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PHD THESIS

The role of Methylglyoxal in the impairment of angiogenic process in endothelial cells

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LIST OF PUBLICATIONS:

This dissertation is based upon the following publications:

Nigro C, Leone A, Raciti GA, Longo M, Mirra P, Formisano P, Beguinot F, Miele C. *Methylglyoxal-Glyoxalase 1 Balance: The Root of Vascular Damage.* Int J Mol Sci. 2017 Jan 18;18(1).

Mirra P, Nigro C, Prevenzano I, Procopio T, Leone A, Raciti GA, Andreozzi F, Longo M, Fiory F, Beguinot F, Miele C. *The role of miR-190a in methylglyoxal-induced insulin resistance in endothelial cells.* Biochim Biophys Acta. 2017 Feb;1863(2):440-449.

Nigro C, Raciti GA, Leone A, Fleming TH, Longo M, Prevenzano I, Fiory F, Mirra P, D'Esposito V, Ulianich L, Nawroth PP, Formisano P, Beguinot F, Miele C. *Methylglyoxal impairs endothelial insulin sensitivity both in vitro and in vivo.* Diabetologia. 2014 Jul;57(7):1485-94.

LIST OF ABBREVIATIONS:

AGEs: Advanced glycation end-products
BM: Basement membrane
CML: Nε-carboxymethyl-Lysine
DM: Diabetes mellitus
DR: Diabetic retinopathy
ECs: Endothelial cells
ECD: Endothelial cells dysfunction
ECM: Extracellular matrix
eNOS: Endothelial nitric oxide synthase
ET-1: Endothelin-1
FBS: Fetal bovine serum
FGFs: Fibroblast growth factors
Glo1: Glyoxalase 1
Glo2: Glyoxalase 2
GSH: Glutathione
G3P: Glyceraldeide-3 phosphate
HbA1c: Glycosylated hemoglobin
IKK: IκB kinase
IL-1: Interleukin-1
KDR: VEGF receptor-2
MAECs: Mouse aortic endothelial cells
MCP-1: Monocyte chemoattractant protein-1
MGO: Methylglyoxal
MGO-H1: Methylglyoxal hydroimidazolone
MMPs: Matrix metalloproteinases
NF-κB: Transcription factor nuclear-κB
NO: Nitric oxide
PARP: Poly(ADP-ribose) polymerase
PDGF: Platelet-derived growth factor
PGI2: Prostacyclin
PKC: Protein kinase C
RAGEs: Receptor of Advanced Glycation End-products
ROIs: Reactive oxygen intermediates
ROSs: Reactive oxygen species
TGF: Transforming growth factor
TNFα: Tumor necrosis factor-α
TSPs: Thrombospondins
T2DM: Type 2 diabetes
uPA: Urokinase-type plasminogen activator
VCAM-1: Vascular cell adhesion molecule-1
VEGFs: Vascular endothelial growth factors
VSMCs: Vascular smooth muscle cells

ABSTRACT

Much of the morbidity and mortality associated with diabetes mellitus (DM) reflects its deleterious effect on micro- and macro-circulation. DM impairs physiological angiogenesis, leading to long-term complications, by molecular mechanisms that are not fully understood. The generation of Advanced Glycation End-products (AGEs) has an important role in the development of hyperglycemia-induced endothelial damage. Moreover, previous evidence demonstrated that exposure of endothelial cells to hyperglycemia induces sustained activation of the transcription factor nuclear-κB (NF-κB), at least in part by the AGEs/RAGE pathways, leading to accelerated vascular disease.

One of the main precursors of AGEs in endothelial cells is Methylglyoxal (MGO), a highly reactive dycarbonyl detoxified by the Glyoxalase System, of which Glyoxalase 1 (Glo1) is the rate limiting enzyme.

In this study, we aim at evaluating the effect of MGO on the angiogenic ability of aortic endothelial cells isolated from *Glo1* knock-down mice (Glo1KD MAECs) and their WT littermates (WT MAECs).

Glo1KD MAECs show a reduced expression of *Glo1* that is paralleled to an increase of MGO accumulation. This leads to an impairment of angiogenic ability of Glo1KD MAECs characterized by a reduced proliferation, migration and invasion.

Both protein and mRNA levels of the antiangiogenic *HoxA5* gene are increased in Glo1KD MAECs compared to WT MAECs. Interestingly, *HoxA5* silencing, is able to improve migration and invasion of Glo1KD MAECs.

Moreover, MGO accumulation in Glo1KD MAECs causes the overexpression of *NF-κB-p65* that is also associated to a higher cytoplasmic and nuclear protein levels. Interestingly, there is an increased binding of NF-κB-p65 to *HoxA5* promoter in Glo1KD MAECs compared to WT MAECs, and NF-κB inhibition results in the reduction of *HoxA5* expression.

This study demonstrates that, through NF-κB-p65 activation, high levels of MGO impair angiogenic ability of MAECs via a mechanism involving the antiangiogenic factor *HoxA5*. Further investigations will allow to identify new strategies for the prevention and treatment of microvascular complications associated to diabetes.

1. BACKGROUND

1.1 Endothelium and vascular function.

Endothelium is a monolayer of cells covering the internal lumen of all the vasculature. For many years, this cell layer has been thought to be relatively inert, a mere physical barrier between circulating blood and the underlying tissues. However, it has now been recognized that endothelium is a complex organ constituted by metabolically active endothelial cells (ECs) with important paracrine, endocrine and autocrine functions, necessary for the maintenance of vascular homeostasis (Vallance 2001, Bonetti, Lerman et al. 2003, Hadi and Suwaidi 2007, Sena, Pereira et al. 2013).
ECs are covered by a glycocalyx that contributes to the selectivity of their barrier function (van Haaren, VanBavel et al. 2003), and serves as an interface between circulating blood and vascular smooth muscle cells (VSMCs) wherewith interact, as well as cells within the blood compartment, facilitating a complex array of functions (De Meyer and Herman 1997, Mombouli 1997) (Figure 1).

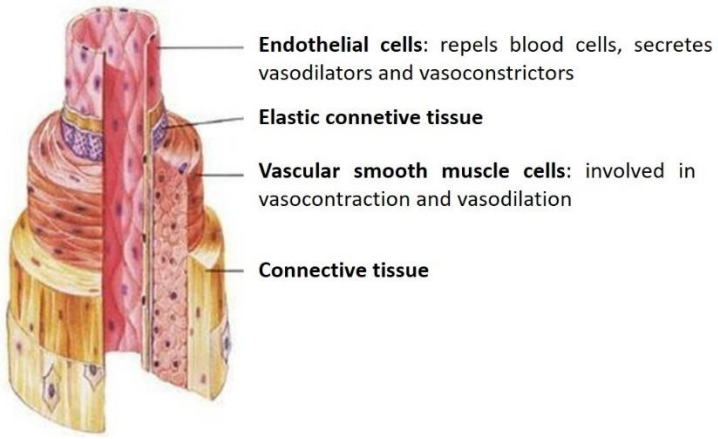

Figure 1. Vessel structure.

Indeed, the endothelium is responsible of the regulation of vessel integrity, vascular growth and remodeling, as well as, tissue growth and metabolism; it mediates the immune responses, cell adhesion, hemostasis, vascular permeability. Moreover, it plays a pivotal role in the regulation of vascular tone, controlling tissue blood flow, inflammatory responses and maintaining blood fluidity (Feletou and Vanhoutte 2006, Moncada and Higgs 2006). Finally, the endothelium has a pivotal role in the process of angiogenesis, occurring during development, growth, tissue repair, but also in capillary tissue perfusion. In several pathological conditions, an improper angiogenic response causes

unwanted growth, risk for local haemorrhage by immature vessels, or insufficient blood supply (Carmeliet 2005) (Figure 2).

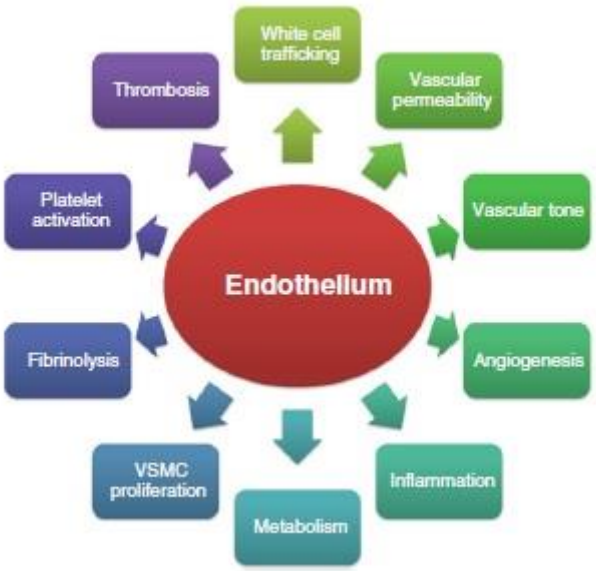

Figure 2. Physiological functions of endothelium. Endothelium guarantees vascular homeostasis through the regulation of several biological processes that are summarized by rectangles in figure. (Sena, Pereira et al. 2013)

ECs guarantee the regulation of all these functions through the production of a wide range of factors. Indeed, they produce endothelium-derived hyperpolarization factor (EDHF), nitric oxide (NO) (Ignarro, Buga et al. 1987, Palmer, Ferrige et al. 1987) and prostacyclin (PGI2) that exert vasodilatory and antiproliferative effects, while endothelin-1 (ET-1) (Yanagisawa, Kurihara et al. 1988), angiotensin II and reactive oxygen species (ROSs) are mediators exerting vasoconstrictor effects (Endemann and Schiffrin 2004, Just, Whitten et al. 2008). ECs also produce anti-thrombotic (NO and PGI2) and pro-thrombotic molecules, such as vonWillebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1), which promotes platelet aggregation and inhibits fibrinolysis, respectively (Moncada and Higgs 2006).
In this way, the endothelium maintains the balance between vasodilation (NO) and vasoconstriction (ET-1), inhibition and promotion of migration and proliferation of VSMCs (NO, angiotensin II, ROSs), fibrinolysis and thrombogenesis as well as prevention and stimulation of platelets adhesion and aggregation (Moncada and Higgs 2006). Inflammatory activation of the endothelium can be caused by bacterial lipopolysaccaride and inflammatory cytokines exposure, of which the potent inducers interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα) have drawn major attention. Inflammatory activation can also be induced by reactive oxygen intermediates (ROIs), which can be

generated by the inflammation process itself and by disturbed metabolic conditions, like diabetes (Gimbrone 1999). Therefore, alterations of this tightly regulated equilibrium leads to dysfunction of endothelial cells (Figure 3) (Sena, Pereira et al. 2013).

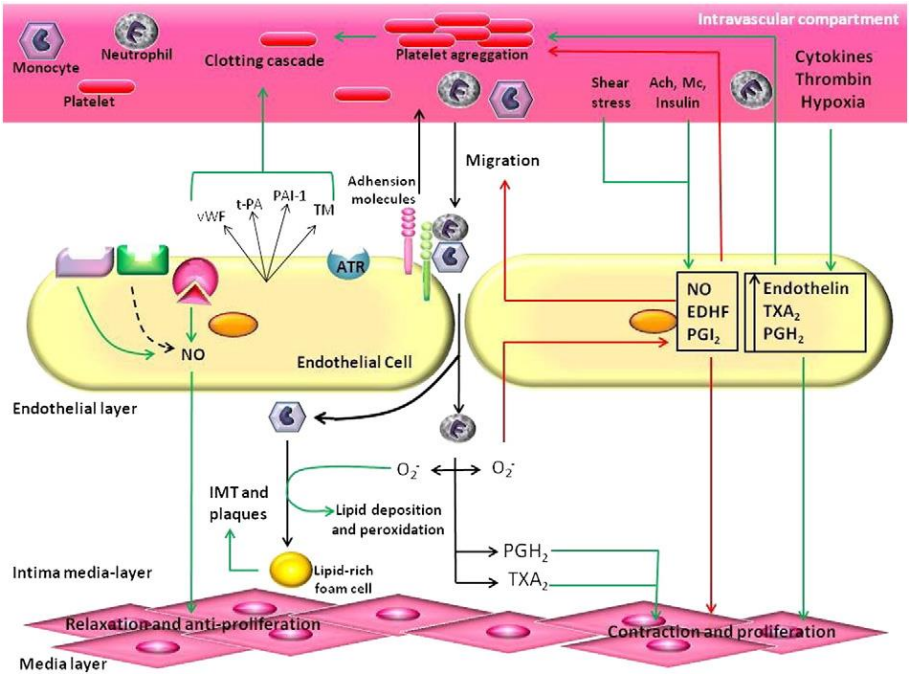

Figure 3. Molecules released by endothelial cells and their role in vascular function. ACh, acetylcholine; ATR, angiotensin-II receptor; BK, bradykinin; EDHF, endothelium-derived hyperpolarization factor; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PGH2, prostaglandin H2; PGI2, prostacyclin; O2•−, superoxide anion; t-PA, tissue plasminogen activator; TM, thrombomodulin; TxA2, thromboxane A2; vWF, vonWillebrand factor. (Sena, Pereira et al. 2013)

1.1.1 Angiogenesis.

Among the functions exerted by endothelium, angiogenesis is one of the most important. Angiogenesis is a global term which indicates the new blood vessel formation from pre-existing ones and it is distinguished from vasculogenesis, which is *de novo* vessel formation from hematopoietic progenitor cells (Nyberg, Xie et al. 2005, Xu, Kanasaki et al. 2012). It is a multistep process that generally begins when endothelial cells switch from "quiescent" to "angiogenic" phenotype in response to angiogenic stimuli (Bastaki, Nelli et al. 1997, Ribatti, Nico et al. 2009, Neve, Cantatore et al. 2014).
Angiogenesis is a vital process for embryological growth, tissue development, reproduction and wound healing in damaged tissues. Under these conditions, it is a highly regulated process, i.e. turned on for brief periods and then completely

inhibited (Folkman and Shing 1992). Indeed, in humans or vertebrates, normal vascularization is quiescent, suggesting that *in vivo* there are anti-angiogenic factors that act against angiogenic factors. Among the endogenous anti-angiogenic factors, the most important include thrombospondins (TSPs), angiostatin and endostatin (Huang and Bao 2004).
Angiogenesis requires extensive interaction between a variety of cells and molecules and it is controlled by several peptides and other modulating factors. In order for vascular sprouting to occur, a cascade of events have to be completed (Tomanek and Schatteman 2000).
First, enzymatic degradation of capillary basement membrane (BM) occurs and vascular permeability increases leading to extravasation of blood proteins and their accumulation into interstitial collagen matrix to form a new, provisional extracellular matrix (ECM). Then, endothelial cells begin to proliferate, invade the ECM, and take part in the formation of an immature capillary structure and deposition of a new complex BM. Finally, pericytes are recruited, thereby providing stabilization for the new vessel. The soluble growth factors, membrane-bound proteins, cell-matrix and cell-cell interactions, together with hemodynamic forces all act in concert to control and influence angiogenesis. The balanced activity between specific angiogenic molecules which can initiate this process and specific inhibitory molecules which can stop it are thought to be critical for an optimal angiogenic response (Neve, Cantatore et al. 2014) (Figure 4).

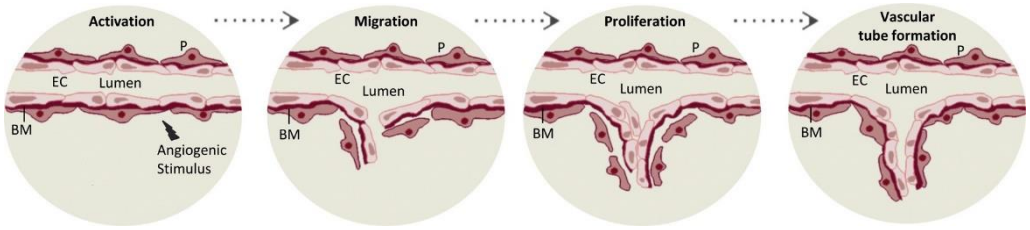

Figure 4. Steps of angiogenic process. Angiogenesis is a multistep process in which, following pro-angiogenic stimulation, endothelial cells begin to migrate, proliferate and to form tubular structure. P: pericyte; BM: basement membrane; EC: endothelial cell. Modified from Blois SM. (Blois, Conrad et al. 2015)

1.1.2 Angiogenesis regulating factors.

There are several angiogenic factors which are involved in stimulation, promotion, and stabilization of new blood vessels such as: vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), angiopoietins, platelet-derived growth factor (PDGF), monocyte chemoattractant protein-1 (MCP-1), transforming growth factor (TGF), various integrins, VE-cadherin, ephrins NO, and others (Figure 5).

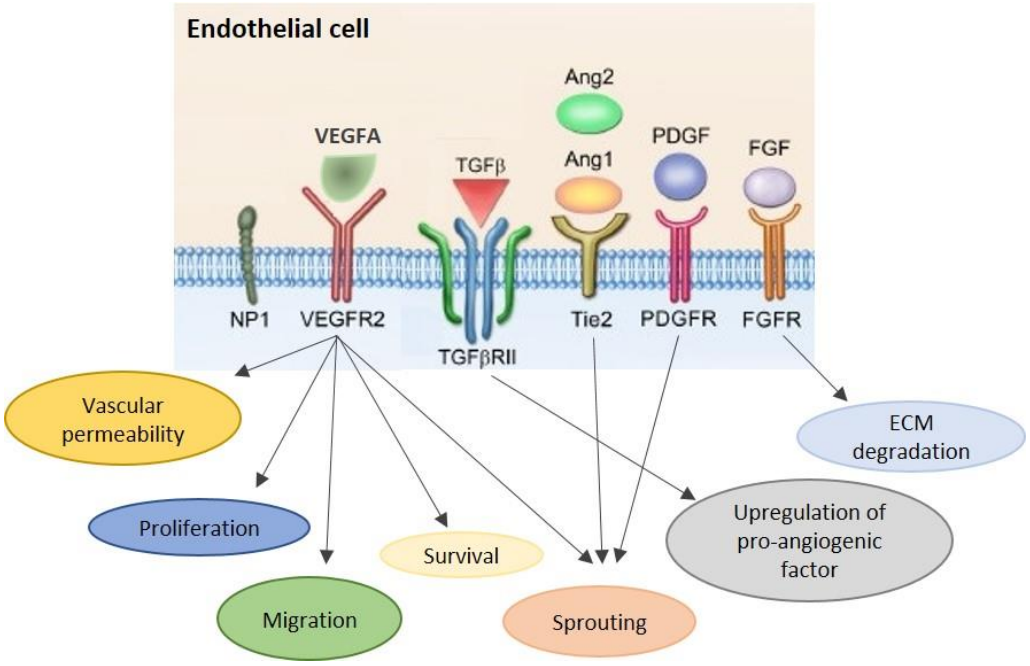

Figure 5. Pro-angiogenic factors involved in angiogenesis. Following binding to their receptors on the endothelial cell membrane, pro-angiogenic factors regulate cellular processes (oval boxes) that are essential for angiogenesis.

Likewise, mechanical stimulation such as physiological shear stress is also important particularly in arteriogenesis, that refers to maturation and enlargement of smaller preexisting arterial vessels through vascular remodeling or collateral growth (Kolluru, Bir et al. 2012).

The VEGF family consists of few members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PlGF) and distinguishes itself from other angiogenic superfamilies by the largely non-redundant roles of its members (Carmeliet and Jain 2011). VEGF-A is the best characterized and the most studied of the VEGF family members. It is a dimeric glycoprotein of 23-kD subunits, mitogenic for endothelial cells and able to induce vessel permeability (Mustonen and Alitalo 1995). The *VEGF* gene, which is located on the short arm of chromosome 6 is composed of eight exons and it is differentially spliced to yield four mature isoforms (VEGF121, VEGF165, VEGF189 and VEGF206) (Tischer, Mitchell et al. 1991, Otrock, Mahfouz et al. 2007).

VEGF-A is the most potent pro-angiogenic protein described to date. It exerts its biologic effect through interaction with cell surface receptors. These receptors are transmembrane tyrosine receptors and they include VEGF receptor-1 (Flt1), VEGF receptor-2 (KDR), selectively expressed on vascular ECs, and VEGF receptor-3 (FLT4). Moreover, there are also the neuropilin receptors (NP-1 and NP-2, also known as NRP-1 and NRP-2), that are expressed on neurons and vascular endothelium and act as co-receptors for the VEGFRs, increasing the binding affinity of VEGF to VEGRs (Ellis and Hicklin 2008).

In any case, the VEGF-A binding to extracellular domain of KDR receptor causes the dimerization and autophosphorylation of the intracellular tyrosine kinases domain that leads to the activation of a cascade of downstream proteins (Otrock, Mahfouz et al. 2007). In this way, VEGF-A induces proliferation, sprouting and tube formation of ECs. It affects an important number of angiogenic processes including wound healing, ovulation, maintenance of blood pressure, menstruation and pregnancy. In addition, it causes vasodilation by inducing the endothelial nitric oxide synthase (eNOS) and so increasing NO production (Otrock, Mahfouz et al. 2007). VEGF-A binds many receptors on hematopoietic stem cells (HSCs), monocytes, osteoblasts and neurons. It induces HSC mobilization from the bone marrow, monocyte chemoattraction and osteoblast-mediated bone formation. In mice, deletion of the *VEGF-A* gene is lethal, resulting in vascular defects and cardiovascular abnormalities (Otrock, Mahfouz et al. 2007).

Other important pro-angiogenic factors are the heparin-binding protein mitogens, acidic and basic fibroblast growth factors (aFGF and bFGF).

Following the binding to their high affinity tyrosine kinase FGF receptors (FGFRs) on the surface of target cells, FGFs lead to ECs proliferation, migration and production of collagenase and plasminogen activator. Indeed, FGFs upregulate the urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs) production in ECs, thus promoting ECM degradation that represents an important step of the angiogenic process (Otrock, Mahfouz et al. 2007).

Angiopoietins and their receptors have an important role in vessel maintenance, growth and stabilization. Angiopoietins are a family of secreted proteins that bind two Tie receptors, Tie1 and Tie2. Angiopoietin-1 (Ang1) is the most studied angiopoietin. Studies in mice demonstrated the important role of Tie receptors in angiogenesis. Tie1-deficient mice die either perinatally (Sato and Rifkin 1989) or between days 13.5 and 14.5 of gestation (Puri, Rossant et al. 1995) after they develop edema and hemorrhage. In these mice, the vascular integrity is severely compromised, implying that Tie1 is necessary for the integrity and survival of ECs during angiogenesis (Puri, Rossant et al. 1995). Tie2-deficient mice die before birth and display a reduction in ECs in their blood vessels and the formation of abnormal vasculature including lack of branching and sprouting vessels (Sato and Rifkin 1989, Dumont, Yamaguchi et al. 1992), suggesting its crucial role for sprouting and branching of vessels (Otrock, Mahfouz et al. 2007).

PDGF exists as heterodimer (PDGF-AB) or homodimer (PDGF-AA or -BB) composed of chains A and B. PDGF receptors are made up of complexes between α and β subtypes. In literature, it is known the role of PDGF-β receptor in the promotion of angiogenic sprouts *in vitro*. Moreover, PDGF also stimulates the proliferation of cultured pericytes (essential for normal microvessels stability) and smooth muscle cells, both of which have been shown to express PDGF-β receptor (Otrock, Mahfouz et al. 2007).

Finally, the transforming growth factor-βs (TGF-β) represent a family of highly conserved cytokines of which TGF-β1 is one of the most common member.

TGF-β family ligands stimulate type II receptors that phosphorylate type I receptors (such as activin receptor-like kinase (ALK)) and activate the downstream signaling SMADs. Endoglin is a type III receptor, which facilitates binding of TGF-β1 to the type II receptors.
Conversely, to the other factors described, the role of TGF-β1 is dual. Indeed, at low doses, TGF-β1 contributes to the angiogenic switch by upregulating angiogenic factors and proteinases, whereas at high doses TGF-β1 inhibits EC growth, promotes basement membrane reformation and stimulates smooth muscle cells differentiation and recruitment. Loss of TGF-β signaling components in mice is responsible for decreased vessel wall integrity; moreover, inactivation of the TGF-β1 caused lethality due to defects in the hematopoietic system and yolk sac vasculature (Goumans, Lebrin et al. 2003, Otrock, Mahfouz et al. 2007).

1.1.3 *Homeobox* genes in neovascularization: specific role of *HoxA5*.

Several studies have been recently involved in the definition of the role of *homeobox* genes in the regulation of neovascularization. *Homeobox* genes encode for a family of transcription factors originally discovered in Drosophila (Lewis 1978) and found to be highly conserved throughout the species. They contain the homeobox region of 183-bp coding for a 61-amino acid domain, containing a helix-turn-helix motif, defined as the homeodomain (HD). During normal development, *homeobox* genes act as master regulators of tissue and organ patterning (Krumlauf 1994, Kachgal, Mace et al. 2012).
In mice (*Hox* genes) and humans (*HOX* genes) there are 39 genes organized into four Hox loci, each localized on a different chromosome (*HOXA* at 7p15.3, *HOXB* at 17p21.3, *HOXC* at 12q13.3 and *HOXD* at 2q31) and containing from 9 to 11 genes (Apiou, Flagiello et al. 1996, Cantile, Schiavo et al. 2008) (Figure 6). On the basis of sequence similarity and position on the locus, corresponding genes in the four clusters can be aligned with each other into 13 paralogous groups (Scott 1992). During mammalian development, *Hox* gene expression controls the identity of various regions along the body axis according to the rules of temporal and spatial co-linearity. Accordingly, the genes located mostly at 3' are expressed earlier and in more anterior domains along the embryonic axes than the 5' located ones (Krumlauf 1994).
Considering the powerful role of *Hox* genes in normal and pathological tissue responses, strict regulatory mechanisms are necessary to guarantee their correct spatiotemporal expression pattern (Kachgal, Mace et al. 2012).
It is widely known that *homeobox* genes regulate the transcription of genes necessary for many cell processes such as differentiation, migration and proliferation in both vertebrate and invertebrate (Krumlauf 1994, Gorski and Walsh 2000). Moreover, they play an essential role in regulating the function of vascular system (Douville and Wigle 2007). In particular, they coordinate the

processes required for proper vascular formation during development, as well as the maintenance and repair of the vasculature system throughout life (Murthi, Abumaree et al. 2014).

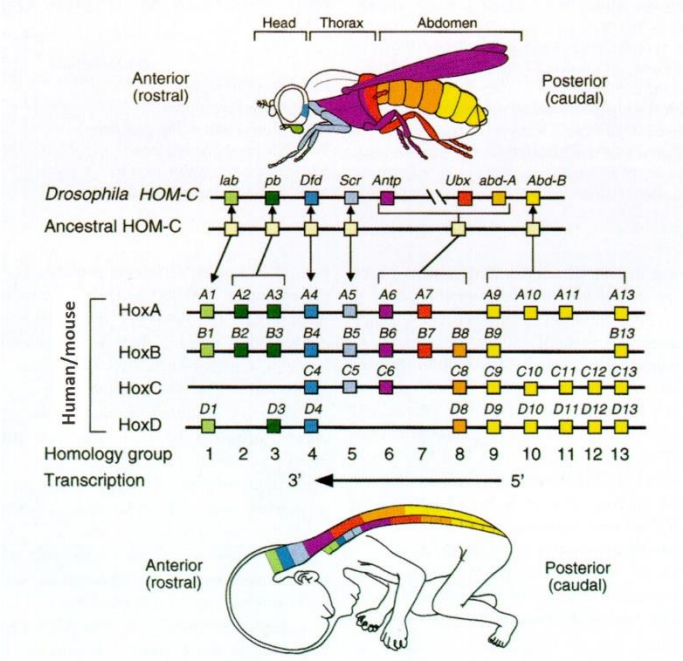

Figure 6. Genomic organization of *Drosophila* homeotic genes and mammalian *Hox* genes. Each gene is represented by a colored box. Expression position on chromosome is in inverted order relative to body plan. Homeotic genes: lab labial, pb proboscipedia, Dfd Deformed, Scr Sex combs reduced, Antp Antennapedia, Ubx Ultrabithorax, abd-A abdominal-A, Abd-B Abdominal-B **(**Modified from Horabin 2013).

Among the *Hox* genes with pro-angiogenic function there are genes belonging to the paralogue group 3. *HoxA3* knockout mice died at or shortly after birth, with abnormalities including an absent right carotid artery and irregularly sized heart compartments (Chisaka and Capecchi 1991). These effects are ameliorated by *HoxD3* insertion in the *HoxA3* locus, suggesting that paralogs are interchangeable if expressed "properly" in space and time (Kachgal, Mace et al. 2012). *HoxD3*, highly expressed in active proliferating ECs, promotes endothelial cell invasion of extracellular matrix early in angiogenesis and the subsequent capillary morphogenesis of these new vascular sprouts (Murthi, Abumaree et al. 2014); while, constitutes *HoxB3* expression increases capillary morphogenesis (Cantile, Schiavo et al. 2008).
In contrast, overexpression of *HoxD10* in ECs attenuates their angiogenic response to FGF2 and VEGFA. Furthermore, *HoxD10*-expressing ECs showed a reduction of the angiogenic factors RHOC, MMP14, uPAR and cyclin D1 (Kachgal, Mace et al. 2012).

Among the anti-angiogenic *Hox* genes, *HoxA5* has been widely studied for its central role in embryo development and its involvement in diseases occurring during adulthood as well.
HoxA5 gene is located on mouse chromosome 6 (and human chromosome 7) and encodes a 270-amino acid ANTP-class homeodomain protein (Odenwald, Taylor et al. 1987).
In the mouse embryo, there is a complex organization of overlapping transcriptional units, which results from alternative splicing and the use of three different promoters: a proximal one producing 1.8kb transcript and two distal ones (D1 and D2) giving rise to four long RNAs (5,0(2x), 9,5 and 11,0 kb). The distal promoter D1 corresponds to the putative *HoxA6* promoter, while the most distal one (D2) is located in the *HoxA6–HoxA7* intergenic region downstream the *HoxA7* gene (Jeannotte, Gotti et al. 2016) (Figure 7).
All the HoxA5-associated transcripts include the ORF encoding HoxA5 protein, but only the 1.8-kb form produces the protein. However, the 5-kb HoxA6/A5 transcript can produce the HOXA6 protein as shown by transfection assays in HEK293 cells. Thus, the 5-kb HoxA5, the 9.5-kb and the 11.0-kb transcripts, all transcribed from the D2 promoter, can be considered as long non-coding (lnc) RNAs (Coulombe, Lemieux et al. 2010).

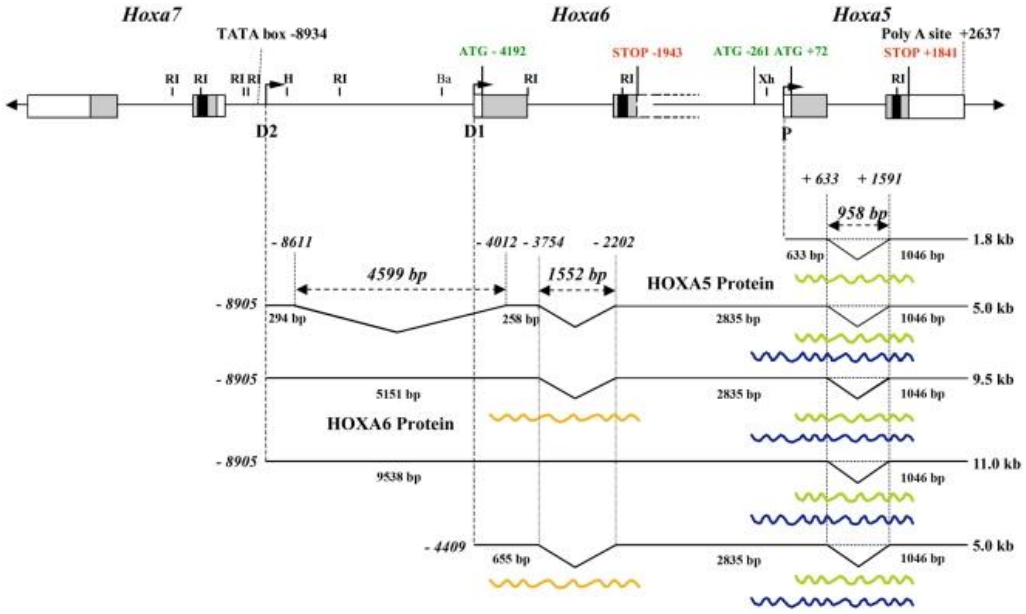

Figure 7. Schematic representation of the *HoxA5, HoxA6 and HoxA7* gene promoter. Black, grey and open boxes indicate homeobox, translated and transcribed sequences, respectively. The two known exons of *HoxA5* and the two in-frame ATG are represented. Position +1 corresponds to the transcription initiation site of *HoxA5* exon 1. The 3′ non-coding sequences of *HoxA6* exon 2 extend further downstream into the *HoxA6-HoxA5* intergenic region and the adjacent *HoxA5* coding sequences and they are indicated by dotted lines. P, D1 and D2 represent the proximal, distal 1 and distal 2 promoters, respectively. *HoxA5* intron is represented by a dotted line to indicate the non-spliced isoforms. The longest ORFs deduced from the sequence of each transcript are represented by waved lines: the 270 a.a. HOXA5 protein, the 381 a.a.

HOXA5 isoform and the HOXA6 protein. Ba, *Bam*HI; H, *Hind*III; RI, *Eco*RI; Xh, *Xho*I. (Coulombe, Lemieux et al. 2010)

Studies performed in mutant mice indicate that the loss of HoxA5 function is related to serious abnormalities in embryo development and often to death at birth. While heterozygous mutants ($HoxA5^{+/-}$) survive with a normal lifespan and no obvious phenotype, HoxA5 homozygous null mutant pups ($HoxA5^{-/-}$) show high mortality rate at birth for respiratory distress due to tracheal and lung dysmorphogenesis and impaired diaphragm innervation. These mutants show an undeveloped lung microvasculature characterized by fewer endothelial cells trapped within dense mesenchyme. HoxA5 is also involved in digestive tract, thyroid and mammary glands development and ovary homeostasis (Aubin, Lemieux et al. 1997, Aubin, Chailler et al. 1999, Meunier, Aubin et al. 2003, Garin, Lemieux et al. 2006, Gendronneau, Boucherat et al. 2012).
Besides its central role in embryo development, HoxA5 is also expressed in adulthood where it is reported to have an important role in regulation of angiogenesis (Jeannotte, Gotti et al. 2016).
Several studies demonstrate that *HoxA5* expression is able to block angiogenesis *in vivo* and cell migration *in vitro*. It is expressed in quiescent, but not activated, endothelium (Rhoads, Arderiu et al. 2005), where stabilizes adherens junctions through β-catenin retention and increasing Akt1, preventing the first step of angiogenesis (Kachgal, Mace et al. 2012). Moreover, sustained expression of *HoxA5* in activated cultured ECs down-regulates expression of *VEGFR2*, *ephrin-A1* and *IL-6* and increases the expression of the anti-angiogenic *TSP-2* (Rhoads, Arderiu et al. 2005).

1.2 Endothelial cells dysfunction.

Endothelium is a flexible organ, whose functioning well adapts to various types of metabolic, mechanical and inflammatory stress (Cines, Pollak et al. 1998, Pober and Sessa 2007). However, when this functioning becomes inadequate, endothelial cells dysfunction (ECD) occurs.
ECD is a broad term which implies dysregulation of endothelial cell functions. It is a systemic pathological condition which can be defined as an imbalance between vasodilator and vasoconstrictor substances produced by the endothelium or overall its functions (Deanfield, Donald et al. 2005). A major hallmark of ECD is the impaired NO bioavailability, which is due to impaired NO production by the endothelium and/or increased NO inactivation by ROSs (Witting, Rayner et al. 2007, Kaneto, Katakami et al. 2010). Moreover, ECD is associated to reduced anticoagulant properties, increased platelet aggregation, increased expression of adhesion molecules, chemokines and cytokines, and increased ROSs production (Al-Isa, Thalib et al. 2010).

It is widely known that ECD is associated with several risk factors including hypercholesterolemia, smoking, hypertension, diabetes mellitus, insulin resistance and obesity (Brook, Bard et al. 2001). It is regarded as an important and early factor in the pathogenesis of atherothrombosis (Gimbrone 1999, Ross 1999) and vascular complications of diabetes (Stehouwer, Lambert et al. 1997). Importantly, ECD has been shown to be of prognostic significance in predicting vascular events (Perticone, Ceravolo et al. 2001, Corrado, Rizzo et al. 2008), so endothelial function testing may potentiate the detection of cardiovascular diseases such as myocardial infarction, peripheral vascular disease, ischemic stroke, and others (Bucala, Tracey et al. 1991, Suuronen, Hazra et al. 2010, Kolluru, Bir et al. 2012).
In diabetes, BM is thickened and altered in composition, due to the enhanced synthesis of matrix proteins by TGF-β activity (Chen, Hong et al. 2001). At the same time, the thickness of the glycocalyx, which contains large amounts of heparan sulfate proteoglycans, is markedly reduced (Nieuwdorp, Mooij et al. 2006, Nieuwdorp, van Haeften et al. 2006). Loss of the glycocalyx leads to the adhesion of mononuclear cells and platelets to the endothelial surface, attenuated NO availability, and to an increased leakage of macromolecules through the endothelium of many vessels in hyperglycemia and diabetes (van den Berg, Nieuwdorp et al. 2006).
The generation of ROIs and advanced glycation end-products (AGEs) also activates the nuclear factor kappa-B (NF-κB) pathway with, subsequently, the activation of numerous genes involved in inflammation, including C reactive protein (CRP), platelet endothelial cell adhesion molecule-1 (PECAM-1), vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule (ICAM-1) (Pober and Sessa 2007). As they represent major receptors controlling the influx of monocytes and other inflammatory cells into the arterial wall, their expression is considered as a hallmark in the etiology of atherosclerosis (Gimbrone 1999).
Finally, the regeneration function of endothelial cells, represented by angiogenesis, is dysfunctional in hyperglycemia and diabetes.

1.3 Diabetes Mellitus.

Diabetes mellitus (DM) is a metabolic disorder representing a leading health problem worldwide (Chawla, Chawla et al. 2016). According to Diabetes Atlas (7^{th} edition), the global prevalence of diabetes is estimated at 415 million which is predicted to rise to 642 million in the next 24 years (IDF Diabetes Atlas, 7th edn, 2015). For this reason, DM is considered as an "epidemic" condition, nowadays without a cure and representing the cause of enormous human and economic losses (Costa and Soares 2013).
Type 2 diabetes (T2DM) is caused by a combination of genetic factors related to impaired insulin secretion and insulin resistance and environmental factors such as obesity, overeating, lack of exercise and stress as well as ageing (Kaku,

2010). Chronic hyperglycemia, the main characteristic of T2DM, develops as a consequence of defect in insulin secretion, insulin action or both (Chawla, Chawla et al. 2016). It is considered as an etiological factor of endothelial barrier injury, as it can stimulate crosslinking and modification of matrix proteins by glyco-oxidation (Naka, Bucciarelli et al. 2004).
Chronic hyperglycemia leads to micro- and macro-vascular alterations that are the principal causes of mortality and morbidity of diabetics.
Indeed, diabetic patients often show poor wound healing, impaired collateral formation after vascular occlusion or myocardial infarction, and also an increased risk of rejection of transplanted organs (Martin, Komada et al. 2003).
For this reason, DM is considered not only a metabolic but also a vascular disease characterized by the so called "angiogenic paradox" in which both anti- and pro-angiogenic conditions co-exist in different tissues. This paradox indicates that local microenvironment of each organ definitely plays a very important role in the angiogenic process (Costa and Soares 2013).

1.3.1 Vascular complications associated to diabetes: micro- vs macro-vascular disease.

Microvessels are the basic functional unit of the cardiovascular system comprising of arterioles, capillaries, and venules (Orasanu and Plutzky 2009). They differ from macrovessels in both their architecture and cellular components. In contrast to macrovessels that supply blood to organs, microvessels play important roles in maintaining blood pressure and proper nutrient delivery (Orasanu and Plutzky 2009). The microcirculation also has regulatory systems controlling vascular permeability and myogenic responses that can adapt blood flow according to local metabolic needs.
Microvascular disease affects microvessels and it is manifested as retinopathy, nephropathy and neuropathy, clinically resulting in blindness, kidney failure and nerve damage. Macrovascular disease, instead, affects medium and large caliper vessels, often leading to atherosclerosis and thromboembolism. Clinically, this translates into coronary, carotid, cerebrovascular disease as well as diseases of peripheral vessels, which are more prevalent in the arteries of the distal segment of the lower limbs (Costa and Soares 2013) (Figure 8).
Patients with DM appear particularly at higher risk of accelerated atherosclerosis which ultimately culminates in cerebrovascular and cardiovascular events and premature death (Kalofoutis, Piperi et al. 2007).
Several studies show that intensive treatment of hyperglycemia is able to reduce the progression of microvascular disease, but not macrovascular (Ahmad, He et al. 2005). This is explained by the different response of large and small vessel endothelium to insulin and glucose (Wang, Chen et al. 2009). It is reported that hyperglycemia is not a determining factor for macrovascular disease since an increase in glycosylated hemoglobin (HbA1c) from 5.5 to 9.5 % implies a two-fold increase in the incidence of macrovascular disease, but the same increase in

HbA1c implies a ten-fold increase in microvascular disease (Wang, Chen et al. 2009, Costa and Soares 2013).

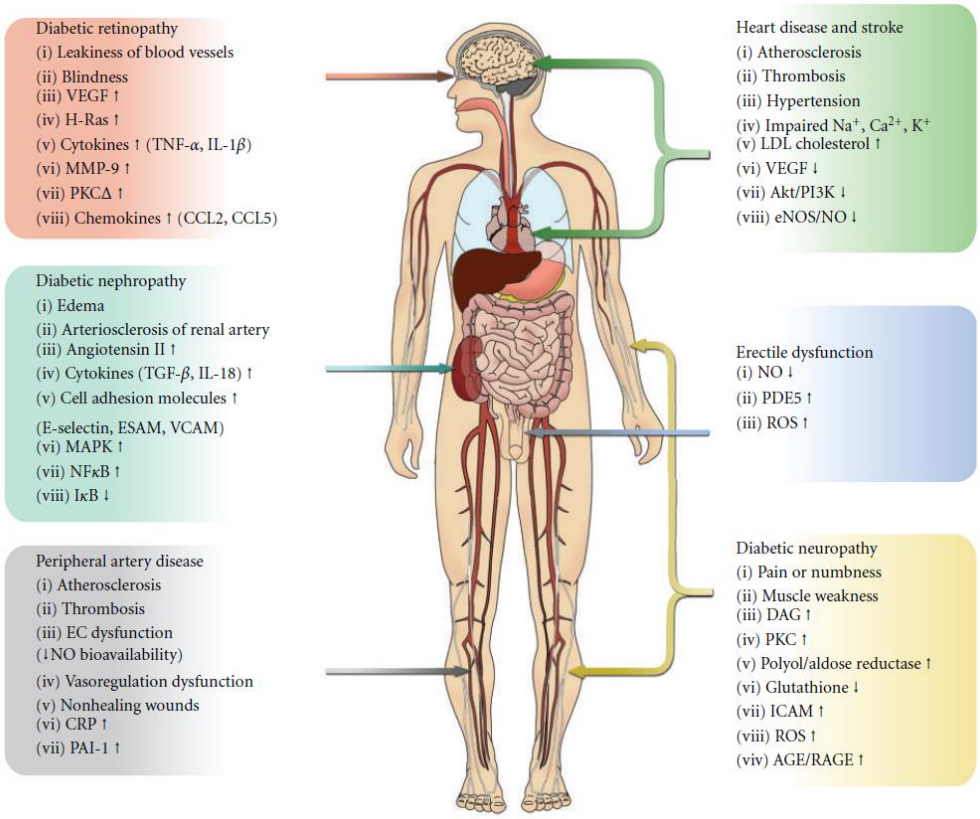

Figure 8. Diabetic vascular diseases. Diabetes affects vasculature in different body tissues leading to micro and macrovascular complications. Main symptoms are indicated for each condition. (Kolluru, Bir et al. 2012)

- *Macrovascular complications*

Atherosclerotic-associated coronary artery disease is a major cause of mortality in diabetics. The first step in the development of atherosclerotic plaque, is the endothelial damage followed by the formation of a fatty streak, a yellow lesion-fatty plaque, which develops well demarcated edges, and evolves to fibrous plaques, which are whitish lesions with a lipid-rich core (Rosenfeld 2000). The enlargement of the necrotic core followed by the rupture of these plaques causes luminal thrombosis in acute coronary syndrome, which occurs in 75% of patients who die of an acute myocardial infarction (Davies and Thomas 1984).

Among the causes leading to destabilization and plaque rupture an important role is played by intra-plaque hemorrhage (Kolodgie, Gold et al. 2003, Costa and Soares 2013). Indeed, red blood cell (RBC) membranes are rich in phospholipids and free cholesterol. Moreover, most intraplaque vasa vasorum are

endothelialized, but only few of them have mural cells such as pericytes and VSMCs. This lack of mural cells may contribute to vessel leakiness because such vessels are fragile and therefore easily damaged. This damage results in the development of immature vessels within the lesion, which are associated with abnormal angiogenesis (Costa and Soares 2013).

- *Microvascular complications*

Several are the microvascular alteration that may occur in diabetes, and these involve several tissues such as retina, nerve, skin and kidney.

- Diabetic Retinopathy

Diabetic retinopathy (DR) is one of the comorbidities of type 1 and 2 diabetes.
It consists of two phases: the first, characterized by an alteration of retina microvasculature and capillary degeneration (Durham and Herman 2011), with capillary pericyte loss and chronic hypoxia; the second in which there is a "super-compensatory" response (Durham and Herman 2011), announced by neovascularization and proliferative changes. Ultimately, DR results in the loss of visual acuity secondary to macular edema, formation of neo-vessels and bleeding or neo-vascular glaucoma (Costa and Soares 2013).
The etiopathogenesis of retinopathy is multifactorial (Costa and Soares 2013) and the risk of development, in patients with T2DM, is related to both severities of hyperglycemia and presence of hypertension (Chawla, Chawla et al. 2016).
Besides the brain, the retina is one of the tissues in human body using the higher amount of oxygen. Hyperglycemia may cause a hypoxic state, thus triggering compensatory mechanisms; however, this only partially explains the development of retinopathy in diabetic patients, indeed not all diabetics show proliferative lesions in their retinas. Moreover, there is increasing evidence that oxidative stress can cause retina damage, leading to diabetic retinopathy and macular edema (Costa and Soares 2013).

- Diabetic nephropathy

Diabetic nephropathy is the leading cause of end-stage renal disease worldwide. Moreover, many associated cardiovascular events can develop following renal degeneration (Xu, Kanasaki et al. 2012). Indeed, in many studies it has been well-established a direct association between the presence of microalbuminuria and macrovascular complications (Hagg, Thorn et al. 2013).
Similarly to what happen in DR, diabetic nephropathy is characterized by abnormal angiogenesis due to *VEGF* overexpression (Xu, Kanasaki et al. 2012). The pathogenic mechanisms underlying diabetic nephropathy involve generation of ROSs, accumulation of AGEs and activation of intracellular signaling molecules, such as protein kinase C (PKC) (Chawla, Chawla et al. 2016). Among the causes leading to the onset and progression of nephropathy, the involvement of pro-inflammatory cytokine, such as IL-1, IL-6, IL-18, TNF-α and TGF-β1, play a pivotal role. Inflammatory activity increases vascular

permeability, thickening of glomerular BM, ECs apoptosis and chronic inflammation. Moreover, oxidative stress, hyperglycemia and diacylglycerol increase TGF-β1 production, facilitating ECM formation through mesangial cells, contributing to hypertrophy of renal cells and increasing collagen synthesis (Costa and Soares 2013).

- Neuropathy and impaired skin wound healing

Diabetic neuropathy is one of the complications induced by microvascular changes and affecting almost half of diabetic patients. Diabetic neuropathy is associated with foot ulcers, gangrene and limb amputation (Costa and Soares 2013, Chawla, Chawla et al. 2016).

The risk of diabetic neuropathy development is directly proportional to both duration and magnitude of hyperglycemia. Although the precise nature of the injury to the peripheral nerves due to hyperglicemia is not certain yet, activation of the polyol pathway, AGEs accumulation and enhanced oxidative stress induced by hyperglycemia have been implicated in the pathogenesis of neuropathy. The peripheral nerves damage could be due to injury on nerve tissue or on endothelial cells or vascular dysfunction (Chawla, Chawla et al. 2016).

In diabetics, chronic ulcers are also associated to an impairment of wound healing capacity. In this process, microangiopathy may also contribute to defects in the nutrient/oxygen supply, thus inhibiting normal healing process (Xu, Kanasaki et al. 2012).

1.4 Molecular mechanisms underlying endothelial damage: role of chronic hyperglycemia.

A wide number of studies performed both in animals and in humans proved that chronic hyperglycemia, as well as acute hyperglycemia, impairs endothelial function in both macro- and micro-vasculature (Ceriello 2010, Grassi, Desideri et al. 2012, Sena, Pereira et al. 2013).

Hyperglycemia causes vascular damage in different cell types of the vascular wall. There are several mechanisms involved in this damaging effect including: 1) increased flux of glucose and other sugars through the polyol pathway; 2) increased intracellular formation of AGEs; 3) increased expression of the receptor for AGEs (RAGE) and its activating ligands; 4) activation of PKC isoforms and 5) overactivation of the hexosamine pathway (Figure 9).

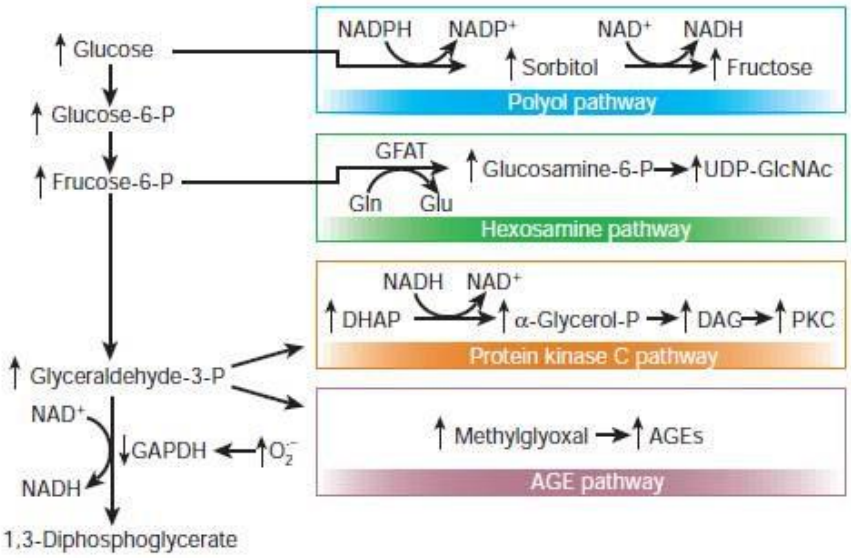

Figure 9. Molecular pathways activated by hyperglycemia. Excess of superoxide partially inhibits the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), thereby directing upstream metabolites from glycolysis into pathways of glucose overutilization. The result is an increase of dihydroxyacetone phosphate (DHAP) to diacylglycerol (DAG), that activates PKC, and of triose phosphates, that are converted to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDP-N-acetylglucosamine increases modification of proteins by O-linked N-acetylglucosamine (GlcNAc) and increased glucose flux through the polyol pathway consumes NADPH and depletes GSH (Brownlee 2001).

Oxidative stress has been identified as the common leading cause of these pathways (Brownlee 2001). Indeed, ROSs decrease the metabolism of glucose through glycolysis increasing the flux through the alternative polyol and hexosamine pathways. Moreover, oxidative stress leads to DNA damage and activation of nuclear poly(ADP-ribose) polymerase (PARP) that in turn increases production of polymers of ADP-ribose reducing glyceraldehyde 3-phosphate dehydrogenase activity. Ultimately the levels of all upstream glycolytic intermediates increase. The accumulation of glycolytic intermediates activates damaging mechanisms: PKC pathway, hexosamine and polyol pathways and AGEs formation. The overall effects of these mechanisms are increased oxidative stress, apoptosis and vascular permeability (Sena, Pereira et al. 2013). Additionally, glucotoxicity induces a low-grade proinflammatory condition, due to the activation of transcription factors such as NF-κB (Creager, Luscher et al. 2003, Brownlee 2005, D'Souza, Hussain et al. 2009).

- Nuclear Factor-κB (NF-κB) activation.

One of the intracellular targets of hyperglycemia and oxidative stress is the transcription factor NF-κB (Evans, Goldfine et al. 2002).

NF-κB is a key mediator regulating multiple proinflammatory and pro-atherosclerotic target genes in ECs, VSMCs and macrophages (Sena, Pereira et al. 2013). As demonstrated by Bierhaurs et al., a sustained activation of NF-κB is reached by activation of AGEs/RAGE pathway (Bierhaus, Schiekofer et al. 2001).

In absence of stimuli, NF-κB is confined in the cytoplasm as an inactive heterodimer, consisting of the p50 and p65 subunits complexed with an inhibitor protein subunit, IκB. Following stimulation, a signaling cascade activates NF-κB-inducing kinase (NIK) and NF-κB-activating kinase (NAK) that activate IκB kinase (IKK) by phosphorylation on serine residues.

IKK is a heterotrimeric complex consisting of two catalytic subunits IKK-α (also called IKK1) and IKK-β (also called IKK2) and a regulatory subunit, IKK-γ. Although both IKK-α and IKK-β subunits are subjected to serine phosphorylation, only substitution of these sites in IKK-β completely prevents the activation of total IKK activity (Delhase, Hayakawa et al. 1999, Evans, Goldfine et al. 2002).

Once activated, IKK phosphorylates IκB leading to its ubiquitination and subsequent degradation. In this way, NF-κB is free to translocate to the nucleus where it mediates the expression of a large number of genes, including growth factors (*e.g.*, VEGF), proinflammatory cytokines (*e.g.*, TNF-α and IL-1β), RAGE, adhesion molecules (*e.g.*, VCAM-1) and others.

Enzymes that catalyze the ubiquitination and degradation of phospho-IκB are constitutively active, indicating that the principal regulatory step in the activation of NF-κB is IκB phosphorylation (Evans, Goldfine et al. 2002) (Figure 10).

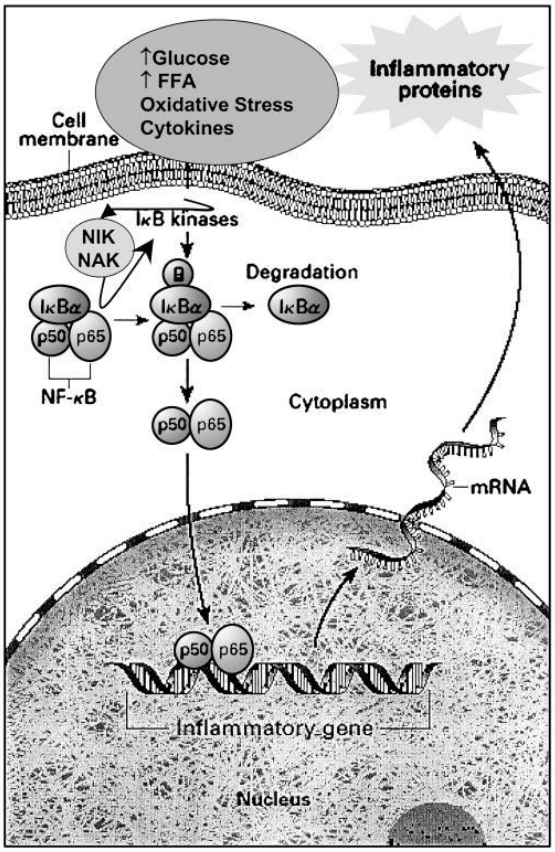

Figure 10. NF-κB activation. Several stimuli (i.e. glucose, FFA, oxidative stress) contribute to NF-κB activation. NF-κB is confined in the cytoplasm in an inactive form complexed with its inhibitor subunit protein IκB. Following IκB phosphorylation, NF-κB translocate to the nucleus where activates the transcription of pro-inflammatory genes. (Evans, Goldfine et al. 2002)

- Advanced Glycation End products (AGEs) pathways activation.

AGEs constitute a heterogeneous group of compounds formed by a combination of glycation, oxidation and/or carbonylation, which can be divided into three distinct pathways (de Vos, Lefrandt et al. 2016) (Figure 11).

The Maillard reaction, the classical mechanism of AGEs formation, is a non-enzymatic process in which glucose or other reducing sugar such as galactose and fructose spontaneously react with aminoacids, nucleotide bases or fatty acids forming glycated molecules.

In particular, in these slow non-enzymatic reactions, carbonyl groups of reducing sugars react with amino group of proteins to form Schiff bases within a few hours. This is followed by intramolecular rearrangement of Schiff bases that results in more stable, but still reversible, Amadori products.

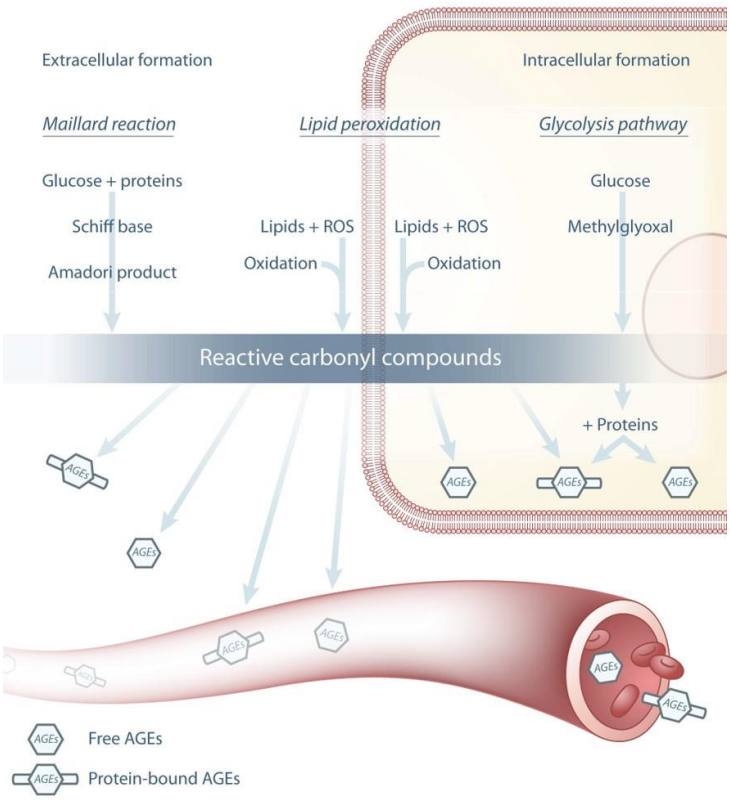

Figure 11. Formation of advanced glycation end products (AGEs). AGEs are formed through three different pathways. The Maillard reaction takes place extracellularly; lipid peroxidation takes place both extra and intracellularly and glycolysis pathway occurs intracellularly (de Vos, Lefrandt et al. 2016).

An example of an Amadori product is glycated hemoglobin, widely used in clinical practice as glycation index and for diagnosis of diabetes mellitus.
Then, the slow process of oxidation of the Amadori products lead to reactive carbonyl compounds and subsequently to the formation of AGEs within weeks to months. The best-known AGEs derived from this glycoxidation process are pentosidine, Nε-carboxymethyl-lysine (CML) and glucosepane (de Vos, Lefrandt et al. 2016).
Other and much faster evolving processes leading to AGEs formation are lipid peroxidation and glycolysis.
The lipid peroxidation pathway take place both intracellularly and extracellularly. In this process, ROSs alter lipids into reactive carbonyl compounds under influence of oxidation. This formation results into AGEs or advanced lipid end products (ALEs), for example malondialdehyde.
Finally, during the intracellular glycolysis pathway, glucose is altered into reactive carbonyl compounds, of which the best-known is Methylglyoxal (MGO). The chemical reaction between reactive carbonyl compounds and

proteins can result in AGEs. An example of AGEs formed by this pathway is MGO-derived hydroimidazolone (MGO-H1) (Jaisson and Gillery 2010).
Besides endogenous formation of AGEs, a certain amount of them can be absorbed through two mechanisms. The first is the inhalation of tobacco smoke, considering that it contains highly reactive glycation products which rapidly form AGEs *in vitro* and *in vivo*. Indeed, it has been shown that serum AGEs are significantly elevated in smokers who smoke at least a package a day as compared to nonsmokers (Koschinsky, He et al. 1997).
The second is the intake of high-AGE food products. Indeed, the temperature at which food products are prepared is of major importance for the amount of AGEs formed in processed food, with oven frying as the most severe inducer (Goldberg, Cai et al. 2004). Approximately 10% of the AGEs from food products and beverages are absorbed from the gastrointestinal tract into the blood (Koschinsky, He et al. 1997). For example, serum CML increased after a 6-week high-AGE diet and decreased in low-AGE diet in patients with diabetes mellitus (Cai, He et al. 2004, de Vos, Lefrandt et al. 2016).
Several studies demonstrate that AGEs have an important role on vasculature contributing to the development of micro- and macro-vascular complication.
Many are the mechanisms by which accumulation of AGEs lead to the alteration of structure and function of different cell types.
First of all, AGEs form cross-link with molecules of ECM. As an example, collagen and elastin cross-link causes tissue stiffness.
Following the binding with their cell membrane receptor, RAGE, AGEs activate intracellular pathways inducing the activation of NF-κB and its target genes, thus promoting the consequent release of pro-inflammatory molecules (de Vos, Lefrandt et al. 2016). Moreover, AGE-bound RAGE increases endothelial permeability to macromolecules.
AGEs are also responsible for the impairment of the balanced release of NO and ET-1. In particular, AGEs decrease NO bioavailability and eNOS expression while increase expression of ET-1 in ECs to favor vasoconstriction and endothelial dysfunction (Sena, Pereira et al. 2013).
Besides the vasculature, accumulation of AGEs due to hyperglycemic condition impair insulin sensitivity of skeletal muscle. Previously in our lab, it has been demonstrated that, in both L6 myotubes and in mice fed a high AGEs diet, human glycated albumin (HGA) induces insulin resistance by activating protein kinase C α(PKCα), through the formation of a multimolecular complex including RAGE/IRS-1/Src and PKCα (Miele, Riboulet et al. 2003, Cassese, Esposito et al. 2008).
Finally, AGEs are also associated to physiological aging and neurodegenerative diseases (Bierhaus and Nawroth 2009, Schlotterer, Kukudov et al. 2009).

1.5 Methylglyoxal metabolism.

The most important AGE precursor is MGO, a α-oxoaldehyde defined as dicarbonyl for the presence in its structure of 2 adjacent carbonyl groups that make this compound highly reactive. Endogenous MGO is derived from metabolic intermediates of carbohydrates, proteins and fatty acids (Fleming, Humpert et al. 2011).
In mammals, the major amount of MGO is formed at relatively high flux as a byproduct of glycolysis by non-enzymatic degradation of triosephosphates, glyceraldeide-3 phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Phillips and Thornalley 1993), with a formation rate of about 120 μM/day in normoglycemic conditions (Thornalley 1988). This rate is increased in conditions leading to higher triosephosphate concentration, like happens in hyperglycemia where glucose metabolism is increased, impairment of pentose pathway with decreased G3P or increased anaerobic glycolysis occurring in hypoxia (Nigro, Leone et al. 2017).
Even if MGO formation through the glycolysis constitute only 0.1% of the glucotriose flux, its biological effect is important considering the high reactivity with proteins and nucleic acids (Maessen, Stehouwer et al. 2015).
Besides glycolysis, minor sources of MGO are: the oxidation of acetone and aminoacetone, the catabolism of ketone bodies, that are increased in diabetic ketoacidosis, the catabolism of threonine, autoxidation of glucose, degradation of proteins glycated and lipid peroxidation (Rabbani, Xue et al. 2016).
Despite endogenous MGO formation, a certain amount of exogenous MGO derives from dietary. In particular, extremely high levels of MGO were found in natural products, such as honey and processed food and beverages such as sweetened soft drink, fruit juice, bread/cakes and others foodstuff. Moreover, also the heating process, prolonged storage and fermentation or polluted air, like tobacco smoke, contribute to MGO formation (Rabbani, Xue et al. 2016, Nigro, Leone et al. 2017).
Anyway, the contribution of exogenous MGO on human health is still controversial. Indeed, some studies report that during digestive process, free MGO is rapidly degraded in the intestine (Degen, Vogel et al. 2013) while others report that in rodents fed with MGO-supplemented diet there are a brain and plasma MGO accumulation (Cai, Uribarri et al. 2014).
Although MGO concentration have frequently been measured in tissues and plasma of various species, the values are highly variable in dependence of the different analytical methods used (Maessen, Stehouwer et al. 2015). Anyway, in physiological conditions, MGO levels have been estimated to be about 50-150 nM in the plasma and 1-4 μM in tissues (Rabbani and Thornalley 2014). When dicarbonyl concentrations increase beyond these, protein and cell dysfunction occur leading to impaired health and pathological conditions.
Effectively, MGO concentrations are increased by 5-6-fold in patients affected by T1DM and by 2-3-fold in T2DM patients (McLellan, Thornalley et al. 1994).

The accumulation of MGO can be prevented by several pathways of detoxification. Among these, the most important enzymatic detoxification is accomplished by the glyoxalase system, which major role is to suppress dicarbonyl stress in physiological systems, keeping dicarbonyl metabolites at low, tolerable levels. Under physiological conditions, >99% of MGO is detoxified by the glyoxalase system. Minor pathways of MGO detoxification include aldehyde dehydrogenase (ALDH) and aldose reductase (AR) converting MGO to pyruvate and hydroxyacetone, respectively (Maessen, Stehouwer et al. 2015) (Figure 12).

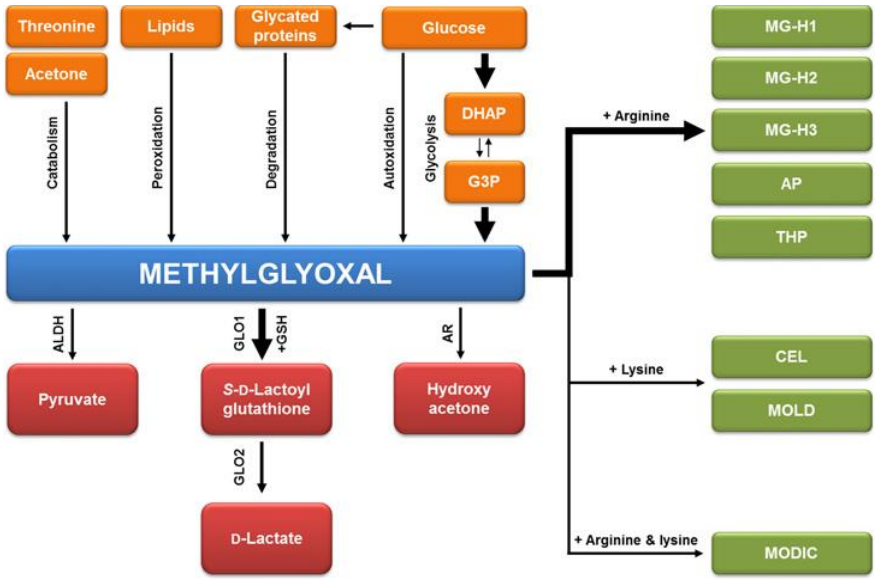

Figure 12. Pathways leading to MGO formation and detoxification. MGO is mainly formed as a byproduct of glycolysis. Other sources of MGO are constitute by: autoxidation of glucose, catabolism of threonine and acetone, lipid peroxidation and degradation of glycated proteins. The glyoxalase system is the most important pathway of MGO detoxification. Other minor pathways are aldehyde dehydrogenase (ALDH) and aldose reductase (AR). When MGO accumulates, it reacts with arginine to form MG-H1, H2, H3, argpyrimidine (AP), tetrahydropyrimidine (THP). When MGO reacts with lysine forms *N*ε-(1-carboxyethyl)lysine (CEL) and 1,3-di(*N*ε-lysino)-4-methyl-imidazolium (MOLD), while it forms 2-ammonio-6-{(2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene) amino}hexanoate (MODIC) following dimer crosslink with arginine and lysine. (Maessen, Stehouwer et al. 2015)

The glyoxalase system is present in the cytosol of all cells and consists of two enzymes, glyoxalase 1 (Glo1) and glyoxalase 2 (Glo2), and a catalytic amount of glutathione (GSH). The rate limiting enzyme Glo1 converts the spontaneously formed MGO-GSH hemithioacetale to the thioester *S*-D-lactoylglutathione. Its activity is directly proportional to GSH concentration, thus in oxidative stress, where cellular GSH concentration is reduced, there is an associated impairment

of Glo1 activity. Glo2 catalyzes the hydrolysis of *S*-D-lactoylglutathione to D-lactate and GSH (Thornalley 1993, Nigro, Leone et al. 2017) (Figure 13).

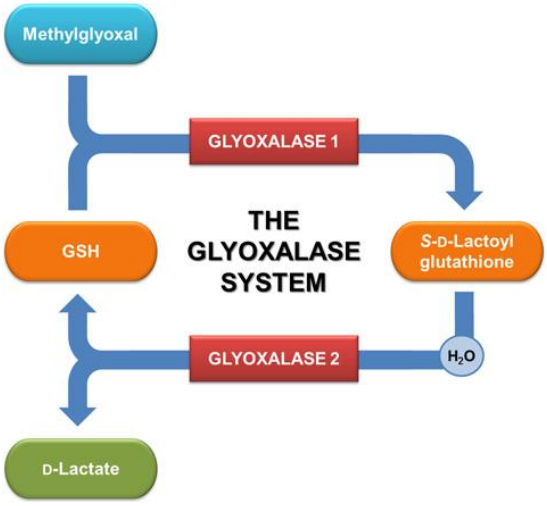

Figure 13. The Glyoxalase System. Glyoxalase system is formed by two enzymes. Glyoxalase 1 catalyzes the conversion of the spontaneously formed MGO-GSH hemithioacetal to the thioester S-D-lactoylglutathione. Glyoxalase 2 catalyzes the hydrolysis of S-D-lactoylglutathione to form the end-product D-lactate. (Maessen, Stehouwer et al. 2015)

The *GLO1* gene is ubiquitously expressed in all tissues of prokaryotic and eukaryotic organisms (Thornalley 2003). Mouse *Glo1* is located on chromosome 17, while human *GLO1* locus is on chromosome 6 and encodes for a homodimeric Zn^{2+} -dependent isomerase of which the two monomers are formed by means of non-covalent bonds. The dimer has molecular mass of 46 kDa and exists as three alloenzymes: Glo1-1, Glo1-2 and Glo2-2 (Thornalley 2003).
The activity of Glo1 is variable during the life. In fetal tissue, Glo1 activity is about 3 times higher than in corresponding adult tissues (Thornalley 2003). Moreover, studies performed at first in *C. Elegans* (Morcos, Du et al. 2008) and then confirmed in rodents (Fleming, Theilen et al. 2013), indicate that there is a physiological decrease of Glo1 activity and expression with age.
In literature, there are several studies demonstrating that Glo1 activity can be modulated by both gene expression regulation and post-translational modifications. For example, Glo1 activity is reduced following the interaction with the GSH-NO adduct S-Nitrosoglutathione that converts Glo1 into an inactive form (Mitsumoto, Kim et al. 2000). Moreover, Glo1 activity is also modified by phosphorylation on Thr^{107} and nitrosylation on Cys^{139} (Maessen, Stehouwer et al. 2015).
In hypoxic conditions, hypoxia inducible factor 1-α (HIF1-α) negatively regulates *Glo1* expression binding an antioxidant- response element (ARE) located on its promoter. In this way, hypoxia is an important driver of dicarbonyl

stress increasing anaerobic glycolysis and thus MGO formation and decreasing *Glo1* expression (Seagroves, Ryan et al. 2001, Nigro, Leone et al. 2017).
In contrast, basal and inducible *Glo1* expression is controlled by nuclear factor erythroid 2-related factor (Nrf2) through the binding to AREs (Xue, Rabbani et al. 2012). The regulation mediated by Nrf2, is antagonized by NF-κB-p65. Indeed, in pro-inflammatory state, typical condition of diabetes and obesity, there is a constitutive activation of NF-κB that, through the inhibition of Nrf2, impairs *Glo1* expression (Liu, Qu et al. 2008). Moreover, the latter is also negatively regulated by the activation of RAGE and, again, NF-κB (Rabbani, Xue et al. 2014).
It is also reported that *GLO1* gene is a hotspot for functional copy number variation (CNV) that leads to an increase of 2-4-fold of *Glo1* expression (Redon, Ishikawa et al. 2006, Cahan, Li et al. 2009). Anyway, further investigations are crucial to understand if these variations are useful against MGO accumulation and damage.

1.5.1 Damaging effect of Methylglyoxal on vascular function.

MGO accumulation is linked to age-related disease such as diabetes, obesity, central nervous disorders and cardiovascular disease, in which endothelial dysfunction is a common feature (Nigro, Leone et al. 2017). Emerging evidence have been highlighted the harmful effect of MGO accumulation on vasculature; it is known that MGO is crucial in the development of diabetic complications playing a damaging effect both on macro- and micro-vasculature.
MGO interacts mainly with arginine residues to form MGO-H1 that is the most prevalent MGO-derived AGE found *in vivo*, leading to structural change, inactivation and degradation of target proteins (Schalkwijk 2015, Nigro, Leone et al. 2017). The interaction of MGO with arginine on serum albumin is responsible for the inhibition of antioxidant capacity and induces an increased synthesis and secretions of proinflammatory markers (TNF-α, IL-1β) in monocyte. Other important MGO-H1 protein are haemoglobin, lens proteins and collagen. In particular, modification of type IV collagen leads to decreased integrin binding, detachment of endothelial cells from vascular wall and inhibition of angiogenesis. Moreover, MGO causes the impairment of physiological angiogenesis through a RAGE-mediated autophagy-induced VEGFR2 degradation (Liu, Yu et al. 2012).
An increase of MGO-H1 modifications of mitochondrial proteins are linked to increased oxidative stress (Maessen, Stehouwer et al. 2015). In turn, oxidative stress induced by MGO is linked to the impairment of endothelium-dependent vasodilation. Indeed, a study performed in rat aortic tissue indicates that acetylcholine-induced vasodilation is impaired by MGO and this effect is attenuated by treatment with MGO scavengers (aminoguanidine and N-acetyl-cisteine) (Dhar, Dhar et al. 2010) and by *Glo1* overexpression (Brouwers, Niessen et al. 2010).

Another target of MGO is represented by eNOS; indeed, it has been demonstrated that long-term MGO treatment of rat isolated mesenteric artery induces a reduction of protein levels and phosphorylation on the active site (Ser^{1177}) of this protein (Mukohda, Morita et al. 2013).
In contrast, antioxidants like polyphenols prevent MGO-dependent impairment of NO release, improving vasodilation in mouse corpora cavernosa (Goueslard, Cottenet et al. 2016). Moreover, age-related glycative and oxidative stress is reduced in Glo1 transgenic rats (Jo-Watanabe, Ohse et al. 2014).
The involvement of MGO in the pathogenesis of atherosclerosis and development of macrovascular diabetic complications is demonstrated by several studies performed in animal models and humans. Vulesevic B et al. demonstrate how MGO, in diabetic mice, is involved in development of cardiomyopathy increasing inflammation and endothelial cell loss (Vulesevic, McNeill et al. 2016). In line with this, MGO treatment of Goto-Kakizaki rats exacerbate endothelial dysfunction in part increasing oxidative stress, AGEs formation and inflammation (Sena, Matafome et al. 2012). Moreover, MGO high levels induce vascular contractile dysfunction in arterial walls of spontaneously hypertensive rats (Mukohda, Okada et al. 2012) and activates NF-κB through RAGE, thus increasing rennin-angiotensin levels and blood pressure in Sprague-Dawley rats (Dhar, Dhar et al. 2014, Nigro, Leone et al. 2017) (Figure 14).

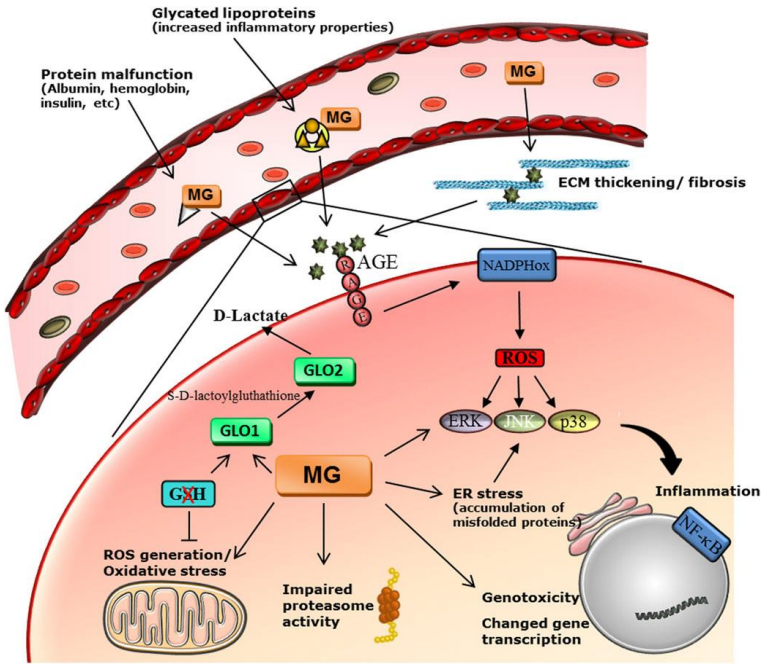

Figure 14. Main mechanisms of MGO action. AGEs, advanced glycation end products; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; GLO1, glyoxalase 1; GLO2, glyoxalase 2; GSH, reduced glutathione; JNK, c-jun N-terminal kinase; MG, methylglyoxal; NADPHox, nicotinamide adenine dinucleotide phosphate oxidase; NF-κB, nuclear factor -κB; ROSs, reactive oxygen species.(Matafome, Rodrigues et al. 2016)

Besides macro-vasculature, MGO has a harmful effect on microvasculature, contributing to the pathogenesis of diabetic nephropathy, retinopathy and neuropathy. In particular, exposure of cultured cells and kidneys from diabetic mice to high levels of MGO sensitize microvascular ECs to inflammatory effects of TNF-α (Yao, Taguchi et al. 2007). Moreover, overexpression of *Glo1* in streptozotocin-induced diabetic rats improves renal function and protects against albuminuria (Liu, Zhu et al. 2013, Geoffrion, Du et al. 2014). MGO induces blood brain barrier damage by increasing permeability of brain endothelial cells (Toth, Walter et al. 2014).
Increased serum levels of MGO-H1 are associated to the development of diabetic retinopathy (Fosmark, Torjesen et al. 2006), while Glo1 overexpression protects against premature death of pericytes and ECs (Miller, Smith et al. 2006). Moreover, MGO also induces cell death via NF-κB activation in diabetic lens (Kim, Kim et al. 2012, Maessen, Stehouwer et al. 2015). Finally, Fleming et al. (Fleming, Theilen et al. 2013) demonstrated in a study performed in mice that aging-dependent reduction of Glo1 delays wound healing.
In light of this evidence, appears clear that the regulation of MGO concentrations is crucial for preserving vascular function.

2. AIM OF THE STUDY

DM is a metabolic disease representing a leading health problem worldwide. Diabetic patients are exposed to a higher risk to develop micro-and macro-vascular complications responsible for the high mortality of associated to DM.
Chronic hyperglycemia is the main characteristic of diabetes and constitutes a primary factor in the development of diabetic vascular complications. A common feature of these complications is the alteration of angiogenesis.
In the last decades, extensive research has been focused on the identification of the causes that lead to the onset and progression of diabetic complications. However, given the complexity of these phenomena, the underling molecular mechanisms are not fully understood yet.
Previously, we and others have demonstrated that MGO, a product of chronic hyperglycemia, is responsible for endothelial dysfunction.
The aim of this study is to evaluate the effect of MGO on the angiogenic process of endothelial cells in order to identify the molecular mechanisms by which this reactive dycarbonyl may impair the vascular function.

3. MATERIALS AND METHODS

Reagents. Media, sera and antibiotics for cell culture were from Lonza (Walkersville, MD, USA). Protein electrophoresis and western blot reagents were from Bio-Rad (Richmond, VA, USA) and ECL reagents from Pierce (Rockford, USA). The used antibodies were anti-HoxA5 (Abcam, Cambridge, UK), anti- NF-κB -p65, anti-GAPDH, anti-Cyclin-G1 from Santa Cruz Biotecnology (CA, USA), anti-α-Tubulin (Sigma-Aldrich, St Luis, MO, USA). TRIzol and SuperScript III were from Invitrogen (Carlsbad, California, USA). SYBR Green Supermix was from Bio-Rad (Hercules, California, USA). DNA Purification Kit and pGEM-T EASY Vector were from Promega (Madison, Wisconsin, USA). NF-κB Activation Inhibitor (JSH-23) was from Santa Cruz Biotecnology (CA, USA). The QIAquick PCR Purification kit was from QIAGEN (Hilden, Germany). All other chemicals were from Sigma (St Louis, MO, USA).

Cell culture procedure. Mouse aortic endothelial cells (MAECs) isolated by C57/bl6 (WT) and Glyoxalase 1 heterozygous knockdown (Glo1KD) mice were kindly provided by Dr. T.H. Fleming (Department of Medicine I and Clinical Chemistry, University Hospital Heidelberg). MAECs were plated in T75 flask and grown in Dulbecco's modified Eagle's medium (DMEM) containing 1 g/l glucose supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine and 0.1 mM non-essential amino acids. Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2.

Cytoplasmic and nuclear fractionation. Nuclear and cytoplasmic proteins were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Kit purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA), according to the manufacturer's instructions. Where indicated, cells were treated overnight with 60μM JSH-23.

Western blot analysis. Cells were solubilized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM Na2P2O7, 2 mM Na3VO4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride and 10 μg/ml aprotinin) for 2 h at 4°C. Cell lysates were clarified by centrifugation at 16,000 *g* for 20 min. Cell lysates were then separated by SDS-PAGE and transferred into 0.45-μm Protran Nitrocellulose Membrane (Sigma-Aldrich, St Luis, MO, USA). Upon incubation with primary and secondary antibodies, immmunoreactive bands were detected by chemiluminescence and densitometric analysis was performed using ImageJ software.

RNA isolation, reverse transcription and Real Time-PCR. Total RNA was isolated from MAECs using TRIzol reagent, according to the manufacturer's protocol. After quantification with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), 1 μg of total RNA was reverse transcribed using SuperScript III according to the manufacturer's instructions. The cDNA obtained was used as a template for Real Time-PCR, performed in triplicate by using iQ SYBR Green Supermix on iCycler Real Time detection system (Bio-Rad). Relative quantification of gene expression was calculated by the $\Delta\Delta^{-Ct}$ method. Each *Ct* value was first normalized to the respective *Cyclophilin A Ct* value of a sample to account for variability in the concentration of RNA and in the conversion efficiency of the reverse transcription reaction. Specific primers used for amplification were purchased from QIAGEN (Hilden, Germany) and listed in the table below (Table 1):

Cyclophilin A	Forward 5'-GCAGACAAAGTTCCAAAGACAG- 3' Reverse 5'-CACCCTGGCACATGAATCC- 3'
Glo1	Forward 5'-CCCTCGTGGATTTGGTCACA-3' Reverse 5'-AGCCGTCAGGGTCTTGAATG-3'
HoxA5	Forward 5'-CCCAGATCTACCCCTGGATG-3' Reverse 5'-CAGGGTCTGGTAGCGAGTGT-3'
NF-κB-p65	Forward 5'-CTGATGTGCATCGGCAAGTG -3' Reverse 5'-CTCTTCAATCCGGTGGCGAT-3'

Table 1: Primer sequences used for Real Time-PCR analysis.

Cell transfection. $1x10^5$ MAECs were seeded in 60-mm plates. After 18 hours, HoxA5 expression was modulated by transfecting MAECs with HoxA5-siRNA 5 nM (Origene, Rockville, MD, USA) and negative control (scramble). TransIT-X2 purchased from Mirus Bio LCC (Madison, WI, USA) was used as transfection reagent, according to the manufacturer's instructions. Cells were incubated with complete medium for 48 hours before starvation.

Quantification of MGO. Intracellular MGO concentration was measured in WT and Glo1KD MAECs extracts by HPLC after derivatization with 1,2-diamino-4,5-dimethoxybenzene as previously described (McLellan, Phillips et al. 1992).

Cell growth assay. $5x10^4$ cells were seeded in MW-6 and cell growth was monitored at 0, 12, 24, 48, 72, 96 hours by automated cell count with TC10™ Automated Cell Counter (Bio-Rad).

Transwell migration and invasion assay. Cells were serum starved for 5 hours. After trypsinization, $1x10^5$ cells/well were plated on the upper side of 12-well cell culture inserts (BD Falcon, Franklin Lakes, NJ) with 5.0 μm pores

polycarbonate membrane. To perform invasion assay, 12-well cell culture inserts with 8.0 µm pores polyethylene terephthalate (PET) membrane were coated with 100 µL 1µg/µl Matrigel (BD Biosciences, San Jose, CA, USA). Culture medium with 10% FBS was added in the bottom of the well as chemotactic stimulus. Following an overnight incubation, the top of the insert was cleared of cells (and Matrigel) with cotton swab; then, the top and the bottom of insert were washed 3 times with 1X PSB.
Inserts were incubated 30 minutes at room temperature with 11% glutaraldehyde to fix cells migrated on the bottom, and then washed 3 times with 1X PBS. After this, cells were stained with Crystal Violet and washed with 1X PBS. Cells from 4 representative fields (10X and 4X magnification) from each insert were counted.

Chromatin immunoprecipitation assay. $5x10^5$ cells were fixed for 10 minutes at room temperature with 1% (v/v) formaldehyde and crosslinking reaction was stopped with 125 mM glycine for 5 minutes at room temperature. Formaldehyde and glycine were removed by centrifugation (3 minutes at 13,000 rpm). SDS and HBSS buffer were added to cells and DNA was sonicated using the Bioruptor® sonicator (Diagenode, Seraing, Belgium).
Cellular debris were removed by centrifugation, then equal amounts of DNA were immunoprecipitated using anti-NF-κB-p65 antibody (Santa Cruz Biotecnology CA, USA) with anti-mouse IgG beads (Thermo Scientific, Waltham, MA, USA).
Precipitated fragments were washed with dialysis buffer (2mM EDTA, 50mM Tris-Cl pH 8.00, 0.2% Sarkosyl), IP wash buffer (100mM Tris-Cl pH:9.0, 500mM LiCl, 1% NP-40, 1% deoxycholic acid) and TE (1M Tris-HCl/ 0.1M EDTA) buffer. Fragments were eluted at 37 °C for 45 minutes with elution buffer (1% SDS, Sodium Bicarbonate 0.1 M) and, then, de-crosslinked with 5M NaCl at 65 °C overnight. Proteins were removed by samples using proteinase K and 1M Tris-HCl/ 0.5M EDTA. After that, immunoprecipitated DNA was purified with QIAquick PCR Purification Kit according to the manufacturer's instructions, and quantified by Real-Time PCR using specific primer sets flanking the expected p65 binding site:

NF-κB-p65/HoxA5	Forward 5'-ACACACAGGTTTTCTCCCCA -3'
	Reverse 5'-GAATTTGATGGCGAGGGACC-3'

Statistical procedures. Data are expressed as means ± SD and means ± SEM of three independent experiments. Comparison between groups were performed using Student's *t*-test. P values of less than 0.05 were considered statistically significant.

4. RESULTS

4.1 Effect of MGO accumulation on angiogenesis in endothelial cells.

Angiogenesis is a multistep process that generally begins in response to pro-angiogenic stimulation when endothelial cells start to proliferate, migrate and invade the ECM to take part in the formation of an immature capillary structure and deposition of a new complex BM (Neve, Cantatore et al. 2014).
It is known that increased MGO levels impair the endothelial function in various way and in different body district (Nigro, Leone et al. 2017), leading to development of micro- and macro-angiopathy associated to diabetes (Stehouwer, Lambert et al. 1997, De Caterina 2000, Schalkwijk and Stehouwer 2005).
In order to evaluate the MGO effect on angiogenesis, we have analyzed the angiogenic ability of endothelial cells isolated from the aortae of mice knockdown for the *Glo1* gene (Glo1KD MAECs) and their wild type littermates (WT MAECs).
To validate our model, we have analyzed the *Glo1* mRNA levels in WT and Glo1KD MAECs by Real Time-PCR. In figure 15A, it is shown that *Glo1* mRNA levels are reduced by a 50% in Glo1KD MAECs compared to WT MAECs. This reduction is paralleled to a 5-fold increase of MGO intracellular concentration in Glo1KD MAECs compared to WT MAECs (figure 15B).

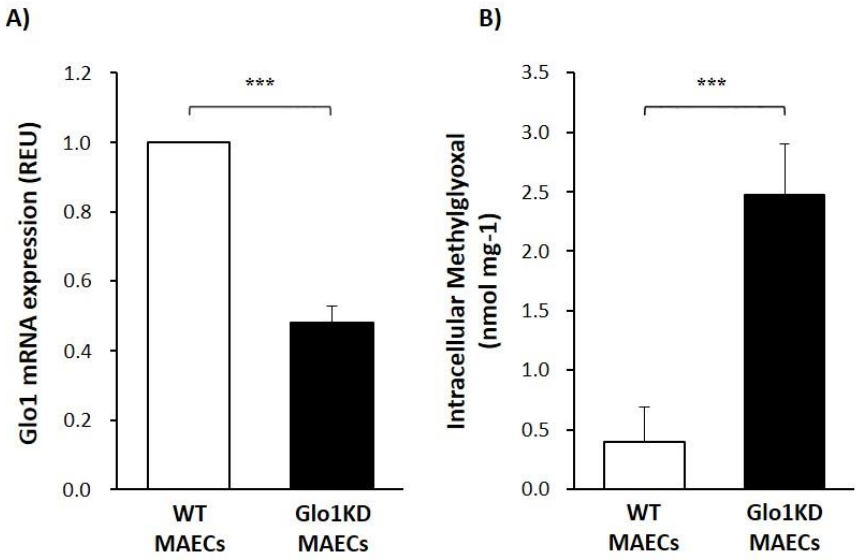

Figure 15. *Glo1* gene expression and MGO intracellular concentration in Glo1KD and WT MAECs. A) *Glo1* mRNA levels were assessed by Real Time-PCR in Glo1KD and WT MAECs. The bars in the graph represent the relative expression unit (REU) ± SD of three independent experiments. Experimental normalization was performed using *Cyclophilin A* gene as housekeeping control. **B)** MGO levels were measured by HPLC. Bars in the graph show MGO intracellular concentrations (nmol) normalized on milligrams of proteins. Statistical significance was evaluated using Student's *t* test (*** $p \leq 0.001$).

In order to test the angiogenic ability of these cells, we have evaluated their proliferation, migration and invasion ability.
Glo1KD and WT MAECs cell growth was monitored up to 96 hours. Then, to test the invasion and migration ability, both Glo1KD and WT MAECs were seeded on the upper side of porous membrane transwell insert coated or not with Matrigel, respectively. FBS (10%) was used as pro-angiogenic stimulus.
As shown in figure 16A, Glo1KD MAECs are characterized by a slower cell growth compared to WT MAECs. The proliferation, indicated as fold of cell number over time 0, is significantly reduced starting from 48 hours. Moreover, in response to 10% FBS, migration and invasion ability of WT MAECs is ~10-fold and ~6-fold increased, respectively. Conversely, Glo1KD MAECs show a strong impairment of these ability in presence of 10% FBS (figure 16B, 16C).
Together, these results indicate that MGO accumulation is responsible for an impairment of angiogenesis in Glo1KD MAECs.

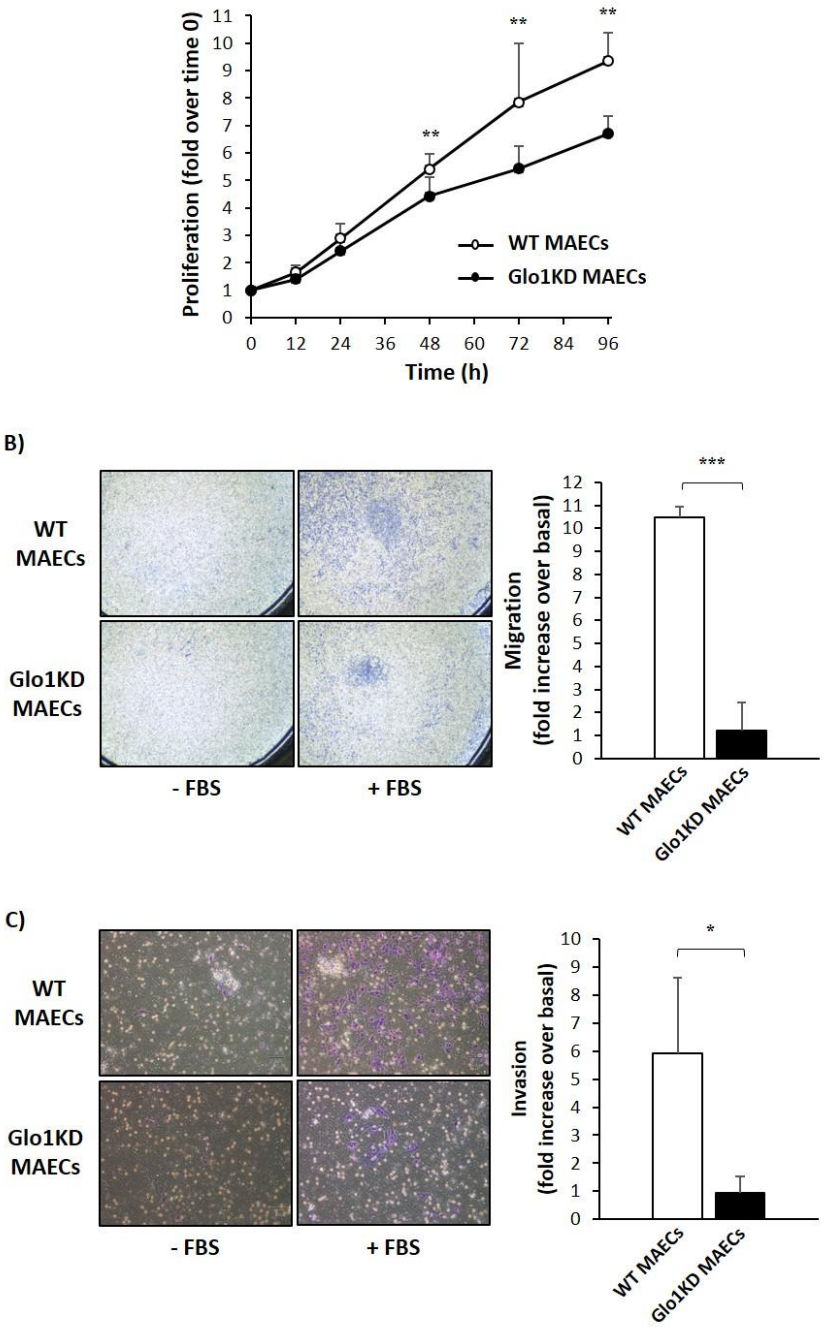

Figure 16. Glo1KD and WT MAECs angiogenic phenotype. **A)** $5 \cdot 10^4$ MAECs were seeded in MW-6 plates and the proliferation was tested from time 0 to 96 hours by automated cell count of Glo1KD and WT MAECs. Proliferation in the graph is reported as fold of cell number over time 0. **B)** MAECs were seeded on the upper side of 5.0 µm polycarbonate membrane transwell inserts and the migration ability was tested in presence or not of 10% FBS by staining cells with crystal violet on the lower side of transwell insert. Migrated cells are visible in violet in the 4X magnification images on the left. The graph on the right represents the mean ± SEM of the fold increase of migrated cells in response to 10% FBS over the basal migration of cells in absence of 10% FBS. **C)** MAECs were seeded on the upper side of 8.0 µm PET membrane transwell

inserts coated with Matrigel and the invasion ability was tested as reported in figure legend 16B. Representative 10X magnification images are on the left of panel C. The graph on the right represents the mean ± SEM of the fold increase of migrated cells in response to 10% FBS over the basal migration of cells in absence of 10% FBS. Statistical significance was evaluated using Student's *t* test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

4.2 *HoxA5* involvement in MGO-mediated impairment of angiogenesis in MAECs.

To identify the molecular mechanisms leading to the impaired angiogenesis in Glo1KD MAECs, we looked at candidate genes that are known to have a pivotal role in neovascularization. Among these, we analyzed the involvement of the antiangiogenic *HoxA5* gene, that is the most representative *Homeobox* gene expressed in adulthood (Kachgal, Mace et al. 2012).
Both protein (figure 17A) and mRNA (figure 17B) levels of *HoxA5* are increased ~2-fold in Glo1KD compared to WT MAECs.
To demonstrate the involvement of *HoxA5* in MGO-mediated angiogenesis impairment, *HoxA5* expression was transiently silenced by the use of specific siRNAs. We used the transfection conditions leading to *HoxA5* mRNA reduction in Glo1KD MAECs at similar levels to those of WT MAECs (data not shown). Interestingly, *HoxA5* silencing results in the improvement of both migration (figure 17C) and invasion (figure 17D) ability of Glo1KD MAECs.

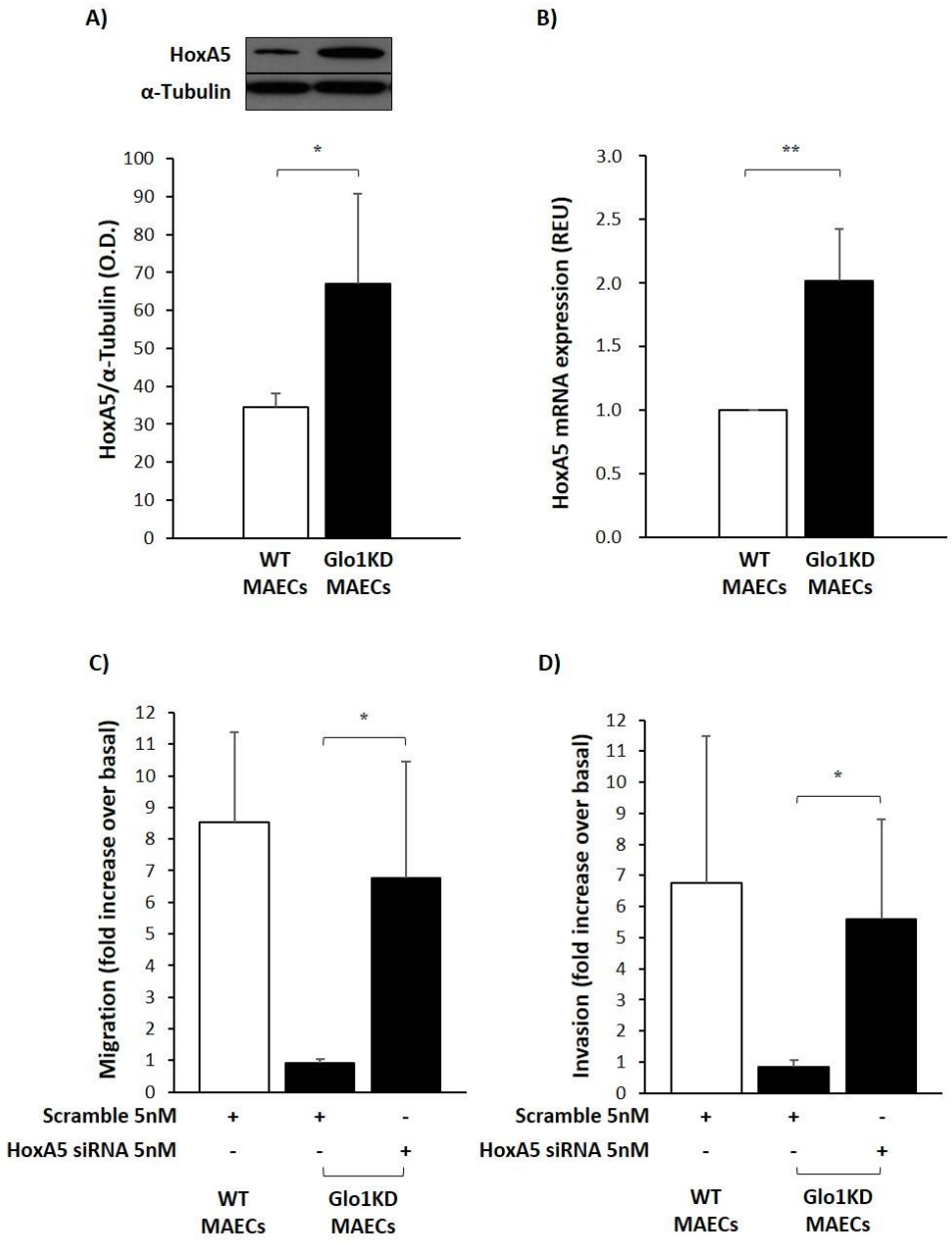

Figure 17. *HoxA5* expression and its involvement in angiogenesis of Glo1KD and WT MAECs. A) Protein lysate were obtained by Glo1KD and WT MAECs and tested by western blot assay with anti-HoxA5 antibody. Anti-α-tubulin antibody was used as loading control. The bars in the graph represent the mean ± SD of the values obtained by the densitometric analysis of at least three independent experiments. **B)** *HoxA5* mRNA expression was measured by Real Time-PCR. *Cyclophilin A* was used as housekeeping control gene. The bars in the graph represent the relative expression unit (REU) ± SD of three independent experiments. HoxA5-siRNA was transfected in Glo1KD MAECs. Scramble siRNA was transfected as negative control. Bars in graphs show the fold increase of migration **(C)** and invasion **(D)** over the basal condition (w/o FBS). Statistical significance was evaluated using the Student's *t* test (*$p \leq 0.05$, **$p \leq 0.01$).

4.3 NF-κB-p65 role in the regulation of MGO-mediated *HoxA5* overexpression.

Evidence in literature indicates that exposure of ECs to hyperglycemia induces sustained activation of the transcription factor NF-κB, at least in part, by the AGEs/RAGE pathways (Nishikawa, Edelstein et al. 2000, Bierhaus, Schiekofer et al. 2001).
Wondering how MGO may alter *HoxA5* expression, we investigated the involvement of NF-κB-p65.
To this aim, we evaluated NF-κB-p65 activation in Glo1KD and WT MAECs analyzing the translocation of p65 subunit into the nucleus. Cytoplasmic and nuclear proteins were fractionated and NF-κB-p65 levels were evaluated by western blot. Our results indicate that NF-κB-p65 is higher in both the cytosol and the nucleus (figure 18A) of Glo1KD MAECs compared to WT cells.
In line with this, Glo1KD MAECs also show a ~2-fold increase of *NF-κB-p65* expression (figure 18B).
Thus, these data demonstrate that a higher amount of activated NF-κB-p65 is present in ECs with high MGO levels.

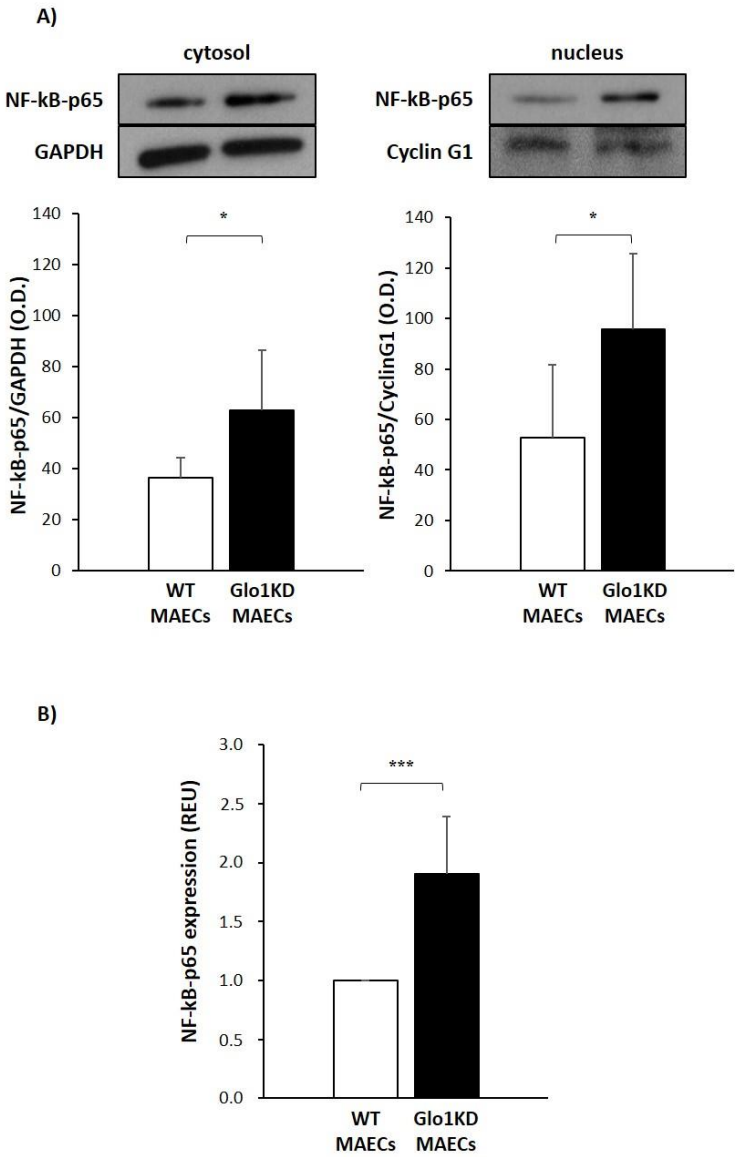

Figure 18. NF-κB-p65 protein and mRNA levels in Glo1KD and WT MAECs. A) NF-κB-p65 protein levels were evaluated on protein lysate from cytosol/nuclear fractionating by western blot with anti-NF-κB-p65 antibody. Cytoplasmic normalization was performed by using anti-GAPDH antibody, and nuclear normalization with anti-CyclinG1 antibody. The bars in the graph represent the mean ± SD of the values obtained by the densitometric analysis of at least three independent experiments. **B)** *NF-κB-p65* mRNA expression levels were measured by Real Time-PCR. *Cyclophilin A* was used as housekeeping control gene. Statistical significance was evaluated using the Student's *t* test (*$p \leq 0.05$, ***$p \leq 0.001$).

Next, we performed a chromatin immunoprecipitation assay to test the binding of NF-κB-p65 to *HoxA5* promoter.

First of all, we looked for predicted p65 binding sites on *HoxA5* promoter through the use of Jaspar software. The consensus sequence with the highest

score was amplified by Real Time-PCR, following the chromatin immunoprecipitation with a NF-κB-p65 specific antibody, to quantify the binding of NF-κB-p65 to the *HoxA5* promoter.
As shown in figure 19A, the binding of NF-κB-p65 to *HoxA5* is higher in Glo1KD MAECs compared to WT MAECs. Interestingly, the inhibition of p65 obtained by the use of JSH-23 is able to reduce the mRNA expression of *HoxA5* in Glo1KD MAECs (figure 19B).

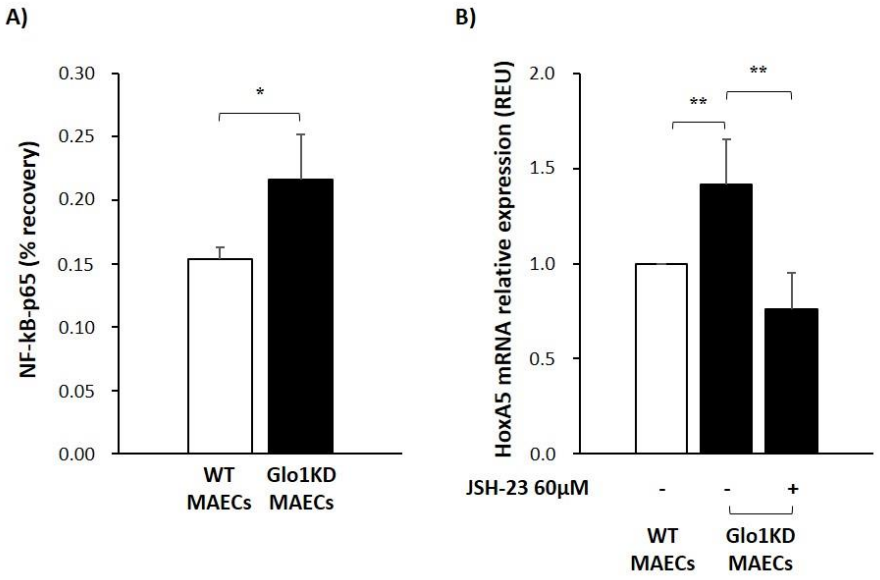

Figure 19. *HoxA5* transcription regulation by NF-κB-p65 binding to its promoter. A) NF-κB-p65 binding on *HoxA5* promoter was tested by chromatin immunoprecipitation assay (ChIP) followed by quantization by Real Time-PCR of a putative high scored NF-κB-p65-consensus sequence on *HoxA5* promoter. Bars in the graph represent a quantitation of the *HoxA5* promoter region bound by NF-κB-p65 and are shown as the % of chromatin recovered by ChIP ± SD of three independent experiments. **B)** Cells were treated with 60 μM JSH-23 and *HoxA5* mRNA levels were analyzed by Real Time-PCR. *Cyclophilin A* was used as housekeeping control gene. Statistical significance was evaluated using the Student's *t* test (*$p \leq 0.05$, **$p \leq 0.01$).

Taken together, these data demonstrate that MGO accumulation in Glo1KD MAECs is associated to a higher transcription and activation of NF-κB-p65 that, binding *HoxA5* promoter, is able to positively regulate *HoxA5* expression.

5. DISCUSSION

T2DM is a chronic metabolic disease characterized by the presence of hyperglycemia. It is considered as an epidemic condition worldwide, nowadays without a cure (Dornadula, Elango et al. 2015).
Over the years, hyperglycemia leads to the development of long-term micro- and macro-vascular complications. These complications are responsible of the high mortality associated to DM, affecting heart, blood vessels, eyes, kidney, nerves and wound healing process (Ribot, Caliaperoumal et al. 2016).
Diabetic vascular disease is associated with impaired vascularization and/or aberrant angiogenesis. Indeed, an excessive angiogenesis is typical of diabetic retinopathy and nephropathy (Abu El-Asrar, Nawaz et al. 2013), while a reduced angiogenesis contributes to impaired wound healing and coronary collateral vessel development (Tahergorabi and Khazaei 2012).
Giving the complexity of these alterations, although extensive research has been performed, the mechanisms underlying the abnormal angiogenesis occurring in diabetic patients are still not fully understood (Ribot, Caliaperoumal et al. 2016).
Endothelial cell function is an essential requirement to guarantee a physiological angiogenesis (Kolluru, Bir et al. 2012). It is widely known that hyperglycemia induces endothelial dysfunction through the activation of several molecular pathways (Brownlee 2005). Among these, great attention has been paid to the increased formation of AGEs, playing a central role in the glucose-mediated vascular damage (Goh and Cooper 2008).
In endothelial cells, one of the most important AGE precursor is MGO, a highly reactive dycarbonyl primarily formed by the intermediates of glycolysis (Baynes and Thorpe 1999, Thornalley 2005) and found to be increased from 2 to 5-fold in diabetic patients (McLellan, Thornalley et al. 1994).
Emerging evidence have highlighted the harmful effect of MGO on endothelial function. We have previously demonstrated that treatment of both ECs *in vitro* and mice with non-toxic concentration of MGO impairs insulin sensitivity by changing the balance of NO and ET-1 release (Nigro, Raciti et al. 2014). Moreover, we have also recently found that MGO-induced down-regulation of miR-190a plays a role in this effect (Mirra, Nigro et al. 2017).
In a study performed in rats, Berlanga et al. showed how MGO exposure leads to the impairment of wound healing and the development of diabetes-like vascular damage. In particular, MGO treatment is associated to the arrest of cell growth, hypercholesterolemia, impaired vasodilation and degenerative changes in cutaneous microvessels with loss of ECs (Berlanga, Cibrian et al. 2005).
In physiological conditions, MGO is mainly detoxified by the glyoxalase system, of which Glo1 is the rate-limiting enzyme, representing the first line in the defense of cellular damage induced by the glycation process (Carmeliet 2005).
It has been demonstrated that aging-dependent reduction of Glo1 delays wound healing in old mice (Fleming, Theilen et al. 2013); moreover, reduced Glo1

activity is associated with painful peripheral neuropathy in patients with T2DM (Skapare, Konrade et al. 2013).
In line with this, the overexpression of *Glo1* improves endothelial function and attenuates early renal impairment in a rat model of diabetes (Brouwers, Niessen et al. 2014). Moreover, *Glo1* overexpression inhibits AGEs formation in bovine ECs (Shinohara, Thornalley et al. 1998) and improves hyperglycemia-induced impairment of angiogenesis in human microvascular ECs (Ahmed, Dobler et al. 2008).
Although several studies have demonstrated that high levels of MGO contribute to endothelial dysfunction and vascular complications, as indicated above, further investigations are still needed to clarify the underlying molecular mechanisms.
Angiogenesis is a complex process highly regulated, in which new vessels are formed from pre-existing ones. In particular, this process involves endothelial cell proliferation, matrix degradation, cell migration, tube formation and vessel maturation (Carmeliet 2005).
In the present study, we sought to investigate the MGO effect on angiogenesis and the underling molecular mechanisms, using an *in vitro* model of ECs characterized by endogenous MGO accumulation.
In detail, ECs were isolated from aortae of mice knock-down for the *Glo1* gene (Glo1KD MAECs) and their WT littermates (WT MAECs).
The reduced expression of *Glo1* in Glo1KD MAECs is paralleled to increased endogenous MGO levels.
To evaluate whether and how the angiogenesis may be altered in these cells, we focused on their ability to perform the main steps of this process.
Endothelial cell proliferation, migration and invasion are essential to angiogenesis. These motile processes are directionally regulated by several stimuli and involve the degradation of ECM to enable progression of migrating cells to form the new vessel (Lamalice, Le Boeuf et al. 2007).
We here demonstrate that Glo1KD MAECs have an impaired angiogenic ability compared to WT cells. In particular, Glo1KD MAECs show a reduced proliferation. The observed impairment of cell growth seems to be not affected by cell apoptosis, but by a decrease in cell proliferation. Indeed, the cleavage of PARP, used as marker of apoptotic activation, was not revealed in Glo1KD nor in WT MAECs, differently from H_2O_2-treated MAECs (data not shown).
Exposure to FBS (10%), used as pro-angiogenic stimulus, is able to induce migration of WT MAECs. Conversely, the migration ability is strongly impaired in Glo1KD cells in response to FBS. Furthermore, Glo1KD MAECs show an impairment of the ability to degrade an extracellular matrix extract and invade a porous membrane in response to FBS, differently from WT MAECs.
In order to identify the molecular mechanisms leading to this phenotype, we focused our attention on the involvement of the anti-angiogenic *HoxA5* gene.
HoxA5 belongs to a family of highly conserved genes coding for transcription factors originally discovered for their role in the regulation of organ patterning during development (Murtha, Leckman et al. 1991). More recently, several

homeobox genes have been shown to affect physiological processes in adult tissues, including angiogenesis and wound healing (Kachgal, Mace et al. 2012). In particular, emerging evidence have highlighted the *HoxA5* involvement on angiogenesis. Arderiu et al. demonstrated that sustained *HoxA5* expression in murine hemangioma cell line (EOMA) reduces their growth *in vivo* (Arderiu, Cuevas et al. 2007). *HoxA5* is also associated to retinoic acid-induced apoptosis and cell growth inhibition acting as a downstream mediator of retinoic acid receptor-β (Chen, Zhang et al. 2007). Furthermore, Cuevas et al. proved that sustained *HoxA5* expression in murine ECs prevents angiogenesis by inhibiting the expression of the pro-angiogenic *VEGFR2* and, at the same time, promoting the expression of anti-angiogenic *TSP-2* (Cuevas, Layman et al. 2015).
We also confirmed a reduction of VEGFR2 protein levels in Glo1KD MAECs compared to WT MAECs (data not shown). In line with this, Liu et al. show that, in bovine aortic endothelial cells (BAECs), MGO impairs endothelial angiogenesis through VEGFR2 protein degradation via a mechanism involving autophagy (Liu, Yu et al. 2012).
These evidence support the idea that *HoxA5* may represent a good target to look for answering the question of which mediator may be involved in the angiogenesis impairment observed in our model.
Interstingly, Glo1KD MAECs show an increase of both protein and mRNA levels of *HoxA5*. This suggests that *HoxA5* could be involved in the altered angiogenic phenotype found in Glo1KD cells. Thus, to validate this hypothesis, *HoxA5* was transiently silenced in Glo1KD MAECs at similar levels of WT MAECs.
Both migration and invasion ability of Glo1KD MAECs is improved by *HoxA5* silencing, highlighting *HoxA5* as possible mediator of the harmful effect played by MGO on MAECs angiogenic function.
Hyperglycemia-mediated vascular complications involve the activation of several biochemical cascades that have been largely investigated in the last decades, including pro-inflammatory pathways (Evans, Goldfine et al. 2002).
In order to clarify how MGO could regulate *HoxA5* expression we looked at the potential involvement of the pro-inflammatory transcription factor NF-κB.
Activation of NF-κB has been suggested to contribute to the development of chronic disorders, such as diabetes and its associated complications. Indeed, Bierhaus et al. demonstrated that ligands of RAGE induce sustained activation of NF-κB in VSMCs, promoting the production of factors involved in the accelerated vascular disease (Bierhaus, Schiekofer et al. 2001). Furthermore, it has been recently demonstrated that MGO directly activates NF-κB translocation in synovial cells (Lin, Chan et al. 2016).
In absence of stimuli, the heterodimer p65-p50 NF-κB is confined in the cytoplasm complexed with the inhibitory subunit, IκB. After stimulation, IκB is phosphorylated, marked for ubiquitination and then degraded. IκB degradation unmasks the nuclear localization signal (NLS) of NF-κB, allowing the translocation of p65 to the nucleus in order to induce the expression of several genes (Palombella, Rando et al. 1994).

In this study, we evaluated NF-κB-p65 protein levels after fractionating nuclear components from cytoplasmic protein lysate. Our data demonstrate that, NF-κB-p65 protein levels are higher in both cytoplasmic and nuclear compartment of Glo1KD MAECs, compared to WT MAECs. Furthermore, also *NF-κB-p65* mRNA levels are overexpressed in Glo1KD MAECs. The increase of p65 in the nuclei of Glo1KD MAECs indicates that a major amount of this transcription factor is present in its active form in these cells compared to WT cells.

To evaluate whether *HoxA5* expression may be regulated by NF-κB, a search for a NF-κB-p65 consensus on murine *HoxA5* promoter was performed by a bioinformatic software, following used to test the presence of a NF-κB-p65-*HoxA5* gene promoter binding by chromatin immunoprecipitation. This study allowed us to demonstrate for the first time that NF-κB-p65 binds *HoxA5* promoter in ECs and, interestingly, this binding is higher in Glo1KD MAECs compared to WT MAECs, suggesting that *NF-κB-p65* overexpression may, in turn, mediate *HoxA5* overexpression in Glo1KD MAECs.

At this point, we sought to prove if this binding is functional to *HoxA5* expression. To this aim, one strategy has been to inhibit NF-κB activation using the aromatic diamine 4-Methyl-N^1-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23) that interferes with nuclear translocation, without affecting IκB degradation (Shin, Kim et al. 2004).

In our hands, JSH-23 treatment is able to reduce NF-κB-p65 translocation into the nucleus of MAECs and this inhibition results in the reduction of *HoxA5* expression, indicating that the transcription factor NF-κB-p65 regulates the expression of the anti-angiogenic *HoxA5* in MAECs. Ongoing experiments will further confirm this regulation by proving the concept that NF-κB regulates the expression of a reporter gene, via the identified promoter region of *HoxA5*, by the performance of luciferase assays.

6. CONCLUSIONS

This study provides a novel mechanism in the understanding of the damaging effect of MGO on endothelium. Through the activation of the transcription factor NF-κB-p65, MGO accumulation induces the overexpression of the anti-angiogenic *HoxA5* gene which in turn impairs, at least in part, the angiogenic process in Glo1KD MAECs.

Thus, this study highlights *HoxA5* as a new mediator of the harmful effect played by MGO on angiogenic ability of endothelial cells. Further investigations will be useful in the identification of novel strategies for the prevention and treatment of diabetes associated microvascular complications.

7. REFERENCES

Abu El-Asrar, A. M., M. I. Nawaz, D. Kangave, M. M. Siddiquei, M. S. Ola and G. Opdenakker (2013). "Angiogenesis regulatory factors in the vitreous from patients with proliferative diabetic retinopathy." Acta Diabetol **50**(4): 545-551.

Ahmad, F. K., Z. He and G. L. King (2005). "Molecular targets of diabetic cardiovascular complications." Curr Drug Targets **6**(4): 487-494.

Ahmed, U., D. Dobler, S. J. Larkin, N. Rabbani and P. J. Thornalley (2008). "Reversal of hyperglycemia-induced angiogenesis deficit of human endothelial cells by overexpression of glyoxalase 1 in vitro." Ann N Y Acad Sci **1126**: 262-264.

Al-Isa, A. N., L. Thalib and A. O. Akanji (2010). "Circulating markers of inflammation and endothelial dysfunction in Arab adolescent subjects: reference ranges and associations with age, gender, body mass and insulin sensitivity." Atherosclerosis **208**(2): 543-549.

Apiou, F., D. Flagiello, C. Cillo, B. Malfoy, M. F. Poupon and B. Dutrillaux (1996). "Fine mapping of human HOX gene clusters." Cytogenet Cell Genet **73**(1-2): 114-115.

Arderiu, G., I. Cuevas, A. Chen, M. Carrio, L. East and N. J. Boudreau (2007). "HoxA5 stabilizes adherens junctions via increased Akt1." Cell Adh Migr **1**(4): 185-195.

Aubin, J., P. Chailler, D. Menard and L. Jeannotte (1999). "Loss of Hoxa5 gene function in mice perturbs intestinal maturation." Am J Physiol **277**(5 Pt 1): C965-973.

Aubin, J., M. Lemieux, M. Tremblay, J. Berard and L. Jeannotte (1997). "Early postnatal lethality in Hoxa-5 mutant mice is attributable to respiratory tract defects." Dev Biol **192**(2): 432-445.

Bastaki, M., E. E. Nelli, P. Dell'Era, M. Rusnati, M. P. Molinari-Tosatti, S. Parolini, R. Auerbach, L. P. Ruco, L. Possati and M. Presta (1997). "Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium. A study of aortic and microvascular endothelial cell lines." Arterioscler Thromb Vasc Biol **17**(3): 454-464.

Baynes, J. W. and S. R. Thorpe (1999). "Role of oxidative stress in diabetic complications: a new perspective on an old paradigm." Diabetes **48**(1): 1-9.

Berlanga, J., D. Cibrian, I. Guillen, F. Freyre, J. S. Alba, P. Lopez-Saura, N. Merino, A. Aldama, A. M. Quintela, M. E. Triana, J. F. Montequin, H. Ajamieh, D. Urquiza, N. Ahmed and P. J. Thornalley (2005). "Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds." Clin Sci (Lond) **109**(1): 83-95.

Bierhaus, A. and P. P. Nawroth (2009). "The Alzheimer's disease-diabetes angle: inevitable fate of aging or metabolic imbalance limiting successful aging. Preface." J Alzheimers Dis **16**(4): 673-675.

Bierhaus, A., S. Schiekofer, M. Schwaninger, M. Andrassy, P. M. Humpert, J. Chen, M. Hong, T. Luther, T. Henle, I. Kloting, M. Morcos, M. Hofmann, H. Tritschler, B. Weigle, M. Kasper, M. Smith, G. Perry, A. M. Schmidt, D. M. Stern, H. U. Haring, E. Schleicher and P. P. Nawroth (2001). "Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB." Diabetes **50**(12): 2792-2808.

Blois, S. M., M. L. Conrad, N. Freitag and G. Barrientos (2015). "Galectins in angiogenesis: consequences for gestation." J Reprod Immunol **108**: 33-41.

Bonetti, P. O., L. O. Lerman and A. Lerman (2003). "Endothelial dysfunction: a marker of atherosclerotic risk." Arterioscler Thromb Vasc Biol **23**(2): 168-175.

Brook, R. D., R. L. Bard, M. Rubenfire, P. M. Ridker and S. Rajagopalan (2001). "Usefulness of visceral obesity (waist/hip ratio) in predicting vascular endothelial function in healthy overweight adults." Am J Cardiol **88**(11): 1264-1269.

Brouwers, O., P. M. Niessen, G. Haenen, T. Miyata, M. Brownlee, C. D. Stehouwer, J. G. De Mey and C. G. Schalkwijk (2010). "Hyperglycaemia-induced impairment of endothelium-dependent vasorelaxation in rat mesenteric arteries is mediated by intracellular methylglyoxal levels in a pathway dependent on oxidative stress." Diabetologia **53**(5): 989-1000.

Brouwers, O., P. M. Niessen, T. Miyata, J. A. Ostergaard, A. Flyvbjerg, C. J. Peutz-Kootstra, J. Sieber, P. H. Mundel, M. Brownlee, B. J. Janssen, J. G. De Mey, C. D. Stehouwer and C. G. Schalkwijk (2014). "Glyoxalase-1 overexpression reduces endothelial dysfunction and attenuates early renal impairment in a rat model of diabetes." Diabetologia **57**(1): 224-235.

Brownlee, M. (2001). "Biochemistry and molecular cell biology of diabetic complications." Nature **414**(6865): 813-820.

Brownlee, M. (2005). "The pathobiology of diabetic complications: a unifying mechanism." Diabetes **54**(6): 1615-1625.

Bucala, R., K. J. Tracey and A. Cerami (1991). "Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes." J Clin Invest **87**(2): 432-438.

Cahan, P., Y. Li, M. Izumi and T. A. Graubert (2009). "The impact of copy number variation on local gene expression in mouse hematopoietic stem and progenitor cells." Nat Genet **41**(4): 430-437.

Cai, W., J. C. He, L. Zhu, M. Peppa, C. Lu, J. Uribarri and H. Vlassara (2004). "High levels of dietary advanced glycation end products transform low-density lipoprotein into a potent redox-sensitive mitogen-activated protein kinase stimulant in diabetic patients." Circulation **110**(3): 285-291.

Cai, W., J. Uribarri, L. Zhu, X. Chen, S. Swamy, Z. Zhao, F. Grosjean, C. Simonaro, G. A. Kuchel, M. Schnaider-Beeri, M. Woodward, G. E. Striker and H. Vlassara (2014). "Oral glycotoxins are a modifiable cause of dementia and the metabolic syndrome in mice and humans." Proc Natl Acad Sci U S A **111**(13): 4940-4945.

Cantile, M., G. Schiavo, L. Terracciano and C. Cillo (2008). "Homeobox genes in normal and abnormal vasculogenesis." Nutr Metab Cardiovasc Dis **18**(10): 651-658.

Carmeliet, P. (2005). "Angiogenesis in life, disease and medicine." Nature **438**(7070): 932-936.

Carmeliet, P. and R. K. Jain (2011). "Molecular mechanisms and clinical applications of angiogenesis." Nature **473**(7347): 298-307.

Cassese, A., I. Esposito, F. Fiory, A. P. Barbagallo, F. Paturzo, P. Mirra, L. Ulianich, F. Giacco, C. Iadicicco, A. Lombardi, F. Oriente, E. Van Obberghen, F. Beguinot, P. Formisano and C. Miele (2008). "In skeletal muscle advanced glycation end products (AGEs) inhibit insulin action and induce the formation of multimolecular complexes including the receptor for AGEs." J Biol Chem **283**(52): 36088-36099.

Ceriello, A. (2010). "Point: postprandial glucose levels are a clinically important treatment target." Diabetes Care **33**(8): 1905-1907.

Chawla, A., R. Chawla and S. Jaggi (2016). "Microvasular and macrovascular complications in diabetes mellitus: Distinct or continuum?" Indian J Endocrinol Metab **20**(4): 546-551.

Chen, H., H. Zhang, J. Lee, X. Liang, X. Wu, T. Zhu, P. K. Lo, X. Zhang and S. Sukumar (2007). "HOXA5 acts directly downstream of retinoic acid receptor beta and contributes to retinoic acid-induced apoptosis and growth inhibition." Cancer Res **67**(17): 8007-8013.

Chen, S., S. W. Hong, M. C. Iglesias-de la Cruz, M. Isono, A. Casaretto and F. N. Ziyadeh (2001). "The key role of the transforming growth factor-beta system in the pathogenesis of diabetic nephropathy." Ren Fail **23**(3-4): 471-481.

Chisaka, O. and M. R. Capecchi (1991). "Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5." Nature **350**(6318): 473-479.

Cines, D. B., E. S. Pollak, C. A. Buck, J. Loscalzo, G. A. Zimmerman, R. P. McEver, J. S. Pober, T. M. Wick, B. A. Konkle, B. S. Schwartz, E. S. Barnathan, K. R. McCrae, B. A. Hug, A. M. Schmidt and D. M. Stern (1998). "Endothelial cells in physiology and in the pathophysiology of vascular disorders." Blood **91**(10): 3527-3561.

Corrado, E., M. Rizzo, G. Coppola, I. Muratori, M. Carella and S. Novo (2008). "Endothelial dysfunction and carotid lesions are strong predictors of clinical events in patients with early stages of atherosclerosis: a 24-month follow-up study." Coron Artery Dis **19**(3): 139-144.

Costa, P. Z. and R. Soares (2013). "Neovascularization in diabetes and its complications. Unraveling the angiogenic paradox." Life Sci **92**(22): 1037-1045.

Coulombe, Y., M. Lemieux, J. Moreau, J. Aubin, M. Joksimovic, F. A. Berube-Simard, S. Tabaries, O. Boucherat, F. Guillou, C. Larochelle, C. K. Tuggle and L. Jeannotte (2010). "Multiple promoters and alternative splicing: Hoxa5 transcriptional complexity in the mouse embryo." PLoS One **5**(5): e10600.

Creager, M. A., T. F. Luscher, F. Cosentino and J. A. Beckman (2003). "Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part I." Circulation **108**(12): 1527-1532.

Cuevas, I., H. Layman, L. Coussens and N. Boudreau (2015). "Sustained endothelial expression of HoxA5 in vivo impairs pathological angiogenesis and tumor progression." PLoS One **10**(3): e0121720.

D'Souza, A., M. Hussain, F. C. Howarth, N. M. Woods, K. Bidasee and J. Singh (2009). "Pathogenesis and pathophysiology of accelerated atherosclerosis in the diabetic heart." Mol Cell Biochem **331**(1-2): 89-116.

Davies, M. J. and A. Thomas (1984). "Thrombosis and acute coronary-artery lesions in sudden cardiac ischemic death." N Engl J Med **310**(18): 1137-1140.

De Caterina, R. (2000). "Endothelial dysfunctions: common denominators in vascular disease." Curr Opin Lipidol **11**(1): 9-23.

De Meyer, G. R. and A. G. Herman (1997). "Vascular endothelial dysfunction." Prog Cardiovasc Dis **39**(4): 325-342.

de Vos, L. C., J. D. Lefrandt, R. P. Dullaart, C. J. Zeebregts and A. J. Smit (2016). "Advanced glycation end products: An emerging biomarker for adverse outcome in patients with peripheral artery disease." Atherosclerosis **254**: 291-299.

Deanfield, J., A. Donald, C. Ferri, C. Giannattasio, J. Halcox, S. Halligan, A. Lerman, G. Mancia, J. J. Oliver, A. C. Pessina, D. Rizzoni, G. P. Rossi, A. Salvetti, E. L. Schiffrin, S. Taddei, D. J. Webb, E. Working Group on and H. Endothelial Factors of the European Society of (2005). "Endothelial function and dysfunction. Part I: Methodological issues for assessment in the different vascular beds: a statement by the Working Group on Endothelin and Endothelial Factors of the European Society of Hypertension." J Hypertens **23**(1): 7-17.

Degen, J., M. Vogel, D. Richter, M. Hellwig and T. Henle (2013). "Metabolic transit of dietary methylglyoxal." J Agric Food Chem **61**(43): 10253-10260.

Delhase, M., M. Hayakawa, Y. Chen and M. Karin (1999). "Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation." Science **284**(5412): 309-313.

Dhar, A., I. Dhar, K. M. Desai and L. Wu (2010). "Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose." Br J Pharmacol **161**(8): 1843-1856.

Dhar, I., A. Dhar, L. Wu and K. M. Desai (2014). "Methylglyoxal, a reactive glucose metabolite, increases renin angiotensin aldosterone and blood pressure in male Sprague-Dawley rats." Am J Hypertens **27**(3): 308-316.

Dornadula, S., B. Elango, P. Balashanmugam, R. Palanisamy and R. Kunka Mohanram (2015). "Pathophysiological insights of methylglyoxal induced type-2 diabetes." Chem Res Toxicol **28**(9): 1666-1674.

Douville, J. M. and J. T. Wigle (2007). "Regulation and function of homeodomain proteins in the embryonic and adult vascular systems." Can J Physiol Pharmacol **85**(1): 55-65.

Dumont, D. J., T. P. Yamaguchi, R. A. Conlon, J. Rossant and M. L. Breitman (1992). "tek, a novel tyrosine kinase gene located on mouse chromosome 4, is

expressed in endothelial cells and their presumptive precursors." Oncogene **7**(8): 1471-1480.

Durham, J. T. and I. M. Herman (2011). "Microvascular modifications in diabetic retinopathy." Curr Diab Rep **11**(4): 253-264.

Ellis, L. M. and D. J. Hicklin (2008). "VEGF-targeted therapy: mechanisms of anti-tumour activity." Nat Rev Cancer **8**(8): 579-591.

Endemann, D. H. and E. L. Schiffrin (2004). "Endothelial dysfunction." J Am Soc Nephrol **15**(8): 1983-1992.

Evans, J. L., I. D. Goldfine, B. A. Maddux and G. M. Grodsky (2002). "Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes." Endocr Rev **23**(5): 599-622.

Feletou, M. and P. M. Vanhoutte (2006). "Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture)." Am J Physiol Heart Circ Physiol **291**(3): H985-1002.

Fleming, T. H., P. M. Humpert, P. P. Nawroth and A. Bierhaus (2011). "Reactive metabolites and AGE/RAGE-mediated cellular dysfunction affect the aging process: a mini-review." Gerontology **57**(5): 435-443.

Fleming, T. H., T. M. Theilen, J. Masania, M. Wunderle, J. Karimi, S. Vittas, R. Bernauer, A. Bierhaus, N. Rabbani, P. J. Thornalley, J. Kroll, J. Tyedmers, R. Nawrotzki, S. Herzig, M. Brownlee and P. P. Nawroth (2013). "Aging-dependent reduction in glyoxalase 1 delays wound healing." Gerontology **59**(5): 427-437.

Folkman, J. and Y. Shing (1992). "Angiogenesis." J Biol Chem **267**(16): 10931-10934.

Fosmark, D. S., P. A. Torjesen, B. K. Kilhovd, T. J. Berg, L. Sandvik, K. F. Hanssen, C. D. Agardh and E. Agardh (2006). "Increased serum levels of the specific advanced glycation end product methylglyoxal-derived hydroimidazolone are associated with retinopathy in patients with type 2 diabetes mellitus." Metabolism **55**(2): 232-236.

Garin, E., M. Lemieux, Y. Coulombe, G. W. Robinson and L. Jeannotte (2006). "Stromal Hoxa5 function controls the growth and differentiation of mammary alveolar epithelium." Dev Dyn **235**(7): 1858-1871.

Gendronneau, G., O. Boucherat, J. Aubin, M. Lemieux and L. Jeannotte (2012). "The loss of Hoxa5 function causes estrous acyclicity and ovarian epithelial inclusion cysts." Endocrinology **153**(3): 1484-1497.

Geoffrion, M., X. Du, Z. Irshad, B. C. Vanderhyden, K. Courville, G. Sui, V. D. D'Agati, S. Ott-Braschi, N. Rabbani, P. J. Thornalley, M. Brownlee and R. W. Milne (2014). "Differential effects of glyoxalase 1 overexpression on diabetic atherosclerosis and renal dysfunction in streptozotocin-treated, apolipoprotein E-deficient mice." Physiol Rep **2**(6).

Gimbrone, M. A., Jr. (1999). "Endothelial dysfunction, hemodynamic forces, and atherosclerosis." Thromb Haemost **82**(2): 722-726.

Goh, S. Y. and M. E. Cooper (2008). "Clinical review: The role of advanced glycation end products in progression and complications of diabetes." J Clin Endocrinol Metab **93**(4): 1143-1152.

Goldberg, T., W. Cai, M. Peppa, V. Dardaine, B. S. Baliga, J. Uribarri and H. Vlassara (2004). "Advanced glycoxidation end products in commonly consumed foods." J Am Diet Assoc **104**(8): 1287-1291.

Gorski, D. H. and K. Walsh (2000). "The role of homeobox genes in vascular remodeling and angiogenesis." Circ Res **87**(10): 865-872.

Goueslard, K., J. Cottenet, A. S. Mariet, M. Giroud, Y. Cottin, J. M. Petit and C. Quantin (2016). "Early cardiovascular events in women with a history of gestational diabetes mellitus." Cardiovasc Diabetol **15**: 15.

Goumans, M. J., F. Lebrin and G. Valdimarsdottir (2003). "Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways." Trends Cardiovasc Med **13**(7): 301-307.

Grassi, D., G. Desideri, S. Necozione, F. Ruggieri, J. B. Blumberg, M. Stornello and C. Ferri (2012). "Protective effects of flavanol-rich dark chocolate on endothelial function and wave reflection during acute hyperglycemia." Hypertension **60**(3): 827-832.

Hadi, H. A. and J. A. Suwaidi (2007). "Endothelial dysfunction in diabetes mellitus." Vasc Health Risk Manag **3**(6): 853-876.

Hagg, S., L. M. Thorn, J. Putaala, R. Liebkind, V. Harjutsalo, C. M. Forsblom, D. Gordin, T. Tatlisumak, P. H. Groop and G. FinnDiane Study (2013). "Incidence of stroke according to presence of diabetic nephropathy and severe diabetic retinopathy in patients with type 1 diabetes." Diabetes Care **36**(12): 4140-4146.

Horabin, J.I. (2013). "Long noncoding RNAs as metazoan developmental regulators." Chromosome Research **21**(6):673-684.

Huang, Z. and S. D. Bao (2004). "Roles of main pro- and anti-angiogenic factors in tumor angiogenesis." World J Gastroenterol **10**(4): 463-470.

International Diabetes Federation. *IDF Diabetes Atlas, 7th edn.* Brussels, Belgium: International Diabetes Federation, 2015. http://www.diabetesatlas.org.

Ignarro, L. J., G. M. Buga, K. S. Wood, R. E. Byrns and G. Chaudhuri (1987). "Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide." Proc Natl Acad Sci U S A **84**(24): 9265-9269.

Jaisson, S. and P. Gillery (2010). "Evaluation of nonenzymatic posttranslational modification-derived products as biomarkers of molecular aging of proteins." Clin Chem **56**(9): 1401-1412.

Jeannotte, L., Gotti, F., Landry-Truchon, K. (2016). "HoxA5: A key player in Development and Disease." J. Dev. Biol. **4**(2):13.

Jo-Watanabe, A., T. Ohse, H. Nishimatsu, M. Takahashi, Y. Ikeda, T. Wada, J. Shirakawa, R. Nagai, T. Miyata, T. Nagano, Y. Hirata, R. Inagi and M. Nangaku (2014). "Glyoxalase I reduces glycative and oxidative stress and prevents age-related endothelial dysfunction through modulation of endothelial nitric oxide synthase phosphorylation." Aging Cell **13**(3): 519-528.

Just, A., C. L. Whitten and W. J. Arendshorst (2008). "Reactive oxygen species participate in acute renal vasoconstrictor responses induced by ETA and ETB receptors." Am J Physiol Renal Physiol **294**(4): F719-728.

Kachgal, S., K. A. Mace and N. J. Boudreau (2012). "The dual roles of homeobox genes in vascularization and wound healing." Cell Adh Migr **6**(6): 457-470.

Kaku, K. (2010)."Pathophysiology of Type 2 Diabetes and its treatment policy."JMAJ **53**(1):41-46.

Kalofoutis, C., C. Piperi, A. Kalofoutis, F. Harris, D. Phoenix and J. Singh (2007). "Type II diabetes mellitus and cardiovascular risk factors: Current therapeutic approaches." Exp Clin Cardiol **12**(1): 17-28.

Kaneto, H., N. Katakami, M. Matsuhisa and T. A. Matsuoka (2010). "Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis." Mediators Inflamm **2010**: 453892.

Kim, J., O. S. Kim, C. S. Kim, E. Sohn, K. Jo and J. S. Kim (2012). "Accumulation of argpyrimidine, a methylglyoxal-derived advanced glycation end product, increases apoptosis of lens epithelial cells both in vitro and in vivo." Exp Mol Med **44**(2): 167-175.

Kolluru, G. K., S. C. Bir and C. G. Kevil (2012). "Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing." Int J Vasc Med **2012**: 918267.

Kolodgie, F. D., H. K. Gold, A. P. Burke, D. R. Fowler, H. S. Kruth, D. K. Weber, A. Farb, L. J. Guerrero, M. Hayase, R. Kutys, J. Narula, A. V. Finn and R. Virmani (2003). "Intraplaque hemorrhage and progression of coronary atheroma." N Engl J Med **349**(24): 2316-2325.

Koschinsky, T., C. J. He, T. Mitsuhashi, R. Bucala, C. Liu, C. Buenting, K. Heitmann and H. Vlassara (1997). "Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy." Proc Natl Acad Sci U S A **94**(12): 6474-6479.

Krumlauf, R. (1994). "Hox genes in vertebrate development." Cell **78**(2): 191-201.

Lamalice, L., F. Le Boeuf and J. Huot (2007). "Endothelial cell migration during angiogenesis." Circ Res **100**(6): 782-794.

Lewis, E. B. (1978). "A gene complex controlling segmentation in Drosophila." Nature **276**(5688): 565-570.

Lin, C. C., C. M. Chan, Y. P. Huang, S. H. Hsu, C. L. Huang and S. J. Tsai (2016). "Methylglyoxal activates NF-kappaB nuclear translocation and induces COX-2 expression via a p38-dependent pathway in synovial cells." Life Sci **149**: 25-33.

Liu, G.H.; Qu, J.; Shen, X. (2008). "NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK". Biochim. Biophys. Acta **1783**: 713–727.

Liu, H., S. Yu, H. Zhang and J. Xu (2012). "Angiogenesis impairment in diabetes: role of methylglyoxal-induced receptor for advanced glycation endproducts, autophagy and vascular endothelial growth factor receptor 2." PLoS One **7**(10): e46720.

Liu, Y. W., X. Zhu, L. Zhang, Q. Lu, J. Y. Wang, F. Zhang, H. Guo, J. L. Yin and X. X. Yin (2013). "Up-regulation of glyoxalase 1 by mangiferin prevents

diabetic nephropathy progression in streptozotocin-induced diabetic rats." Eur J Pharmacol **721**(1-3): 355-364.

Maessen, D. E., C. D. Stehouwer and C. G. Schalkwijk (2015). "The role of methylglyoxal and the glyoxalase system in diabetes and other age-related diseases." Clin Sci (Lond) **128**(12): 839-861.

Martin, A., M. R. Komada and D. C. Sane (2003). "Abnormal angiogenesis in diabetes mellitus." Med Res Rev **23**(2): 117-145.

Matafome, P., T. Rodrigues, C. Sena and R. Seica (2016). "Methylglyoxal in Metabolic Disorders: Facts, Myths, and Promises." Med Res Rev.

McLellan, A. C., S. A. Phillips and P. J. Thornalley (1992). "The assay of methylglyoxal in biological systems by derivatization with 1,2-diamino-4,5-dimethoxybenzene." Anal Biochem **206**(1): 17-23.

McLellan, A. C., P. J. Thornalley, J. Benn and P. H. Sonksen (1994). "Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications." Clin Sci (Lond) **87**(1): 21-29.

Meunier, D., J. Aubin and L. Jeannotte (2003). "Perturbed thyroid morphology and transient hypothyroidism symptoms in Hoxa5 mutant mice." Dev Dyn **227**(3): 367-378.

Miele, C., A. Riboulet, M. A. Maitan, F. Oriente, C. Romano, P. Formisano, J. Giudicelli, F. Beguinot and E. Van Obberghen (2003). "Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C alpha-mediated mechanism." J Biol Chem **278**(48): 47376-47387.

Miller, A. G., D. G. Smith, M. Bhat and R. H. Nagaraj (2006). "Glyoxalase I is critical for human retinal capillary pericyte survival under hyperglycemic conditions." J Biol Chem **281**(17): 11864-11871.

Mirra, P., C. Nigro, I. Prevenzano, T. Procopio, A. Leone, G. A. Raciti, F. Andreozzi, M. Longo, F. Fiory, F. Beguinot and C. Miele (2017). "The role of miR-190a in methylglyoxal-induced insulin resistance in endothelial cells." Biochim Biophys Acta **1863**(2): 440-449.

Mitsumoto, A., K. R. Kim, G. Oshima, M. Kunimoto, K. Okawa, A. Iwamatsu and Y. Nakagawa (2000). "Nitric oxide inactivates glyoxalase I in cooperation with glutathione." J Biochem **128**(4): 647-654.

Mombouli, J. V. (1997). "ACE inhibition, endothelial function and coronary artery lesions. Role of kinins and nitric oxide." Drugs **54 Suppl 5**: 12-22.

Moncada, S. and E. A. Higgs (2006). "Nitric oxide and the vascular endothelium." Handb Exp Pharmacol(176 Pt 1): 213-254.

Morcos, M., X. Du, F. Pfisterer, H. Hutter, A. A. Sayed, P. Thornalley, N. Ahmed, J. Baynes, S. Thorpe, G. Kukudov, A. Schlotterer, F. Bozorgmehr, R. A. El Baki, D. Stern, F. Moehrlen, Y. Ibrahim, D. Oikonomou, A. Hamann, C. Becker, M. Zeier, V. Schwenger, N. Miftari, P. Humpert, H. P. Hammes, M. Buechler, A. Bierhaus, M. Brownlee and P. P. Nawroth (2008). "Glyoxalase-1 prevents mitochondrial protein modification and enhances lifespan in Caenorhabditis elegans." Aging Cell **7**(2): 260-269.

Mukohda, M., T. Morita, M. Okada, Y. Hara and H. Yamawaki (2013). "Long-term methylglyoxal treatment causes endothelial dysfunction of rat isolated mesenteric artery." J Vet Med Sci **75**(2): 151-157.

Mukohda, M., M. Okada, Y. Hara and H. Yamawaki (2012). "Methylglyoxal accumulation in arterial walls causes vascular contractile dysfunction in spontaneously hypertensive rats." J Pharmacol Sci **120**(1): 26-35.

Murtha, M. T., J. F. Leckman and F. H. Ruddle (1991). "Detection of homeobox genes in development and evolution." Proc Natl Acad Sci U S A **88**(23): 10711-10715.

Murthi, P., M. Abumaree and B. Kalionis (2014). "Analysis of homeobox gene action may reveal novel angiogenic pathways in normal placental vasculature and in clinical pregnancy disorders associated with abnormal placental angiogenesis." Front Pharmacol **5**: 133.

Mustonen, T. and K. Alitalo (1995). "Endothelial receptor tyrosine kinases involved in angiogenesis." J Cell Biol **129**(4): 895-898.

Naka, Y., L. G. Bucciarelli, T. Wendt, L. K. Lee, L. L. Rong, R. Ramasamy, S. F. Yan and A. M. Schmidt (2004). "RAGE axis: Animal models and novel insights into the vascular complications of diabetes." Arterioscler Thromb Vasc Biol **24**(8): 1342-1349.

Neve, A., F. P. Cantatore, N. Maruotti, A. Corrado and D. Ribatti (2014). "Extracellular matrix modulates angiogenesis in physiological and pathological conditions." Biomed Res Int **2014**: 756078.

Nieuwdorp, M., H. L. Mooij, J. Kroon, B. Atasever, J. A. Spaan, C. Ince, F. Holleman, M. Diamant, R. J. Heine, J. B. Hoekstra, J. J. Kastelein, E. S. Stroes and H. Vink (2006). "Endothelial glycocalyx damage coincides with microalbuminuria in type 1 diabetes." Diabetes **55**(4): 1127-1132.

Nieuwdorp, M., T. W. van Haeften, M. C. Gouverneur, H. L. Mooij, M. H. van Lieshout, M. Levi, J. C. Meijers, F. Holleman, J. B. Hoekstra, H. Vink, J. J. Kastelein and E. S. Stroes (2006). "Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo." Diabetes **55**(2): 480-486.

Nigro, C., A. Leone, G. A. Raciti, M. Longo, P. Mirra, P. Formisano, F. Beguinot and C. Miele (2017). "Methylglyoxal-Glyoxalase 1 Balance: The Root of Vascular Damage." Int J Mol Sci **18**(1).

Nigro, C., G. A. Raciti, A. Leone, T. H. Fleming, M. Longo, I. Prevenzano, F. Fiory, P. Mirra, V. D'Esposito, L. Ulianich, P. P. Nawroth, P. Formisano, F. Beguinot and C. Miele (2014). "Methylglyoxal impairs endothelial insulin sensitivity both in vitro and in vivo." Diabetologia **57**(7): 1485-1494.

Nishikawa, T., D. Edelstein, X. L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M. A. Yorek, D. Beebe, P. J. Oates, H. P. Hammes, I. Giardino and M. Brownlee (2000). "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage." Nature **404**(6779): 787-790.

Nyberg, P., L. Xie and R. Kalluri (2005). "Endogenous inhibitors of angiogenesis." Cancer Res **65**(10): 3967-3979.

Odenwald, W. F., C. F. Taylor, F. J. Palmer-Hill, V. Friedrich, Jr., M. Tani and R. A. Lazzarini (1987). "Expression of a homeo domain protein in noncontact-inhibited cultured cells and postmitotic neurons." Genes Dev **1**(5): 482-496.

Orasanu, G. and J. Plutzky (2009). "The pathologic continuum of diabetic vascular disease." J Am Coll Cardiol **53**(5 Suppl): S35-42.

Otrock, Z. K., R. A. Mahfouz, J. A. Makarem and A. I. Shamseddine (2007). "Understanding the biology of angiogenesis: review of the most important molecular mechanisms." Blood Cells Mol Dis **39**(2): 212-220.

Palmer, R. M., A. G. Ferrige and S. Moncada (1987). "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor." Nature **327**(6122): 524-526.

Palombella, V. J., O. J. Rando, A. L. Goldberg and T. Maniatis (1994). "The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B." Cell **78**(5): 773-785.

Perticone, F., R. Ceravolo, A. Pujia, G. Ventura, S. Iacopino, A. Scozzafava, A. Ferraro, M. Chello, P. Mastroroberto, P. Verdecchia and G. Schillaci (2001). "Prognostic significance of endothelial dysfunction in hypertensive patients." Circulation **104**(2): 191-196.

Phillips, S. A. and P. J. Thornalley (1993). "The formation of methylglyoxal from triose phosphates. Investigation using a specific assay for methylglyoxal." Eur J Biochem **212**(1): 101-105.

Pober, J. S. and W. C. Sessa (2007). "Evolving functions of endothelial cells in inflammation." Nat Rev Immunol **7**(10): 803-815.

Puri, M. C., J. Rossant, K. Alitalo, A. Bernstein and J. Partanen (1995). "The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells." EMBO J **14**(23): 5884-5891.

Rabbani, N. and P. J. Thornalley (2014). "Measurement of methylglyoxal by stable isotopic dilution analysis LC-MS/MS with corroborative prediction in physiological samples." Nat Protoc **9**(8): 1969-1979.

Rabbani, N., M. Xue and P. J. Thornalley (2014). "Activity, regulation, copy number and function in the glyoxalase system." Biochem Soc Trans **42**(2): 419-424.

Rabbani, N., M. Xue and P. J. Thornalley (2016). "Dicarbonyls and glyoxalase in disease mechanisms and clinical therapeutics." Glycoconj J **33**(4): 513-525.

Redon, R., S. Ishikawa, K. R. Fitch, L. Feuk, G. H. Perry, T. D. Andrews, H. Fiegler, M. H. Shapero, A. R. Carson, W. Chen, E. K. Cho, S. Dallaire, J. L. Freeman, J. R. Gonzalez, M. Gratacos, J. Huang, D. Kalaitzopoulos, D. Komura, J. R. MacDonald, C. R. Marshall, R. Mei, L. Montgomery, K. Nishimura, K. Okamura, F. Shen, M. J. Somerville, J. Tchinda, A. Valsesia, C. Woodwark, F. Yang, J. Zhang, T. Zerjal, J. Zhang, L. Armengol, D. F. Conrad, X. Estivill, C. Tyler-Smith, N. P. Carter, H. Aburatani, C. Lee, K. W. Jones, S. W. Scherer and M. E. Hurles (2006). "Global variation in copy number in the human genome." Nature **444**(7118): 444-454.

Rhoads, K., G. Arderiu, A. Charboneau, S. L. Hansen, W. Hoffman and N. Boudreau (2005). "A role for Hox A5 in regulating angiogenesis and vascular patterning." Lymphat Res Biol **3**(4): 240-252.

Ribatti, D., B. Nico and E. Crivellato (2009). "Morphological and molecular aspects of physiological vascular morphogenesis." Angiogenesis **12**(2): 101-111.

Ribot, J., G. Caliaperoumal, J. Paquet, C. Boisson-Vidal, H. Petite and F. Anagnostou (2016). "Type 2 diabetes alters mesenchymal stem cell secretome composition and angiogenic properties." J Cell Mol Med.

Rosenfeld, M. E. (2000). "An overview of the evolution of the atherosclerotic plaque: from fatty streak to plaque rupture and thrombosis." Z Kardiol **89 Suppl 7**: 2-6.

Ross, R. (1999). "Atherosclerosis--an inflammatory disease." N Engl J Med **340**(2): 115-126.

Sato, Y. and D. B. Rifkin (1989). "Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture." J Cell Biol **109**(1): 309-315.

Schalkwijk, C. G. (2015). "Vascular AGE-ing by methylglyoxal: the past, the present and the future." Diabetologia **58**(8): 1715-1719.

Schalkwijk, C. G. and C. D. Stehouwer (2005). "Vascular complications in diabetes mellitus: the role of endothelial dysfunction." Clin Sci (Lond) **109**(2): 143-159.

Schlotterer, A., G. Kukudov, F. Bozorgmehr, H. Hutter, X. Du, D. Oikonomou, Y. Ibrahim, F. Pfisterer, N. Rabbani, P. Thornalley, A. Sayed, T. Fleming, P. Humpert, V. Schwenger, M. Zeier, A. Hamann, D. Stern, M. Brownlee, A. Bierhaus, P. Nawroth and M. Morcos (2009). "C. elegans as model for the study of high glucose- mediated life span reduction." Diabetes **58**(11): 2450-2456.

Scott, M. P. (1992). "Vertebrate homeobox gene nomenclature." Cell **71**(4): 551-553.

Seagroves, T. N., H. E. Ryan, H. Lu, B. G. Wouters, M. Knapp, P. Thibault, K. Laderoute and R. S. Johnson (2001). "Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells." Mol Cell Biol **21**(10): 3436-3444.

Sena, C. M., P. Matafome, J. Crisostomo, L. Rodrigues, R. Fernandes, P. Pereira and R. M. Seica (2012). "Methylglyoxal promotes oxidative stress and endothelial dysfunction." Pharmacol Res **65**(5): 497-506.

Sena, C. M., A. M. Pereira and R. Seica (2013). "Endothelial dysfunction - a major mediator of diabetic vascular disease." Biochim Biophys Acta **1832**(12): 2216-2231.

Shin, H. M., M. H. Kim, B. H. Kim, S. H. Jung, Y. S. Kim, H. J. Park, J. T. Hong, K. R. Min and Y. Kim (2004). "Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation." FEBS Lett **571**(1-3): 50-54.

Shinohara, M., P. J. Thornalley, I. Giardino, P. Beisswenger, S. R. Thorpe, J. Onorato and M. Brownlee (1998). "Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis." J Clin Invest **101**(5): 1142-1147.

Skapare, E., I. Konrade, E. Liepinsh, I. Strele, M. Makrecka, A. Bierhaus, A. Lejnieks, V. Pirags and M. Dambrova (2013). "Association of reduced glyoxalase 1 activity and painful peripheral diabetic neuropathy in type 1 and 2 diabetes mellitus patients." J Diabetes Complications **27**(3): 262-267.

Stehouwer, C. D., J. Lambert, A. J. Donker and V. W. van Hinsbergh (1997). "Endothelial dysfunction and pathogenesis of diabetic angiopathy." Cardiovasc Res **34**(1): 55-68.

Suuronen, E. J., S. Hazra, P. Zhang, R. Vincent, P. Kumarathasan, Y. Zhang, J. Price, V. Chan, F. W. Sellke, T. G. Mesana, J. P. Veinot and M. Ruel (2010). "Impairment of human cell-based vasculogenesis in rats by hypercholesterolemia-induced endothelial dysfunction and rescue with L-arginine supplementation." J Thorac Cardiovasc Surg **139**(1): 209-216 e202.

Tahergorabi, Z. and M. Khazaei (2012). "Imbalance of angiogenesis in diabetic complications: the mechanisms." Int J Prev Med **3**(12): 827-838.

Thornalley, P. J. (1988). "Modification of the glyoxalase system in human red blood cells by glucose in vitro." Biochem J **254**(3): 751-755.

Thornalley, P. J. (1993). "The glyoxalase system in health and disease." Mol Aspects Med **14**(4): 287-371.

Thornalley, P. J. (2003). "Glyoxalase I--structure, function and a critical role in the enzymatic defence against glycation." Biochem Soc Trans **31**(Pt 6): 1343-1348.

Thornalley, P. J. (2005). "Dicarbonyl intermediates in the maillard reaction." Ann N Y Acad Sci **1043**: 111-117.

Tischer, E., R. Mitchell, T. Hartman, M. Silva, D. Gospodarowicz, J. C. Fiddes and J. A. Abraham (1991). "The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing." J Biol Chem **266**(18): 11947-11954.

Tomanek, R. J. and G. C. Schatteman (2000). "Angiogenesis: new insights and therapeutic potential." Anat Rec **261**(3): 126-135.

Toth, A. E., F. R. Walter, A. Bocsik, P. Santha, S. Veszelka, L. Nagy, L. G. Puskas, P. O. Couraud, F. Takata, S. Dohgu, Y. Kataoka and M. A. Deli (2014). "Edaravone protects against methylglyoxal-induced barrier damage in human brain endothelial cells." PLoS One **9**(7): e100152.

Vallance, P. (2001). "Importance of asymmetrical dimethylarginine in cardiovascular risk." Lancet **358**(9299): 2096-2097.

van den Berg, B. M., M. Nieuwdorp, E. S. Stroes and H. Vink (2006). "Glycocalyx and endothelial (dys) function: from mice to men." Pharmacol Rep **58 Suppl**: 75-80.

van Haaren, P. M., E. VanBavel, H. Vink and J. A. Spaan (2003). "Localization of the permeability barrier to solutes in isolated arteries by confocal microscopy." Am J Physiol Heart Circ Physiol **285**(6): H2848-2856.

Vulesevic, B., B. McNeill, F. Giacco, K. Maeda, N. J. Blackburn, M. Brownlee, R. W. Milne and E. J. Suuronen (2016). "Methylglyoxal-Induced Endothelial Cell Loss and Inflammation Contribute to the Development of Diabetic Cardiomyopathy." Diabetes **65**(6): 1699-1713.

Wang, X. H., S. F. Chen, H. M. Jin and R. M. Hu (2009). "Differential analyses of angiogenesis and expression of growth factors in micro- and macrovascular endothelial cells of type 2 diabetic rats." Life Sci **84**(7-8): 240-249.

Witting, P. K., B. S. Rayner, B. J. Wu, N. A. Ellis and R. Stocker (2007). "Hydrogen peroxide promotes endothelial dysfunction by stimulating multiple sources of superoxide anion radical production and decreasing nitric oxide bioavailability." Cell Physiol Biochem **20**(5): 255-268.

Xu, L., K. Kanasaki, M. Kitada and D. Koya (2012). "Diabetic angiopathy and angiogenic defects." Fibrogenesis Tissue Repair **5**(1): 13.

Xue, M., N. Rabbani, H. Momiji, P. Imbasi, M. M. Anwar, N. Kitteringham, B. K. Park, T. Souma, T. Moriguchi, M. Yamamoto and P. J. Thornalley (2012). "Transcriptional control of glyoxalase 1 by Nrf2 provides a stress-responsive defence against dicarbonyl glycation." Biochem J **443**(1): 213-222.

Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki (1988). "A novel potent vasoconstrictor peptide produced by vascular endothelial cells." Nature **332**(6163): 411-415.

Yao, D., T. Taguchi, T. Matsumura, R. Pestell, D. Edelstein, I. Giardino, G. Suske, N. Rabbani, P. J. Thornalley, V. P. Sarthy, H. P. Hammes and M. Brownlee (2007). "High glucose increases angiopoietin-2 transcription in microvascular endothelial cells through methylglyoxal modification of mSin3A." J Biol Chem **282**(42): 31038-31045.

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Review

Methylglyoxal-Glyoxalase 1 Balance: The Root of Vascular Damage

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Abstract: The highly reactive dicarbonyl methylglyoxal (MGO) is mainly formed as byproduct of glycolysis. Therefore, high blood glucose levels determine increased MGO accumulation. Nonetheless, MGO levels are also increased as consequence of the ineffective action of its main detoxification pathway, the glyoxalase system, of which glyoxalase 1 (Glo1) is the rate-limiting enzyme. Indeed, a physiological decrease of Glo1 transcription and activity occurs not only in chronic hyperglycaemia but also with ageing, during which MGO accumulation occurs. MGO and its advanced glycated end products (AGEs) are associated with age-related diseases including diabetes, vascular dysfunction and neurodegeneration. Endothelial dysfunction is the first step in the initiation, progression and clinical outcome of vascular complications, such as retinopathy, nephropathy, impaired wound healing and macroangiopathy. Because of these considerations, studies have been centered on understanding the molecular basis of endothelial dysfunction in diabetes, unveiling a central role of MGO-Glo1 imbalance in the onset of vascular complications. This review focuses on the current understanding of MGO accumulation and Glo1 activity in diabetes, and their contribution on the impairment of endothelial function leading to diabetes-associated vascular damage.

Keywords: methylglyoxal; glyoxalase; vascular function; insulin-resistance

1. Introduction

Methylglyoxal (MGO) is a dicarbonyl aldehyde mainly formed as byproduct of glycolysis [1]. By its nature, MGO efficiently reacts with other molecules in the organism and causes cell and tissue dysfunction [2]. While cells use glucose as major source of energy and store most of their glucose in non-reactive molecules, as glycogen, a spontaneous and unavoidable leakage from the Embden-Meyerhof pathway occurs in mammalian cells in the form of MGO [1]. It has been estimated that about 0.1% of the glucotriose flux generates MGO by the non-enzymatic degradation of glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) [3]. Thus, glycolysis contributes to the formation of MGO with a rate of about 125 μmol/kg cell mass per day under normoglycaemic conditions [4]. This situation may be further increased under conditions that lead to higher triosephosphate concentrations, like increased glucose metabolism in hyperglycaemia [5], impairment of the pentose pathway decreasing GA3P disposal, or increased anaerobic glycolysis occurring in hypoxia [6]. A minor amount of MGO is generated by acetone oxidation in the catabolism of ketone bodies [7], aminoacetone oxidation in the catabolism of threonine [8], lipid peroxidation and degradation of glycated proteins and monosaccharides [9,10].

At variance, the contribution of exogenous sources is still controversial. Extremely high levels of MGO were found in natural products, such as honey, and especially processed food and beverages like soft drinks, coffee, and dairy products [11,12]. Indeed, MGO levels in foodstuffs is strongly affected by heating [13], prolonged storage and fermentation, when microorganisms release MGO in alcoholic drinks and cheese [11].Unexpectedly, variable levels of MGO can be found even in drinking water that underwent treatments like ozonation and chlorination, which easily result in the formation of MGO from humic substances [14]. It should be further considered that polluted air also represents an exogenous source of MGO. In particular, smoking is a major source of indoor air contamination [15]. However, whether these exogenous sources of MGO are significant for plasma MGO levels has not been clarified yet. Several studies report that free MGO is rapidly degraded during digestion in the intestine and, thus, does not influence MGO level in vivo [16]. But there is also conflicting evidence indicating brain and serum MGO accumulation in rodents fed MGO-supplemented diets and suggesting that at least 10% of MGO-derived AGEs is absorbed and then accumulated in tissues like vessels, heart, liver, kidney and adipose tissue [17,18].

MGO levels in healthy humans have been estimated to be about 50–150 nM in the plasma and 1–4 μM in tissues [19]. When the accumulation of MGO exceeds these levels, dicarbonyl stress occurs as a consequence of the imbalance between the generation/exposure and MGO metabolism [20].

Under physiological conditions, >99% of MGO is detoxified by the glyoxalase system [21], with minor metabolism by aldoketo reductases (AKRs) and aldehyde dehydrogenases (ADHs) which convert MGO to hydroxyacetone and pyruvate, respectively [22]. The glyoxalase system consists of glyoxalase 1 (Glo1), glyoxalase 2 (Glo2) and a catalytic amount of glutathione (GSH). Acting as the rate-limiting enzyme, Glo1 catalyses the primary detoxification step by converting the spontaneously formed MGO-GSH hemithioacetal to the thioester *S*-D-lactoylglutathione [23]. Its activity is directly proportional to GSH concentration. Thus when cellular GSH concentration declines, as in oxidative stress, Glo1 activity is impaired. Glo2 catalyses the hydrolysis of *S*-D-lactoylglutathione to D-lactate and GSH [24]. With the exception of certain protozoa [25], both Glo1 and 2 are expressed in most eukaryotes and prokaryotes [26,27], and are localized in the cytoplasmic compartment [28].

The modulation of Glo1 activity is dependent on both regulation of gene expression and post-translational modifications. The Glo1 gene contains regulatory elements including ARE (antioxidant-response element) in exone 1 [29], and its expression is negatively regulated by HIF1α (hypoxia inducible factor 1α) binding to ARE under hypoxic conditions [30]. Indeed, hypoxia is an important physiological driver of dicarbonyl stress as it facilitates MGO formation by increasing metabolic flux through anaerobic glycolysis [6]. Hypoxia decreases Glo1 expression as well [3]. Furthermore, basal and inducible expression of Glo1 is subjected to stress-responsive control by nuclear factor erythroid 2-related factor 2 (Nrf2) through regulatory AREs [29]. Current evidence indicates that NF-κB constitutive activation in chronic hyperglycaemia, and related inflammation, mediates the impairment in Glo1 expression and activity [31]. It has been further demonstrated that NF-κB antagonizes the transcriptional activity of Nrf2 [32], suggesting that in a pro-inflammatory state, typical of diabetes and obesity, NF-κB activation impairs Glo1 expression by inhibiting Nrf2 action. Glo1 expression is also negatively regulated by the activation of the receptor of advanced glycated end products (RAGE) and, again, NF-κB has an important role in this pathway [33].

While genetic polymorphisms of Glo2 are extremely rare, different SNPs (single nucleotide polymorphisms) have been identified in the Glo1 gene and found to be associated with reduced enzyme activity [34], increased prevalence of diabetic neuropathy [35], and increased risk of cardiovascular complications [36]. Moreover, Glo1 is a hotspot for functional copy number variation (CNV) causing 2 to 4-fold increases in Glo1 expression in approximately 2% of the human population [37,38]. However, further studies are needed to understand whether these patients are protected from MGO accumulation and damage.

A physiological decrease of Glo1 activity and expression occurs with ageing, as demonstrated by Morcos et al. in the nematode *Caenorhabditis elegans*, where an inverse correlation was found

between ageing and Glo1 expression [39]. This same effect has been confirmed in rodents [40–42]. Glo1 expression is therefore likely linked to healthy ageing [43].

Thus, abnormal cellular accumulation of the dicarbonyl metabolite MGO occurs upon exposure to high glucose concentrations, oxidative stress, inflammation, cell aging and senescence. It is associated with increased MGO-adduct content of proteins susceptible to dicarbonyl modification, collectively defined as dicarbonyl proteome [20], and with DNA instability and mutations [36,43]. An adequate balance between MGO levels and Glo1 activity is necessary to ensure detoxification of MGO from different sources and cell survival (Figure 1).

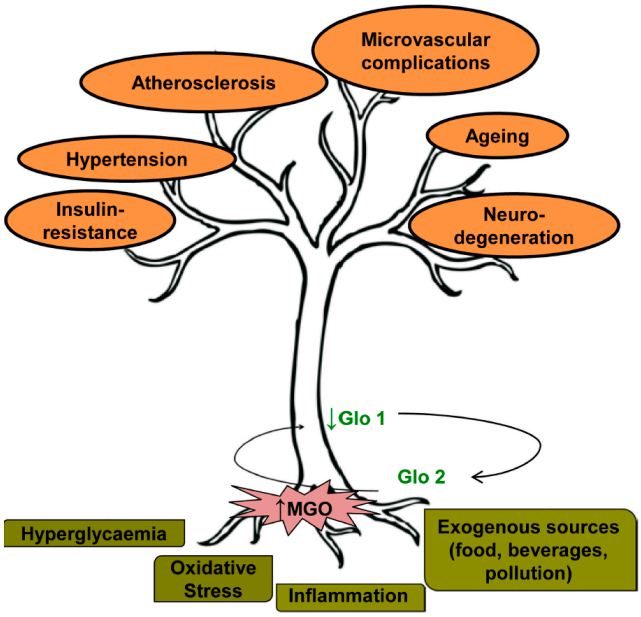

Figure 1. Sources of methylglyoxal (MGO) accumulation contributing to vascular dysfunction. Hyperglycaemia, oxidative stress, inflammation and exogenous sources of MGO contribute both to the increase of MGO levels and the decrease of glyoxalase 1 (Glo1) activity. MGO-Glo1 imbalance leads to vascular dysfunction contributing to endothelial insulin-resistance, hypertension, atherosclerosis, microvascular complications, ageing and neuro-degeneration.

2. Effect of MGO Accumulation on Vascular Function

In the last decades, an increasing number of studies claiming a central role for MGO in vascular dysfunction have been published [44]. Endothelial dysfunction is one of the major factors in the development of cardiovascular disease. It has been recently reviewed how different noxious agents induce endothelial dysfunction, promoting an amplified endoplasmic reticulum stress response [45]. Vascular endothelium behaves as an autocrine and paracrine organ regulating vascular homeostasis [46]. This is ensured by the tight control of the vascular tone, cell–cell interaction, permeability and coagulation systems through the production of the extracellular matrix components, and soluble factors in response to various stimuli.

When this balance is impaired, vasoconstriction may occur, along with leukocyte adherence, platelet activation, mitogenesis, pro-oxidation, thrombosis and impaired coagulation, vascular inflammation, and atherosclerosis [47]. Among the bioactive molecules generated by the endothelium, nitric oxide (NO) plays a pivotal role in the maintenance of vascular homeostasis. NO controls vascular tone by maintaining basal and induced vascular smooth muscle relaxation and opposing the action of potent contraction factors, including Angiotensin II (Ang II) and Endothelin-1 (ET-1). In addition, NO inhibits platelet activation and leukocyte adhesion and rolling, as well as cytokine-induced expression of VCAM (vascular cell adhesion molecule) and MCP-1 (monocyte chemotactic protein-1), thus promoting an anti-inflammatory action [47].

MGO accumulation, perpetuated by Glo1 down-regulation, is linked to age-related diseases, such as diabetes, obesity, disorders of the central nervous system and cardiovascular disease, which share endothelial dysfunction as a common pathological denominator [47–50]. MGO reacts predominantly with arginine residues on proteins, with methylglyoxal hydroimidazolone (MG-H1) being the most prevalent MGO-derived AGE modification found in vivo, leading to structural change, inactivation and degradation of target proteins [51]. Thus, increased levels of MGO impair endothelial function in various way and in different districts in the organism. Several studies have shown an impairment of endothelium-dependent vasorelaxation by MGO, mostly mediated by oxidative stress. Indeed, acetylcholine-induced vasorelaxation is impaired in aortic tissue from rats by both high glucose and MGO. This effect is attenuated by the MGO scavengers aminoguanidine (AG) and *N*-acetyl-cisteine (NAC) [52], via the inhibition of NADPH oxidase [53], and is prevented by Glo1 over-expression [54]. A protective effect of anti-oxidants such as polyphenols has been reported to prevent MGO-dependent impairment of NO release, improving endothelium-mediated relaxation in mouse *corpora cavernosa* [55]. A proposed mechanism by which MGO increases reactive oxygen species (ROS), promoting the apoptotic process, involves the reduced transcription of the cytoprotective protein thioredoxin [56]. Moreover, accumulating evidence suggests that physiological angiogenesis is impaired by MGO through RAGE-mediated and autophagy-induced vascular endothelial growth factor receptor 2 (VEGFR2) degradation [57]. Our preliminary data indicate that Glo1 down-regulation in mouse aortic endothelial cells (MAECs) impairs the angiogenic process via a mechanism involving NFκB (unpublished data).

A study performed in Goto-Kakizaki diabetic rats demonstrates that endothelial dysfunction is worsened by MGO treatment, which increases oxidative stress, AGE formation and inflammation with a decline in NO bioavailability [58]. Moreover, MGO accumulation in arterial walls causes vascular contractile dysfunction in spontaneously hypertensive rats [59], and Dhar et al. have demonstrated that MGO treatment activates NFκB through RAGE, thereby increasing renin-angiotensin levels and blood pressure in Sprague-Dawley rats [60]. These findings provide further evidence that MGO is a causative factor in the pathogenesis of atherosclerosis and development of macrovascular diabetic complications. In humans, an association between MGO-derived AGEs and endothelial dysfunction markers has been found in individuals with type 1 diabetes [61,62]. In addition, reduced Glo1 activity in atherosclerotic lesions associates with increased HbA1c levels in non-diabetic patients [63].

As a pivotal mediator of endothelial-dependent release of NO and resulting smooth muscle relaxation, endothelial nitric oxide synthase (eNOS) represents a target of the harmful effect of MGO. Indeed, eNOS protein levels and its active phosphorylated form at the serine 1177 site are decreased in long-term MGO treatment of rat isolated mesenteric artery [53] and in thoracic aortic rings [64]. Also, eNOS uncoupling, found to be associated with $O^{-}{}_{2}$ generation and eNOS cofactor biopterin depletion [65], contributes to redox-sensitive leukocyte recruitment and microvascular leakage elicited by MGO [66]. In addition, age-related glycative and oxidative stress, resulting in endothelial dysfunction, is reduced in Glo1 transgenic rats [67].

Besides macrovascular function, MGO and MGO-derived AGEs also play a harmful effect on microvascular function, contributing to the onset of nephropathy and neuropathy. Indeed, regulation of the Glo1 enzyme has been proved to be important in prevention of early renal impairment in experimental diabetes [68], but also independently of hyperglycaemia in apoE$^{-/-}$ mice [69]. This is also confirmed by the evidence that MGO accumulation in Wistar normal rats impairs several renal disease markers progressively observed in diabetic Goto-Kakizaki rats [70]. MGO induces blood–brain barrier damage by reducing the integrity and increasing the permeability of brain endothelial cells [71]. Recently, the generation of an occludin-MGO adduct, which leads to dysfunctional tight junctions and increased brain microvascular endothelial cell (BMEC) permeability, has been identified as a mechanism potentially involved in these abnormalities [72]. BMECs are crucial for brain vascular repair and maintenance. Recent evidence obtained from both in vitro [73,74] and in vivo [75] models of

brain ischemia demonstrates that MGO induces BMEC injuries and exaggerates ischemia-reperfusion injury in diabetic rats.

Based on these findings, it became clearer that an effective reduction of MGO accumulation is crucial for preserving vascular function. Several attempts to alleviate dicarbonyl stress have been made in the last few decades, with MGO scavengers such as AG. But trials had to be terminated due to the lack of efficacy, safety concerns or undesired side effects [18]. A promising strategy is the development of Glo1 inducers through the activation and binding of Nrf2 to the Glo1 functional ARE. Up to now, a Glo1 inducer combination of *trans*-resveratrol and hesperetin (tRES-HESP) has been evaluated in a Phase 1 clinical trial and is now available for evaluation in Phase 2. In highly overweight subjects, tRES-HESP improves arterial dilation and decreases the vascular inflammation marker VCAM-1 [76]. Further efforts in the development of pharmacological intervention to prevent dicarbonyl stress are needed to provide new therapeutic options aimed at preventing vascular dysfunction in diabetes and other age-related diseases.

3. MGO-Induced Insulin-Resistance: A Link to Endothelial Dysfunction

Because of its highly reactive nature, MGO rapidly binds protein residues, with arginine and lysine representing the sites with the higher binding affinity [77]. This is particularly insidious as arginine and lysine are amino acid residues with a high probability of being located in the functional sites of proteins, including kinases involved in insulin signal transduction [3]. We and others have provided evidence about the role of MGO on insulin-resistance in major target tissues for insulin action.

MGO interferes with insulin signaling in L6 skeletal muscle and beta cells. In INS-1E beta cells it decreases insulin secretion through the inhibition of the insulin receptor substrate (IRS)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway activation and independently from reactive oxygen species production. The inhibitory effect of MGO and the formation of AGE adducts on IRS are reversed by AG [78,79]. In addition, MGO administration in vivo inhibits insulin secretion from isolated beta cells, due to decreased PDX-1 levels, and results in higher levels of MGO-derived AGEs and insulin-resistance in muscle, liver, and adipose tissue [80–82]. Such effects are inhibited by the MGO scavenger Alagebrium, indicating the specificity of MGO effects. In support of animal data and studies in animal models, a very recent human study in healthy overweight individuals demonstrates that a diet with low AGE content decreases risk of type 2 diabetes by improving insulin sensitivity [83]. Besides the canonical target tissue of insulin action, MGO induces neuronal cell death in the central nervous system, and the impairment of insulin signaling was found in astrocytes exposed to MGO [84]. In both mice and humans, dietary MGO and AGEs were positively correlated with cognitive deficit, and inversely associated with the survival factor sirtuin-1 (SIRT1) levels and other markers of insulin sensitivity, suggesting MGO dependent SIRT1 down-regulation as a possible link between insulin-resistance and neurodegeneration [17].

Insulin also plays important haemodynamic actions. Insulin receptors were identified on endothelial cells of both large and small blood vessels by Jialal and colleagues in 1985 [85]. The hemodynamic effect of insulin was suggested by pivotal experiments reporting an insulin-mediated increase in blood flow into leg skeletal muscle during a hyperinsulinemic euglycaemic clamp [86]. Indeed, insulin action on endothelium is an integration of increased capillary recruitment and increased total blood flow. Both of these effects are reduced and delayed in insulin-resistance [87].

At the molecular level, insulin binding to its cognate receptor on endothelial cells activates IRS, PI3K and Akt, leading to the activation of eNOS [88]. eNOS catalyses the conversion of arginine to citrulline and NO. Its activity is regulated by phosphorylation at multiple sites; two of the best characterized sites are serine 1177 and threonine 495. Serine 1177 is rapidly phosphorylated by Akt in response to insulin, whereas threonine 495 is constitutively phosphorylated in endothelial cells and is thought to be a negative regulatory site [89,90]. Insulin is also able to cause rapid release of ET-1 via the mitogen-activated protein kinase (MAPK) cascade, which induces the activation of endothelin converting enzyme (ECE) in endothelial cells [50], followed by vasoconstriction and proliferation

of vascular smooth muscle cells (VSMCs). Studies enabling the understanding of the causal role of insulin signal transduction in vasodilation were carried out in genetically modified animal models. Mice lacking IRS-1 feature hypertension and impaired endothelium-dependent vasorelaxation [91]. Consistently, a polymorphism at arginine 972 IRS-1 is associated with decreased eNOS expression in endothelial cells and plasma NO levels in human subjects [92]. Moreover, eNOS knock-out mice are hypertensive and have a 40% lower insulin-stimulated glucose uptake than control mice [93], indicating that insulin-mediated vasodilation and capillary recruitment are required for insulin delivery and action at target tissues.

Besides the above mentioned harmful effect of MGO on insulin-sensitivity of insulin target tissues, we have recently identified MGO as an endothelial insulin-resistance-inducing molecule (Figure 2) [94]. Increased MGO levels are responsible for the impairment of insulin action in mouse aortic endothelial cells (MAECs) exposed to exogenous MGO, or treated with the Glo1 inhibitor *S-p-bromobenzylglutathione cyclopentyl diester* (SpBrBzGSHCp2), and in vivo in healthy mice chronically treated with MGO. MGO exposure renders insulin unable to induce IRS-1 tyrosine phosphorylation, its association to p85, Akt activation and the subsequent eNOS phosphorylation on serine 1177, while the inhibitory phosphorylation of eNOS on threonine 497 is prevented by MGO accumulation. eNOS inactivation results in loss of the insulin-dependent increase in NO production. Conversely, ERK 1/2 activation is enhanced by MGO in both MAECs and aortic tissue from MGO-treated mice, accompanied by increased phosphorylation of IRS-1 on its inhibitory site serine 616. The rescue of IRS-1/Akt/eNOS pathway activation following the chemical inhibition of ERK 1/2 indicates that ERK 1/2 mediates, at least in part, the detrimental effect exerted by MGO on IRS-1/Akt pathway activation by phosphorylating IRS-1 at serine 616. Furthermore, ERK 1/2 hyperactivation does not result in increased MAEC proliferation, but rather in the increase of ET-1 production both in the absence and presence of insulin, suggesting that MGO alters endothelial insulin signaling and causes an imbalance between NO and ET-1 production, which facilitates vasoconstriction. Studies by Raoch et al. showed that NO regulates endothelin converting enzyme 1 ECE-1 expression [95]. It is therefore possible that the reduced NO levels also contribute to the activation of ECE-1 and the subsequent release of ET-1.

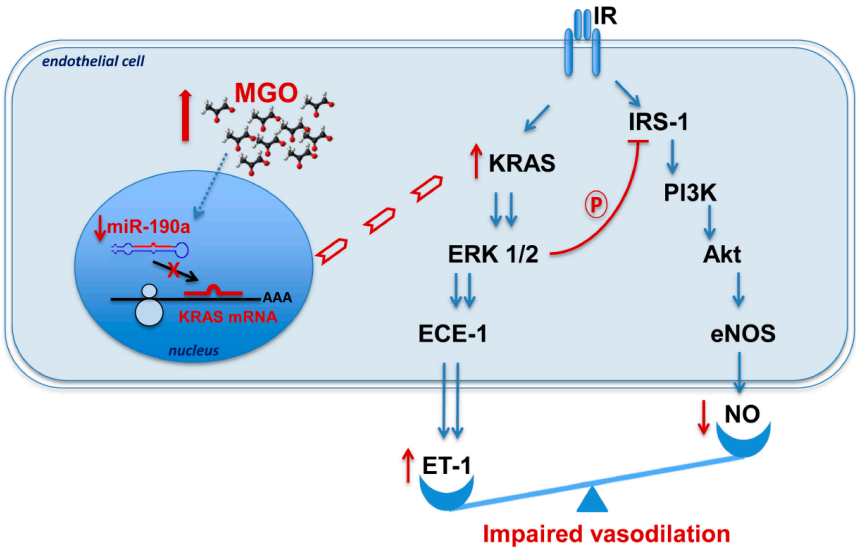

Figure 2. MGO-mediated endothelial insulin-resistance. MGO accumulation causes the reduction of miR-190a. Protein levels of miR-190a target KRAS increased, resulting in the hyperactivation of ERK 1/2. The latter phosphorylates IRS-1 (insulin receptor substrate 1) on serine 616 inhibiting its activation and the downstream pathway PI3K/Akt/eNOS. These effects result in the impairment of insulin stimulated NO production by endothelial cells, together with increased ET-1 release. Red ↑: increased molecule levels; red ↓: decreased molecule levels; blue ↓: protein activation; blue ↓↓: protein hyperactivation. KRAS (Kirsten rat sarcoma viral oncogene homolog).

Emerging evidence suggests that MGO may alter gene expression through miRNA regulation [96,97], besides changes in DNA methylation [98] and histone modifications [99,100]. To answer the question of how MGO increases ERK 1/2 activation in MAECs, we have carried on studies which led to the recognition of miR-190a as an important modulator. The expression of this miRNA is down-regulated in both MAECs treated with MGO and aortic tissue from mice where Glo1 expression has been knocked down (Glo1-KD mice) [101]. The modulation of miR-190a inversely correlated with Kirsten rat sarcoma viral oncogene homolog (KRAS) levels and ERK 1/2 activation. Furthermore, pretreatment of MAECs with the inhibitor of class I and II histone deacetylase (HDAC), trichostatin A, was able to abolish the MGO effect on miR-190a expression, suggesting HDAC involvement. Consistent with previous findings proving the role of MGO in insulin-sensitivity and vascular function [53,55,78], the rescue of miR-190a levels we observed in MAECs upon the co-treatment with MGO and AG, revealed that MGO-modified proteins are critical in determining MGO-induced down-regulation of miR-190a.

Despite demonstration that MGO promotes insulin-resistance and hypertension by increasing oxidative stress [81], other investigators reported MGO-mediated insulin-resistance in skeletal muscle cells in the absence of ROS increases [79]. Whether oxidative stress may also play a part in MGO-mediated endothelial insulin-resistance remains to be further addressed.

4. Mouse Models of MGO Accumulation

To study the impact of MGO on vascular function, animal models that allow observation of the systemic implications of an imbalanced accumulation/detoxification ratio of MGO have been investigated. Important insights in this field have been generated by studies carried out in rodents that received MGO in drinking water [81], subcutaneous infusion with osmotic minipumps [80] or by chronic intraperitoneal infusion [94,102]. However, exogenous sources of MGO are only partially absorbed and not completely accumulated in tissues as free MGO, thus limiting the interpretation of these studies. Indeed, MGO rapidly reacts generating in situ adducts that, depending on the nature and half-life of the specific protein, can move in different ways to other districts in the organism or remain confined to the site of administration [22]. Experimental studies have often used MGO levels 10–20-fold higher than physiological concentrations. These conditions are similar to or exceed the upper limit of severe dicarbonyl stress in poorly controlled diabetes [103]. Moreover, MGO is physiologically formed intracellularly, as also indicated by the cellular MGO concentration exceeding that in plasma of human subjects by 100-fold [104]. Measuring tissue and plasma levels in experimental models is therefore necessary to ensure the patho-physiological relevance of the animal model.

A possibility to bypass these limitations is to modulate Glo1 activity and/or expression. Indeed, reduced activity of Glo1, inducible by treatment with the chemical Glo1 inhibitor SpBrBzGSHCp2 or by silencing Glo1 expression [105,106], mimics the physiological condition of endogenous accumulation of MGO as a consequence of MGO/Glo1 imbalance, occurring in both diabetes and ageing [107]. Conversely, Glo1 over-expression reduces hyperglycaemia-induced carbonyl stress [108], and has a protective effect on vascular dysfunction [68]. Chemical inhibition of Glo1 in apoE$^{-/-}$ mice is able to increase endothelial inflammation and atherogenesis in the absence of hyperglycaemia, to a similar extent as in hyperglycaemic mice with diabetes [109]. Other studies using Glo1 genetically modified mice demonstrate its implication in microvascular complications. As reported by Giacco F. and colleagues, modulation of Glo1 in mice influences the risk of nephropathy development [106]. Indeed, in non-diabetic mice, knockdown of Glo1 increases both MGO modification of glomerular proteins and oxidative stress to diabetic levels, and causes alterations in kidney morphology which are indistinguishable from those caused by diabetes. Conversely, in streptozotocin (STZ)-induced diabetic mice, Glo1 over-expression prevents diabetes-induced increases in MGO modification of glomerular proteins, increased oxidative stress, and the development of diabetic kidney pathology, despite unchanged levels of diabetic hyperglycemia [106]. Recently, beneficial effects of Glo1 over-expression were also found in tubular cell survival, resulting in nephroprotective effects in renal ischemia-perfusion injury [110]. Another study of Bierhaus A. et al., demonstrated that both chemical

Glo1 inhibition in wild-type mice and non-diabetic Glo1 knockdown mice develop signs of peripheral neuropathy, including thermal and mechanical hyperalgesia [105]. Strain dependent differences in Glo1 copy numbers also confirm that a lower expression of Glo1 promotes the development of diabetic neuropathy symptoms [111]. Very recent evidence obtained in Glo1 over-expressing mice has shown that MGO increases inflammation in diabetes, leading to endothelial cell loss and, thus, contributing to the development of diabetic cardiomyopathy [112]. Moreover, systemic Glo1 over-expression in rats prevents age-related impairment of endothelium-dependent vasorelaxation through modulation of eNOS phosphorylation, proving that blunting glycative stress prevents the long-term impact of endothelial dysfunction on vascular ageing [67]. In line with these findings, our preliminary data obtained in Glo1 knockdown mice indicated that Glo1 down-regulation is sufficient to increase blood pressure with ageing, likely as a consequence of impaired endothelium-dependent vasorelaxation. By contrast, previous studies in the same model did not reveal any differences in blood pressure compared to wild-type mice [106]. In this study, however, blood pressure was recorded in young mice where, even in presence of early signs of kidney damage, hypertension seemed to be not clinically evident yet.

5. Conclusions

It is undeniable that MGO accumulation has harmful effects on vascular function, by inducing insulin-resistance, hypertension, atherosclerosis, neurodegenerative disease and diabetic microvascular complications. A large body of literature supports the concept that maintaining the MGO/Glo1 balance is crucial to guarantee the containment of MGO levels under the toxic threshold. To this purpose, it is necessary to adopt a multi-task strategy. It is indeed not sufficient to minimize MGO intake. Also, improving glucose control to treat vascular dysfunction is likely hampered by metabolic memory. In this context, a promising contribution might be provided by modulating Glo1 activity. At the moment, it is necessary to use optimal experimental models to further uncover the pathways contributing to MGO accumulation and its effects, as well as its real impact on physiological systems, fostering the development of novel and effective pharmacological interventions to prevent vascular dysfunction.

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References

1. Phillips, S.A.; Thornalley, P.J. The formation of methylglyoxal from triose phosphates. Investigation using a specific assay for methylglyoxal. *Eur. J. Biochem.* **1993**, *212*, 101–105. [CrossRef] [PubMed]
2. Rabbani, N.; Xue, M.; Thornalley, P.J. Dicarbonyls and glyoxalase in disease mechanisms and clinical therapeutics. *Glycoconj. J.* **2016**, *33*, 513–525. [CrossRef] [PubMed]
3. Rabbani, N.; Xue, M.; Thornalley, P.J. Methylglyoxal-induced dicarbonyl stress in aging and disease: First steps towards glyoxalase 1-based treatments. *Clin. Sci.* **2016**, *130*, 1677–16796. [CrossRef] [PubMed]
4. Thornalley, P.J. Modification of the glyoxalase system in human red blood cells by glucose in vitro. *Biochem. J.* **1988**, *254*, 751–755. [CrossRef] [PubMed]
5. Babaei-Jadidi, R.; Karachalias, N.; Ahmed, N.; Battah, S.; Thornalley, P.J. Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamine. *Diabetes* **2003**, *52*, 2110–2120. [CrossRef] [PubMed]
6. Seagroves, T.N.; Ryan, H.E.; Lu, H.; Wouters, B.G.; Knapp, M.; Thibault, P.; Laderoute, K.; Johnson, R.S. Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. *Mol. Cell. Biol.* **2001**, *21*, 3436–3444. [CrossRef] [PubMed]
7. Beisswenger, B.G.; Delucia, E.M.; Lapoint, N.; Sanford, R.J.; Beisswenger, P.J. Ketosis leads to increased methylglyoxal production on the Atkins diet. *Ann. N. Y. Acad. Sci.* **2005**, *1043*, 201–210. [CrossRef] [PubMed]
8. Lyles, G.A.; Chalmers, J. The metabolism of aminoacetone to methylglyoxal by semicarbazide-sensitive amine oxidase in human umbilical artery. *Biochem. Pharmacol.* **1992**, *43*, 1409–1414. [CrossRef]

9. Kalapos, M.P. Methylglyoxal in living organisms: Chemistry, biochemistry, toxicology and biological implications. *Toxicol. Lett.* **1999**, *110*, 145–175. [CrossRef]
10. Thornalley, P.J.; Langborg, A.; Minhas, H.S. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem. J.* **1999**, *344 Pt 1*, 109–116. [CrossRef] [PubMed]
11. Nemet, I.; Varga-Defterdarovic, L.; Turk, Z. Methylglyoxal in food and living organisms. *Mol. Nutr. Food. Res.* **2006**, *50*, 1105–1117. [CrossRef] [PubMed]
12. Degen, J.; Hellwig, M.; Henle, T. 1,2-dicarbonyl compounds in commonly consumed foods. *J. Agric. Food Chem.* **2012**, *60*, 7071–7079. [CrossRef] [PubMed]
13. Martins, S.I.; van Boekel, M.A. Kinetic modelling of Amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part II—kinetic analysis. *Carbohydr. Res.* **2003**, *338*, 1665–1678. [CrossRef]
14. Do Rosario, P.M.; Cordeiro, C.A.; Freire, A.P.; Nogueira, J.M. Analysis of methylglyoxal in water and biological matrices by capillary zone electrophoresis with diode array detection. *Electrophoresis* **2005**, *26*, 1760–1767. [CrossRef] [PubMed]
15. Fujioka, K.; Shibamoto, T. Determination of toxic carbonyl compounds in cigarette smoke. *Environ. Toxicol.* **2006**, *21*, 47–54. [CrossRef] [PubMed]
16. Degen, J.; Vogel, M.; Richter, D.; Hellwig, M.; Henle, T. Metabolic transit of dietary methylglyoxal. *J. Agric. Food Chem.* **2013**, *61*, 10253–10260. [PubMed]
17. Cai, W.; Uribarri, J.; Zhu, L.; Chen, X.; Swamy, S.; Zhao, Z.; Grosjean, F.; Simonaro, C.; Kuchel, G.A.; Schnaider-Beeri, M.; et al. Oral glycotoxins are a modifiable cause of dementia and the metabolic syndrome in mice and humans. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 4940–4945. [CrossRef] [PubMed]
18. Maessen, D.E.; Stehouwer, C.D.; Schalkwijk, C.G. The role of methylglyoxal and the glyoxalase system in diabetes and other age-related diseases. *Clin. Sci.* **2015**, *128*, 839–861. [CrossRef] [PubMed]
19. Rabbani, N.; Thornalley, P.J. Measurement of methylglyoxal by stable isotopic dilution analysis LC-MS/MS with corroborative prediction in physiological samples. *Nat. Protoc.* **2014**, *9*, 1969–1979. [CrossRef] [PubMed]
20. Rabbani, N.; Thornalley, P.J. Dicarbonyl stress in cell and tissue dysfunction contributing to ageing and disease. *Biochem. Biophys. Res. Commun.* **2015**, *458*, 221–226. [CrossRef] [PubMed]
21. Rabbani, N.; Thornalley, P.J. Dicarbonyl proteome and genome damage in metabolic and vascular disease. *Biochem. Soc. Trans.* **2014**, *42*, 425–432. [CrossRef] [PubMed]
22. Baba, S.P.; Barski, O.A.; Ahmed, Y.; O'Toole, T.E.; Conklin, D.J.; Bhatnagar, A.; Srivastava, S. Reductive metabolism of AGE precursors: A metabolic route for preventing AGE accumulation in cardiovascular tissue. *Diabetes* **2009**, *58*, 2486–2497. [CrossRef] [PubMed]
23. Sousa Silva, M.; Gomes, R.A.; Ferreira, A.E.; Ponces Freire, A.; Cordeiro, C. The glyoxalase pathway: The first hundred years and beyond. *Biochem. J.* **2013**, *453*, 1–15. [CrossRef] [PubMed]
24. Thornalley, P.J. The glyoxalase system in health and disease. *Mol. Aspects. Med.* **1993**, *14*, 287–371. [CrossRef]
25. Silva, M.S.; Ferreira, A.E.; Gomes, R.; Tomás, A.M.; Freire, A.P.; Cordeiro, C. The glyoxalase pathway in protozoan parasites. *Int. J. Med. Microbiol.* **2012**, *302*, 225–229. [CrossRef] [PubMed]
26. Aronsson, A.C.; Marmstal, E.; Mannervik, B. Glyoxalase I, a zinc metalloenzyme of mammals and yeast. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 1235–1240. [CrossRef]
27. He, M.M.; Clugston, S.L.; Honek, J.F.; Matthews, B.W. Determination of the structure of *Escherichia coli* glyoxalase I suggests a structural basis for differential metal activation. *Biochemistry* **2000**, *39*, 8719–8727. [CrossRef] [PubMed]
28. Rabbani, N.; Thornalley, P.J. Glyoxalase, Centennial conference: Introduction, history of research on the glyoxalase system and future prospects. *Biochem. Soc. Trans.* **2014**, *42*, 413–418. [CrossRef] [PubMed]
29. Xue, M.; Rabbani, N.; Momiji, H.; Imbasi, P.; Anwar, M.M.; Kitteringham, N.; Park, B.K.; Souma, T.; Moriguchi, T.; Yamamoto, M.; et al. Transcriptional control of glyoxalase 1 by Nrf2 provides a stress-responsive defence against dicarbonyl glycation. *Biochem. J.* **2012**, *443*, 213–222. [CrossRef] [PubMed]
30. Zhang, H.; Li, H.; Xi, H.S.; Li, S. HIF1α is required for survival maintenance of chronic myeloid leukemia stem cells. *Blood* **2012**, *119*, 2595–2607. [CrossRef] [PubMed]
31. Bierhaus, A.; Schiekofer, S.; Schwaninger, M.; Andrassy, M.; Humpert, P.M.; Chen, J.; Hong, M.; Luther, T.; Henle, T.; Klöting, I.; et al. Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. *Diabetes* **2001**, *50*, 2792–2808. [CrossRef] [PubMed]
32. Liu, G.H.; Qu, J.; Shen, X. NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim. Biophys. Acta* **2008**, *1783*, 713–727. [CrossRef] [PubMed]

33. Rabbani, N.; Xue, M.; Thornalley, P.J. Activity, regulation, copy number and function in the glyoxalase system. *Biochem. Soc. Trans.* **2014**, *42*, 419–424. [CrossRef] [PubMed]
34. Peculis, R.; Konrade, I.; Skapare, E.; Fridmanis, D.; Nikitina-Zake, L.; Lejnieks, A.; Pirags, V.; Dambrova, M.; Klovins, J. Identification of glyoxalase 1 polymorphisms associated with enzyme activity. *Gene* **2013**, *515*, 140–143. [CrossRef] [PubMed]
35. Groener, J.B.; Reismann, P.; Fleming, T.; Kalscheuer, H.; Lehnhoff, D.; Hamann, A.; Roser, P.; Bierhaus, A.; Nawroth, P.P.; Rudofsky, G. C332C genotype of glyoxalase 1 and its association with late diabetic complications. *Exp. Clin. Endocrinol. Diabetes* **2013**, *121*, 436–439. [CrossRef] [PubMed]
36. Rabbani, N.; Thornalley, P.J. Glyoxalase in diabetes, obesity and related disorders. *Semin. Cell Dev. Biol.* **2011**, *22*, 309–317. [CrossRef] [PubMed]
37. Cahan, P.; Li, Y.; Izumi, M.; Graubert, T.A. The impact of copy number variation on local gene expression in mouse hematopoietic stem and progenitor cells. *Nat. Genet.* **2009**, *41*, 430–437. [CrossRef] [PubMed]
38. Redon, R.; Ishikawa, S.; Fitch, K.R.; Feuk, L.; Perry, G.H.; Andrews, T.D.; Fiegler, H.; Shapero, M.H.; Carson, A.R.; Chen, W.; et al. Global variation in copy number in the human genome. *Nature* **2006**, *444*, 444–454. [CrossRef] [PubMed]
39. Morcos, M.; Du, X.; Pfisterer, F.; Hutter, H.; Sayed, A.A.; Thornalley, P.; Ahmed, N.; Baynes, J.; Thorpe, S.; Kukudov, G.; et al. Glyoxalase-1 prevents mitochondrial protein modification and enhances lifespan in *Caenorhabditis elegans*. *Aging Cell* **2008**, *7*, 260–269. [CrossRef] [PubMed]
40. Fleming, T.H.; Theilen, T.M.; Masania, J.; Wunderle, M.; Karimi, J.; Vittas, S.; Bernauer, R.; Bierhaus, A.; Rabbani, N.; Thornalley, P.J.; et al. Aging-dependent reduction in glyoxalase 1 delays wound healing. *Gerontology* **2013**, *59*, 427–437. [CrossRef] [PubMed]
41. Ikeda, Y.; Inagi, R.; Miyata, T.; Nagai, R.; Arai, M.; Miyashita, M.; Itokawa, M.; Fujita, T.; Nangaku, M. Glyoxalase I retards renal senescence. *Am. J. Pathol.* **2011**, *179*, 2810–2821. [CrossRef] [PubMed]
42. Gu, Q.; Wang, B.; Zhang, X.F.; Ma, Y.P.; Liu, J.D.; Wang, X.Z. Contribution of receptor for advanced glycation end products to vasculature-protecting effects of exercise training in aged rats. *Eur. J. Pharmacol.* **2014**, *741*, 186–194. [CrossRef] [PubMed]
43. Xue, M.; Rabbani, N.; Thornalley, P.J. Glyoxalase in ageing. *Semin. Cell Dev. Biol.* **2011**, *22*, 293–301. [CrossRef] [PubMed]
44. Shamsaldeen, Y.A.; Mackenzie, L.S.; Lione, L.A.; Benham, C.D. Methylglyoxal, A Metabolite Increased in Diabetes is Associated with Insulin Resistance, Vascular Dysfunction and Neuropathies. *Curr. Drug Metab.* **2016**, *17*, 359–367. [CrossRef] [PubMed]
45. Cimellaro, A.; Perticone, M.; Fiorentino, T.V.; Sciacqua, A.; Hribal, M.L. Role of endoplasmic reticulum stress in endothelial dysfunction. *Nutr. Metab. Cardiovasc. Dis.* **2016**, *26*, 863–871. [CrossRef] [PubMed]
46. Szmitko, P.E.; Wang, C.H.; Weisel, R.D.; de Almeida, J.R.; Anderson, T.J.; Verma, S. New markers of inflammation and endothelial cell activation: Part I. *Circulation* **2003**, *108*, 1917–1923. [CrossRef] [PubMed]
47. Verma, S.; Anderson, T.J. Fundamentals of endothelial function for the clinical cardiologist. *Circulation* **2002**, *105*, 546–549. [CrossRef] [PubMed]
48. Dhananjayan, R.; Koundinya, K.S.; Malati, T.; Kutala, V.K. Endothelial Dysfunction in Type 2 Diabetes Mellitus. *Indian J. Clin. Biochem.* **2016**, *31*, 372–379. [CrossRef] [PubMed]
49. Dimassi, S.; Chahed, K.; Boumiza, S.; Canault, M.; Tabka, Z.; Laurant, P.; Riva, C. Role of eNOS- and NOX-containing microparticles in endothelial dysfunction in patients with obesity. *Obesity* **2016**, *24*, 1305–1312. [CrossRef] [PubMed]
50. Eringa, E.C.; Stehouwer, C.D.; van Nieuw Amerongen, G.P.; Ouwehand, L.; Westerhof, N.; Sipkema, P. Vasoconstrictor effects of insulin in skeletal muscle arterioles are mediated by ERK1/2 activation in endothelium. *Am. J. Physiol. Heart Circ. Physiol.* **2004**, *287*, H2043–H2048. [CrossRef] [PubMed]
51. Schalkwijk, C.G. Vascular AGE-ing by methylglyoxal: The past, the present and the future. *Diabetologia* **2015**, *58*, 1715–1719. [CrossRef] [PubMed]
52. Dhar, A.; Dhar, I.; Desai, K.M.; Wu, L. Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose. *Br. J. Pharmacol.* **2010**, *161*, 1843–1856. [CrossRef] [PubMed]
53. Mukohda, M.; Morita, T.; Okada, M.; Hara, Y.; Yamawaki, H. Long-term methylglyoxal treatment causes endothelial dysfunction of rat isolated mesenteric artery. *J. Vet. Med. Sci.* **2013**, *75*, 151–157. [CrossRef] [PubMed]

54. Brouwers, O.; Niessen, P.M.; Haenen, G.; Miyata, T.; Brownlee, M.; Stehouwer, C.D.; de Mey, J.G.; Schalkwijk, C.G. Hyperglycaemia-induced impairment of endothelium-dependent vasorelaxation in rat mesenteric arteries is mediated by intracellular methylglyoxal levels in a pathway dependent on oxidative stress. *Diabetologia* **2010**, *53*, 989–1000. [CrossRef] [PubMed]
55. Boydens, C.; Pauwels, B.; van den Daele, L.; van de Voorde, J. Protective effect of resveratrol and quercetin on in vitro-induced diabetic mouse corpus cavernosum. *Cardiovasc. Diabetol.* **2016**, *15*, 46. [CrossRef] [PubMed]
56. Oba, T.; Tatsunami, R.; Sato, K.; Takahashi, K.; Hao, Z.; Tampo, Y. Methylglyoxal has deleterious effects on thioredoxin in human aortic endothelial cells. *Environ. Toxicol. Pharmacol.* **2012**, *34*, 117–126. [CrossRef] [PubMed]
57. Liu, H.; Yu, S.; Zhang, H.; Xu, J. Angiogenesis impairment in diabetes: Role of methylglyoxal-induced receptor for advanced glycation endproducts, autophagy and vascular endothelial growth factor receptor 2. *PLoS ONE* **2012**, *7*, e46720. [CrossRef] [PubMed]
58. Sena, C.M.; Matafome, P.; Crisostomo, J.; Rodrigues, L.; Fernandes, R.; Pereira, P.; Seica, R.M. Methylglyoxal promotes oxidative stress and endothelial dysfunction. *Pharmacol. Res.* **2012**, *65*, 497–506. [CrossRef] [PubMed]
59. Mukohda, M.; Okada, M.; Hara, Y.; Yamawaki, H. Methylglyoxal accumulation in arterial walls causes vascular contractile dysfunction in spontaneously hypertensive rats. *J. Pharmacol. Sci.* **2012**, *120*, 26–35. [CrossRef] [PubMed]
60. Dhar, I.; Dhar, A.; Wu, L.; Desai, K.M. Methylglyoxal, a reactive glucose metabolite, increases renin angiotensin aldosterone and blood pressure in male Sprague-Dawley rats. *Am. J. Hypertens* **2014**, *27*, 308–316. [CrossRef] [PubMed]
61. Van Eupen, M.G.; Schram, M.T.; Colhoun, H.M.; Hanssen, N.M.; Niessen, H.W.; Tarnow, L.; Parving, H.H.; Rossing, P.; Stehouwer, C.D.A.; Schalkwijk, C.G. The methylglyoxal-derived AGE tetrahydropyrimidine is increased in plasma of individuals with type 1 diabetes mellitus and in atherosclerotic lesions and is associated with sVCAM-1. *Diabetologia* **2013**, *56*, 1845–1855. [CrossRef] [PubMed]
62. Heier, M.; Margeirsdottir, H.D.; Torjesen, P.A.; Seljeflot, I.; Stensaeth, K.H.; Gaarder, M.; Brunborg, C.; Hanssen, K.F.; Dahl-Jørgensen, K. The advanced glycation end product methylglyoxal-derived hydroimidazolone-1 and early signs of atherosclerosis in childhood diabetes. *Diab. Vasc. Dis. Res.* **2015**, *12*, 139–145. [CrossRef] [PubMed]
63. Peters, A.S.; Lercher, M.; Fleming, T.H.; Nawroth, P.P.; Bischoff, M.S.; Dihlmann, S.; Böckler, D.; Hakimi, M. Reduced glyoxalase 1 activity in carotid artery plaques of nondiabetic patients with increased hemoglobin A1c level. *J. Vasc. Surg.* **2016**, *64*, 990–994. [CrossRef] [PubMed]
64. Turkseven, S.; Ertuna, E.; Yetik-Anacak, G.; Yasa, M. Methylglyoxal causes endothelial dysfunction: The role of endothelial nitric oxide synthase and AMP-activated protein kinase α. *J. Basic. Clin. Physiol. Pharmacol.* **2014**, *25*, 109–115. [CrossRef] [PubMed]
65. Ravassa, S.; Barba, J.; Coma-Canella, I.; Huerta, A.; Lopez, B.; Gonzalez, A.; Díez, J. The activity of circulating dipeptidyl peptidase-4 is associated with subclinical left ventricular dysfunction in patients with type 2 diabetes mellitus. *Cardiovasc. Diabetol.* **2013**, *12*, 143. [CrossRef] [PubMed]
66. Su, Y.; Qadri, S.M.; Hossain, M.; Wu, L.; Liu, L. Uncoupling of eNOS contributes to redox-sensitive leukocyte recruitment and microvascular leakage elicited by methylglyoxal. *Biochem. Pharmacol.* **2013**, *86*, 1762–1774. [CrossRef] [PubMed]
67. Jo-Watanabe, A.; Ohse, T.; Nishimatsu, H.; Takahashi, M.; Ikeda, Y.; Wada, T.; Shirakawa, J.; Nagai, R.; Miyata, T.; Nagano, T.; et al. Glyoxalase I reduces glycative and oxidative stress and prevents age-related endothelial dysfunction through modulation of endothelial nitric oxide synthase phosphorylation. *Aging Cell* **2014**, *13*, 519–528. [CrossRef] [PubMed]
68. Brouwers, O.; Niessen, P.M.; Miyata, T.; Ostergaard, J.A.; Flyvbjerg, A.; Peutz-Kootstra, C.J.; Sieber, J.; Mundel, P.H.; Brownlee, M.; Janssen, B.J.; et al. Glyoxalase-1 overexpression reduces endothelial dysfunction and attenuates early renal impairment in a rat model of diabetes. *Diabetologia* **2014**, *57*, 224–235. [CrossRef] [PubMed]
69. Geoffrion, M.; Du, X.; Irshad, Z.; Van der hyden, B.C.; Courville, K.; Sui, G.; D'Agati, V.D.; Ott-Braschi, S.; Rabbani, N.; Thornalley, P.J.; et al. Differential effects of glyoxalase 1 overexpression on diabetic atherosclerosis and renal dysfunction in streptozotocin-treated, apolipoprotein E-deficient mice. *Physiol. Rep.* **2014**, *2*, e12043. [CrossRef] [PubMed]

70. Rodrigues, L.; Matafome, P.; Crisostomo, J.; Santos-Silva, D.; Sena, C.; Pereira, P.; Seiça, R. Advanced glycation end products and diabetic nephropathy: A comparative study using diabetic and normal rats with methylglyoxal-induced glycation. *J. Physiol. Biochem.* **2014**, *70*, 173–184. [CrossRef] [PubMed]
71. Toth, A.E.; Walter, F.R.; Bocsik, A.; Santha, P.; Veszelka, S.; Nagy, L.; Puskás, L.G.; Couraud, P.O.; Takata, F.; Dohgu, S.; et al. Edaravone protects against methylglyoxal-induced barrier damage in human brain endothelial cells. *PLoS ONE* **2014**, *9*, e100152. [CrossRef] [PubMed]
72. Li, W.; Maloney, R.E.; Aw, T.Y. High glucose, glucose fluctuation and carbonyl stress enhance brain microvascular endothelial barrier dysfunction: Implications for diabetic cerebral microvasculature. *Redox. Biol.* **2015**, *5*, 80–90. [CrossRef] [PubMed]
73. Li, W.; Chen, Z.; Yan, M.; He, P.; Chen, Z.; Dai, H. The protective role of isorhamnetin on human brain microvascular endothelial cells from cytotoxicity induced by methylglyoxal and oxygen-glucose deprivation. *J. Neurochem.* **2016**, *136*, 651–659. [CrossRef] [PubMed]
74. Fang, L.; Li, X.; Zhong, Y.; Yu, J.; Yu, L.; Dai, H.; Yan, M. Autophagy protects human brain microvascular endothelial cells against methylglyoxal-induced injuries, reproducible in a cerebral ischemic model in diabetic rats. *J. Neurochem.* **2015**, *135*, 431–440. [CrossRef] [PubMed]
75. Alomar, F.; Singh, J.; Jang, H.S.; Rozanzki, G.J.; Shao, C.H.; Padanilam, B.J.; Mayhan, W.G.; Bidasee, K.R. Smooth muscle-generated methylglyoxal impairs endothelial cell-mediated vasodilatation of cerebral microvessels in type 1 diabetic rats. *Br. J. Pharmacol.* **2016**. [CrossRef] [PubMed]
76. Xue, M.; Weickert, M.O.; Qureshi, S.; Kandala, N.B.; Anwar, A.; Waldron, M.; Shafie, A.; Messenger, D.; Fowler, M.; Jenkins, G.; et al. Improved Glycemic Control and Vascular Function in Overweight and Obese Subjects by Glyoxalase 1 Inducer Formulation. *Diabetes.* **2016**, *65*, 2282–2294. [CrossRef] [PubMed]
77. Rabbani, N.; Thornalley, P.J. Methylglyoxal, glyoxalase 1 and the dicarbonyl proteome. *Amino. Acids* **2012**, *42*, 1133–1142. [CrossRef] [PubMed]
78. Fiory, F.; Lombardi, A.; Miele, C.; Giudicelli, J.; Beguinot, F.; van Obberghen, E. Methylglyoxal impairs insulin signalling and insulin action on glucose-induced insulin secretion in the pancreatic beta cell line INS-1E. *Diabetologia* **2011**, *54*, 2941–2952. [CrossRef] [PubMed]
79. Riboulet-Chavey, A.; Pierron, A.; Durand, I.; Murdaca, J.; Giudicelli, J.; Van Obberghen, E. Methylglyoxal impairs the insulin signaling pathways independently of the formation of intracellular reactive oxygen species. *Diabetes* **2006**, *55*, 1289–1299. [CrossRef] [PubMed]
80. Dhar, A.; Dhar, I.; Jiang, B.; Desai, K.M.; Wu, L. Chronic methylglyoxal infusion by minipump causes pancreatic beta-cell dysfunction and induces type 2 diabetes in Sprague-Dawley rats. *Diabetes* **2011**, *60*, 899–908. [CrossRef] [PubMed]
81. Guo, Q.; Mori, T.; Jiang, Y.; Hu, C.; Osaki, Y.; Yoneki, Y.; Sun, Y.; Hosoya, T.; Kawamata, A.; Ogawa, S.; et al. Methylglyoxal contributes to the development of insulin resistance and salt sensitivity in Sprague-Dawley rats. *J. Hypertens* **2009**, *27*, 1664–1671. [CrossRef] [PubMed]
82. Cai, W.; Ramdas, M.; Zhu, L.; Chen, X.; Striker, G.E.; Vlassara, H. Oral advanced glycation endproducts (AGEs) promote insulin resistance and diabetes by depleting the antioxidant defenses AGE receptor-1 and sirtuin 1. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 15888–15893. [CrossRef] [PubMed]
83. De Courten, B.; de Courten, M.P.; Soldatos, G.; Dougherty, S.L.; Straznicky, N.; Schlaich, M.; Sourris, K.C.; Chand, V.; Scheijen, J.L.; Kingwell, B.A.; et al. Diet low in advanced glycation end products increases insulin sensitivity in healthy overweight individuals: A double-blind, randomized, crossover trial. *Am. J. Clin. Nutr.* **2016**, *103*, 1426–1433. [CrossRef] [PubMed]
84. Chu, J.M.; Lee, D.K.; Wong, D.P.; Wong, R.N.; Yung, K.K.; Cheng, C.H.; Yue, K.K. Ginsenosides attenuate methylglyoxal-induced impairment of insulin signaling and subsequent apoptosis in primary astrocytes. *Neuropharmacology* **2014**, *85*, 215–223. [CrossRef] [PubMed]
85. Jialal, I.; Crettaz, M.; Hachiya, H.L.; Kahn, C.R.; Moses, A.C.; Buzney, S.M.; King, G.L. Characterization of the receptors for insulin and the insulin-like growth factors on micro and macrovascular tissues. *Endocrinology* **1985**, *117*, 1222–1229. [CrossRef] [PubMed]
86. Baron, A.D. Hemodynamic actions of insulin. *Am. J. Physiol.* **1994**, *267*, E187–E202. [PubMed]
87. Jahn, L.A.; Hartline, L.; Rao, N.; Logan, B.; Kim, J.J.; Aylor, K.; Gan, L.M.; Westergren, H.U.; Barrett, E.J. Insulin Enhances Endothelial Function Throughout the Arterial Tree in Healthy But Not Metabolic Syndrome Subjects. *J. Clin. Endocrinol. Metab.* **2016**, *101*, 1198–1206. [CrossRef] [PubMed]

88. Montagnani, M.; Ravichandran, L.V.; Chen, H.; Esposito, D.L.; Quon, M.J. Insulin receptor substrate-1 and phosphoinositide-dependent kinase-1 are required for insulin-stimulated production of nitric oxide in endothelial cells. *Mol. Endocrinol.* **2002**, *16*, 1931–1942. [CrossRef] [PubMed]
89. Andreozzi, F.; Laratta, E.; Procopio, C.; Hribal, M.L.; Sciacqua, A.; Perticone, M.; Sesti, G. Interleukin-6 impairs the insulin signaling pathway, promoting production of nitric oxide in human umbilical vein endothelial cells. *Mol. Cell. Biol.* **2007**, *27*, 2372–2383. [CrossRef] [PubMed]
90. Fleming, I.; Busse, R. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2003**, *284*, R1–R12. [CrossRef] [PubMed]
91. Abe, H.; Yamada, N.; Kamata, K.; Kuwaki, T.; Shimada, M.; Osuga, J.; Shionoiri, F.; Yahagi, N.; Kadowaki, T.; Tamemoto, H.; et al. Hypertension, hypertriglyceridemia, and impaired endothelium-dependent vascular relaxation in mice lacking insulin receptor substrate-1. *J. Clin. Investig.* **1998**, *101*, 1784–1788. [CrossRef] [PubMed]
92. Huang, C.; Li, G.; Dong, H.; Sun, S.; Chen, H.; Luo, D.; Sun, L.; Li, X.; Chen, Z.; Yang, H.; et al. Arg(9)(7)(2) insulin receptor substrate-1 inhibits endothelial nitric oxide synthase expression in human endothelial cells by upregulating microRNA-155. *Int. J. Mol. Med.* **2015**, *36*, 239–248. [PubMed]
93. Duplain, H.; Burcelin, R.; Sartori, C.; Cook, S.; Egli, M.; Lepori, M.; Vollenweider, P.; Pedrazzini, T.; Nicod, P.; Thorens, B.; et al. Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. *Circulation* **2001**, *104*, 342–345. [CrossRef] [PubMed]
94. Nigro, C.; Raciti, G.A.; Leone, A.; Fleming, T.H.; Longo, M.; Prevenzano, I.; Fiory, F.; Mirra, P.; D'Esposito, V.; Ulianich, L.; et al. Methylglyoxal impairs endothelial insulin sensitivity both in vitro and in vivo. *Diabetologia* **2014**, *57*, 1485–1494. [CrossRef] [PubMed]
95. Raoch, V.; Rodriguez-Pascual, F.; Lopez-Martinez, V.; Medrano-Andres, D.; Rodriguez-Puyol, M.; Lamas, S.; Rodríguez-Puyol, D.; López-Ongil, S. Nitric oxide decreases the expression of endothelin-converting enzyme-1 through mRNA destabilization. *Arterioscler. Thromb. Vasc. Biol.* **2011**, *31*, 2577–2585. [CrossRef] [PubMed]
96. Liu, H.; Zhang, N.; Tian, D. MiR-30b is involved in methylglyoxal-induced epithelial-mesenchymal transition of peritoneal mesothelial cells in rats. *Cell. Mol. Biol. Lett.* **2014**, *19*, 315–329. [CrossRef] [PubMed]
97. Li, S.S.; Wu, Y.; Jin, X.; Jiang, C. The SUR2B subunit of rat vascular KATP channel is targeted by miR-9a-3p induced by prolonged exposure to methylglyoxal. *Am. J. Physiol. Cell. Physiol.* **2015**, *308*, C139–C145. [CrossRef] [PubMed]
98. Palsamy, P.; Bidasee, K.R.; Ayaki, M.; Augusteyn, R.C.; Chan, J.Y.; Shinohara, T. Methylglyoxal induces endoplasmic reticulum stress and DNA demethylation in the Keap1 promoter of human lens epithelial cells and age-related cataracts. *Free. Radic. Biol. Med.* **2014**, *72*, 134–148. [CrossRef] [PubMed]
99. El-Osta, A.; Brasacchio, D.; Yao, D.; Pocai, A.; Jones, P.L.; Roeder, R.G.; Cooper, M.E.; Brownlee, M. Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. *J. Exp. Med.* **2008**, *205*, 2409–2417. [CrossRef] [PubMed]
100. Mir, A.R.; uddin, M.; Alam, K.; Ali, A. Methylglyoxal mediated conformational changes in histone H2A-generation of carboxyethylated advanced glycation end products. *Int. J. Biol. Macromol.* **2014**, *69*, 260–266. [CrossRef] [PubMed]
101. Mirra, P.; Nigro, C.; Prevenzano, I.; Procopio, T.; Leone, A.; Raciti, G.A.; Andreozzi, F.; Longo, M.; Fiory, F.; Beguinot, F.; et al. The role of miR-190a in methylglyoxal-induced insulin resistance in endothelial cells. *Biochim. Biophys. Acta* **2016**, *1863*, 440–449. [CrossRef] [PubMed]
102. Berlanga, J.; Cibrian, D.; Guillen, I.; Freyre, F.; Alba, J.S.; Lopez-Saura, P.; Merino, N.; Aldama, A.; Quintela, A.M.; Triana, M.E.; et al. Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds. *Clin. Sci.* **2005**, *109*, 83–95. [CrossRef] [PubMed]
103. Masania, J.; Malczewska-Malec, M.; Razny, U.; Goralska, J.; Zdzienicka, A.; Kiec-Wilk, B.; Gruca, A.; Stancel-Mozwillo, J.; Dembinska-Kiec, A.; Rabbani, N.; et al. Dicarbonyl stress in clinical obesity. *Glycoconj. J.* **2016**, *33*, 581–589. [CrossRef] [PubMed]
104. McLellan, A.C.; Thornalley, P.J.; Benn, J.; Sonksen, P.H. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin. Sci.* **1994**, *87*, 21–29. [CrossRef] [PubMed]
105. Bierhaus, A.; Fleming, T.; Stoyanov, S.; Leffler, A.; Babes, A.; Neacsu, C.; Sauer, S.K.; Eberhardt, M.; Schnölzer, M.; Lasitschka, F.; et al. Methylglyoxal modification of Nav1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy. *Nat. Med.* **2012**, *18*, 926–933. [CrossRef] [PubMed]

106. Giacco, F.; Du, X.; D'Agati, V.D.; Milne, R.; Sui, G.; Geoffrion, M.; Brownlee, M. Knockdown of glyoxalase 1 mimics diabetic nephropathy in nondiabetic mice. *Diabetes* **2014**, *63*, 291–299. [CrossRef] [PubMed]
107. Skapare, E.; Konrade, I.; Liepinsh, E.; Strele, I.; Makrecka, M.; Bierhaus, A.; Lejnieks, A.; Pirags, V.; Dambrova, M. Association of reduced glyoxalase 1 activity and painful peripheral diabetic neuropathy in type 1 and 2 diabetes mellitus patients. *J. Diabetes Complicat.* **2013**, *27*, 262–267. [CrossRef] [PubMed]
108. Brouwers, O.; Niessen, P.M.; Ferreira, I.; Miyata, T.; Scheffer, P.G.; Teerlink, T.; Schrauwen, P.; Brownlee, M.; Stehouwer, C.D.; Schalkwijk, C.G. Overexpression of glyoxalase-I reduces hyperglycemia-induced levels of advanced glycation end products and oxidative stress in diabetic rats. *J. Biol. Chem.* **2011**, *286*, 1374–1380. [CrossRef] [PubMed]
109. Tikellis, C.; Pickering, R.J.; Tsorotes, D.; Huet, O.; Cooper, M.E.; Jandeleit-Dahm, K.; Thomas, M.C. Dicarbonyl stress in the absence of hyperglycemia increases endothelial inflammation and atherogenesis similar to that observed in diabetes. *Diabetes* **2014**, *63*, 3915–3925. [CrossRef] [PubMed]
110. Kumagai, T.; Nangaku, M.; Kojima, I.; Nagai, R.; Ingelfinger, J.R.; Miyata, T.; Fujita, T.; Inagi, R. Glyoxalase I overexpression ameliorates renal ischemia-reperfusion injury in rats. *Am. J. Physiol. Renal. Physiol.* **2009**, *296*, F912–F921. [CrossRef] [PubMed]
111. Jack, M.M.; Ryals, J.M.; Wright, D.E. Protection from diabetes-induced peripheral sensory neuropathy—A role for elevated glyoxalase I? *Exp. Neurol.* **2012**, *234*, 62–69. [CrossRef] [PubMed]
112. Vulesevic, B.; McNeill, B.; Giacco, F.; Maeda, K.; Blackburn, N.J.; Brownlee, M.; Milne, R.W.; Suuronen, E.J. Methylglyoxal-Induced Endothelial Cell Loss and Inflammation Contribute to the Development of Diabetic Cardiomyopathy. *Diabetes* **2016**, *65*, 1699–1713. [CrossRef] [PubMed]

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The role of miR-190a in methylglyoxal-induced insulin resistance in endothelial cells

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ABSTRACT

Methylglyoxal (MGO) is a reactive dicarbonyl produced as by-product of glycolysis, and its formation is heightened in hyperglycaemia. MGO plasma levels are two-fold to five-fold increased in diabetics and its accumulation promotes the progression of vascular complications. Impairment of endothelium-derived nitric oxide represents a common feature of endothelial dysfunction in diabetics. We previously demonstrated that MGO induces endothelial insulin resistance. Increasing evidence shows that high glucose and MGO modify vascular expression of several microRNAs (miRNAs), suggesting their potential role in the impairment of endothelial insulin sensitivity. The aim of the study is to investigate whether miRNAs may be involved in MGO-induced endothelial insulin resistance in endothelial cells.

MGO reduces the expression of miR-190a both in mouse aortic endothelial cells (MAECs) and in aortae from mice knocked-down for glyoxalase-1. miR-190a inhibition impairs insulin sensitivity, whereas its overexpression prevents the MGO-induced insulin resistance in MAECs. miR-190a levels are not affected by the inhibition of ERK1/2 phosphorylation. Conversely, ERK1/2 activation is sustained by miR-190a inhibitor and the MGO-induced ERK1/2 hyper-activation is reduced by miR-190a mimic transfection. Similarly, protein levels of the upstream KRAS are increased by both MGO and miR-190a inhibitor, and these levels are reduced by miR-190a mimic transfection. Interestingly, silencing of KRAS is able to rescue the MGO-impaired activation of IRS1/Akt/eNOS pathway in response to insulin.

In conclusion, miR-190a down-regulation plays a role in MGO-induced endothelial insulin resistance by increasing KRAS. This study highlights miR-190a as new candidate for the identification of strategies aiming at ameliorating vascular function in diabetes.

1. Introduction

Methylglyoxal (MGO) is an endogenous dicarbonyl metabolite formed mainly as by-product of the glycolitic pathway, from the degradation of triose phosphates [1]. A minor contribution to its formation is also given by lipid peroxidation, threonine catabolism [2] and non-enzymatic degradation of glucose and glycated proteins [3]. MGO is a highly potent glycating agent, representing one of the most reactive advanced glycated end product (AGE) precursor. It has been estimated that 99.7% of MGO formed inside the cell is mostly metabolized by the glyoxalase 1 (Glo1) enzyme [4]. In diabetic patients, the flux of formation of MGO is increased two-fold to five-fold, depending on glycemic control [5], and its accumulation may be heightened because of down-regulation and increased degradation of Glo1 [1,6,7].

The increased formation of MGO is one of the mechanisms proposed as the cause of insulin resistance [8–11] and important contributor to the development of vascular complications in chronic hyperglycaemia [12–15]. Indeed, studies in cellular and rodent experimental models have demonstrated that high MGO exposure affects endothelial cell

Abbreviations: AG, aminoguanidine; AGE, advanced glycation end-product; Akt, protein kinase B; eNOS, endothelial NO synthase; ERK, extracellular signal-regulated protein kinase; Glo1, glyoxalase 1; HDAC, histone deacetylase; HUVECs, human umbilical vein endothelial cells; IRS-1, insulin receptor substrate 1; KRAS, kirsten rat sarcoma viral oncogene homolog; MAECs, mouse aortic endothelial cells; MAPK, mitogen-activated protein kinase; MEK, MAPK ERK kinase; MGO, methylglyoxal; miRNAs, microRNAs; NO, nitric oxide; PHLPP, leucine-rich repeat protein phosphatase; PI3K, phosphatidylinositol 3-kinase; TSA, trichostatin A.

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function including angiogenesis [16–19], contributes to inflammatory-induced cardiomyopathy [20] and induces hypertension [21] and arteriolar atherogenicity by low density lipoprotein (LDL) glycation [22].

Impairment of endothelium-derived nitric oxide (NO) represents a common feature of endothelial dysfunction in diabetic individuals [23–25]. We have recently shown that MGO inhibits the insulin-mediated activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/endothelial NO synthase (eNOS) pathway, increases the phosphorylation of Extracellular Signal-Regulated Kinase 1/2 (ERK 1/2), and reduces NO release in mouse aortic endothelial cells (MAECs) [19]. However, the underlying molecular mechanisms remain to be further investigated.

microRNAs (miRNAs) represent an evolutionarily well-conserved class of small, non-coding RNAs that negatively regulate gene expression at the post-transcriptional level in a sequence-specific manner [26]. Several studies have highlighted the role of miRNAs in maintaining the endothelial function and the impact of their alteration in endothelial dysfunction [27,28]. Increasing evidence has demonstrated that miRNA expression is affected by high glucose and MGO in vascular tissue and this associates with the alteration of vascular reactivity [29–32]. In the present study, we sought to investigate whether miRNAs may be involved in endothelial insulin resistance induced by MGO in endothelial cells.

2. Materials and methods

2.1. Reagents

Media, sera and antibiotics for cell culture were from Lonza (Walkersville, MD, USA). MGO (40% in water), aminoguanidine bicarbonate salt (AG) and trichostatin A (TSA) were from Sigma-Aldrich (St Louis, MO, USA). D-(+)-Glucose and *D*-(+)-Xylose were also from Sigma-Aldrich (St Louis, MO, USA). U0126 was from ENZO Lifescience (Florence, Italy). Insulin was from Eli Lilly (Florence, Italy). The antibodies used were anti-p-thr308-Akt, anti-p-tyr-IRS1 (Millipore, Billerica, MA, USA), anti-p-ser616-IRS1 (Life Technologies, Carlsbad, CA, USA), anti-p-tyr-IR, anti-Akt, anti-p-ser473-Akt, anti-eNOS, anti-p-ser1177-eNOS, anti-p-thr497-eNOS (Cell Signaling Technology, Beverly, MA, USA), anti-ERK1/2, anti-p-ERK1/2, anti-14-3-3 and anti-β-actin (Santa Cruz, CA, USA), anti-KRAS (Abcam, Cambridge, UK) and anti-α-tubulin (Sigma-Aldrich, St Louis, MO, USA). All other chemicals were from Sigma-Aldrich (St Louis, MO, USA). Protein electrophoresis and western blot reagents were from Bio-Rad (Richmond, VA, USA) and electrochemiluminescence reagents from Pierce (Rockford, IL, USA).

2.2. Cell culture

Mouse aortic endothelial cells (MAECs) were kindly provided by T.H Fleming (University of Heidelberg, Heidelberg, Germany). MAECs were plated in T75 flasks and grown in Dulbecco's Modified Medium (DMEM) containing 1 g/l glucose supplemented with 10% (v/v) FBS, 2 mmol/l L-glutamine and 0.1 mmol/l non-essential amino acids. Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. Cells were starved in serum-free medium containing 0.25% (w/v) albumin bovine serum (BSA) for 16 h, treated with MGO 500 μmol/l and then exposed or not to 100 nmol/l insulin for 10 min. Treatments with different concentrations of MGO for 16 h were also performed in MAECs (Fig. S2a). AG 4 mM was used in presence of MGO 500 μmol/l for 16 h. Where indicated, cells were pre-treated for 30 min with U0126 15 μmol/l and then treated with MGO 500 μmol/l for 16 h. In addition, cells were pre-treated for 30 min with TSA 330 nmol/l and then treated with MGO 500 μmol/l for 16 h, where indicated. Cells were exposed to 5 mM glucose (normal glucose control), 25 mM glucose (high glucose condition) or 5 mM glucose plus 20 mM Xylose (osmotic control) for 24, 48 and 72 h.

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in EGM-2 Bullet Kit from Lonza (Walkersville, MD, USA). Cells were starved in serum-free medium containing 0.25% (w/v) albumin bovine serum (BSA) for 16 h and treated with MGO 500 μmol/l.

2.3. Cell transfection

miR-190a levels were modulated by transfecting MAECs with miR-190a-mimic (mmu-miR-190a-5p miRIDIAN Mimic) 0.5 nM or with miR-190a-inhibitor (mmu-miR-190a-5p miRIDIAN Harpin Inhibitor) 5 nM using DharmaFECT 4 (Dharmacon, part of GE Healthcare, Lafayette, CO). miRIDIAN microRNA Mimic negative control #1 and miRIDIAN microRNA Harpin negative control #1 (Dharmacon) were used as negative controls (scrambles) of miR-190a-mimic and miR-190a-inhibitor transfection, respectively. After 48 h, the cells were directly harvested in the case of miR-190a-inhibitor or harvested after MGO treatment, and analyzed as described below. KRAS protein levels were reduced in MAECs transfected with a specific siRNA 50 nM or a siRNA with a scrambled sequence used as a negative control, both of which purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). TransIT-TKO from Mirus Bio LLC (Madison, WI, USA) was used as transfection reagent, according to the manufacturer's instructions.

2.4. Mice

Glo1-knockdown (Glo1-KD) mice were generated and characterized as previously described [34,6,15]. Mice were housed in a temperature-controlled (22 °C) room with a 12 h light/dark cycle, in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-23, revised 1996), and experiments were approved by the ethics committee of the MIUR. Reduced Glo1 mRNA levels were confirmed by quantitative PCR. For vascular insulin signaling, a bolus of insulin (0.15 U/g body weight) was i.p. administrated to mice 10 min before they were killed by cervical dislocation. Aortic tissues were then collected and processed as follow.

2.5. Western blot analysis

Cells and mouse aorta samples after homogenization were solubilised in lysis buffer (50 mmol/l HEPES pH 7.5, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $Na_2P_2O_7$, 2 mmol/l Na_3VO_4, 100 mmol/l NaF, 10% glycerol, 1% Triton X-100) for 2 h at 4 °C. Protein lysates were clarified by centrifugation at 16,000*g* for 20 min. Upon incubation with primary and secondary antibodies (for a full list of the antibodies, see above), immunoreactive bands were detected by electrochemiluminescence according to the manufacturer's instructions and densitometric analysis was performed using ImageJ software.

2.6. Measurement of NO

NO was measured in cell culture medium. After treatments, culture medium was collected, centrifuged at 1000*g* for 15 min and the supernatant fraction was used as a sample solution for the detection, using Nitrate/Nitrite Assay Kit Colorimetric (Sigma-aldrich). This assay uses the Griess reaction, resulting in the formation of a chromophoric azo-derivative, which absorbs light at 540–570 nm.

2.7. miRNA reverse transcription, miScript PCR Array and Real Time-PCR

Total RNA was isolated from MAECs, HUVECs and mouse aorta samples after homogenization in Qiazol using miRNeasy mini kit (QIAGEN, Hilden, Germany), according to manufacturer's instructions. After quantification with NanoDrop 2000 spectrophotometer (Thermo Scientific,

Waltham, MA), total RNA was reverse transcribed using the miScript II RT Kit (QIAGEN) and analyzed by the Diabetes miRNA PCR Array (QIAGEN; *http://sabiosciences.com/mirna_pcr_product/HTML/MIMM-115Z*), according to the manufacturer's instructions. The PCR array was provided in ready-to-use 96 well-plate, which contains 84 miRNA-specific primers and different snoRNA/snRNA specific primers, used as normalization controls. One plate was used for each experimental condition. Resulting data were analyzed by the free data analysis software, available at *http://pcrdataanalysis.sabiosciences.com/mirna*. The differential expression of miRNAs was then validated by Real Time-PCR using the miScript SYBR Green PCR Kit (QIAGEN) and specific miScript Primer Assays. The levels of all differential expressed miRNAs were quantified as expression units relative to U6 snRNA, used as housekeeping small RNA. Specific primers used for amplification were purchased from QIAGEN:

Mm_miR-190_3 miScript Primer Assay, MS00032438
Mm_miR-214_2 miScript Primer Assay, MS00032571
Mm_miR-34a_1 miScript Primer Assay, MS00001428
Mm_miR-126-5p_1 miScript Primer Assay, MS00006006
Mm_miR-129-3p_1 miScript Primer Assay, MS00006013
Mm_miR-199a-5p_1 miScript Primer Assay, MS00032529
Mm_miR-214_2 miScript Primer Assay, MS00032571
Mm_miR-320_3 miScript Primer Assay, MS00011767
Mm_miR-335_1 miScript Primer Assay, MS00002142
Mm_miR-375_2 miScript Primer Assay, MS00032774
Mm_miR-381_2 miScript Primer Assay, MS00032802
Mm_miR-450a_1 miScript Primer Assay, MS00025886
Mm_miR-451_1 miScript Primer Assay, MS00002408
RNU6B_13 miScript Primer Assay, MS00014000

2.8. Reverse transcription and Real Time-PCR

Reverse transcription of 1 μg of total RNA was performed using SuperScript III (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Quantitative real-time PCR was performed in triplicate by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Relative quantification of gene expression was calculated by the ΔΔCt method (28). Each Ct value was first normalized to the respective *Cyclophilin* Ct value of a sample to account for variability in the concentration of RNA and in the conversion efficiency of the RT reaction. Data were presented relative to the untreated control condition and reported as arbitrary units (REU). Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers specific, which were then purchased from Sigma-Aldrich (St Louis, MO, USA).

Primers used for real-time RealTime-PCR are as follow:

Cyclophilin	Forward 5′-GCAGACAAAGTTCCAAAGACAG-3′ Reverse 5′-CACCCTGGCACATGAATCC-3′
Kras	Forward 5′-CTCTAAGGGAGTGGGCTCCT-3′ Reverse 5′-GATCATTTGTGGATGCCGCC-3′
Tgfβ	Forward 5′-ATACGCCTGAGTGGCTGTCT-3′ Reverse 5′-TGGGCCTGATCCCCTTGATT-3′

2.9. Statistic procedures

Data are expressed as means ± SEM and means ± SD, as indicated in figure legends. Comparison between groups were performed using Student's *t*-test or the one-way analysis of variance followed by Tukey multiple comparison tests, as appropriate, using GraphPad Prism 6.01 software (GraphPad, San Diego, CA, USA): a p-value ≤ 0.05 was considered significant.

3. Results

3.1. MGO exposure affects the expression of miR-190a in endothelial cells

The expression profile of miRNAs experimentally identified and/or bioinformatically predicted to be involved in diabetes-related biological processes (see Materials and methods) has been evaluated by a miRNA PCR Array in endothelial cells (MAECs) treated with 500 μmol/l MGO for 16 h [19]. Twelve out of 84 tested miRNAs resulted to be over or under the arbitrary chosen cut-off (±log 0.5 fold changes vs untreated cells). Among these, 5 miRNAs are up-regulated and 7 are down-regulated in MGO treated MAECs compared to control untreated cells (Fig. 1).

The expression of these 12 miRNAs was then tested by Real Time-PCR and the differential expression of miR-126, miR-190a, miR-214 and miR-450a was confirmed. We then focused our attention on miR-190a as its expression resulted to be the most significantly reduced in MGO-treated MAECs compared to control cells (Fig. 1).

Furthermore, the downregulation of miR-190a is also confirmed in human umbilical vein endothelial cells (HUVECs) exposed to 500 μmol/l MGO (Fig. S1). Conversely, high glucose (up to 72 h) does not lead to a significant reduction of miR-190a (Fig. S2c).

As expected, the MGO scavenger AG is able to prevent the decrease of miR-190a levels in MAECs treated with MGO (Fig. S2b), indicating that the formation of MGO adducts is detrimental for miR-190a expression.

3.2. The inhibition of miR-190a impairs insulin sensitivity in endothelial cells

To establish whether miR-190a is involved in the MGO-induced endothelial insulin resistance, its expression was antagonized by transfecting the miR-190a inhibitor in MAECs (miR-190a inhibitor: 0.42 ± 0.16 vs scramble: 1.0 REU, $p < 0.001$). Similarly to MGO-treated cells, the inhibition of miR-190a significantly decreases the insulin-induced tyrosine phosphorylation of IRS1 (Fig. 2a) and Akt phosphorylation, both on ser473 and thr308 (Fig. 2b), compared to control MAECs transfected with a scramble miRNA, without affecting the insulin-receptor (IR) phosphorylation (Fig. 2a). In addition, insulin-dependent eNOS activation is impaired by the inhibition of miR-190a, as shown by the reduction of phosphorylation on ser1177 and the loss of de-phosphorylation on thr497 in response to insulin, compared to scramble treated cells (Fig. 2c). Consistent with eNOS activation, insulin stimulation induces a ~1.4 fold increase of NO release in scramble-treated cells, as expected, while no increase of NO levels is revealed in the culture medium from MAECs transfected with miR-190a inhibitor (Fig. 2d). Furthermore, as in MGO-treated cells, the inhibition of miR-190a increases basal ERK 1/2 phosphorylation (Fig. 2e). These data indicate that, independently from MGO exposure, the reduction of miR-190a levels is sufficient to affect ERK 1/2 activation and insulin-dependent IRS1/Akt/eNOS pathway in MAECs.

3.3. Overexpression of miR-190a prevents MGO-induced inhibition of insulin sensitivity in endothelial cells

To investigate whether the reduced expression of miR-190a may be crucial in mediating MGO effect on endothelial cells, miR-190a levels were increased by transfecting cells with a miR-190a mimic. As expected, insulin-induced IRS1, but not IR, phosphorylation (Fig. 3a), ser473 and thr308-Akt phosphorylation (Fig. 3b), and the downstream ser1177-eNOS phosphorylation (Fig. 3c) are impaired in MAECs treated with MGO. The over-expression of miR-190a in MGO-treated cells prevents MGO deleterious effects, sustaining the insulin-mediated IRS1/Akt/eNOS pathway activation even in presence of MGO (Fig. 3a–c). Interestingly, the over-expression of miR-190a also rescues the insulin-induced NO release in MGO-treated MAECs (Fig. 3d). Moreover, the

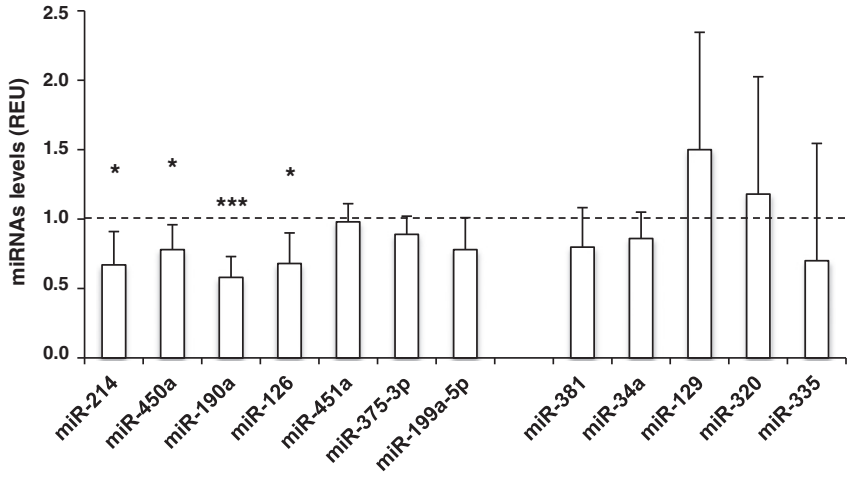

Fig. 1. MGO effect on miRNAs expression. The expression of miRNAs resulted to be differentially expressed in the PCR array was tested by Real Time-PCR in MAECs treated with 500 μmol/l MGO. The bars in the graph represent the mean ± SD of the expression units relative to U6 snRNA levels, used as housekeeping gene. Control levels of miRNAs in not treated MAECs are referred as 1 (dotted line). Statistical significance was evaluated using the Student's *t*-test; $^{*}p < 0.05$, $^{***}p < 0.001$.

MGO-induced ERK 1/2 hyper-activation is prevented by transfection of the miR-190a mimic (Fig. 3e). These data indicate that miR-190a is a key player in the MGO-dependent impairment of insulin sensitivity in MAECs.

3.4. miR-190a down-regulation plays a role in MGO-induced endothelial insulin resistance by increasing KRAS protein levels

MGO exposure affects insulin sensitivity in MAECs through the hyper-activation of ERK 1/2 [19]. Thus, to establish whether ERK 1/2 activation could be up- or down-stream the MGO-induced miR-190a down-regulation, it was chemically inhibited. Pre-treatment with the MAPK ERK Kinase (MEK) inhibitor U0126 prevents ERK 1/2 phosphorylation (data not shown) without affecting miR-190a expression (Fig. 4a). Interestingly, the total protein levels of the GTPase Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS), one of the validated targets of miR-190a [33], are increased by both MGO exposure and miR-190a inhibitor transfection (Fig. 4b), and reduced when miR-190a expression is increased by miR-190a mimic transfection, even in presence of MGO (Fig. 4c). Furthermore, the role of KRAS on MGO effect on insulin signalling was evaluated in MAECs transfected with a specific siRNA for KRAS. KRAS protein levels are reduced by siKRAS compared to MGO-treated cells transfected with a scramble siRNA used as negative control (Fig. 4d). Silencing of KRAS prevents ERK 1/2 basal activation as well as IRS1 phosphorylation on ser616 (Fig. 4d), rescuing the insulin-dependent Akt and eNOS activation (Fig. 4e). Taken together, these data indicate that miR-190a down-regulation mediates MGO effect on endothelial insulin sensitivity, at least in part, by increasing the levels of its target KRAS.

3.5. miR-190a and insulin sensitivity are reduced in aortae of Glo1-KD mice

To validate the effect of MGO on miR-190a *in vivo*, aortae were isolated from non-diabetic Glo1-KD mice, described to have high levels of MGO-modified proteins (34; 15), and miR-190a levels were analyzed by Real Time-PCR. Vascular miR-190a expression is reduced in Glo1-KD mice compared to their WT littermates (Fig. 5a). In aortae of WT mice, insulin stimulation induces a 8- and 3-fold increase of ser473-Akt and ser1177-eNOS phosphorylation, respectively. By contrast, aortae from Glo1-KD mice show a 60% decrease of insulin-dependent Akt activation and a 50% decrease of eNOS activation, compared to WT mice (Fig. 5b). These data indicate that also in a mouse model that is prone to accumulate MGO-modified proteins (15), miR-190a and insulin sensitivity are reduced in aortae.

3.6. Regulation of miR-190a expression by MGO

In order to address the mechanism by which MGO may reduce miR-190a levels, we started to look at the epigenetic regulation of miR-190a [45]. Interestingly, the reduced expression of miR-190a is paralleled by a consistent decrease of its host gene *talin*, suggesting that a possible MGO-dependent modification may occur at the level of *talin* promoter. While the inhibitor of methyltransferases 5-azacytidine does not modify the MGO-induced miR-190a downregulation (data not shown), treatment of MGO-exposed MAECs with the inhibitor of histone deacetyltransferases TSA is able to rescue the expression of both miR-190a and *talin* gene at levels similar to untreated cells (Fig. 6a and b), suggesting that miR-190a downregulation is potentially dependent by histone modifications in presence of high MGO concentration.

4. Discussion

MGO is a highly reactive dicarbonyl compound, which is formed as a by-product of glycolysis [1], and is a major cell-permeant precursor of AGEs, reacting non-enzymatically with amino acid residues (e.g., Lys and Arg) located in the active sites of proteins and thereby causing their loss of function [35–37]. In condition of hyperglycemia, the plasma levels of MGO are increased [5] and its abnormal accumulation contributes to the damage of various tissues in patients with diabetes [38,39], also disturbing the insulin signaling pathway [8–11]. There is increasing evidence that MGO may contribute to the pathogenesis of insulin resistance by means of direct functional modifications in the molecular components of the insulin signaling pathway. For instance, an impairment in the insulin-stimulated activation of IRS-1 has been observed in the adipose tissue of fructose-fed rats and in 3T3-L1 adipocytes [40], in L6 muscle cells [11] and in INS-1E β-cells treated with MGO [9]. Moreover, in our previous study, we proved that the exposure of both MAECs and C57bl6 mice to MGO impairs the insulin-dependent IRS-1/Akt/eNOS pathway and the consequent release of NO. In addition, we demonstrated that the MGO-dependent ERK 1/2 hyper-activation is responsible, at least in part, for the negative effect of MGO on the IRS-1/Akt/eNOS signaling pathway [19].

Emerging evidence suggests that MGO alters gene expression by modifying transcription factors [41–43]. An interesting new concept concerns the possibility that MGO may also affect gene expression through changes in DNA methylation [44], histone modifications [45, 46] and miRNA expression [47,32]. Concerning the regulation of miRNA levels, miR-30b was significantly up-regulated in peritoneal

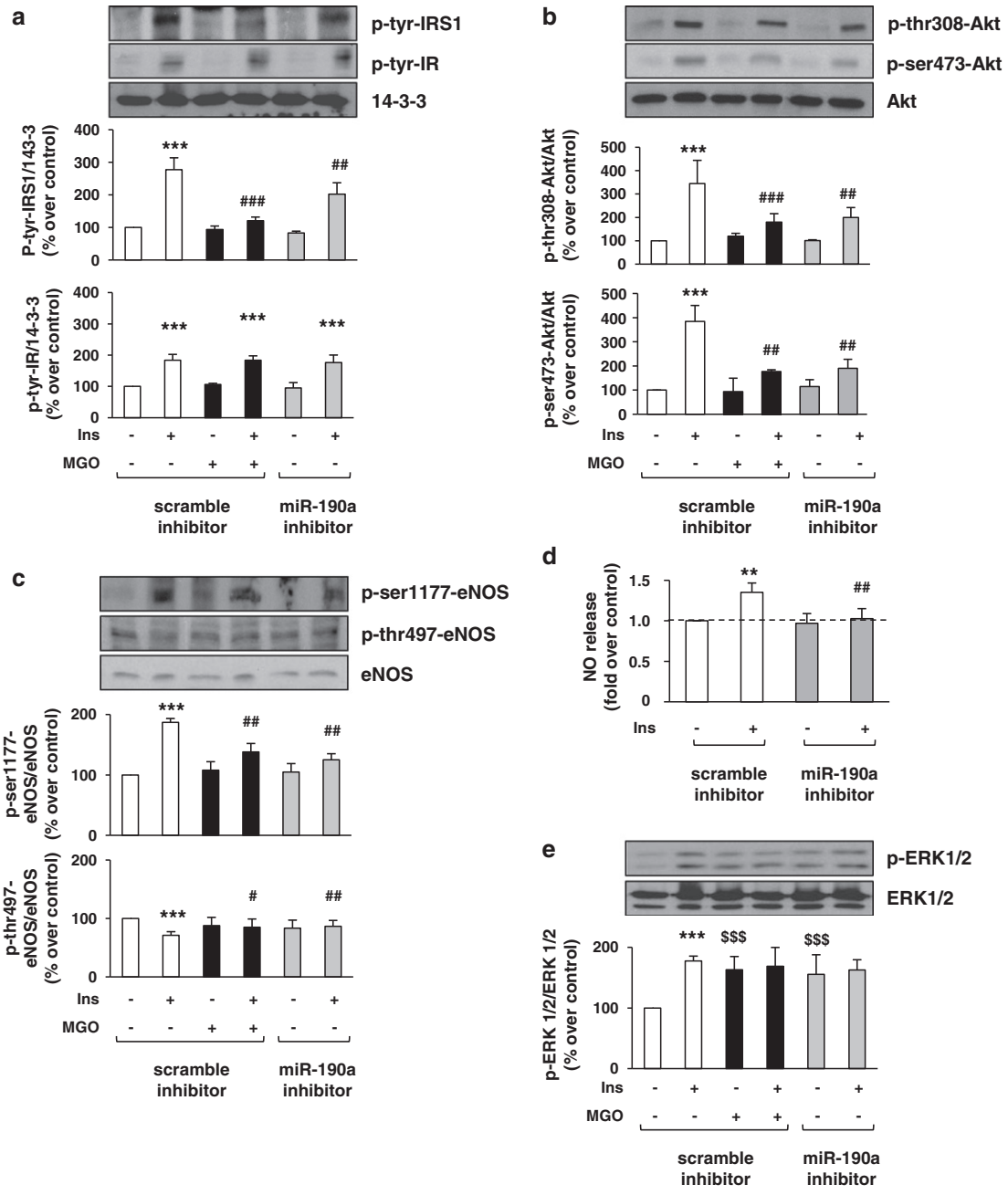

Fig. 2. Effect of miR-190a inhibitor on insulin sensitivity in MAECs. MAECs were transfected with a negative control (scramble; white and black bars) or with the inhibitor of miR-190a (gray bars) and incubated with 500 μmol/l MGO (black bars) where indicated. They were then stimulated or not with 100 nmol/l insulin (Ins) for 10 min. Protein lysates obtained from these cells were analyzed by Western Blot with anti-p-tyr-IR, anti-p-tyr-IRS1, anti 14-3-3 (a), anti-p-ser473-Akt, anti-p-thr308-Akt, anti-Akt (b), anti-p-ser1177-eNOS, anti-p-thr497-eNOS, anti-eNOS (c), anti-p-ERK1/2 and anti-ERK1/2 antibodies (e). Protein levels were quantified by the densitometric analysis of at least 3 independent experiments. The bars in the graph represent the means ± SEM of the percent (%) over control. (d) The culture medium was collected and tested for NO concentration by a colorimetric kit assay. The bars in the graph represent the increase of NO medium content after insulin stimulation over the control. The basal NO medium concentration is referred as 1.0 (dotted line). Values are expressed as means ± SD of triplicate determinations. Statistical analysis was evaluated using the Student's *t*-test; $^{**}p < 0.01$, $^{***}p < 0.001$ (+insulin vs −insulin); $^{\#\#}p < 0.01$ (MGO scramble +insulin and miR-190a inhibitor +insulin vs scramble untreated +insulin); $^{\$\$\$}p < 0.001$ (MGO scramble −insulin and miR-190a inhibitor −insulin vs scramble untreated −insulin).

mesothelial cells from MGO-injected rats [47]. In the A10 VSM line following MGO exposure, a significant increase in the miR-9a-3p levels was observed [32].

In line with these recent findings, we have examined here the involvement of miRNAs as potential culprits behind the action of MGO on the endothelium in order to explain the molecular mechanisms responsible for the MGO-dependent impairment of the endothelial insulin sensitivity previously demonstrated [19]. Therefore, we have evaluated the expression profile of 84 diabetes-associated miRNAs through a diabetic-specific array platform and we have provided the first evidence that MGO down-regulates the expression of miR-190a in MAECs, as well as in HUVECs, and in aortae from Glo1-KD mice. Furthermore, the aortae of these mice showed an impaired insulin sensitivity compared to their WT littermates. We performed the in vivo study in Glo1-KD mice because they represent a suitable animal model to study MGO-dependent effects, as in these mice the reduced levels of Glo1 are responsible for the accumulation of MGO-modified proteins and the development of diabetic complications in the absence of hyperglycaemia

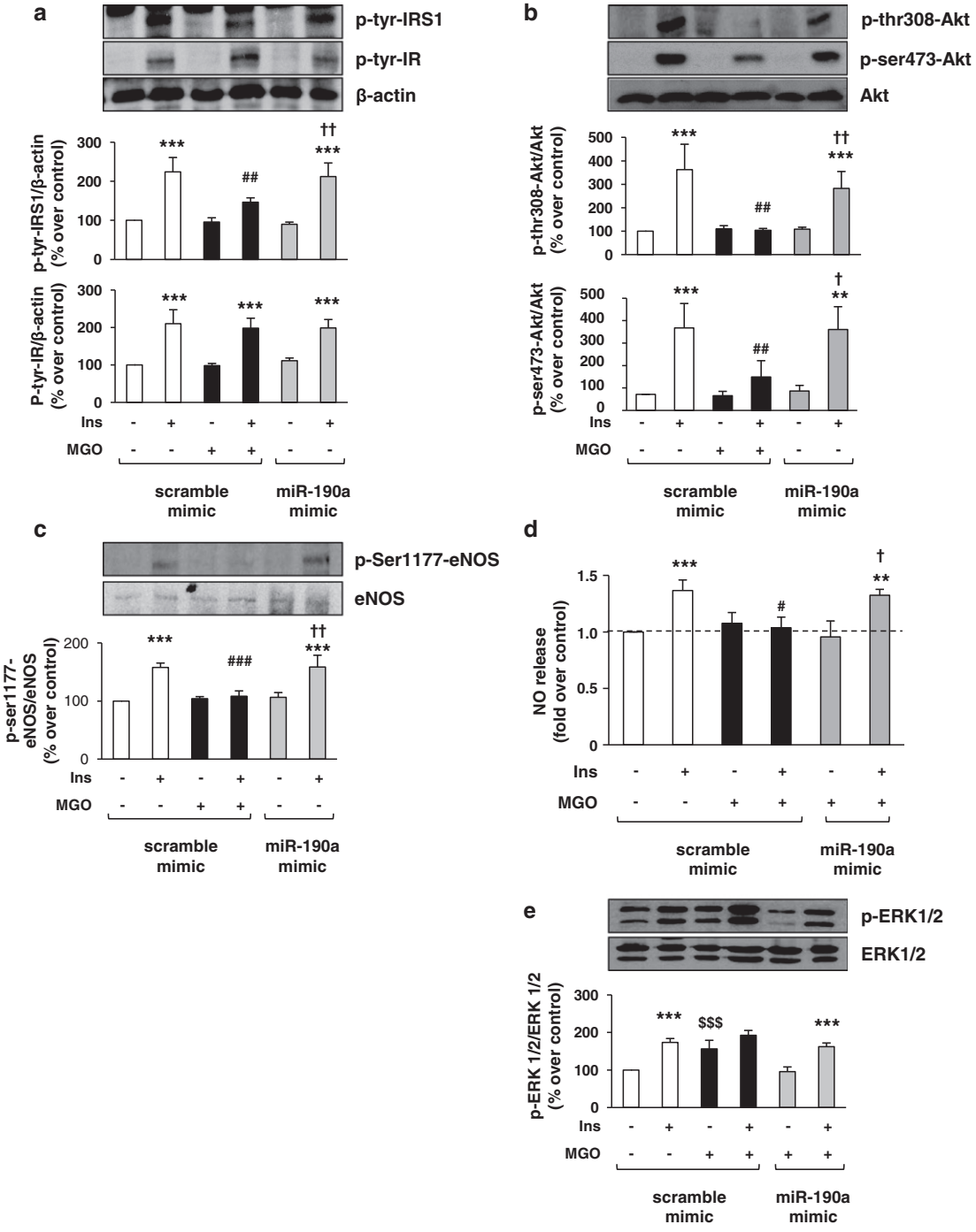

Fig. 3. Effect of miR-190a mimic on insulin sensitivity in MAECs. MAECs were transfected with a negative control (scramble; white and black bars) or with the mimic of miR-190a (gray bars), and incubated with 500 μmol/l MGO, where indicated. They were then stimulated or not with 100 nmol/l insulin for 10 min. (a, b, c and e) Protein lysates obtained from these cells were analyzed by Western Blot as described in figure legend 2. The bars in the graph represent the means ± SEM of the percent (%) over control. (d) NO production was evaluated as reported in figure legend 2. The bars in the graph represent the increase of NO medium content after insulin stimulation over the control. The basal NO medium concentration is referred as 1.0 (dotted line). Values are expressed as means ± SD of triplicate determinations. Statistical analysis was evaluated using the Student's *t*-test; $^{**}p < 0.01$, $^{***}p < 0.001$ (+insulin vs −insulin); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (MGO scramble +insulin vs scramble untreated +insulin); $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ (miR-190a mimic +insulin vs MGO scramble +insulin); $^{\$\$\$}p < 0.001$ (MGO scramble −insulin vs scramble untreated −insulin).

[6,15,34]. The rescue of miR-190a levels observed in MAECs upon the co-treatment with MGO and AG provided evidence that MGO-modified proteins are critical in the down-regulation of miR-190a due to MGO. We also observed that the exposure to chronic high glucose caused a mild negative effect on the expression of miR-190a (Fig. S2c). According to literature, miR-190a is differentially expressed in 3 tissues related to glucose homeostasis, namely liver, adipose tissue and islets of diabetic mice [48]. In lung adenocarcinoma A549 cells, miR-190a inhibits the TGFβ (transforming growth factor-beta) signaling pathway [49]. In addition, by repressing the expression of the PH domain leucine-rich repeat protein phosphatase (PHLPP), a PH domain-containing serine phosphatase which specifically dephosphorylates and inactivates Akt [50], increased levels of miR-190 are able to enhance the activation of Akt in BEAS-2B cells [51].

In our cell model, we have observed that the over-expression of miR-190a prevented the MGO-dependent inhibition of both the insulin-

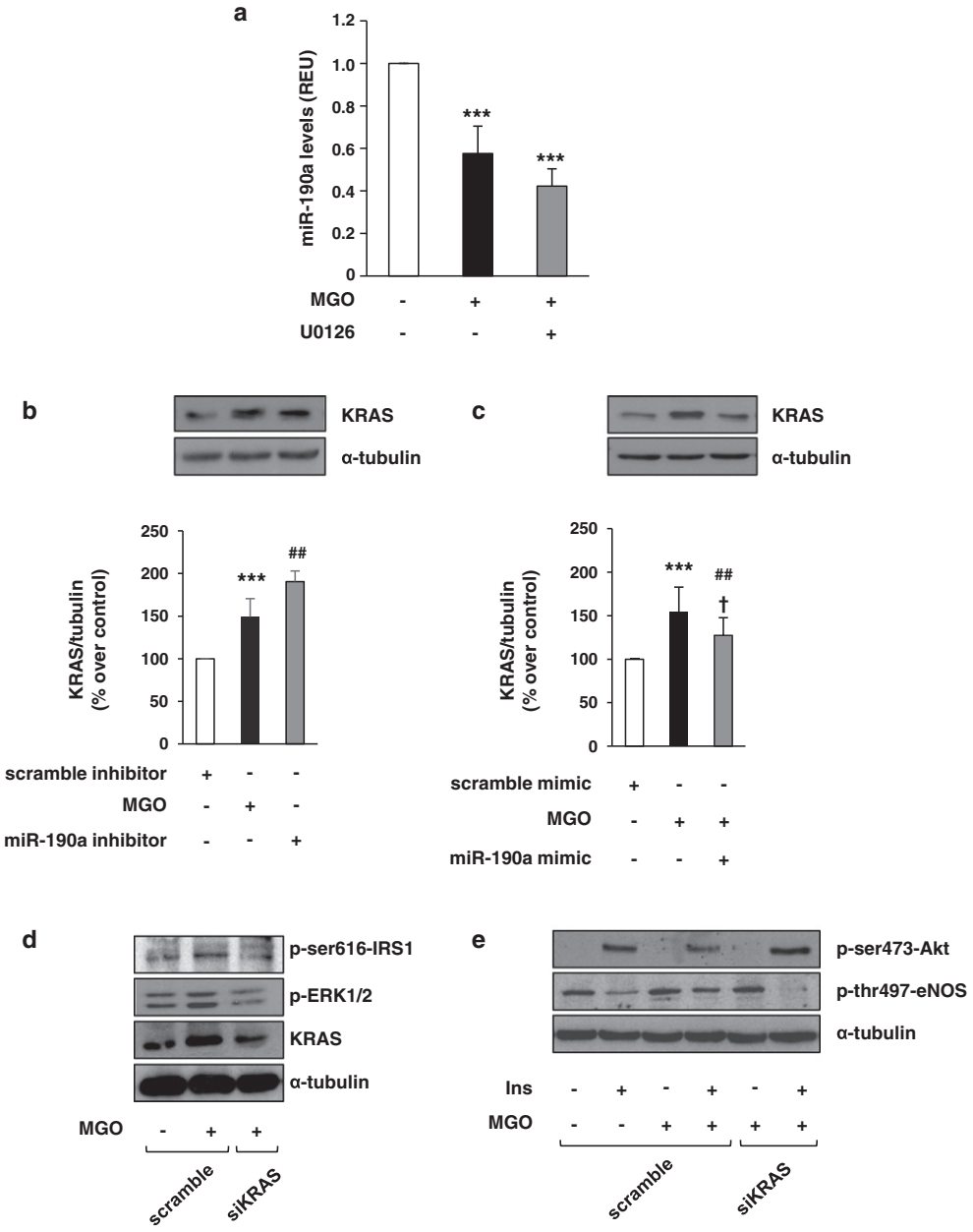

Fig. 4. The role of KRAS in MGO-mediated effect. (a) MAECs were pre-treated with the MEK inhibitor U0126 15 μmol/l and then treated with MGO 500 μmol/l. Total RNA was extracted from these cells and analyzed by Real Time-PCR. The bars in panel a represent the means ± SD of miR-190a expression units relative to U6 snRNA levels. (b, c) MAECs were transfected with a negative control (scramble inhibitor or scramble mimic; white bars), or treated with 500 μmol/l MGO (black bars) and transfected with the inhibitor (b) and the mimic (c) of miR-190a (gray bars), were indicated. Protein lysates obtained from these cells were analyzed by Western Blot with anti-KRAS and anti-α-tubulin antibodies. Protein levels were quantified by the densitometric analysis of at least 3 independent experiments. The bars in the graph represent the means ± SEM of the percent (%) over control. (d, e) MAECs were transfected with a negative control, treated or not with MGO 500 μmol/l, or transfected with an interfering RNA specific for KRAS, as indicated. Protein lysates obtained from these cells were analyzed by Western Blot with anti-p-ser616-IRS1, anti-p-ERK1/2, anti-KRAS (d), anti-p-ser473-Akt, anti-p-thr497-eNOS antibodies (e). Anti-α-tubulin antibody was used as loading control. Statistical analysis was evaluated using the Student's *t*-test; $^{***}p < 0.001$ (MGO vs control untreated or scramble); $^{\#\#}p < 0.01$ (miR-190a inhibitor and mimic vs scramble); $^{\dagger}p < 0.05$ (MGO + miR190a mimic vs MGO).

stimulated IRS-1/AKT/eNOS pathway and the NO release. The effect of MGO on Akt seemed to be PHLPP-independent, as MGO showed no effect on the PHLPP protein levels in MAECs (data not shown). Furthermore, no effect on TGFβ was observed in the MGO-treated MAECs (Fig. S3).Interestingly, the inhibition of miR-190a was sufficient to cause insulin resistance and impair the insulin-induced NO secretion, thus mimicking the MGO-dependent effect on the insulin sensitivity in the endothelial cells.

With the aim of establishing how MGO mediates the miR-190a down-regulation in MAECs, we have investigated the ERK 1/2 pathway, since: i. a decreased transcription of miR-190a has recently been described to be dependent on ERK 1/2 nuclear activity in rat primary hippocampal neuron cultures and in mouse hippocampi [52,53]; and *ii.* the hyper-activation of ERK 1/2 mediates the MGO-dependent insulin resistance in MAECs, as we have previously demonstrated [19]. In the presence of U0126, a highly selective inhibitor of MEK kinases, MGO still affected the miR-190a expression in MAECs, thus indicating that MGO modulates the miR-190a expression independently of ERK 1/2 in the endothelial cells. Conversely, when we induced alterations in the miR-190a levels, an inverse correlation between the miR-190a and ERK 1/2 phosphorylation levels was observed, suggesting that the MGO-dependent hyper-activation of ERK 1/2 might be mediated by miR-190a.

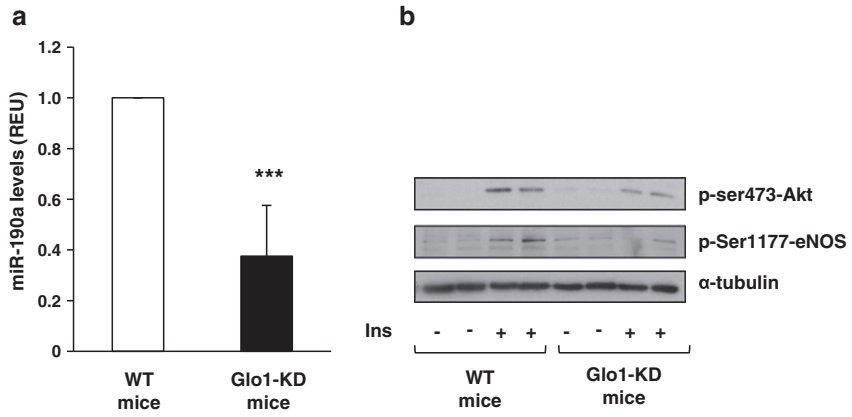

Fig. 5. Effect of MGO on miR-190a and vascular insulin sensitivity *in vivo*. (a) miR-190a expression was measured by Real Time-PCR in aortae from Glo1-KD ($n = 4$) and WT mice ($n = 4$). The bars in the graphs represent the mean ± SD of the expression units relative to U6 snRNA levels, used as housekeeping gene. Statistical significance was evaluated using the Student's *t*-test; $^{***}p < 0.001$. (b) Proteins were isolated from aortae of Glo1-KD and WT mice, receiving or not an i.p. bolus of insulin, and then analyzed by Western Blot with anti- p-ser473-Akt, anti-p-ser1177-eNOS and anti-α-tubulin. Blots are representative of at least four mice.

To better clarify how miR-190a mediates the hyper-activation of ERK 1/2, we initially tested whether alterations in the miR-190a levels were able to modulate the expression of ERK 1/2. However, we did not observe any difference in the protein levels of ERK 1/2 in MAECs when alterations in the miR-190a levels were induced. Subsequently, in accordance with the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis [54], we identified the MAPK signaling pathway as one of the most enriched molecular pathways in the miR-190a target set, achieved through the combined results of 4 well-known tools for miRNA target prediction (miRanda [55], Targetscan [56], RNA22 [57] and miRWalk [58]). Among the 42 potential targets of miR-190a related to the MAPK signaling pathway, we found KRAS, a protein with an intrinsic GTPase activity which engages the canonical RAF (Rapidly Accelerated Fibrosarcoma)/MEK/ERK as one of its downstream effectors [59]. For this reason, we tested the protein levels of KRAS and we observed that alterations in the miR-190a levels were able to modulate this target in MAECs. Consistent with our data, Hao et al. have demonstrated by luciferase assays that KRAS is directly regulated by miR-190a in HCT166 cells via a highly conserved binding site [33]. In addition, the silencing of KRAS in our cell model prevented the MGO effect on both the ERK 1/2 phosphorylation and the insulin-stimulated IRS-1/PI3K/Akt/eNOS signaling pathway.

To address the mechanism by which MGO regulates miR-190a in MAECs, as the miR-190a expression is associated with its host gene talin [60], we investigated a possible involvement of the GC-rich region with clusters of CpG dinucleotides in talin gene. No reversion of the MGO effect on miR-190a levels was observed after the addition of 5-azacytidine, a pyrimidine nucleoside analogue of cytidine that inhibits DNA methyltransferase and impairs DNA methylation, thus excluding the hypothesis of changes in DNA methylation for the MGO-dependent down-regulation of miR-190a (data not shown). Conversely, TSA, which selectively inhibits the class I and II mammalian histone deacetylase (HDAC), was able to abolish the MGO effect on the miR-190a expression, suggesting that HDACs may be involved. In agreement with our results, others have shown the increased interaction between O-Linked N-Acetylglucosamine (GlcNAc) Transferase (OGT) and transcription factors modified by MGO, such as mSin3A [61], and the consequent increased recruitment of HDACs in the complex of OGT and the modified transcription factor to the promoter [62]. However, the question of how MGO mediates the miR-190a down-regulation in MAECs still remains unanswered and further experiments are necessary to solve this issue.

5. Conclusion

This study shows for the first time that MGO modulates miR-190a levels and the miR-190a down-regulation contributes to the MGO-dependent endothelial insulin resistance. Thus, in light of the

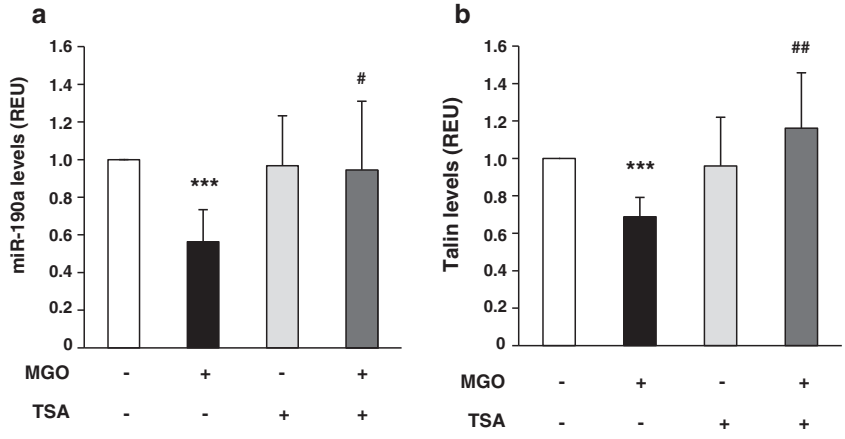

Fig. 6. Effect of TSA on miR-190a and *talin* expression. mRNA was isolated from MAECs pretreated or not with 330 nmol/l TSA and then exposed to MGO where indicated. The expression of miR-190a (a) and *talin* (b) were analyzed by Real Time-PCR. Bars in the graphs represent the means ± SD of miR-190a (a) or *talin* (b) expression units relative to U6 snRNA or 18S levels, respectively. Statistical significance was evaluated using the Student's *t*-test; $^{***}p < 0.001$ (MGO vs control untreated), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (TSA + MGO vs MGO).

observations that: *i.* a range of drugs, including the AGE inhibitor aminoguanidine, have failed in clinical trials as a result of side effects or limited efficacy [63–66], and *ii.* alternative strategies to modulate the AGE pathway, including approaches to reduce the accumulation of MGO and enhance the Glo1 activity, are currently under investigation [67,68], this study suggests miR-190a as a new player in the MGO-dependent negative effect on the endothelial cell function, encouraging scientists to address further efforts to prove that miR-190a may represent a novel therapeutic target to ameliorate the endothelial insulin sensitivity in diabetes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2016.11.018.

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Author contributions

PM and CN conceived and designed the experiments, acquired data, analysed data and wrote the manuscript. IP, TP and AL acquired data and revised the manuscript. FA provided reagents/materials and revised the manuscript. GAR, ML and FF interpreted data and reviewed the manuscript. FB reviewed/edited the manuscript. CM designed the research and supervised the project, reviewed/edited the manuscript and contributed to the discussion. CM is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version of the manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

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References

[1] N. Rabbani, P.J. Thornalley, The critical role of methylglyoxal and glyoxalase 1 in diabetic nephropathy, Diabetes 63 (2014) 50–52, http://dx.doi.org/10.2337/db13-1606.
[2] M.P. Kalapos, Methylglyoxal in living organisms: chemistry, biochemistry, toxicology and biological implications, Toxicol. Lett. 110 (1999) 145–175, http://dx.doi.org/10.1016/S0378-4274(99)00160-5.
[3] N. Rabbani, P.J. Thornalley, Dicarbonyls, glyoxal, methylglyoxal, and 3-deoxyglucosone, Uremic Toxins, John Wiley & Sons, New Jersey 2012, pp. 177–192, http://dx.doi.org/10.1002/9781118424032.ch12.
[4] N. Rabbani, P.J. Thornalley, Methylglyoxal, glyoxalase 1 and the dicarbonyl proteome, Amino Acids 42 (2012) 1133–1142, http://dx.doi.org/10.1007/s00726-010-0783-0.
[5] P.J. Beisswenger, et al., α-Dicarbonyls increase in the postprandial period and reflect the degree of hyperglycemia, Diabetes Care 24 (2001) 726–732, http://dx.doi.org/10.2337/diacare.24.4.726.
[6] A. Bierhaus, T. Fleming, S. Stoyanov, et al., Methylglyoxal modification of Nav1.8 facilitates nociceptive neuron firing and causes hyperalgesia indiabetic neuropathy, Nat. Med. 18 (2012) 926–933, http://dx.doi.org/10.1038/nm.2750.
[7] D. Yao, M. Brownlee, Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation endproducts (RAGE) and RAGE ligands, Diabetes 59 (2009) 249–255, http://dx.doi.org/10.2337/db09-0801.
[8] Q. Guo, et al., Methylglyoxal contributes to the development of insulin resistance and salt sensitivity in Sprague–Dawley rats, J. Hypertens. 27 (2009) 1664–1671, http://dx.doi.org/10.1097/HJH.0b013e32832c419a.
[9] F. Fiory, et al., Methylglyoxal impairs insulin signalling and insulin action on glucose-induced insulin secretion in the pancreatic beta cell line INS-1E, Diabetologia 54 (11) (2011) 2941–2952, http://dx.doi.org/10.1007/s00125-011-2280-8.
[10] C.G. Schalkwijk, O. Brouwers, C.D. Stehouwer, Modulation of Insulin Action by Advanced Glycation Endproducts: a new Player in the Field, 2008 614–619, http://dx.doi.org/10.1055/s-0028-1082085.
[11] A. Riboulet-Chavey, et al., Methylglyoxal impairs the insulin signaling pathways independently of the formation of intracellular reactive oxygen species, Diabetes 55 (5) (2006) 1289–1299, http://dx.doi.org/10.2337/db05-0857.
[12] M. Bourrajjaj, et al., Role of methylglyoxal adducts in the development of vascular complications in diabetes mellitus, Biochem. Soc. Trans. 31 (2003) 1400–1402, http://dx.doi.org/10.1042/bst0311400.
[13] M. Brownlee, Biochemistry and molecular cell biology of diabetic complications, Nature 414 (2001) 813–820, http://dx.doi.org/10.1038/414813a.
[14] M. Brownlee, The pathobiology of diabetic complications — a unifying mechanism, Diabetes 54 (2005) 1615–1625, http://dx.doi.org/10.2337/diabetes.54.6.1615.
[15] F. Giacco, et al., Knockdown of glyoxalase 1 mimics diabetic nephropathy in nondiabetic mice, Diabetes 63 (1) (2014) 291–299, http://dx.doi.org/10.2337/db13-0316.
[16] D. Dobler, et al., Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification, Diabetes 55 (2006) 1961–1969, http://dx.doi.org/10.2337/db05-1634.
[17] C.G. Schalkwijk, et al., Heat-shock protein 27 is a major methylglyoxal-modified protein in endothelial cells, FEBS Lett. 580 (2006) 1565–1570, http://dx.doi.org/10.1016/j.febslet.2006.01.086.
[18] P.E. Morgan, et al., Perturbation of human coronary artery endothelial cell redox state and NADPH generation by methylglyoxal, PLoS One 9 (1) (2014), e86564 http://dx.doi.org/10.1371/journal.pone.0086564.
[19] C. Nigro, et al., Methylglyoxal impairs endothelial insulin sensitivity both in vitro and in vivo, Diabetologia 57 (2014) 1485–1494, http://dx.doi.org/10.1007/s00125-014-3243-7.
[20] B. Vulesevic, B. McNeill, F. Giacco, et al., Methylglyoxal-induced endothelial cell loss and inflammation contribute to the development of diabetic cardiomyopathy, Diabetes (2016) http://dx.doi.org/10.2337/db15-0568 (pii: db150568).
[21] X. Wang, et al., Attenuation of hypertension development by scavenging methylglyoxal in fructose-treated rats, J. Hypertens. 26 (4) (2008) 765–772, http://dx.doi.org/10.1097/HJH.0b013e3282f4a13c.
[22] N. Rabbani, et al., Glycation of LDL by methylglyoxal increases arterial atherogenicity: a possible contributor to increased risk of cardiovascular disease in diabetes, Diabetes 60 (2011) 1973–1980, http://dx.doi.org/10.2337/db11-0085.
[23] M. Montagnani, et al., Insulin receptor substrate-1 and phosphoinositide-dependent kinase-1 are required for insulin-stimulated production of nitric oxide in endothelial cells, Mol. Endocrinol. 16 (2002) 1931–1942, http://dx.doi.org/10.1210/me.2002-0074.
[24] H.O. Steinberg, et al., Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance, J. Clin. Invest. 97 (1996) 2601–2610, http://dx.doi.org/10.1172/JCI118709.
[25] S. Mäkimattila, et al., Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus, Circulation 94 (1996) 1276–1282, http://dx.doi.org/10.1161/01.CIR.94.6.1276.
[26] N. Tetreault, V. De Guire, miRNAs: their discovery, biogenesis and mechanism of action, Clin. Biochem. 46 (2013) 842–845, http://dx.doi.org/10.1016/j.clinbiochem.2013.02.009.
[27] J.E. Fish, A primer on the role of microRNAs in endothelial biology and vascular disease, Semin. Nephrol. 32 (2) (2012) 167–175, http://dx.doi.org/10.1016/j.semnephrol.2012.02.003.
[28] A. Magenta, et al., Oxidative stress and microRNAs in vascular diseases, Int. J. Mol. Sci. 14 (9) (2013) 7319–7346, http://dx.doi.org/10.3390/ijms140917319.
[29] Y. Li, et al., MicroRNA-221 regulates high glucose-induced endothelial dysfunction, Biochem. Biophys. Res. Commun. 381 (1) (2009) 81–83, http://dx.doi.org/10.1016/j.bbrc.2009.02.013.
[30] J. Long, et al., Identification of microRNA-93 as a novel regulator of vascular endothelial growth factor in hyperglycemic conditions, J. Biol. Chem. 285 (30) (2010) 23457–23465, http://dx.doi.org/10.1074/jbc.M110.136168.
[31] C. Piperi, et al., AGE/RAGE signalling regulation by miRNAs: associations with diabetic complications and therapeutic potential, Int. J. Biochem. Cell Biol. 60 (2015 Mar) 197–201, http://dx.doi.org/10.1016/j.biocel.2015.01.009.
[32] S.S. Li, et al., The SUR2B subunit of rat vascular KATP channel is targeted by miR-9a-3p induced by prolonged exposure to methylglyoxal, Am. J. Phys. Cell Physiol. 308 (2) (2015) C139–C145, http://dx.doi.org/10.1152/ajpcell.00311.2014.
[33] Y. Hao, et al., The synergistic regulation of VEGF-mediated angiogenesis through miR-190 and target genes, RNA 20 (8) (2014) 1328–1336, http://dx.doi.org/10.1261/rna.044651.114.
[34] M.A. Queisser, et al., Hyperglycemia impairs proteasome function by methylglyoxal, Diabetes 59 (3) (2010 Mar) 670–678, http://dx.doi.org/10.2337/db08-1565.
[35] P.J. Thornalley, Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems–role in ageing and disease, Drug Metabol. Drug Interact. 23 (1–2) (2008) 125–150.
[36] N. Ahmed, P.J. Thornalley, Advanced glycation endproducts: what is their relevance to diabetic complications? Diabetes Obes. Metab. 9 (3) (2007) 233–245, http://dx.doi.org/10.1111/j.1463-1326.2006.00595.x.
[37] F.W. Chaplen, Incidence and potential implications of the toxic metabolite methylglyoxal in cell culture, Rev. Cytotechnology 26 (3) (1998) 173–183, http://dx.doi.org/10.1023/A:1007953628840.

[38] P.J. Beisswenger, Methylglyoxal in diabetes: link to treatment, glycaemic control and biomarkers of complications, Biochem. Soc. Trans. 42 (2) (2014) 450–456, http://dx.doi.org/10.1042/BST20130275.
[39] M. Bourajjaj, C.D. Stehouwer, V.W. Van Hinsbergh, C.G. Schalkwijk, Role of methylglyoxal adducts in the development of vascular complications in diabetes mellitus, Biochem. Soc. Trans. 31 (Pt 6) (2003) 1400–1402, http://dx.doi.org/10.1042/bst0311400.
[40] X. Jia, L. Wu, Accumulation of endogenous methylglyoxal impaired insulin signaling in adipose tissue of fructose-fed rats, Mol. Cell. Biochem. 306 (2007) 133–139, http://dx.doi.org/10.1007/s11010-007-9563-x.
[41] D. Yao, T. Taguchi, T. Matsumura, R. Pestell, D. Edelstein, I. Giardino, G. Suske, N. Rabbani, P.J. Thornalley, V.P. Sarthy, H.P. Hammes, M. Brownlee, High glucose increases angiopoietin-2 transcription in microvascular endothelial cells through methylglyoxal modification of mSin3A, J. Biol. Chem. 282 (42) (2007) 31038–31045, http://dx.doi.org/10.1074/jbc.M704703200.
[42] H. Thangarajah, D. Yao, E.I. Chang, Y. Shi, L. Jazayeri, I.N. Vial, R.D. Galiano, X.L. Du, R. Grogan, M.G. Galvez, M. Januszyk, M. Brownlee, G.C. Gurtner, The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues, Proc. Natl. Acad. Sci. U. S. A. 106 (32) (2009) 13505–13510, http://dx.doi.org/10.1073/pnas.0906670106.
[43] M. Laga, A. Cottyn, F. Van Herreweghe, W. Vanden Berghe, G. Haegeman, P. Van Oostveldt, J. Vandekerckhove, K. Vancompernolle, Methylglyoxal suppresses TNF-alpha-induced NF-kappaB activation by inhibiting NF-kappaB DNA-binding, Biochem. Pharmacol. 4 (4) (2007) 579–589, http://dx.doi.org/10.1016/j.bcp.2007.05.026.
[44] P. Palsamy, K.R. Bidasee, M. Ayaki, R.C. Augusteyn, J.Y. Chan, T. Shinohara, Methylglyoxal induces endoplasmic reticulum stress and DNA demethylation in the Keap1 promoter of human lens epithelial cells and age-related cataracts, Free Radic. Biol. Med. 72 (2014) 134–148, http://dx.doi.org/10.1016/j.freeradbiomed.2014.04.010.
[45] A. El-Osta, D. Brasacchio, D. Yao, A. Pocai, P.L. Jones, R.G. Roeder, M.E. Cooper, M. Brownlee, Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia, J. Exp. Med. 205 (2008) 2409–2417, http://dx.doi.org/10.1084/jem.20081188.
[46] A.R. Mir, M. Uddin, K. Alam, A. Ali, Methylglyoxal mediated conformational changes in histone H2A-generation of carboxyethylated advanced glycation endproducts, Int. J. Biol. Macromol. 69 (2014) 260–266, http://dx.doi.org/10.1016/j.ijbiomac.2014.05.057.
[47] H. Liu, N. Zhang, D. Tian, MiR-30b is involved in methylglyoxal-induced epithelial-mesenchymal transition of peritoneal mesothelial cells in rats, Cell. Mol. Biol. Lett. 19 (2) (2014) 315–329, http://dx.doi.org/10.2478/s11658-014-0199-z.
[48] E. Zhao, M.P. Keller, M.E. Rabaglia, A.T. Oler, et al., Obesity and genetics regulate microRNAs in islets, liver, and adipose of diabetic mice, Mamm. Genome 20 (8) (2009) 476–485, http://dx.doi.org/10.1007/s00335-009-9217-2.
[49] V.A. Gennarino, G. D'Angelo, G. Dharmalingam, et al., Identification of microRNA-regulated gene networks by expression analysis of target genes, Genome Res. 22 (6) (2012) 1163–1172, http://dx.doi.org/10.1101/gr.130435.111.
[50] T. Gao, F. Furnari, A.C. Newton, PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth, Mol. Cell 18 (2005) 13–24, http://dx.doi.org/10.1016/j.molcel.2005.03.008.
[51] K. Beezhold, J. Liu, H. Kan, et al., miR-190-mediated downregulation of PHLPP contributes to arsenic-induced Akt activation and carcinogenesis, Toxicol. Sci. 123 (2) (2011) 411–420, http://dx.doi.org/10.1093/toxsci/kfr188.
[52] H. Zheng, Y. Zeng, X. Zhang, J. Chu, H.H. Loh, P.Y. Law, mu-Opioid receptor agonists differentially regulate the expression of miR-190 and NeuroD, Mol. Pharmacol. 77 (1) (2010) 102–109, http://dx.doi.org/10.1124/mol.109.060848.
[53] H. Zheng, J. Chu, Y. Zeng, H.H. Loh, P.Y. Law, Y. Yang, 1 Phosphorylation contributes to the differential effects of mu-opioid receptor agonists on microRNA-190 expression, J. Biol. Chem. 285 (29) (2010) 21994–22002, http://dx.doi.org/10.1074/jbc.M110.112607.
[54] E. Altermann, T.R. Klaenhammer, Pathwayvoyager: pathway mapping using the Kyoto encyclopedia of genes and genomes (KEGG) database, BMC Genomics 6 (2005) 60, http://dx.doi.org/10.1186/1471-2164-6-60.
[55] A.J. Enright, B. John, U. Gaul, T. Tuschl, C. Sander, D.S. Marks, MicroRNA targets in drosophila, Genome Biol. 5 (1) (2003) R1, http://dx.doi.org/10.1186/gb-2003-5-1-r1.
[56] B.P. Lewis, I.H. Shih, M.W. Jones-Rhoades, D.P. Bartel, C.B. Burge, Prediction of mammalian microRNA targets, Cell 115 (7) (2003) 787–798, http://dx.doi.org/10.1016/S0092-8674(03)01018-3.
[57] K.C. Miranda, T. Huynh, Y. Tay, Y.S. Ang, W.L. Tam, A.M. Thomson, B. Lim, I. Rigoutsos, A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes, Cell 126 (6) (2006) 1203–1217, http://dx.doi.org/10.1016/j.cell.2006.07.031.
[58] H. Dweep, C. Sticht, P. Pandey, N. Gretz, miRWalk–database: prediction of possible miRNA binding sites by "walking" the genes of three genomes, J. Biomed. Inform. 44 (5) (2011) 839–847, http://dx.doi.org/10.1016/j.jbi.2011.05.002.
[59] A.D. Cox, C.J. Der, Ras history: the saga continues, Small GTPases 1 (1) (2010) 2–27, http://dx.doi.org/10.4161/sgtp.1.1.12178.
[60] E. Debrand, et al., Talin 2 is a large and complex gene encoding multiple transcripts and protein isoforms, FEBS J. 276 (6) (2009 Mar) 1610–1628, http://dx.doi.org/10.1111/j.1742-4658.2009.06893.
[61] D1. Yao, et al., High glucose increases angiopoietin-2 transcription in microvascular endothelial cells through methylglyoxal modification of mSin3A, J. Biol. Chem. 282 (42) (2007 Oct 19) 31038–31045, http://dx.doi.org/10.1074/jbc.M704703200.
[62] S.Y. Hwang, et al., O-GlcNAc transferase inhibits LPS-mediated expression of inducible nitric oxide synthase through an increased interaction with mSin3A in RAW264.7 cells, Am. J. Phys. Cell Physiol. 305 (6) (2013 Sep 15) C601–C608, http://dx.doi.org/10.1152/ajpcell.00042.2013.
[63] P.J. Thornalley, Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts, Arch. Biochem. Biophys. 419 (1) (2003) 31–40, http://dx.doi.org/10.1016/j.abb.2003.08.013.
[64] B.I. Freedman, J.P. Wuerth, K. Cartwright, et al., Design and baseline characteristics for the amino guanidine clinical trial in overt type 2 diabetic nephropathy (ACTION II), Control. Clin. Trials 20 (1999) 493–510, http://dx.doi.org/10.1016/S0197-2456(99)00024-0.
[65] R.G. Khalifah, Y. Chen, J.J. Wassenberg, Post-Amadori AGE inhibition as a therapeutic target for diabetic complications: a rational approach to second-generation Amadorin design, Ann. N. Y. Acad. Sci. 1043 (2005) 793–806, http://dx.doi.org/10.1196/annals.1333.092.
[66] L. Engelen, C.D. Stehouwer, C.G. Schalkwijk, Current therapeutic interventions in the glycation pathway: evidence from clinical studies, Diabetes Obes. Metab. 15 (8) (2013) 677–689, http://dx.doi.org/10.1111/dom.12058.
[67] I. Dhar, K. Desai, Aging: drugs to eliminate methylglyoxal, a reactive glucose metabolite, and advanced glycation endproducts, Pharmacol. Can. (2012) 681–708, http://dx.doi.org/10.5772/34337 (chapter 30).
[68] F. Song, A.M. Schmidt, Glycation and insulin resistance: novel mechanisms and unique targets? Arterioscler. Thromb. Vasc. Biol. 32 (8) (2012) 1760–1765, http://dx.doi.org/10.1161/ATVBAHA.111.241877.

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ARTICLE

Methylglyoxal impairs endothelial insulin sensitivity both in vitro and in vivo

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Abstract
Aims/hypothesis Insulin exerts a direct action on vascular cells, thereby affecting the outcome and progression of diabetic vascular complications. However, the mechanism through which insulin signalling is impaired in the endothelium of diabetic individuals remains unclear. In this work, we have evaluated the role of the AGE precursor methylglyoxal (MGO) in generating endothelial insulin resistance both in cells and in animal models.
Methods Time course experiments were performed on mouse aortic endothelial cells (MAECs) incubated with 500 μmol/l MGO. The glyoxalase-1 inhibitor *S-p*-bromobenzylglutathione-cyclopentyl-diester (SpBrBzGSHCp2) was used to increase the endogenous levels of MGO. For the in vivo study, an MGO solution was administrated i.p. to C57BL/6 mice for 7 weeks.
Results MGO prevented the insulin-dependent activation of the IRS1/protein kinase Akt/endothelial nitric oxide synthase (eNOS) pathway, thereby blunting nitric oxide (NO) production, while extracellular signal-regulated kinase (ERK1/2) activation and endothelin-1 (ET-1) release were increased by MGO in MAECs. Similar results were obtained in MAECs treated with SpBrBzGSHCp2. In MGO- and SpBrBzGSHCp2-exposed cells, inhibition of ERK1/2 decreased IRS1 phosphorylation on S616 and rescued insulin-dependent Akt activation and NO generation, indicating that MGO inhibition of the IRS1/Akt/eNOS pathway is mediated, at least in part, by ERK1/2. Chronic administration of MGO to C57BL/6 mice impaired whole-body insulin sensitivity and induced endothelial insulin resistance.
Conclusions/interpretation MGO impairs the action of insulin on the endothelium both in vitro and in vivo, at least in part through an ERK1/2-mediated mechanism. These findings may be instrumental in developing novel strategies for preserving endothelial function in diabetes.

Keywords Endothelial dysfunction · Glyoxalase-1 · Insulin resistance · Methylglyoxal · Nitric oxide

Abbreviations

ECE	Endothelin-converting enzyme
ENOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
Glo1	Glyoxalase-1
MAEC	Mouse aortic endothelial cell
MAPK	Mitogen-activated protein kinase
MGO	Methylglyoxal
NO	Nitric oxide
PI3K	Phosphatidylinositol 3-kinase
SpBrBzGSHCp2	*S-p*-Bromobenzylglutathione-cyclopentyl-diester

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Introduction

The decreased availability of endothelium-derived nitric oxide (NO) accompanied by impaired endothelium-dependent vasorelaxation represents a common feature of endothelial dysfunction in diabetic individuals and is an important cause of vascular disease [1]. Several studies have demonstrated impaired endothelium-dependent vasorelaxation in diabetic patients [2, 3] as well as in animal models of diabetes [4, 5]. In addition to its effects on glucose metabolism, insulin has important haemodynamic actions. Insulin acts on endothelium, inducing the dilatation of arteries and arterioles by a receptor-dependent mechanism which activates IRS1/phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial nitric oxide synthase (eNOS) signalling leading to NO generation and vasodilation [6, 7]. Insulin is also able to cause rapid release of endothelin-1 (ET-1) via the mitogen-activated protein kinase (MAPK) cascade and activation of endothelin-converting enzyme (ECE) [8, 9]. Defects in insulin's action on endothelial cells may therefore lead to a shift in the balance between the insulin-induced vasodilator NO and vasoconstrictor ET-1, with vasodilatation representing the normal response and impaired vasodilatation, or even net vasoconstriction, representing the abnormal responses associated with endothelial dysfunction [10]. However, the mechanism(s) by which insulin signalling becomes impaired in the vascular endothelium of diabetic individuals remains poorly understood.

Hyperglycaemia, associated with impaired glucose tolerance and diabetes, causes insulin resistance and endothelial dysfunction [11]. The entry of glucose into endothelial cells occurs by facilitated diffusion and is insulin independent. Increased blood glucose concentration will therefore lead to increased intracellular accumulation of glucose and its associated metabolites, making the endothelium highly susceptible to hyperglycaemia-induced damage. Generation of AGEs has an important role in the development of these abnormalities and the accompanying vascular complications [12]. AGEs are a heterogeneous family of non-enzymatically modified proteins, the levels of which are increased in tissues and plasma from patients with diabetes [13]. The highly reactive dicarbonyl methylglyoxal (MGO) has been found to be the most reactive AGE precursor in endothelial cells [14, 15]. MGO is an α-oxoaldehyde formed at a higher rate in hyperglycaemic conditions by the fragmentation of triosephosphates, the metabolism of ketone bodies or the degradation of threonine [16]. MGO is detoxified to D-lactate by the glyoxalase system, which consists of glyoxalase-1 (Glo1), glyoxalase-2 (Glo2) and a catalytic amount of glutathione (GSH) [17]. Glo1 activity prevents the accumulation of reactive dicarbonyls, such as MGO, thereby suppressing dicarbonyl-mediated glycation reactions and is therefore a key enzyme in anti-glycation defences [18, 19]. MGO reacts non-enzymatically with the basic amino acids lysine and arginine in proteins to form AGEs both extra- and intracellularly [20]. Studies have demonstrated that increased MGO-derived AGE levels in diabetic patients are associated with diabetic vascular complications [21–23].

In this study, the effect of MGO on endothelial insulin sensitivity was evaluated both in vitro and in vivo, and the molecular mechanisms involved in the impairment of insulin signalling and endothelium-dependent NO production following exposure to MGO were investigated.

Methods

Reagents Media, sera and antibiotics for cell culture were from Lonza (Walkersville, MD, USA). Protein electrophoresis and western blot reagents were from Bio-Rad (Richmond, VA, USA) and electrochemiluminescence reagents from Pierce (Rockford, IL, USA). Protein-A sepharose and MGO (40% in water) were from Sigma-Aldrich (St Louis, MO, USA). Insulin was from Eli Lilly (Florence, Italy). The antibodies used were anti-p-tyrosine, anti-Akt T308, anti-IRS-1 (Millipore, Billerica, MA, USA), anti-IRS1 S616 (Life Technologies, Carlsbad, CA, USA), anti-p85 (Upstate biotechnology, Lake Placid, NY, USA), anti-Akt, anti-p-Akt S473, anti-eNOS, anti-p-eNOS S1177, anti-p-eNOS T497, anti-14-3-3 ε (Cell Signaling Technology, Beverly, MA, USA), anti-ERK1/2, anti-p-ERK1/2 (Santa Cruz, CA, USA) and anti-α-tubulin (Sigma-Aldrich). U0126 was from ENZO Lifescience (Florence, Italy). PD98059 was from Sigma-Aldrich. The inhibitor of glyoxalase 1, *S-p*-bromobenzylglutathione-cyclopentyl-diester (SpBrBzGSHCp2), was kindly provided by A. Bierhaus (University of Heidelberg, Heidelberg, Germany). All other chemicals were from Sigma-Aldrich.

Cell culture procedure Mouse aortic endothelial cells (MAECs) were plated in T75 flasks and grown in DMEM containing 1 g/l glucose supplemented with 10% (vol./vol.) FBS, 2 mmol/l L-glutamine and 0.1 mmol/l non-essential amino acids. Cultures were maintained at 37°C in a humidified atmosphere containing 5% (vol./vol.) CO_2. Cells were starved in serum-free medium containing 0.25% (wt/vol.) BSA, pre-treated (or not) with MGO 500 μmol/l for the appropriate times, and then exposed (or not) to 100 nmol/l insulin. Where indicated, cells were pre-treated for 30 min with U0126 15 μmol/l or PD98059 30 μmol/l or for 48 h with 10 μmol/l SpBrBzGSHCp2.

Western blot analysis Cells were solubilised in lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $Na_2P_2O_7$, 2 mmol/l Na_3VO_4, 100 mmol/l NaF, 10% glycerol, 1% Triton X-100, 1 mmol/l phenylmethylsulfonylfluoride and 10 μg/ml aprotinin) for 2 h at 4°C. Cell lysates were clarified by centrifugation at

16,000 *g* for 20 min. To analyse the phosphorylation of IRS-1 and its interaction with p85, 250 μg of protein lysates were incubated with IRS-1 antibodies and then precipitated with protein-A sepharose. Cell lysates or immunoprecipitated proteins were then separated by SDS-PAGE and transferred onto 0.45 μm Immobilon-P membranes (Millipore). Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by electrochemiluminescence according to the manufacturer's instructions.

Measurement of NO NO was measured in cell culture medium and mouse serum. After treatments, culture medium was collected, centrifuged at 1,000 *g* for 15 min, and the supernatant fraction was used as a sample solution for the detection. Serum was collected from the mice after cervical dislocation, treated with Centricon 10 (Amicon, Beverly, MA, USA) at 4,000 *g* for 1 h at 4°C to remove haemoglobin and proteins. NO concentration was detected by the use of Nitrate/nitrite Assay Kit Colorimetric (Sigma-Aldrich). This assay uses the Griess reaction, resulting in the formation of a chromophoric azo-derivative, which absorbs light at 540–570 nm.

Measurement of ET-1 ET-1 was measured in cell culture medium and mouse serum with a solid-phase sandwich

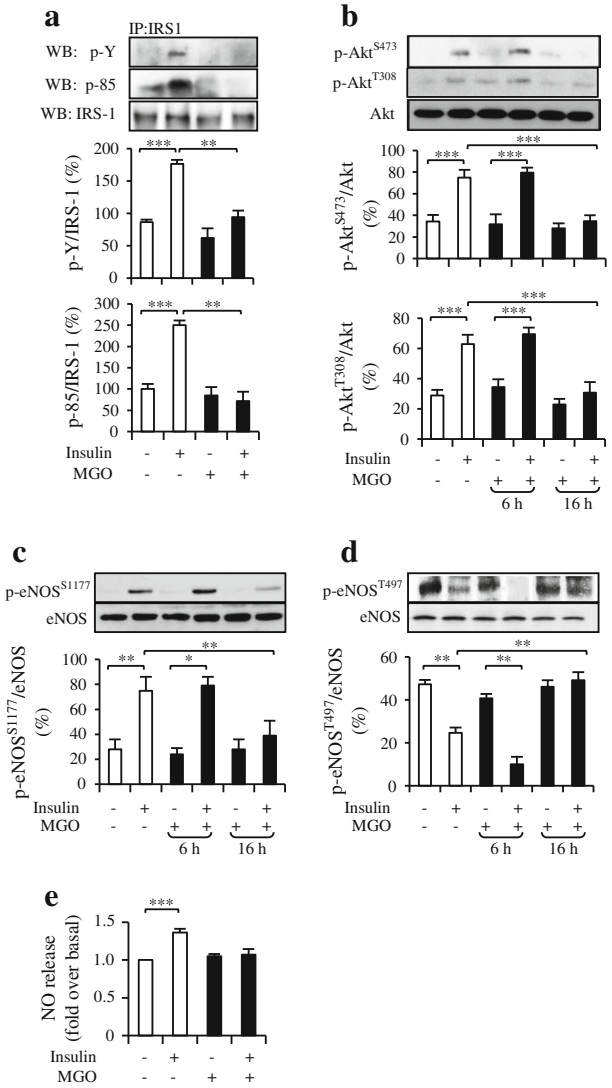

Fig. 1 MGO impairs insulin signalling and NO release in endothelial cells. MAECs were pre-treated (black bars) or not (white bars) with 500 μmol/l MGO for up to 16 h and stimulated or not with 100 nmol/l insulin for 10 min. (**a**) Immunoblots of immunoprecipitated proteins (IP) from whole-cell lysates. Immunoprecipitation was performed using the anti-IRS-1 antibody as described in Methods. The blots (WB) were probed with antibodies to p-tyrosine (p-Y), p85 and IRS-1. (**b**–**d**) Immunoblots of whole-cell lysates. The blots were probed with antibodies to p-Akt S473, p-Akt T308 and Akt (**b**) and to p-eNOS S1177, p-eNOS T497 and eNOS (**c**, **d**). Each bar graph represents the densitometric quantification in three independent immunoblots. (**e**) NO release in the culture medium. Values are expressed as fold over basal of triplicate determinations. Statistical analysis was evaluated using Student's *t* test; **p*<0.05, ***p*<0.01, ****p*<0.001

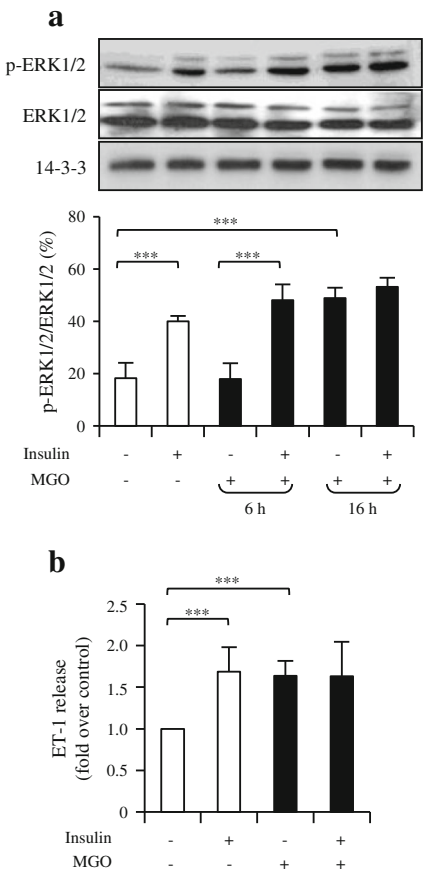

Fig. 2 MGO affects ERK1/2 and ET-1 release in endothelial cells. MAECs were treated as reported in Fig. 1. (**a**) Immunoblots of whole-cell lysates. The blots were probed with antibodies to p-ERK1/2 and ERK1/2. To ensure accurate normalisation, the same blots were probed with anti-14-3-3 antibody. Each bar graph represents the densitometric quantification in three independent immunoblots. (**b**) ET-1 release in the culture medium from these cells. Values are expressed as fold over basal of triplicate determinations. Statistical analysis was evaluated using Student's *t* test; ****p*<0.001

ELISA (Immuno-Biological Laboratories, Hamburg, Germany) according to the manufacturer's instructions.

Animals and MGO administration Twelve-week-old C57/BL6 male mice (n=30) were purchased from the Charles River Laboratories (Milan, Italy). The mice were housed in a temperature-controlled (22°C) room with a 12 h light/dark cycle, in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-23, revised 1996), and experiments were approved by the ethics committee of the Federico II University. MGO was administered i.p. over five consecutive days each week for 7 consecutive weeks. The initial dose administered was 50 mg/kg of body weight for the first 2 weeks, followed by a dose of 60 mg/kg for weeks 3 and 4 and a dose of 75 mg/kg for the last 3 weeks [24]. The same protocol was performed twice in two different groups of mice. Body weight was recorded weekly throughout the study. The mice were divided into two groups and injected with MGO solution (15 mice) or physiological solution (15 mice). After 7 weeks of treatment, mice from each group were killed by cervical dislocation, the sera were collected and the aortas were isolated and homogenised as previously reported [25].

Determination of MGO MGO in mouse serum samples or cell extracts was determined by HPLC after derivatisation with 1,2-diamino-4,5-dimethoxybenezene as previously described [26].

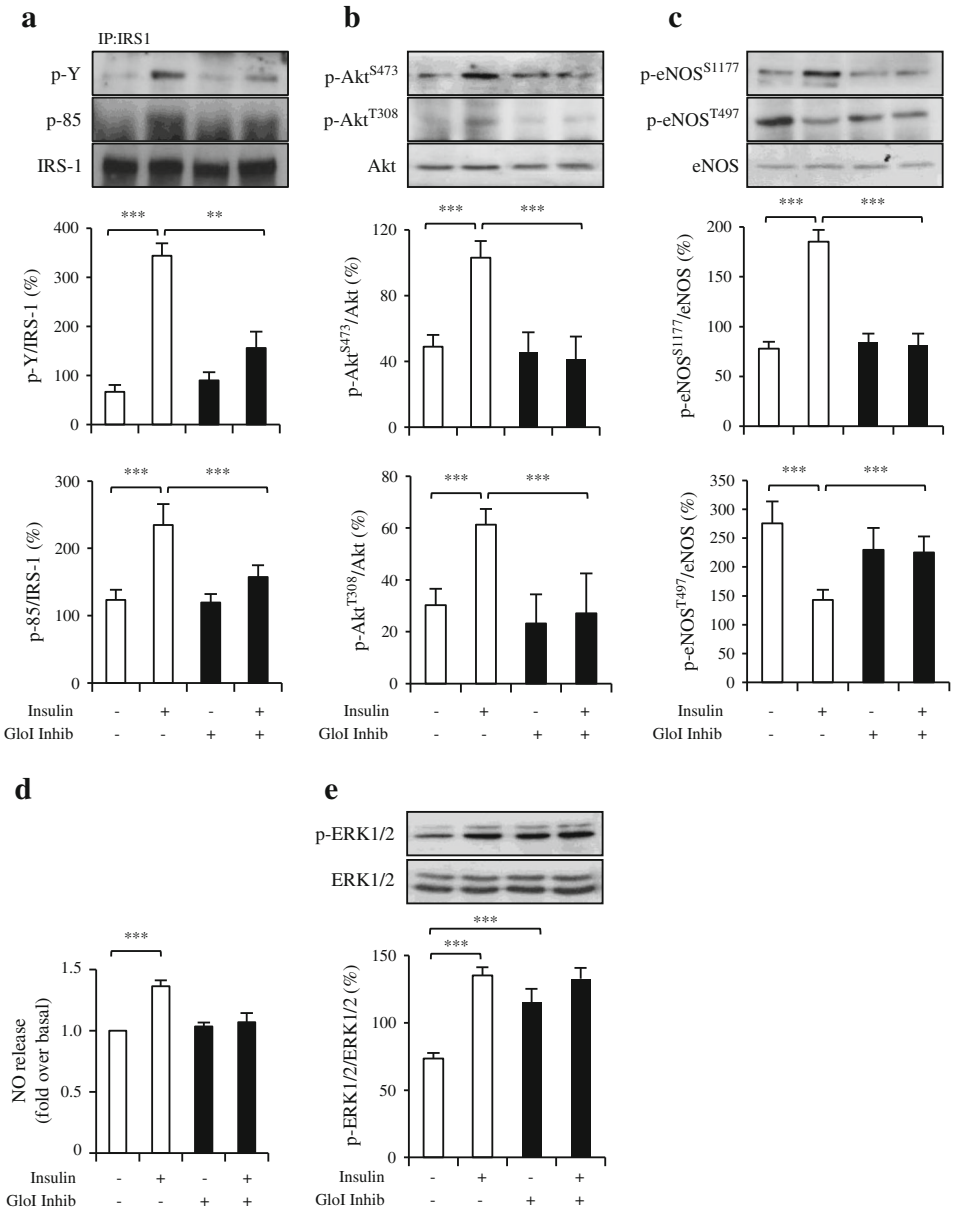

Fig. 3 GloI inhibitor impairs insulin signalling and NO release in endothelial cells. MAECs were pre-treated (black bars) or not (white bars) with 10 μmol/l of the Glo1 inhibitor SpBrBzGSHCp2 (Glo1 Inhib) for 48 h and stimulated or not with 100 nmol/l insulin for 10 min. (**a**) Immunoblots of immunoprecipitated proteins (IP) from whole-cell lysates. Immunoprecipitation was performed using the anti-IRS-1 antibody as described in Methods. The blots were probed with antibodies to p-Y, p85 and IRS-1. (**b**, **c**, **e**) Immunoblots of whole-cell lysates. The blots were probed with antibodies to p-Akt S473, p-Akt T308 and Akt (**b**), to p-eNOS S1177, p-eNOS T497 and eNOS (**c**) and to p-ERK1/2 and ERK1/2 (**e**). Each bar graph represents the densitometric quantification in three independent immunoblots. (**d**) NO release in the culture medium. Values are expressed as fold over basal of triplicate determinations. Statistical analysis was evaluated using Student's t test; $^{**}p<0.01$, $^{***}p<0.001$

Insulin tolerance test Mice were fasted for 4 h followed by insulin injection (0.75 U/kg body weight, i.p.). Whole venous blood was obtained from the tail vein at 0, 15, 30, 60, 90 and 120 min after the injection. Blood glucose was measured using an automatic glucometer (One Touch Lifescan, Milan, Italy).

Statistical procedures Data were analysed with Statview software (Abacus Concepts Piscataway, NJ, USA) by one-factor analysis of variance. A p value of less than 0.05 was considered statistically significant.

Results

MGO impairs PI3K-dependent pathway in endothelial cells To determine whether MGO impairs insulin sensitivity in endothelial cells, we pre-treated MAECs with 500 μmol/l of MGO for up to 16 h and then stimulated the cells with 100 nmol/l insulin for 10 min. Pre-treatment of MAECs with MGO suppressed insulin-induced IRS-1 tyrosine phosphorylation (Fig. 1a), as well as the interaction between the PI3K regulatory subunit p85 and IRS-1 (Fig. 1a). The phosphorylation of the PI3K downstream target Akt at both T308 and S473 sites, as well as its substrate glycogen synthase kinase 3 (ESM Fig. 1), was also suppressed by 16 h MGO treatment (Fig. 1b). Pre-treatment with MGO for a shorter time period (6 h) did not affect the insulin-induced Akt activation. Cell viability was not affected by either the concentration of MGO or the time used for cell exposure (ESM Fig. 2).

MGO impairs insulin-dependent NO production in endothelial cells Insulin stimulation of MAECs resulted in a twofold increase in the phoshorylation of S1177, the activation site of eNOS (Fig. 1c), and a twofold decrease in phosphorylation of T497, the inhibitory site (Fig. 1d). Pre-treatment with MGO for 16 h suppressed insulin's effect on the phosphorylation at both of the eNOS sites (Fig. 1c, d), while pre-treatment with MGO for 6 h did not affect the insulin-induced eNOS activation (Fig. 1c, d). Consistent with eNOS activation, the insulin-induced NO release in the culture medium of MAECs was suppressed by 16 h MGO pre-treatment (Fig. 1e). At variance, insulin induced an increase in NO release of ~40% in control cells (Fig. 1e).

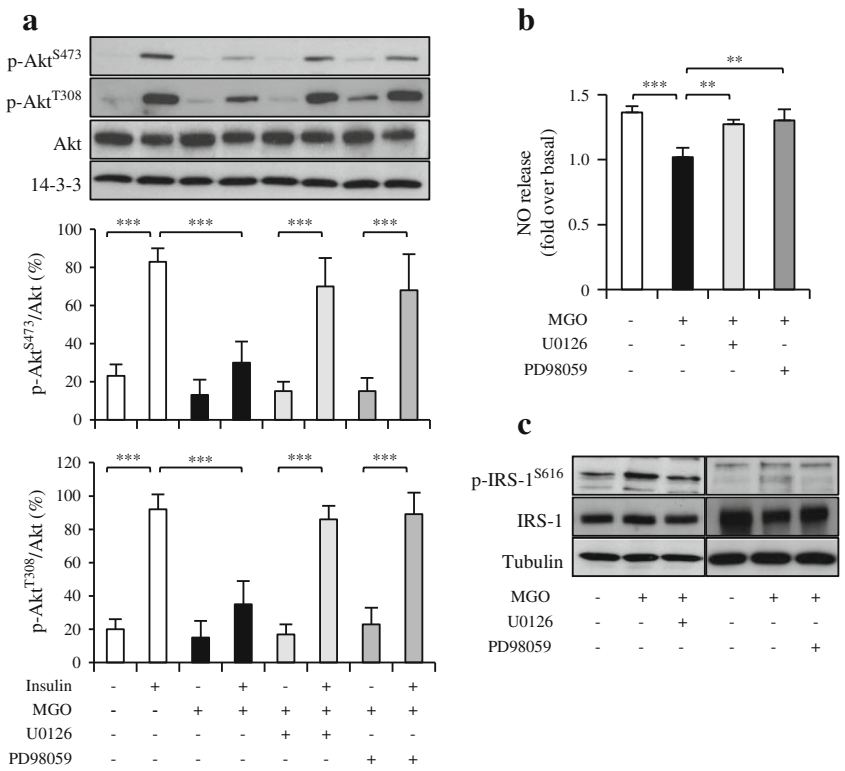

Fig. 4 MGO-induced endothelial insulin resistance is mediated by ERK1/2 basal activation. MAECs were pre-incubated with 15 μmol/l of U0126 (light-grey bars) or with 30 μmol/l PD98059 (dark-grey bars) for 30 min. Cells were then treated or not with 500 μmol/l MGO for 16 h and stimulated or not with 100 nmol/l insulin for 10 min. (**a**, **c**) Immunoblots of whole-cell lysates. The blots were probed with antibodies to p-Akt S473, p-Akt T308 and Akt (**a**) and to p-IRS-1S616 and IRS-1 (**c**). To ensure accurate normalisation, the same blots were probed with anti-14-3-3 or anti-tubulin antibodies. Each bar graph represents the densitometric quantification in three independent immunoblots. (**b**) NO release in the culture medium from these cells. Values are expressed as fold over basal of triplicate determinations. Statistical analysis was evaluated using Student's t test; $^{**}p<0.01$, $^{***}p<0.001$

MGO induces extracellular signal-regulated kinase 1/2 activation in endothelial cells Insulin stimulation resulted in a twofold increase in the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in control MAECs and in 6 h pre-treated MAECs (Fig. 2a). Pre-treatment with MGO for 16 h significantly increased both basal ERK1/2 phosphorylation and ET-1 release in the culture medium of MAEC cells (Fig. 2a, b).

No further insulin-dependent increase in either ERK1/2 phosphorylation or ET-1 level occurred in cells exposed to MGO for 16 h (Fig. 2a, b). At variance, insulin induced a 50% increase of ET-1 release in control cells (Fig. 2b).

Glo1 inhibition impairs insulin signal transduction We then aimed to increase the endogenous MGO levels by inhibiting its detoxifying enzyme Glo1 [27]. Treatment of MAECs with 10 μmol/l of the Glo1 inhibitor SpBrBzGSHCp2 for 48 h reduced insulin-induced IRS-1 tyrosine phosphorylation, as well as IRS1/p85 interaction (Fig. 3a). Furthermore, SpBrBzGSHCp2 caused a complete loss of insulin-induced phosphorylation of Akt at both T308 and S473 sites (Fig. 3b) and suppressed the effect of insulin on the phosphorylation of eNOS at both S1177 and T497 sites (Fig. 3c). Impairment of eNOS activation was accompanied by the loss of insulin-dependent increase of NO production by the cells (Fig. 3d). In contrast, in cells treated with the Glo1 inhibitor, ERK1/2 activation was increased in the basal condition compared with control cells and its phosphorylation remained unchanged after insulin stimulation (Fig. 3e). Thus, high levels of MGO, whether induced by exogenous MGO exposure or by inhibition of Glo1, reduce insulin sensitivity in endothelial cells.

MGO-induced activation of ERK1/2 impairs insulin-dependent Akt activation and NO production via IRS-1 phosphorylation on S616 Since ERK1/2 phosphorylates IRS-1 on specific serine inhibitory sites [28, 29], we addressed the issue of whether the activation of ERK1/2 induced by either MGO or by Glo1 inhibitor could also have a role in mediating its effect on the insulin-dependent IRS-1/PI3K/Akt/eNOS pathway. Inhibition of ERK1/2 with both MEK inhibitors U0126 and PD98059 (ESM Fig. 3a, b) prevented MGO-dependent suppression of insulin-induced effects on the phosphorylation of Akt (Fig. 4a) and on NO release (Fig. 4b). Furthermore, the effect of MGO on ET-1 release was also prevented in cells treated with the MEK inhibitor U0126 (ESM Fig. 3c). Interestingly, MGO increased IRS-1 phosphorylation on S616, and this increase was lost upon MEK inhibition with both U0126 and PD98059 (Fig. 4c). As shown in Fig. 5a, b, the inhibition of ERK1/2 by U0126 was also able to restore the Akt activation and NO release in response to insulin, which were lost in MAECs treated with SpBrBzGSHCp2. Moreover, as found for MGO-treated cells,

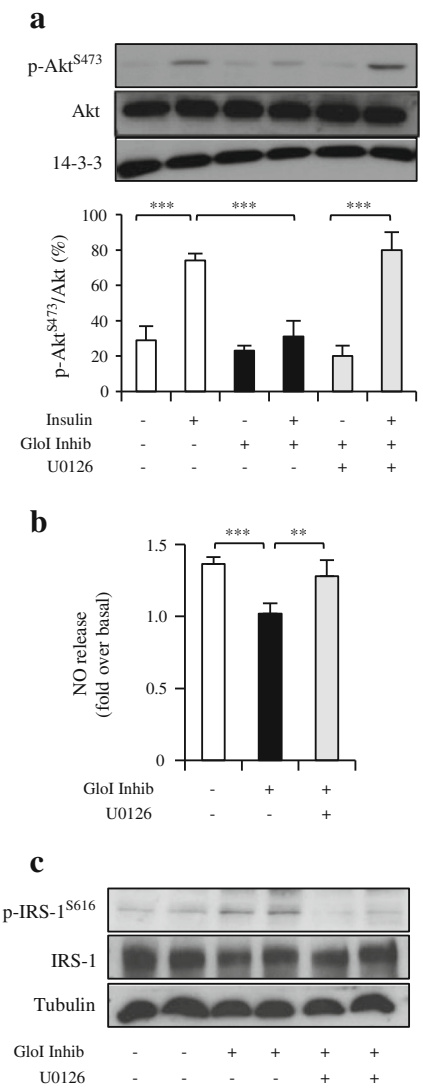

Fig. 5 Glo1 inhibitor-induced endothelial insulin resistance is mediated by ERK1/2 basal activation. MAECs were pre-incubated with 15 μmol/l of U0126 (light-grey bars) for 30 min. Cells were then treated or not with 10 μmol/l of the Glo1 inhibitor SpBrBzGSHCp2 (Glo1 Inhib) for 48 h and stimulated or not with 100 nmol/l insulin for 10 min. (**a**, **c**) Immunoblots of whole-cell lysates. The blots were probed with antibodies to p-Akt S473 and Akt (**a**) and to p-IRS-1 S616 and IRS-1 (**c**). To ensure accurate normalisation, the same blots were probed with anti-14-3-3 or anti-tubulin antibodies. Each bar graph represents the densitometric quantification in three independent immunoblots. (**b**) NO release in the culture medium from these cells. Values are expressed as fold over basal of triplicate determinations. Statistical analysis was evaluated using Student's *t* test; $^{**}p<0.01$, $^{***}p<0.001$

IRS-1 phosphorylation on S616, increased by SpBrBzGSHCp2 exposure, was abolished by U0126 (Fig. 5c). These data indicate that high levels of MGO impair PI3K-dependent signal transduction, at least in part, via ERK1/2-induced inhibition of IRS-1.

MGO administration causes insulin resistance in C57BL/6 mice To determine whether MGO affects insulin sensitivity

in vivo, C57BL/6 mice were treated with MGO for 7 weeks. At the end of the treatment, MGO serum concentration was increased by ~2.6-fold in MGO-treated mice compared with vehicle-treated mice (Table 1). Insulin tolerance tests showed a significant increase in systemic insulin resistance in mice treated with MGO compared with vehicle-treated mice (Fig. 6a and Table 1). Western blots of the aortic tissue lysates from MGO-treated mice showed a reduction of insulin-induced activation of both Akt and eNOS (Fig. 6b, c). Moreover, insulin-stimulated NO release was completely lost in MGO-treated mice compared with the control mice (Fig. 6d). As previously observed in MAECs in vitro, ERK1/2 activation and ET-1 release were increased at basal level with no further significant increase upon insulin stimulation in the aortic tissue and serum, respectively, from MGO-treated mice compared with vehicle-treated mice (Fig. 6e, f). Furthermore, MGO treatment increased IRS-1 phosphorylation on S616 in the aorta lysates (Fig. 6g). These data indicate that in vivo MGO treatment impairs whole-body insulin sensitivity and induces endothelial insulin resistance.

Discussion

Type 2 diabetes is an important risk factor for the development of cardiovascular disease [1]. Several studies have highlighted how insulin, besides its function as regulator of glucose homeostasis, has important haemodynamic actions [30]. Alterations in the endothelial insulin signalling pathway cause endothelial dysfunction by modifying the balance between ET-1 and NO production [31]. Hyperglycaemia itself plays an important role in the onset and progression of vascular dysfunction [32]. A widely accepted hypothesis on the mechanisms implicated in glucose-mediated vascular damage includes the increased production of AGEs [12].

In the present study, MGO has been identified as an endothelial insulin resistance-inducing molecule. It has been shown that higher serum and adipose tissue MGO concentrations are closely related to systemic insulin resistance [33]. Furthermore, it has been shown that MGO treatment in vitro impairs insulin signalling activation in L6 skeletal muscle cells [34] and glucose uptake in 3T3-L1 adipocytes [33]. However, the molecular mechanism(s) of MGO action on insulin signalling in endothelial cells remains unclear.

In this study, we have shown that when endothelial cells are pre-treated with a non-toxic concentration of MGO, insulin is unable to induce IRS-1 tyrosine phosphorylation. The protein level of IRS-1 was unchanged by MGO, suggesting that alterations in the synthesis and/or degradation of IRS-1 are not responsible for the impairment of its tyrosine phosphorylation. Consistent with the reduction in IRS-1 tyrosine phosphorylation, MGO treatment impaired the association of p85 with IRS-1, together with the activation of the downstream PI3K effector, Akt. Insulin binding to its receptor on endothelial cells also triggers the activation of the MAPK cascade, which leads to the rapid release of ET-1 [32]. Despite the impaired insulin-dependent induction of the IRS-1/Akt pathway caused by MGO, ERK1/2 activity was enhanced by MGO. MGO-induced ERK1/2 activation was accompanied by increased phosphorylation of IRS-1 on its inhibitory site Ser^{616}. Interestingly, ERK1/2 inhibition by U0126 or PD98059 resulted in both the reduction of IRS1 Ser^{616} phosphorylation and the rescue of insulin-dependent Akt activation and NO production. Collectively, these findings indicate that ERK1/2 mediates, at least in part, the detrimental effect exerted by MGO on PI3K/Akt pathway activation by phosphorylating IRS-1 on Ser^{616}.

Despite the constitutive activation of ERK1/2, we did not observe any effects on MAEC proliferation (data not shown). Indeed, in endothelial cells ERK1/2 modulates the interaction between the ET-1 precursor (Big-ET-1) and ECE in the

Table 1 Metabolic characteristics of MGO-treated mice

Variable	Before treatment		After 7 weeks of treatment	
	Vehicle	MGO	Vehicle	MGO
Body weight (g)	26.2±2.71	26.5±2.19	27.5±2.89	26.8±1.16
Food intake (g/day per mouse)	3.7±0.87	3.8±0.99	3.9±0.24	3.8±0.47
Fasting glucose (mmol/l)	5.3±1.0	5.4±1.0	4.9±0.6	5.2±1.0
Fed glucose (mmol/l)	9.3±1.6	9.1±1.9	9.3±1.9	9.2±1.8
ITT AUC (mmol/l × min)	–	–	6,344±1,621	9,814±3,537*
Serum MGO (nmol/l)	–	–	322±143	835±303***

Data are means ± SD of determinations in >10 mice per group

C57BL/6 mice were injected i.p. with either MGO or physiological solution as control of vehicle injection. Body weight, food intake, fasting glucose and fed blood glucose were monitored throughout the treatment period. At the end of treatment, serum MGO was determined, an insulin tolerance test (ITT) was performed on the two groups of mice and the AUCs are shown in the table

*p<0.01, ***p<0.001, MGO vs vehicle

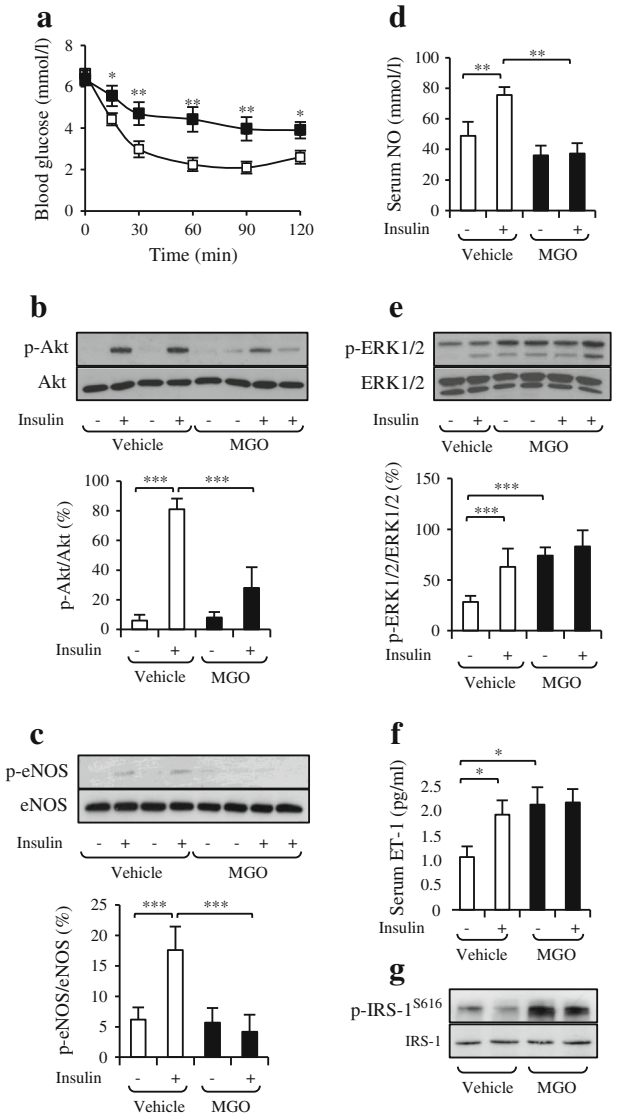

Fig. 6 Effect of MGO administration on whole-body and endothelial insulin sensitivity in mice. C57BL/6 mice were injected with MGO i.p. (black squares, black bars), or with physiological solution (white squares, white bars) as control vehicle injection. (**a**) MGO-treated and vehicle-treated mice were subjected to insulin tolerance test. Values are expressed as means ± SEM of determinations in >10 mice per group. (**b**–**g**) At the end of treatment, five mice for each group were injected with insulin i.p. (0.15 U/g body weight) 10 min before they were killed. Aortas were isolated and the protein extracts were analysed by western blot. The blots were probed with antibodies to p-Akt S473, Akt (**b**), p-eNOS S1177, eNOS (**c**), p-ERK1/2, ERK1/2 (**e**), p-IRS-1 S616 and IRS-1 (**g**). Each bar graph represents the mean ± SD of the densitometric quantification of the blots obtained from at least three mice. (**d**, **e**) Serum NO (**d**) and ET-1 concentrations (**f**) in MGO-treated and control mice are expressed as means ± SE for five mice in each group. Statistical significance was evaluated using Student's *t* test; *$p<0.05$; **$p<0.01$, ***$p<0.001$

cytosol, resulting in ET-1 production [35]. Several studies have reported that hyperglycaemia can induce glucotoxicity by oxidative stress [12, 36], which is known to cause ERK1/2 activation [37]. It has been demonstrated that MGO induces insulin resistance and salt sensitivity by increasing oxidative stress in Sprague–Dawley rats [38]. However, MGO leads to oxidative stress in a time- and dose-dependent manner; indeed, it has been found that MGO causes insulin resistance in L6 cells at earlier times and at lower concentrations than those necessary to induce oxidative stress [34]. Further studies will be necessary to determine whether oxidative stress causes ERK1/2 activation in MGO-exposed endothelial cells.

NO is a key molecule secreted by endothelium and acts as the major mediator of insulin action on vascular homeostasis. Impaired NO availability is pivotal in the development and progression of vascular complications of diabetes. Indeed, it has been shown that patients with either hypertension or diabetes have relatively low NO plasma levels. Reduced NO availability is of relevance to the development of atherosclerotic complications in diabetes and to insulin-mediated postprandial glucose disposal and also contributes to the development of insulin resistance [39]. eNOS catalyses the conversion of arginine to citrulline and NO. Its activity is regulated by phosphorylation at multiple sites, but two of the better-characterised sites are Ser^{1177} and Thr^{495}. Ser^{1177} is rapidly phosphorylated by Akt in response to insulin, resulting in increased eNOS activity and NO production, whereas Thr^{495} is constitutively phosphorylated in endothelial cells and is thought to be a negative regulatory site, causing decreased enzymatic activity [7, 40]. In the present study, it was shown that MGO inhibits insulin-induced phosphorylation on eNOS at Ser^{1177} and prevents the effect of insulin on the inhibitory phosphorylation of Thr^{497}. Furthermore, the reduced activation of eNOS is paralleled by the loss of insulin-dependent increase in NO production. ET-1 production was also significantly increased in MGO-treated cells both in the absence and presence of insulin. These results suggest that MGO impairs insulin signalling thereby causing an imbalance between NO and ET-1 production, which facilitates vasoconstriction. Recent findings that NO regulates ECE-1 expression [41] support our results. It is therefore possible that the reduced NO levels also contribute to the activation of ECE-1 and the subsequent release of ET-1.

Estimates of the cellular and whole-blood concentrations of MGO in healthy individuals are in the range of 1–5 μmol/l and 120 nmol/l, respectively. The whole-blood MGO concentration is increased by five- to sixfold in patients with type 1 diabetes and by two- to threefold in patients with type 2 diabetes [17]. Therefore, the MGO concentration we used in this study may not be considered of (patho)physiological relevance. To overcome this issue, endogenous levels of MGO were increased by inhibition of Glo1 with the cell-permeable inhibitor $SpBrBzGSHCp_2$. Previous studies report a twofold increase in the intracellular concentration of MGO in SpBrGSHCp2-treated HL60 cells [27]. Treatment of MAECs with this inhibitor reduced the insulin-dependent IRS-1 Tyr phosphorylation and its interaction with p85, caused the complete loss of insulin-stimulated Akt

phosphorylation, eNOS activation and NO production and also caused a significant increase of ERK1/2 activation in the basal condition as compared with control cells, confirming our previous finding with exogenous MGO.

The findings of this study provide further evidence that increased MGO levels affect insulin sensitivity and insulin-dependent NO production in vivo. Previous studies have shown a positive correlation between MGO levels and insulin resistance [34] as well as glucose intolerance in rats [40, 42]. Brouwers et al have shown that hyperglycaemia-induced impairment of endothelium-dependent vasorelaxation is mediated by increased intracellular MGO levels [43], supporting the need for further studies regarding the specific role of MGO in endothelial function. In this study, it was found that healthy mice chronically treated with MGO became insulin resistant. Furthermore, IRS-1 phosphorylation on Ser^{616} was increased, while insulin-induced Akt and eNOS activation were reduced, in aortas from these mice. When compared with control mice, MGO-treated mice also featured the loss of insulin-induced increase in serum levels of NO. As found in vitro, this was accompanied by increased ERK1/2 activation and ET-1 serum levels, indicating that MGO is able to induce vascular insulin resistance and endothelial dysfunction in vivo.

In conclusion, this work shows that increased concentration of MGO impairs the action of insulin on vascular endothelium both in vitro and in vivo, leading to an imbalance between NO and ET-1 production. Furthermore, we provide evidence that ERK1/2 activation mediates, at least in part, this effect in endothelial cells through the phosphorylation of IRS-1 on Ser^{616}. Our findings highlight MGO as an important culprit of endothelial dysfunction associated with insulin resistance, providing new insights for the development of strategies to preserve endothelial function in diabetic individuals.

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Contribution statement CN conceived and designed the experiments, acquired data, analysed data and wrote the manuscript. GAR reviewed/edited the manuscript and collected and analysed data. AL, IP, ML, FF, PM, LU and VD collected data and revised the manuscript. THF and PPN provided reagents/materials, acquired data and critically reviewed the manuscript. PF and FB interpreted data and reviewed/edited the manuscript. CM designed the research and supervised the project, reviewed/edited the manuscript and contributed to the discussion. CM is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version of the manuscript.

References

1. Schalkwijk CG, Stehouwer (2005) CDA: vascular complications in diabetes mellitus: the role of endothelial dysfunction. Clin Sci 109: 143–159
2. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD (1996) Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. J Clin Invest 97:2601–2610
3. Mäkimattila S, Virkamäki A, Groop PH et al (1996) Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus. Circulation 94:1276–1282
4. Diederich D, Skopec J, Diederich A, Dai FX (1994) Endothelial dysfunction in mesenteric resistance arteries of diabetic rats: role of free radicals. Am J Physiol 266:H1153–H1161
5. Pflueger AC, Osswald H, Knox FG (1999) Adenosine-induced renal vasoconstriction in diabetes mellitus rats: role of nitric oxide. Am J Physiol 276:F340–F346
6. Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P (1994) Nitric oxide release accounts for insulin's vascular effects in humans. J Clin Invest 94:2511–2515
7. Andreozzi F, Laratta E, Procopio C et al (2007) Interleukin-6 impairs the insulin signaling pathway, promoting production of nitric oxide in human umbilical vein endothelial cells. Mol Cell Biol 27:2372–2383
8. Kubota T, Kubota N, Moroi M et al (2003) Lack of insulin receptor substrate-2 causes progressive neointima formation in response to vessel injury. Circulation 107:3073–3080
9. Montagnani M, Ravichandran LV, Chen H, Esposito DL, Quon MJ (2002) Insulin receptor substrate-1 and phosphoinositide-dependent kinase-1 are required for insulin-stimulated production of nitric oxide in endothelial cells. Mol Endocrinol 16:1931–1942
10. Eringa EC, Stehouwer CD, Nieuw Amerongen GP, Ouwehand L, Westerhof N, Sipkema P (2004) Vasoconstrictor effects of insulin in skeletal muscle arterioles are mediated by ERK1/2 activation in endothelium. Am J Physiol Heart Circ Physiol 287:H2043–H2048
11. Muniyappa R, Iantorno M, Quon MJ (2001) An integrated view of insulin resistance and endothelial dysfunction. Endocrinol Metab Clin N Am 37:685–711
12. Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414:813–820
13. Goh SY, Cooper ME (2008) Clinical review: the role of advanced glycation end products in progression and complications of diabetes. J Clin Endocrinol Metab 93:1143–1152
14. Shinohara M, Thornalley PJ, Giardino I et al (1998) Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. J Clin Invest 101: 1142–1147
15. Bourajjaj M, Stehouwer CD, van Hinsbergh VW, Schalkwijk CG (2003) Role of methylglyoxal adducts in the development of vascular complications in diabetes mellitus. Biochem Soc Trans 31:1400–1402
16. Thornalley PJ (2005) Dicarbonyl intermediates in the maillard reaction. Ann N Y Acad Sci 1043:111–117

17. Rabbani N, Thornalley PJ (2011) Glyoxalase in diabetes, obesity and related disorders. Semin Cell Dev Biol 22:309–317
18. Fleming TH, Humpert PM, Nawroth PP, Bierhaus A (2011) Reactive metabolites and AGE/RAGE-mediated cellular dysfunction affect the aging process: a mini-review. Gerontology 57:435–443
19. Thornalley PJ (2003) Glyoxalase I—structure, function and a critical role in the enzymatic defence against glycation. Biochem Soc Trans 31:1343–1348
20. Thornalley PJ (2008) Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems—role in ageing and disease. Drug Metabol Drug Interact 23:125–150
21. Wang X, Desai K, Chang T, Wu L (2005) Vascular methylglyoxal metabolism and the development of hypertension. J Hypertens 23: 1565–1573
22. Wang X, Chang T, Jiang B, Desai K, Wu L (2007) Attenuation of hypertension development by aminoguanidine in spontaneously hypertensive rats: role of methylglyoxal. Am J Hypertens 20:629–636
23. Wang X, Jia X, Chang T, Desai K, Wu L (2008) Attenuation of hypertension development by scavenging methylglyoxal in fructose-treated rats. J Hypertens 26:765–772
24. Berlanga J, Cibrian D, Guillén I et al (2005) Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds. Clin Sci (Lond) 109:83–95
25. Cassese A, Raciti GA, Fiory F et al (2013) Adenoviral gene transfer of PLD1-D4 enhances insulin sensitivity in mice by disrupting phospholipase D1 interaction with PED/PEA-15. PLoS One 8(4):e60555
26. McLellan AC, Phillips SA, Thornalley PJ (1992) The assay of methylglyoxal in biological systems by derivatization with 1,2-diamino-4,5-dimethoxybenzene. Anal Biochem 206:17–23
27. Thornalley PJ, Edwards LG, Kang Y et al (1996) Antitumor activity of S-p-bromobenzylglutathione cyclopentyl diester in vitro and in vivo. Biochem Pharmacol 51:1365–1372
28. Gual P, Le Marchand-Brustel Y, Tanti JF (2005) Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. Biochimie 87:99–109
29. Bard-Chapeau EA, Hevener AL, Long S, Zhang EE, Olefsky JM, Feng GS (2005) Deletion of Gab1 in the liver leads to enhanced glucose tolerance and improved hepatic insulin action. Nat Med 11: 567–571
30. Cersosimo E, DeFronzo RA (2006) Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases. Diabetes Metab Res Rev 22:423–436
31. Federici M, Pandolfi A, de Filippis EA et al (2004) G972R IRS-1 variant impairs insulin regulation of endothelial nitric oxide synthase in cultured human endothelial cells. Circulation 109:399–405
32. Bakker W, Eringa EC, Sipkema P, van Hinsbergh VW (2009) Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. Cell Tissue Res 335:165–189
33. Jia X, Wu L (2007) Accumulation of endogenous methylglyoxal impaired insulin signaling in adipose tissue of fructose-fed rats. Mol Cell Biochem 306:133–139
34. Riboulet-Chavey A, Pierron A, Durand I, Murdaca J, Giudicelli J, van Obberghen E (2006) Methylglyoxal impairs the insulin signaling pathways independently of the formation of intracellular reactive oxygen species. Diabetes 55:1289–1299
35. Sen U, Tyagi N et al (2009) Fibrinogen-induced endothelin-1 production from endothelial cells. Am J Physiol Cell Physiol 296:C840–C847
36. Nishikawa T, Edelstein D, Du XL et al (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787–790
37. Glotin AL, Calipel A, Brossas JY, Faussat AM, Tréton J, Mascarelli F (2006) Sustained versus transient ERK1/2 signaling underlies the anti- and proapoptotic effects of oxidative stress in human RPE cells. Invest Ophthalmol Vis Sci 47:4614–4623
38. Guo Q, Mori T, Jiang Y et al (2009) Methylglyoxal contributes to the development of insulin resistance and salt sensitivity in Sprague-Dawley rats. Hypertension 27:1664–1671
39. Shiekh GA, Ayub T, Khan SN, Dar R, Andrabi KI (2011) Reduced nitrate level in individuals with hypertension and diabetes. J Cardiovasc Dis Res 2:172–176
40. Fleming I, Busse R (2003) Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 284:R1–R12
41. Raoch V, Rodríguez-Pascual F, López-Martínez V et al (2011) Nitric oxide decreases the expression of endothelin-converting enzyme-1 through mRNA destabilization. Arterioscler Thromb Vasc Biol 31: 2577–2585
42. Dhar A, Desai KM, Wu L (2010) Alagebrium attenuates acute methylglyoxal-induced glucose intolerance in Sprague-Dawley rats. Br J Pharmacol 159:166–175
43. Brouwers O, Niessen PM, Haenen G et al (2010) Hyperglycaemia-induced impairment of endothelium-dependent vasorelaxation in rat mesenteric arteries is mediated by intracellular methylglyoxal levels in a pathway dependent on oxidative stress. Diabetologia 53:989–1000