Analysis of the ghrelin receptor-independent vascular actions of ulimorelin

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ABSTRACT

Ulimorelin (TZP101) is a ghrelin receptor agonist that stimulates intestinal motility, but also reduces blood pressure in rodents and humans and dilates blood vessels. It has been proposed as a treatment for intestinal motility disorders. Here we investigated the mechanisms through which ulimorelin affects vascular diameter. Actions of ulimorelin on wall tension of rodent arteries were investigated and compared with other ghrelin receptor agonists.

Saphenous, mesenteric and basilar arteries were obtained from Sprague-Dawley rats (male, 8 weeks) and saphenous arteries were obtained from wild type or ghrelin receptor null mice. These were mounted in myography chambers to record artery wall tension.

Ulimorelin (0.03-30 μ M) inhibited phenylephrine-induced contractions of rat saphenous (IC_{50} =0.6 μ M; I_{max} =66 $\pm$ 5%; n=3-6) and mesenteric arteries (IC_{50} =5 μ M, I_{max} =113 $\pm$ 16%; n=3-4), but not those contracted by U46619, ET-1 or 60 mM $[K^+]$. Relaxation of phenylephrine-constricted arteries was not observed with ghrelin receptor agonists TZP102, capromorelin or AZP-531. In rat saphenous and basilar arteries, ulimorelin (10-100 μ M) and TZP102 (10-100 μ M) constricted arteries (EC_{50} =9.9 μ M; E_{max} =50 $\pm$ 7% and EC_{50} =8 μ M; E_{max} =99 $\pm$ 16% respectively), an effect not attenuated by the ghrelin receptor antagonist YIL 781 3 μ M or mimicked by capromorelin or AZP-531. In mesenteric arteries, ulimorelin, 1-10 μ M, caused a surmountable rightward shift in the response to phenylephrine (0.01-1000 μ M; pA_2 =5.7; n=3-4). Ulimorelin had similar actions in mouse saphenous artery from both wild type and ghrelin receptor null mice.

We conclude that ulimorelin causes vasorelaxation through competitive antagonist action at α_1 -adrenoceptors and a constrictor action not mediated via the ghrelin receptor.

KEY WORDS

Ulimorelin, ghrelin, myography, vasodilatation, peripheral arteries

1. INTRODUCTION

The peptide hormone, ghrelin, has major roles in the control of appetite, growth hormone release and metabolic functions (Kojima and Kangawa 2005; Kojima and Kangawa 2010; Delhanty and van der Lely 2011). It reduces blood pressure in human and animals when administered intravenously (Nagaya et al. 2001; Okumura et al. 2002) and reduces sympathetic nerve activity when administered into the lower brain stem of rats and rabbits (Matsumura et al. 2002; Lin et al.

2004). Ghrelin has no direct vasodilator action on vessels from rat and the ghrelin receptor, growth hormone secretagogue receptor 1a (GHSR1a), is not expressed in rat vessels (Callaghan et al. 2012).

Ulimorelin, also known as TPZ101, is a macrocyclic molecule that is a potent agonist of the ghrelin receptor (Hoveyda et al. 2011). Due to its ability to stimulate gastrointestinal motility, ulimorelin has been evaluated as a possible treatment in gastroparesis (Ejskjaer et al. 2010), ileus (Fraser et al. 2009) and constipation (Pustovit et al. 2014).

We previously showed, in rats, that ulimorelin caused a biphasic reduction in blood pressure with an initial rapid decrease (resistant to ghrelin receptor antagonists), followed by a slower decrease (Callaghan et al. 2014). Ulimorelin also relaxed rat mesenteric arteries precontracted with phenylephrine, an action not blocked by ghrelin receptor antagonists. Whether ulimorelin relaxes vessels constricted with other agents, or affects arteries in other vascular beds was not investigated, and mechanisms of action were also not investigated. In the current study, we investigated the mechanism(s) by which ulimorelin mediates its effects on vascular constriction, and investigated arteries supplying the viscera, limbs and central nervous system.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats, mice with knockout of the gene for the ghrelin receptor, and wild-type C57BL6 mice were used. The knockout was created by inserting a transcriptional blocking cassette into intron 1 of GHSR1a in C57BL6 mice, creating a GHSR1a-null allele (Zigman et al. 2005). All animal procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee. The procedures abided by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

25 adult male Sprague-Dawley rats (300 – 400 g) and 8 adult mice (30 – 40 g) were killed by increasing atmospheric CO₂, followed by exsanguination. The saphenous artery was dissected from its origin at the femoral artery to a point just above the knee joint. Second/third order mesenteric arteries supplying the distal 15 cm of ileum were isolated from the mesentery. The whole of the basilar artery was isolated. After dissection, all vessels were maintained in physiological saline of the following composition (in mM): Na⁺, 150.6; K⁺, 4.7; Ca²⁺, 2; Mg²⁺, 1.2; Cl⁻, 144.1; H₂PO₄²⁻, 1.3; HCO₃⁻, 16.3; glucose, 7.8. This solution was gassed with 95% O₂/5% CO₂.

The arteries were cut into 1.2 – 2 mm length sections, and mounted isometrically onto stainless steel wires. The rat saphenous arteries were mounted onto 50 µm diameter wires, whereas the rat mesenteric, basilar, and mouse saphenous arteries were mounted onto 40 µm diameter wires. The basal conditions were normalized by gradually stretching the vessel in small steps until the effective transmural pressure calculated using Laplace's equation [transmural pressure = wall

tension/(internal circumference/ 2π), where wall tension = force/ $2 \times$ vessel segment length] was 13.3 mN/mm² (100 mmHg; see Mulvany and Halpern, 1977). The rat and mouse saphenous arteries were suspended at an effective transmural pressure of 100 mmHg (Rummery et al. 2010). The rat mesenteric and basilar arteries were suspended at 90% of lumen circumference measured at an effective transmural pressure 100 mmHg (see Mulvany and Halpern, 1977). Under these conditions the vessels are mounted at close to the peak of their length-tension relationship. The baths were filled with 6 ml of physiological saline that was continuously bubbled with 95% O₂/5% CO₂ and heated to ~36.5 C. Following mounting, the arteries were allowed to recover for 30 min.

At the start of all experiments, the tissues were stimulated with three applications of phenylephrine (3 or 10 μ M) to confirm viability and when the contraction to the third application of phenylephrine had plateaued, carbachol (1 μ M) was applied to determine if the endothelium was intact. Different tissues were used to assess the effects of each concentration of ghrelin receptor agonist on contractions induced by phenylephrine, methoxamine, U46619, ET-1 and 60 mM [K⁺]. The 60 mM [K⁺] experiments were performed in the presence of the α -adrenoceptor agonists prazosin 100 nM and idazoxan 1 μ M to prevent the actions of noradrenaline released from the perivascular sympathetic nerve terminals. The test agonists (ghrelin, desacyl ghrelin, ulimorelin, capromorelin, or AZP-351) were applied and left in contact with the tissue for at least 5 min or until the relaxation produced had plateaued. When applied on their own, the concentration of ghrelin receptor agonists was increased cumulatively, with at least 8 min between each increase in concentration. For cumulative concentration curves to phenylephrine, at least 4 min was allowed to elapse between each addition of phenylephrine. When the effects of receptor antagonists, L-NAME or ion channel blockers were investigated, they were applied at least 30 min prior to application of ulimorelin.

Data were collected using a Powerlab and Chart 5 software (ADInstruments, Bella Vista, NSW, Australia) and were analysed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Responses to ghrelin receptor agonists in phenylephrine, methoxamine, endothelin-1, U46619 and 60 mM [K⁺] contracted vessels were measured as the % change in active wall tension from that immediately prior to their addition. Contractions to ghrelin receptor agonists were measured as a % of the peak force of contraction to phenylephrine (3 μ M for saphenous, 10 μ M for mesenteric artery) or 60 mM [K⁺] (in basilar artery). Both cumulative and non-cumulative curves were fitted using three parameter models. Data are reported as mean $\pm$ S.E.M or, for data generated by the three parameter models, the best-fit parameter determined by nonlinear regression and the standard error is presented; n is the number of animals. For the Schild plot a linear regression was performed using the EC₅₀ values obtained from the phenylephrine concentration response curves. 1- or 2- way

ANOVAs, followed by Dunnett's post-tests were used where indicated. $P < 0.05$ was considered significant.

The following compounds were used: ulimorelin and TZP102 from Tranzyme Pharma; capromorelin (CP424391) from Pfizer Pharmaceuticals, Sandwich, UK and synthesised by us; carbamylcholine chloride (carbachol), phenylephrine hydrochloride, methoxamine hydrochloride, propranolol hydrochloride, prazosin, idazoxan, atropine, glibenclamide, nifedipine, L-NAME, and ET-1 (from Sigma Aldrich, Sydney, Australia); rat ghrelin (Auspep, Melbourne, Victoria, Australia); rat desacyl ghrelin (GL Biochem, China), AZP-531 (ChinaPeptides, Shanghai, China); YIL 781 and U46619 (Tocris, Bristol, UK). Stock solutions were prepared as follows; ghrelin, desacyl ghrelin, AZP-531 (all 1 mM pH 5.6 acetic acid buffer solution), U46619 (100 μ M 70% ethanol), glibenclamide (10 mM in DMSO), nifedipine (1 mM 70% ethanol), YIL 781 (10 mM 10% DMSO). All other drugs used were prepared in dH₂O. The carrier solvents, acetic acid buffer, DMSO and ethanol, added alone in the same amounts as in experiments with drugs, had no effects on arterial wall tension in the vessels studied.

3. RESULTS

3.1 Rat saphenous artery

In rat saphenous artery, ulimorelin (100 nM – 30 μ M) caused a concentration-dependent inhibition of vasoconstriction elicited by the α_1 -adrenoceptor agonist phenylephrine (3 μ M; $pIC_{50} = 6.2 \pm 0.2$; $I_{max} = 66 \pm 5\%$; $n = 3 - 6$; Figure 1). This effect was not mimicked by other ghrelin receptor agonists investigated, including ghrelin (1 nM – 1 μ M; $n = 3 - 4$), desacyl ghrelin (1 nM – 1 μ M; $n = 3 - 4$), TZP102 (10 nM – 30 μ M; $n = 3 - 4$), capromorelin (10 nM – 10 μ M), and AZP-531 (1 nM – 1 μ M; $n = 3 - 4$; Table 1) or the vehicles used (data not shown). Table 2 shows that the inhibition of constriction caused by ulimorelin (1 – 10 μ M) was not reduced by the ghrelin receptor antagonist YIL 781 (3 μ M, $n = 4$), the nitric oxide synthase inhibitor L-NAME (100 μ M; $n = 4$), or the β -adrenoceptor antagonist propranolol (1 μ M; $n = 4$; table 2). The muscarinic acetylcholine receptor antagonist atropine (1 μ M) caused a significant attenuation of the response to ulimorelin (10 μ M; $n = 4$; $P < 0.01$; Table 2). The L-type calcium channel blocker nifedipine (1 μ M; $n = 4$) and the K_{ATP} channel blocker glibenclamide (10 μ M; $n = 4$) did not reduce the effects of ulimorelin (Table 2).

Ulimorelin (1 – 10 μ M) also inhibited responses to the α_1 -adrenoceptor agonist methoxamine (3 μ M) by $42 \pm 4\%$ ($P > 0.05$) but did not inhibit contractions to the thromboxane A₂ agonist U46619 (100 nM, $n = 4$) or endothelin 1 (ET-1; 100 nM, $n = 3 - 4$, Table 3). The slow onset of the plateau phase of contraction to ET-1 is likely to be responsible for the apparent increase in artery tension following application of ulimorelin. In addition, ulimorelin did not reduce contractions to 60 mM

[K⁺] (Table 3). Ghrelin (1 μ M) and desacyl ghrelin (1 μ M) also had no effect on contractions to ET-1 or U46619 (both 100 nM; n = 3; Table 3).

At higher concentrations (10 – 100 μ M), ulimorelin on its own caused an increase in artery tension ($pEC_{50} = 5.0 \pm 0.2$; $E_{max} = 50 \pm 7\%$ n = 4; Figure 2), an effect that was attenuated by nifedipine (1 μ M; $P < 0.001$ at 30 and 100 μ M ulimorelin; n = 3). TZP102 (10 – 100 μ M) also caused an increase in artery tension (estimated $pEC_{50} = 4.4$; n = 4). In contrast, ghrelin (1 nM – 1 μ M; n = 3), desacyl ghrelin (1 nM – 1 μ M; n = 3), capromorelin (10 nM – 10 μ M; n = 3), and AZP-531 (1 nM – 1 μ M; n = 3; Table 4), or the vehicles used (data not shown), did not produce this effect (Table 4). The contraction to a single application of a relatively high concentration of ulimorelin (30 μ M; $19 \pm 1\%$ of the contraction to 3 μ M phenylephrine; n = 3) was not significantly affected by pre-incubation with the α_1 -adrenoceptor antagonist prazosin (100 nM; $46 \pm 12\%$ n = 3; $P > 0.05$) or YIL 781 (3 μ M; $29 \pm 9\%$ n = 3; $P > 0.05$).

3.2 Rat mesenteric and basilar artery

In the rat mesenteric arteries, ulimorelin (100 nM – 30 μ M) also caused concentration-dependent relaxation of phenylephrine (10 μ M)-induced contractions ($pIC_{50} = 5.3 \pm 0.2$, $I_{max} = 113 \pm 16\%$; n = 3 – 4; Figure 1), but did not cause constriction on its own (100 nM – 100 μ M; n = 3). This allowed the construction of cumulative concentration response curves to phenylephrine in the presence of ulimorelin (100 nM – 10 μ M; n = 3 – 4; Figure 3). Ulimorelin caused a surmountable, concentration dependent shift in the concentration response curve to phenylephrine with a pA_2 value of 5.7 (slope of Schild plot = -0.7 ± 0.2 ; Figure 3).

Rat basilar arteries did not respond to phenylephrine (3 μ M; n = 4), as previously reported for noradrenaline (Chang et al. 1988). Ulimorelin (10 μ M; n = 3) caused a small, but consistent inhibition of 60 mM [K⁺] stimulated contractions (by $24 \pm 12\%$; n = 3; $P > 0.05$), and on its own increased in basilar artery tension ($pEC_{50} = 5.1 \pm 0.3$; $E_{max} = 99 \pm 16$ of 60 mM [K⁺]-induced contraction; n = 3; Figure 2).

3.3 Saphenous artery from wild-type and ghrelin receptor null mice

In the saphenous artery from mice lacking the ghrelin receptor, and wild type littermates, ulimorelin had similar effects to those observed in rat saphenous artery. Ulimorelin (1 – 10 μ M) relaxed phenylephrine (3 μ M) contracted vessels (by $61 \pm 17\%$ and $51 \pm 16\%$ respectively at 10 μ M; $P > 0.05$; n = 3). On its own, ulimorelin increased saphenous artery tension (in wild type $pEC_{50} = 4.7 \pm 0.3$; $E_{max} = 15 \pm 3\%$ of contraction to phenylephrine 3 μ M; n = 3; in mice lacking the

ghrelin receptor $pEC_{50} = 4.8 \pm 0.4 \mu M$; $E_{max} = 23 \pm 6\%$ of contraction to phenylephrine $3 \mu M$; $n = 3$; $P > 0.05$; Figure 4).

4. DISCUSSION

In a previous study of ulimorelin's effects, a vasodilator effect on arteries precontracted with phenylephrine was reported (Callaghan et al. 2014). Ghrelin receptor antagonists, JMV2959 and YIL 781 (Esler et al. 2007), did not inhibit this effect and ghrelin receptor expression was not detected in the arteries. In the current work, we observed that ulimorelin caused vasodilatation in saphenous arteries from rats, as well as wild type and *Ghsr* null mice, which supports that conclusion that vasodilatation is mediated through a receptor different to the ghrelin receptor. Two classes of ghrelin receptors, other than GHSR1a, have been pharmacologically identified at which ghrelin and compounds related to ghrelin act, the ghrelin receptor-like receptors (GRLRs), where ghrelin and desacyl ghrelin are both active, and the unacylated ghrelin (UAG) receptors at which desacyl ghrelin but not ghrelin is an effective agonist (Callaghan and Furness, 2014). The sites identified in the present study do not match GRLRs or UAG receptors as neither ghrelin nor desacyl ghrelin have a vasodilator action in the arteries investigated.

The potencies of ulimorelin at mediating the effects described in this study were quite different from those at the ghrelin receptors. At rat and human ghrelin receptors, ulimorelin has an EC_{50} in the range of 1-2 nM (Hoveyda et al. 2011; Callaghan et al. 2014). For relaxation of precontracted arteries, the EC_{50} of ulimorelin was 0.5 - 5 μM and for contraction the EC_{50} was 10 - 30 μM , providing further evidence that these effects are not mediated by the ghrelin receptor. The concentrations of ulimorelin we studied are within the range achieved *in vivo* following infusion of 160 - 600 $\mu g/kg$ ulimorelin which produced peak plasma concentrations in the range 6 - 14 μM (Lasseter et al. 2008). However, the approximately 1000-fold lower potency at the off-target sites identified in the current study suggests that they can be avoided in therapeutic applications of ulimorelin targeting the ghrelin receptor.

The experiments indicate that a major component of the vasodilatation is due to antagonism of α_1 -adrenoceptors. Evidence for α_1 -adrenoceptor antagonism is that vasodilatation was observed in arteries precontracted with α_1 -adrenoceptor agonists, phenylephrine or methoxamine, but not in vessels precontracted with endothelin-1 or the thromboxane A_2 agonist U46619. Moreover, in rat mesenteric artery, ulimorelin shifted the concentration-response curve for phenylephrine to the right with a pA_2 of 5.7. Performing these studies in mice lacking α_1 -adrenoceptors may assist in confirming these conclusions.

A number of off-target effects for other ghrelin receptor ligands at known non-ghrelin receptors have been identified, including β_3 -adrenoceptor agonist activity by (4-[(aminocarbonyl)amino]-N-[4-(2-aminoethyl)phenyl]benzenesulfonamides (Pasternak et al. 2009) and muscarinic receptor agonist activity by spiro-azetidine–piperidine analogues (McClure et al. 2013). In our study, propranolol did not reduce the vasodilator action of ulimorelin, excluding a role for β_3 -adrenoceptors. However, atropine did reduce the vasodilator action of ulimorelin, suggesting it may in part be mediated via endothelial muscarinic receptors. Previously it has been reported that in rat mesenteric artery endothelial denudation reduced but did not abolish vasodilation to ulimorelin (Callaghan et al. 2014).

In addition to its vasodilator effect, relatively high concentrations of ulimorelin caused constriction of rat and mouse saphenous and rat basilar artery. Constriction was observed in saphenous artery segments from *Ghsr* null mice suggesting this effect was not mediated by the ghrelin receptor. Similar concentrations of TZP102 also caused constriction of rat saphenous artery, but other ghrelin receptor agonists investigated had no constrictor effect in this vessel. This suggests that the macrocyclic molecules, ulimorelin and TZP102, may possess a structurally related molecular motif that is responsible for their contractile effects and that is absent in the other ghrelin receptor agonists investigated. Comprehensive structure-activity relationship investigations will be required to identify the particular motif responsible.

The contractile effects of ulimorelin observed in the rat saphenous and basilar arteries were not observed in rat mesenteric arteries. These observations suggest the expression of a common molecular entity (possibly an ion channel or receptor) mediating the contractile effects of ulimorelin in the saphenous and basilar arteries that is not expressed in the mesenteric arteries. Determining the particular entity mediating this effect is beyond the scope of this investigation.

The basilar artery was contracted using 60 mM $[K^+]$ as there was no contraction to phenylephrine. This absence of α -adrenoceptor-mediated contractions in rat basilar arteries has been previously reported (Chang et al. 1988). Only a small vasodilation of the basilar artery contracted with 60 mM $[K^+]$ was observed following application of ulimorelin, making this vessel an ideal place to investigate the vasoconstriction. The reduced vasodilation to ulimorelin observed in this artery adds further evidence to support the α -adrenoceptor antagonist effect of ulimorelin observed in both the saphenous and mesenteric arteries.

5. CONCLUSION

Here, for the first time, the ghrelin receptor agonist ulimorelin has been shown to have two distinct, regionally selective, non-ghrelin receptor mediated actions on arteries in rodents, namely a

reduction in α_1 -adrenoceptor-mediated constriction and, at a higher concentrations, a vasoconstrictor action. The vasodilator mechanisms are unknown, however the evidence presented suggests inhibition of α_1 -adrenoceptors, and activation of muscarinic acetylcholine receptors, mediates this action of ulimorelin. The mechanism of constriction is currently unknown although opening of L-type calcium channels appears to be required. As the concentrations of ulimorelin producing these non-ghrelin receptor-mediated effects may be achieved *in vivo*, this may aid interpretation of studies of the effects of ulimorelin on blood pressure.

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FIGURE LEGENDS

Fig. 1 Ulimorelin causes a decrease in saphenous and mesenteric artery tension following stimulation with phenylephrine. Panels A and B display representative traces of the inhibition of phenylephrine-induced contractions of rat (A) saphenous and (B) mesenteric arteries by ulimorelin. Panels C and D display non-cumulative concentration response curves showing the inhibition of phenylephrine-induced contractions in (C) saphenous and (D) mesenteric arteries.

Fig. 2 Ulimorelin contracts rat saphenous and basilar arteries. Panels A and B display representative traces of ulimorelin-induced contractions of rat (A) saphenous and (B) basilar arteries. Arrowheads indicate times and concentrations of ulimorelin added. Panels C and D display cumulative concentration contraction curves for ulimorelin in (C) saphenous and (D) basilar arteries.

Fig. 3 Ulimorelin alters the concentration-response relationship for phenylephrine in rat mesenteric arteries. Panel A displays cumulative concentration contraction curves for phenylephrine in the presence of 0, 100 nM, 1 μ M, and 10 μ M ulimorelin. A surmountable shift in the concentration contraction curve for phenylephrine was observed in the presence of 10 μ M ulimorelin. Panel B shows a Schild plot of these data with a pA_2 of 5.7 for ulimorelin.

Fig. 4 Ulimorelin is equally efficacious in saphenous arteries taken from wild type mice and mice lacking the ghrelin receptor. The histogram in panel A shows there is no difference in the magnitude of the inhibition of phenylephrine induced responses by 1 – 10 μ M ulimorelin in either genotype (2-way ANOVA). Panel B displays cumulative concentration contraction curves for ulimorelin, showing there is no difference in these responses between mice lacking the ghrelin receptor and wild-type mice.

Table 1. The effect of ulimorelin, TZP102, capromorelin, ghrelin, desacyl ghrelin, and AZP-531 on phenylephrine-constricted rat saphenous arteries

Drug (concentration)	% Δ of phenylephrine response (n)	<i>P</i> value (compared to ulimorelin 30 μ M)
Ulimorelin (10 nM – 30 μ M)	-66 $\pm$ 5% (3-6)	NA
TZP102 (100 nM – 30 μ M)	-2 $\pm$ 4% (3-4)	< 0.001
Capromorelin (100 nM – 10 μ M)	1 $\pm$ 9% (3-4)	< 0.001
Ghrelin (1 nM – 1 μ M)	0 $\pm$ 3% (3-4)	< 0.001
Desacyl Ghrelin (1 nM – 1 μ M)	-1 $\pm$ 5% (3-4)	< 0.001
AZP-531 (1 nM – 1 μ M)	-10 $\pm$ 6% (3-4)	< 0.001

Data are expressed as % phenylephrine contraction. For ulimorelin $E_{\max} \pm$ standard error (best-fit parameters determined by nonlinear regression) is reported; for others mean $\pm$ S.E.M. is reported. Data were analysed using 1-way ANOVA followed by Dunnett's post-tests.

Table 2. Effects of ghrelin receptor antagonists, and other inhibitors and antagonists, on ulimorelin-induced inhibition of phenylephrine contractions in rat saphenous arteries

Antagonist pre-incubation (concentration)	% Δ of phenylephrine response by ulimorelin 10 μ M (n)	<i>P</i> value	% Δ of phenylephrine response by ulimorelin 1 μ M (n)	<i>P</i> value
None	-63 $\pm$ 5 (5)	NA	-40 $\pm$ 6 (6)	NA
YIL 781 (3 μ M)	-48 $\pm$ 8 (4)	<i>P</i> > 0.05	-32 $\pm$ 9 (4)	<i>P</i> > 0.05
Nifedipine (1 μ M)	-58 $\pm$ 2 (4)	<i>P</i> > 0.05	-44 $\pm$ 6 (4)	<i>P</i> > 0.05
Glibenclamide (10 μ M)	-66 $\pm$ 6 (4)	<i>P</i> > 0.05	-31 $\pm$ 12 (4)	<i>P</i> > 0.05
L-NAME (100 μ M)	-43 $\pm$ 3 (4)	<i>P</i> > 0.05	-22 $\pm$ 11 (4)	<i>P</i> > 0.05
Atropine (1 μ M)	-30 $\pm$ 4 (4)	<i>P</i> < 0.01	-42 $\pm$ 13 (4)	<i>P</i> > 0.05
Propranolol (1 μ M)	-44 $\pm$ 12 (4)	<i>P</i> > 0.05	-28 $\pm$ 13 (4)	<i>P</i> > 0.05

Data were compared to the inhibition of phenylephrine-induced contractions by ulimorelin using 1-way ANOVA with Dunnett's post-tests. Note only pre-incubation of atropine 1 μ M caused a significant change in the magnitude of ulimorelin-induced inhibition of phenylephrine contractions.

Table 3. The % change in saphenous artery tension induced by ghrelin receptor agonists on contractions induced by phenylephrine, methoxamine, 60 mM [K⁺], U46619 and ET-1.

Stimulus	% Δ ulimorelin 10 μM (n)	% Δ ulimorelin 1 μM (n)	% Δ ghrelin 1 μM (n)	% Δ desacyl ghrelin 1 μM (n)
Phenylephrine (3 μM)	-63 ± 5 (5)	-41 ± 6 (6)	-1 ± 3 (4)	-2 ± 5 (4)
Methoxamine (3μM)	-42 ± 4 (3)	-24 ± 10 (3)	NA	NA
[K ⁺] (60 mM)	6 ± 3 (4) ^b	14 ± 3 (3) ^b	NA	NA
U46619 (100 nM)	-1 ± 6 (3) ^a	-2 ± 1 (3) ^a	-12 ± 15 (3)	0 ± 9 (3)
ET-1 (100 nM)	47 ± 40 (4) ^c	54 ± 8 (3) ^c	30 ± 6 (3) ^a	32 ± 10 (3) ^a

Data compared to the inhibition of phenylephrine-induced contractions using 1-way ANOVA with Dunnett’s post-test. a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$.

Table 4. The contractile effects of ulimorelin, TZP102, capromorelin, ghrelin, desacyl ghrelin, and AZP-531 on rat saphenous arteries

Drug (concentration)	Drug-induced contraction as % Δ of phenylephrine response (n)	<i>P</i> value compared to ulimorelin 30 μ M
Ulimorelin (10 nM – 100 μ M)	50 $\pm$ 7 (4)	NA
TZP102 (100 nM – 100 μ M)	68 $\pm$ 5 (4)	< 0.01
Capromorelin (100 nM – 100 μ M)	0 $\pm$ 1 (3)	< 0.001
Ghrelin (1 nM – 1 μ M)	-2 $\pm$ 0 (3)	< 0.001
Desacyl Ghrelin (1 nM – 1 μ M)	-2 $\pm$ 0 (3)	< 0.001
AZP-531 (1 nM – 1 μ M)	-2 $\pm$ 1 (3)	< 0.001

Data are expressed as % phenylephrine contraction. For ulimorelin and TZP102 $E_{\max} \pm$ standard error is reported; for others mean $\pm$ S.E.M. is reported. Data were analysed using 1-way ANOVA followed by Dunnett's post-tests.

Figure 1

A

B

Figure 2

C

D

Figure 3

A

Schild analysis

B

Figure 4

A

B

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