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RESEARCH PAPER

The gastrointestinal peptide obestatin induces vascular relaxation via specific activation of endothelium-dependent NO signalling

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BACKGROUND AND PURPOSE
Obestatin is a recently discovered gastrointestinal peptide with established metabolic actions, which is linked to diabetes and may exert cardiovascular benefits. Here we aimed to investigate the specific effects of obestatin on vascular relaxation.

EXPERIMENTAL APPROACH
Cumulative relaxation responses to obestatin peptides were assessed in rat isolated aorta and mesenteric artery ($n \geq 8$) in the presence and absence of selective inhibitors. Complementary studies were performed in cultured bovine aortic endothelial cells (BAEC).

KEY RESULTS
Obestatin peptides elicited concentration-dependent relaxation in both aorta and mesenteric artery. Responses to full-length obestatin(1–23) were greater than those to obestatin(1–10) and obestatin(11–23). Obestatin(1–23)-induced relaxation was attenuated by endothelial denudation, L-NAME (NOS inhibitor), high extracellular K^+, GDP-β-S (G-protein inhibitor), MDL-12,330A (adenylate cyclase inhibitor), wortmannin (PI3K inhibitor), KN-93 (CaMKII inhibitor), ODQ (guanylate cyclase inhibitor) and iberiotoxin (BK_{Ca} blocker), suggesting that it is mediated by an endothelium-dependent NO signalling cascade involving an adenylate cyclase-linked GPCR, PI3K/PKB, Ca^{2+}-dependent eNOS activation, soluble guanylate cyclase and modulation of vascular smooth muscle K^+. Supporting data from BAEC indicated that nitrite production, intracellular Ca^{2+} and PKB phosphorylation were increased after exposure to obestatin(1–23). Relaxations to obestatin(1–23) were unaltered by inhibitors of candidate endothelium-derived hyperpolarizing factors (EDHFs) and combined SK_{Ca}/IK_{Ca} blockade, suggesting that EDHF-mediated pathways were not involved.

CONCLUSIONS AND IMPLICATIONS
Obestatin produces significant vascular relaxation via specific activation of endothelium-dependent NO signalling. These actions may be important in normal regulation of vascular function and are clearly relevant to diabetes, a condition characterized by endothelial dysfunction and cardiovascular complications.

Abbreviations
BAEC, bovine aortic endothelial cells; BK_{Ca}, high-conductance Ca^{2+}-activated K^+ channel; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial NOS; ETI, 5,8,11-eicosatriynoic acid; GDP-β-S, guanosine 5′-(β-thio)diphosphate trilithium salt; GPR39, G-protein-coupled receptor 39; GTP-γ-S, guanosine 5′-(γ-thio)triphosphate tetralithium salt; IDM, indomethacin; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channel; KHB, Krebs–Henseleit buffer; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MDL-12,330A, *cis*-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride; ODQ, 1*H*-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one; PI3K, phosphoinositide-3 kinase; PE, phenylephrine; pPKB, phosphorylated PKB; PPOH, 6-(2-proparagylloxyphenyl) hexanoic acid; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel

Introduction

Gastrointestinal hormones are secreted from the epithelium of the stomach and intestine and have wide-ranging physiological actions. These effects are pleiotropic in nature and are frequently involved in the regulation of digestion, nutrient ingestion, appetite and energy metabolism. It is becoming increasingly apparent that some gastrointestinal hormones also have important vascular actions. For example, vasoactive intestinal peptide, glucagon-like peptide-1 and cholecystokinin have been shown to induce concentration-dependent vascular relaxation and to modulate blood pressure (Henning and Sawmiller, 2001; Sanchez-Fernandez *et al.*, 2004; Grieve *et al.*, 2009).

Obestatin is a recently discovered ghrelin-related peptide, comprising a 23-amino-acid sequence that is C-terminally amidated and adopts an α-helical conformation in solution (Zhang *et al.*, 2005; Subasinghage *et al.*, 2010). It is expressed in several tissues throughout the gastrointestinal tract, most notably the stomach, pancreas, duodenum, jejunum and colon (Zhao *et al.*, 2008). However, the majority of obestatin-producing cells appear to be concentrated in the oxyntic mucosa of the stomach. Indeed, surgical removal of the stomach by gastrectomy has been demonstrated to reduce circulating obestatin levels by 50–80% in rats (Furnes *et al.*, 2008).

It was originally suggested that obestatin functioned as an endocrine hormone with several physiological actions, but this concept has since proved to be controversial. Shortly after its discovery, obestatin was reported to exert potent metabolic actions, resulting in inhibition of gastric motility, suppression of food intake and body weight reduction (Zhang *et al.*, 2005). These initial findings have subsequently been confirmed by several other groups (Chartrel *et al.*, 2007; Green *et al.*, 2007; Nagaraj *et al.*, 2008), whilst others dispute claims that obestatin influences food intake and/or body weight (Bassil *et al.*, 2007; Gourcerol and Tache, 2007; Unniappan *et al.*, 2008). Interestingly, our group recently reported that obestatin may also play a role in modulation of physiological lipid metabolism (Agnew *et al.*, 2011). In addition to its proposed metabolic effects, other studies have suggested that obestatin acts centrally to inhibit thirst, alter sleep patterns and improve memory (Szentirmai and Krueger, 2006; Carlini *et al.*, 2007; Samson *et al.*, 2007), although it is thought to not cross the blood-brain barrier (Pan *et al.*, 2006). Furthermore, the receptor originally proposed to mediate the actions of obestatin, the GPCR 39 (GPR39) (Zhang *et al.*, 2005), has also been disputed (Holst *et al.*, 2007; Unniappan *et al.*, 2008); therefore, the cognate receptor for obestatin remains to be determined.

Interestingly, a number of preliminary studies suggest that there may be a link between obestatin and type 2 diabetes. Circulating obestatin levels have been reported to be significantly lower in both glucose intolerant and type 2 diabetic patients compared with age-matched control subjects (Qi *et al.*, 2007) together with marked alterations in their postprandial secretory responses (St-Pierre *et al.*, 2010). However, it should be noted that other studies have failed to find differences in circulating obestatin levels between type 2 diabetic patients and normoglycaemic controls (Lippl *et al.*, 2008; St-Pierre *et al.*, 2010). Nonetheless, studies in streptozotocin-treated rats, used as an experimental model of diabetes, are supportive of an important role for obestatin in this setting. Concentrations of obestatin in the hypothalamus of these animals were 34% lower compared with controls (Wang *et al.*, 2010), and obestatin administration has been shown to counter streptozotocin-induced diabetes, resulting in improved pancreatic morphology (Grala *et al.*, 2010; Granata *et al.*, 2010). The same group also reported that incubation of human beta cells and islets with obestatin promoted cell survival and stimulation of beta-cell regulatory genes, therefore indicating likely anti-diabetic actions (Granata *et al.*, 2008).

Although the precise nature of the ascribed metabolic actions of obestatin and its relationship to type 2 diabetes remains unclear, it is becoming evident that this peptide exerts important physiological effects on the cardiovascular system. Obestatin administration has been found to reduce infarct size, cardiac contractile dysfunction and cardiomyocyte apoptosis in a rat model of ischaemia–reperfusion injury, effects that may be mediated by phosphoinositide-3 kinase (PI3K), PKC and ERK1/2 (Kellokoski *et al.*, 2009; Alloatti *et al.*, 2010). It has also been reported that obestatin decreases TNF-α-induced vascular cell adhesion molecule-1 expression in endothelial cells, whilst promoting binding of oxidized low-density lipoprotein to macrophages, suggesting that obestatin may differentially modulate early atherogenic processes (Kellokoski *et al.*, 2009). Furthermore, plasma concentrations of obestatin and the ghrelin to obestatin ratio are lower in patients with untreated mild to moderate hypertension compared with normotensive controls (Li *et al.*, 2010b) and negatively correlated with systolic blood pressure in patients with insulin resistance (Anderwald-Stadler *et al.*, 2007). Conversely, both circulating obestatin and the ghrelin to obestatin ratio have been shown to be increased in spontaneously hypertensive rats and positively correlated with both systolic and diastolic blood pressure (Li *et al.*, 2010a), whilst bolus obestatin injection in these animals had no acute effect on blood pressure (Li *et al.*, 2009). Although it appears that obestatin may play a role in the regulation of vascular function and blood pressure, the precise nature of its involvement remains unknown. In the present study, we show for the first time that obestatin relaxes both rat isolated aorta and mesenteric artery in a dose-dependent manner and describe detailed underlying mechanisms. This may have implications for the normal regulation of blood pressure and vascular function and indicates that obestatin may be beneficial in the setting of diabetes, which is frequently associated with cardiovascular complications.

Methods

Experimental animals

Male Sprague–Dawley rats (8–12 weeks) were used throughout this study. They were housed under constant climatic conditions with free access to food and water. All animal care and experimental procedures were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK).

Isolated vessel studies

Rats were killed with a sodium pentobarbitone overdose (200 mg·kg^{-1} body weight i.p.), and the thoracic aorta or superior mesenteric artery was excised, cleared of surrounding connective tissue and cut into 2–3 mm rings, taking care to leave the endothelium intact. Rings were suspended between a force transducer and a fixed support in organ bath chambers containing 5 mL modified Krebs–Henseleit buffer (KHB, composition in mmol·L^{-1}: NaCl 118.5, KCl 3.8, KH_2P0_4 1.18, $NaHCO_3$ 25, $MgSO_4$ 1.19, glucose 10, and $CaCl_2$ 1.25), bubbled with 95% O_2/5% CO_2 at 37°C. Data were recorded using a PowerLab 8/30 acquisition system (ADInstruments Ltd., Chalgrove, UK). Vessels were held at a resting tension of 1 g (which was found to be optimal in preliminary experiments) and allowed to equilibrate for 45 min before the maximal contractile response to 80 mmol·L^{-1} KCl was assessed. Following washout and re-equilibrium, a bolus dose of phenylephrine (PE, 10 μmol·L^{-1}) was added to produce maximal contraction. After further washout rings were then pre-constricted with PE to 70% of their maximal PE-induced contraction before relaxation protocols were performed.

Organ bath protocols

In order to study the direct effects of obestatin peptides on vascular function, cumulative relaxation responses were performed to full-length obestatin(1–23) and the peptide fragments, obestatin(11–23) and obestatin(1–10) (0.1 pmol·L^{-1}–0.1 μmol·L^{-1}), in both thoracic aorta and superior mesenteric artery, in parallel with appropriate time controls. These fragments were chosen on the basis of previous reports, indicating that they are physiological breakdown products of obestatin(1–23), which may possess differential bioactivity (Green *et al.*, 2007; Nagaraj *et al.*, 2008). Potential mechanisms of action of obestatin were further investigated in thoracic aorta only, by performing cumulative relaxation responses to obestatin(1–23) in endothelium-denuded vessels (by gentle rubbing of the luminal surface) and in the presence or absence of the following specific inhibitors of candidate pathways (n = 8–14): (i) N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 0.3 mmol·L^{-1}), non-selective NOS inhibitor (Rees *et al.*, 1990); (ii) high extracellular K^+ (KCl, 30 mmol·L^{-1}), to inhibit membrane hyperpolarization (Green *et al.*, 2008); (iii) indomethacin (IDM, 10 μmol·L^{-1}), COX inhibitor (Vane, 1971); (iv) guanosine 5′-(β-thio)diphosphate trilithium salt (GDP-β-S, 10 μmol·L^{-1}), G-protein inhibitor (Bolego *et al.*, 1995); (v) MDL-12,330A (30 μmol·L^{-1}), adenylate cyclase inhibitor (Hunt and Evans, 1980); (vi) wortmannin (0.1 μmol·L^{-1}), PI3K inhibitor (Davies *et al.*, 2000); (vii) KN-93 (10 μmol·L^{-1}), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor (Schneider *et al.*, 2003); (viii) 1*H*-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ, 10 μmol·L^{-1}), guanylate cyclase inhibitor (Garthwaite *et al.*, 1995); (ix) 6-(2-proparagylloxyphenyl) hexanoic acid (PPOH, 0.1 μmol·L^{-1}), cytochrome P450 inhibitor (Wang *et al.*, 1998); (x) catalase (1250 U·mL^{-1}), hydrogen peroxide scavenger (Mian and Martin, 1995); (xi) 5,8,11-eicosatriynoic acid (ETI, 10 μmol·L^{-1}), lipoxygenase inhibitor (Hammarstrom, 1977); (xii) a combination of the small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) blocker, apamin (0.1 μmol·L^{-1}) (Marchenko and Sage, 1996) and the intermediate-conductance Ca^{2+}-activated K^+ channel (IK_{Ca}) blocker, TRAM-34 (10 μmol·L^{-1}) (Wulff *et al.*, 2000), which has been shown to block endothelium-derived hyperpolarizing factor (EDHF)-mediated responses (Busse *et al.*, 2002); and (xiii) iberiotoxin (0.1 μmol·L^{-1}) (Galvez *et al.*, 1990), high-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) blocker. Cumulative relaxation-response curves were also performed to guanosine 5′-(γ-thio)triphosphate tetralithium salt (GTP-γ-S, 0.1 pmol·L^{-1}–0.1 μmol·L^{-1}), a G-protein-activating analogue of GTP (Bolego *et al.*, 1995). At the end of all experimental protocols, a bolus dose of ACh (0.1 mmol·L^{-1}) was added to assess endothelial integrity.

Cell culture

Bovine aortic endothelial cells (BAEC) were isolated from the descending aorta using 0.01% collagenase, as previously described (Booyse *et al.*, 1975). The cell suspension was then cultured in DMEM (Sigma, Gillingham, UK) supplemented with 10% fetal bovine serum and 1% penicillin (100 U·mL^{-1}) and maintained in a humidified atmosphere at 37°C and 5% CO_2. BAEC were then grown to confluence, and cells from passages 2–8 were used for experiments.

NO production

In order to investigate whether obestatin can stimulate endothelial NO production directly, confluent BAEC were separated into six-well plates and incubated with or without obestatin(1–23) (0.1 μmol·L^{-1}) for 24 h, using ACh (10 μmol·L^{-1}) as a positive control. At the end of the experiment, supernatants were collected, and nitrite concentrations were quantified (as a reliable indicator of NO production) by the Greiss reaction using a commercially available assay kit (Promega, Southampton, UK), and absorbance was determined at 550 nm (Tecan Ltd., Reading, UK). All experiments and assays were performed in triplicate.

Western blotting

To investigate whether obestatin stimulates phosphorylation of PKB (Akt), confluent BAEC were separated into six-well plates and incubated with obestatin(1–23) (0.1 μmol·L^{-1}) for 0, 15, 30, 60 and 180 min. Cell lysates were then prepared with RIPA buffer, as previously described (Looi *et al.*, 2008), and total protein (20 μg) was applied to a 10% SDS-PAGE gel and blotted onto a PVDF membrane (Immobilon-FL; Millipore, Watford, UK). Membranes were probed overnight at 4°C with rabbit polyclonal PKB or phosphorylated PKB (pPKB) antibodies (Cell Signaling Technology, Boston, MA) using a mouse monoclonal β-actin antibody as a loading control (Sigma-Aldrich, Poole, UK). This was followed by incubation with IRDye 800 goat anti-rabbit and IRDye 680CW goat anti-mouse secondary antibodies (Li-COR, Lincoln, NE) for 60 min at room temperature, before the membrane was scanned and quantified by densitometry (Odyssey; Li-COR).

Intracellular Ca^{2+} measurements

Measurements of intracellular Ca^{2+} were performed in BAEC, as previously described (Banumathi *et al.*, 2011). Briefly, cells were seeded on to gelatin-coated coverslips and loaded with fura-2 AM (5 μmol·L^{-1}) for 20 min at 37°C. They were then placed on to the stage of an inverted microscope (Eclipse TE2000; Nikon, Tokyo, Japan), superfused with Hanks solu-

tion at 37°C and illuminated alternately at 340/380 nm by a dual monochromator (5 nm bandwidth) and light chopper (Cairn Research Ltd., Faversham, UK). Emitted fluorescence was measured before and after exposure to obestatin(1–23) (0.1 μmol·L^{-1}), and data were analysed using acquisition software (Acquisition Engine, v1.1.5; Cairn Research Ltd.). At the end of each experiment, background fluorescence was quantified by incubating BAEC with $MnCl_2$ (10 mmol·L^{-1}) in Ca^{2+}-free solution, and changes in the ratio of the normalized fluorescence at each excitation wavelength ($R = F340/F380$) were used as a measure of changes in intracellular Ca^{2+}.

Drugs and reagents

Obestatin(1–23), obestatin(1–10) and obestatin(11–23) (>95% purity) were custom made by GL Biochem Ltd. (Shanghai, China). L-phenylephrine hydrochloride (PE), ACh, L-NAME, IDM, GDP-β-S, MDL-12,330A, wortmannin, KN-93, ODQ, PPOH, catalase, ETI, apamin, TRAM-34, iberiotoxin, and GTP-γ-S were all purchased from Sigma-Aldrich. All drugs, with the exception of IDM, MDL-12,330A, wortmannin, KN-93, ODQ, ETI, apamin and TRAM-34, were initially dissolved in de-ionized water (at 10 mmol·L^{-1}) and diluted in KHB. IDM, MDL-12,330A, wortmannin, KN-93, ODQ, ETI, apamin and TRAM-34 were initially dissolved in dimethyl sulphoxide (at 100 mmol·L^{-1}), which had no effect on vascular function at its final concentration (0.01%). All solutions were freshly prepared on the day of the experiment. Concentrations are expressed as the final concentration of each drug in the organ bath.

Statistical analysis

Data are expressed as a mean ± SEM. For organ bath studies, data are expressed as decrease in tension calculated as a % of the initial PE-induced tone, and plotted against log agonist

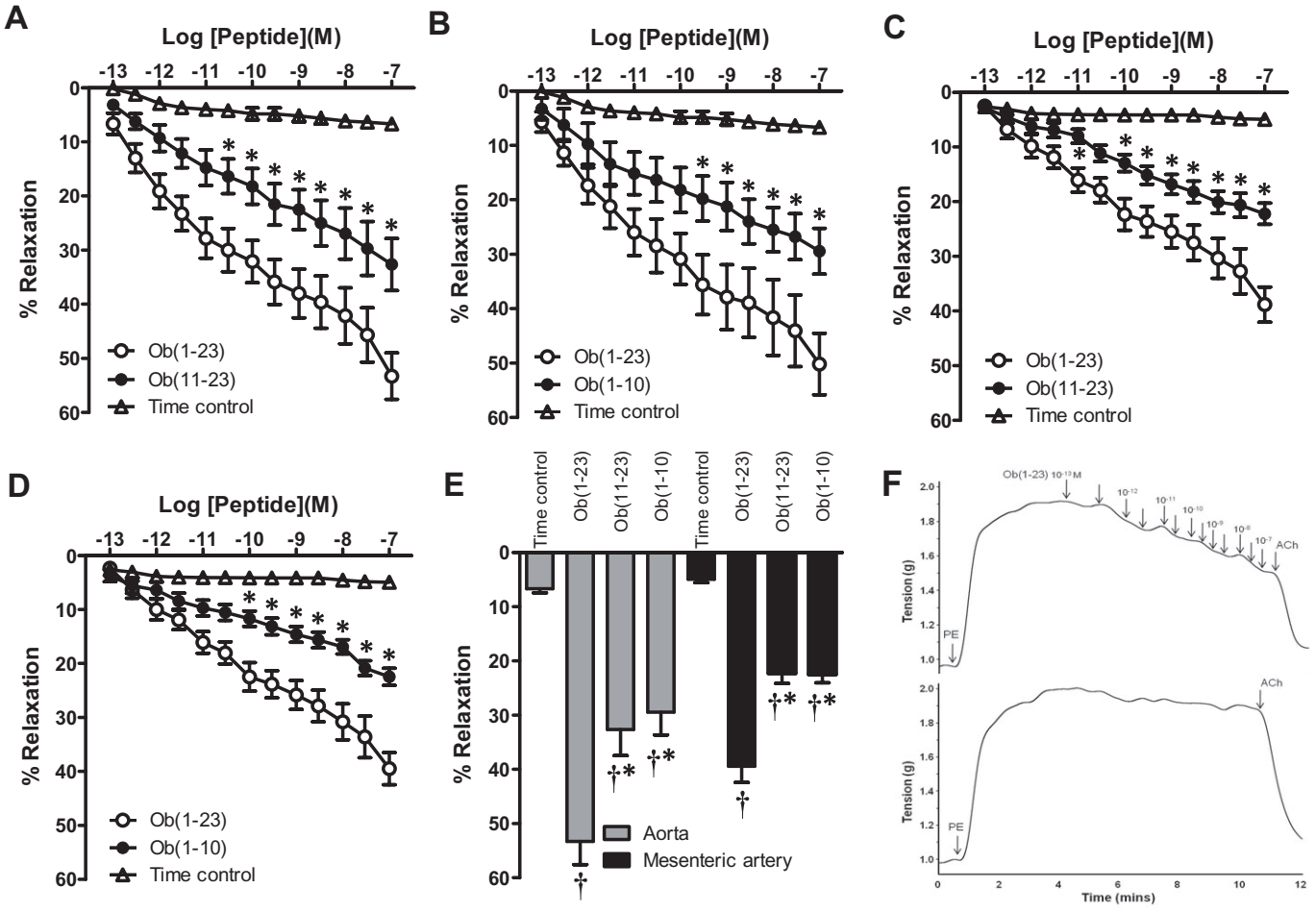

Figure 1

Effect of obestatin peptides on vascular relaxation. Cumulative relaxation-response curves to obestatin(1–23), obestatin(11–23) and obestatin (1–10) in (A and B) rat aorta and (C and D) mesenteric artery, compared with appropriate time controls. (E) Histogram comparing maximal relaxations between groups. (F) Representative organ bath traces showing a typical relaxation response to obestatin(1–23) in rat aorta (upper panel) after preconstriction with PE, together with a time control (lower panel). Experiments were followed by addition of ACh to assess endothelial integrity. Relaxations are expressed as a percentage of the initial PE-induced contraction. Each data point or column represents the mean ± SEM for ≥8 experiments. †$P < 0.05$ versus corresponding time control, *$P < 0.05$ versus corresponding obestatin(1–23) control, two-way repeated-measures ANOVA (A–D) or one-way ANOVA (E) with Bonferroni *post hoc* test.

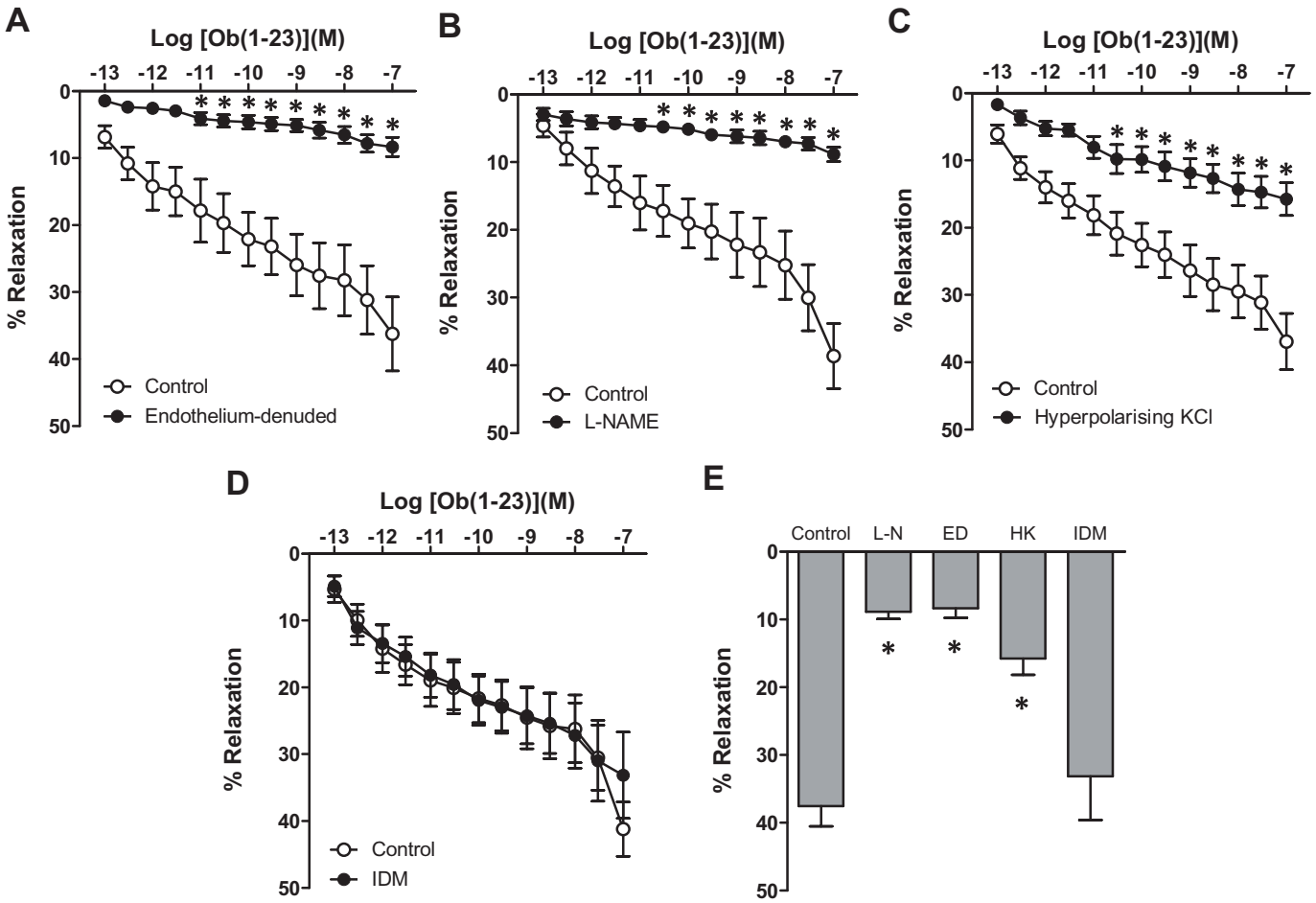

Figure 2

Investigation of candidate mediators of obestatin-induced vascular relaxation. Cumulative relaxation responses to obestatin(1–23) in (A) endothelium-denuded (ED) vessels and in the presence or absence of (B) L-NAME (L-N, 0.3 mmol·L^{-1}), (C) hyperpolarizing K^+ (HK, 30 mmol·L^{-1}) and (D) IDM (10 μmol·L^{-1}). (E) Histogram comparing maximal relaxations between groups. Results are expressed as a percentage of the initial PE-induced contraction. Each data point or column represents the mean ± SEM for ≥8 experiments. *P < 0.05 versus corresponding control, two-way repeated-measures ANOVA with Bonferroni *post hoc* test (A–D) or one-way ANOVA with Dunnett's *post hoc* test (E).

concentration. Data were analysed by a two-way repeated-measures ANOVA, one-way ANOVA with Bonferroni or Dunnett's *post hoc* test, or Student's unpaired *t*-test, as appropriate. $P < 0.05$ was considered to be significant.

Results

Effect of obestatin peptides on vascular relaxation in rat isolated aorta and mesenteric artery

Figure 1B–E shows cumulative relaxation response curves to obestatin(1–23), obestatin(11–23) and obestatin(1–10) in both rat aorta and mesenteric artery. All three peptides elicited concentration-dependent vasorelaxation in both vessels, with obestatin(1–23) causing a significantly greater response compared with both obestatin(11–23) and obestatin(1–10) in both aorta (53.3 ± 4.3 vs. 32.7 ± 4.8 vs. 29.5 ± 4.2%, respectively; $P < 0.05$) and mesenteric artery (39.5 ± 3.0 vs. 22.2 ± 1.9 vs. 22.5 ± 1.6%, respectively; $P < 0.05$). Minimal relaxations were observed in parallel time control preparations (aorta, 6.7 ± 0.8%; mesenteric artery, 5.0 ± 0.6%). A summary of the mean maximal relaxation data is presented in Figure 1E, and representative organ bath traces showing a typical relaxation response to obestatin are shown in Figure 1F.

Identification of candidate mediators of obestatin-induced vascular relaxation

As obestatin(1–23) was found to cause the greatest vasorelaxation and the pattern of responses to obestatin peptides was similar between vessels, it was decided to perform further detailed mechanistic studies using only obestatin(1–23) in aorta. Relaxation to obestatin(1–23) was significantly attenuated by endothelial denudation (8.3 ± 1.5 vs. 36.3 ± 5.5%; $P < 0.05$; Figure 2A) and in the presence of either the NOS inhibitor, L-NAME (8.8 ± 1.0 vs. 38.6 ± 4.8%; $P < 0.05$; Figure 2B), or high extracellular K^+ (15.8 ± 2.4 vs. 36.9 ±

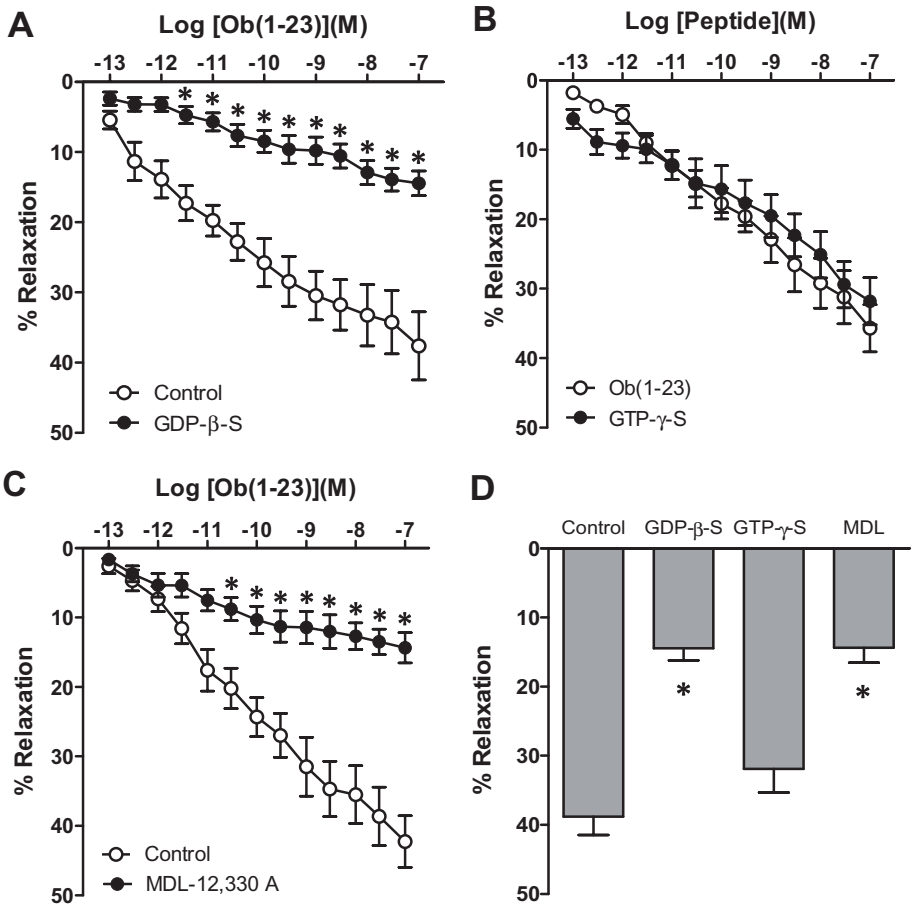

Figure 3

Characterization of the receptor involved in obestatin-induced vascular relaxation. Cumulative relaxation responses to obestatin(1–23) (A) in the presence or absence of GDP-β-S (10 μmol·L^{-1}) (B) compared with GTP-γ-S and (C) in the presence or absence of MDL-12 330 A (30 μmol·L^{-1}). (D) Histogram comparing maximal relaxations between groups. Results are expressed as a percentage of the initial PE-induced contraction. Each data point or column represents the mean ± SEM for ≥8 experiments. *$P < 0.05$ versus corresponding control, two-way repeated-measures ANOVA with Bonferroni *post hoc* test (A–C) or one-way ANOVA with Dunnett's *post hoc* test (D).

4.2%; $P < 0.05$; Figure 2C), which causes membrane hyperpolarization. However, the COX inhibitor, IDM, had no significant effect on obestatin(1–23)-induced relaxation (Figure 2D). Maximal relaxation responses for these studies are summarized in Figure 2E.

Characterization of the receptor involved in obestatin-induced vascular relaxation

Although the cognate receptor for obestatin remains to be determined, we performed a series of studies to attempt to identify the receptor family and downstream signalling pathways involved in obestatin-mediated vasorelaxation. Relaxation to obestatin(1–23) was significantly attenuated by the G-protein inhibitor, GDP-β-S (14.5 ± 1.7 vs. 37.6 ± 4.8%; $P < 0.05$; Figure 3A) and, importantly, was mirrored by relaxation in response to the G-protein-activating analogue, GTP-γ-S (Figure 3B). In addition, obestatin(1–23)-induced relaxation was significantly reduced by the adenylate cyclase inhibitor, MDL-12,330A (14.3 ± 2.2 vs. 42.6 ± 3.7%; $P < 0.05$; Figure 3C). Taken together, these data strongly implicate an adenylate cyclase-linked GPCR in the observed vascular relaxation to obestatin. Maximal relaxation responses for these receptor studies are summarized in Figure 3D.

Mechanisms underlying NO activation in response to obestatin

In order to confirm that obestatin directly activated endothelial NO production, studies were performed in primary BAEC incubated with or without obestatin(1–23) for 24 h. Importantly, nitrite concentrations measured in the supernatants from these cells were significantly increased after incubation with both obestatin(1–23) and ACh, used as a positive control (Figure 4A), supporting our initial findings in isolated vessels. To further characterize the precise mechanisms underlying endothelium-dependent NO signalling in response to obestatin, specific inhibitors of established pathways were employed in the organ bath. Relaxation to obestatin(1–23) was significantly attenuated by in the presence of the PI3K inhibitor, wortmannin (19.9 ± 3.3 vs. 42.5 ±

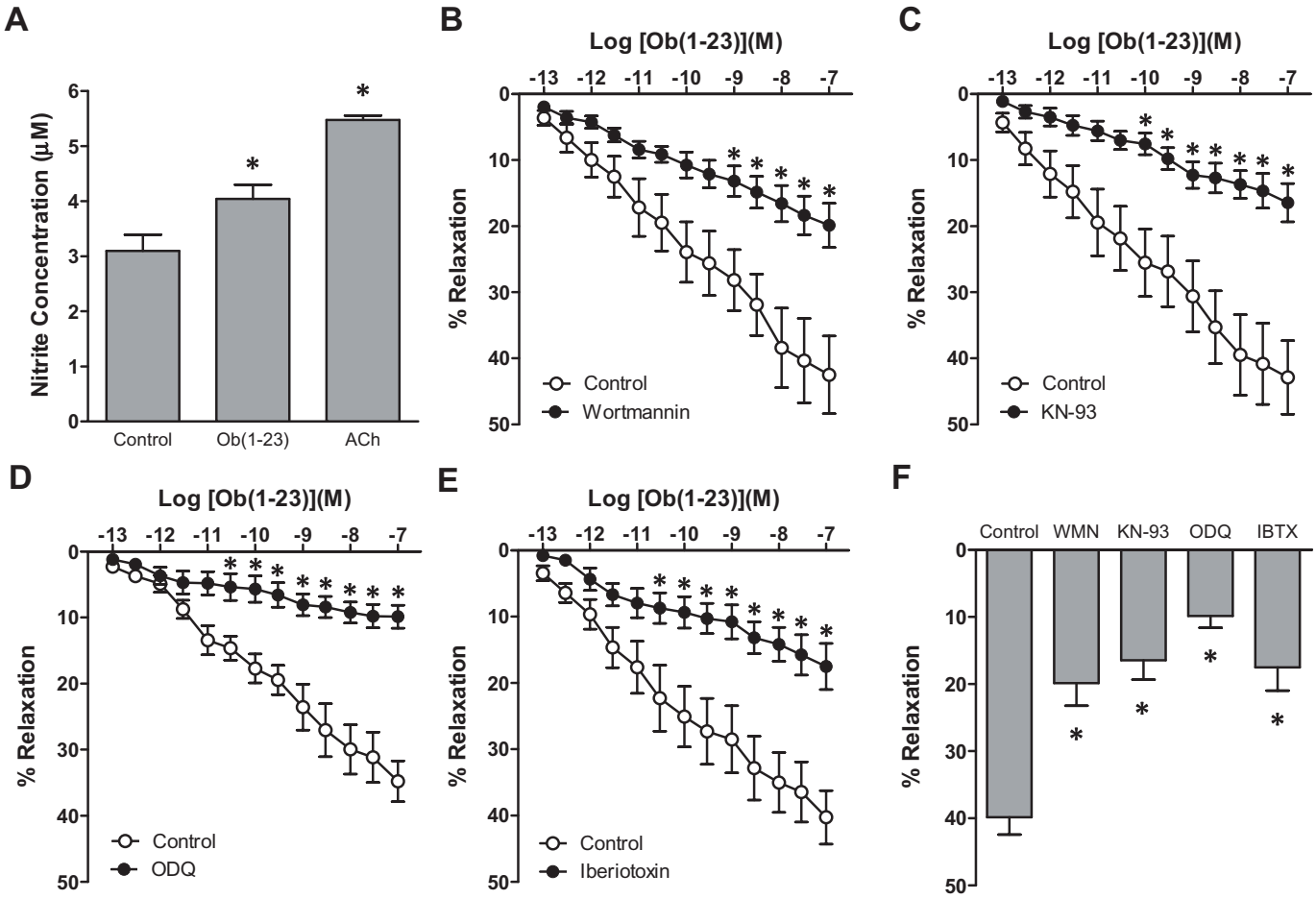

Figure 4

Mechanisms underlying obestatin-induced NO signalling. (A) Nitrite concentration in supernatants of BAEC incubated with or without obestatin(1–23) (0.1 μmol·L^{-1}) for 24 h, compared with ACh (10 μmol·L^{-1}) as a positive control. Cumulative relaxation responses to obestatin (1–23) in the presence or absence of (B) wortmannin (WMN, 0.1 μmol·L^{-1}), (C) KN-93 (10 μmol·L^{-1}), (D) ODQ (10 μmol·L^{-1}) and (E) iberiotoxin (IBTX, 0.1 μmol·L^{-1}). (F) Histogram comparing maximal relaxations between groups. Results are expressed as a percentage of the initial PE-induced contraction. Each data point or column represents the mean ± SEM for 4 (cell culture) or ≥8 (organ bath) experiments. *P < 0.05 versus corresponding control, two-way repeated-measures ANOVA with Bonferroni *post hoc* test (B–E) or one-way ANOVA with Dunnett's *post hoc* test (A and F).

5.9%; P < 0.05; Figure 4B), the CaMKII inhibitor, KN-93 (16.5 ± 2.9 vs. 42.9 ± 5.6%; P < 0.05; Figure 4C), the guanylate cyclase inhibitor, ODQ (9.9 ± 1.7 vs. 34.8 ± 3.1%; P < 0.05; Figure 4D) and the BK_{Ca} blocker, iberiotoxin (17.5 ± 3.5 vs. 40.3 ± 4.0%; P < 0.05; Figure 4E). Maximal relaxation responses for these organ bath studies are summarized in Figure 4F. In addition, incubation of BAEC with obestatin(1–23) resulted in a transient alteration of PKB protein phosphorylation, which was significantly increased after 30 min, whilst total PKB expression remained unaltered (Figure 5A and B). Furthermore, Ca^{2+} fluorescence experiments in BAEC indicated that intracellular [Ca^{2+}] was significantly increased by obestatin(1–23) (Figure 5C and D). Taken together, these data confirm the involvement of NO in obestatin-induced vascular relaxation and suggests that endothelial NOS (eNOS) activation is Ca^{2+}-dependent and is mediated via acute PI3K/PKB activation, which is linked to downstream guanylate cyclase and BK_{Ca} activation.

Investigation of potential role of endothelium-dependent hyperpolarization in obestatin-induced vascular relaxation

Further to our finding that vascular relaxation to obestatin was attenuated by high extracellular K^+, a series of detailed studies were conducted to investigate the potential role of several putative EDHFs. Obestatin(1–23)-induced relaxations were unaltered in the presence of either the cytochrome P450 inhibitor, PPOH, the hydrogen peroxide scavenger, catalase and the lipoxygenase inhibitor, ETI (Figure 6A–C), in addition to the COX inhibitor, IDM (Figure 2D), suggesting that these candidate EDHFs were not involved. In addition, a combination of the small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) blocker, apamin, and the intermediate-conductance Ca^{2+}-activated K^+ channel (IK_{Ca}) blocker, TRAM-34 (Figure 6D) had no effect on relaxations to obestatin(1–23), further indicating that EDHFs do not play a

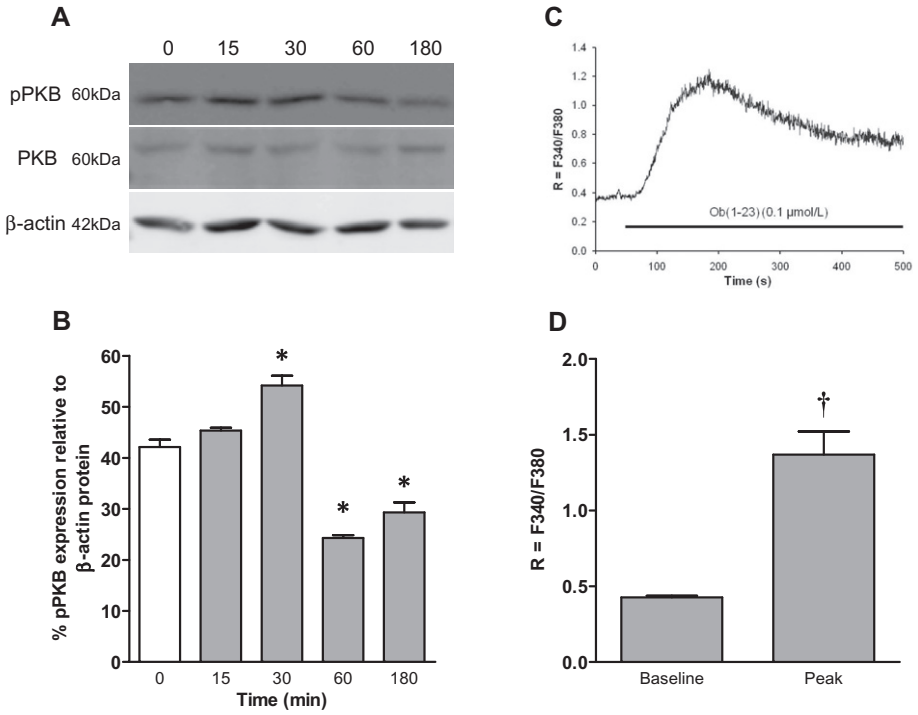

Figure 5

Mechanisms underlying NO activation in response to obestatin. (A) Representative Western blots for PKB, phosphorylated PKB (pPKB) and β-actin in BAEC incubated with obestatin(1–23) (0.1 μmol·L^{-1}) for 0–180 min. (B) Mean quantified data ± SEM for pPKB/PKB protein expression normalized to β-actin from three experiments. (C) Representative Ca^{2+} fluorescence trace showing a typical response to obestatin(1–23) in a BAEC. (D) Mean quantified data ± SEM for normalized Ca^{2+} fluorescence from seven cells. *P < 0.05 versus 0 min, one-way ANOVA with Dunnett's *post hoc* test. †P < 0.05 versus baseline, Student's unpaired *t*-test.

significant role in this setting. Maximal relaxation responses from these studies are summarized in Figure 6E.

Discussion and conclusions

In this study, we demonstrated that the gastrointestinal peptide, obestatin, causes significant and dose-dependent vascular relaxation. This appears to be mediated by endothelium-dependent NO release via a signalling cascade involving an adenylate cyclase-linked GPCR, PI3K/PKB and Ca^{2+}-dependent eNOS activation, which is linked to downstream soluble guanylate cyclase and BK_{Ca} activation. Although several previous studies have suggested that obestatin may exert important effects in the cardiovascular system (Anderwald-Stadler *et al.*, 2007; Kellokoski *et al.*, 2009; Alloatti *et al.*, 2010; Li *et al.*, 2010a,b), this is the first definitive demonstration that this peptide produces direct vascular actions. Importantly, these effects occurred at physiologically relevant concentrations, which are reported to be in the pg·mL^{-1} range in both rat and human plasma (Beck *et al.*, 2010; Li *et al.*, 2010a,b), equivalent to organ bath concentrations of 10–100 pmol·L^{-1}. The finding that these appear to be mediated via endothelium-dependent NO production is particularly interesting given the potential links of obestatin to type 2 diabetes (Qi *et al.*, 2007; Granata *et al.*, 2010; Wang *et al.*, 2010), a condition characterized by endothelial dysfunction and reduced NO production (Hamilton *et al.*, 2007). However, further studies are clearly required to assess whether obestatin induces similar relaxant actions in resistance vessels and the microcirculation, before any conclusions on its therapeutic potential in this context can be drawn.

In our initial experiments, we chose to investigate the effects of obestatin(1–23) and two of its peptide fragments, obestatin(1–10) and obestatin(11–23). Both of these fragments have previously been identified and may occur in stomach extracts (Zhang *et al.*, 2005), suggesting that processing of the full-length obestatin(1–23) peptide occurs either during biosynthesis or upon secretion (Scrima *et al.*, 2007). As the obestatin(11–23) fragment had been previously reported to reduce both food intake and body weight in mice (Green *et al.*, 2007; Nagaraj *et al.*, 2008), suggesting that the bioactive region of obestatin may reside in this region, we surmised that this may also apply to its vascular actions. Indeed, in the present study, we found that relaxations in response to obestatin(11–23) were significantly reduced as compared with obestatin(1–23), although they were comparable with those observed in response to obestatin(1–10). Our data are therefore inconclusive as to the precise nature of the interaction between obestatin and its receptor, although they indicate that the intact peptide may be required in order to elicit a maximal relaxation response. In this regard, the identity of the cognate receptor for obestatin remains to be determined, and this has proved to be a controversial issue. GPR39 was originally put forward as a strong

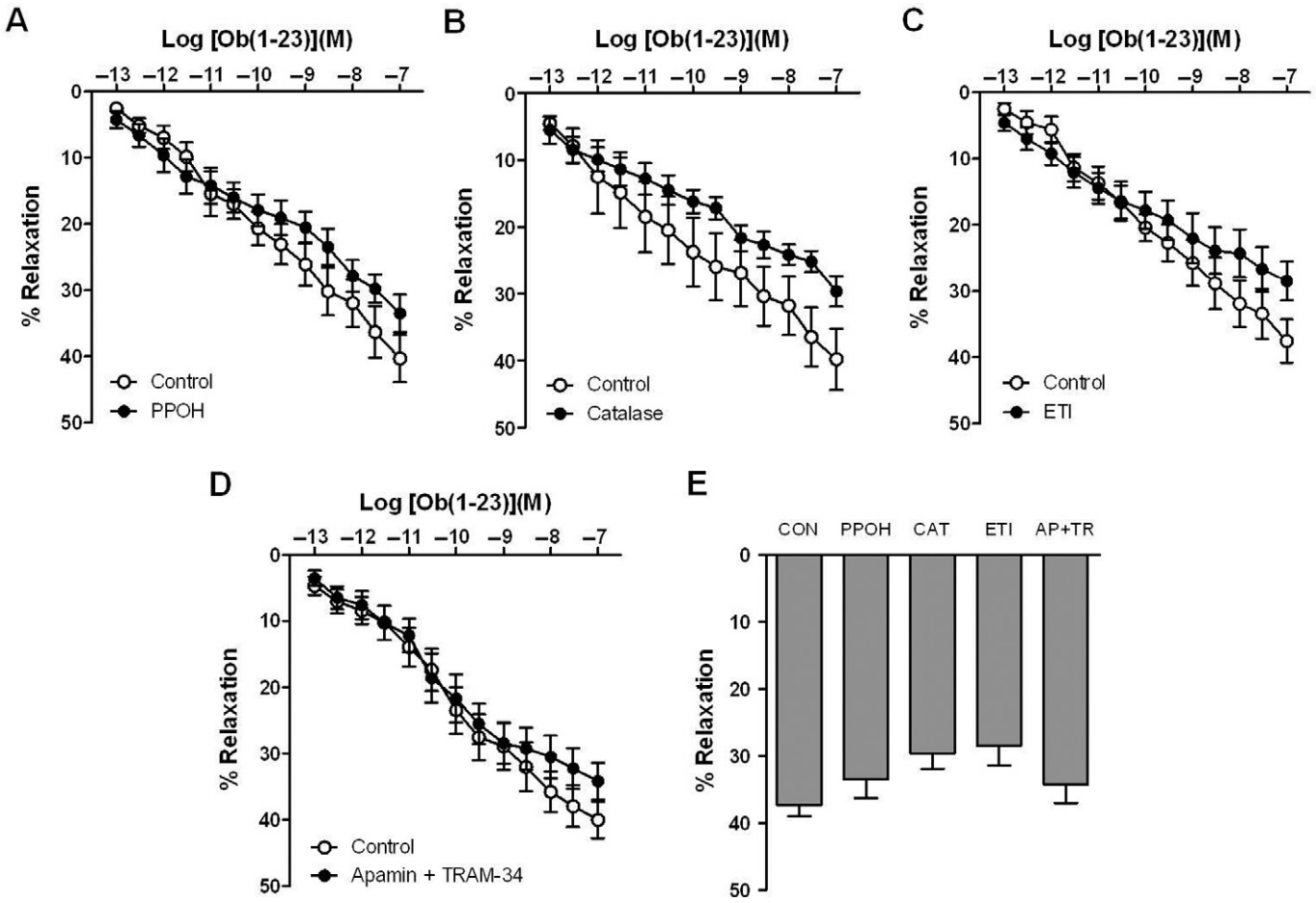

Figure 6

Investigation of potential role of endothelium-dependent hyperpolarization in obestatin-induced vascular relaxation. Cumulative relaxation responses to obestatin(1–23) in the presence or absence of (A) PPOH (0.1 μmol·L^{-1}), (B) catalase (CAT, 1250 U·mL^{-1}), (C) ETI (10 μmol·L^{-1}), (D) both apamin (AP, 0.1 μmol·L^{-1}) and TRAM-34 (TR, 10 μmol·L^{-1}). (E) Histogram comparing maximal relaxations between groups. Results are expressed as a percentage of the initial PE-induced contraction. Each data point or column represents the mean ± SEM for ≥7 experiments.

candidate on the basis of plasma membrane [^{125}I]-obestatin binding studies (Zhang *et al.*, 2005); however, the assay used by these investigators was subsequently shown to be unreliable (Holst *et al.*, 2007), and their findings could not be corroborated (Chartrel *et al.*, 2007). Further studies have also cast considerable doubt on the original claim that obestatin was a ligand for GPR39. Gene-modified mice lacking GPR39 were found to exhibit similar body weight and feeding behaviour compared with wild-type controls (Scrima *et al.*, 2007). In addition, Lauwers *et al.* (2006) failed to demonstrate activation of GPR39 by obestatin in HEK293 cells via either cAMP or PLC signal transduction pathways, which are both known to be linked to this GPCR. Nonetheless, our experiments using GDP-β-S and MDL-12,330A presented here strongly indicate that, at least in the endothelium, obestatin exerts its actions via binding to a heterotrimeric GPCR, which links to and activates adenylate cyclase, mostly likely through the $G_{\alpha s}$ subunit. Indeed, this suggestion is supported by previous work showing that obestatin increases intracellular cAMP production in human pancreatic beta cells (Granata *et al.*, 2008). However, it should be noted that in other cell types such as human retinal pigment epithelium, rat tumour growth cells and cardiac muscle, obestatin has been linked to G_i activation (Camina *et al.*, 2007; Pazos *et al.*, 2009; Sazdova *et al.*, 2009), indicating potential tissue-specific differences in GPCR signalling, which could underlie the diverse physiological actions of obestatin.

Our mechanistic experiments targeting established mediators of vascular relaxation in rat aorta (Grieve *et al.*, 1998; Feletou and Vanhoutte, 2009) clearly demonstrate that obestatin-induced relaxation is endothelium-dependent, involves production of NO (but not COX) and may be mediated via membrane hyperpolarization. Complementary experiments conducted in cultured BAEC confirmed that NO production is markedly increased in response to obestatin. Further organ bath studies using the selective CaMKII inhibitor, KN-93, together with BAEC Ca^{2+} fluorescence measurements suggested that eNOS activation may occur in a Ca^{2+}-dependent manner. Wortmannin was also demonstrated to significantly inhibit obestatin-induced relaxation, indicating that obestatin signalling involves PI3K/PKB, an established regulator of eNOS activation (Dimmeler *et al.*, 1999),

findings that were supported by our Western blotting data for pPKB. Indeed, it is well established that obestatin induces PI3K/PKB signalling in other settings. For example, obestatin has previously been shown to stimulate PI3K/PKB phosphorylation in human pancreatic beta cells and in rat cardiomyocytes and adipocytes (Granata *et al.*, 2008; Alloatti *et al.*, 2010; Gurriaran-Rodriguez *et al.*, 2010). Furthermore, it has been reported that obestatin stimulates the proliferation of human retinal pigment epithelial and gastric cancer cells via a PI3K/PKB-dependent mechanism (Camina *et al.*, 2007; Pazos *et al.*, 2009), supporting the involvement of this signal transduction pathway in obestatin-induced vascular relaxation. Relaxations mediated by NO are generally associated with stimulation of the cytosolic soluble guanylate cyclase in the vascular smooth muscle and the subsequent cGMP-dependent activation of PKG (Moncada *et al.*, 1991), and the involvement of this pathway in obestatin-induced relaxation was confirmed by our experiments with ODQ. PKG is then thought to mediate smooth muscle relaxation through several actions on Ca^{2+} signalling, which either lower cytosolic Ca^{2+} (e.g. increased uptake by the sarcoplasmic reticulum, inactivation of plasma membrane voltage-gated Ca^{2+} channels) or desensitize the contractile apparatus (e.g. stimulation of myosin light chain phosphatase, inhibition of Rho kinase) (Lincoln *et al.*, 2001). In this regard, our finding that relaxations to obestatin were inhibited by the selective BK_{Ca} blocker, iberiotoxin, indicates that activation of BK_{Ca}, which are preferentially expressed on smooth muscle (Feletou and Vanhoutte, 2009) and are thought to modulate activity of voltage-gated Ca^{2+} channels, may mediate relaxation in this setting (Lincoln *et al.*, 2001). However, it is important to note that a role for desensitization of the contractile apparatus cannot be ruled out on the basis of our data; indeed, this may explain the incomplete blockade of obestatin-induced relaxations by iberiotoxin (Figure 4E).

Further to our finding that vascular relaxation to obestatin was attenuated by high extracellular K^+, we chose to investigate the potential involvement of an endothelium-dependent hyperpolarization component by performing a series of studies in the presence of several inhibitors of the main putative EDHFs (Feletou and Vanhoutte, 2009). Obestatin-induced relaxations were unaffected by either (i) IDM, an inhibitor of COX, which is thought to cause hyperpolarization via its major metabolite, prostacyclin; (ii) PPOH, an inhibitor of cytochrome P450 mono-oxygenases, which cause hyperpolarization via the formation of epoxyeicosatrienoic acids; (iii) catalase, a scavenger of hydrogen peroxide, which hyperpolarizes vascular smooth muscle; and (iv) ETI, an inhibitor of lipooxygenase, which has been shown to evoke vascular smooth muscle relaxation via activation of BK_{Ca}. Furthermore, relaxations to obestatin were unaltered by inhibition of both SK_{Ca} and IK_{Ca} (using a combination of apamin and TRAM-34), which are largely localized to the endothelium and mediate EDHF-dependent responses (Feletou and Vanhoutte, 2009), further indicating that endothelium-dependent hyperpolarization does not appear to play a significant role in this setting.

In conclusion, the major novel finding of this study is that obestatin exerts significant vasorelaxant effects via activation of a specific endothelial NO signalling cascade (which is summarized in Figure 7). This may not only have relevance to normal regulation of blood pressure but is likely to also extend to type 2 diabetes, a condition characterized by reduced endothelial NO production and the frequent development of macrovascular and microvascular complications. Taken together with the emerging anti-obesogenic and anti-diabetic actions of this endogenous peptide, this presents obestatin as an attractive therapeutic option worthy of further investigation.

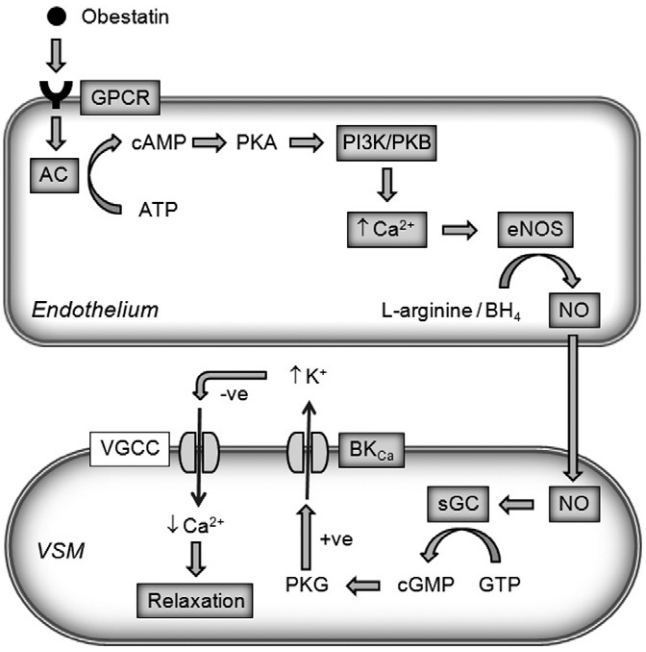

Figure 7

Schematic diagram of proposed signalling pathways mediating obestatin-induced vascular relaxation. Our data suggest that obestatin binds to an adenylate cyclase (AC)-linked GPCR, thereby promoting PI3K/PKB-, Ca^{2+}-dependent eNOS activation. NO released from the endothelium then induces soluble guanylate cyclase (sGC) in the vascular smooth muscle (VSM) and PKG-dependent activation of BK_{Ca}, leading to K^+ efflux. The resultant inhibition of voltage-gated Ca^{2+} channels (VGCC) is then likely to decrease intracellular Ca^{2+} concentration, causing vascular relaxation. Shaded boxes signify signalling components for which we present good experimental evidence for their involvement in obestatin-induced relaxation. BH_4, tetrahydrobiopterin.

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Conflict of interests

None.

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