VASODILATOR ACTION OF GHRELIN

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By

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ABSTRACT

Ghrelin is a 28-amino acid peptide predominantly produced in the stomach and secreted into the circulation. Ghrelin is found in plasma and tissues in two major forms of n-octanoyl-modified at its N-terminal third serine residue and des-acyl ghrelin. The n-octanoyl group of ghrelin is essential for its growth hormone (GH)-releasing activity and appetite regulation mediated through growth hormone secretagogue receptor (GHS-R). We demonstrated that both ghrelin and des-acyl ghrelin evoke vasodilatation at remarkably low concentrations compared to acetylcholine (ACh) in phenylephrine (PE)-constricted perfused rat mesenteric vascular bed (MVB). This was abolished in endothelium-denuded preparations and in endothelium-intact preparations exposed to either a calcium-activated potassium channel (K_{Ca}) blocker or a depolarizing stimulus. While K_{ATP} channel blockade, nitric oxide synthase and cyclooxygenase inhibition had no effect, the responses were abolished in the presence of combinations of apamin and charybdotoxin, apamin and TRAM-34, and ouabain and Ba^{2+}. The GHS-R antagonist, [D-Lys^3]-GHRP-6, *per se* evoked vasodilatation. Inclusion of L-756867, a peptide antagonist of classical GHS-R, failed to evoke any vasodilator response or to affect vasodilatation evoked by ghrelin. Both non-peptide agonists of GHS-R, L-166446 and L-163255, demonstrated concentration-dependent decreases in perfusion pressure. All short peptides encompassing the first 20, 16, 10, 6, 4, and 3 residues of des-acyl ghrelin were able to evoke vasodilator responses to the same extent as des-acyl ghrelin. However, vasodilatation to single amino acids, L-serine and glycine, were significantly attenuated. Streptozotocin (STZ)-induced diabetes increased plasma ghrelin concentration. Diabetes for 4-weeks did not cause any significant reduction in ghrelin-

evoked vasodilatation, whereas 8-weeks diabetes significantly reduced ghrelin-evoked vasodilatation. In contrast to ghrelin, there was a duration-dependent fall in vasodilator response to ACh from 4- to 8-weeks diabetes. These data suggest that the vasodilatation evoked by ghrelin is mediated by endothelium-dependent hyperpolarization (EDHF) by mechanism(s) that are independent of classical GHS-R activation. In addition, EDHF-dependent ghrelin-evoked vasodilator responses may not be affected, at least in the early stages of STZ diabetes, whereas the responses to ACh, predominantly mediated through nitric oxide, are progressively diminished right from the early stages of endothelial dysfunction in STZ diabetic rats.

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DEDICATED TO MY BELOVED HUSBAND

MANOUCHEHR HASHEMI

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LIST OF KEY ABBREVIATIONS

AA	Arachidonic Acid
ACE	Angiotensin Converting Enzyme
ACh	Acetylcholine
ACTH	Adrenocorticotropin Hormone
AGRP	Agouti Gene-Related Protein
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ARC	Arcuate Nucleus
ATP	Adenosine Triphosphate
Ba^{2+}	Barium Chloride Dehydrate
BH_4	Tetrahydrobiopterine
BK_{Ca}	Big Conductance Calcium-Activated Potassium Channels
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular Free Calcium Concentration
cGMP	Cyclic 3', 5'-Guanosine Monophosphate
ChTX	Charybdotoxin
CNP	C-Type Natriuretic Peptide
COX	Cyclooxygenase
CR curve	Concentration-Response Curve
CYP 450	Cytochrome P450
[D-Lys^3]-GHRP-6	[D-Lys^3]-Growth Hormone Releasing Peptide-6

DMSO	Dimethyl Sulfoxide
EC	Endothelial Cells
EC_{50}	Concentration of Agonist Required to Evoke Half Maximal Response
EDHF	Endothelium-Derived Hyperpolarizing Factor
EDRF	Endothelium-Derived Relaxing Factor/Nitric Oxide
EDTA	Ethylene Diaminetetraacetic Acid
EET	Epoxyeicosatrienoic Acid
E_{max}	Maximum Effect
Endo +	Endothelium-Intact
Endo -	Endothelium-Denuded
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
GH	Growth Hormone
GHRH	Growth Hormone Releasing Hormone
GHS	Growth Hormone Secretagogue
GHS-R	Growth Hormone Secretagogue Receptor
GHSR-1a	Growth Hormone Secretagogue Receptor Type 1a
GIRK	G Protein-Gated Inwardly Rectifying Potassium Channel
GPCR	G Protein-Coupled Receptor
G protein	Guanosine Nucleotide-Binding Protein
IGF-1	Insulin-Like Growth Factor-1
IgG	Immunoglobulin G
IK_{Ca}	Intermediate Conductance Calcium-Activated Potassium

	Channel
i.p.	Intraperitoneal
IP_3	Inositol 1, 4, 5-triphosphate
IRAG	IP_3 Receptor-Associated Protein Kinase G-I Substrate
K^+	Potassium
K_{ATP}	Adenosine Triphosphate-Sensitive Potassium Channels
K_{ir}	Inward Rectifying Potassium Channels
L-NAME	N(G)-Nitro-L-Arginine Methyl Ester
MAP	Mean Arterial Pressure
MBS	Myosin-Binding Subunit
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
mRNA	Messenger Ribonucleic Acid
MVB	Mesenteric Vascular Bed
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
NOSI	Nitric Oxide Synthase Inhibitor
NPR-C	Natriuretic Peptide Receptor-C
NPY	Neuropeptide Y
PE	Phenylephrine Hydrochloride
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PKG	cGMP-Dependent Protein Kinase

POMC	Proopiomelanocortin
PP	Perfusion Pressure
PRL	Prolactin
RIA	Radioimmunoassay
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RYR	Ryanodine Receptor
SD	Sprague Dawley
Ser3	Third Serine Residue
sGC	Soluble Guanylyl Cyclase
SK_{Ca}	Small Conductance Calcium-Activated Potassium Channels
SNP	Sodium Nitroprusside
SOD	Superoxide Dismutase
SR	Sarcoplasmic Reticulum
STOCs	Spontaneous Transient Outward Currents
STZ	Streptozotocin
TBA	Tetrabutylammonium
TM	Transmembrane
TP receptors	Endoperoxide-Thromboxane A_2 Receptors
TRAM-34	1-(2-Chlorophenyl)Diphenyl Methyl)-1 H-Pyrazole
TXA_2	Thromboxane A_2
VMN	Ventromedial Nucleus
VSMC	Vascular Smooth Muscle Cells

1. INTRODUCTION

1.1. Endothelium and Regulation of Vascular Tone

The entire blood vessel consists of a number of layers of different cells and tissues, each performing different functions in different arteries, capillaries, and veins. Large arteries are typified by an outermost layer of smaller arteriols and venules (adventitia) that provide nutrient, and connective tissue components to provide support and anchorage. The middle layer (media) is composed mostly of smooth muscle cells (that provide vascular tone) with a smaller number of fibroblasts. The innermost layer, the intima, which interfaces directly with the blood, is composed entirely of endothelial cells (EC) sitting on a basement membrane that separates it from the media. The EC do not form a passive layer between the blood and the rest of the blood vessel, but quite the opposite, they perform vital functions in haemostasis, in managing tissue fluid and leukocyte movement into the vessel wall, and in regulating vascular tone (Esper et al., 2006).

The endothelium plays a primary role in the local control of vascular tone by releasing contracting and relaxing factors both under basal conditions and when activated by neurotransmitters, hormones, autocoids, or physical stimuli (Thomas et al., 1993; Taddei et al., 2006). Endothelium-derived contracting factors are partly produced by the cyclooxygenase (COX) pathway from arachidonic acid (AA). The products of COX mediating the contractions are thromboxane A_2 (TXA_2) and endoperoxides, such as prostaglandin H_2 (PGH_2), which activate endoperoxide-thromboxane A_2 receptors (TP receptors) on vascular smooth muscle cells (VSMC) (Vanhoutte, 1993; 1996; 2001).

The COX pathway is also a source of superoxide anions, which can mediate endothelium-dependent contractions either by the breakdown of nitric oxide (NO) or by direct effects on VSMC. EC also produce the 21-amino acid peptide endothelin-1 (ET-1). Among the three forms of this peptide, ET-1, ET-2, and ET-3, ECs appear to produce exclusively ET-1 (Luscher and Vanhoutte, 1990). The peptide ET-1 is a potent vasoconstrictor that under normal conditions circulates at low levels. The renin-angiotensin system (RAS) is another cascade of enzymatic reactions that ends in the production of a potent vasoconstrictor, angiotensin II (Ang II). Renin, produced by the kidney, acts on angiotensinogen, produced by the liver, degrading it to angiotensin I (Ang I), which in turn is acted on by circulating or tissue angiotensin converting enzyme (ACE) and hydrolyzed to Ang II. The EC release Ang II, which through the activation of Ang I receptors causes contraction of blood vessels (Esper et al., 2006).

Endothelium-dependent relaxation, however, is mediated through three different mechanisms: endothelium-derived relaxing factor (EDRF, NO), prostacyclin (PGI_2), and an as yet unidentified endothelium-derived hyperpolarizing factor (EDHF) (He, 2005). The contribution of these three vasodilators to vascular relaxation varies among different vessels and vascular beds (Shimokawa et al., 1996). It has been reported that the contribution of EDHF to endothelium-dependent vasodilatation increases as the vessel size decreases (Tomioka et al., 1999; Fitzgerald et al., 2005).

1.1.1. Endothelium-Derived Relaxing Factor (NO)

Furchgott and Zawadzki (1980) postulated that upon stimulation by acetylcholine (ACh), EC release a diffusible vasodilator substance, which relaxes the blood vessels. Ignarro and co-workers (1987) clarified the chemical identity of EDRF as NO. NO is formed in EC from the amino acid L-arginine by oxidation of its guanidine-nitrogen terminal by the enzyme endothelial NO synthase (eNOS), which requires calmodulin, calcium (Ca^{2+}), nicotinamide adenine dinucleotide phosphate (NADPH), and tetrahydrobiopterine (BH_4) (He, 2005). Several isoforms of the enzyme occur not only in EC, but also in platelets, macrophages, VSMC, and in the brain (Radomski et al., 1990). It appears that the endothelial enzyme is a constitutive Ca^{2+}-dependent enzyme and produces picomoles of NO, whereas the enzyme in VSMC is an inducible Ca^{2+}-independent enzyme, which produces nanomoles of NO under stimulation by endotoxin, interleukin-1, and tumor necrosis factor (Luscher and Tanner, 1993). In the vascular system, the endothelium-derived NO diffuses out of EC and a portion of it arrives at the underlying smooth muscle cell layer (Rubanyi et al., 1986). It is generally conceded that vasodilatation to NO is associated with stimulating the soluble enzyme guanylyl cyclase (sGC), and in turn, the formation of cyclic 3', 5'-guanosine monophosphate (cGMP) in smooth muscle cells, which results in the activation of a family of serine/thereonine protein kinases, cGMP-dependent protein kinase (PKG) (Lincoln et al., 2001). Intracellular events downstream activation of PKG, however, are still much debated.

There appears to be three major pathways regulated by NO/cGMP/PKG signaling that induce relaxation in smooth muscle cells: (1) Decrease in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$), (2) Ca^{2+} desensitization, and (3) Thin filament regulation. It is likely that no single pathway acts exclusively or independently in any one type of smooth muscle cell. However, the relative importance of the various pathways leading to cGMP-induced relaxation is likely to be different in cells from large arteries compared to those from microvessels (Lincoln et al., 2001). A comprehensive picture demonstrating all the proposed mechanisms of NO/cGMP/PKG-mediated relaxation of smooth muscle cells is shown in Figure 1.

(1) Decrease in $[Ca^{2+}]_i$. The first mechanism proposed for cGMP-dependent relaxation of smooth muscle cells was the reduction of $[Ca^{2+}]_i$ (Johnson and Lincoln, 1985). So far, as increases in Ca^{2+} were required for myosin light chain (MLC) phosphorylation and contraction, the reduction in cytosolic free Ca^{2+} by cGMP was seen primarily as a reversal of the contractile mechanism. Several sites of action have been proposed to account for NO/cGMP/PKG-mediated reduction of cytosolic Ca^{2+} (Hofmann et al., 2000; Lincoln et al., 2001):

(a) PKG has been demonstrated to directly inhibit voltage-gated Ca^{2+} channels (Hofmann et al., 2000).

(b) Phosphorylation of sarcoplasmic reticulum (SR) protein, phospholamban, has been suggested as another mechanism through which PKG contributes to the influx of Ca^{2+} into the SR (SR Ca^{2+} loading) through Ca^{2+}-ATPase pump and thus decreased $[Ca^{2+}]_i$ and relaxation (Mundian-Weilenmann et al., 2000).

(c) cGMP/PKG is involved in the regulation of the inositol 1, 4, 5-triphosphate (IP_3) receptor. Previous studies demonstrated that the smooth muscle IP_3 receptor (IP_3R) is phosphorylated by PKG (Haug et al., 1999). PKG-dependent phosphorylation of IP_3R decreases Ca^{2+} release from the SR, and this effect is dependent on the targeting of PKG to the SR (Schlossman et al., 2000). A targeting protein for PKG, termed IP_3 receptor-associated PKG-I substrate (IRAG), has been characterized and shown to be necessary for IP_3R phosphorylation (Lincoln et al., 2001).

(d) Several types of potassium (K^+) channels have been shown to be involved in NO/PKG-mediated relaxation. K^+ channel activation stabilizes the cell by increasing efflux of K^+ ions, leading to hyperpolarization, and thus decreased cell excitability (He, 2005; Ghatta et al., 2006). NO/cGMP/PKG has been shown to activate adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels in rat mesenteric arteries as well as in guinea pig coronary arteries (Katakam et al., 1999; Triggle et al., 2004). In addition, NO also relaxes various blood vessels by increasing the gating of big conductance calcium-activated potassium (BK_{Ca}) channels either directly or via PKG (Mistry and Garland, 1998; Lincoln et al., 2001). It has been reported that PKG regulates the phosphorylation of the BK_{Ca} channels by acting as a direct catalyst for phosphorylation (Fukao et al., 1999). Increased BK_{Ca} activity also appears to arise from PKG-mediated increased activity of spontaneous transient outward currents (STOCs) produced by small bursts of Ca^{2+} released from the superficial SR through ryanodine receptors (RYR) (Nelson et al., 1995). Increased BK_{Ca} channel activity hyperpolarizes the smooth muscle membrane and reduces $[Ca^{2+}]_i$ through inhibition of voltage-gated Ca^{2+} channels. As a result, a decrease in global Ca^{2+} occurs, leading to the dephosphorylation of MLC and relaxation (Lincoln et al., 2001).

(2) Ca^{2+} desensitization. Ca^{2+} sensitization is the term used to describe the increase in contractile activity produced by G protein (guanine nucleotide binding protein)-coupled agonists. MLC phosphorylation at serine-19 is necessary for actin activation of myosin ATPase, and subsequent cross-bridge cycling and contraction. Two key enzymes involved in the control of MLC phosphorylation are MLC kinase (MLCK), a Ca^{2+}/calmodulin-activated kinase, and a MLC phosphatase (MLCP), a serine/thereonine protein phosphatase type I. With respect to the regulation of MLCK, there has been no firm evidence that PKG-dependent phosphorylation of MLCK inhibits its activity (Van Riper et al., 1997). Most of the recent work suggests that PKG activates MLCP, thereby inhibiting MLC phosphorylation and contraction (Lincoln et al., 2001). An important component of the Ca^{2+} sensitization mechanism is the Rho-Rho kinase-dependent inhibition of MLCP following agonist stimulation of contraction (Gong et al., 1996). PKG apparently opposes Rho kinase-induced inhibition of the phosphatase by phosphorylating the myosin-binding subunit (MBS) of MLCP, thus activating the catalytic subunit of the phosphatase (Surks et al., 1999; Torrecillas et al., 2000). These studies have also shown that PKG is targeted to the MBS, thus placing it in an ideal location to activate MLCP. There are further reports that suggest that cGMP/PKG may interfere with the Rho-dependent activation of Rho kinase, possibly through the phosphorylation of Rho (Sauzeau et al., 2000).

(3) Thin-filament regulation. The role of the thin filament in the regulation of smooth muscle cell contraction has been somewhat controversial. Thin filament binding proteins regulate and contribute to the contractile activity of the cell, but there have been few reports regarding the role of second messenger regulation of thin filament protein function. More recently, the thin filament/actin binding protein, 20-KDa heat shock-related protein (HSP20), gained some attention as a potential target for PKG action (Yamboliev et al., 2000). HSP20 was reported to be a specific target for PKG-dependent phosphorylation. Phosphorylation of HSP20 serine-16 by PKG is associated with relaxation of smooth muscle.

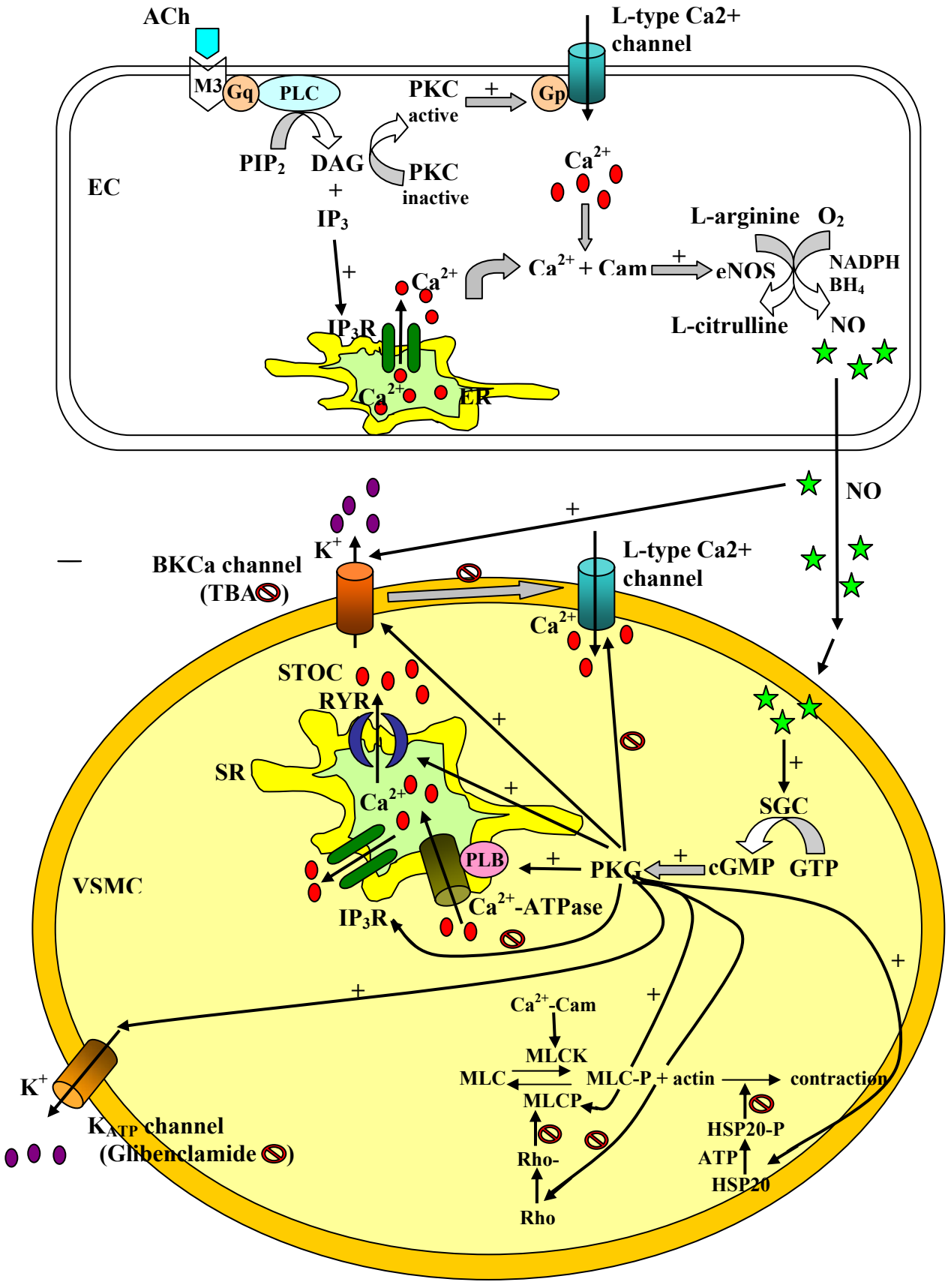

Figure 1. Schematic view of NO formation in EC and its effects on VSMC tone leading to endothelium-dependent vasodilatation.

1.1.2. Prostacyclin (PGI_2)

PGI_2 was the first defined relaxing factor derived from endothelium (Moncada et al., 1976). When activated by stimuli, the enzyme phospholipase A_2 (PLA_2) in EC converts membrane phospholipids to AA that is subsequently metabolized to PGI_2 by the enzyme COX. The vasodilatation induced by PGI_2 is mediated by the rise of cyclic 3', 5'-adenosine monophosphate (cAMP) through increased activity of adenylyl cyclase (AC), and the subsequent activation of cAMP-dependent protein kinase (PKA) in VSMC (Luscher and Tanner, 1993; Hecker, 2000). Similar to NO/cGMP/PKG, activation of PGI_2/cAMP/PKA pathway also leads to the reduction of $[Ca^{2+}]_{i,}$ decreased sensitivity of the contractile apparatus to Ca^{2+}, and regulation of the thin filament (Luscher and Tanner, 1993; Hecker, 2000; Lincoln et al., 2001; Pfitzer, 2001; He, 2005). PKA has been shown to phosphorylate, and thus directly inhibit voltage-gated Ca^{2+} channels. The involvement of different types of K^+ channels has also been demonstrated in the PGI_2-mediated hyperpolarization in different vascular beds (He, 2005). PGI_2 has been shown to activate BK_{Ca} channels in three different ways: (1) Direct channel activation through its G_s-coupled receptor, which is cAMP-independent; (2) Phosphorylation and activation of the channel by PKA; and (3) Phosphorylation of RYR channels on the SR, and therefore increased small bursts of Ca^{2+} near BK_{Ca} channels on the plasma membrane (Schubert et al., 1996; Lincoln et al., 2001; Ghatta et al., 2006). In addition to the activation of BK_{Ca} channels, PGI_2 has been shown to activate smooth muscle cell K_{ATP} and inward-rectifier K^+ (K_{ir}) channels through the secondary rise in cAMP/PKA after binding to its corresponding cell surface receptor (Li et al., 1997).

Similar to cGMP/PKG, it is well established that cAMP/PKA also decreases Ca^{2+} sensitivity of contraction in both intact and permeabilized smooth muscles (Pfitzer, 2001). *In vitro*, PKA was shown to phosphorylate MLC kinase (MLCK) at two sites, site A and B, located carboxy terminal to the calmodulin binding domain. Phosphorylation of site A, but not site B, decreased the affinity of MLCK for the Ca^{2+}/calmodulin complex. However, agonists that elevated cAMP *in vivo* had negligible effects on the phosphorylation of site A and Ca^{2+} activation of MLCK (Pfitzer, 2001). This suggested that cAMP may desensitize smooth muscle by an alternative, yet not defined, mechanism. Activity of MLCP is increased by cGMP/PKG, and perhaps also cAMP/PKA (Pfitzer, 2001). PKA has also been involved in the regulation of thin filaments; PKA activation, like PKG, also phosphorylates the thin filament binding protein, HSP20 (Brophy et al., 1999). Phosphorylation of HSP20 is associated with relaxation of VSMC (Lincoln et al., 2001).

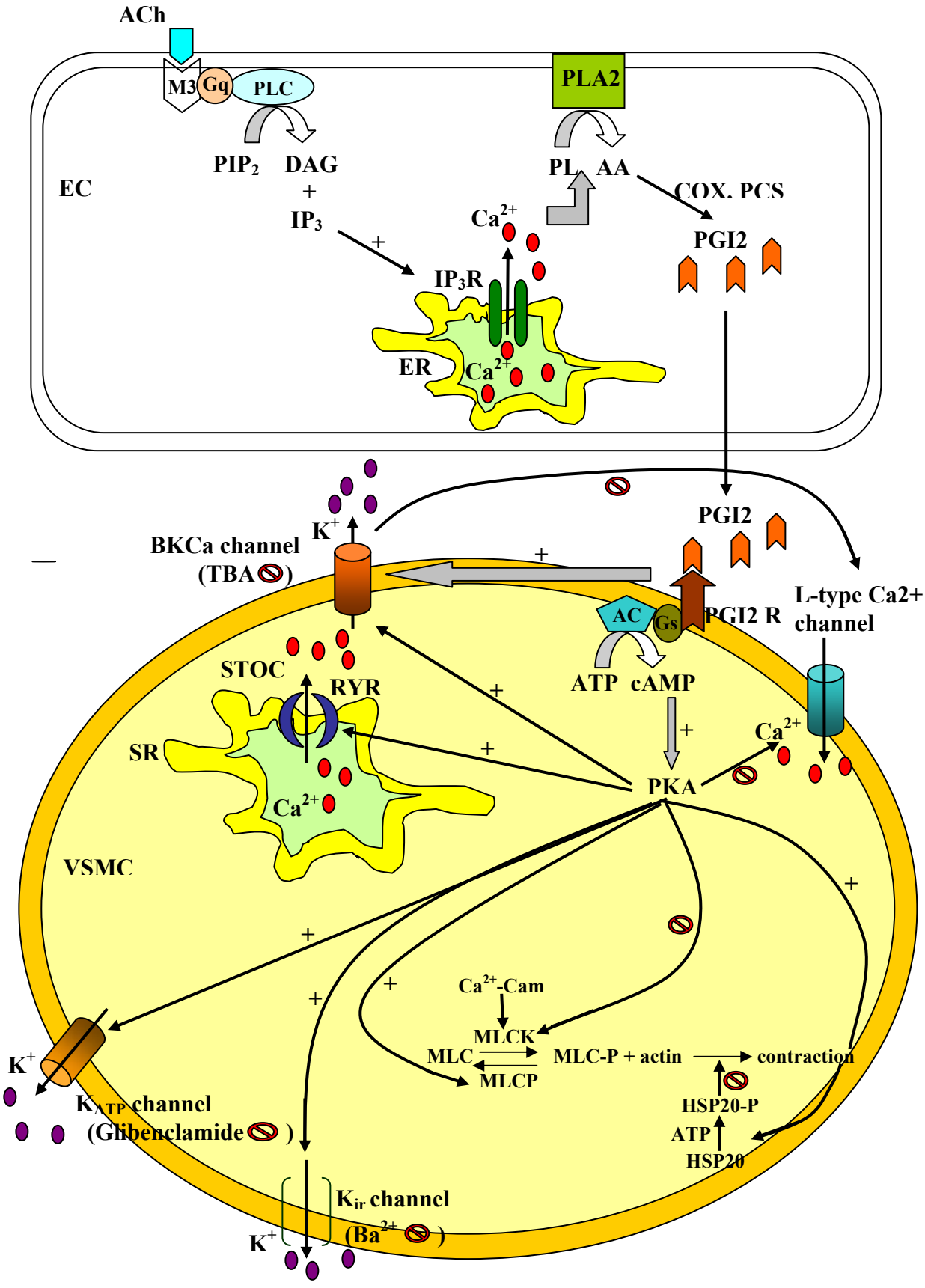

Figure 2. Schematic view of PGI_2 formation in EC and its effects on VSMC leading to endothelium-dependent vasodilatation.

1.1.3. Endothelium-Derived Hyperpolarizing Factor (EDHF)

Endothelium-dependent vasodilatation to different vasodilator agonists in various isolated blood vessels and vascular preparations is only partially inhibited, or not changed, by a combination of a NOS inhibitor and a COX inhibitor (Luscher and Tanner, 1993). EC, therefore, appear to release another relaxing factor distinct from NO and PGI_2. Endothelium-derived hyperpolarizing factor (EDHF) is defined as the non-NO/PGI_2 endothelium-derived vasodilator factor that mediates VSMC hyperpolarization and therefore relaxation (Ge et al., 2000; Triggle et al., 2004). It has been suggested that, in most instances, the agonist (eg. ACh)-stimulation of EC increases $[Ca^{2+}]_i$, which in turn results in the extrusion of K^+ ions through both small- and intermediate-conductance calcium-activated potassium (SK_{Ca} and IK_{Ca}, respectively) channels. Increased K^+ concentration in the myo-endothelial space activates Na^+/K^+-ATPase pump and inward rectifying K^+ (K_{ir}) channels on VSMC, leading to hyperpolarization and relaxation of VSMC (Triggle et al., 2004). Thus, the hypothesis is that it is the apamin-sensitive SK_{Ca} and the charybdotoxin (ChTX)-sensitive IK_{Ca} channels on the EC that regulate EDHF release, and the ouabain-sensitive Na^+/K^+-ATPase and Ba^{2+}-sensitive K_{ir} channels on the VSMC that mediate vasodilatation of EDHF (Campbell et al., 1996; Mombouli and Vanhoutte, 1997; Edwards et al., 1998; Triggle et al., 2004). In most cases, an EDHF-mediated vasodilatation lacks sensitivity to either BK_{Ca} blocker, iberiotoxin, or K_{ATP} channel blocker, glibenclamide (Edwards et al., 1998; Hecker, 2000).

However, the identity or chemical nature of EDHF is still under much discussion. It is still not clear if EDHF is K^+ ions themselves, which accumulate in myo-endothelial space upon stimulation of SK_{Ca} and IK_{Ca}, or if it is just the agonist-induced hyperpolarization that is propagated from EC to the underlying VSMC via myoendothelial gap junctions. It may also be a substance or molecule that is released from EC upon their agonist stimulation into myoendothelial space and activates its receptors on EC and VSMC, leading to their hyperpolarization and thus relaxation. The potential role of K^+ ions as EDHF was first suggested by Edwards et al. (1998) who reported that in the rat hepatic artery, ACh mediated an increase in K^+ concentration, as measured by a K^+-sensitive microelectrode. They also recorded membrane potential with sharp glass microelectrodes and illustrated that ACh can hyperpolarize both VSMC and EC. An increase in extracellular K^+ concentration by 5 mmol/L mimicked the effects of ACh and comparable data was reported for the rat mesenteric artery preparation. Hence the data from Edwards et al. (1998) provided strong support that small changes in extracellular K^+, which were already known to cause VSMC relaxation, may represent the putative EDHF. However, up to now, the proposed idea has not been universally accepted (Ding and Trrigle, 2000). Moreover, Hecker (2000) also represented that an increase in myo-endothelial K^+ ion level to an extent of double its normal concentration is unlikely to be effective in thick-walled arteries such as coronary arteries. It is noteworthy to mention that, however, the identity of EDHF may differ in different vasculatures and vascular beds (He, 2005).

Myo-endothelial gap junctions have been reported to be able, via intercellular channels, to provide a pathway for the passage of small water-soluble molecules (<1000 Da), including cAMP, cGMP, IP_3, as well as inorganic ions including Ca^{2+} , but not proteins, to pass between adjacent connecting cells (Kumar and Gilula, 1996). Electrical coupling between EC and VSMC should also be considered, especially for the microcirculation, in which the relative contribution of EDHF to endothelium-dependent vasodilatation appears to be much more prominent than in conduit arteries (Mombouli and Vanhoutte, 1997; Hecker, 2000).

In addition to the above, there are several candidate molecules that, to varying extents, fulfill some of the criteria that would be expected of an EDHF and a number of these are enzymatically derived from AA (Triggle et al., 2004): (1) Epoxyeicosatrienoic acids, EETs. EETs are generated via an epoxygenase from AA and are attractive candidates for EDHF in a number of vascular beds, notably in the coronary vascular bed (Fleming, 2001). Cytochrome P450 (CYP 450) enzymes can metabolize AA to vasoactive substances and the CYP 2J2 isoform, that is expressed in both human EC and VSMC, can generate EETs and an accumulation of evidence suggests that these products have important physiological and pathophysiological effects in the cardiovascular system (Fleming, 2001). Nevertheless, the role of EETs as EDHF has been questioned because their action does seem to be primarily mediated via iberiotoxin-sensitive BK_{Ca} channels and not the apamin- and ChTX-sensitive mechanism that is typical of EDHF (McGuire et al., 2001).

(2) Isoprostanes. Isoprostanes have been hypothesized by Janssen (2002) to be candidate molecules for EDHF. Isoprostanes are generated via the action of PLA_2, from polyunsaturated fatty acids, such as AA, under circumstances when oxygen free radicals are elevated. Isoprostanes, such as thromboxanes, may serve as endothelium-derived contracting factors, as well as EDHF, as for instance 8-isoPGE_2 is a vasoconstrictor. EDHF-mediated responses have been reported to be sensitive to PLA_2 inhibitors (McGuire et al., 2001; Janssen, 2002).

(3) Anandamide. Anandamide, an endogenous cannabinoid, is another AA product via a transacylase and also has been advanced as a possible EDHF in some vascular beds (Randal et al., 1996). The main reason for this hypothesis was that the EDHF-mediated vasodilatation of the rat mesenteric artery is impaired by the CB1 cannabinoid receptor antagonist SR-141716A, and that the anandamide-induced vasodilatation in this vascular bed is sensitive both to SR-141716A and to membrane depolarization through raising K^+ concentration (Chataigneau et al., 1998; Randall and Kendall, 1998). Subsequent studies in this, as well as in several other vascular preparations, have demonstrated, however, that anandamide is a very poor hyperpolarizing factor and that its effects on K^+ channel activity in VSMC can be clearly distinguished from those of EDHF (Chataigneau et al., 1998). It thus seems unlikely that anandamide *per se* is an EDHF.

(4) Hydrogen peroxide (H_2O_2). H_2O_2 may be produced in the endothelium from superoxide anions via superoxide dismutase (SOD) and in situations where the eNOS substrate, L-arginine, or co-factor, tetrahydrobiopreine (BH4), have been depleted, for

instance in disease states where there is high oxidative stress (Pannirselvam et al., 2002). eNOS can also generate superoxide anions as can CYP and NADPH (Stroes et al., 1998). Matoba et al. (2000) determined ACh-mediated vasodilatation of small mesenteric arteries from eNOS-/- mice and reported that catalase, an enzyme that dismutates H_2O_2 to water and oxygen, inhibited EDHF-mediated hyperpolarization and vasodilatation. It must be noted, however, that catalase has been shown to inhibit COX-dependent 8-iso $PGF_{2\alpha}$, thus suggesting that H_2O_2 may not be the final mediator of EDHF (Watkins et al., 1999). In addition, H_2O_2-mediated vasodilatation was shown to lack the sensitivity to the combination of apamin and ChTX (Beny and der Weid, 1991).

(5) C-type natriuretic peptide (CNP). It has been recently demonstrated that in the rat mesenteric vasculature, the release of CNP accounts for the biological activity of EDHF (Chauhan et al., 2003; Sandow and Tare, 2007). EDHF-dependent vasodilatation is concomitant with liberation of endothelial CNP. CNP release and EDHF responses are profoundly suppressed after endothelial denudation (Chauhan et al., 2003). In addition, similar to EDHF, CNP produces VSMC hyperpolarization that is attenuated in the presence of high K^+ concentration, G_i protein inhibitor pertusis toxin, the G protein-gated inwardly rectifying K^+ channel (GIRK) inhibitor tertiapin, and a combination of Ba^{2+} (K_{ir} channel blocker) plus ouabain (Na^+/K^+-ATPase inhibitor) (Chauhan et al., 2003). Responses to both EDHF and CNP are mimicked by the selective natriuretic peptide receptor-C (NPR-C) agonist, $cANF^{4-23}$. Therefore, the data demonstrate that ACh-evoked release of endothelial CNP activates NPR-C on VSMC that via G_i coupling promotes Ba^{2+}/ouabain-sensitive hyperpolarization (Chauhan et al., 2003). However, in addition to the information above, key data supporting the hypothesis that CNP is EDHF

are still required. In the rat mesenteric artery, a crucial investigation is whether the combination of ChTX-TRAM-34 and apamin prevents CNP release from the endothelium, since blockade of the EDHF response by these agents is a defining feature of EDHF in this vessel (Grane et al., 2003; Sandow and Tare, 2007).

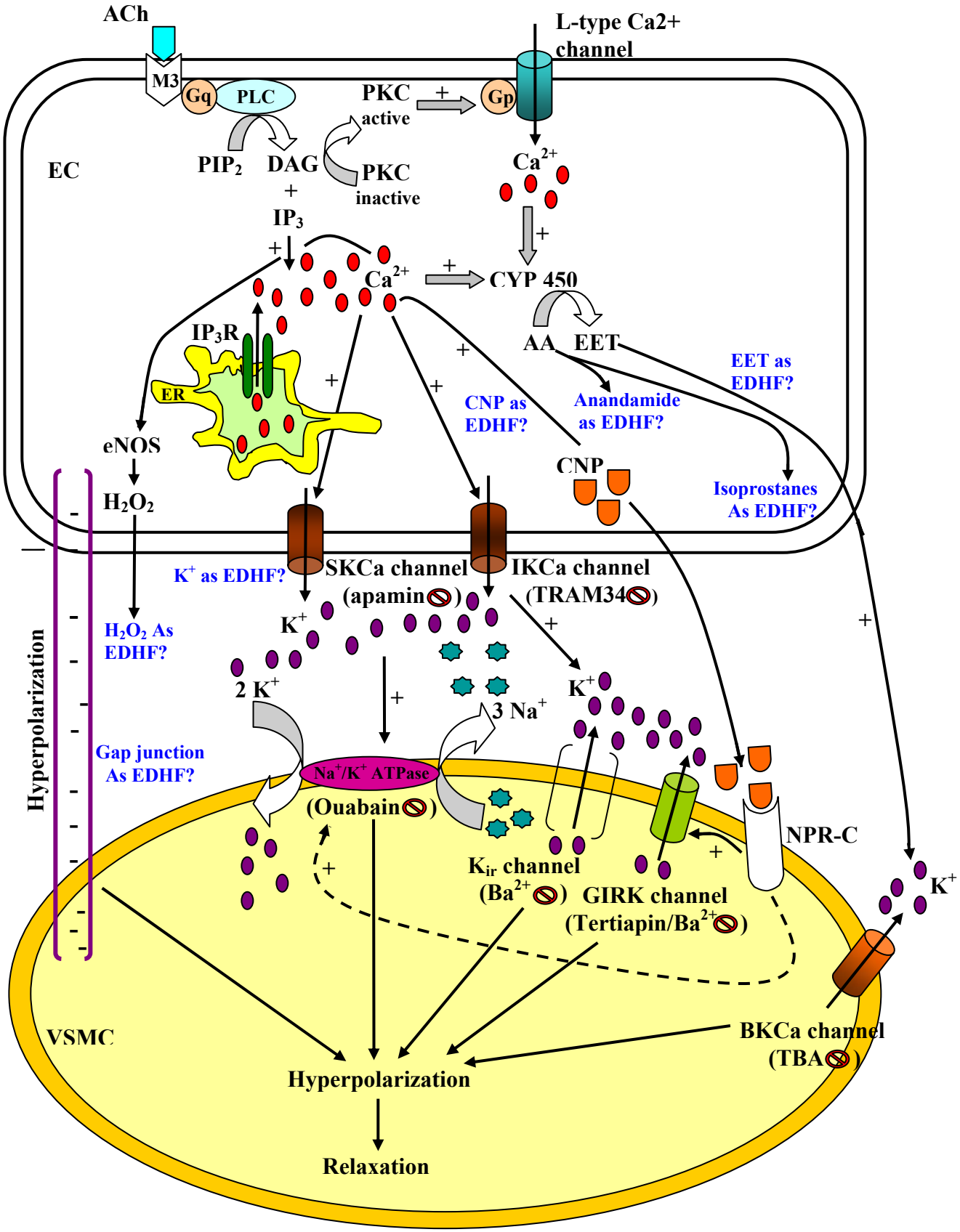

Figure 3. Schematicview of EDHF formation in EC and its effects on VSMC tone leading to endothelium-dependent vasodilatation.

1.2. ENDOTHELIAL DYSFUNCTION AND CARDIOVASCULAR DISEASE

Because of its strategic anatomical position, endothelium is a favorite early target organ of cardiovascular risk factors and cardiovascular disease (Thuillez and Richard, 2005). Intact endothelial function is essential to maintain an adequate vascular tone, to prevent platelet aggregation in the intimal surface of blood vessels, to prevent smooth muscle proliferation, and to prevent atherosclerosis (He, 2005). Correct vascular tone is regulated by a balance between endothelial-derived vasodilators and vasoconstrictors that act on the smooth muscle cells of the intima (Blann, 2003). Endothelial dysfunction, however, is defined as an impaired endothelium-dependent vasodilatation (for instance to ACh) and also is associated with the release of contracting factors, including prostanoids and ET-1 (Taddei et al., 2006). A number of cardiovascular disease conditions, such as diabetes mellitus and hypertension, share the specific pathophysiological feature of endothelial dysfunction.

1.2.1. Diabetes Mellitus Type I

Macro- and microvascular complications are the main cause of morbidity and mortality in patients with diabetes mellitus (Panus et al., 2003). Endothelial dysfunction is considered to be a critical initiating factor leading to the vascular complications of diabetes (Triggle et al., 2004). Impaired vasodilator responses to different endothelium-dependent agonists have been reported and consistently demonstrated in different isolated vessels, isolated perfused vascular beds/organs, as well as by *in vivo* studies that involve chemically-induced and genetic models of type I diabetes (Piper and Gross,

1988; Endo et al., 1995; Poston and Taylor, 1995). Similarly, impaired endothelium-dependent vasodilatation has been demonstrated in patients with type I diabetes in the absence of clinical complications (Johnstone et al., 1993; Nitenberg et al., 1993; Clarkson et al., 1996). Tables 1 (A-C) and 2 summarize some of the studies demonstrating endothelial dysfunction in both human and animal models of type I diabetes.

There have been many reports concerning the endothelial factors that contribute to the attenuation of endothelium-dependent vasodilatation in diabetic models. The effects of diabetes on NO-mediated responses have been extensively studied. Several studies have clearly demonstrated that endothelial dysfunction can occur despite blockade of EDHF and PGI_2 synthesis, suggesting a role for deficits of endothelium-derived NO (Pieper, 1998). Basal NO production has been shown to be diminished in diabetes (Pieper, 1997). Additionally, it has been previously reported that agonist-evoked NO-mediated responses are also reduced in type I diabetes mellitus in both animal models and humans (El-Kashef, 1996; Pieper, 1998; De Vriese et al., 2000). To our knowledge, there has been no direct measurement of NO from diabetic arteries using NO electrode or analysis of NO level in tissues by sensitive assay procedures. According to indirect measurements, however, measurement of cGMP levels in rat, and rabbit aorta as well as in mesenteric arteries isolated from diabetic animals treated with alloxan demonstrated that diabetes decreased both basal and ACh-stimulated cGMP production (Abiru et al., 1990; Miller et al., 1994). Treatment with L-NAME was shown to promote a greater degree of rightward shift in the dose-response curve to ACh in diabetic rats compared to age-matched control rats (Endo et al., 1995).

Despite many investigations reporting impaired NO-mediated responses in diabetes, only few studies have focused on the contribution of EDHF to endothelial dysfunction in diabetes. It has been reported that diabetes mellitus affects EDHF-mediated vasodilatation. In the absence of specific inhibitors of EDHF, all evidence to date is inevitably indirect. NOS- and COX-resistant vasodialatation to ACh or bradykinin was profoundly impaired in the Langendroff perfused hearts, in the isolated perfused kidneys, in the isolated renal arteries, as well as in the renal microcirculation *in vivo* (Dae et al., 1993; Fulton et al., 1996; Quilley et al., 1996; De Vriese et al., 1999). Other authors, interestingly, observed pronounced deficits of ACh-induced hyperpolarization simultaneously with decreased ACh-induced vasodilatation in the presence of NOS- and COX-blockade in isolated mesenteric arteries from streptozotocin (STZ)-induced diabetic rats and mice (Fukao et al., 1997; Makino et al., 2000; Morikawa et al., 2005). In the isolated rat aorta, however, no evidence was found concerning a decreased contribution of EDHF to the endothelium-dependent vasodilatation (Endo et al., 1995). Since the contribution of EDHF is most prominent in smaller vessels, it is not suprising that evidence for a role for EDHF in diabetic endothelial dysfunction is restricted to resistance artery and whole organ studies.

Table 1. Experimental studies demonstrating endothelium-dependent and -independent vasodilatation (EDVD/EIVD) and resroration of the defect.

A. Isolated Vessels **Reference**	**Diabetic Model**	**Vessel**	**EDVD**	**EIVD**	**Restoration**
Tesfamariam et al., 1989; 1993	Rabbit, AL, 6w	Abdominal aorta	ACh ↓	SNP ↔	COX blockade (T) TP-RA (T)
Hattori et al., 1991	Rat, STZ, 8-12w	Thoracic aorta	ACh ↓	SNP ↔	SOD (T) catalase
Cameron & Cotter 1992	Rat, STZ, 3m	Thoracic aorta	ACh ↓		
Shimizu et al., 1993	Rat, STZ, 10w	Thoracic aorta	ACh ↓		TP-RA (T)
Otter & Chess-Williams 1994	Rat, STZ, 2w	Thoracic aorta	Carbachol ↓	SNP ↔	
Pieper & Peltier 1995	Rat, STZ, 2m	Thoracic aorta	ACh ↓	Nitro glycerin ↔	L-arginine (T)
Keegan et al 1995	Rat. STZ, 2m	Thoracic aorta	ACh ↓		
Pieper et al., 1996	Rat, BB, 2m	Thoracic aorta	ACh ↓		SOD (P)
Pieper et al., 1997	Rat, STZ, 2m	Thoracic aorta	ACh ↓	Nitro glycerin ↔	SOD + catalase (T)
Pieper & Siebeneich 1998	Rat, STZ, 2m	Thoracic aorta	ACh ↓	SNP ↔	
Taylor et al., 1992	Rat, STZ, 5-6w	Mesenteric artery	ACh ↓	SNP ↔	
Diederich et al., 1994	Rat, STZ, 6-24w	Mesenteric artery	ACh ↓	SNP ↔	SOD (P)
Heygate et al., 1995	Rat, BB, 6-8w	Mesenteric artery	ACh , BK ↓	SNP ↔	SOD catalase (P)
Fukao et al., 1997	Rat, STZ, 8-12w	Mesenteric artery	ACh ↓	Pinacidil ↔	
Palmer et al., 1998a, b	Rat, STZ, 4-5w	Mesenteric artery	ACh ↓	SNP ↔	Vitamin (C)(P) Vitamin (C+E) (P)

Dai et al., 1993	Rat, STZ, 6-24w	Interlobar artery	ACh ↓	SNP ↔	SOD (N)
Hill & Ege 1994	Rat, STZ, 4-6w	Skeletal Muscle artery	ACh ↓		
Morikawa et al., 2005	Mice, STZ, 10w	Mesenteric artery	ACh ↓	SNP ↔	COX blockade (P)
Endo et al., 1995	Rat, STZ, 8w	Abdominal aorta	ACh ↓	SNP ↔	
Hopfner et al., 1999	Rat,STZ, 14w	Thoracic aorta	ACh ↓	SNP ↔	
Matsumoto et al., 2006	Mice, STZ, 10w	Mesenteric artery	ACh ↓	SNP ↔	
Shi et al., 2006	Rats, STZ, 12w	Carotid, Femoral arteries	ACh ↓		L-arginine (T)
B: Isolated Perfused Organs References	**Diabetes Model**	**Organ**	**EDVD**	**EIVD**	**Restoration**
Taylor et al., 1994b	Rat, STZ, 2-10w	Mesenteric bed	ACh ↓		
Quilley et al., 1996	Rat, STZ, 4-6w	Heart	BK ↓		COX blockade (N)
Fulton et al., 1996	Rat, STZ, 4-6w	Kidney	ACh, BK ↓	SNP ↔	L-arginine (P)
Makino et al., 2000	Rat,STZ, 10w	Mesenteric bed	ACh ↓	Nitro glycerin ↔	
Misurski & Gopalakrishnan, 2002	Rat, STZ, 14w	Mesenteric bed	ACh ↓		

Kamata et al., 2006	Rat, STZ, w	Kidney	ACh ↓		
Rosen et al., 1996	Rat, STZ, W	Heart	5-HT ↓	SNP ↔	SOD (T)

C: In Vivo Studies **References**	**Diabetic Model**	**Vascular Bed**	**EDVD**	**EIVD**	**Restoration**
Bucala et al., 1991	Rat, STZ, 0.5-12 m	Blood Pressure	ACh ↓	Nitro glycerin ↔	Aminoguanidine(P)
Mayhan et al., 1991	Rat, STZ, 2.5-3.5 m	Pial arteriols	ACh ↓		COX blockade (T)
Mayhan et al., 1997	Hamster, STZ, 2w	Cheek pouch arteriols	Substance P ↓ Histamine ↓	Nitro glycerin ↔	L-arginine (N)
Mayhan, 1997	Rat, STZ, 2-2.5 m	Basilar artery	ACh , BK ↓	Nitro glycerin ↔	SOD (P)
Mayhan & Pate, 1998	Rat, STZ, 3-4 w	Basilar artery	Substance P ↓ ACh ↓	SNP ↔	
Pelligrino et al., 1994	Rat, STZ, 6 m	Pial arteriols	ACh ↓	SNP ↔	PKC blockade (P)
Matsunaga et al., 1996	Dog, AL., 4w	Coronary circulation	ACh ↓		L-arginine (P)
Koltai et al., 1997	Dog, AL., 3 m	Coronary circulation	ACh ↓	Sodium nitrite ↔	L-arginine (N)
Angulo et al., 1998	Rat, STZ, 8w	Hindlimb circulation	ACh ↓	SNP ↔	L-arginine (P) SOD (P)
Crijns et al., 1998	Rat, STZ, 6w	Skeletal muscle arteriols	ACh ↓		Aminoguanidine (N)
De Vriese et al., 1999	Rat, STZ, 6w	Renal circulation	ACh ↓	Pinacidil ↔	COX blockade (N)

Table 2. Clinical studies on endothelium-dependent vasodilatation in diabetes.

Reference	Subjects	Vascular bed	EDVD	EIVD
Jorgensen et al., 1988	38 Type I 21 Control	Forearm	FMD↓	
Johnstone et al., 1993	15 Type I 16 Control	Forearm	FMD↔ MCh↓	SNP↔
Nitenberg et al., 1993	6 Type I 5 Control	Coronary circulation	ACh↓	ISDN↔
Zenere et al., 1995	18 Type I 16 Control	Femoral artery	FMD↓	GTN↓
Khan et al., 1996	16 Type I 20 Control	Forearm	FMD↓ MCh↔	SNP↔
Clarkson et al., 1996	80 Type I 80 Control	Brachial artery	FMD↓	GTN↓
Lekakis et al., 1997	31 Type I 26 Control	Brachial artery	FMD↓	
Arcaro et al., 1999	9 Type I 17 Control	Femoral artery	FMD↓	GTN↔

1.3. GHRELIN: A DISCOVERED PEPTIDE DERIVED FROM STOMACH

1.3.1. Endogenous Source, Structure, and Major Forms of the Peptide Ghrelin

The purified rat ghrelin is a 28-amino acid peptide that is predominately produced in the stomach by the enteroendocrine X/A-like cells that represent a major endocrine population in the oxyntic mucosal layer of the fundus (Kojima et al., 1999). These cells are associated with capillary networks that transport secreted ghrelin into the bloodstream for its endocrine actions. Substantially lower amounts of the peptide ghrelin have been detected in bowel, pancreas, kidneys, the immune system, placenta, testes, pituitary, lung, and hypothalamus (Kojima et al., 1999; Tena-Sempere et al., 2002). Ghrelin is the first natural hormone to be identified, in which the hydroxyl group of its N-terminal third serine residue (Ser3) is acylated by a hydrophobic moiety, n-octanoic acid (Figure 4A, Kojima et al., 1999). An enzyme that catalyzes the acyl-modification of ghrelin has not yet been identified. However, a number of acyltransferases have previously been identified in mammals; the only reported enzymes that use medium-chain fatty acids as substrates are carnitine octanoyltransferases, which function in the β-oxidation of fatty acids (Ramsay and Naismith, 2003). Members of the serine acyltransferase family that transfer acyl groups to serine residues of target molecules have been identified, including two serine palmitoyltransferases functioning in the biosynthesis of sphingolipids in mammals and a plant *O*-acetyltransferase gene family in *Arabidopsis thalaiana* (Hanada, 2003; Howarth et al., 2003). An acyltransferase has also been purified from the gastric mucosa of rat (Kasinathan et al., 1990). This enzyme is an

integral rough microsomal protein, catalyzing the transfer of acyl-CoA to mucosal proteins. The putative ghrelin Ser *O*-acyltransferase may have structural homology with these acyltransferases (Kojima and Kangawa, 2005).

In mammals, ghrelin homologs have been identified in rat, human, rhesus monkey, mouse, mongolian gerbil, cow, pig, sheep, and dog (Kojima and Kangawa, 2005). The amino acid sequences of mammalian ghrelins are well conserved, in particular, the 10 amino acids in their NH_2 termini are identical. This structural conservation, and the universal requirement for acyl-modification of the third residue, indicate that the NH_2-terminal region is of central importance to the activity of the peptide. The amino acid sequence of human ghrelin differs from rat ghrelin at residues 11 and 12 with Arg substituted to Lys and Val substituted to Ala (Hosoda et al., 2003).

In the endocrine mucosa of the rat stomach, a second type of ghrelin peptide has been also purified and identified as desGln14-ghrelin (Hosoda et al., 2000). DesGln14-ghrelin has undergone the same process of acylation with n-octanoic acid at its Ser3 residue as ghrelin and is homologous to ghrelin except that it lacks one glutamine at position 14 from the N-terminal. DesGln14-ghrelin is the result of alternative splicing of the ghrelin gene, and it seems to possess the same hormonal activities as ghrelin (Hosoda et al., 2000). However, desGln14-ghrelin is only present in low amounts in the stomach, indicating that ghrelin is the major active form. Both types of the ghrelin peptide, either 28- or 27-amino acid residue, are found in tissues and plasma in two major forms: n-octanoyl-modified and des-acyl ghrelin (Figure 4B, Hosoda et al., 2000). In blood, des-acyl ghrelin circulates in amounts far greater than the acylated ghrelin.

Ghrelin in plasma binds to high-density lipoproteins that contain a plasma esterase, paraoxonase, and clusterine (Beaumont et al., 2003). Because a fatty acid is attached to the Ser3 of ghrelin via an ester bond, paraoxonase, a potent esterase, may be involved in deacylation of acyl-modified ghrelin. Thus, des-acyl form of ghrelin may represent either a pre-form of acyl-modified ghrelin or the product of its deacylation.

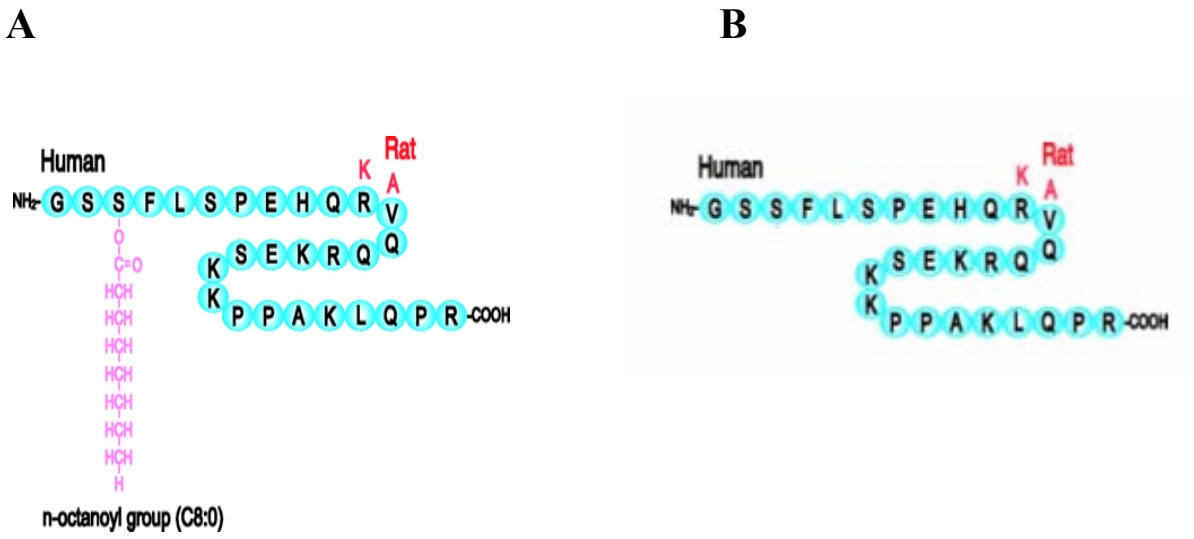

Figure 4. Schematic presentation of the molecules A) ghrelin and B) des-acyl ghrelin.

1.3.2. Ghrelin Receptor and its Distribution

Ghrelin actions are mediated through the ghrelin receptor, also called the growth hormone secretagogue receptor (GHS-R), which is a typical G protein-coupled receptor (GPCR) with seven transmembrane domains (7-TM) (Howard et al., 1996; Smith et al., 1999). Two distinct ghrelin receptor cDNAs have been isolated (Howard et al., 1996). The first, GHS-R type 1a (GHS-R 1a), encodes a 7-TM GPCR with binding and functional properties consistent with its role as ghrelin's receptor. This type 1a receptor

has features characteristic of a typical GPCR, including conserved cysteine residues in the first two extracellular loops, several potential sites for posttranslational modifications, and an aromatic triplet sequence located immediately after TM-3 in the second intracellular loop. Another GHS-R cDNA type 1b, is produced by an alternative splicing mechanism (Howard et al., 1996). The GHS-R gene consists of two exons. The first exon encodes TM-1 to TM-5, and the second exon encodes TM-6 to TM-7. Type 1b is derived from only the first exon and encodes only five of the seven predicted TM domains. The type 1b receptor is thus a COOH-terminal truncated form of the type 1a receptor and is pharmacologically inactive.

The active ghrelin receptor (GHS-R 1a) is well conserved across a number of mammals. This strict conservation suggests that ghrelin and its receptor serve important physiological functions (Kojima and Kangawa, 2005). However, it is suggested that a novel unidentified subtype of ghrelin receptor also exists. Des-acyl ghrelin does not displace radiolabeled ghrelin at the binding sites of acylated ghrelin in hypothalamus and pituitary, and therefore shows no endocrine activities in either humans or rats. On the other hand, both ghrelin and des-acyl ghrelin bind to H9C2 cardiomyocytes, which do not express the ghrelin receptor (Baldanzi et al., 2002).

GHS-R 1a messenger ribonucleic acid (mRNA) is mainly expressed in multiple hypothalamic nuclei, including arcuate (ARC), ventromedial (VMN), dorsomotor, and solitary tract, and in the anterior pituitary somatotrophs. However, lower levels of the receptor has been detected in other areas of the brain such as the dentate gyrus, CA2, and CA3 regions of the hippocampus, the substantia nigra, the ventral tegmental area,

and the dorsal and median raphe nuclei (Howard et al., 1996; Guan et al., 1997). Reverse transcriptase polymerase chain reaction (RT-PCR) analyses also demonstrated ghrelin receptor mRNA expression in many peripheral organs, including cardiovascular, lung, liver, kidney, pancreas, stomach, small and large intestines, adipose tissue, and immune cells, indicating that ghrelin has multiple functions in these tissues (Broglio et al., 2001; Kojima et al., 2001).

1.3.3. Physiological Functions of Ghrelin

Physiological fuctions of ghrelin could be classified into three different categories:

(1) Hypothalamic-Pituitary actions of ghrelin: growth hormone (GH)-releasing activity, prolactin (PRL)- and adrenocorticotropin hormone (ACTH)-releasing activities.

(2) Central actions of ghrelin: food intake and appetite regulation, effects on sleep, effects on behavior.

(3) Peripheral activities of ghrelin: cardiovascular and hemodynamic effects, gastroenteropancreatic actions, modulation of proliferation of neoplastic cells.

1.3.3.1. GH-Releasing Activity

GH secretion is regulated by the interaction between the hypothalamic peptide growth hormone releasing hormone (GHRH), which stimulates, and somatostatin, which inhibits, GH secretion from the pituitary. These two factors also influence GH pulses, as human GH is released in 10-20 pulses in each 24 hr cycle (Roelfsema et al., 2001). The timing of these pulses is controlled by intermittent somatostatin secretion whereas the

amplitude by GHRH (Roelfsema et al., 2001). Recently, ghrelin was identified as the third endogenous factor responsible for regulation of GH release. Ghrelin stimulates GH release both *in vitro* and *in vivo* in a dose-dependent manner. It has been shown that ghrelin stimulates GH release from pituitary cells *in vitro* (Kojima et al., 1999). Similarly, intracerebroventricular, as well as intravenous, ghrelin administration in rats resulted in increase in GH secretion (Date et al., 2000; Seoane et al., 2000). In humans, ghrelin also specifically induced GH secretion and the observed effect was stronger as compared to that of GHRH (Arvat et al., 2000; Takaya et al., 2000).

The cellular mechanisms and the signal transduction pathway involved in the GH-releasing activity of ghrelin appear to be distinct from the one of GHRH. Ghrelin stimulates GH secretion via activation of GHS-R 1a receptors (Lazarczyk et al., 2003). In contrast to GHRH, which activates AC resulting in increased intracellular cAMP levels in pituitary cells upon activation of GHRH receptors, ghrelin causes a transient increase in $[Ca^{2+}]_i$ in somatotrophs (Chen et al., 1996). Binding of ghrelin to its receptor activates the phospholipase C signaling pathway, leading to increased inositol phosphate turnover and protein kinase C activation, followed by the release of Ca^{2+} from intracellular stores. GHS-R 1a activation also leads to an inhibition of K^+ channels, allowing the entry of Ca^{2+} through voltage-gated L-type channels (Chen et al., 1996).

The n-octanoyl group at the Ser3 residue of the ghrelin molecule seems to be essential for the binding to the receptor and the bioactivity of the hormone, at least in terms of GH release. In fact, the non-acylated ghrelin is known to be devoid of any GH-releasing activity in both rats and humans (Bowers 2001; Broglio et al., 2003).

1.3.3.2. Food Intake and Appetite Regulation

Feeding is a basic behavior that is necessary for life. Long-term lack of food results in death. It is well accepted that appetite is controlled by the brain and that feeding behavior is regulated by complex mechanisms in the central nervous system, in particular the hypothalamus (Schwartz et al., 2000). Removal of the lateral hypothalamus causes hypophagia, leading to death due to severe weight loss. On the other hand, removal of the ventromedial hypothalamus causes hyperphagia and obesity.

Recent identification of appetite-regulating humoral factors reveals regulatory mechanisms not only in the central nervous system, but also mediated by factors secreted from peripheral tissues (Neary et al., 2004; Ukkola, 2004). Leptin, produced in adipose tissues, is an appetite-suppressing factor that transmits satiety signals to the brain (Friedman, 2002). Hunger signals from peripheral tissues, however, had remained unidentified until the discovery of ghrelin. Plasma ghrelin levels increase immediately before each meal and fall to minimum levels within 1h after eating (Tschop et al., 2001). The clear preprandial rise and postprandial fall in plasma ghrelin levels support the hypothesis that ghrelin is an initiation signal for meal consumption. Indeed, ghrelin levels and hunger scores have shown to be positively correlated. Furthermore, the postprandial suppression of plasma ghrelin level is proportional to the ingested caloric load, further reinforcing the hypothesis that ghrelin is a hunger signal (Callahan et al., 2004). Additionally, ghrelin gene expression in the stomach as well as plasma ghrelin concentrations are increased in fasting conditions and reduced after habitual feeding

(Tschop et al., 2001). Ghrelin has been found to be the most powerful orexigenic peptide.

Chronic intracerebroventricular injection of ghrelin in rats increases their cumulative food intake and decreases energy expenditure, resulting in body weight gain. Ghrelin-treated mice also increase their fat mass, both absolutely and as a percentage of total body weight (Tschop et al., 2000; Nakazato et al., 2001; Wren et al., 2001). Not only intracerebroventricular injection, but also intravenous and subcutaneous injections of ghrelin have been shown to increase food intake and body weight gain (Tschop et al., 2000). Plasma ghrelin levels were lower in obese subjects than the age-matched lean controls (Tschop et al., 2001; Shiiya et al., 2002). Moreover, plasma ghrelin concentrations were significantly lower in Pima Indians, who are prone to develop insulin resistance and obesity, than in Caucasians (Tschop et al., 2001).

The mechanism of appetite stimulation by ghrelin is not yet fully identified. Immunohistochemical analyses indicate that ghrelin-containing neurons are present in the ARC nucleus of the hypothalamus, a region involved in appetite regulation (Kojima et al., 1999). In the ARC, these ghrelin-containing neurons send efferent fibers onto neuropeptide Y (NPY)- and agouti gene-related peptide (AGRP)-expressing neurons to stimulate the release of these orexigenic peptides, and onto proopiomelanocortin (POMC) neurons to suppress the release of this anorexigenic peptide. Therefore, at least part of the orexigenic effect of ghrelin is suggested to be mediated by inducing the genes encoding the potent appetite stimulants NPY and AGRP (Kojima and Kangawa, 2005). The appetite-stimulating effects of ghrelin are blocked by an antagonist of NPY

receptor. In addition, intracerebroventricular injection of an AGRP inhibitor, anti-NPY immunoglobulin G (IgG), or anti-AGRP IgG, inhibits the appetite-stimulating effect of ghrelin (Cowley et al., 2003). Studies with knockout mice confirmed these results. Although deletion of either NPY or AGRP caused a modest or no effect on the orexigenic action of ghrelin, the double knockout mice lacked the action of ghrelin completely (Chen et al., 2004).

Peripherally injected ghrelin also is shown to stimulate hypothalamic neurons and stimulate food intake (Wren et al., 2001; Date et al., 2002). However, the rate at which peripheral ghrelin passes the blood-brain barrier has been shown to be very low. Thus, peripheral ghrelin must activate the appropriate hypothalamic regions via an indirect pathway (Kojima and Kangawa, 2005). The detection of ghrelin receptors on vagal afferent neurons in the rat nodose ganglion suggests that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve (Date et al., 2002). Vagotomy has been shown to inhibit the ability of ghrelin to stimulate food intake (Andrews and Sanger, 2002; Date et al., 2002).

1.3.3.3. Cardiovascular and Hemodynamic Effects

Expression of mRNA encoding both ghrelin and its receptor GHS-R 1a has been demonstrated in heart and aorta (Nagaya et al., 2001). Specific binding sites for radiolabeled ghrelin, [^{125}I-His9] ghrelin, have been identified in rat heart and human peripheral arteries, where the density of ghrelin receptors is up-regulated with atherosclerosis (Katugampola et al., 2001). This evidence may suggest the involvement

of ghrelin in cardiovascular function. In agreement with the presence of ghrelin receptors in the cardiovascular system, there is already evidence that ghrelin mediates GH-independent cardiovascular activities, both in animals and humans. Chronic administration of ghrelin is able to improve cardiac contractility in GH-deficient rats (Nagaya et al., 2001). In humans, intra-arterial infusion of ghrelin increased forearm blood flow in a dose-dependent manner, in spite of unchanged serum level of insulin-like growth factor-1 (IGF-1) (Okumura et al., 2002). An intravenous bolus injection of ghrelin into human volunteers decreased mean arterial pressure (MAP) without changing the heart rate (Nagaya et al., 2001 a, and b). Ghrelin also increased the cardiac index and stroke volume indices. Rats with chronic heart failure that were treated with ghrelin showed higher cardiac output, stroke volume, and left ventricular function compared with afflicted, but placebo-treated controls (Nagaya and Kangawa, 2003). Furthermore, ghrelin increased the diastolic thickness of the noninfarcted posterior wall, inhibited left ventricle enlargement, and increased left ventricular fractional shortening in these chronic heart failure rats (Nagaya et al., 2001). Thus, ghrelin improves left ventricular dysfunction and attenuates the development of left ventricular remodeling and cardiac cachexia (Nagaya and Kangawa, 2003 b). Moreover, prolonged administration of ghrelin in chronic heart failure rats is associated with a reduction of systemic vascular resistance. The decrease in systemic vascular resistance in response to ghrelin was accompanied by a concomitant reduction in MAP in patients with chronic heart failure (Enomoto et al., 2003).

The decrease in MAP induced by ghrelin seems to occur, at least in part, through its indirect action on the nucleus of the solitary tract (Matsumura et al., 2002; Lin et al.,

2004). Microinjection of ghrelin into this nucleus significantly decreased MAP and heart rate. This injection also suppressed sympathetic activity. The effect of ghrelin on the local vasodilator factors produced in the cardiovascular system, however, can not be neglected. Ghrelin-evoked decrease in MAP was much greater in rats subjected to NOS inhibition (Shinde et al., 2005). In addition, intravenous infusion of apamin and ChTX , a combination that is known to block K_{Ca} channels or the EDHF process, attenuated the decrease in MAP evoked by ghrelin in both control and NOS-inhibited rats (Shinde et al., 2005).

2. PRESENT INVESTIGATION

2.1. Working Hypothesis

(1) Both ghrelin and des-acyl ghrelin possess potent endothelium-dependent vasodilator responses in mesenteric vascular bed (MVB). Vasodilator effects in MVB in response to the natural ligand of the GHSR-1a receptors, ghrelin, are possibly mediated through the generation of EDHF. The released EDHF stimulates K_{Ca} channels and increases K^+ conductance, resulting in smooth muscle relaxation. Therefore, inclusion of the NO synthase inhibitor (NOSI) N (G)-nitro-L-arginine methyl ester (L-NAME), COX inhibitor indomethacin, or the K_{ATP} channel blocker glibenclamide, fails to abolish the vasodilator responses to ghrelin. However, pretreatment of MVB with either a high extracellular K^+ concentration (80 mmol/L KCl), or different selective and nonselective K_{Ca} channel blockers [TBA (tetrabutylammonium), apamin + ChTX, apamin + TRAM-34 (1-(2-chlorophenyl) diphenylmethyl)-1H-pyrazole]] abolishes the vasodilator responses to ghrelin.

(2) The vasodilator action of ghrelin is not mediated by the classical GHSR-1a receptors present in the pituitary. But, it is mediated by the activation of a novel receptor present on the endothelium. Treatment of MVB with antagonists of the GHSR-1a receptors, such as [D-Lys^3]-growth hormone releasing peptide-6 ([D-Lys^3]-GHRP-6) or the peptide L-756867, does not block the vasodilator actions of ghrelin.

(3) The whole ghrelin molecule, which is a 28-amino acid peptide with an n-octanoyl group on the Ser3, is not necessary for its vasodilator activity. Des-acyl ghrelin, which is

a form of ghrelin without n-octanoic acid on the Ser3 residue, can also cause vasodilatation to the same extent as ghrelin in MVB.

(4) Taking into account the direct cardiovascular effects of ghrelin, it would be interesting if ghrelin could be administered orally in patients with cardiovascular diseases and chronic heart failure. However, since ghrelin is a peptide hormone, it is mostly degraded in the stomach by peptidases. Thus, due to the lower bioavailability of the peptidyl growth hormone secretagogues (GHSs), the development of selective non-peptide agonists directed to the novel vascular receptors of ghrelin would be a new drug investigation for the management of cardiovascular diseases. Besides ghelin, orally active non-peptide agonists of the classical GHSR-1a receptors, L-166446 and L-163255, are also effective vasodilators in MVB. These non-peptides, therefore, could be used as orally administered agents in the management of cardiovascular disease.

(5) In STZ-induced diabetic rats, plasma ghrelin levels are elevated due to low plasma insulin levels. The decreased MVB resistance and exaggerated vasodilator response to agonists in early stages of STZ diabetic rats are due to increased ghrelin levels, which promote enhanced vasodilatation via activation of novel vascular ghrelin receptors. At later stages of STZ diabetes, the enhanced effect due to ghrelin is countered by down-regulation of vascular ghrelin receptors. In addition, in later stages of STZ diabetes, EDHF response is impaired, which itself would affect the vascular responses to ghrelin as ghrelin response is dependent on EDHF. The same condition seems to happen in fasted rats. Plasma ghrelin levels are elevated during fasting because of decreased

plasma glucose and insulin concentrations. Ghrelin, then, acts on its vascular receptors and induces vasodilatation.

2.2 Rationale for the Study

Six major pieces of evidence provide the basis and foundation for undertaking investigations on ghrelin-evoked vasodilator responses in MVB of Sprague Dawley (SD) rats. They are as follows:

(1) Intravenous bolus injection of ghrelin (10 μg/Kg) led to a significant decrease in MAP with increased cardiac output within 15 min that lasted for about one hr in both normal volunteers and chronic heart failure patients (Nagaya et al., 2001 a). Systemic administration of ghrelin in conscious rats led to a dose-dependent decrease in blood pressure (20 mmHg by 10 min-10 nmol/L/rat) that lasted for 30 min (Nagaya et al., 2001b). Ghrelin was shown to be an effective endothelium-independent vasodilator of the long-lasting constrictor ET-1 in human mammary arteries (Wiley and Davenport, 2002). In contrast, 3-week treatment with ghrelin improved endothelial dysfunction demonstrated by impaired ACh-induced vasodilatation in GH-deficient rats (Shimizu et al., 2003). On the other hand, intra-arterial infusion of ghrelin reduced the forearm vascular resistance that was not accompanied by changes in plasma cyclic nucleotide (cAMP and cGMP) levels. Since a NOSI failed to block the responses, it was concluded that the vasodilator effect of ghrelin was not mediated by NO generation (Okumura et al., 2002). Previous data from our laboratory also demonstrated that ghrelin-evoked decrease in MAP was much greater in rats subjected to NOS inhibition. In addition,

intravenous infusion of apamin + ChTX attenuated the decrease in MAP evoked by ghrelin in both control and NOS-inhibited rats (Shinde et al., 2005).

(2) In addition to ghrelin, a natural ligand of the classical GHSR-1a receptors, synthetic GHSs, also feature a variety of cardiovascular activities (Locatelliot et al., 1999; Nagaya et al., 2001 a and b; Baldanzi et al., 2002; Okumura et al., 2002; Benso et al., 2004). Hexarelin, a peptidyl GHS, has been shown to improve cardiac function and decrease peripheral vascular resistance in rats with myocardial infarction and to protect the isolated heart from ventricular dysfunction (Rossoni et al., 2000; Tivesten et al., 2001). Hexarelin was also demonstrated to induce a dose-dependent increase in coronary perfusion pressure in isolated perfused hearts (Bodart et al., 2003). In contrast, Bedendi et al. (2003) showed a negative ionotropic effect induced by hexarelin in the rat and guinea pig papillary muscles. A stimulatory effect on cell proliferation and an antiapoptotic action of GHSs on the cardiomyocyte cell line H9C2 and EC have also been shown (Filigheddu et al., 2001; Baldanzi et al., 2002; Petterson et al., 2002). Both MK-0677 and hexarelin, a non-peptidyl and peptidyl synthetic GHS, respectively, inhibited the cell death in cardiomyocyte and EC lines (Baldanzi et al., 2002). In hearts subjected to 30 min of ischemia followed by 120 min of reperfusion, however, the synthetic peptidyl secretagogue hexarelin significantly reduced infarct size, while the non-peptidyl secretagogue MK-0677 was ineffective (Frascarelli et al., 2003).

(3) Ghrelin strongly stimulates GH secretion acting on a specific GPCR, namely GHSR-1a receptor, which is different from the GHRH-receptor (Muccioli et al., 2002). Expression of GHSR-1a receptors has been demonstrated at the myocardial level and

within the vasculature (Nagaya and Kangawa, 2003). Bedendi et al. (2003) found unexpectedly that the non-acylated form of ghrelin was able to exert a negative ionotropic effect on the rat as well as guinea pig papillary muscles to even a greater extent than the acylated ghrelin, even though unacylated ghrelin is generally assumed to be devoid of biological activity, as it can not bind to or activate the GHSR-1a receptors (Bednarek et al., 2001). The binding studies performed by Bednarek et al. (2003) with either acylated and unacylated ghrelin confirmed that there is a specific receptor recognized by all ghrelin forms and by hexarelin as well. These findings therefore indicate the existence of a cardiac non-GHS type 1a receptor involved in the cardiovascular actions of ghrelin and des-acyl ghrelin. It has been reported previously that both ghrelin and des-acyl ghrelin bind to a common receptor in breast cancer cells (Cassoni et al., 2001). In these cells, ghrelin and des-acyl ghrelin inhibit cell proliferation, whereas, on the other hand, GHSR-1a receptors appeared to mediate a proliferative signal (Murata et al., 2002). Additionally, the recent finding that H9C2 cardiomyocytes do not express GHSR-1a receptors, but have high affinity binding sites, common for ghrelin and des-acyl ghrelin, involved in mediating their antiapoptotic activity, provides further support to the hypothesis that multiple ghrelin and GHS receptors exist in the cardiovascular system (Baldanzi et al., 2002). Each receptor may then contribute independently to the wide array of cardiovascular activities induced by ghrelin, synthetic GHSs, and endogenous ghrelin-derived molecules (Torsello et al., 2003). Furthermore, the recent data from our laboratory demonstrating the characterization of [^{125}I] ghrelin binding sites in mesenteric artery EC, provided another piece of evidence suggesting that the vasodilatory effect of ghrelin may not be mediated by GHSR-1a receptors. Des-acyl ghrelin was shown to bind to pituitary GHSR-1a

receptors with much lower affinity (3500 nmol/L) compared to the high affinity (1.5 nmol/L) interaction by ghrelin (Matsumoto et al., 2001). On the contrary, our laboratory reported that des-acyl ghrelin displaced [^{125}I] ghrelin specific binding to EC with a higher affinity (215 pmol/L) than ghrelin (330 pmol/L).

The existence of different GHS receptor subtypes in the heart has already been demonstrated (Muccioli et al., 2000 and 2002). GHSR-1b receptors, a GHSR-1-truncated splicing variant, although expressed in the heart, did not appear to be functional (Howard et al., 1996). CD36, a fatty acid receptor, has been identified recently as a myocardial receptor for hexarelin. However, neither ghrelin nor the synthetic non-peptidyl GHS, MK-0677, recognize such a receptor (Bodart et al., 2002). In contrast, Bedendi et al. (2003) demonstrated that there is a non-GHSR-1a receptor that is recognized by all ghrelin forms and by hexarelin as well.

(4) Several studies elucidated the structural features of the peptide ghrelin necessary for its efficient binding to and activation of the classical GHSR-1a receptors (Bednarek et al., 2000; Matsumoto et al., 2001). The n-octanoic acid modification of ghrelin on the third amino acid residue appeared to be necessary for its GH-releasing activity (Kojima et al., 1999 and 2000). The study by Bednarek et al. (2000) demonstrated that the entire sequence of ghrelin is not necessary for activation of GHSR-1a receptors. The short peptides encompassing the first four or five residues of ghrelin were found to activate the GHSR-1a receptors about as efficiently as the full-length ghrelin, thus implying that the N-terminal Gly-Ser-Ser(n-octanoyl)-Phe segment constitutes the “essential core” required for efficient binding to and activation of GHSR-1a receptors.

(5) Early type I diabetes is associated with changes in vascular function leading to over-perfusion of the vasculature, whereas the later stages are associated with atherosclerotic and hypertensive changes, promoting the well-known late stage complications of the disease (Epstein and Sowers, 1995; Pieper, 1998). While the mechanisms of these changes are not completely understood, it is possible that shifts in the actions of endothelial derived relaxing and contracting factors might contribute. Impaired endothelial-dependent vasodilatation is considered as a major risk factor of cardiovascular complications of type I diabetes (Triggle et al., 2004). Impaired endothelium-dependent vasodilatation can be directly linked to a reduction in the bioavailability/bioactivity of endothelium-derived NO (Pieper, 1998; De Vriese et al., 2000). There is now accumulating a body of evidence in type I diabetic mice, rats and humans showing impaired EDHF activity, especially in small resistance vessels (Fukao et al., 1997; Makino et al., 2000; Fitzgerald et al., 2005; Morikawa et al., 2005). Fukao et al. (1997) showed that the amplitude and duration of the hyperpolarization produced by ACh was significantly decreased in mesenteric arteries from diabetic rats. Inhibition of NOS did not affect the hyperpolarization response to ACh in mesenteric arteries from control and diabetic rats, thus concluding that the functional EDHF response is decreased in the rat mesenteric artery as a result of diabetes.

(6) It has been demonstrated that plasma ghrelin levels are elevated in STZ-treated diabetic rats (Ishii et al., 2002; Gellings et al., 2004). While plasma ghrelin levels and the gastric preproghrelin mRNA expression levels in the stomach are significantly higher in STZ diabetic rats, their gastric levels and the number of ghrelin-

immunoreactive cells in the gastric fundus are relatively lower in STZ-induced diabetic rats (Masaoka et al., 2003).

2.3. Objectives

Based on the following objectives, I will attempt to validate my working hypothesis:

(1) To evaluate the vasodilator responses evoked by ghrelin and des-acyl ghrelin in MVB, with an emphasis on the underlying mechanisms by which ghrelin produces vasodilatation in the perfused rat MVB.

(2) To evaluate if the vasodilator responses evoked by ghrelin in the MVB are mediated through the activation of GHSR-1a receptors present on vascular tissues.

(3) To evaluate the vasodilator activities of the synthetic non-peptide agonists of GHSR-1a receptors, L-166446 and L-163255 (Spiroindanyl piperidines).

(4) To evaluate the structure-activity relationship of the ghrelin-related peptides in relation to their vasodilatory effects.

(5) To evaluate the vasodilator responses to ghrelin, in both conditions of fasting and STZ-induced diabetes, in relation to the plasma ghrelin concentrations.

3. MATERIALS AND METHODS

3.1. Animals

The care and use of animals used in the present study conformed to the regulations stipulated by the University of Saskatchewan Animal Care Committee. We followed similar guidelines as established by the US National Institutes of Health for the maintenance and use of experimental animals in our laboratories (NIH Publication No. 85: 23, 1996). Male SD rats were purchased from Charles River (St. Constant, Quebec, Canada) at an age of 11 weeks. They were housed in our animal quarters assigned to the Department of Pharmacology with due care under standardized conditions with a light/dark cycle of 12 hr and a constant temperature of 22 ± 1ºC. The rats were fed *ad libitum* with food pellets (Purina Rat Chow) and tap water. The animals were utilized for experiments when they reached the age of 12 weeks.

3.2. Chemicals and Reagents

3.2.1. Chemicals for Evaluation of Vasodilator Responses to Agonists

ACh, phenylephrine hydrochloride (PE: a-(1-Aminoethyl)-2,5-imethoxybenzylalcohol), indomethacin, L-NAME, barium chloride dehydrate (Ba^{2+}), glibenclamide, ouabain, TBA, and sodium nitroprusside (SNP), were all purchased from Sigma-Aldrich Chemical Co., Oakville, ON., Canada. Apamin and ChTX were obtained from EMD Biosciences Inc. (La Jolla, CA, USA). TRAM-34 was purchased from Sigma (St. Louis, MO). [D-Lys3]-GHRP-6 and synthetic rat Ser3-(n-octanoyl)-ghrelin were from either Bachem (Torrance, CA, USA) or from Phoenix Pharmaceuticals Inc.

(Belmont, CA, USA), while the des-acyl form of ghrelin, rat Ser3(des-octanoyl)-ghrelin, was obtained from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA).

3.2.2. Chemicals for STZ-Induced Diabetes

STZ was obtained from Sigma-Aldrich (Milwaukee, Wis., USA). Radioimmunoassay (RIA) kits for the detection of ghrelin and insulin in samples were purchased from Phoenix Pharmaceuticals (Belmont, CA) and Amersham (Oakville, ON), respectively. One Touch Basic Glucometer and the One Touch Basic Glucose Strips were from Lifescan (Vancouver, BC).

3.3. Methods

3.3.1. Rat MVB Preparation

3.3.1.1. Isolation of MVB

Experiments were performed when animals reached the age of 12 weeks. Rats were anesthetized with thiopental sodium (100 mg/Kg, i.p.), laprotomy was performed by a mid-line incision and the heart was removed. Immediately after the removal of the heart, the superior mesenteric artery was identified and cleared of any adherent tissue using a cotton swab. Using forceps, two pieces of sutures were placed under the superior mesenteric artery at the level of the T-junction where the superior mesenteric artery branches off from the abdominal aorta. A small incision was made at the T-junction and the superior mesenteric artery was cannulated with polyethylene tubing fitted on to the 20 gauge, dissected from the gastrointestinal tract, and placed in a warm (37 ± 1ºC)

Krebs solution. The MVB was then transferred and connected to the perfusion apparatus (Misurski, Tatchum-Talom et al., 2000; Shastri, McNeill et al., 2001).

3.3.1.2. Perfusion of MVB

The MVB was perfused via the cannula with a modified Krebs bicarbonate solution (pH 7.4) by means of Gilson constant flow pump, at a constant flow rate of 5 ml/min. The composition of the Krebs buffer solution (in mM) was: NaCl 118, KCl 4.7, $MgCl_2$ $6H_2O$ 1.2, $CaCl_2.2H_2O$ 1.8, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 11.1. The buffer was continuously oxygenated using carbogen (O_2 95% oxygen + with 5% CO_2) and maintained at a steady temperature 37 ± 1ºC with the thermocirculator (from Harvard Apparatus). An air pocket was incorporated in the circuit for air entrapment in the perfusate system to prevent denudation of the endothelium (Misurski, Tatchum-Talom et al., 2000; Shastri, McNeill et al., 2001).

3.3.1.3. Measurement of Perfusion Pressure Changes to Agonists and Antagonists

The changes in perfusion pressure (PP) monitored as mmHg in response to agonists and antagonists, were measured using a strain gauge pressure transducer placed in the circuit, before monitoring the MVB preparation. A chart program (Chart V4.0.1) using a PowerLab/8SP Series system (AD Instruments Pty. Ltd., Castlehill, NSW, Australia) electronically integrated and recorded the pressure signals. The pressure change was subjected to an initial external calibration using a sphygmomanometer.

3.3.2. Experimental Protocol

3.3.2.1. Equilibration of MVB Preparation

The preparations were allowed to equilibrate for 1 hr with normal Krebs buffer to attain steady state baseline PP. Then, two bolus doses of PE (80 μmol/L), a non-selective α_1 agonist, were given in succession to ensure reproducible constrictor responses could be attained. In all preparations, before performing the concentration-response (CR) curves or single-dose responses to different vasodilator agonists, PE (80 μmol/L) was perfused for a period of about 20 min to evoke a consistent and stable vasoconstrictor response.

3.3.2.2. Concentration-Response Curves to Vasodilator Agonists

Graded dilator responses to serially increasing concentrations of either ACh (10 pmol/L-10 μmol/L), ghrelin (100 fmol/L-100 nmol/L), or des-acyl ghrelin (100 fmol/L-100 nmol/L) were determined by perfusing appropriate concentrations of the respective agonist in a maximum volume of 20-25 ml. The experiments were performed each day by perfusing two MVBs isolated from two SD rats in parallel under identical conditions using the three mentioned agonists. The order in which the agonists were given did not affect the pattern of their responses. A minimum of 45 min was provided for the tissues to recover after completing the CR protocol to one agonist before a similar protocol was initiated with a serially increasing concentration of the second agonist. In several experiments, to confirm that no desensitization had occured to these vasodilators, we conducted the CR curves to each of these dilators more than once in the same MVB preparation. The results demonstrated that the same CR curves could be repeated as long

as the concentrations of ghrelin or des-acyl ghrelin did not exceed 1 nmol/L. After completing the control responses, the CR protocols to the two vasodilators ghrelin and ACh were repeated in the presence of different inhibitors of NO- and EDHF-mediated pathways of vasodilatation including L-NAME (100 μmol/L), TBA (0.5 mmol/L), L-NAME + TBA, and KCl (80 mmol/L). These different inhibitors of the NO- and EDHF-mediated pathways of vasodilatation were present in the perfusion buffer for a period of 30 min and also during the serial addition of increasing concentrations of either ghrelin or ACh in PE-containing buffer.

In addition to evaluating the responses to ghrelin, des-acyl ghrelin, and ACh, CR curves to the increasing concentrations (100 fmol/L-100 nmol/L) of the two synthetic non-peptide agonists of the classical GHSR-1a receptors, L-166446 and L-163255, were also determined in PE-contracted MVB preparations. Both non-peptide agonists, were H_2O-soluble. However, we tend to solubilize all small molecule compounds in a very small quantity of dimethyl sulfoxide (DMSO).

3.3.2.3. Responses to a Single Dose of Vasodilator Agonists

The vasodilator response to a single dose of either ghrelin (1 nmol/L) or ACh (1 μmol/L), which produces maximal relaxation in PE-contracted MVB preparations, were assessed in the presence and in the absence of different inhibitors of COX, NO, and EDHF pathways of vasodilatation including: indomethacin (10 μmol/L), apamin (1 μmol/L) + ChTX (100 nmol/L), apamin + TRAM-34 (10 μmol/L), ouabain (10 μmol/L) + Ba^{2+} (30 mmol/L), and glibenclamide (10 μmol/L). The stock concentration of

indomethacin was dissolved in sodium carbonate (0.01 mol/L). TRAM-34 stock concentration was dissolved in DMSO. To make up the final concentrations, further dilutions were made using Krebs buffer. Vasodilator responses to a single concentration of ghrelin were also evaluated in the presence of different blockers of the classical GHSR-1a receptors, [D-Lys^3]-GHRP-6 (10 nmol/L) as well as L-756867 (a synthetic peptide antagonist, 1 μmol/L). L-756867 was best solubilized in dilute acidified H_2O (0.01N acetic acid).

In addition to evaluating the vasodilator responses in the normal SD rats, vasodilator responses to single concentrations of ghrelin and ACh were also determined in either fully fed or fasted rats that were subjected to STZ-induced diabetes for 4 or 8 weeks. In each day, the experiments were performed by perfusing two MVBs isolated from either 4- or 8-weeks fed/fasted diabetic versus control rats in parallel under the same conditions using both agonists.

3.3.2.4. Removal of Endothelium

CR curves to ghrelin (10 pmol/L-1 nmol/L), des-acyl ghrelin (10 pmol/L-1 nmol/L), and ACh (10 nmol/L-1 μmol/L) were also done in the absence of endothelium. Removal of endothelium was achieved by slow and intermittent infusion of 10 ml air at 5 min intervals through a port for 1hr. In the MVB, endothelium removal led to a rise in the tone of the MVB. The injection of air into the MVB led to the removal of 85-90% of the EC from the vascular bed. In each experiment, the removal of endothelium was

confirmed by demonstrating a lack of responsiveness to a test dose of ACh (100 μmol/L) in the MVB constricted with 80 μmol/L of PE subsequent to the exposure to air.

3.3.3. STZ Treatment

At 12 weeks of age, SD rats were fasted overnight and divided into two groups of diabetic and control rats. Diabetic animals were given a single intraperitoneal (i.p.) injection of STZ in citrate buffer (65 mg/Kg, pH 4.5). Control rats, in contrast, were treated with a single i.p. injection of citrate buffer alone. Blood glucose levels were assessed one week post-treatment to verify hyperglycemia (>15 mmol/L). Following the treatment with STZ or citrate buffer, STZ-treated as well as control rats were kept for the period of either 4 or 8 weeks. After 4 or 8 weeks of treatment, the rats in each group were again divided into two groups of either fasted (4- or 8-weeks diabetic fasted, control fasted) or fed (4- or 8-weeks diabetic fed, control fed) for overnight. The experiments were then performed on the following day, after the rats were anesthetized using thiopental sodium (100 mg/Kg, i.p.).

3.3.4. Blood Glucose Monitoring

Using a scalpel blade, a small incision was made on the tail vein. A drop of blood was then applied to a glucose strip (One Touch Basic Glucose Strips, Lifescan, Vancouver, B.C.) placed on the glucometer (One Touch Basic Glucometer). Glucose in the drop of blood was measured by the glucose oxidase method. Pressure was subsequently applied to the incision to promote clotting. With this system, blood glucose levels were recorded accurately within 45 seconds.

3.3.5. Plasma Sample Collection

Animals were killed cutting the heart under anesthesia with thiopental sodium (100 mg/Kg, i.p.). Blood samples were subsequently collected from the dead animals in tubes containing 15% ethylene diaminetetraacetic acid (EDTA). A 5 ml sample of combined arterial and venous blood was easily obtained by this method. These samples were then centrifuged at 2000 x g, 10 min at 4ºC. Aliquots (100 µl) of the supernatant plasma were stored at -70ºC.

3.3.6. Assay for Plasma Insulin Levels

Plasma insulin concentrations were measured from frozen 100 µl aliquots using a commercially available RIA kit based on the competition between unlabeled insulin and a fixed quantity of [^{125}I]-labeled human insulin for a limited number of binding sites on an insulin antibody. Using fixed amounts of antibody and radioactive ligand, the amount of added non-radioactive ligand is inversely proportional to the concentration of radioactive ligand bound by the antibody. All reagents (standard, antiserum, and [^{125}I] insulin radiotracer) were diluted with assay buffer (0.052 mol/L phosphate buffer, pH 7.5, containing 0.1% sodium azide). Both the antiserum and radiotracer were then combined with the insulin samples and standards, vortexed, and allowed to incubate for 4hr at room temperature. After this, the Amerlex-M second antibody reagent (which contains second antibody that is bound to polymer particles) was added to the antibody bound to insulin, vortexed, and allowed to incubate at room temperature for a further 10 min. Separation of the antibody bound fraction was then performed by centrifugation (1500 x g, 10 min, 3ºC) followed by decantation of the supernatant by inversion of the

tubes in decantation racks and draining for 5 min. Radioactivity (cpm) in the pellet from each sample was then counted for a period of 60 seconds in a gamma-counter. This enabeled the amount of labeled insulin in the bound fraction to be calculated. Non-specific binding was first subtracted from all counts. The amount bound for each standard was then plotted against insulin concentration on a standard curve. The concentration of unlabeled insulin in the sample was then determined by interpolation from this standard curve. The assay was completely cross-reactive with insulin from a variety of species including rat and human. The sensitivity was 48 pg/ml.

3.3.7. Assay for Plasma Ghrelin Levels

Ghrelin concentrations were measured from plasma samples kept at -70ºC using a commercial RIA kit, which uses ^{125}I-labeled ghrelin as a tracer molecule. The principle of the assay is the same as the one described for plasma insulin measurement. The assay is based on the competition of ^{125}I-ghrelin and ghrelin (either standard or sample) binding to the limited quantity of antibodies specific for the peptide in each reaction mixture. As the quantity of standard or unknown sample in the reaction increases, the amount of ^{125}I-ghrelin able to bind to the antibody decreases. The samples, as well as standards, were re-suspended in the assay buffer and assayed at 100 µl/sample in duplicates. Diluted samples or standard peptide solutions (100 µl) were incubated for 24 hr with 100 µl of antiserum diluent. A tracer solution (100 µl) was added, and the mixture was incubated for another 24 hr. The bound and free ligands were separated using a second antibody (200 µl). All the procedures were performed at 4ºC. 500 µl of RIA buffer was added and the samples and standards were centrifuged at 1700 x g for 20

min at 4ºC. The supernatant was aspirated off and the amount of radioactivity (cpm) in the pellet for each standard and sample was counted, for a period of 60 seconds, using a gamma-counter. By measuring the amount of ^{125}I-ghrelin bound as a function of the concentration of the peptide in standard reaction mixtures, it is possible to construct a standard curve, from which the concentrations of peptide in unknown samples can be determined, as described for plasma insulin measurement.

3.3.8. Statistical Analysis

CR curves as well as responses to a single dose of agonists were analyzed individually. The results (mean ± S.E.M.) were expressed as a percentage of the maximal vasodilatation (E_{max}) of PE-evoked vasoconstrictions. The potencies of ghrelin-, des-acyl ghrelin-, ACh-, and synthetic non-peptide agonists-induced vasodilatation were expressed as the concentrations required for half-maximal vasodilatation (EC_{50}) evoked by each agonist. Plasma insulin and ghrelin levels were also analyzed individually and the results were expressed as pmol/L for plasma insulin and pg/ml for plasma ghrelin. Comparison of mean values amongst various groups was performed by analysis of variance (ANOVA, Superanova program-SAS Institute, San Francisco, CA). Simultaneous multiple comparisons were examined by Scheffe's F-test. Level of significance was set at $p<0.05$.

4. RESULTS

4.1. Ghrelin and Des-acyl Ghrelin Evoked Vasodilatation in Mesenteric Arteries Compared to ACh.

4.1.1. Ghrelin Evoked Concentration-Dependent Vasodilatation in MVB Compared to ACh

After one hr of equilibration of MVB *ex vivo*, the mean ± S.E.M. basal PP values under control conditions averaged 15.0 ± 1.2 mmHg in SD rats. PE (80 μmol/L) evoked a sustained tonic steady-state contraction in all MVBs after three minutes of perfusion. In MVBs, the PP after contraction with PE averaged 120 ± 2.5 mmHg. Cumulative concentration-response (CR) curves for ghrelin (100 fmol/L-100 nmol/L) and ACh (10 pmol/L-10 μmol/L) in PE-contracted MVB demonstrated concentration-dependent decreases in PP. A representative experiment comparing the CR patterns between ghrelin and ACh to increasing concentrations of ghrelin and ACh is shown in Figure 5A and 5B. The maximal vasodilator response (E_{max}) to ghrelin (45 ± 5% attained at 1 nmol/L) was much lower (2 fold; $p<0.01$) than the response evoked by ACh (90 ± 6% attained at 1 μmol/L). The addition of ACh to the perfusion medium led to almost complete relaxation of MVB (Figures 5B and 6B). The data from several CR curves, however, revealed that ghrelin is more potent (EC_{50} 55 ± 6 pmol/L; E_{max} 45 ± 5%) than ACh (EC_{50} 3 ± 2 nmol/L; E_{max} 90 ± 6%). The differences in mean ± S.E.M. for both EC_{50} and E_{max} values between ghrelin and ACh are significant ($P<0.01$), and the data are shown in Table 3.

The order in which ghrelin and ACh were used in the same preparation, did not affect the responses to either agonist. The time course of ghrelin-evoked vasodilator responses, however, was relatively slower in onset (between 20-30 seconds after the addition of the peptide), and took a relatively longer time to reach the maximal effect (2-3 minutes). This is while ACh-evoked responses were relatively much more rapid (approximately 10 seconds after the addition of the muscarinic agonist, and 20 seconds to reach the plateau) (Figure 6A and 6B). It is noteworthy to mention that the responses to ghrelin could be reproduced in the same preparation as long as the concentration of the peptide did not exceed 1 nmol/L. This allowed the repeated administration, and therefore determination of the responses to ghrelin in the same preparation.

4.1.2. Ghrelin Evoked Endothelium-Dependent Vasodilatation in MVB Compared to ACh

To evaluate whether the concentration-dependent decreases in PP evoked by increasing concentrations of either ghrelin or ACh are endothelium-dependent, endothelium was removed by injection of 10 ml air at 5 minute intervals for a maximum period of 1 hr. Ghrelin- and ACh-evoked vasodilator responses were then evaluated in parallel in both endothelium-intact (Endo +) or endothelium-denuded (Endo -) preparations. Ghrelin, at concentrations of 10 pmol/L-1 nmol/L, evoked concentration-dependent decreases in PP in PE-constricted MVBs (Figure 7A). ACh, at concentrations of 10 nmol/L-1 μmol/L, also induced concentration-dependent decreases in PP in PE-constricted MVBs. In comparison to ghrelin, addition of ACh (1 μmol/L) led to almost complete relaxation of MVB (90%, Figure 7B).

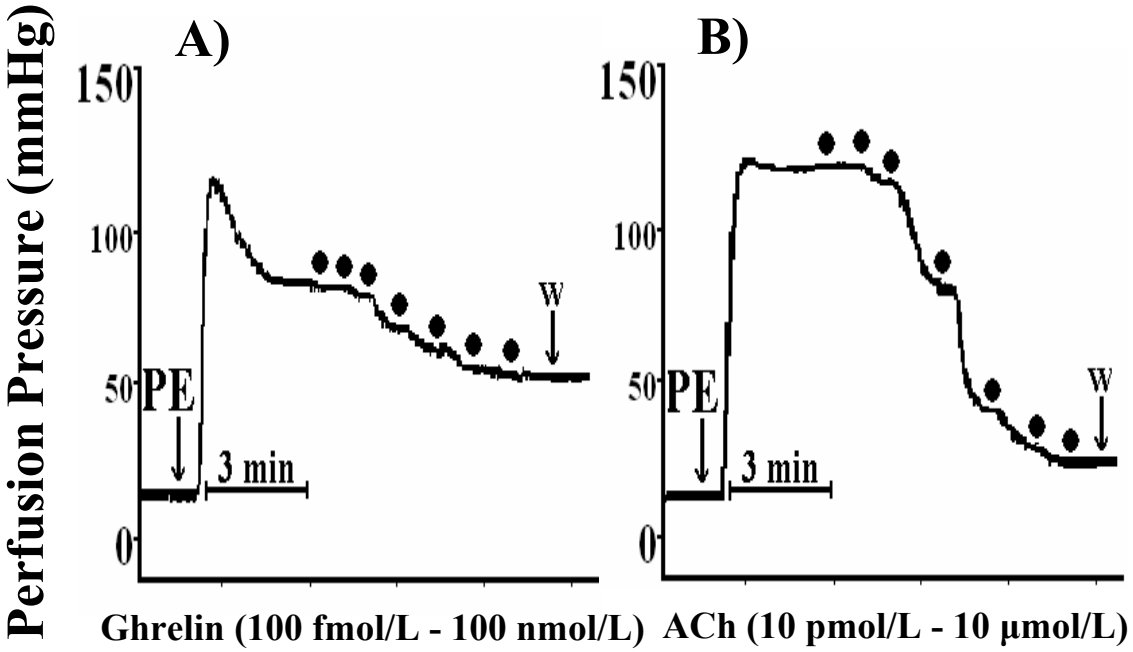

Figure 5. A) ghrelin, and B) ACh induce reduction of PP (mmHg) in PE (80 µmol/L)-constricted MVB in a dose-dependent manner.

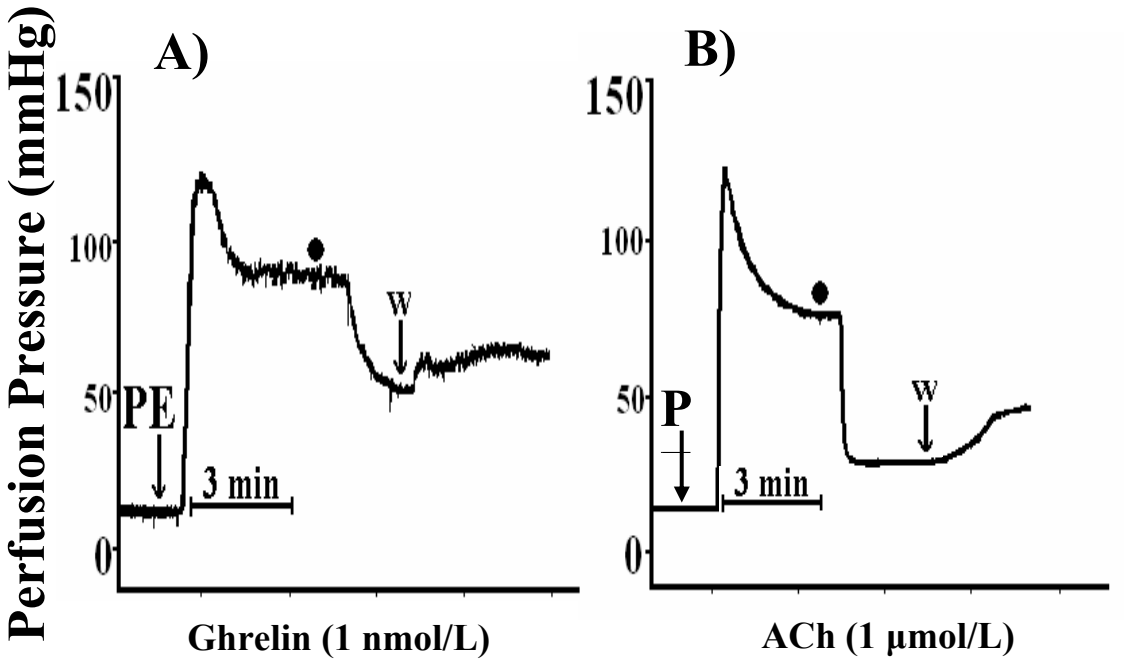

Figure 6. A) Ghrelin (1 nmol/L), and B) ACh (1 µmol/L) induce maximal reduction of PP (mmHg) in PE (80 µmol/L)-constricted MVB.

Denudation of endothelium led to an increase in basal PP as well as an increase in the vasoconstor response to a submaximal dose of PE (80 μmol/L). The basal PP increased to 24.0 ± 0.7 mmHg in endothelium-denuded preparations compared to the level seen in endothelium-intact preparations (15.0 ± 1.2 mmHg). However, the difference in basal PP between endothelium-intact and endothelium-denuded preparations did not reach statistical significance. PE-induced increases in PP were significantly higher in endothelium-denuded (163 ± 8 mmHg) compared to endothelium-intact (120 ± 3 mmHg) preparations ($p<0.05$; Figure 7). Endothelium-removal completely abolished the vasodilator responses to increasing concentrations of both ghrelin (10 pmol/L-1 nmol/L) and ACh (10 nmol/L-1 μmol/L) (Figure 7A and 7B). While vasodilator responses to ghrelin and ACh were not seen in endothelium-denuded preparations, addition of sodium nitroprusside (SNP, 100 μmol/L) led to a remarkable degree of vasodilatation in both endothelium-intact as well as endothelium-denuded preparations to a similar extent (>95%) confirming that removal of endothelium did not affect VSM sensitivity to SNP, a directly acting vasodilator agonist. A representative experiment demonstrating vasodilator responses to SNP in parallel endothelium-intact and -denuded preparations is shown in Figure 8.

Concentration-dependent decreases in PP in response to increasing concentrations of both ghrelin (100 fmol/L-100 nmol/L) and ACh (1 pmol/L- 10 μmol/L) in endothelium-intact and -denuded preparations were also measured as a percentage of maximal PE (80 μmol/L)-induced contractions (Figure 9A and 9B). As explained before, whereas both ghrelin (EC_{50} 55 ± 6 pmol/L; E_{max} 45 ± 5% at 1 nmol/L) and ACh (EC_{50} 3 ± 2 nmol/L; E_{max} 90 ± 6% at 1μmol/L) evoked concentration-

dependent vasodilatation of MVB in endothelium-intact preparations, increasing concentrations of either ghrelin or ACh failed to evoke vasodilatation in endothelium-denuded preparations.

4.1.3. Effects of L-NAME on Vasodilator Responses to Ghrelin in MVB Compared to ACh

As the vasodilator responses evoked by ghrelin and ACh were shown to be endothemium-dependent, detailed experiments were performed to determine the relative contributions of endothelium-derived relaxing factors NO, EDHF, and PGI_2 to both ghrelin- and ACh-evoked vasodilator responses in perfused MVB. To investigate the role of NO, studies were performed in the presence or the absence of L-NAME in the perfusion medium and the data is presented in Figure 10A and 10B. Ghrelin, at concentration range of 100 fmol/L-100 nmol/L, caused concentration-dependent vasodilatation of PE-constricted perfused MVB with an EC_{50} value of 55 ± 6 pmol/L and an E_{max} value of 45 ± 5% obtained at a concentration of 1 nmol/L. Increasing the concentration of ghrelin to 10 and 100 nmol/L did not evoke further relaxation. The presence of L-NAME (100 μmol/L) alone in the perfusion buffer for 15 minutes did not affect the basal PP, but after addition of PE (80 μmol/L), the PP was increased significantly ($p<0.05$) compared to PP attained with the addition of PE alone (from 120 ± 2.5 mmHg in PE alone compared to 154 ± 6.1 mmHg in PE + L-NAME). However, inclusion of a high concentration of L-NAME (100 μmol/L) failed to affect the vasodilator responses to increasing concentrations of ghrelin in PE-precontracted MVB (Figure 10A).

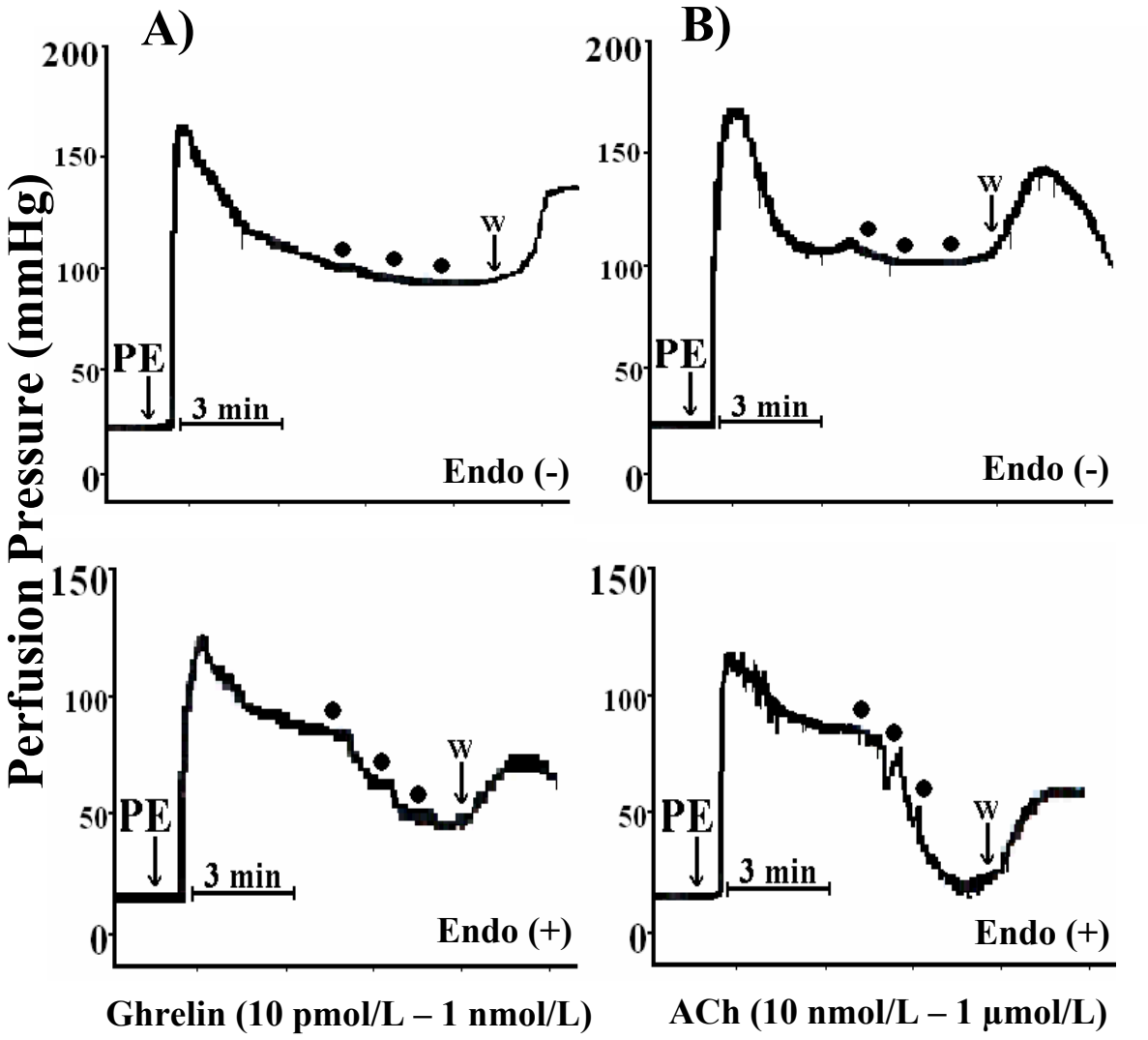

Figure 7. A) Ghrelin, and B) ACh evoke reduction of PP (mmHg) in PE (80 µmol/L)-constricted MVB in an endothelium-dependent manner.

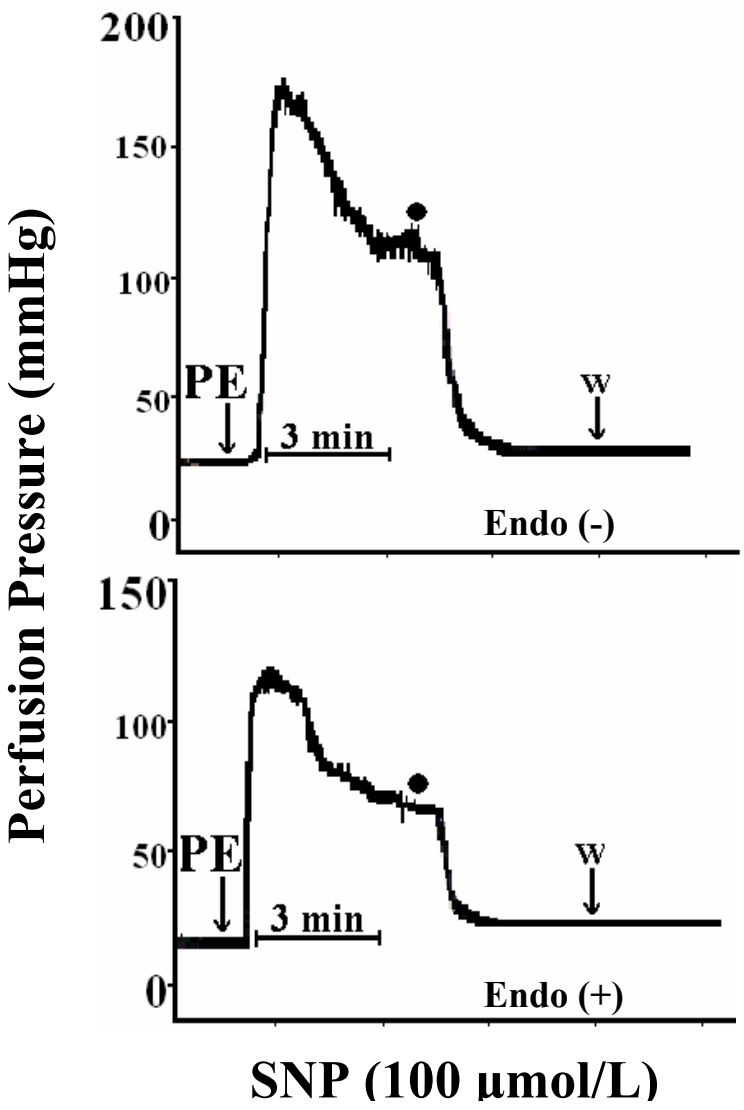

Figure 8. Reduction of PP (mmHg) in PE (80 µmol/L)-constricted MVB in response to SNP (100 µmol/L) in the presence and absence of endothelium.

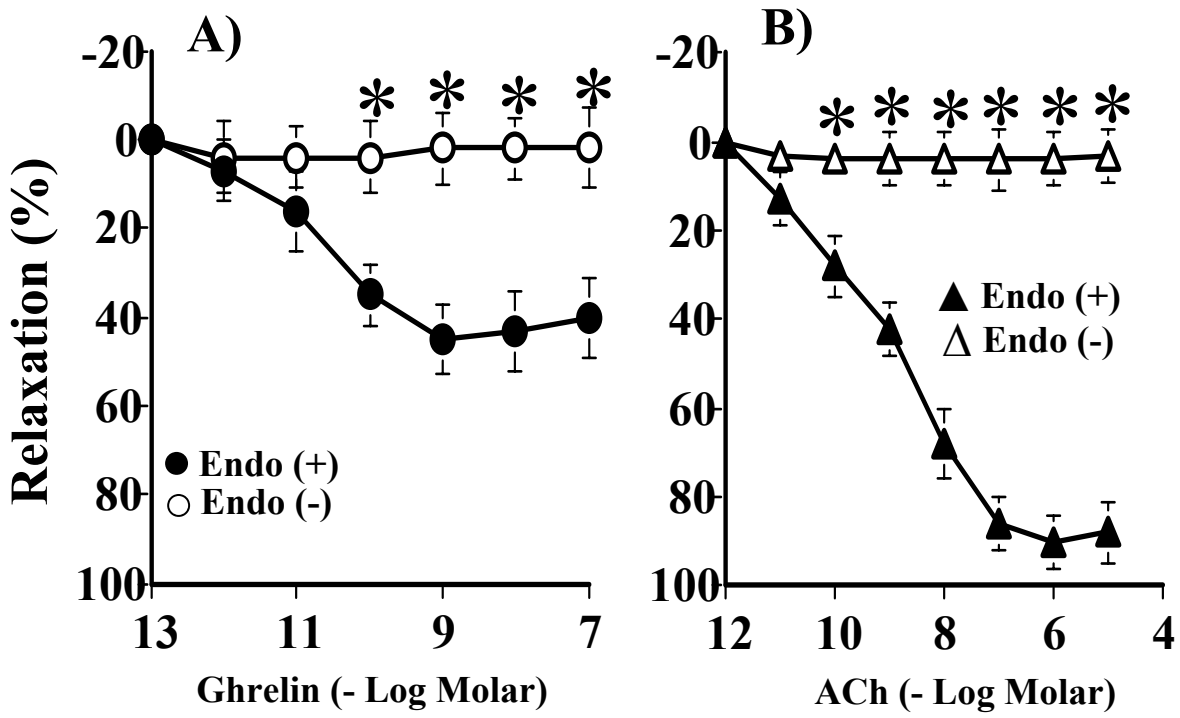

Figure 9. Comparison of CR curves to A) ghrelin and B) ACh in PE-constricted MVB preparations with or without endothelium. Relaxations were measured as a percentage of maximal PE (80 μmol/L)-induced constrictions. Each data point is a mean ± S.E.M. of 6 different determinations. (* $p<0.01$ Endo (-) v/s the respective Endo (+) group).

Similar to ghrelin, ACh (1 pmol/L-10 μmol/L) also produced a concentration-dependent vasodilatation in PE-constricted MVBs with EC_{50} of 3 ± 2 nmol/L and E_{max} value of 90 ± 6% obtained at 1 μmol/L. Increasing the concentration of ACh to 10 μmol/L did not evoke further vasodilatation. Maximal vasodilatation to ACh was significantly different than ghrelin (90 ± 6% for ACh compared to 45 ± 5% for ghrelin, $p<0.01$). Ghrelin, however, produced its half maximal effect (EC_{50}) at much lower concentrations than ACh (EC_{50} 3 ± 2 nmol/L for ACh compared to 55 ± 6 pmol/L; $p<0.01$). Presence of L-NAME (100 μmol/L) caused a significant ($p<0.05$) rightward shift in the CR curve to ACh (control EC_{50} of 3 ± 2 nmol/L compared to L-NAME treated EC50 of 9 ± 3 nmol/L). This was also accompanied by a significant ($p<0.05$) reduction in the E_{max} to ACh in PE-constricted MVBs (E_{max} 90 ± 6% for ACh compared to 75 ± 6% for ACh in the presence of L-NAME).

4.1.4. Effects of TBA on Vasodilator Responses to Ghrelin in the Presence and Absence of L-NAME in MVB Compared to ACh

Since vasodilator responses evoked by ghrelin were insensitive to the blockade by L-NAME, the effect of other distinct mediators of vasodilatation was considered. Several experiments were designed to evaluate the involvement of EDHF in ghrelin- and ACh-evoked vasodilator responses in PE-constricted MVBs. Figure 10 demonstrates the vasodilator responses to ghrelin and ACh in PE-constricted MVBs in the presence and absence of TBA (0.5 mmol/L). Inclusion of TBA, a non-selective K_{Ca} channel inhibitor, in the perfusion medium did not significantly alter either the basal PP or the maximal

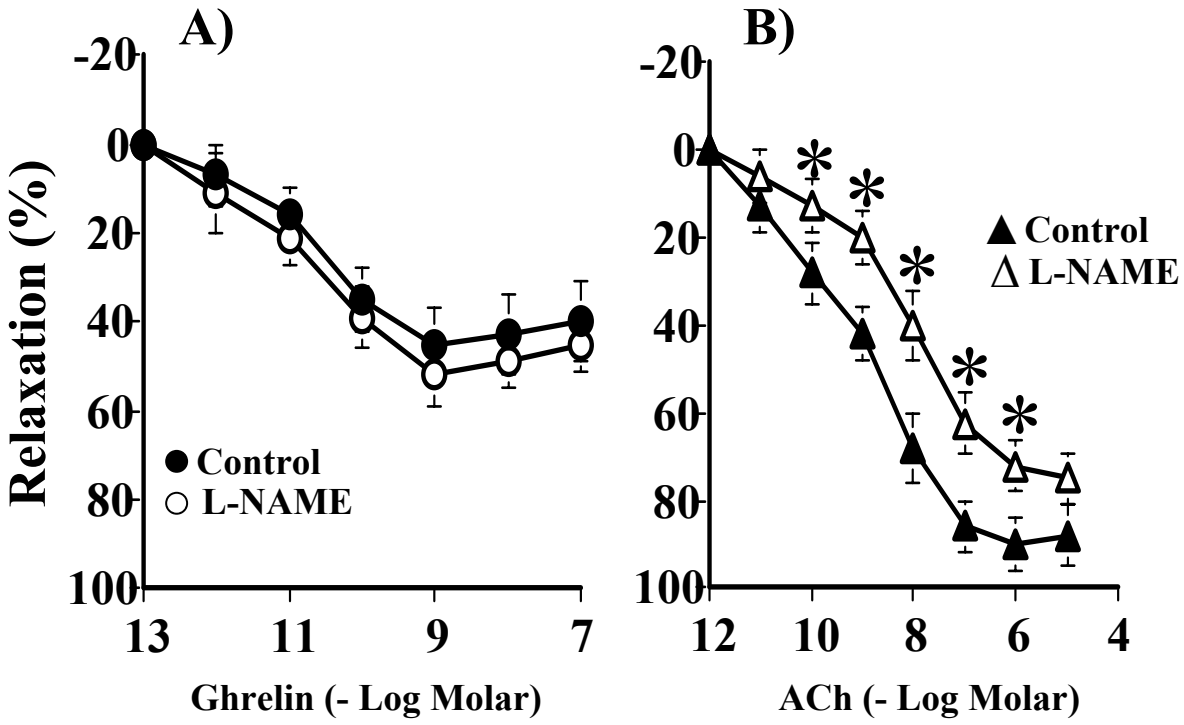

Figure 10. Comparison of CR curves to A) ghrelin and B) ACh in PE-constricted MVB in the presence and absence of NOS inhibitor, L-NAME (100 μmol/L). Relaxations were measured as a percentage of maximal PE (80 μmol/L)-induced constrictions. Each data point is a mean ± S.E.M. of 6 different determinations. (* $p<0.01$ L-NAME v/s control).

vasoconstrictor response to PE (80 μmol/L). Ghrelin-induced vasodilatation (100 fmol/L-100 nmol/L) was completely abolished in the presence of TBA (Figure 11A). The inclusion of L-NAME and TBA together did not affect ghrelin-induced relaxation compared to the presence of TBA alone (Figure 12A).

ACh-induced maximal vasodilatation of MVB was significantly ($p<0.01$) higher than that induced by ghrelin (90 ± 6% for ACh compared to 45 ± 5% for ghrelin). However, ACh was 54.5 fold less potent compared to ghrelin (EC_{50} 3 ± 2 nmol/L for ACh compared to 55 ± 6 pmol/L for ghrelin). In the presence of TBA, the CR curve to ACh was significantly ($p<0.01$) shifted to the right (EC_{50} 3 ± 2 nmol/L in control versus 30 ± 4 nmol/L in the presence of TBA), with a significant ($p<0.05$) reduction in the maximal relaxation (90 ± 6% in control versus 78 ± 6% in the presence of TBA; Figure 11B, Table 3). When TBA was added in combination with L-NAME (100 μmol/L) together, there was a significant ($p<0.01$) rightward shift in the CR curve to ACh (EC_{50} 3 ± 2 nmol/L in control versus 100 ± 6 nmol/L in the presence of TBA + L-NAME). This was accompanied by a significant ($p<0.01$) reduction in the maximal vasodilator response (Emax 90 ± 6% in control versus 20 ± 7% in the presence of TBA + L-NAME; Figure 12B). It is noteworthy to mention that the rightward shift in the CR curve to ACh in PE-constricted MVB in the presence of TBA alone was increased by 3.3 fold compared to the shift in the presence of L-NAME alone (30 ± 4 nmol/L for TBA compared to 9 ± 3 nmol/L for L-NAME, Table 3).

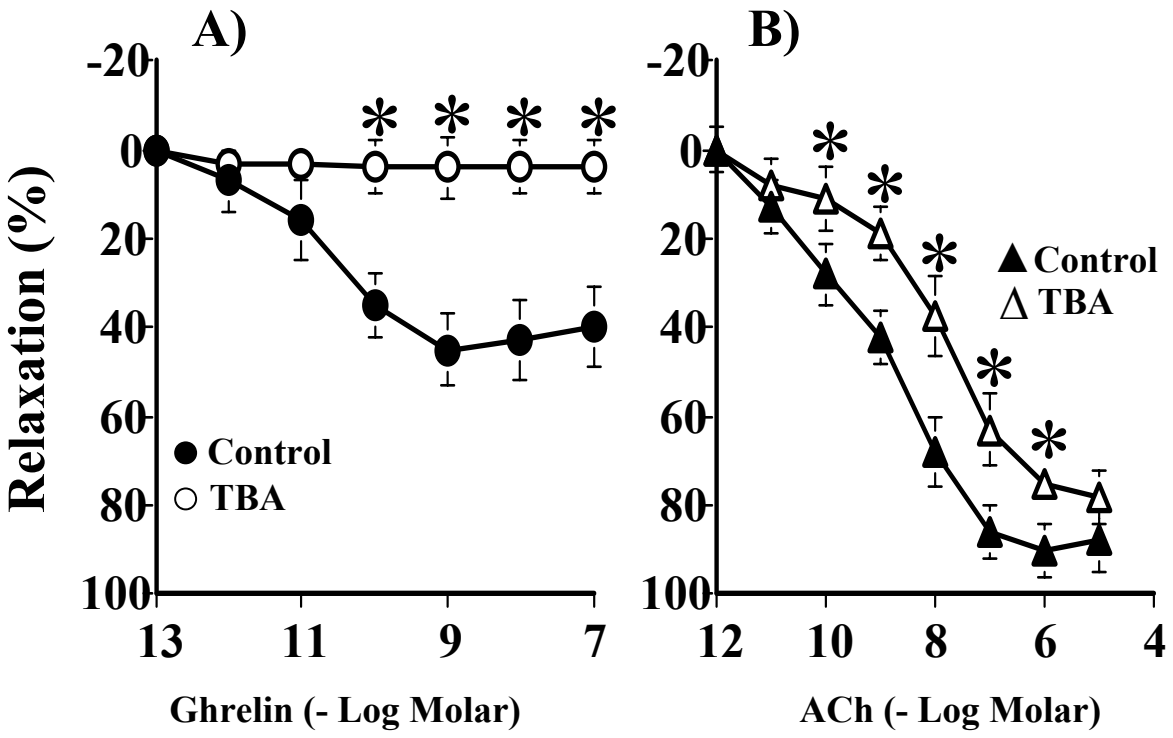

Figure 11. Comparison of CR curves to A) ghrelin and B) ACh in PE-constricted MVB in the presence and absence of a non-selective K_{ca} channel inhibitor, TBA (0.5 mmol/L). Relaxations were measured as a percentage of maximal PE (80μmol/L)-induced constrictions. Each data point is a mean ± S.E.M. of 6 different determinations. (* $p<0.01$ TBA v/s control group).

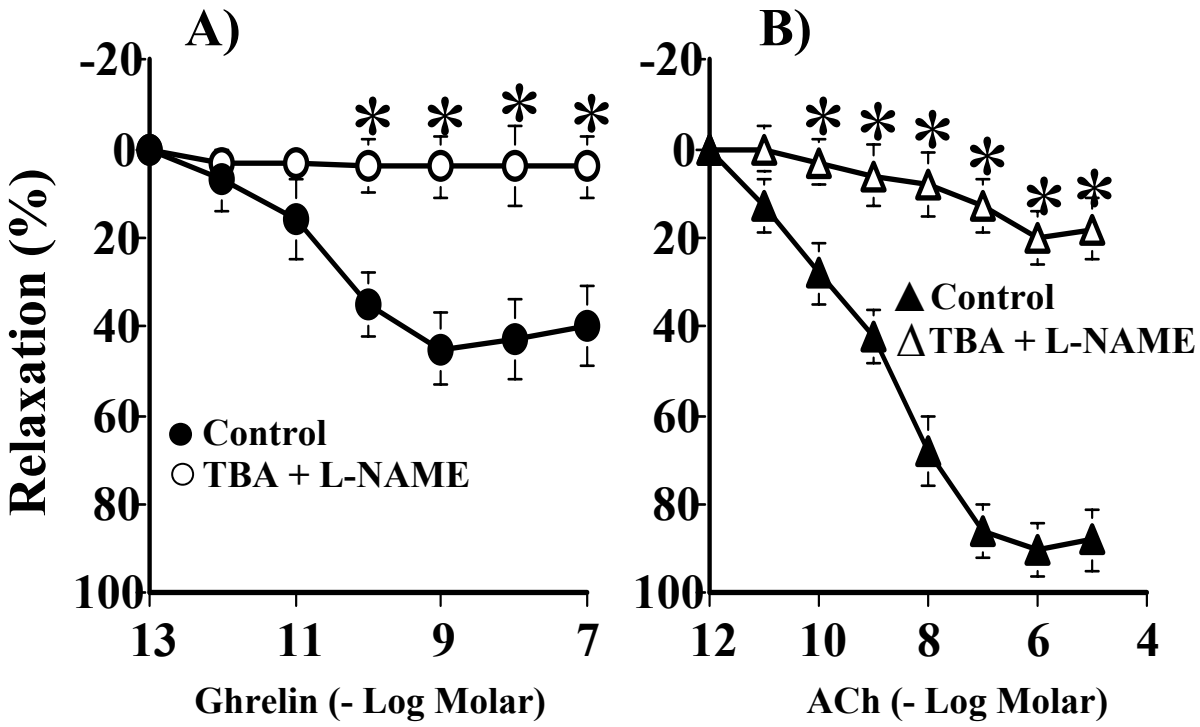

Figure 12. Comparison of CR curves to A) ghrelin and B) ACh in PE-constricted MVB in the presence or absence of a combination of NOS inhibitor (L-NAME, 100 μmol/L)) and a non-selective K_{ca} channel blocker (TBA, 0.5 mmol/L). Relaxations were measured as a percentage of maximal PE (80μmol/L)-induced constrictions. Each data point ia a mean ± S.E.M. of 6 different determinations. (* $p<0.01$ TBA + L-NAME v/s control group).

4.1.5. Effects of High Extracellular K^+ Concentration on Vasodilator Responses to Ghrelin in MVB Compared to ACh

The vasodilator responses to ghrelin and ACh were determined either in the presence or the absence of high K^+ (80 mmol/L)-containing depolarizing Krebs buffer. The PP in high K^+ (80 mmol/L)-containing buffer measured up to 91.3 ± 4 mmHg. Increasing ghrelin concentrations from 100 fmol/L to 100 nmol/L failed to evoke vasodilator responses in K^+-contracted MVB (Figure 13A). It was not possible to determine the EC_{50} value, but the E_{max} value for ghrelin-evoked vasodilatation was 9 ± 3% in the presence of 80 mmol/L K^+ compared to the dilator response of 45 ± 5% seen in PE-constricted preparations. The ACh-evoked vasodilator responses (EC_{50}; E_{max}) were also significantly lower in high K^+ (80 mmol/L)-containing depolarizing buffer compared to the responses to ACh seen in PE-constricted MVB preparations with intact-endothelium. ACh-induced vasodilator responses measured an EC_{50} value of 3 ± 2 nmol/L in the presence of PE compared to 30 ± 5 nmol/L in the presence of 80 mmol/L [K^+]. In addition, the E_{max} value for ACh-evoked dilatation was 50 ± 5% in the presence of 80 mmol/L K^+ compared to the dilator response of 90 ± 6% seen in PE-constricted preparations (Figure 13B, Table 3).

4.1.6. Effects of Different Specific K^+ Channel Blockers on Vasodilator Activities of Ghrelin in MVB Compared to ACh

Vasodilator responses to 1 nmol/L ghrelin and 1 μmol/L ACh were also examined in the presence and absence of different inhibitors of NO, PGI_2, and EDHF pathways. A ghrelin concentration of 1nmol/L and an ACh concentration of 1 μmol/L

were shown by previous experiments in our study to produce maximal vasodilatation in PE-constricted MVB. Under control conditions, 1 nmol/L ghrelin evoked 45 ± 5% vasodilatation in perfused PE-constricted MVB. This was significantly ($p<0.01$) different from 90 ± 6% vasodilatation evoked by 1 μmol/L ACh (Figure 14, Table 3). Inclusion of L-NAME (100 μmol/L) in the perfusion buffer containing PE, failed to affect the maximal response evoked by ghrelin (48 ± 6% in the presence of L-NAME versus 45 ± 5% in control) (Figure 14, Table 3). E_{max} to ACh was significantly ($p<0.05$, 1.2 fold) reduced in the presence of L-NAME (75 ± 6% in the presence of L-NAME versus 90 ± 6% in control). Treatment with the COX inhibitor indomethacin (10 μmol/L) produced similar degree of vasodilatations to both ghrelin and ACh compared to their respective values under control conditions (40 ± 4% in the presence of indomethacin versus 45 ± 5% in control for ghrelin, 81 ± 5% in the presence of indomethacin versus 90 ± 6% in control for ACh). In the presence of TBA alone, there was a significant ($p<0.01$) reduction in the maximal relaxation to ghrelin (5 ± 0% in the presence of TBA versus 45 ± 5% in control) (Figure 14, Table 3). TBA in combination with L-NAME failed to have any further effect on maximal vasodilator responses to ghrelin (5 ± 2% in the presence of TBA + L-NAME versus 5 ± 0% in the presence of TBA alone). Maximal vasodilator responses to ACh were also significantly ($p<0.05$) reduced in the presence of TBA alone (78 ± 5% in the presence of TBA versus 90 ± 6% in control). However, when TBA was added in combination with L-NAME, there was a further significant ($p<0.01$) decrease in maximum vasodilator response to ACh (20 ± 7% in the presence of TBA + L-NAME versus 78 ± 5% in the presence of TBA alone, and 90 ± 6% in control).

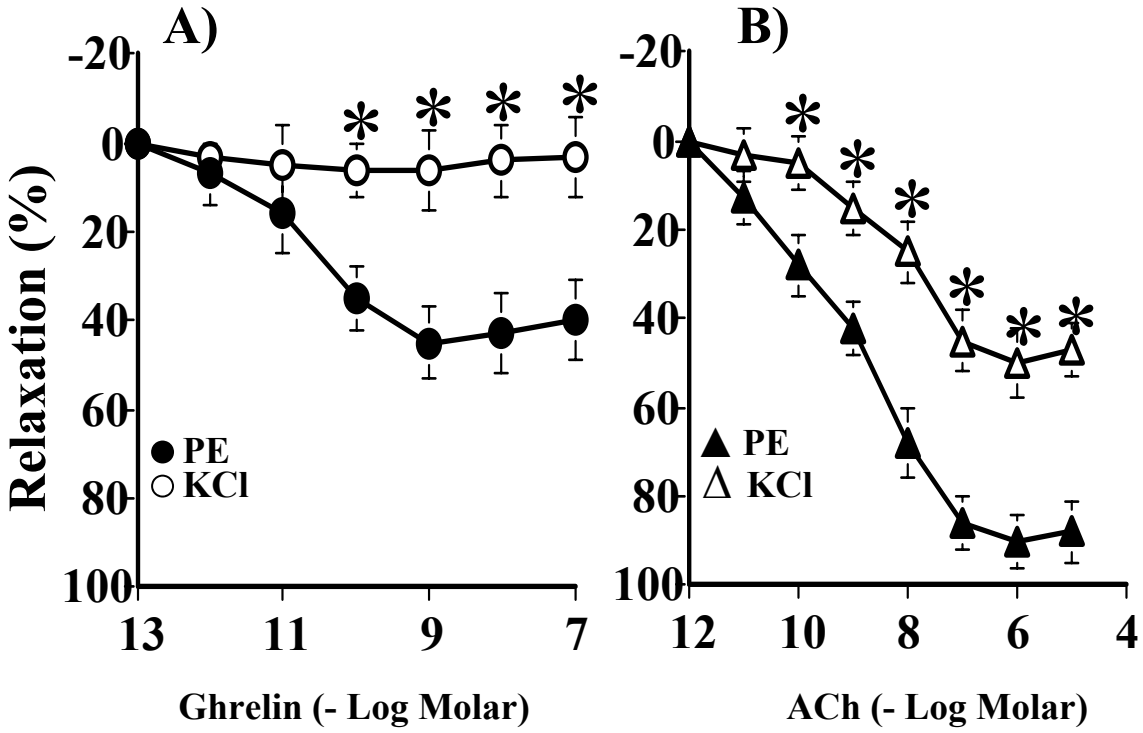

Figure 13. Comparison of CR curves to A) ghrelin and B) ACh in constricted MVB in the presence of either PE or KCl (80 mmol/L). Relaxations were measured as a percentage of maximal PE (80 μmol/L)-induced constrictions. Each data point is a mean ± S.E.M. of 6 different determinations. (* $p<0.01$ KCl v/s PE preparations).

Apamin is a selective blocker of SK_{Ca} channels and ChTX is a non-selective blocker of IK_{Ca}, BK_{Ca}, and some voltage-dependent K^+ channels. The maximal vasodilatation evoked by ghrelin was significantly ($p<0.01$) reduced in the presence of apamin + ChTX (10 ± 4 % in the presence of apamin + ChTX versus 45 ± 5% in control). Similarly, ACh-evoked vasodilatation was also significantly ($p<0.05$) lower in the presence of apamin + ChTX (73 ± 5 % in the presence of apamin + ChTX versus 90 ± 6% in control). The decrease in ghrelin-evoked vasodilator responses in the presence of apamin + ChTX was much higher compared to the blockade of ACh-evoked vasodilator responses (Figure 14). To assess the effect of endothelial IK_{Ca} channels specifically, an experiment was designed to perform ghrelin- and ACh-evoked maximal vasodilator responses in the combined presence of apamin (1 μmol/L) and TRAM-34 (a selective IK_{Ca} channel blocker, 10 μmol/L). A combination of apamin + TRAM-34 resulted in significant reductions in both ghrelin- and ACh-evoked maximal vasodilatation (ghrelin: 2 ± 0% in the presence of apamin + TRAM-34 versus 45 ± 5% in control [$p<0.01$], ACh: 68 ± 6% in the presence of apamin + TRAM-34 versus 90 ± 6% in control [$p<0.05$]) (Figure 14). The decrease in ghrelin-evoked vasodilator responses in the presence of apamin + TRAM-34 was higher than that in ACh-evoked vasodilator responses in MVB (43% decrease for ghrelin versus 22% decrease for ACh; Figure 14). Ghrelin-evoked maximal vasodilator responses were reduced more in the presence of apamin + TRAM-34 than in the presence of apamin + ChTX; a combination of apamin + TRAM-34 resulted in almost a complete abolition of ghrelin-evoked responses (45 ± 5% in control versus 10 ± 4% in the presence of apamin + ChTX, and 2 ± 0% in the presence of apamin + TRAM-34).

To evaluate the involvement of EDHF in ghrelin- and ACh-evoked vasodilatations in MVB in more detail, our study was to assess ghrelin- and ACh-induced maximal vasodilatations in the presence of ouabain (10 μmol/L) and Ba^{2+} (30 mmol/L) (Figure 14). Ouabain is an inhibitor of Na^{+}/K^{+}-ATPase pump and Ba^{2+} is a blocker of K_{ir} channels. Treatment with ouabain + Ba^{2+} diminished ghrelin-evoked maximal vasodilatations significantly ($p<0.01$; 13 ± 5% in the presence of ouabain + Ba^{2+} versus 45 ± 5% in control). Similarly, ACh-evoked maximal vasodilatations were significantly ($p<0.01$) reduced in the presence of ouabain + Ba^{2+} (52 ± 6% in the presence of ouabain + Ba^{2+} versus 90 ± 6% in control). However, the decrease in ghrelin-evoked responses in the presence of ouabain + Ba^{2+} was not significantly different from that of ACh (32% decrease in ghrelin-evoked response versus 38% decrease in ACh-evoked response).

To validate the minimal involvement of K_{ATP} channels in EDHF-mediated pathways, an experiment was performed to assess ghrelin- and ACh-induced maximal vasodilatation in the presence of glibenclamide (10 μmol/L), a specific blocker of K_{ATP} channels. Unlike apamin + ChTX and apamin + TRAM-34, inclusion of glibenclamide in the perfusion medium caused no inhibition of ghrelin-mediated maximal response in PE-constricted MVB (40 ± 5 in the presence of glibenclamide versus 45 ± 5% in control) (Figure 14). In contrast, maximal vasodilatation to ACh was significantly ($p<0.05$) attenuated in the presence of glibenclamide (70 ± 4% in the presence of glibenclamide versus 90 ± 6% in control). However, it is noteworthy to mention that the decrease in ACh-evoked maximal vasodilatation in the presence of glibenclamide was to

the same extent as that in the presence of L-NAME (20% in the presence of glibenclamide versus 15% in the presence of L-NAME).

4.1.7. Des-acyl Ghrelin Evokes Concentration-Dependent Vasodilatation in MVB in an Endothelium-Dependent Manner

After 1hr equilibration, the basal PP values under control conditions averaged 15 ± 1.2 mmHg. Three minutes of perfusion with PE (80 μmol/L) resulted in a sustained tonic steady-state contraction of 120 ± 2.5 mmHg in MVB. Cumulative CR curves for des-acyl ghrelin (100 fmol/L-100 nmol/L) in PE-constricted MVB demonstrated concentration-dependent decreases in PP. A representative experiment demonstrating CR patterns to increasing concentrations of des-acyl ghrelin is shown in Figure 15A. The CR curve pattern to des-acyl ghrelin (100 fmol/L-100 nmol/L) was closely similar to the CR curve to ghrelin with similar E_{max} values (E_{max} values of 45 ± 5% for ghrelin and 43 ± 6% for des-acyl ghrelin). The E_{max} values for both peptides were reached at the same concentration level (1 nmol/L) (Figure 16A and 16B). In addition, the data from several CR curves confirmed that the EC_{50} values for ghrelin and des-acyl ghrelin were also closely similar (EC_{50} 49 ± 7 pmol/L for des-acyl ghrelin and EC_{50} 55 ± 6 pmol/L for ghrelin). The time-course of des-acyl ghrelin-evoked vasodilator responses also followed the same pattern as ghrelin with a “slower onset” and a relatively “longer time” to reach maximal effect compared to the other agonist ACh, which was much more rapid.

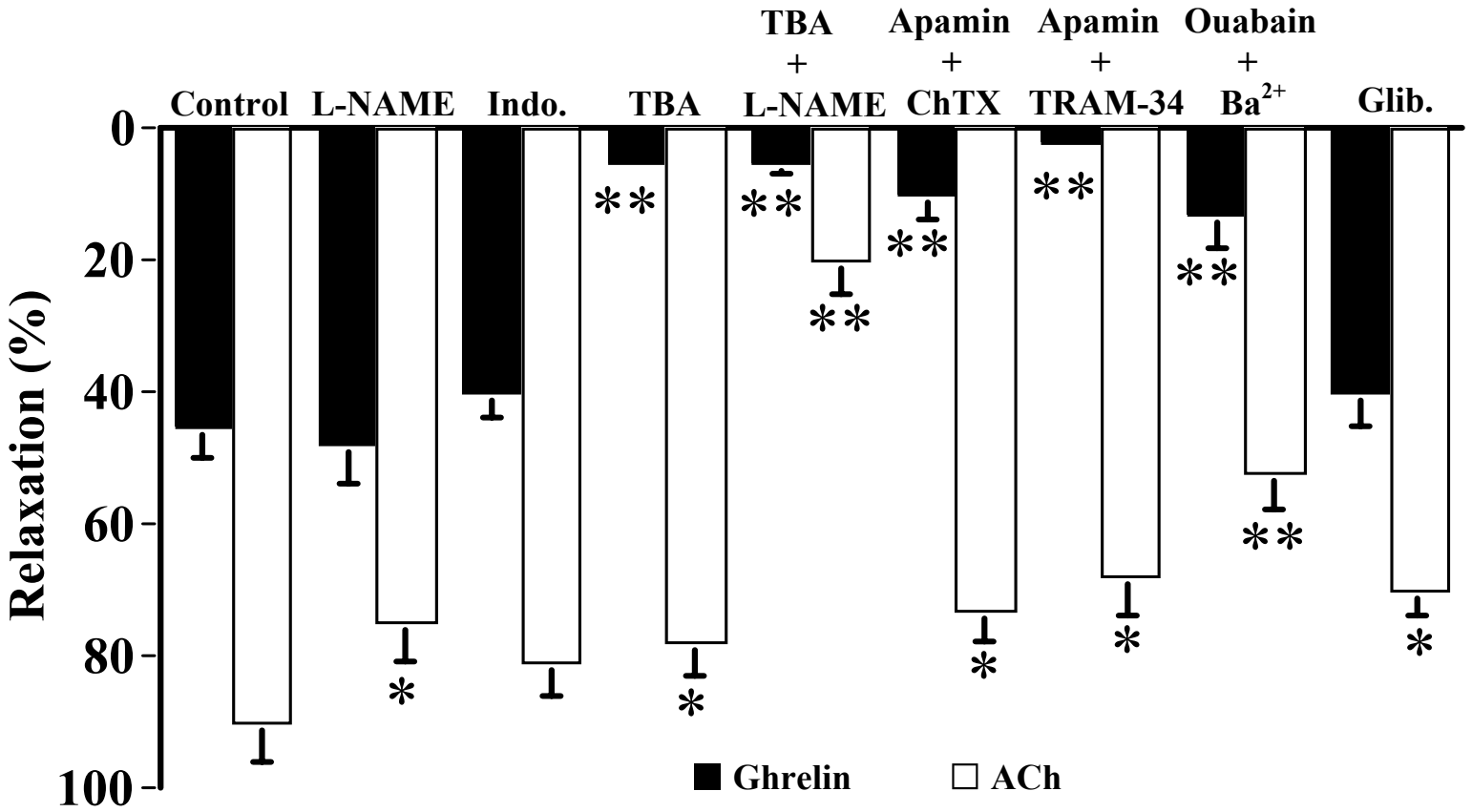

Figure 14. Ghrelin (1 nmol/L) and ACh (1 μmol/L)-evoked maximal relaxations of PE (80 μmol/L)-constricted MVB in the presence of NOS inhibitor (L-NAME, 100 μmol/L), non-selective K_{Ca} channel blocker (TBA, 0.5 mmol/L), a combination of L-NAME + TBA, a combination of selective SK_{Ca} channel blocker (apamin, 1 μmol/L) and a non-selective blocker of BK_{Ca} and IK_{Ca} (charybdotoxin ChTX, 100 nmolL), a combination of apamin and a selective blocker of IK_{Ca} (TRAM-34, 10 μmol/L), K_{ATP} channel blocker (glibenclamide, 10μmol/L), a combination of Na^+/K^+-ATPase pump inhibitor (ouabain, 10 μmol/L) and K_{ir} channel blocker (Ba^{2+}, 30 mmol/L), and COX inhibitor (indomethacin, 10 μmol/L). Relaxations were measured as a percentage of maximal PE-induced contractions. Each data point is a mean±S.E.M. of 6 different preparations (* $p<0.05$ and ** $p<0.01$ v/s control group).

Table 3. EC_{50} and E_{max} values for Ghrelin- and ACh-evoked relaxations of MVB in the presence or absence of endothelium, as well as in pretreated preparations with NOS inhibitor (L-NAME, 100 μmol/L), non-selective K_{ca} channel blocker (TBA, 0.5 mmol/L), a comnination of L-NAME + TBA, and a depolarizing agent KCl (80 mmol/L).

Group	Ghrelin		ACh	
	EC_{50} (pM)	**E_{max}**	**EC_{50} (nM)**	**E_{max}**
Control	55±6	45±5	3±2§§	90±6§§
Endo (-)	-------	4±0**	-------	7±8**
L-NAME	55±2	48±6	9±3*	75±6*
TBA	-------	5±0**	30±4**	78±5*
TBA + L-NAME	-------	5±2**	100±6**	20±5**
KCl	-------	9±3**	30±5**	50±5**

* $p<0.05$, ** $p<0.01$ endothelium-denuded or different inhibitors v/s the respective control group.
§§ $p<0.01$ control ghrelin v/s control ACh.

The importance of endothelium in the regulation of vascular tone by increasing concentrations of des-acyl ghrelin was also demonstrated. In comparison to the intact-endothelium, endothelium denudation increased both basal PP and PP after contraction with PE (80 μmol/L). Basal PPs of 15 ± 1.2 mmHg in intact-endothelium preparations were increased to 24 ± 0.7 mmHg in endothelium-denuded preparations. PE-evoked contractions of 120 ± 2.5 mmHg in endothelium-intact preparations were increased to 163 ± 8 mmHg in endothelium-denuded preparations. Similar to ghrelin, endothelium-denudation completely abolished vasodilator responses to increasing concentrations of des-acyl ghrelin (100 fmol/L-100 nmol/L) (Figure 15A and 15B).

4.2. Ghrelin Evokes Vasodilatation in the MVB in the Presence of Antagonists of GHSR1a receptors

4.2.1. [D-Lys^3]-GHRP-6, a competitive Antagonist of GHSR-1a Receptors, Evokes Vasodilatation in MVB

In order to determine the involvement of GHSR-1a receptors in ghrelin- and des-acyl ghrelin-evoked vasodilatations, an experiment was designed to assess ghrelin- and des-acyl ghrelin-evoked vasodilatation of MVB in the presence of [D-Lys^3]-GHRP-6, a competitive antagonist of the classical GHSR-1a receptors. A representative tracing of a single experiment is shown in Figure 17. Basal PP in MVB averaged 15 ± 1.2 mmHg. PE (80 μmol/L) induced increases in PP up to 120 ± 2.5 mmHg. Addition of a high concentration (10 μmol/L) of [D-Lys^3]-GHRP-6 *per se* evoked decreases in PP in PE-constricted MVBs, which were not statistically different from those induced by ghrelin (E_{max} 45 ± 5% for ghrelin and 37 ± 4% for [D-Lys^3]-GHRP-6).

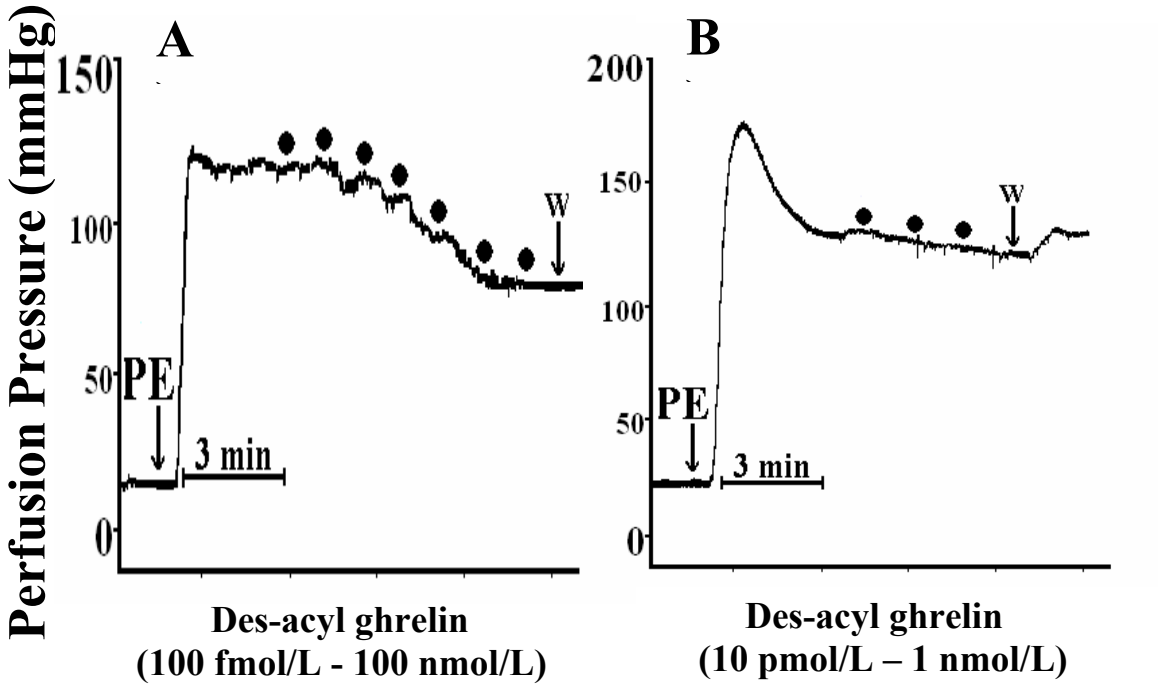

Figure 15. Des-acyl ghrelin induces reduction of PP (mmHg) in PE (80 μmol/L)-constricted MVB in an endothelilum-dependent manner: A) Preparation with intact-endothelium, B) Preparation with denuded-endothelium.

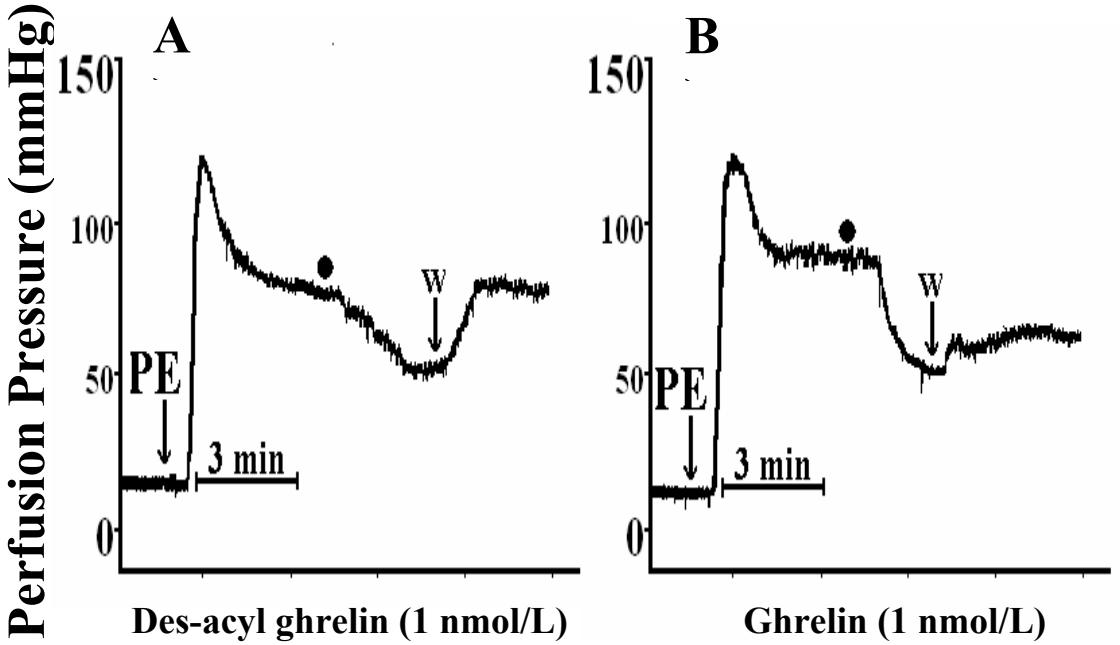

Figure 16. A) Des-acyl ghrelin (1 nmol/L), and B) ghrelin (1 nmol/L) induce maximal reduction of PP (mmHg) in PE (80 μmol/L)-constricted MVB.

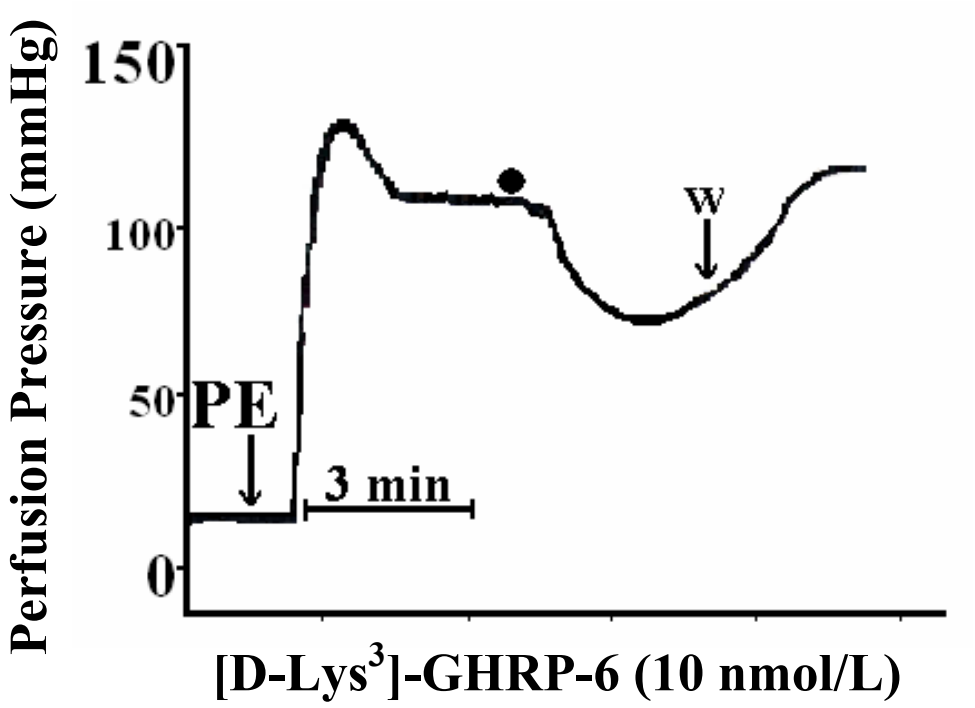

Figure 17. [D-Lys^3]-GHRP-6, an antagonist of GHSR-1a receptors, reduces PP (mmHg) in PE (80 μmol/L)-constricted MVB.

4.2.2. L-756867, a Peptide Antagonist of GHSR-1a receptors, does not Block Ghrelin-Evoked Vasodilator Responses in MVB

Different antagonists of the classical GHSR-1a receptors may have different structural and chemical properties, and therefore may influence the receptor and both the potency and efficacy of ghrelin signaling in different manners. Vasodilatation in response to ghrelin was also evaluated in the presence of L-756867, a peptide antagonist of the classical GHSR-1a receptors. Inclusion of L-756867 in the perfusion buffer, up to a concentration of 1 μmol/L, failed to alter the basal PP or evoke any vasodilator response (Figure 18A). Moreover, L-756867 (1 μmol/L) failed to affect the vasodilator responses evoked by ghrelin. Similar to the control conditions (absence of the antagonist), where ghrelin induced maximal vasodilatation of E_{max} 45 ± 5%, ghrelin evoked maximal vasodilatation of E_{max} 42 ± 3% in MVBs treated with antagonist (Figures 18B and 18C).

4.3. Non-Peptide Agonists of GHSR-1a receptors, L-166446 and L-163255, Evoke Concentration-Dependent Vasodilatation in MVB

Similar to ghrelin, synthetic peptidyl GHSs have also been demonstrated to exert direct and GH-independent cardiac actions (Locatelli et al., 1999; Imazio et al., 2002). However, to our knowledge, there has not been any study performed investigating cardiovascular actions of synthetic non-peptidyl GHSs. Therefore, based on the above information, our purpose was to investigate if the two synthetic non-peptidyl agonists of GHSR1a, L-166446 and L-163255, can promote vasodilator responses in constricted MVBs. Cumulative CR curves for both non-peptide agonists L-166446 and L-163255

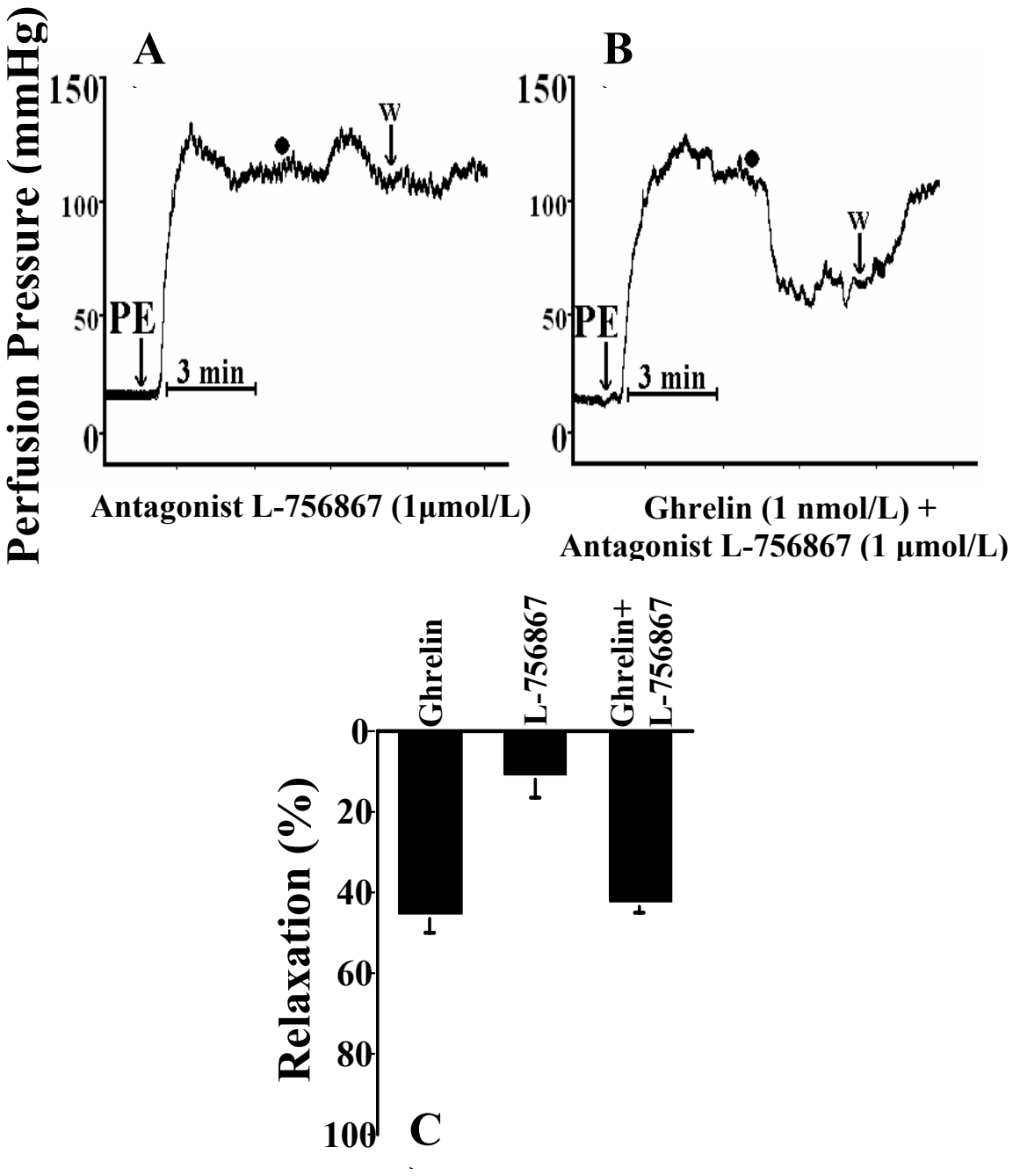

Figure 18. L-756867 (1 µmol/L), an antagonist of GHSR-1a receptors, A) does not induce reduction in PP (mmHg), and B) does not block ghrelin (1 nmol/L)-evoked reduction in PP in PE (80 µmol/L)-constricted MVBs. C) Reductions in PP by ghrelin in the presence and absence of L-756867 were also measured as percentages of maximal PE-induced contractions. Results are mean ± S.E.M. of 5 different determinations.

(100 fmol/L-100 nmol/L) in PE-constricted MVBs demonstrated concentration-dependent decreases in PP. Representative experiments demonstrating CR patterns to increasing concentrations of L-166446 and L-163255 are shown in Figure 19A and 19B. Both L-166446- and L-163255-evoked concentration-dependent decreases in PP were also measured as a percentage of maximal PE-induced contractions (Figure 21). Similar to ghrelin, both compounds reached their maximal effects at the concentration of 1 nmol/L. However, the maximal vasodilatation in response to L-166446 (E_{max} 70 ± 6%, 1.6 fold, $p<0.01$) and L-163255 (E_{max} 60 ± 5%, 1.3 fold, $p<0.05$) were significantly higher than the response evoked by ghrelin (E_{max} 45 ± 5%) (Figure 19A and 19B, Figure 20A, 20B, and 20C). A comparison of the data from several CR curves for the two non-peptides demonstrated that L-166446 was more potent (EC_{50} 4 ± 2 pmol/L; E_{max} 70 ± 6%) than L-163255 (EC_{50} 6 ± 3 pmol/L; E_{max} 60 ± 5%). However, the comparison of EC_{50} and E_{max} values for the two non-peptides did not reach statistical significance (Figure 21, Table 4). Both non-peptide agonists were shown to be much more potent ($p<0.01$) compared to ghrelin (EC_{50} 4 ± 2 pmol/L for L-166446, EC_{50} 6 ± 3 pmol/L for L-163255, EC_{50} 55 ± 6 pmol/L for ghrelin). A comparison of EC_{50} and E_{max} values for L-166446, L-163255, and ghrelin is depicted in Table 4.

4.4. Short Peptide Fragments of Des-acyl Ghrelin Evoke Vasodilatation in MVB

In agreement with the previous data, the present study also showed that the whole molecule of ghrelin may not be necessary for its vasodilator response. Des-acyl

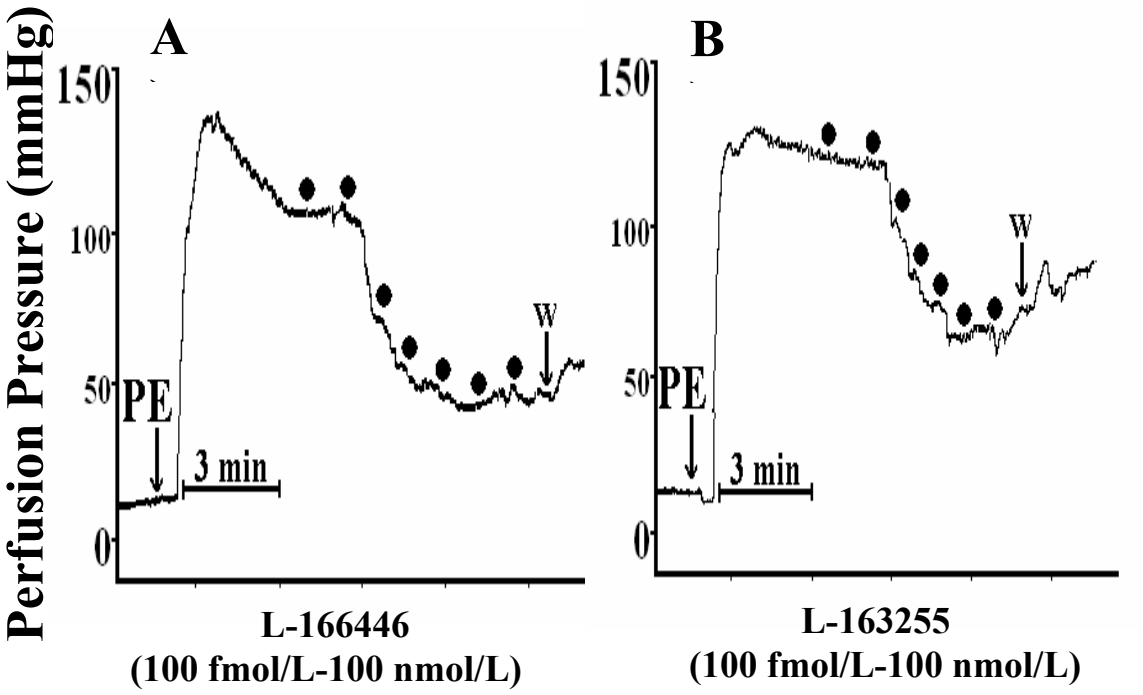

Figure 19. Agonists of GHSR-1a receptors A) L-166446 (100 fmol/L-100 nmol/L), and B) L-163255 (100 fmol/L-100 nmol/L) induce reductions in PP (mmHg) in PE (80 μmol/L)-constricted MVBs in a concentration-dependent manner.

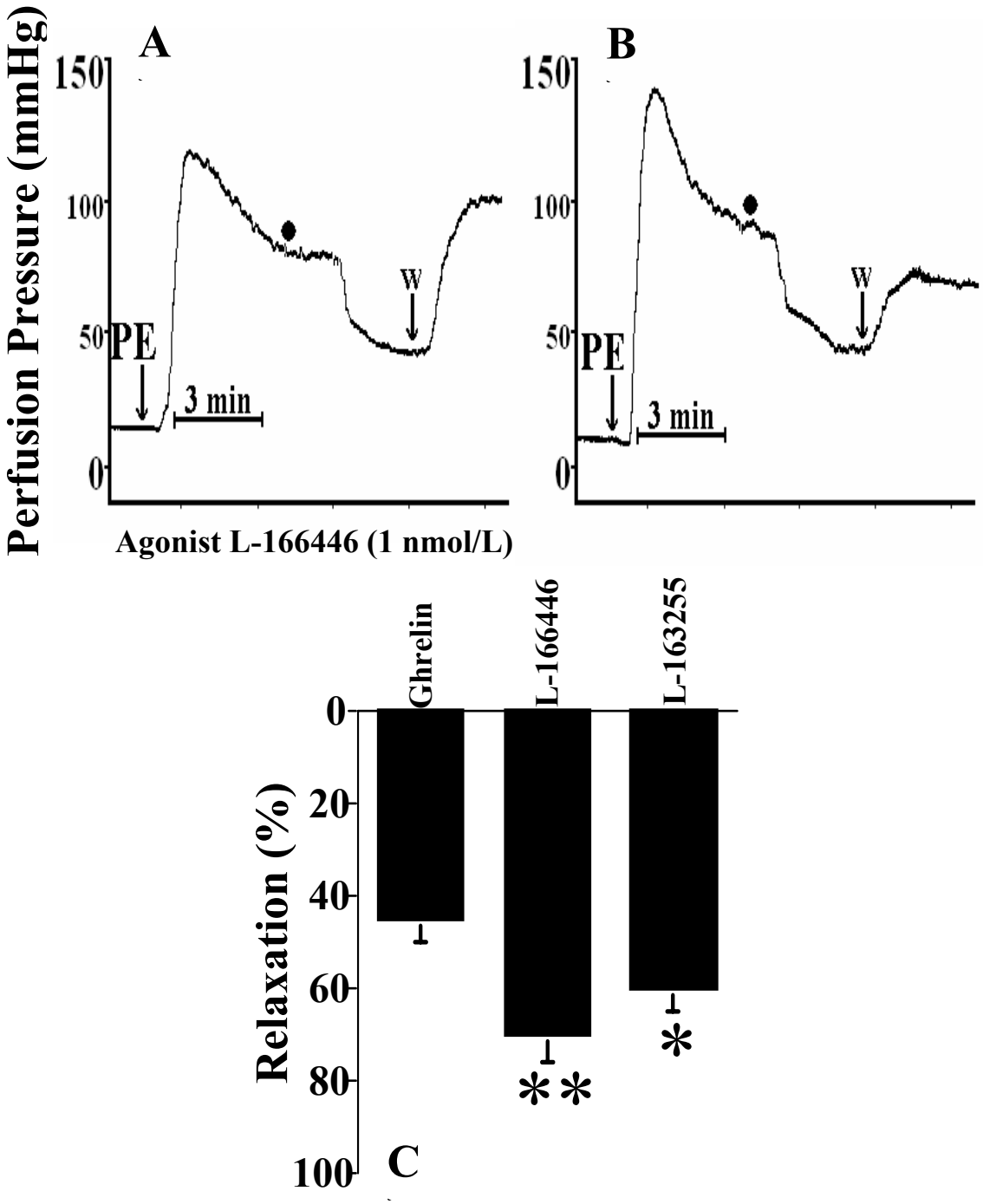

Figure 20. Agonists of GHSR-1a receptor A) L-166446 (1 nmol/L), and B) L-163255 (1 nmol/L) induce maximal reductions in PP (mmHg) in PE (80 μmol/L)-constricted MVBs. C) Reductions in PP by the two agonists were also measured as percentages of maximal PE-induced contractions. Results are mean ± S.E.M. of 5 different determinations. * $p<0.05$ and ** $p<0.01$ agonists v/s ghrelin.

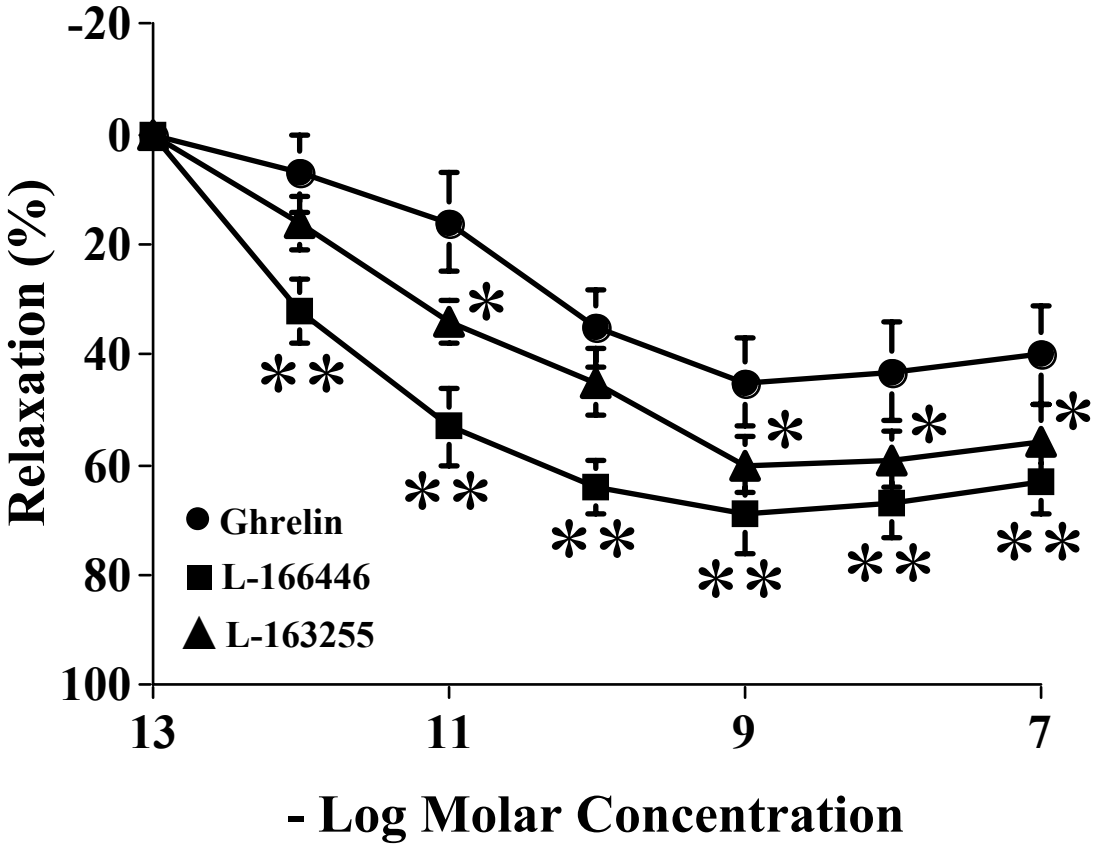

Figure 21. Comparison of CR curves (100 fmol/L-100 nmol/L) for ghrelin, L-166446, and L-163255 in PE (80 μmol/L)-constricted MVBs. Relaxations were measured as percentages of maximal PE-induced contractions at each concentration. Each data point is mean ± S.E.M. of 5 different determinations. * $p<0.05$ and ** $p<0.01$ agonists v/s their respective concentrations of ghrelin.

Table 4. EC_{50} and E_{max} values for Ghrelin-evoked relaxations of PE (80 μmol/L)-constricted MVB compared to the relaxations evoked by the two synthetic nonpeptide agonists of GHSR-1a receptors, L-166446 and L-163255.

Group	EC_{50} (pM)	E_{max}
Ghrelin	55±6	45±5
L-166446	4±2**	70±6**
L-163255	6±3**	60±5*

* $p<0.05$ and ** $p<0.01$ synthetic nonpeptide agonists v/s ghrelin.

ghrelin could dilate MVB to the same extent as ghrelin. Therefore, several fragments of des-acyl ghrelin were prepared by successive deletions of C-terminal amino acid residues to determine the active core required for des-acyl ghrelin-evoked vasodilatation. Vasodilatation or responses to 1 nmol/L concentration of either des-acyl ghrelin or its fragments consisting of 20, 16, 10, 6, 4, and 3 amino acids from the N-terminal were evaluated in PE (80 μmol/L)-constricted MVBs. A comparison of the vasodilator effects of des-acyl ghrelin with other fragmented des-acyl ghrelin peptides is presented in Figure 22. Des-acyl ghrelin induced a maximal vasodilatation of E_{max} 43 ± 6% in PE-constricted MVBs. All the short peptides encompassing the first 20, 16, 10, 6, 4, and 3 residues of des-acyl ghrelin were found to evoke vasodilatation about as efficiently as the full-length des-acyl ghrelin. Vasodilator responses to a 1 nmol/L concentration of single amino acids L-serine or glycine, however, were significantly reduced. While des-acyl ghrelin produced a maximal vasodilatation of E_{max} 43 ± 6%, L-serine-, and glycine-evoked maximal vasodilatation were reduced to 24 ± 4% ($p<0.05$) and 17 ± 3($p<0.01$), respectively. A representative tracing demonstrating reductions in PP in constricted MVBs in response to 1 nmol/L concentrations of L-serine, glycine, and des-acyl ghrelin is demonstrated in Figure 23.

4.5. Ghrelin-Evoked Vasodilatation in MVB in Relation to Plasma Ghrelin Levels in STZ-treated Diabetic rats

4.5.1. Metabolic Parameters in STZ-treated Diabetic rats

Following an overnight fasting, diabetes was induced in 12 weeks old SD rats by

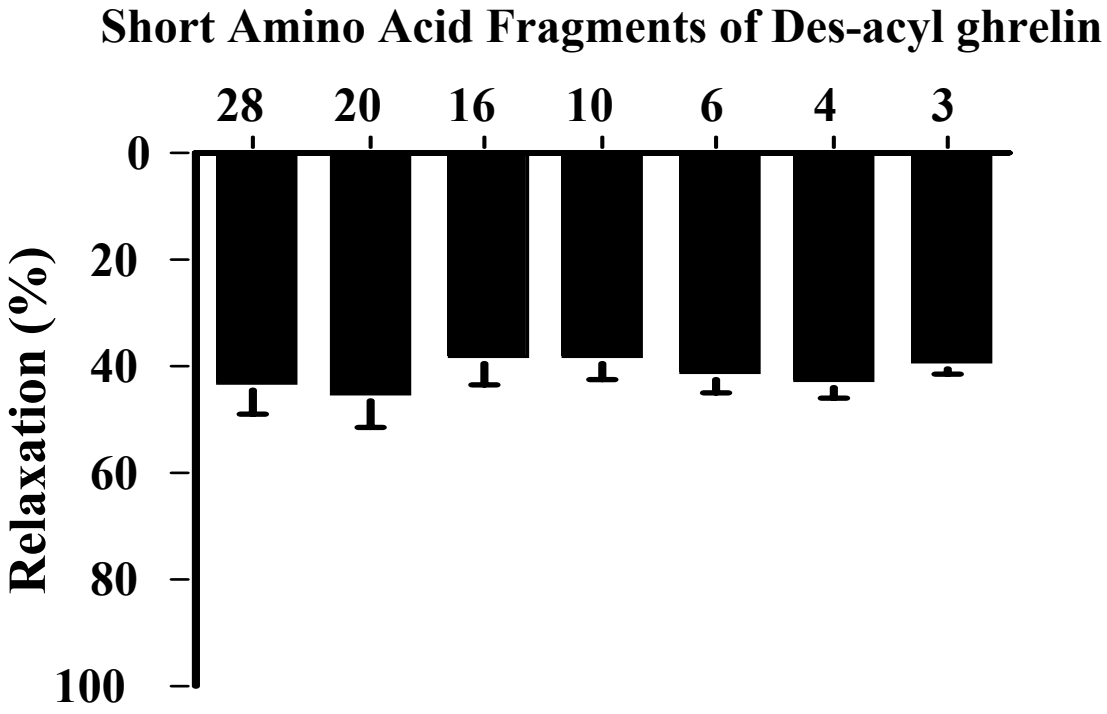

Figure 22. Maximal Relaxation to short amino acid fragments (1 nmol/L) of des-acyl ghrelin in PE (80 μmol/L)-constricted MVB. Relaxations were measured as percentages of maximal PE-induced contraction. Results are mean ± S.E.M. of 6 different determinations.

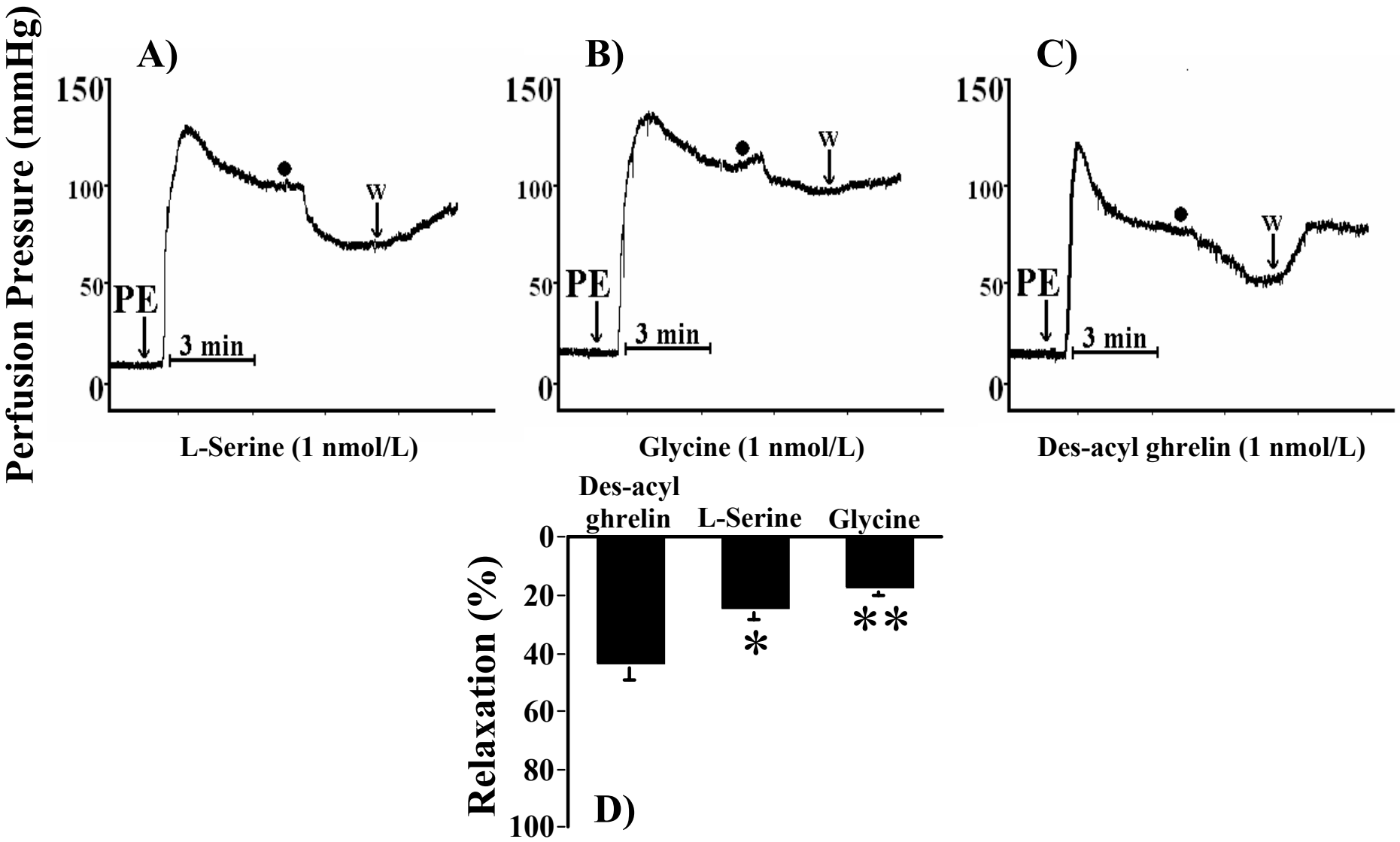

Figure 23. Reductions of PP (mmHg) in PE (80 μmol/L)-constricted MVBs in response to single amino acids A) L-serine (1 nmol/L) and B) Glycine (1 nmol/L) compared to C) des-acyl ghrelin (1 nmol/L). D) Reductions in PP were also measured as percentages of maximal PE-induced contractions. Results are mean ± S.E.M. of 6 different determinations. * $p<0.05$ and ** $p<0.01$ single amino acids v/s des-acylghrelin.

a single i.p. injection of STZ in citrate buffer. Metabolic variables of both STZ-induced diabetic and control rats were then evaluated after 4 and 8 weeks of treatment. Table 5 summarizes metabolic variables of 4- and 8-weeks STZ-induced diabetic and control SD rats. There were no differences in weight at the time of STZ-treatment. All rats weighed approximately between 360-380 grams at the age of 12 weeks. Control animals, both fed and fasted, gained weight after 4 and 8 weeks (after STZ-treatment group versus before STZ-treatment group) resulting in significant weight differences at the time of sacrifice (Table 5). Weight gain in control fed/fasted animals, however, was more pronounced in 8-weeks ($p<0.01$) compared to 4-weeks ($p<0.05$). Control fed rats weighed 372 ± 5 to 462 ± 6 after 4 weeks ($p<0.05$) compared to 380 ± 9 to 511 ± 10 after 8 weeks ($p<0.01$). Similarly, control fasted rats weighed 366 ± 7 to 430 ± 11 after 4 weeks ($p<0.05$) compared to 374 ± 3 to 490 ± 9 after 8 weeks ($p<0.01$). In contrast to control rats, diabetic rats, both fed and fasted, lost weight after 4 and 8 weeks of STZ treatment (after STZ-treatment group versus before STZ-treatment group) resulting in significant weight reductions at the time of sacrifice (Table 5). Weight loss in diabetic fed/fasted animals, was more pronounced in 8-weeks ($p<0.01$) compared to 4-weeks ($p<0.05$). Diabetic fed rats weighed 365 ± 4 to 330 ± 7 after 4 weeks ($p<0.05$) compared to 379 ± 6 to 281 ± 24 after 8 weeks ($p<0.01$). Similarly, diabetic fasted rats weighed 377 ± 2 to 332 ± 4 after 4 weeks ($p<0.05$) compared to 383 ± 9 to 263 ± 15 after 8 weeks ($p<0.01$).

Type 1 diabetes is associated with low plasma insulin and high glucose levels (Hopfner et al., 1999). Accordingly, the mean blood glucose levels after 4 weeks of induced diabetes were significantly higher in both fasted (20.4 ± 0.5 mmol/L) and fed (23.1 ± 1.4 mmol/l) rats compared to their age-matched controls (4.2 ± 0.9 and 5.3 ± 0.2

mmol/L in fasted and fed animals, respectively) (Table 5). Similarly, blood glucose was significantly ($p<0.01$) elevated in the 8-weeks STZ-diabetic group (25 ± 0.9 and 21.1 ± 1.2 mmol/L in fed and fasted animals, respectively) relative to the control group (5.2 ± 0.2 and 4 ± 0.6 mmol/L in fed and fasted animals, respectively) (Table 5). There were no significant differences in mean blood glucose values between 4- and 8-weeks fed and fasted controls or the 4- and 8-weeks fed and fasted STZ-treated group (Table 5).

4.5.2. Plasma Ghrelin Levels are Increased in Fasted as well as in STZ-treated Diabetic Rats

Plasma ghrelin concentrations (pg/ml) were measured using a commercial radio-immunoassay kit in 4- and 8-weeks control and diabetic animals. The mean plasma ghrelin concentration was 390 ± 10 pg/ml in 4-weeks control fed rats (Figure 24A, Table 6). Plasma ghrelin level was significantly higher in rats that were fasted overnight (720 ± 16 pg/ml, $p<0.05$). Fasting overnight increased plasma ghrelin levels in 4-weeks diabetic rats as well. While mean plasma ghrelin concentration was high (583 ± 9 pg/ml) in diabetic fed rats, in 4-weeks STZ-induced rats that were fasted the plasma ghrelin level was much higher (950 ± 15 pg/ml, $p<0.05$). Similarly, plasma ghrelin levels were also augmented by fasting overnight in 8-weeks control and diabetic animals (Figure 24B, Table 6). There were no significant differences in plasma ghrelin concentrations between 8-weeks and 4-weeks fed/fasted control rats. The mean plasma ghrelin concentrations were 410 ± 7 and 752 ± 10 pg/ml in 8-weeks fed and fasted control rats compared to 390 ± 10 and 720 ± 16 pg/ml in 4-weeks fed and fasted control rats (Figure

Table 5. Metabolic variables in 4- and 8-weeks STZ-induced diabetic and control SD rats.

Groups	4-weeks diabetics				8-weeks diabetics			
	Control fed	Control fasted	Diabetic fed	Diabetic fasted	Control fed	Control fasted	Diabetic fed	Diabetic fasted
Weight before STZ treatment	372±5	366±7	365±4	377±2	380±9	374±3	379±6	383±9
Weight after STZ treatment	462±6*	430±11*	330±7*	332±4*	511±10**	490±9**	281±24**	263±15**
Glucose (mM)	5.3±0.2	4.2±0.9	23.1±1.4§§	20.4±0.5§§	5.2±0.2	4.0±0.6	25±0.9§§	21.1±1.2§§
Insulin (pM)	650±73	624±39	104±16§§	98±13§§	596±43	555±32	120±11§§	114±12§§

* $P<0.05$ and ** $p<0.01$ fed versus fed, fasted versus fasted (control versus control, diabetic versus diabetic, weight before STZ treatment versus after STZ treatment).

§ $p<0.05$ and §§ $p<0.01$ fed versus fed, fasted versus fasted (control versus diabetic).

24A and 24B, Table 6). However, plasma ghrelin concentrations were significantly ($p<0.05$) different between 8- and 4-weeks fed/fasted diabetic rats. Mean plasma ghrelin concentration was 806 ± 16 pg/ml in 8-weeks diabetic fed compared to 583 ± 9 pg/ml in 4-weeks diabetic fed. Moreover, plasma ghrelin concentration was 1220 ± 10 pg/ml in 8-weeks STZ diabetic rats under fasting state compared to 950 ± 15 pg/ml in 4-weeks STZ-diabetic fasted rats (Figure 24A and 24B, Table 6).

Similar to fasting, diabetes was also shown to increase plasma ghrelin concentrations in both fed and fasted animals (Figure 24A and 24B, Table 6). As mentioned before, the mean plasma ghrelin concentration was 390 ± 10 pg/ml in 4-weeks control fed rats. Diabetes for 4 weeks increased plasma ghrelin levels significantly (583 ± 9 pg/ml, $p<0.05$). Additionally, while the mean plasma ghrelin concentration was 720 ± 16 pg/ml in control fasted rats, 4 weeks diabetes increased plasma ghrelin levels significantly (950 ± 15 pg/ml, $p<0.05$). Plasma ghrelin levels were also increased significantly ($p<0.01$) after 8 weeks of diabetes in both fed and fasted animals. Plasma ghrelin levels were 806 ± 16 pg/ml in diabetic fed rats compared to 410 ± 7 pg/ml in control fed animals. Additionally, plasma ghrelin concentrations were 1220 ± 10 pg/ml in diabetic fasted rats compared to 752 ± 10 pg/ml in control fasted animals. Plasma ghrelin levels were significantly ($p<0.05$) different between 8-weeks and 4-weeks fed and fasted diabetic animals.

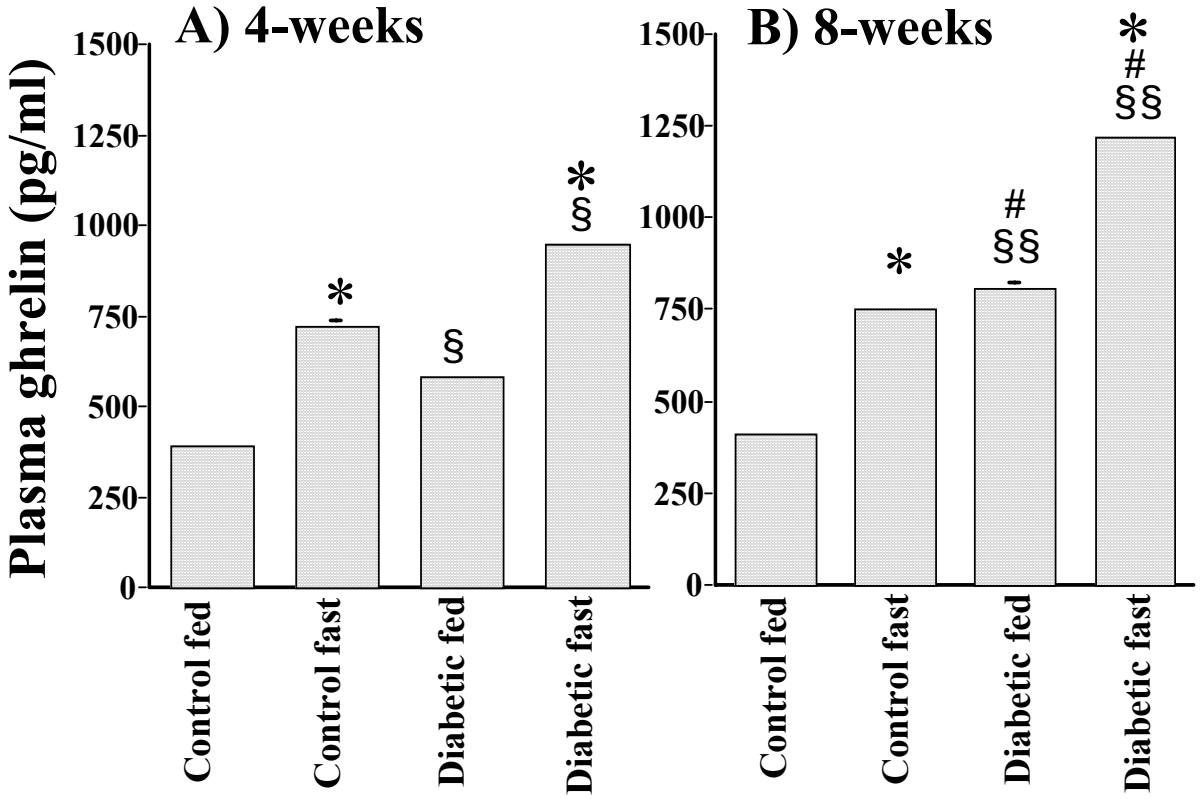

Figure 24. Plasma ghrelin concentrations (pg/ml) in fed/fasted control and diabetic SD rats after 4- and 8-weeks of STZ treatment. Results are mean ± S.E.M. of 5 different experiments. * $p<0.05$ and ** $p<0.01$ fed v/s fasted (control v/s control; diabetic v/s diabetic). § $p<0.05$ and §§ $p<0.01$ fed v/s fed and fasted v/s fasted (control v/s diabetic). # $p<0.05$ and ## $p<0.01$ fed v/s fed and fasted v/s fasted (control v/s control, diabetic v/s diabetic, 4- v/s 8-weeks).

4.5.3. Ghrelin-Evoked Vasodilatation in MVB is Diminished in Fasted as well as STZ-treated Diabetic Rats

Vasodilatation to both ghrelin (1 nmol/L) and ACh (1μmol/L) was measured as a percentage of maximal PE (80 μmol/L)-induced contraction. Ghrelin-evoked vasodilatation in MVB in 4-weeks control fed animals was 45 ± 12% (Figure 25A). The maximal vasodilator responses to ghrelin were significantly lower in overnight fasted rats (26 ± 13%) compared to age-matched rats that were maintained in fully fed state. A similar phenomenon of reduced ($p<0.01$) vasodilator response to ghrelin was noticed in 8-weeks control rats that were subjected to fasting (from 40 ± 15% in control fed to 25 ± 14% in control fasted). Age of the animals, however, did not have significant effect on ghrelin-induced vasodilatation in control rats (Figure 25A and 25B). Ghrelin induced vasodilatation in MVB in 8-weeks control fed/fasted rats to almost the same extent as 4-weeks control fed/fasted animals. A representative tracing comparing ghrelin-evoked decreases in PP in MVBs of 8-weeks control fed versus control fasted rats is shown in Figure 26. Ghrelin induced vasodilatation of MVB in 4-weeks diabetic fed animals by 38 ± 15% (Figure 25A). Fasting 4-weeks diabetic animals for an overnight duration, significantly ($p<0.05$) decreased ghrelin-evoked vasodilator effects in MVB to 28 ± 10%. Similarly, fasting 8-weeks diabetic rats decreased ghrelin-evoked vasodilatations in MVB compared to 8-weeks diabetic fed animals ($p<0.01$; 29 ± 10% in 8-weeks diabetic fed versus 13 ± 9% in 8-weeks diabetic fasted).

Diabetes for 4 weeks did not cause any significant reduction in vasodilator response to ghrelin (Figure 25A). Ghrelin-induced vasodilatation was 38 ± 15% in 4-

weeks diabetic fed rats compared to 45 ± 12% in 4-weeks fed controls. Moreover, ghrelin-induced vasodilatation was 28 ± 10% in 4-weeks diabetic fasted rats compared to 26 ± 13% in 4-weeks fasted controls. Diabetes for 8 weeks, however, induced a very significant ($p<0.01$) reduction in ghrelin-induced vasodilator response (Figure 25B). Ghrelin-induced vasodilatation was 29 ± 10% in 8-weeks diabetic fed compared to 40 ± 15% in 8-weeks fed controls. A representative tracing of one single experiment is shown in Figure 27. In addition, ghrelin induced only 13 ± 9% vasodilatation in MVB in 8-weeks diabetic fasted compared to 25 ± 14% in 8-weeks fasted controls. A representative tracing of one single experiment is shown in Figure 28.

Ghrelin-induced vasodilator responses in 8-weeks diabetic (fed/fasted) animals were significantly different compared to 4-weeks diabetic (fed/fasted) rats (Figure 25A and 25B). While 8-weeks diabetes induced 29 ± 10% vasodilator response to ghrelin in diabetic fed rats, the response was 38 ± 15% in 4-weeks diabetic fed rats ($p<0.05$). Additionally, whereas ghrelin induced only 13 ± 9% vasodilatation in diabetic fasted animals after 8 weeks of diabetes, the response was 28 ± 10% in 4-weeks diabetic fasted rats ($p<0.01$). The relationship between plasma ghrelin concentrations and its vasodilator responses in MVB from 4- and 8-weeks control and diabetic (fed/fasted) rats is summarized in Table 6.

ACh induced vasodilatation of MVB in 4-weeks control fed rats by 98 ± 3% (Figure 25A, Table 6). Fasting did not have any effect on ACh-induced relaxation (98 ± 2%). There were no differences in ACh-evoked vasodilator responses between 4- and 8-weeks fed/fasted animals (97 ± 3% and 98 ± 2% in 8-weeks control fed and fasted,

respectively) (Figure 25B, Table 6). Diabetes significantly ($p<0.01$) reduced ACh-induced vasodilatation in 4-weeks diabetic fed (77 ± 6%) compared to 4-weeks control fed rats (98 ± 3%). ACh-induced vasodilatation in 4-weeks diabetic fasted rats was also attenuated (1.36 fold) to the same extent as diabetic fed rats (1.27 fold) (78 ± 9% in 4-weeks diabetic fasted compared to 98 ± 2% in 4-weeks control fasted animals, $p<0.01$). The same situation was true for 8-weeks diabetic animals. ACh-induced vasodilatation was significantly ($p<0.01$) reduced to 60 ± 10% in 8-weeks diabetic fed compared to 97 ± 3% in control fed rats. Similarly, the vasodilator response to ACh was significantly ($p<0.01$) attenuated to 65 ± 13% in 8-weeks diabetic fasted compared to 98 ± 2% in control fasted rats. It is noteworthy to mention that there was a duration-dependent fall in the vasodilator responses to ACh in STZ-diabetic rats as the vasodilatations were significantly lower in 8-weeks STZ-diabetic animals compared to 4-weeks (77 ± 6% in 4-weeks diabetic fed versus 60 ± 10% in 8-weeks diabetic fed, and 78 ± 9% in 4-weeks diabetic fasted versus 65 ± 13% in 8-weeks diabetic fasted) (Figure 25A and 25B, Table 6). There were significant differences between ACh-induced vasodilatation in 4- and 8-weeks diabetic fed as well as diabetic fasted rats ($p<0.01$). A representative tracing of one single experiment demonstrating progressive decreases in ACh-evoked vasodilatation in MVB of 4- and 8-weeks diabetic fasted rats is shown in Figure 29.

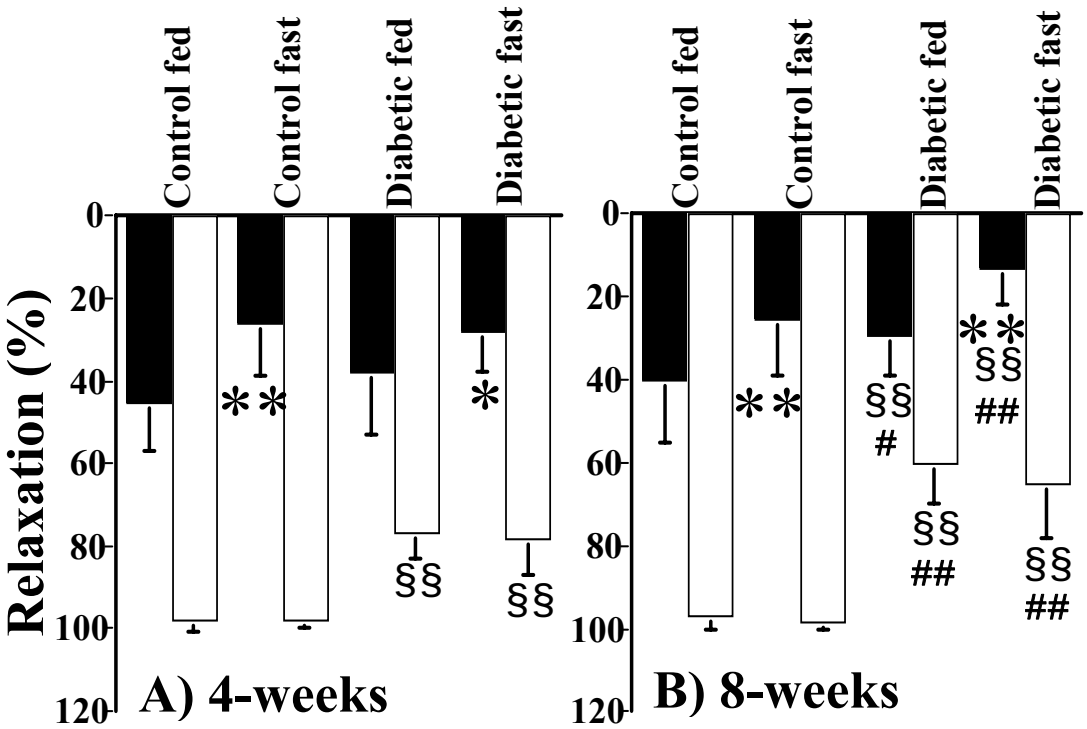

Figure 25. Vascular relaxation (%) of PE (80 μmol/L)-constricted MVB in response to Ach (1 μmol/L) and ghrelin (1 nmol/L) in fed/fasted control and diabetic SD rats. Relaxations were measured as a percentage of maximal PE-induced constractions. Results are mean ± S.E.M. of 5 different determinations. * $p<0.05$ and ** $p<0.01$ fed v/s fasted (control v/s control; diabetic v/s diabetic). § $p<0.05$ and §§ $p<0.01$ fed v/s fed and fasted v/s fasted (control v/s diabetic). # $p<0.05$ and ## $p<0.01$ fed v/s fed and fasted v/s fasted (control v/s control, diabetic v/s diabetic, 4- v/s 8- weeks).

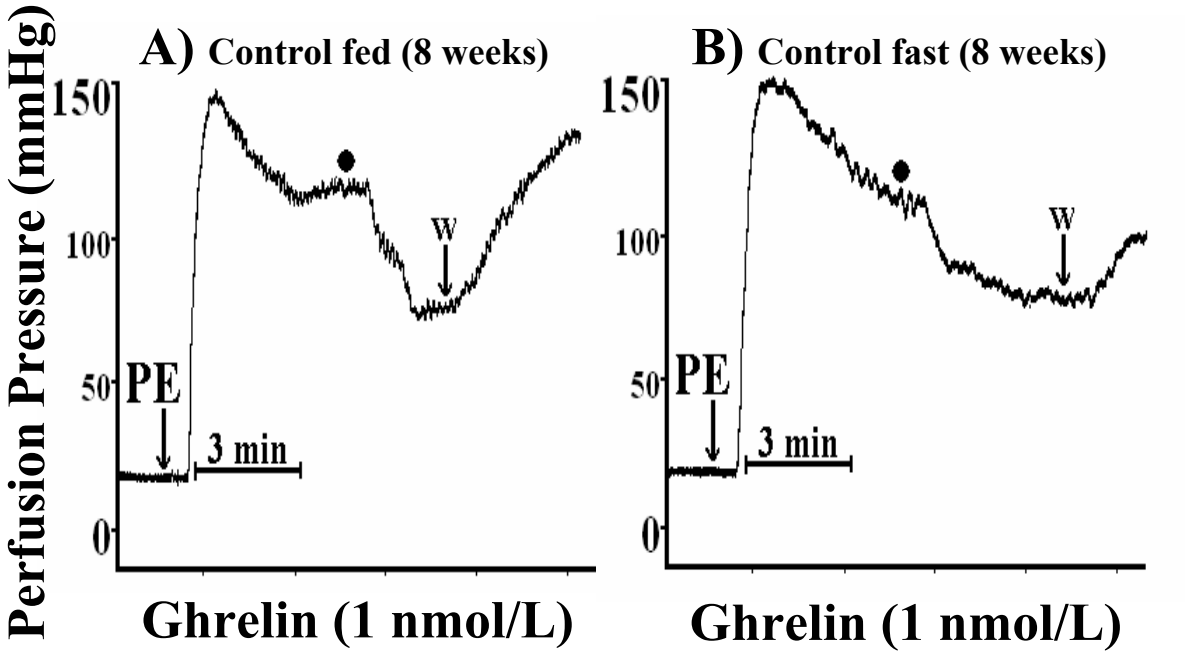

Figure 26. Reductions in PP (mmHg) in PE (80 μmol/L)-constricted MVB in response to ghrelin (1 nmol/L) in 8-weeks A) fed compared to B) fasted control SD rats.

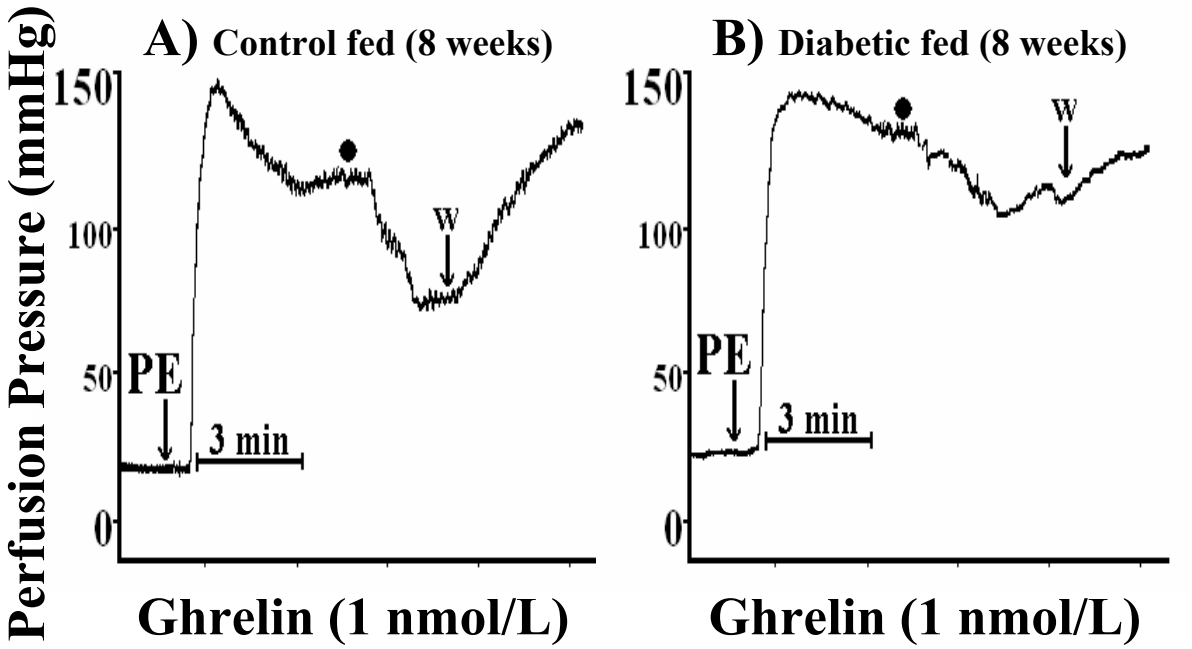

Figure 27. Reductions in PP (mmHg) in PE (80 μmol/L)-constricted MVB in response to ghrelin (1 nmol/L) in 8-weeks STZ-treated A) control fed compared to B) diabetic fed SD rats.

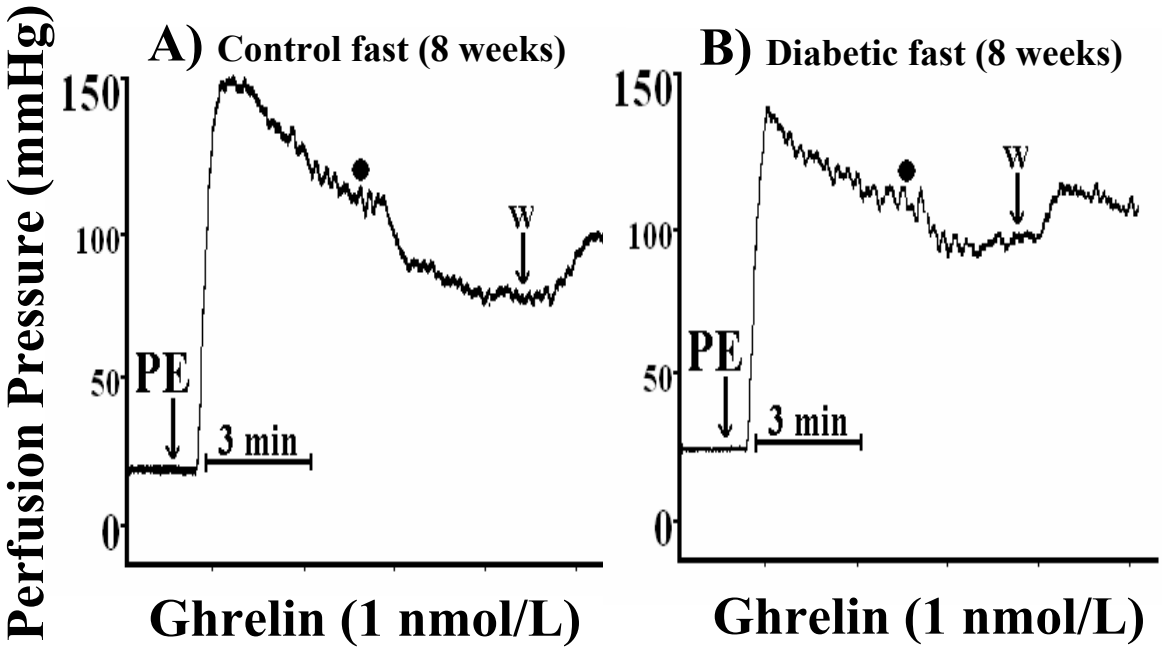

Figure 28. Reductions in PP (mmHg) in PE (80 μmol/L)-constricted MVB in response to ghrelin (1 nmol/L) in 8-weeks STZ-treated A) control fast compared to B) diabetic fast SD rats.

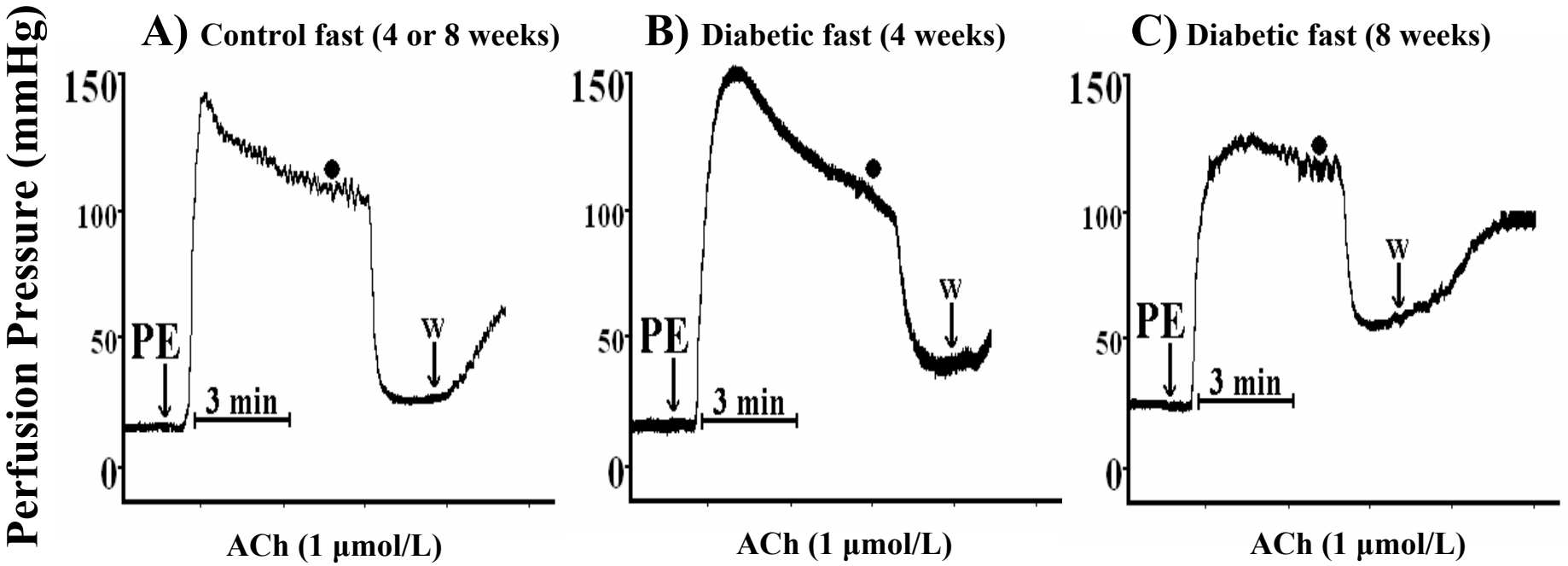

Figure 29. Reductions in PP (mmHg) in PE (80 µmol/L)-constricted MVBs in response to ACh (1 µmol/L) in A) control fast (4- or 8-week), B) diabetic fast (4-week), and C) diabetic fast (8-week).

Table 6. Plasma ghrelin concentrations (pg/ml) and vascular relaxations (%) to ghrelin (1 nmol/L) and acetylcholin (ACh, 1 μmol/L) in PE (80 μmol/L)-constricted MVBs of 4- and 8-weeks fed/fasted STZ-induced diabetic and control SD rats.

Groups	4-weeks diabetes			8-weeks diabetes		
	Plasma ghrelin (pg/ml)	Vascular relaxation (%) Ghr	ACh	Plasma ghrelin (pg/ml)	Vascular relaxation (%) Ghr	ACh
Control fed	390±10	45±12	98±3	410±7	40±15	97±3
Control fasted	720±16*	26±13**	98±2	752±10*	25±14**	98±2
Diabetic fed	583±9§	38±15	77±6§§	806±16$^{\S\S\#}$	29±10$^{\S\S\#}$	60±10$^{\S\S\#\#}$
Diabetic fasted	950±15*§	28±10*	78±9§§	1220±10*$^{\S\S\#}$	13±9**$^{\S\S\#\#}$	65±13$^{\S\S\#\#}$

* P<0.05 and ** p<0.01 fed versus fasted (control versus control, diabetic versus diabetic).
§ p<0.05 and §§ p<0.01 fed versus fed, fasted versus fasted (control versus diabetic).
# p<0.05 and ## p<0.01 fed versus fed and fasted versus fasted (control versus control, diabetic versus diabetic, 4- versus 8-weeks).

5. Discussion

5.1. Mechanisms Underlying ACh- and Ghrelin-Evoked Vasodilatation in MVB

5.1.1. ACh-Evoked Vasodilatation in MVB is Mediated Mainly through both NO and EDHF

The results of the present study clearly demonstrate that, in the rat MVB, dose-dependent vasodilatation to the endothelium-dependent muscarinic agonist, ACh, has two major components, one of which is L-NAME-sensitive and mediated by NO, while the other one is sensitive to TBA, a non-selective K_{Ca} channel blocker. On the basis of these results, it is likely that the second component of the vasodilatation is mediated by putative EDHF. This is in agreement with the conclusions of Adeagbo and Triggle (1993), and McCulloch et al. (1997) that both EDHF and NO mediate the vasodilator response to ACh in the rat MVB. Pretreatment with indomethacin, however, did not have a significant effect on the ACh-induced vasodilatation in perfused MVB. This was in agreement to the previous data by several investigators (Makino et al., 2000; Morikawa et al., 2005). The results of the present study further show that there appears to be interaction, or "cross-talk", between NO and EDHF, such that EDHF activity is enhanced when the NO system is blunted.

Inhibition of NOS with a high concentration of L-NAME (100 μmol/L) decreased the potency as well as maximal vasodilator response to ACh by 3 and 1.2 fold, respectively. The present data is different from the data reported by Makino et al. (2000) and Wigg et al. (2001), who demonstrated that in the mesenteric arteries the

vasodilator response to ACh persisted in the presence of L-NAME, although there was a significant decrease in its potency. These observations, however, suggest that there is a second pathway in addition to NO-mediated vasodilatation that would account for ACh-induced vasodilatation. A similar NO-independent component of vasodilatation was observed in the human forearm (Chowienczyk et al., 1993). The L-NAME-insensitive component has generally been attributed to the existence of an EDHF (Feletou and Vanhoutte, 1988), which acts by increasing membrane K^+ conductance through K_{Ca} channels, thus inducing hyperpolarization of EC with subsequent relaxation of vascular smooth muscle cells. In order to assess the contribution made by EDHF to ACh-induced vasodilatation, K_{Ca} channels were blocked by TBA. In the presence of TBA, both the potency and efficacy of ACh were reduced, thereby implicating the involvement of K_{Ca} channels in ACh-induced vasodilatation. The addition of TBA along with L-NAME further reduced the potency of ACh, by 34 fold relative to the potency seen in the absence of TBA and L-NAME and by 11.1 fold relative to the potency seen in the presence of L-NAME alone, indicating that there are at least two mechanisms by which ACh can induce vasodilatation in rat MVB, one involving NO and the other involving the EDHF pathway. This contrasts with the findings of Garland and McPherson (1992) who concluded that the release of NO was not involved in ACh-induced vasodilatation in isolated small mesenteric arteries. This may perhaps be explained by the size of the vessels being studied as Hilgers et al. (2005) demonstrated that the relative contribution of the EDHF-mediated vasodilatation differs depending on the vessel type and size, being more important in smaller arteries and arterioles. Hilgers et al. (2005) mentioned a greater EDHF-mediated vasodilatation in 4^{th}-degree branches compared to the 1^{st}-degree branches of

the mesenteric arteries. ACh was less potent as a vasodilator in the presence of TBA than in the presence of L-NAME alone, suggesting a greater contribution of EDHF than NO to ACh-induced vasodilatation in the rat mesenteric arterial bed, a conclusion also made by Adeagbo and Triggle (1993). Accordingly, Katakam et al. (1999) reported that, in the presence of L-NAME and indomethacin, ACh induced a maximal vasodilator response of almost 70%, suggesting the EDHF as a primary endothelium-dependent relaxing factor in rat small mesenteric arteries.

Raising extracellular K^+ reduces the electrochemical gradient for K^+ efflux that could occur through all K^+ channels including K_{Ca} channels. Therefore, elevated K^+ inhibits EDHF response, and also depolarizes the VSM cell membrane resulting in vasoconstriction. The substantial decrease in potency and maximal responses to ACh in the presence of high K^+ suggests that a K^+ conductance was important in mediating vasodilatation to ACh and confirms the previous works by others (Adeagbo and Triggle, 1993; Waldron and Garland, 1994; Makino et al., 2000). Although the potency seen for ACh in the presence of KCl is the same as that in the presence of TBA, the maximal response to ACh in the presence of KCl was much more reduced compared to that in the presence of TBA. This may be due to the fact that TBA inhibits K_{Ca} channels, whereas increasing intracellular K^+ concentration inhibits K^+ conductance through all types of K^+ channels. Others have shown that NO itself may act via certain K^+ channel activation (K_{ATP} channel) and hyperpolarization (Tare et al., 1990). Therefore, our hypothesis is that in the presence of TBA, although EDHF response is inhibited, but NO response can, up to a great extent, compensate for the lack of EDHF. Similar to our study, others have shown that there is an interaction

between NO- and EDHF-mediated responses as EDHF-mediated vasodilatation, for example, is particularly apparent when NO production is blocked. However, in the presence of KCl, where EDHF is inhibited, NO response (up to a limited extent) can compensate for the lack of EDHF, since NO response is also partially inhibited due to the blockade of all cellular K^+ channels by high extracellular K^+ concentration. The residual response to ACh in the presence of KCl is attributed to the involvement of NO and PGI_2, since Makino et al. (2000) have demonstrated that the vasodilator responses of the MVBs to ACh in the presence of high K^+ were completely inhibited in the combined presence of NOS inhibitor (L-NAME) and COX-inhibitor (indomethacin).

The present study also investigated the relative contribution of different subtypes of K_{Ca} channels as well as K_{ir}, K_{ATP} channels, and Na^+/K^+-ATPase pump on ACh-induced vasodilatations in MVB. The results strongly suggest that the endothelium-derived relaxing factors released in MVB in response to ACh are predominantly NO and EDHF: (1) Pharmacological inhibition of SK_{Ca} by apamin along with non-selective inhibition of IK_{Ca} and BK_{Ca} by ChTX, caused a reduction in ACh-induced vasodilatation. (2) Similarly, pharmacological inhibition of SK_{Ca} by apamin along with a very selective inhibition of IK_{Ca} by TRAM-34 also reduced ACh-mediated vasodilatation almost to the same extent as apamin plus ChTX. (3) ACh-induced vasodilatation of MVB was also reduced in the presence of Ba^{2+} and ouabain, which inhibit K_{ir} channels and Na^+/K^+-ATPase pump, respectively. These data are consistent with the involvement of EDHF in ACh-mediated vasodilatation of MVB. In general, EDHF-mediated responses involve an increase in the $[Ca^{2+}]_i$ levels in ECs,

the opening of K_{Ca} channels of small and intermediate conductance, and hyperpolarization of the EC. This results in an endothelium-dependent hyperpolarization of the smooth muscle cells, which can be evoked by direct electrical coupling through myo-endothelial junctions and/or the accumulation of K^+ ions in the intracellular space. K^+ ions hyperpolarize the smooth muscle cells by activation of K_{ir} channels and/or Na^+/K^+-ATPase pump (Wang et al., 2006). In agreement with the above, it has been demonstrated that, in the presence of PE, ACh evoked substantial concentration-dependent hyperpolarization of the smooth muscle cells in the mesenteric arteries of control rats (Wigg et al., 2001). It is noteworthy to mention that this hyperpolarization was attributed to EDHF-mediated vasodilatation, since hyperpolarization was not affected by using a combination of NOS-inhibitor (L-NAME) and COX-inhibitor (indomethacin), but was abolished in the presence of a combination of apamin plus ChTX. However, besides the involvement of EDHF, inhibition of K_{ATP} channels by glibenclamide, which reduced ACh-mediated vasodilatation of MVB, suggested the involvement of another vasodilator mechanism in ACh-mediated vasodilatation, namely NO. K_{ATP} channels appeared to be involved in NO-dependent responses and have a very minimal role in EDHF-mediated vasodilatation (Katakam et al., 1999).

5.1.2. Ghrelin-Evoked Vasodilatation in MVB is Mediated Solely through EDHF

GHSR-1a receptors were thought to be exclusively expressed in several nuclei of the brain, particularly in hypothalamus and anterior pituitary gland, and at very low

levels in pancreas (Katugampola and Davenport, 2003). However, recent studies have suggested that the GHSR-1a receptors are expressed in many tissues such as lung, intestine, pancreas, and adipose tissue. The presence of appreciable amounts of GHS binding sites and mRNA in the cardiovascular system of both animals and humans suggests that ghrelin may have direct cardiovascular effects through GH-independent mechanisms (Kojima et al., 2001; Wu et al., 2004). In healthy human volunteers and in patients with dilated cardiomyopathy, the intravenous administration of ghrelin significantly reduced peripheral vascular resistance and increased cardiac output without a significant change in heart rate (Nagaya et al., 2001; Nagaya and Kangawa, 2003). In rats with heart failure and cachexia, chronic ghrelin administration improves left ventricular remodeling (Nagaya et al., 2001). The results of the present study demonstrated, for the first time, that ghrelin evokes a slow concentration-dependent vasodilatation (EC_{50} 55 ± 6 pmol/L) in PE-constricted rat MVB, reaching its maximum effect (E_{max} 45 ± 5) at the concentration of 1 nmol/L. MVB, consisting of resistance sized arteries, roughly having an internal diameter of 400-500 μm (in larger 1^{st}-degree branches) and 150-200 μm (in 4^{th}-degree branches), is an important regulator of vascular tone and hence blood pressure. Our study, therefore suggests that ghrelin could be one of the most potent vasodilators and regulators of vascular tone. In contrast to our data, Pemberton et al. (2004) reported that PP was slightly increased in a dose-dependent manner during ghrelin infusion in isolated perfused rat hearts. Interestingly, similar to our data, the effect was pronounced at the concentration of 1 nmol/L, which resulted in a 44 ± 9% increase in PP compared to the vehicle group. Administration of 1 nmol/L ghrelin also resulted in a slow constriction of coronary arteries. However, the observed discrepancy could be due to the different sites of

ghrelin administration (MVB versus coronary arteries or isolated hearts), different GHSR-1a expression profiles, different GHSR subtypes, and even different ghrelin receptors. Bodart et al. (2002) demonstrated that the increase of the coronary PP induced by hexarelin (a synthetic GHS) in the perfused heart model might result from the direct interaction of this ligand with CD36 receptors, because the lack of this effect was observed in CD36 knockout mice and in genetically CD36-deficient spontaneously hypertensive rats.

The vasodilator responses to ghrelin observed in the present study were slow in onset (20-30 seconds after addition of the peptide), and took between 2-3 minutes to reach the plateau. This is much slower than the response times for ACh, which had an onset of approximately 10 seconds after addition of the agonist and took approximately 20 seconds to reach the plateau. In addition, MVBs perfused with ghrelin took more time to wash and reach their basal PP compared to those perfused with ACh. The slower and more sustained effects of ghrelin may suggest that *in vivo*, pulses of ghrelin could promote vasodilatation evoked by this peptide. It has been reported that plasma concentrations of ghrelin rise progressively during fasting and fall after eating (Eisenstein and Greenberg, 2003).

The vasodilator effect of ghrelin seen in our MVB preparations is a physiological effect, since: (1) The circulating plasma concentration of ghrelin was shown to be about 220 pmol/L (Kojima et al., 2001; Matsumoto et al., 2001). Our results demonstrated that ghrelin evokes its vasodilator effect with an EC_{50} of 55 ± 6 pmol/L. However, it is noteworthy to mention that the plasma concentration of 220

pmol/L represents the "total ghrelin" content in plasma and it does not distinguish between plasma levels of ghrelin, des-acyl ghrelin, or des-Gln^{14} ghrelin. It has been reported that the acylated form of ghrelin released from the stomach could be deacylated in the circulation (Kojima et al., 2001). Des-Gln^{14} ghrelin is also a form of ghrelin with 27 amino acids resulting from alternative splicing of the same ghrelin gene (Hosoda et al., 2000). (2) Ghrelin-evoked vasodilatation of MVB was endothelium-dependent, since the responses were not seen in endothelium-denuded preparations. Bedendi et al. (2003) also reported that the negative ionotropic effect of ghrelin on contractility of the guinea pig papillary muscles was not observed when the endocardial endothelium was removed. In contrast to our data, however, Wiley and Davenport (2002) described ghrelin as a potent endothelium-independent vasodilator of human internal mammary arteries, effectively reversing ET-1-mediated constrictions *in vitro*. It is noteworthy to mention that Wiley and Davenport (2002) employed ghrelin *in vitro* at doses up to 300 nmol/L, nearly 300 times those employed in our study. Our study demonstrated that ghrelin at concentrations more than 1 nmol/L desensitizes its receptors, and therefore its vasodilator effect could not be reproduced. On the other hand, it is well known that ghrelin is a peptide, which promotes Ca^{2+} mobilization upon binding to its G protein-coupled receptors (Kojima et al., 1999; Kojima et al., 2001). Accordingly, previous unpublished data from our laboratory revealed that ghrelin evokes increases in $[Ca^{2+}]_i$ in EC in a dose-dependent manner. Increases in $[Ca^{2+}]_i$ in EC were attenuated by the IP_3 (inositol-triphosphate) antagonist, suggesting Ca^{2+} mobilization from intracellular stores. Thus, the *in vitro* study by Wiley and Davenport (2002) failed to explain how a Ca^{2+} mobilizing agonist, ghrelin, could promote vasodilatation in endothelium-denuded arteries.

Several lines of evidence (present study as well as previous data by our own laboratory and others), confirm that ghrelin-evoked vasodilatation is linked to endothelium-dependent hyperpolarization and EDHF: (1) Endothelium-denudation abolished the responses to ghrelin. (2) Inclusion of either NOSI (L-NAME) or COX inhibitor (indomethacin) failed to block the vasodilator responses to ghrelin in endothelium-intact preparations. (3) The K_{ATP} channel blocker, glibenclamide, failed to inhibit the responses. (4) The response to ghrelin was completely abolished in the presence of a non-selective K_{Ca} blocker (TBA), or in the presence of a depolarizing medium (KCl). (5) Inclusion of a combination of either a selective inhibitor of SK_{Ca} channels (apamin) and a non-selective inhibitor of BK_{Ca} and IK_{Ca} (ChTX), or apamin and a selective inhibitor of IK_{Ca} (TRAM-34) abolished ghrelin-evoked vasodilator responses. 6) Inclusion of a combination of Na^+/K^+-ATPase inhibitor (ouabain) and K_{ir} channel blocker (Ba^{2+}) also abolished the responses. (7) In addition to the above, previous data from our laboratory demonstrated that ghrelin evoked dose-dependent increases in $[Ca^{2+}]$i, with an E_{max} value of 150 ± 16 nmol/L, which was significantly lower than the E_{max} for ACh (640 ± 32 nmol/L). This suggests that a higher level of $[Ca^{2+}]_i$ increase is essential for the Ca^{2+}-dependent increases in eNOS activity evoked by agonists like ACh. In addition, this may also explain why ghrelin fails to evoke NO-dependent vasodilator responses in MVB. It is noteworthy to mention that ghrelin reached its maximal increase in $[Ca^{2+}]_i$ at the concentration of 1 nmol/l, the same concentration at which ghrelin produced its maximal vasodilator response. (8) Using electrophysiological studies, previous data from our laboratory also showed that ghrelin-evoked increases in K_{Ca} currents could induce hyperpolarization in EC isolated from the rat mesenteric artery (which express only IK_{Ca} channels).

EDHF-mediated responses have been demonstrated to be more prominent as the vessel size decreases, from the 1st-degree branch to the 4th-degree branch mesenteric arteriole rings. Hilgers et al. (2006) demonstrated that apamin almost completely abolished ACh-evoked vasodilatation in 4th-degree branch mesenteric arterioles, but only partially blocked vasodilatation in 1st-degree branch arteries. In addition, TRAM-34 caused significantly greater inhibition of the ACh-induced vasodilatation in 4th-degree branch compared to 1st-degree branch mesenteric arteries. Their data demonstrated regional heterogeneity in SK_{Ca} and IK_{Ca} functional gene expression, with the observation that relative mRNA expression levels of SK_{Ca} were significantly higher in 4th-degree branch compared to 1st-degree branch mesenteric rings. These data suggest that the observed vasodilator effect of ghrelin and des-acyl ghrelin arise mostly from the secondary and tertiary branches of the perfused MVB.

The present work, therefore supports the view that ghrelin increases K^+ levels in myo-endothelial junction subsequent to the activation of SK_{Ca} and IK_{Ca} currents and EC hyperpolarization. The elevated K^+ concentration in myo-endothelial junction, then hyperpolarizes underlying smooth muscle cells via activating the influx of K^+ through Na^+/K^+-ATPase pump along with the efflux of the entered K^+ through the k_{ir} channels. Although ghrelin appears to be an agonist that selectively promotes EDHF process or mimickes EDHF, there is no direct evidence for ghrelin-evoked release of EDHF in the present study. This could be very difficult to determine, since various candidates have been suggested to serve the role of EDHF such as EETs, anandamide, hydrogen peroxide, C-type natriuretic peptide (NP), and elevation in K^+ levels in the

myo-endothelial junctions (Edwards et al., 1998; Busse et al., 2002; Mcguire et al., 2002; Chauhan et al., 2003).

5.2. Ghrelin-Evoked Vasodilatation in MVB is not Mediated through the Classical GHSR-1a Receptors.

The results of the present study support the notion that ghrelin evokes its vasodilator activity in MVB through binding to a specific receptor. These include: (1) Ghrelin evoked slow vasodilator responses in MVB in a dose-dependent manner, starting at concentrations as low as 1 pmol/L (indicating specificity and selectivity). (2) The vasodilator response evoked by ghrelin reached its peak effect at a concentration range of 1 nmol/L (indicating saturability). (3) Ghrelin-evoked vasodilatation could be reproduced as long as the concentration of the peptide in the perfusion buffer did not exceed 1nmol/L (indicating reproducibility and desensitization). The expected ghrelin receptor seems to be present on the endothelium, since ghrelin-evoked vasodilator responses in MVB were abolished completely following endothelium removal. Kojima et al. (1999) and Katugampola et al. (2001) identified the presence of GHSR-1a receptor in cardiac EC. Baldanzi et al. (2002) also demonstrated the presence of ghrelin binding sites in membrane fractions of cultured porcine aortic EC. Accordingly, previous work (unpublished) by our laboratory identified the presence of much higher affinity sites (Kd 0.256 nmol/L) for [^{125}I] ghrelin in cultured EC from the mesenteric artery compared to the affinity sites (Kd 8 nmol/L) for ghrelin in cultured porcine aortic EC reported by Baldanzi et al. (2002). In support of this data, the presence of only a single class of ghrelin binding

sites with similar Kd value to the data from our laboratory (0.220-0.570 nmol/L) has been characterized in membrane fractions of human and rat vascular tissues as well as human ventricle (Katugampola et al., 2001). However, despite all of the above information, our study can not rule out the possibility that ghrelin may also have receptors on the VSMC, and therefore may act directly to regulate channels (such as K_{ir} channels) on VSMC. Davenport et al. (2006) reported the existence of ghrelin receptors on VSMC and cardiomyocytes using specific antisera.

Combining all the data together, there is a possibility for defining the presence of differential distribution of GHSR-1a receptors such that more receptors are present on EC than smooth muscle cells. Thus, in the presence of endothelium, ghrelin evokes its maximal vasodilator responses at the lower concentrations of about 1 nmol/L, upon binding to its receptors present on the EC. However, in the absence of endothelium in internal mammary arteries, as reported by Wiley and Davenport (2002), ghrelin produces its maximal vasodilator responses in the ranges up to 300 nmol/l upon binding to its receptors present on VSMC.

The evidence to date suggests that ghrelin is synthesized, produced, and released from the stomach and circulates at reasonable concentrations (220 pmol/L) to act on the pituitary promoting GH release (Kojima et al., 1999). More recently, the data by Hosoda et al. (2000) as well as Pemberton et al. (2004) demonstrated that cardiac tissue extracts also contained measurable amounts of ghrelin, with the atrium containing approximately twice as much as the ventricles. However, despite the presence of quantifiable amounts of ghrelin in cardiac tissue extracts, they could not

detect immunoreactive ghrelin in isolated heart perfusates, suggesting that if ghrelin has an endogenous role in mediating cardiac function, it might act in a paracrine/autocrine manner. There is also the possibility that ghrelin/des-acyl ghrelin are expressed in the EC to serve the role of EDHF and that the exogenous addition of these peptides mimics the response of these endogenous peptides/EDHF. To our knowledge, there is only one published study investigating the expression of ghrelin contents in EC. Davenport et al. (2006) demonstrated for the first time the expression of ghrelin in EC of human arteries and veins and in secretory vesicles of cultured EC using immunocytochemistry. However, the results of the present study are more in agreement with the hypothesis that, at least under normal conditions, ghrelin-evoked MVB relaxation is mediated mostly by circulating ghrelin binding to the receptors on the EC. This is because: (1) Ghrelin/des-acyl ghrelin produced their vasodilator responses with the potencies of 55 ± 6 pmol/L and 49 ± 7 pmol/L respectively, which are within their physiological range of plasma concentrations. (2) Ghrelin/des-acyl ghrelin did not produce any vasodilator responses in extremely low concentrations of 100 fmol/L, thus putting a question mark on the possibility of the peptides regulating VSMC tone in a paracrine or autocrine manner.

The vasodilator responses to ghrelin and des-acyl ghrelin reported in the present study are likely mediated through a distinctly different receptor from the classical GHSR-1a receptors expressed in the anterior pituitary and hypothalamus for the following reasons: (1) Des-acyl ghrelin has been reported to bind very weakly or not at all to the classical GHSR-1a receptors, and is therefore devoid of any endocrine action (Bednarek et al., 2001; Kojima et al., 2001). In the present study, however, des-

acyl ghrelin evoked endothelium-dependent/concentration-dependent vasodilatation in MVBs with a potency and maximal effect (EC_{50} 49 ± 7 pmol/L; E_{max} 43 ± 6%) similar to those of ghrelin (EC_{50} 55 ± 6 pmol/L; E_{max} 45 ± 5%). Both ghrelin and des-acyl ghrelin reached their maximal response at a concentration of 1 nmol/L. These observations are in agreement with the data by Bedendi et al. (2003), who reported that non-acylated ghrelin was also able to exert a negative ionotropic effect on the contractility of guinea pig papillary muscle similar to ghrelin. (2) [D-Lys^3]-GHRP-6, an antagonist of the classical GHSR-1a receptor, failed to antagonize the vasodilator response to ghrelin. It *per se* evoked a vasodilator response in contracted MVBs. (3) L-756867, another peptide antagonist of the classical GHSR-1a receptor, which did not evoke vasodilatation in MVB by itself, did not block vasodilator responses evoked by ghrelin in contracted MVBs. In agreement with the above findings, previous data from our laboratory showed that des-acyl ghrelin was also able to evoke increases in $[Ca^{2+}]_i$ in EC (E_{max} 162 ± 18 nmol/L; EC_{50} 48 ± pmol/L) to almost the same extent as ghrelin (E_{max} 150 ± 16; EC_{50} 58 ± pmol/L). This finding was very different from previous studies reporting the potencies of des-acyl ghrelin (EC_{50} 3500 nmol/L) and ghrelin (EC_{50} 1.5 nmol/L) in increasing $[Ca^{2+}]_i$ in somatotrophs upon binding to their classical GHSR-1a receptors (Matsumoto et al., 2001). Taken together, all of these data confirm that ghrelin and des-acyl ghrelin evoke their vasodilator responses in MVBs by interacting at the same novel receptor that is distinctly different from the classical GHSR-1a receptor present in the pituitary and hypothalamus.

The data reported by Baldanzi et al. (2002) support our observations. They have observed that H9C2 cardiomyocytes do not express GHSR-1a receptors, but have

high affinity binding sites, both for ghrelin and des-acyl ghrelin, the activation of which results in preventing cardiomyocyte apoptosis. This provides further support to the hypothesis that multiple ghrelin receptors exist in the cardiovascular system. However, whether this novel receptor is encoded by alternative splicing of GHSR-1a gene or by distinct gene is still unknown. The existence of GHS receptor subtypes in the heart has already been shown (Muccioli et al., 2002). GHSR-1b receptor, which is a splicing variant of the GHSR-1a, although expressed in the heart, does not appear to be functional (Howard et al., 1996). The study by Bodart et al. (2002) led to the discovery of a distinct type of binding sites in cardiac membranes from different mammalian species. Using N-terminal sequencing, the purified receptor was identified as CD36, a glycoprotein belonging to the scavenger receptor type-B family of proteins. This receptor is specifically expressed in adipose tissue, platelets, monocytes/macrophages, dendritic cells, and microvascular endothelium (Dawson et al., 1997).

5.3. L-166446 and L-163255, two Synthetic Non-Peptide Agonists of GHSR-1a Receptors, Evoke Vasodilatation in MVB

GH secretion is well known to be regulated by GHRH and somatostatin at the hypothalamic level. The discovery of GH-releasing peptides or GHSs has revealed the existence of a third pathway for the modulation of GH release. In the late 1970s, Bowers et al. (1980) discovered that certain peptides from a series of synthetic opioid-like peptides were able to release GH from isolated pituitary cells. GH-releasing peptide (GHRP)-6 was shown to induce GH secretion in a dose-dependent manner,

independent of the GHRH receptor (Holst et al., 2005). Several pharmaceutical companies initiated drug discovery projects based on this peptide and its putative receptor. A series of potent and efficient peptide as well as non-peptide analoges and antagonists were consequently developed (Holst et al., 2005). Several peptide and non-peptide compounds exhibited strong dose-dependent GH-releasing activity that was mediated through activation of GHSR-1a receptors in both *in vitro* and *in vivo* models (Moulas et al., 2002; Smith et al., 2005).

Theoretically, synthetic GHSs would influence cardiac structure and function via enhanced GH secretion. However, several studies have demonstrated that these molecules, at least the peptidyl ones, exert also direct and GH-independent cardiac actions: (1) Long-term treatment of GH-deficient rats with hexarelin, a hexapeptide member of the synthetic peptidyl GHS family, provided protective effect on hearts from ischemia/reperfusion damages and prevented alterations of the vascular endothelium-dependent relaxtant function (Bossni et al., 1998). (2) Hexarelin decreased peripheral resistance in rats with myocardial infarction (Tivesten et al., 2001). (3) Hexarelin increased left ventricular ejection in patients with severe GH-deficiency (Imazio et al., 2002). (4) Hexarelin also inhibited cell death in H9C2 cardiomyocytes (Baldanzi et al., 2002), and (5) Hexarelin was shown to have a negative ionotropic effect on both rat and guinea pig papillary muscle (Bedendi et al., 2003).

Despite a wide spectrum of cardiac and vascular actions of synthetic peptidyl GHSs, the effects of synthetic non-peptidyl GHSs on the cardiovascular function have

not been much studied. Frascarelli et al. (2003) reported that, while hexarelin significantly reduced infarct size in rat hearts subjected to 30 minutes of ischemia followed by 120 minutes of reperfusion, the synthetic non-peptidyl GHS, MK-0677, was ineffective. However, like hexarelin, MK-0677 was able to inhibit cell death in H9C2 cardiomyocytes (Baldanzi et al., 2002). Therefore, in the present study, we were interested to characterize the two synthetic non-peptidyl GHSs, L-166446 and L-163255, in respect to their ability to stimulate vasodilator responses in PE-constricted MVB. This could have a very important advantage. For many years, investigators have been searching for new therapeutic molecules to treat patients with cardiovascular diseases such as hypertension. The oral bioavailability of the peptidyl GHSs is limited (Moulas et al., 2002). However, recently available non-peptide secretagogues have been reported to be orally active and effective. Therefore, if the non-peptidyl GHSs, L-166446 and L-163255, could evoke vasodilatation in PE-constricted MVB, it could be investigated for potential use in the management of cardiovascular disease states. However, it should be noted that synthetic GHS compounds have been found to have different molecular pharmacological properties on their own as agonists. That is, the non-endogenous ghrelin receptor agonists can act both as positive as well as negative allosteric modulators of ghrelin signaling (Holst et al., 2005). It is, therefore, proposed that compounds, which act both as agonists and as positive allosteric modulators of the signaling of the endogenous ligand ghrelin could be the optimal type of compound that could be considered for use in clinical settings.

Very interestingly, the results of the present study clearly demonstrate that the synthetic non-peptidyl GHSs, L-166446 and L-163255, evoke concentration-

dependent vasodilatation in PE-constricted MVB. As mentioned earlier, similar to ghrelin, synthetic peptidyl and non-peptidyl GHSs were shown to release GH through binding to their GHSR-1a receptors. Our results suggest the involvement of a different class of GHS receptors than GHSR-1a in vasodilator responses evoked by synthetic non-peptides, because both non-peptidyl GHSs, L-166446 (EC_{50} 4 ± 2 pmol/L) and L-163255 (EC_{50} 6 ± 3 pmol/L), were very potent in inducing their vasodilator responses in MVB. Both L-166446 and L-163255 evoked their vasodilator responses in the same concentration range as ghrelin and des-acyl ghrelin, with a threshold concentration at 1-10 pmol/L reaching a maximal effect at concentrations of about 1 nmol/L. Similar to ghrelin and des-acyl ghrelin, the non-peptides were also slow in evoking their dilator responses. However, both L-166446 (EC_{50} 4 ± 2 pmol/L; E_{max} 70 ± 6%) and L-163255 (EC_{50} 6 ± 3 pmol/L; E_{max} 60 ± 7%) are more potent than ghrelin (EC_{50} 55 ± 6 pmol/L; E_{max} 45 ± 5%) and des-acyl ghrelin (EC_{50} 49 ± 7 pmol/L; E_{max} 43 ± 6%) in evoking their vasodilator responses in MVB. Previous studies have demonstrated the existence of a receptor in cultured cardiomyocytes and endothelial cells that binds hexarelin, as well as ghrelin either in acylated or unacylated form (Baldanzi et al., 2002; Bedendi et al., 2003). On the other hand, some others have reported that, besides GHSR-1a receptor, there are other specific binding sites labeled by [125I] His9-ghrelin in animal and human myocardium that are specific for peptidyl GHSs and that these binding sites do not recognize the non-peptidyl GHS, MK-0677 (Bodart et al., 1993; Papotti et al., 2000). These suggest the hypothesis that, from multiple ghrelin and GHS receptors identified in the cardiovascular system, each receptor may then contribute independently to the wide array of cardiovascular activities induced by synthetic

peptidyl GHSs, synthetic nonpeptidyl GHSs, ghrelin, and endogenous ghrelin-derived molecules.

5.4. Active Core of Ghrelin, Necessary for its Vasodilator Activity, Consists of an N-terminal three Amino Acid Segment Without n-octanoyl Modification

The results of the present study clearly demonstrate that the n-octanoyl modification at the hydroxyl group of the Ser^3 residue of 28-amino acid ghrelin is not necessary for induction of its vasodilator activity, because des-acyl ghrelin evoked concentration-dependent vasodilator responses similar to ghrelin. This was understandable, since to date no octanoyl modifications have been considered to be a prerequisit for bioactive peptides and proteins in the mammalian system. It is noteworthy to mention that our present finding was in contrast to the previous studies reporting the essentiality of n-octanoyl modification for ghrelin binding to its receptor GHSR-1a, and therefore the release of GH (Kojima et al., 1999). However, the identity of the portions of the des-acyl ghrelin peptide necessary for its vasodilator activity remains unknown. To address this issue, several des-acyl fragments with varying lengths were prepared by successive deletions of amino acids from the C-terminal while the N-terminal end was kept intact. This is because of the previous observations that 10 N-terminal amino acids of ghrelin are identical among the different mammalian species. The C-terminal portions of ghrelin, however, are varied, suggesting that the N-terminal portion of ghrelin contains the active core (Matsumoto

et al., 2001). Moreover, Matsumoto et al. (2001) demonstrated that the C-terminal fragment of ghrelin, ghrelin [16-28], was completely inactive.

The evaluation of the vasodilator activity of the prepared short peptide fragments of des-acyl ghrelin demonstrated for the first time that all short peptide fragments of des-acyl ghrelin (des[1-20], des[1-16], des[1-10], des[1-6], des[1-4], des[1-3]) up to three amino acid length evoked vasodilator responses with an efficacy similar to that of full-length des-acyl ghrelin. Previous studies by Bednarek et al. (2000) and Matsumoto et al. (2001), however, indicated that successive deletions of the C-terminal amino acids of ghrelin led to successive decreases in the potency of the peptides to elevate $[Ca^{2+}]_i$. They have also reported that when the peptide length was shortened to a tri-peptide, no activity was observed, even in the presence of the octanoyl modification. Their results demonstrated that the minimum core of ghrelin mediating GHSR-1a GH stimulating activity resides in the N-terminal tetra-peptide, absolutely requiring the octanoyl group at the third residue. This is in comparison to our study identifying the minimum active core of ghrelin as the N-terminal tri-peptide without the octanoyl modification at the third serine residue, or the three-amino acid sequence of des-acyl ghrelin from the N-terminal. The difference between our results and the results from the previous studies can be explained as follows: Ghrelin/Des-acyl ghrelin-evoked vasodilator responses are mediated through a different class of receptors distinctly different from the classical GHSR-1a receptors involved in GH-releasing activity.

The present study also discovered that even single amino acids such as L-serine and glycine can induce vasodilator responses in MVB. These effects do not seem to be artifacts, as maximal vasodilator responses evoked by single amino acids were significantly reduced compared to ghrelin/des-acyl ghrelin. The vasodilator responses evoked by glycine were very negligible compared to those evoked by L-serine. Single amino acids produced their dilator effects at the concentration of 1 nmol/L. Involvement of amino acids in the cardiovascular function has been mentioned for a long time. L-arginine, for example, is a precursor amino acid for the synthesis of NO by NOS. NO accounts for a major portion of the endothelium-dependent vasodilatation produced in a variety of blood vessels. Therefore, a defect in the L-arginine supply may produce inadequate levels of NO, and thus impaired endothelium-dependent vasodilatation (Pieper and Dondlinger, 1997). In addition to L-arginine, animal and human studies have shown a homocysteine-lowering effect of serine treatment (Verhoef et al., 2004). Increased homocysteine is usually associated with the cardiovascular disease.

Besides vasodilator responses evoked by single amino acids observed in the present study, it is noteworthy to mention that if single amino acids could provoke hypotensive effects in the whole animal, it could be a major finding towards new therapeutic agents for the treatment of hypertension. Amino acid therapy with L-serine and glycine in patients with congenital microcephaly and seizures has been reported to be well tolerated, beneficial, and without side effects (Koning et al., 2002; Asechi et al., 2006).

5.5. Diabetes Mellitus Type 1

5.5.1. Plasma Ghrelin Concentrations Are Markedly Elevated in STZ-Treated Diabetic Rats

In agreement with the previous data, our experiments demonstrated that fasting led to a significant increase in plasma total ghrelin levels in both control and diabetic rats. A significant increase in plasma ghrelin levels also occurred in a progressive duration-dependent manner in STZ-diabetic rats. Increased plasma ghrelin levels in STZ-diabetic rats may be due to an increase in ghrelin synthesis and release by the stomach into the blood stream. Increased plasma ghrelin levels in STZ-diabetes coincides with the decreased gastric ghrelin levels (Masaoka et al., 2003). The number of ghrelin-immunoreactive cells in the gastric fundus of diabetic rats was also decreased significantly compared to control rats. Increased plasma ghrelin levels can not be due to a direct irreversible toxicity of STZ on the gastric mucosa and a simultaneous release of the cell contents, since plasma ghrelin concentrations were shown to be quickly normalized by insulin treatment in the STZ-diabetic rats (Ishii et al., 2002; Masaoka et al., 2003). Although the changes in plasma ghrelin concentrations in fasting and diabetes compared to respective control groups are consistent with the data in the literature, there is a substantial degree of variability amongst these observations. There are a few possibilities that may account for the variations including: (1) Duration of the fasting time in both animals and humans. To our knowledge, there is no study reporting the effect of different fasting times on plasma ghrelin levels. However, in the study done by Tschop et al. (2000), where very high basal and fasting plasma ghrelin levels were reported, the animals were fasted for 48 hr compared to the overnight fasting state

maintained in our experiments. A comparison of fasting plasma ghrelin levels between 47 individuals with chronic alcoholism during a period of abstinence and 50 control subjects, observed a positive correlation between ghrelin levels and the duration of abstinence. (2) Animal species, strains, and categories of human subjects. It is possible that rodents may not be ideal for these types of studies. Rats, for example, appear to exhibit a paradoxical fall in GH levels with hypoglycemia (Flanagan et al., 2003). (3) Animal sex and human gender. Our experiments were performed using male rats. However, there are some studies showing the existence of sexual dimorphism for ghrelin. Plasma ghrelin levels were significantly higher in female than in male infants (Ng et al., 2004). Similar observations were reported in adult human subjects under fed as well as fasting states (Greeman et al., 2004). (4) Age of animals and human subjects. Plasma ghrelin levels were found to be inversely correlated with age (Haqq et al., 2003). There was no signs of difference in plasma ghrelin levels in our control group of rats between the age of 16 (4-weeks control) to 20 weeks (8-weeks control). In contrast, plasma ghrelin levels were significantly higher in 8-weeks STZ-treated rats compated to 4-weeks STZ-diabetic rats.

Increased plasma ghrelin levels may be beneficial in diabetes, as ghrelin has been shown to serve as a physiological regulator of insulin release (Date et al., 2002). Both ghrelin and its receptors are present in the pancreatic islets (Date et al., 2002). Additionally, it was also demonstrated that glucose-stimulated insulin release was significantly increased by the addition of 1 pmol/l ghrelin, and ghrelin at the same concentration increased $[Ca^{2+}]_i$ in single rat pancreatic β cells (Date et al., 2002). This

effect of ghrelin, however, may not be important in our STZ-induced diabetic rats, a type I diabetic rat model. This may have a more beneficiary effect in type II diabetes, where the cells develop a resistant state to insulin. Contradictory to the proposed hypothesis, however, previous data have demonstrated that plasma ghrelin levels are decreased in type II diabetes (Poykko et al., 2003). Moreover, as an orexigenic peptide, ghrelin promotes increases in plasma concentration of glucose, and thus may affect glucose homeostasis in different levels, which may be harmfull in type II diabetes.

The mechanisms by which plasma ghrelin concentrations are elevated in STZ-induced diabetes are largely unknown. Reduced plasma GH levels could be a possible mechanism. Tschop et al. (2002) demonstrated that circulating levels of endogenous ghrelin were reduced by the administration of GH in normal rats, and were markedly increased in hypophysectomized rats. Reduced plasma leptin levels were also shown to increase the concentration of ghrelin. The gastric ghrelin mRNA expression was elevated in *ob/*ob mice, which are deficient in leptin and thereby manifest hyperphagia and obesity (Lee et al., 2002). Additionally, in rodents, fasting and hypoglycemia increase both ghrelin mRNA levels and plasma concentrations, whereas enhanced food intake and elevated plasma glucose decrease ghrelin secretion (Lee et al., 2002). Furthermore, plasma ghrelin concentrations of normal human subjects decrease significantly after oral or intravenous glucose administration (Shiiya et al., 2002). In contrast, the reports on the effects of insulin on ghrelin synthesis or release are conflicting. Insulin treatment increased or did not affect the expression of the ghrelin gene in the stomach of rodents and humans (Toshina et al., 2001; Caixas et al., 2002; Shiiya et al., 2002). Gelling et al. (2004) drew some important conclusions. They

demonstrated that meal-induced suppression of plasma ghrelin is intact in STZ rats, and that this response occurs despite the absence of any postprandial increase in plasma insulin levels. This provides unequivocal evidence that meal-related increases in insulin are not required for acute nutrient-induced lowering of circulating ghrelin. However, meal-induced suppression of plasma ghrelin levels was relatively short-lived in STZ rats, suggesting that the inhibitory effects of postprandial insulin release are a key determinant of the duration of meal-induced inhibition of ghrelin release, and thus the time course over which plasma levels of ghrelin are restored to pre-prandial values. This interpretation is compatible with findings from studies in humans with type I diabetes suggesting that insulin has a physiological inhibitory role, and is a determinant of the duration of meal-induced ghrelin suppression (Spranger et al., 2003).

In agreement with previous data suggesting an inhibitory role of insulin on plasma ghrelin levels, increased plasma ghrelin levels in our STZ-treated diabetic rats were in the presence of decreased insulin concentrations. However, our diabetic data still leaves the question of "whether or not insulin plays an inhibitory role on ghrelin secretion?" unanswered, as we did not measure plasma insulin and ghrelin contents over a certain period of time. STZ injection is shown to cause a marked decrease in plasma insulin concentrations. Gelling et al. (2004) also demonstrated that within one day of STZ injection, plasma insulin levels drop more than half compared to the control situation. This effect was accompanied by elevated glucose within one day. However, they demonstrated that food intake decreased initially up to two days and the onset of hyperphagia was not evident until day three after STZ administration. Therefore, according to the above explanation and our finding of increased plasma ghrelin levels in

diabetes, we hypothesize that very early (up to three days) after STZ injection, insulin deficiency stimulates ghrelin secretion and raises its plasma concentrations to a very high level, when the hyperphagia is not yet started and blood glucose levels still are not that high to inhibit ghrelin secretion. But, as the food intake increases and blood glucose levels start to increase more and sustain in that elevated level, nutrient-induced inhibition of ghrelin secretion partially offsets the stimulatory effect of uncontrolled diabetes and lower plasma levels of this hormone. Finally, the two opposing effects of insulin deficiency in uncontrolled STZ-diabetes and high blood glucose levels result in the steady-state values of plasma ghrelin that are still markedly higher than in non-diabetic animals, but are constrained by hyperphagic feeding. This proposed hypothesis, however, needs further investigation.

5.5.2. Ghrelin- and ACh-Evoked Vasodilatations in MVB are Diminished in STZ-treated Diabetic Rats.

As mentioned earlier, the results of the present study clearly demonstrate that ghrelin is a potent vasodilator agonist in the MVB. This was confirmed in our control fed rats, where ghrelin relaxed MVB by 40-45% compared to 98% vasodilatation evoked by ACh. However, the vasodilator effect of ghrelin in MVB has never been studied in diabetes mellitus. Relating plasma ghrelin levels with its vasodilator action in MVB, our present study for the first time shows that despite increased plasma ghrelin levels, its vasodilator effect was significantly reduced in later stages (8-weeks) of STZ-induced diabetes. The vasodilator effect of ghrelin, however, was preserved in earlier stages (4-weeks) of STZ-induced diabetes, when plasma ghrelin levels were still very

high. In comparison to what was observed for ghrelin, vasodilator response to ACh was decreased gradually with the progression of the disease.

Decrease in endothelium-dependent vasodilatation has been demonstrated as a common feature in both conduit and resistance arteries of not only chemically-induced experimental diabetic animals, but also in genetic models of insulin-dependent diabetes mellitus (IDDM) as well as in patients with type I diabetes (Pieper, 1998). In perfused MVB pre-constricted with PE, infusion of cumulative concentrations of ACh causes concentration-dependent vasodilatation. In agreement with our data, an accumulating body of evidence shows that the vasodilatation induced by ACh (in both conduit and resistance arteries) is attenuated in STZ-diabetic rats (Hopfener et al., 1999; Makino et al., 2000). However, the degree of attenuation reported by various studies differs considerably. Endothelium-dependent vasodilatation to ACh in aortic rings was attenuated in 14-week, by approximately 30%, but not in 2-week diabetic rats (Hopfner et al., 1999). In comparison, our results demonstrated more than 30% decrease in vasodilatation to ACh in MVB after 8 weeks of diabetes. It is important to note that the onset and degree of endothelial dysfunction may vary widely among individual vascular beds. In addition, the duration, and /or the severity of the diabetic model used in any given study are also different.

ACh has been known for a long time to promote vasodilatation of blood vessels via the release of multiple vasodilator substances, EDRF, now identified as NO, PGI_2, and EDHF (Furchgott and Zawadzki, 1980; Palmer et al., 1987; Garland et al., 1995). The component of EDHF versus NO, however, increases with decreasing vessel size.

Whereas NO is the major vasodilator factor in aorta, EDHF has been mentioned as the primary endothelium-mediated relaxing factor in small mesenteric arteries (Katakam et al., 1999).

Several studies support the concept that insulin-dependent diabetes mellitus decreases NO production and bioactivity (Pieper, 1998). The ability of NO inhibition to attenuate ACh-induced vasodilatation was reduced in the 14-week STZ rats relative to the 2-week STZ-treated rats (Misurski and Gopalakrishnan, 2002). Glucose possesses a reactive aldehyde moiety that reacts non-enzymatically with the amino groups of proteins, forming products, termed advanced glycation end-products (AGEs), which remain irreversibly bound to proteins (Bucala and Cerami, 1992). It is noteworthy to mention that AGE modification occurs not only to proteins, but also to DNA and lipids and particularly to components of connective tissue. It is hypothesized that AGE modification of proteins and the AGE binding to its receptors on EC, induce drastic alterations in many endothelial functions, representing a critical and initiating factor in the development of diabetic vascular disease (Rojas et al., 2004). AGEs are known to quench NO (Rojas et al., 2004). In addition, studies have also shown a marked reduction in protein and gene expression of endothelial NOS, by decreasing serine phosphorylation of this enzyme and increasing the rate of mRNA degradation, when EC were cultured in the presence of AGEs (Xu et al., 2003; Rojas et al., 2004). Very interestingly, increased serum concentrations of AGEs in patients with type II diabetes are associated with higher magnitude of endothelial dysfunction (Tan et al., 2002).

Endothelium dysfunction, and thus impaired vasodilatation, was shown to arise from deficits in EDHF in certain blood vessel types. A study by Fukao et al. (1997) demonstrated that while resting membrane potential was normal, the response of the diabetic mesenteric artery to ACh was accompanied by diminished hyperpolarization despite unaltered hyperpolarization in response to the K^+ channel agonist, pinacidil. This study was conducted in the presence of NOS and COX inhibitors, suggesting that the release or synthesis of EDHF in the diabetic state may be reduced by comparison with that in the controls. Involvement of a defective EDHF pathway in an impaired endothelium-dependent vasodilatation of mesenteric arterial bed to ACh in STZ-induced diabetic rats was also seen by Makino et al. (2000). They further suggested that the elevated K^+ levels in the myo-endothelial space serves as EDHF and that the vasodilatation response to K^+ is impaired in the MVB from diabetic rats. However, in the latter study, pretreatment with indomethacin had no effect on the ACh-induced dose-dependent vasodilatation in either control or diabetic rats, suggesting that prostanoids are not involved in the ACh-induced vasodilatation in MVB. EDHF-mediated vasodilatation was also shown severely compromised in mesenteric arteries from insulin-resistant rats fed a fructose-rich diet for seven days (Katakam et al., 1999).

Many studies have demonstrated a marked increase in plasma low density lipoproteins (LDL) in the diabetic state, indicating that the impairment of the EDHF-mediated response may be, at least in part, due to the increase in plasma LDL (Katakam et al., 1999; Kobayashi and Kamata, 1999). Indeed, one possible explanation for the decreased EDHF-mediated vasodilatation could be attributed to the following sequence of events: STZ-diabetes leads to an increase in plasma LDL cholesterol and a decrease

in superoxide dismutase levels in blood vessels (Katakam et al., 1999; Kobayashi and Kamata, 1999). The decreased superoxide dismutase activity causes an increased accumulation of superoxide anions. The accumulated superoxide anions may oxidize LDL (Kobayashi and Kamata, 1999). The oxidized LDL may itself impair the EDHF-mediated response and/or the oxidized LDL may release lysophosphatidylcholine (LPC), which then attenuates the EDHF-mediated hyperpolarization (Fukao et al., 1995). The oxidized LDL or LPC may also inhibit Ca^{2+} influx into the endothelium, thus reducing the production or release of EDHF, since this requires an increase in $[Ca^{2+}]_i$ in the endothelium (Chen and Suzuki, 1990; Kamata and Nakajima, 1998). As a result, ACh-induced vasodilatation in the presence of NOS inhibitors would be significantly impaired in the MVBs from diabetic rats.

Several explanations could be proposed in support of our observation of decreased ghrelin-evoked vasodilatation in MVB in the later stages of diabetes. Firstly, there may be a deficit in the mechanism by which ghrelin evokes vasodilatation in MVB. *In vivo* and *in vitro* studies on the cardiovascular actions of ghrelin have not explored the exact mechanism(s) involved. However, previous data from our own laboratory showed that the hypotensive effect of ghrelin is endothelium-dependent, but NO- and COX-independent (Shinde et al., 2005). This is because pretreatment with indomethacin and L-NAME, either alone or in combination, failed to attenuate the fall in MAP evoked by ghrelin. This is in accordance with the data presented by other investigators who showed that intra-arterial vasodilatation produced by the infusion of ghrelin in human forearm was NO-independent (Okumura et al., 2002). Interestingly, a single bolus administration of ghrelin in TBA-pretreated rats failed to induce a fall in

MAP. These data confirm that the acute hypotensive and vasodilator responses to ghrelin are due to the activation of vascular K_{Ca} channels, that are sensitive to blockade by TBA and promoting the generation of endothelium-dependent hyperpolarization. This is consistent with the observation that the synthetic GHS ligand, hexarelin, evoked K^+ conductance in pituitary somatotrophs (Herrington and Hille, 1994). Following these observations, previous data from our laboratory showed that in adherent EC, the addition of ghrelin evoked rapid concentration-dependent increases in $[Ca^{2+}]_i$. Ca^{2+} mobilization following ghrelin binding to the GHS-R type-1a receptors in somatotrophs of pituitary was also observed (Kojima et al., 1999). Ghrelin, therefore, appears to be an agonist that selectively promotes EDHF process or mimics EDHF, since it seems to fulfill all the criteria defining EDHF or the mechanism of endothelium-dependent hyperpolarization.

Considering all the above information, the acute hypotensive effect of ghrelin in MVB is EDHF-mediated, and that diabetes impairs the EDHF-mediated vasodilatation, making it possible to conclude that the decrease in ghrelin-evoked vasodilatation in the later stages of diabetes in MVB may in part be due to an impaired EDHF release. The possibilities of an impaired production of EDHF or an enhanced breakdown of EDHF are likely. The EDHF pathway, however, seems to be normal at the earlier stages of diabetes, as ghrelin-evoked vasodilatation of MVB was preserved in 4-week diabetic rats.

Reduced ghrelin-evoked vasodilatation in later stages of diabetes could also be attributed to the decrease in the number of ghrelin receptors or to the receptor desensitization, since an increase in the plasma ghrelin concentration could theoretically

lead to a down-regulation of ghrelin receptors. It is also noteworthy to mention that plasma glucose levels may also have some regulatory effect on the number and also the function of ghrelin receptors, since plasma glucose levels are highly elevated in IDDM, and glucose administration has been associated with changes in plasma ghrelin content. Hyperglycemia has been shown to cause hyperactivity of isoforms of protein kinase C (PKC) (Rojas et al., 2004). Hyperglycemia-mediated PKC activation has been demonstrated to selectively regulate the receptors for, and responses to, ET-1 (Xu et al., 1993).

In conclusion, our results demonstrate that despite increased plasma ghrelin levels in both early and later stages of diabetes, the ghrelin vasodilator effect is preserved in the early diabetic stage and vasodilatation to ghrelin is impaired only in the later stages of diabetes. These changes were concomitant with the observation that ACh-induced relaxation of MVB is progressively diminished from early to the later stages of diabetes. Ghrelin-evoked vasodilatation, which is supposed to be mediated through the EDHF pathway, seems to be intact in the early diabetes, when the NO pathway is progressively impaired. This is consistent with the observation that NO inhibits EDHF activity such that EDHF may act as a primary endothelium-dependent relaxing factor only when NO is diminished. Our results suggest that, in early diabetes, when NO-mediated vasodilatation begins to deteriorate, the alternative compensatory vasodilatator pathway by EDHF may be activated, which could mask impaired NO-induced endothelium-dependent vasodilatation to compensate for defective NO synthesis. Our present observation that ghrelin-evoked vasodilatation is preserved in early stage of STZ-induced diabetes, while NO is deteriorated, may contribute to increased capillary

perfusion observed in early type I diabetes. Additionally, the diminished ghrelin-evoked vasodilatation in conjunction with a more attenuated NO pathway in chronic diabetes may contribute to the hypertensive and atherosclerotic changes observed in the late stages of the disease.

6. Summary

The ability of ghrelin and des-acyl ghrelin to produce vasodilator responses in MVB was investigated in the presence and absence of endothelium in 12 week old male SD rats. We first examined agonist-evoked vasodilator responses to ACh, and then compared the results to those evoked by ghrelin and des-acyl ghrelin. In endothelium-intact PE-constricted preparations, increasing concentrations of ghrelin, des-acyl ghrelin, and ACh produced concentration-dependent vasodilator responses. However, we found that ghrelin and des-acyl ghrelin were much more potent, with lower maximal vasodilator responses, compared to ACh. Similar to ACh, endothelium-removal completely abolished vasodilator responses to increasing concentrations of ghrelin and des-acyl ghrelin. Agonist-evoked vasodilator responses to SNP, an NO donor, were unaffected in both endothelium-intact as well as endothelium-denuded preparations.

Having established the vasodilator effect of des-acyl ghrelin to the same extent as ghrelin, our data revealed that the whole molecule of ghrelin may not be necessary for its vasodilator response. To determine the active core required for des-acyl ghrelin-evoked vasodilatation, vasodilator responses to several fragments of des-acyl ghrelin, prepared by successive deletions of the C-terminal amino acid residues, were evaluated. We found that all short peptides encompassing the first 20, 16, 10, 6, 4, and 3 residues of des-acyl ghrelin were able to evoke vasodilator responses to the same extent as the full-length des-acyl ghrelin. Vasodilator responses to single amino acids L-serine or glycine, however, were significantly attenuated.

In terms of understanding the underlying mechanism(s) of agonist-evoked vasodilatation to both ghrelin and ACh, several experiments were performed to determine the relative contributions of distinct mediators of endothelium-dependent vasodilator responses including NO, EDHF, and PGI_2. While inclusion of a high concentration of L-NAME failed to affect vasodilator responses to increasing concentrations of ghrelin, there was a significant rightward shift in the CR curve, accompanied by a significant reduction in E_{max}, to ACh. Ghrelin-induced vasodilatation was completely abolished in the presence of TBA, and a combination of TBA and L-NAME did not affect the response any further. In contrast, vasodilator responses to ACh were significantly shifted to the right with a significant reduction in maximal vasodilatation in the presence of TBA. Vasodilator response to ACh was even further attenuated in the presence of a combination of TBA and L-NAME. Increasing ghrelin concentrations failed to evoke vasodilator responses in the presence of high K^+-containing depolarizing buffer. The ACh-evoked vasodilatations were also significantly lower in the presence of high K^+-containing buffer compared to the response seen in PE-constricted MVB preparations. The functional importance of SK_{Ca} and IK_{Ca} channels in the maximal vasodilatations evoked by ghrelin and ACh was examined in the presence of apamin + ChTX or apamin + TRAM-34. Maximal vasodilator responses evoked by both ghrelin and ACh were significantly reduced in the presence of apamin + ChTX, and even further reduced in the presence of apamin + TRAM-34. Treatment with ouabain and Ba^{2+} diminished ghrelin- and ACh-evoked maximal vasodilatations significantly. Inclusion of glibenclamide in the perfusion buffer revealed minimal involvement of K_{ATP} channels in vasodilator response evoked by ghrelin. However, maximal vasodilatation to ACh was significantly attenuated in the presence of glibenclamide.

Having established the presence of GHSR-1a receptors in the cardiovascular system, we determined the involvement of GHSR-1a receptors in ghrelin-evoked vasodilatation using both antagonists as well as agonists of the classical GHSR-1a receptor. The addition of a high concentration of [D-Lys^3]-GHRP-6, a competitive antagonist of the classical GHSR-1a receptor, to the perfusion buffer induced decreases in PP in PE-constricted MVB. Furthermore, the inclusion of L-756867, a peptide antagonist of the classical GHSR-1a receptor, in the perfusion buffer failed to evoke any vasodilator responses on its own or affect the vasodilator responses evoked by ghrelin in PE-constricted MVB. Both non-peptide agonists of GHSR-1a receptors, L-166446 and L-163255, on the other hand, demonstrated concentration-dependent decreases in PP, which were much more potent with significantly higher maximal vasodilatation compared to the response evoked by ghrelin.

Finally, the ability of diabetes to affect vasodilator responses evoked by ghrelin and ACh was investigated in STZ-induced diabetic rats. We first measured plasma ghrelin concentrations, and then evaluated agonist-evoked vasodilatation to both ghrelin and ACh in PE-constricted MVB of both control and STZ-induced diabetic rats. Given the progressive nature of the disease, we chose an early/intermediate (4 weeks) and later (8 weeks) stage of diabetes, in which to assess these responses. We first found that diabetes increases plasma ghrelin concentrations in both 4- and 8-weeks diabetic (fed/fasted) animals compared to their age-matched control rats. In terms of vasodilator responses, however, diabetes for 4 weeks did not cause any significant reduction in vasodilator response to ghrelin, whereas diabetes for 8 weeks induced a very significant reduction in ghrelin-evoked vasodilator responses. Ghrelin-induced vasodilator

responses in 8-weeks diabetic (fed/fasted) animals were significantly different compared to 4-weeks diabetic (fed/fasted) rats. In contrast to ghrelin-evoked vasodilator responses, our data demonstrated that there was a duration-dependent fall in the vasodilator responses to ACh in STZ-diabetic rats compared to their age-matched control groups. The ACh-evoked vasodilatations were significantly lower in 8-weeks compared to 4-weeks STZ-diabetic rats.

7. Conclusions

The results of the present study demonstrate, for the first time, a role for ghrelin and des-acyl ghrelin in mediating vasodilatory effects in MVB by promoting endothelium-dependent hyperpolarization of VSMC and the EDHF process. These data, in conjunction with the previous data from our own laboratory demonstrating the K_{Ca} channel-mediated ghrelin-evoked decreases in MAP in anaestethized male SD rats, may suggest an important role for ghrelin-evoked vasodilatation, and thus decreases in MAP, in states of endothelial dysfunction associated with reduced NO availability such as diabetes and hypertension. The data presented in this thesis suggest that in early/intermediate stages of STZ-induced diabetes, increased plasma ghrelin levels lead to the decreased MVB resistance and exaggerated vasodilator response via activation of the EDHF process. At later stages of STZ-diabetes, the enhanced vasodilatation due to ghrelin is countered by the impairement of EDHF process. Diabetes mellitus is essentially a metabolic disorder characterized by vascular complications that result in a significant morbidity and mortality. Tight glycemic control is a nobel goal, but often an unrealistic one, given the expense and invasiveness of current methods of treatment and monitoring. Ghrelin, therefore, may become a powerful tool in the management of cardiovascular function in disease states such as diabetes and hypertension.

However, ghrelin, which is by far the most studied GHS, has a limited oral bioavailability. Several pharmaceutical companies initiated drug discovery projects and as a result a series of potent and efficient non-peptide compounds exhibiting GH-releasing activity were developed. Recently, available non-peptide secretagogues have

been reported to be orally active and effective. The effects of synthetic non-peptidyl GHSs on the cardiovascular function have not been much studied. The results of the present study clearly demonstrated that both synthetic non-peptidyl GHSs, L-166446 and L-163255, evoked concentration-dependent vasodilatation in PE-constricted MVB. Therefore, if the non-peptidyl GHSs, L-166446 and L-163255, could also evoke decreases in MAP, they could be investigated for potential use in the management of cardiovascular disease states.

Having established that the n-octanoyl modification at the ser3 residue of the 28-amino acid ghrelin is not necessary for induction of its vasodilator activity, our study, for the first time, identified the minimum active core of des-acyl ghrelin molecule necessary for its vasodilator effect to be the N-terminal tri-peptide. Single amino acids, such as L-serine and glycine, could also induce vasodilator responses in MVB. The involvement of amino acids in the cardiovascular function has been mentioned for a long time. If single amino acids could provoke hypotensive effects in the whole animal, it could be a major finding towards new therapeutic agents for the treatment of cardiovascular disease.

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