banitt P, Rongen GA, Creager MA Endothelial release of nitric oxide contributes to the vasodilator effect of adenosine in humans *Circulation* 1995,in press

362 Balcells E, Suarez J, Rubio R Implications of the coronary vascular endothelium as mediator of the vasodilatory and dromotropic actions of adenosine *J Mol Cell Cardiol* 1993,25 693-706

363 Anderson EA, Hoffman RP, Balon TW, Sinkey CA, Mark AL Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans *J Clin Invest* 1991,87 2246-2252

364 Lembo G, Rendina V, Iaccarino G, Lamenza F, Volpe M, Trimarco B Insulin reduces reflex forearm sympathetic vasoconstriction in healthy humans *Hypertension* 1993,21 1015-1019

365 Elliott TG, Cockcroft JR, Groop P-H, Viberti GC, Ritter JM Inhibition of nitric oxide synthesis in forearm vasculature of insulin-dependent diabetic patients blunted vasoconstriction in patients with microalbuminuria *Clin Sci* 1993,85 687-693

366 Wilson PW, Cupples LA, Kannel WB Is hyperglycemia associated with cardiovascular disease? The Framingham Study *Am Heart J* 1991,121 586-590

367 Kannel WB Lipids, diabetes, and coronary heart disease insights from the Framingham Study *Am Heart J* 1985,110 1100-1107

368 Singer DE, Nathan DM, Anderson KM, Wilson PW, Evans JC Association of HbA_{1c} with prevalent cardiovascular disease in the original cohort of the Framingham Heart Study *Diabetes* 1992,41 202-208

369 Vergauwen L, Hespel P, Richter EA Adenosine receptors mediate synergistic stimulation of glucose uptake and transport by insulin and by contractions in rat skeletal muscle *J Clin Invest* 1994,93 974-981

370 Martin SE, Bockman EL Adenosine regulates blood flow and glucose uptake in adipose tissue of dogs *Am J Physiol* 1986,250 H1127-H1135

371 Baron AD, Brechtel-Hook G, Johnson A, Hardin D Skeletal muscle blood flow A possible link between insulin resistance and blood pressure *Hypertension* 1993,21 129-135

372 Houben AJHM, Schaper NC, de Haan CHA, Huvers FC, Slaaf DW, de Leeuw PW, Nieuwenhuijzen Kruseman AC THe effects of 7-hour local hyperglycaemia on forearm macro and microcirculatory blood flow and vascular reactivity in healthy man *Diabetologia* 1994,37 750-756

373. Baron AD, Brechtel G: Insulin differentially regulates systemic and skeletal muscle vascular resistance. *Am J Physiol* 1993;265:E61-E67

374. Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD: Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J Clin Invest* 1994;94:1172-1179

375. Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P: Nitric oxide release accounts for insulin's vascular effects in humans. *J Clin Invest* 1994;94:2511-2515

376. Storms GEMG: *Human insulin: efficacy and immunogenicity.* Dissertation Nijmegen, 1985. University of Nijmegen, Nijmegen, The Netherlands.

377. Greenfield ADM, Patterson GC: Reactions of the blood vessels of the human forearm to increases in transmural pressure. *J Physiol (Camb)* 1954;125:508-524

378. Kohner EM, Hamilton AM, Saunders SJ, Sutcliffe BA, Bulpitt CJ: The retinal blood flow in diabetes. *Diabetologia* 1975;11:27-33

379. Christiansen JS: Glomerular hyperfiltration in diabetes mellitus. *Diabet Med* 1985;2:235-239

380. Sandeman DD, Shore AC, Tooke JE: Relation of skin capillary pressure in patients with insulin-dependent diabetes mellitus to complications and metabolic control. *N Engl J Med* 1992;327:760-764

381. Morrison PD, Mackinnon MW, Bartrup JT, Skett PG, Stone TW: Changes in adenosine sensitivity in the hippocampus of rats with streptozotocin-induced diabetes. *Br J Pharmacol* 1992;105:1004-1008

382. Chowienczyk PJ, Cockcroft JR, Ritter JM: Differential inhibition by NG-monomethyl-L-arginine of vasodilator effects of acetylcholine and methacholine in human forearm vasculature. *Br J Pharmacol* 1993;110:736-738

383. Bruning TA: *Serotonergic and muscarinic receptors mediating vascular effects in the human forearm.* Dissertation Leiden, 1995. University of Leiden, Leiden, The Netherlands; Chapter 9;151-167.

384. Shen J, Gimbrone MAJ, Luscinskas FW, Dewey CFJ: Regulation of adenine nucleotide concentration at endothelium-fluid interface by viscous shear flow *Biophys J* 1993;64:1323-1330

385. Dibner-Dunlab ME, Kinugawa T, Thames MD. Activation of cardiac sympathetic afferents-effects of exogenous adenosine and adenosine analogues. *Am J Physiol* 1993;265:H395-H400

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Henny, zonder jouw steun en vermogen tot relativeren had ik deze klus nooit kunnen klaren.

CURRICULUM VITAE

Gerard Rongen werd geboren op 5 maart 1965 in Breda Na het behalen van zijn Gymnasium B diploma (Norbertus College, Roosendaal), begon hij in 1983 de studie Geneeskunde aan de Medische Faculteit van de Katholieke Universiteit in Nijmegen In 1987 behaalde hij daar het Doctoraal Examen Geneeskunde. In 1988 begon hij zijn co-assistentschappen in het Academisch Ziekenhuis Nijmegen, St Radboud en geaffilieerde ziekenhuizen In 1990 behaalde hij het artsexamen (Cum Laude) Direkt daarna vervolgde hij zijn loopbaan in de kliniek voor Inwendige Ziekten, afdeling Algemeen Interne Geneeskunde van het Academisch Ziekenhuis Nijmegen (Hoofd Prof Dr. A. van 't Laar) Aanvankelijk was hij hier kortdurend werkzaam als arts-assistent geneeskundige. Vanaf december 1990 was hij aan deze afdeling verbonden als wetenschappelijk medewerker Na een onderzoek naar de effecten van renine-remming bij patienten met essentiele hypertensie (onder leiding van Dr J W M Lenders) begon hij op 1 augustus 1991 aan het het onderzoeksproject getiteld 'de rol van ATP als cotransmitter in het sympathisch zenuwstelsel van de mens' dat uiteindelijk heeft geleid tot dit proefschrift. Op 1 augustus 1994 is hij begonnen aan de opleiding tot internist in de Kliniek voor Inwendige Ziekten van het Rijnstate ziekenhuis te Arnhem (opleider Dr L Verschoor) Van 1 september 1995 tot 31 augustus 1996 is hij als research fellow verbonden aan de afdeling Cardiologie van het Mount Sinai Hospital in Toronto (Hoofd Dr. John S. Floras) Hij is gehuwd met Henny Hesselmans Samen hebben zij een zoon, Sjoerd

BIBLIOGRAPHY

Bos WJW, van Goudoever J, Wesseling KH, Rongen GA, Hoedemaker G, Lenders JWM, van Montfrans GA. Pseudohypertension and the measurement of blood pressure. Hypertension 1992; 20:26-31.

Rongen GA, Jacobs MC, Smits P, Thien Th. Endothelium-dependent vasodilation in hypercholesterolaemic subjects. Lancet 1993; 341:490-491 (letter).

Rongen GA, Smits P, Thien Th. N^G-monomethyl-L-ARG reduces the forearm vasodilator response to acetylcholine but not to methacholine in humans. J Cardiovasc Pharmacol 1993; 22:884-888.

Rongen GA, Lenders JWM, Kleinbloesem CH, Weber C, Welker H, Fahrner E, Pozenel H, Woittiez AJJ, Haug G, Buchmann MS, Hoglund CEK, Thien Th. Efficacy and tolerability of the renin inhibitor Ro 42-5892 in patients with hypertension. Clin Pharmacol Ther 1993; 54:567-577.

Rongen GA, Smits P, Thien Th. Endothelium and the regulation of vascular tone with emphasis on the role of nitric oxide; Physiology, pathophysiology and clinical implications. Neth J Med 1994; 44:26-35.

Rongen GA, Smits P, Thien Th. Role of norepinephrine in ischemic preconditioning in rabbit myocardium. Circulation 1994; 89:2460 (letter).

Rongen GA, Smits P, Thien Th. Characerization of adenosine-5'-triphosphate (ATP)-induced vasodilation in the human forearm vascular bed. Circulation 1994; 90:1891-1898.

Rongen GA, Bos WJW, Lenders JWM, van Montfrans GA, van Lier HJJ, van Goudoever J, Wesseling KH, Thien Th. Comparison of intrabrachial and finger blood pressure in healthy elderly volunteers. Am J Hypertens 1995; 8:237-248

Rongen GA, Smits P, Bootsma G, Ver Donck K, de Vries A, Thien Th. High grade nucleoside transport inhibition stimulates ventilation in man. J Clin Pharmacol 1995; 35:357-361

Rongen GA, Smits P, Ver Donck K, Willemsen JJ, De Abreu RA, Van Belle H, Thien Th. Hemodynamic and neurohumoral effects of various grades of selective adenosine transport inhibition in man; implications for its future role in cardioprotection. J Clin Invest, 1995; 95:658-668.

Rongen GA, Lenders JWM, Smits P, Thien Th. Clinical pharmacokinetics and efficacy of renin inhibitors. Clin Pharmacokinet 1995; 29:6-14

Rongen GA, van Ginneken E, Thien Th, Lutterman JA, Smits P. Preserved vasodilator response to adenosine in insulin-dependent diabetes mellitus. Eur J Clin Invest 1995; in press

Smits P, Williams SB, Lipson DE, Banitt P, Rongen GA, Creager MA. Endothelial release of nitric oxide contributes to the vasodilator effect of adenosine in humans. Circulation 1995; 95:2135-2141.

Rongen GA, Lenders JWM, Lambrou G, Willemsen JJ, Van Belle H, Thien Th, Smits P. Presynaptic inhibition of norepinephrine release from sympathetic nerve endings by endogenous adenosine Hypertension, provisionally accepted for publication

ABSTRACTS:

Rongen GA, Janssen RWMM, Hoefnagels WHL Blood pressure and heart rate responses to head-up tilt in young and elderly healthy volunteers. Neth J Med 1991; 39:A69.

Rongen GA, Smits P, Thien T. Adenosine 5'-triphosphate-induced vasodilation in the human forearm is not mediated by P_1-purinoceptor stimulation. Int J Purine and Pyrimidine Res 1992, 3:101.

Rongen GA, Bos WJW, Lenders JWM, van Montfrans GA, van Lier H, Hoefnagels WHL, Thien Th. In elderly subjects Finapres measures blood pressure correctly during standing but not during valsalva. J Hypertens 1992; 10 (suppl. 4):S92.

Rongen GA, Smits P, Thien T. High doses of intra-arterially infused adenosine-5'-triphosphate do not reveal P_{2x}-purinoceptor-mediated vasoconstriction in man. Pharmacy, World & Science 1993; 15:E7.

Rongen GA, Smits P, Ver Donck K, Van Belle H, Thien T. Hemodynamic and neurohumoral effects of the adenosine transport inhibitor draflazine in man. a dose response trial Pharmacy, World and Science 1993, 14:L5.

Rongen GA, Smits P, Thien T. L-NMMA attenuates the forearm vasodilator response to acetylcholine but not to adenosine-5'-triphosphate in man. J Hypertens 1994; 12 (suppl. 3):S26.

Rongen GA, Smits P, Willemsen J, Ver Donck K, Van Belle H, Thien Th. High grade adenosine uptake inhibition stimulates the sympathetic nervous system in man. J Hypertens 1994; 12 (suppl. 3):S160.

Rongen GA, Smits P, Van Belle H, Thien T. Low grade nucleoside transport inhibition potentiates the in-vivo effect of adenosine in man at a site of increased supply. J Hypertens 1994; 12 (suppl 3):S160.

STELLINGEN

behorende bij het proefschrift:

Vascular pharmacology of adenosine and adenosine-5'-triphosphate in humans

Gerard Rongen

Nijmegen, 5 januari 1996

1 Bij toediening van een selectieve remmer van het nucleosidetransport in het vaatbed van de onderarm worden de lokale effecten van endogeen adenosine gecamoufleerd door een reflexmatige activering van het sympathisch zenuwstelsel (dit proefschrift, hoofdstuk 2 en 4)

2 De nucleosidetransportremmer draflazine is in staat om het effect van endogeen interstitieel adenosine te potentieren, zelfs indien draflazine zo laag gedoseerd wordt dat er geen systemische hemodynamische effecten optreden (dit proefschrift, hoofdstuk 2 en 4)

3 Het toegenomen ademminuutvolume na draflazine-infusie (dit proefschrift, hoofdstuk 3) suggereert dat deze nucleosidetransportremmer slechts matig de bloedhersenbarrière van de mens passeert Gezien het potentieel groot klinisch belang van cerebrale nucleosidetransportremming bij de preventie van cerebrale infarcten dient deze suggestie geverifieerd te worden door bijvoorbeeld de draflazineconcentratie te bepalen in de liquor cerebrospinalis

4 In het vaatbed van de onderarm wordt de afgifte van noradrenaline uit sympathische zenuwvezels presynaptisch geremd door endogeen adenosine (dit proefschrift, hoofdstuk 4)

5 Stimulatie van het sympathisch zenuwstelsel door endogeen adenosine draagt bij aan een optimale aanpassing van de circulatie aan situaties van ischemie of zuurstofgebrek

6 *In-vitro* hebben hoge concentraties draflazine of dipyridamol een dodend effect op de erythrocytaire stadia van de malaria parasiet Plasmodium Falciparum (eigen waarneming, in samenwerking met Ton Lensen, afdeling Medische Parasitologie, Academisch Ziekenhuis Nijmegen) Gezien het wereldwijde belang van een goede malariabestrijding behoort deze waarneming *in-vivo* geverifieerd te worden

7 Tot tenminste 50 minuten na infusie van acetylcholine in de arteria brachialis van de mens dient met carry-over effecten rekening gehouden te worden (dit proefschrift, hoofdstuk 6)

8 In klinisch farmacologisch onderzoek is het proefdier een onacceptabel substituut voor de mens (dit proefschrift, hoofdstuk 2 en 6)

9 Bij een onverwachte observatie dient altijd eerst de kwaliteit van het meetinstrument te worden onderzocht (Rongen GA, Janssen RWMM, Hoetnagels WHL Neth J Med 1991, 39 A69 (abstract) en Rongen GA, Bos WJW, Lenders JWM, van Montfrans GA, van Lier HJJ, van Goudoever J, Wesseling KH, Thien Th Am J Hypertens 1995, 8 237-248)

10 Zowel ten gevolge van farmacodynamische als farmacokinetische tekortkomingen zullen de huidige renineremmers in de dagelijkse patientenzorg nooit een plaats krijgen als antihypertensivum (Rongen GA, Lenders JWM, Smits P, Thien Th. Clin Pharmacokinet 1995, 95 658-668)

11 Onderzoekers kun je zien als een tikje neurotische mensen die het leven niet helemaal aankunnen en het daarom verkleinen tot een onderzoeksprobleempje Als het opgelost is, denken ze dat ze iets belangrijks gedaan hebben (P. Borst, uit rubriek 'Gezegd', Gelderlander, 29 augustus 1992)

12 De impactfactor van een wetenschappelijk tijdschrift is een slechte indicator voor de kwaliteit van het daarin gepubliceerd onderzoek

13 Violisten met hypermobiliteit van vinger- en polsgewrichten hebben minder klachten van deze gewrichten dan hun collega's zonder deze hypermobiliteit (Larsson L-G, Baum J, Mudholkar GS, Kollia GD N Engl J Med 1993,329 1079-1082) De mobiliteit van deze gewrichten is door frequent bespelen van een viool te verhogen (eigen waarneming) Dit is als een vorm van 'preconditioning' te beschouwen

14 Een computer is een essentieel werktuig bij het uitvoeren en publiceren van wetenschappelijk onderzoek Daarom dient iedere promovendus de beschikking te hebben over een computer die gefinancierd wordt door de werkgever en die de promovendus in bruikleen heeft gedurende de periode van onderzoek en bewerking van het proefschrift

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