Relaxin induces rapid, transient vasodilation in the microcirculation of hamster skeletal muscle

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Abstract

Relaxin produces a sustained decrease in total peripheral resistance, but the effects of relaxin on skeletal muscle arterioles, an important contributor to systemic resistance, are unknown. Using the intact, blood-perfused hamster cremaster muscle preparation *in situ*, we tested the effects of relaxin on skeletal muscle arteriolar microvasculature by applying 10^{-10} M relaxin to second-, third- and fourth-order arterioles and capillaries. The mechanisms responsible for relaxin-induced dilations were explored by applying 10^{-10} M relaxin to second-order arterioles in the presence of 10^{-5} M N(G)-nitro-L-arginine methyl ester (L-NAME, nitric oxide (NO) synthase inhibitor), 10^{-5} M glibenclamide (GLIB, ATP-dependent potassium (K^+) channel inhibitor), 10^{-3} M tetraethylammonium (TEA) or 10^{-7} M iberiotoxin (IBTX, calcium-associated K^+ channel inhibitor). Relaxin caused second- (peak change in diameter: $8.3 \pm 1.7 \mu\text{m}$) and third ($4.5 \pm 1.1 \mu\text{m}$)-order arterioles to vasodilate transiently while fourth-order arterioles did not ($0.01 \pm 0.04 \mu\text{m}$). Relaxin-induced vasodilations were significantly inhibited by L-NAME, GLIB, TEA and IBTX. Relaxin stimulated capillaries to induce a vasodilation in upstream fourth-order arterioles ($2.1 \pm 0.3 \mu\text{m}$), indicating that relaxin can induce conducted responses vasodilation that travels through blood vessel walls via gap junctions. We confirmed gap junction involvement by showing that gap junction uncouplers (18- β -glycyrrhetic acid (40×10^{-6} M) or 0.07% halothane) inhibited upstream vasodilations to localised relaxin stimulation of second-order arterioles. Therefore, relaxin produces transient NO- and K^+ channel-dependent vasodilations in skeletal muscle arterioles and stimulates capillaries to initiate conducted responses. The transient nature of the arteriolar dilation brings into question the role of skeletal muscle vascular beds in generating the sustained systemic haemodynamic effects induced by relaxin.

Key Words

- relaxin
- vasodilation
- skeletal muscle
- arteriole
- capillary

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Introduction

Recently, interest has been generated concerning the effects of the peptide hormone relaxin in the cardiovascular system, as numerous clinical trials are proceeding using relaxin as a therapeutic to treat acute human heart failure (Teerlink *et al.* 2009, Metra *et al.* 2010, Teichman *et al.* 2010). In spite of the recent progression of clinical

trials, our understanding of the effects of relaxin in the cardiovascular system remains incomplete. Skeletal muscle resistance arterioles play a critical role in the regulation of systemic vascular resistance (Haddy *et al.* 1968, Folkow *et al.* 1984), and relaxin has been demonstrated to elicit vasodilation in renal (Danielson *et al.*

1999) and mesentery vascular beds (Bigazzi *et al.* 1986) and rapid relaxin-induced relaxation of isolated resistance arterioles (Fisher *et al.* 2002, McGuane *et al.* 2011). Therefore, given the important contribution of skeletal muscle resistance arterioles to systemic vascular resistance and the potential for relaxin to promote vasodilation, it is critical to examine the putative vasodilatory effects of relaxin in the skeletal muscle microvasculature in order to understand the systemic haemodynamic effects of relaxin.

In this study, we used an *in situ* model where blood vessels can be visualised in a live, intact preparation, the anaesthetised, blood-perfused hamster cremaster muscle preparation, to test the vasodilatory effects of relaxin on skeletal muscle arteriolar reactivity. The hamster cremaster muscle preparation is a common model for skeletal muscle microcirculation (for example, see Baez (1973), Gorczynski *et al.* (1978), Meininger (1987), Hester *et al.* (1993), Jackson (1993), Berg *et al.* (1997) and Armstrong *et al.* (2007)). This model is a good template for the study of skeletal muscle microcirculation because it is of mixed fibre types similar to human muscle (Sarelius *et al.* 1983), the muscle is thin and the entire intact arteriolar network can be directly visualised using intravital microscopy, drugs can be applied directly to the arteriole of interest without affecting the entire animal and the vasculature remains within its native tissue with all the neural, humoral and locally released tissue factor influences intact.

The aims of this study were threefold: i) the first was to determine the vasoactive actions of relaxin in the arteriolar microcirculation of skeletal muscle. Previous studies have shown that relaxin stimulates vasodilation through nitric oxide (NO)-mediated mechanisms (Bani-Sacchi *et al.* 1995, Danielson & Conrad 1995, Bani *et al.* 1998, Danielson *et al.* 1999, McGuane *et al.* 2011); therefore, we investigated this possibility in relaxin-induced responses in skeletal muscle arterioles. Furthermore, relaxin has been shown to alter smooth muscle function through the modulation of potassium channels (Anwer *et al.* 1993, Meera *et al.* 1995, Piedras-Renteria *et al.* 1997); thus, we investigated this possibility in skeletal muscle resistance arterioles. ii) The second was to determine whether different branch orders of the terminal microvasculature respond similarly to relaxin. It has been demonstrated that terminal arterioles are less influenced by NO than are larger arterioles (Hester *et al.* 1993); thus, relaxin's effect may not be equal among different branch orders of resistance arteriole. iii) The third was to determine whether capillaries can be stimulated by relaxin. Capillaries have been identified as a locus of

control of blood flow in skeletal muscle (Berg *et al.* 1997, Twynstra *et al.* 2012). Stimulation of capillaries can cause signals to be sent proximally through the blood vessel wall, via gap junctions, to the upstream arterioles controlling resistance and flow through the stimulated capillaries (for review see Segal (2005)). Communication of gap junction-dependent signals from local vascular sites to remote upstream vascular sites are known as conducted responses (Segal & Duling 1986, 1989). Capillary stimulation by relaxin will indicate whether relaxin can alter arteriolar diameter by inducing conducted responses.

Materials and methods

Animals

All surgical and experimental procedures were approved by the Animal Care Committee of the University of Guelph and were completed in accordance with the guidelines of the Canadian Council on Animal Care set out in the Guide to the Care and use of Experimental Animals. Adult male Golden Syrian Hamsters (100–130 g) were purchased from Charles River (Saint-Constant, QC, Canada). Animals had access to food and water *ad libitum* and were housed in a 14 h light:10 h darkness cycle.

General surgical procedures

Hamsters were initially anaesthetised with sodium pentobarbital (70 mg/kg i.p.) and tracheotomised. Polyethylene catheters were placed in the left femoral vein (for supplemental pentobarbital during surgery) and in the left femoral artery to monitor mean arterial pressure (to confirm a stable plane of anaesthesia). Hamsters were placed on a lucite platform fitted with water-filled heating coils to maintain an oesophageal temperature of 37 °C. The right cremaster muscle was prepared for *in situ* microscopy by procedures first described by Baez (1973) and modified by Murrant (2005). Briefly, the right testis was isolated and skin was cut longitudinally, exposing the cremaster muscle. The muscle and scrotum were separated and cleaned of connective tissue; the cremaster muscle was cut longitudinally, separated from the testis and epididymis, and spread over a semi-circular lucite platform. The periphery of the muscle was fixed to the platform by insect pins; care was taken not to stretch the muscle. The exposed muscle was constantly superfused with a bicarbonate-buffered physiological salt solution containing (mmol/l) NaCl 131.9, KCl 4.7, CaCl₂ 2.0, MgSO₄ 1.2, NaHCO₃ 30 (Fisher Scientific, Ottawa, ON,

Canada) and 0.3 mg/l tubarine. The solution was equilibrated with 5% CO₂–95% N₂ gas and final pH was 7.35–7.45. The drip rate of the superfusate was adjusted to maintain a cremaster muscle temperature of 34 °C. The preparation was then moved to the intravital microscope and allowed to equilibrate for 45–60 min before the start of the experimental protocols. During all experimental protocols, pentobarbital sodium (10 mg/ml saline; 0.56 ml/h; i.v.) was constantly administered via an anaesthetic pump.

The microvasculature of the cremaster muscle was visualised with an Olympus BX51WI microscope (Olympus Canada, Inc., Richmond Hill, ON, Canada) using a $\times 20$ long objective (n.a. 0.50) and a $\times 1.6$ magnification changer and transilluminated by a tungsten lamp. The resulting images were recorded using a video camera (DXC-390; Sony Canada Ltd., Toronto, ON, Canada) displayed on a monitor and recorded using a videotape recorder (Sony, SVO-9600 MD). Final magnification of the selected site was approximately $\times 2000$. Data were recorded on videocassettes (Sony, T-120PR) and analysed off-line using calibrated ImageJ software (public domain software, <http://rsb.info.nih.gov>, NIHMH, Bethesda, MD, USA).

Application of relaxin in experimental protocols

As relaxin-2 is the circulating and biologically significant form of relaxin in humans, we used recombinant human relaxin-2 in our studies. In humans, relaxin-2 acts in a similar fashion to relaxin-1 (the biologically active form in rodents). Furthermore, relaxin-3 appears to act primarily as a neuropeptide and there is no evidence that relaxin-3 circulates in the body; therefore, relaxin-3 was not studied (Bathgate *et al.* 2006).

Relaxin was applied to the cremaster preparation either globally over the whole preparation through the superfusate or to specific localised vascular sites using glass micropipettes. Glass tubes were pulled using a pipette puller (PC-10; Narishige International USA, Inc., East Meadow, NY, USA) and bevelled (BV-10; Sutter Instrument Co., Novato, CA, USA) (tip diameter $\sim 10\ \mu\text{m}$). Micropipettes were filled with pharmacological agents or relaxin ($10^{-10}\ \text{M}$) and FITC–dextran ($10^{-4}\ \text{M}$, FITC) as a marker for micropipette flow. Previous studies have confirmed that $10^{-4}\ \text{M}$ FITC is not vasoactive (Frame & Sarelius 1993). The micropipette tip was positioned close to the test arteriole using a micromanipulator. The micropipette was connected to a water manometer and water pressure was adjusted to cause drugs in the pipette to flow onto the test site (Frame & Sarelius 1993). Epifluorescence was used

Figure 1

The peak change in diameter of the TA in response to incremental doses of relaxin. Two dose response experiments were conducted: i) 10^{-10} to $10^{-5}\ \text{M}$ ($n=5$) and ii) 10^{-13} to $10^{-8}\ \text{M}$ ($n=6$). Data from both series of dose response experiments were combined to produce a dose-response curve (total 11 animals; i.e. 10^{-13} to $10^{-11}\ \text{M}$ ($n=6$), 10^{-10} to $10^{-8}\ \text{M}$ ($n=11$), 10^{-7} to $10^{-5}\ \text{M}$ ($n=5$)). *Significantly different values from baseline.

to confirm flow from the micropipette and that flow from the micropipette was approximately perpendicular to the arteriole under investigation and that no other parts of the vasculature under investigation were being exposed. The region of the arteriole that was exposed during micropipette application was $\sim 200\ \mu\text{m}$. Figure 2A shows a schematic representation of the terminal arteriolar microvasculature architecture under study, whereby blood flows through a subsequently diverging network of arterioles from the paired feed arterioles through the transverse (TA), Branch and module inflow arterioles (MI) until it reaches the capillary module of the network.

Experimental protocol 1: relaxin dose response

To determine the reactivity of the microvasculature to relaxin, we performed an initial dose response of 10^{-10} to $10^{-5}\ \text{M}$ ($n=5$) relaxin (from lowest to highest concentration), globally applied to the entire cremaster preparation while observing a TA. Relaxin induced vasodilation at the lowest concentration tested; therefore, a second protocol was performed using 10^{-13} to $10^{-8}\ \text{M}$ relaxin ($n=6$). During the experimental protocol, TA diameter was continuously recorded for 1 min before relaxin application (to obtain a baseline diameter value) and then continuously for each subsequent 5-min relaxin application. We observed a non-significant dilation at $10^{-13}\ \text{M}$, and what appeared to be a maximal response at $10^{-10}\ \text{M}$. Thus, we chose to use $10^{-10}\ \text{M}$ relaxin for the rest of the experimental protocols.

Experimental protocol 2: effects of 10^{-10} M relaxin on specific arteriolar branch orders

MI (fourth-order arterioles), Branches (third-order arterioles) and TAs (second-order arterioles) were identified by counting up one, two or three branch order from the capillaries respectively (nomenclature based on that of Berg *et al.* (1997), see Fig. 2A). Arteriolar diameter at the TA, Branch or MI observation site was continuously recorded for 1 min before relaxin exposure and then continuously during the 5-min micropipette application of 10^{-10} M relaxin. Multiple branch orders were tested per

preparation and care was taken to ensure that each branch order tested was not a vessel from the same terminal microvasculature network previously tested.

Experimental protocol 3: involvement of NO and potassium channels in the relaxin-mediated vasodilation

TA diameter was recorded for 1 min before relaxin application and throughout the 5 min of superfusion of 10^{-10} M relaxin (control). Preparations were then superfused with known pharmacological inhibitors of NO synthase (*N*(G)-nitro-L-arginine methyl ester (L-NAME); 10^{-5} M) (Murrant & Sarelius 2002, Dua *et al.* 2009) or inhibitors of potassium channels, ATP-dependent potassium (K_{ATP}) channel inhibitor glibenclamide (GLIB; 10^{-5} M) (Murrant & Sarelius 2002) or calcium-associated potassium (K_{Ca}) channel inhibitor iberiotoxin (IBTX; 10^{-7} M) (Jackson & Blair 1998) or tetraethylammonium (TEA) (10^{-3} M) (Jackson & Blair 1998). Experiments involving L-NAME application produced significant arteriolar vasoconstriction presumably due to the inhibition of endogenous NO production. Thus, when using L-NAME, we also added S-nitroso-*N*-acetylpenicillamine (10^{-7} M, SNAP), an NO donor, in order to restore the basal levels of endogenous NO and maintain a baseline arteriolar diameter similar to control values. L-NAME + SNAP, GLIB and IBTX were superfused for 30 min while TEA was superfused for 1 h. Relaxin (10^{-10} M) was then re-applied for 5 min in the presence of the inhibitors and arteriolar diameter was recorded as described earlier. Inhibitors were then

Figure 2

The vasodilatory response to relaxin applied directly to different branch orders of the arteriolar network. (A) A schematic representation of the branches of the terminal arteriolar microvasculature (diagram adapted from Murrant & Sarelius (2000)). Blood flows through a subsequently diverging network of arterioles from the paired feed arterioles through the TA, Branch and MI arterioles until it reaches the capillary modules of the network. The schematic is not drawn to scale and all capillary modules from each MI are not shown for the sake of clarity. Arrows represent relaxin micropipette application sites and circles represent observation sites. In this protocol, each of the relaxin micropipette application sites was observed directly ($n=8$ for each type of arteriole). (B) The time course of the change in diameter of each vascular level in response to a 5-min application of 10^{-10} M relaxin. *A significant increase in diameter compared with baseline for the TA and the Branch arterioles. The bracket encompasses all data that are significantly different from baseline values. (C) The peak change in diameter expressed as a per cent of maximum diameter for each vascular level in response to 10^{-10} M relaxin. There was no significant difference in the vasodilatory response shown between the TA and Branch arterioles when comparing change in percentage maximal responses. *A significant increase in diameter compared with baseline. The bracket encompasses all data that are significantly different from baseline values.

Figure 3

The effect of 10^{-5} M L-NAME+SNAP on the peak change in diameter induced by relaxin on the TA. 10^{-10} M relaxin produced a significant control dilation that was inhibited in the presence of 10^{-5} M L-NAME + 10^{-7} M SNAP ($n=8$). Following a 30-min washout period, the vasodilation induced by 10^{-10} M relaxin was restored. *Significantly different values from control experiments.

washed out by 30-min superfusion with control superfusate alone. Following the washout period, 10^{-10} M relaxin was re-applied for 5 min and arteriolar diameter was recorded to ensure the preparation remained responsive to relaxin.

Experimental protocol 4: application of 10^{-10} M relaxin on capillaries

Relaxin (10^{-10} M) was micropipette applied to capillaries and the upstream MI arteriole responsible for perfusion of the stimulated capillaries was observed. A schematic representation of the experimental protocol is provided in Fig. 5A. During the experiment, the MI observation site was continuously recorded for 1 min before relaxin exposure and then continuously during the 5-min application of relaxin on the capillaries.

Experimental protocol 5: 10^{-10} M relaxin-induced conducted responses

Micropipette application of relaxin on capillaries resulted in the upstream vasodilation of connected MI arteriole, indicating that relaxin stimulated capillaries and initiated a conducted response. To determine whether relaxin-induced conducted responses were gap junction dependent, relaxin (10^{-10} M) was locally micropipette applied to a TA for 5 min and the vasodilation at the local site was observed. After 10 min, 10^{-10} M relaxin was re-applied to the local site for 5 min while observing a site ~ 1000 μm upstream along the same TA (for schematic diagram of experimental protocol 5, see Fig. 6A). A gap junction uncoupler (18- β -glycyrrhetic acid (βGA ;

40×10^{-6} M) or 0.07% halothane) was then micropipette applied between the local and the upstream observation site for 30 min and the local relaxin application protocol was repeated, observing both the local and the upstream sites. The gap junction uncoupler application was then

Figure 4

The effect of (A) 10^{-5} M GLIB, (B) 10^{-3} M TEA and (C) 10^{-7} M IBTX on the relaxin-induced vasodilation. (A) The peak change in diameter of the TA in response to 10^{-10} M relaxin alone (control) and in the presence of 10^{-5} M GLIB ($n=8$). Following a 30-min washout period, the vasodilatory response to 10^{-10} M relaxin was returning to control values. (B) The peak change in diameter of the TA in response to 10^{-10} M relaxin alone (control) and in the presence of 10^{-3} M TEA ($n=8$). Following a 1-h washout period, the vasodilatory response to 10^{-10} M relaxin was restored. (C) The peak change in diameter of the TA in response to 10^{-10} M relaxin alone (control) and in the presence of 10^{-7} M IBTX ($n=8$). Following a 30-min washout period, the vasodilatory response to 10^{-10} M relaxin was restored. *Significant differences from control conditions.

stopped, and after a 30-min washout period, the relaxin application protocol was repeated for a third time to confirm that the arteriole maintained viability and responded to relaxin.

At the conclusion of protocols 1 through 5, arteriolar diameters of all observation sites were recorded following 2 min of superfusion with 10^{-4} M sodium nitroprusside, an NO donor, which is considered to produce maximum vasodilation (Sweeney & Sarelius 1989, Murrant & Sarelius 2000, Mihok & Murrant 2004). Animals were then killed with an overdose of sodium pentobarbital (33 mg/ml i.v. to effect).

Figure 5

The peak change in diameter of the MI arteriole in response to relaxin applied to an associated group of capillaries. (A) A schematic representation of the branches of the terminal arteriolar microvasculature (diagram adapted from Murrant & Sarelius (2000)). Blood flows through a subsequently diverging network of arterioles from the paired feed arterioles through the TA, Branch and MI arterioles until it reaches the capillary module of the network. The schematic is not drawn to scale and all capillary modules from each MI are not shown for the sake of clarity. The arrows represent the relaxin micropipette application site over the capillaries and circle represents the MI observation site ($n=8$). (B) The time course of the MI vasodilatory response to 5 min of 10^{-10} M relaxin application to capillaries in the associated capillary module and observed in the MI is shown. Treatment with relaxin (10^{-10} M) started at time zero and lasted throughout the duration of the experiment until 300 s. *A significant increase in diameter compared with baseline. The bracket encompasses all data that are significantly different from baseline values.

Chemicals and reagents

Recombinant human relaxin-2 was a generous gift from Dr E N Unemori, Corthera, Inc. Relaxin was kept in frozen aliquots and solubilised in superfusate on each experimental day. All other pharmacological agents used were obtained from Sigma–Aldrich) unless otherwise indicated and prepared on the day of the experiment.

Data and statistical analysis

All experiments were videotaped and analysed off-line. Images were digitised with Canopus Media Cruise software (Canopus Corp., San Jose, CA, USA) and arteriolar diameter was measured with calibrated ImageJ software. Arteriolar diameter was defined as the distance between each inside wall of the arteriole. Arteriolar diameter was measured just before relaxin application (defined as baseline diameter) and every 10 s throughout the entire 5-min application of relaxin. All data are reported as mean $\pm$ S.E.M.; n represents the number of arterioles observed. Group means were compared with a Student's *t*-test (Fig. 6B and C), an ANOVA (Figs 1, 2C, 3 and 4) or repeated measures ANOVA when comparing data over time (Figs 2B and 5B). When the ANOVA identified a significant difference, a protected LSD was performed *post hoc* in order to determine whether diameter changes were significant (Snedecor & Cochran 1989). Differences were considered statistically significant when $P < 0.05$.

Results

Experimental protocol 1: dose response to relaxin

With the initial dose response range (10^{-10} to 10^{-5} M), all concentrations of relaxin tested produced vasodilation and peak changes in diameter that were not significantly different from each other. Thus, a second dose response using concentrations ranging from 10^{-13} to 10^{-8} M was carried out. The peak changes in vessel diameter in response to 10^{-10} to 10^{-8} M relaxin (concentration overlap between the two dose responses) were not significantly different between the two trials, so the data from the two trials were combined and shown in Fig. 1 (baseline diameter = 20.5 ± 1.1 μ m; maximum diameter = 40.2 ± 1.0 μ m). Significant vasodilation was observed with concentrations ranging from 10^{-12} to 10^{-5} M, but no significant dilation was seen at 10^{-13} M. Relaxin (10^{-10} M) was the first concentration applied in the initial dose response and fourth concentration applied in the

Figure 6

Uncoupling gap junctions with either β GA or halothane significantly reduces conductance of relaxin-induced vasodilation. (A) A schematic representation of the branches of the terminal arteriolar microvasculature (diagram adapted from Murrant & Sarelius (2000)). Blood flows through a subsequently diverging network of arterioles from the paired feed arterioles through the TA, Branch and MI arterioles until it reaches the capillary module of the network. The schematic is not drawn to scale and all capillary modules from each MI are not shown for the sake of clarity. The relaxin arrow represents the relaxin micropipette application site over the TA and circles represents local and upstream observation sites. The β GA or Halothane arrow represents the position of the micropipette application of the gap junction uncouplers in this experiment, approximately 500 μ m from both the local and upstream observation sites. (B) The peak change in diameter of the local and upstream observation sites in response to locally applied 10^{-10} M relaxin in the absence (control) and presence of gap junction inhibitor β GA ($n=8$) and after washout conditions. (C) The peak change in diameter of the local and upstream sites in response to locally applied 10^{-10} M relaxin in the absence (control) and presence of gap junction inhibitor halothane ($n=8$) and after washout conditions.

*A significant difference from control change in diameter at the same site.

second dose response; the consistency of the response at 10^{-10} M relaxin between the two trials shows that there is no change in the sensitivity of the preparation over time or after multiple exposures to relaxin.

Experimental protocol 2: effects of relaxin on different branches of the arteriolar network

Baseline and maximal arteriolar diameters respectively for each branch order were as follows: TA, 24.2 ± 3.1 and 46.3 ± 3.7 μ m ($n=8$); Branch, 13.4 ± 2.6 and 31.3 ± 2.4 μ m ($n=8$); MI, 6.5 ± 1.2 and 12.1 ± 2.1 μ m ($n=8$). Relaxin (10^{-10} M) induced a significant vasodilation in TA and Branch arterioles; however, no significant vasodilation was observed in the MI arterioles (Fig. 2B). The absolute change in diameter of the TA appears to be larger than the Branch arteriole; however, these branch orders differ in terms of their maximum possible diameter (TA maximum diameter is ~ 45 μ m and Branch arteriole maximum diameter is ~ 30 μ m) and it is important to normalise vascular responses to maximum diameter when comparing different vascular branch orders. Once normalised, there is no difference in the vasodilatory response to 10^{-10} M relaxin between the TA and the Branch arterioles (Fig. 2C). The relaxin-induced vasodilation in both the TA and the Branch arterioles was transient, and the vasodilation was not sustained over the entire 5-min relaxin exposure period.

Experimental protocol 3: involvement of NO and potassium channels in the relaxin-mediated vasodilation

Baseline and maximal diameters for vessels studied in experimental protocol 3 are provided in Table 1. There were no significant differences between the baseline values for control and experimental groups, indicating that there was no impact of the blockers alone on arteriolar diameter. There were no significant differences between baseline diameter in control conditions and in the presence of L-NAME+SNAP, indicating that we were successful at restoring the baseline diameter in the presence of L-NAME to control values using SNAP. Figure 3 shows that the peak change in diameter from baseline in response to 10^{-10} M relaxin was significantly attenuated in the presence of L-NAME+SNAP. Following a 30-min washout period, the vasodilatory response to relaxin (10^{-10} M) was restored.

Figure 4 shows the response of relaxin in the presence of potassium channel inhibitors. Figure 4A illustrates the inhibited vasodilatory response to 10^{-10} M relaxin in the

Table 1 Baseline and maximal diameters of arterioles (μm) tested in experimental protocol 3. Baseline diameters reported are baseline diameter before 10^{-10} M relaxin application alone and baseline diameter in the presence of the pharmacological blocking agents just before 10^{-10} M relaxin+inhibitor application. Data are presented as mean $\pm$ S.E.M. in μm

Blocker	Baseline diameter		Maximum diameter	n
	10^{-10} M relaxin alone (control)	10^{-10} M relaxin+inhibitor (experimental)		
L-NAME+SNAP	15.9 ± 1.5	19.1 ± 1.4	46.1 ± 1.6	8
GLIB	15.9 ± 1.8	17.4 ± 1.5	45.6 ± 2.2	8
TEA	20.4 ± 1.8	24.3 ± 1.7	55.7 ± 2.3	8
IBTX	17.6 ± 1.3	18.5 ± 1.6	42.5 ± 3.2	8

presence of the K_{ATP} channel blocker GLIB (10^{-5} M). The 30-min washout period was not long enough to fully restore the relaxin vasodilation to pre-GLIB levels but was partially restored as 10^{-10} M relaxin produced a significantly larger dilation when compared with relaxin and 10^{-5} M GLIB. Inhibition of K_{Ca} channels with either 10^{-3} M TEA (Fig. 4B) or 10^{-7} M IBTX (Fig. 4C) significantly inhibited relaxin-induced vasodilation. Following the washout period for both inhibitors, the vasodilatory response to relaxin was restored.

Experimental protocol 4: application of 10^{-10} M relaxin on capillaries

Application of 10^{-10} M relaxin for 5 min to a group of capillaries caused a significant, transient vasodilation in the associated MI arterioles (baseline diameter: 7.2 ± 1.3 μm ; maximum diameter: 11.3 ± 1.6 μm) (Fig. 5B). Significant vasodilation was observed from 50 to 200 s after which the arteriolar diameter returned to baseline values.

Experimental protocol 5: 10^{-10} M relaxin and conducted vasodilations

Baseline and maximal diameters for TAs studied in protocol 5 are shown in Table 2. Application of 10^{-10} M relaxin for 5 min induced a significant local vasodilation

that was conducted over 1000 μm to the upstream site (Fig. 6B). The magnitude of the local and upstream vasodilation did not significantly differ from each other. Application of the gap junction uncoupler βGA (Fig. 6B) or halothane (Fig. 6C) midway between the local and upstream sites significantly inhibited the conducted vasodilation. Following a 30-min washout period, 10^{-10} M relaxin induced a conducted vasodilation similar to control vasodilations.

Discussion

The data presented in this study show that very low concentrations of relaxin (10^{-12} M) produce a rapid, transient vasodilation in specific branches of the terminal arteriolar network in skeletal muscle *in situ*. The relaxin-induced dilations in TA and Branch arterioles were both NO- and potassium channel-dependent while the smallest ramification of the arteriolar tree was not responsive to relaxin. Capillaries were also responsive to relaxin and affected the arteriolar vasculature through gap junction-mediated conducted responses. The significance of the ability of relaxin to induce conducted responses indicates that localised, paracrine release of relaxin within a tissue may have the ability to alter the tissue vascular network and significantly alter blood flow distribution within the tissue and blood flow to the tissue. The transient

Table 2 Baseline diameters, maximal diameters and distances between local and upstream sites of arterioles used in experimental protocol 5 investigating the possible role for gap junctions in transmitting vasodilation in upstream transverse arterioles in response to 10^{-10} M relaxin. Data are mean $\pm$ S.E.M. in μm

Drug	Control		Blocker		Washout		Maximum diameter		Distance from local to upstream site
	Local	Upstream	Local	Upstream	Local	Upstream	Local	Upstream	
βGA (n=8)	22.5 ± 1.4	24.3 ± 1.1	24.2 ± 2.1	25.6 ± 3.2	23.1 ± 2.2	25.9 ± 3.4	46.6 ± 4.2	48.6 ± 5.2	1154.3 ± 22.4
HAL (n=8)	19.7 ± 0.9	23.1 ± 2.4	22.4 ± 3.1	23.5 ± 2.3	24.1 ± 1.1	25.3 ± 2.3	42.5 ± 3.9	47.4 ± 4.1	1265.6 ± 33.2

nature of the arteriolar vasodilator effect of relaxin within the skeletal muscle vascular bed indicates that relaxin's systemic haemodynamic effects are not derived through sustained changes in skeletal muscle resistance vasculature, indicating a locus of control elsewhere.

Experimental considerations

A number of factors were taken into account to ensure accurate data interpretation. These included the following: i) similar baseline diameters between control and experimental conditions were used to ensure that the experimental treatments had no effects on basal diameter of unstimulated vessels. This allows for direct comparisons to be made between control and experimental groups with pharmacological inhibitors. ii) A pharmacological agent washout process was performed after each experiment to ensure that relaxin-induced vasodilation returned. This proved that the preparation was still viable following experimental interventions and that the preparation responded to relaxin over time. iii) The maximum diameter was determined at the end of the experimental period for each experiment, which allowed us to confirm whether maximum diameter had been achieved during experimental protocols. We have previously determined that there is no difference between the maximal dilation produced by 10^{-4} M sodium nitroprusside and a calcium-free superfusate solution (Ross *et al.* 2013). There was no protocol where the vasodilation induced a maximum dilation; therefore, we did not exceed maximum vasodilatory capacity of our preparation.

Relaxin dose response

Relaxin produced rapid vasodilation across a wide range of doses (10^{-12} to 10^{-5} M). We found that very low concentrations of relaxin (10^{-12} M) induced a significant vasodilation. This is an order of magnitude more sensitive than isolated resistance arterioles in which 10^{-11} M was found to produce a significant vasodilation (Fisher *et al.* 2002). Relaxin (10^{-12} M) is well below the levels detected by ELISA and RIA techniques (Stewart *et al.* 1990, Gedikli *et al.* 2009) in the blood. It is also two to three orders of magnitude lower than the concentrations of acetylcholine (NO-dependent vasodilator) (Clark & Fuchs 1997, Jasperse & Laughlin 1999, Woodman *et al.* 2003, Spier *et al.* 2004, Erdei *et al.* 2006, Newcomer *et al.* 2008, Roseguini *et al.* 2010) and NO donors (Jasperse & Laughlin 1999, Woodman *et al.* 2003, Erdei *et al.* 2006, Hein *et al.* 2010) required to initiate vasodilation.

The largest change in diameter in response to relaxin was observed at 10^{-10} M and no larger vasodilation was observed at higher concentrations and no cumulative vasodilation was observed. This behaviour can be explained by the transient nature of the relaxin-induced vasodilation (Fig. 2B), which was observed at each concentration of relaxin used in the dose response above 10^{-12} M (data not shown); thus, no classic 'cumulative' dose response behaviour was observed. These data indicate that, regardless of relaxin concentration above 10^{-10} M, the maximum capacity of relaxin to vasodilate is ~ 10 μ m. Therefore, it may not be the absolute concentration of relaxin released that is important, but the temporal release of relaxin that may establish the arteriolar vasodilatory state.

This transient vasodilatory behaviour differs from non-transient responses to relaxin found in vascular preparations such as rat renal and human subcutaneous arteries (McGuane *et al.* 2011), human gluteal arteries (Fisher *et al.* 2002), venules of the mesocaecum (Bigazzi *et al.* 1986) and coronary flow in the guinea pig (Bani-Sacchi *et al.* 1995). Thus, our data indicate that the response of the microvascular resistance arterioles of skeletal muscle may be unique and indicates that there might be compensatory mechanisms at play in restoring arterial wall diameter. Certainly, there is evidence that relaxin has specific and varied effects on different vascular beds even within the same species (Dombrowski *et al.* 1986, Petersen *et al.* 1991, Fisher *et al.* 2002, McGuane *et al.* 2011). Refractoriness to relaxin has been reported in other tissues, for example the uterus (Chamley & Parkinson 1984), the mammary gland (Summerlee *et al.* 1984) and the brain (Parry *et al.* 1990), but sustained responses have also been reported (Porter 1971, Bradshaw *et al.* 1981, Norstrom *et al.* 1984). The physiological relevance of a sustained response is self-evident, but the significance of a transient response is less obvious. Given that this transient behaviour is present in the relaxin response in other tissues indicates that there may be two common response types, transient and non-transient, each with their own, yet-to-be-determined, importance.

Different microvascular responses to relaxin

Micropipette application of 10^{-10} M relaxin produced vasodilation when the TA, Branch arterioles and capillaries were stimulated, but no vasodilation was produced in MI arterioles when relaxin was applied directly. These data indicate that a relaxin membrane receptor population (RXFP1) is present at the TA, Branch and capillary

level but not on MI arterioles. There is evidence to support that RXFP1 is located on the endothelium of renal arteries (Novak *et al.* 2006) and VSM of renal (Novak *et al.* 2006) and cardiac tissues (Samuel *et al.* 2003). Studies on isolated vessels have shown that the endothelium is necessary for vasodilation by relaxin in gluteal and subcutaneous arteries (Fisher *et al.* 2002, McGuane *et al.* 2011). It is unclear from our studies whether RXFP1 is present on the VSM, endothelium or both in skeletal muscle resistance arterioles. The capillary response to relaxin provides evidence that implies that capillary endothelial cells express a membrane receptor population for relaxin. There is always a remote possibility that there are indirect effects of relaxin (non-receptor-mediated effects) on the vasculature, but the lack of effect of relaxin on the MI would indicate that there are no indirect effects. Our data show that, although the MI does not respond to relaxin directly, they can respond to relaxin indirectly, through capillary stimulation and conducted responses.

Our data show that the arteriolar vasodilation in response to relaxin was dependent on NO. Previous reports have indicated that relaxin simulates NO-mediated responses in multiple vascular and non-vascular tissues (Bani *et al.* 1995, Bani-Sacchi *et al.* 1995, Novak *et al.* 2002, Failli *et al.* 2005, McGuane *et al.* 2011, van Drongelen 2011). Our data show that TA and Branch arterioles dilate to the same extent relaxin, once the maximum size of the vessels is taken into account; however, MI arterioles do not respond directly to relaxin. As mentioned earlier, this could be due to the lack of the membrane receptor population for relaxin. A second possibility is that there is a reduced importance of NO in the terminal microvasculature tree as vessels branch toward the capillaries. Hester *et al.* (1993) showed that even though all the arterioles in the terminal microvasculature have the ability to produce NO and respond to it, the importance of NO in their function is less as the branch order gets smaller. Thus, the importance of NO in the regulation of MI diameter may be diminished, therefore possibly less responsive to NO-dependent dilators such as relaxin.

We have further demonstrated that relaxin causes vasodilation through K_{ATP} and K_{Ca} channels. Potassium channels have been implicated in many vasodilatory responses in the microcirculation by inducing hyperpolarisation and relaxing vascular smooth muscle (for review see Jackson (2005)). We show that this is consistent within the relaxin-induced stimulation of the vasculature of skeletal muscle arterioles. Whether NO stimulates the opening of the potassium channels is unknown. There is

support for NO and cGMP in opening K_{ATP} channels (Murphy & Brayden 1995) and support for NO in stimulating K_{Ca} channels (Archer *et al.* 1994), but the exact signalling mechanisms for relaxin in skeletal muscle arterioles are yet to be defined. McGuane *et al.* (2011) have shown that phosphatidylinositol-3-kinase and Akt are part of the relaxin signalling pathway to stimulate rapid vasodilation but, as of yet, the connection with potassium channels has not been made.

Relaxin-induced conducted responses

Capillary stimulation with relaxin produced vasodilation in the upstream MI, indicating that relaxin can induce conducted responses. We explored the nature of the conducted responses induced by relaxin at the level of the TA and found that, indeed, relaxin induced gap junction-dependent conducted responses. We demonstrated that 10^{-10} M relaxin, applied locally to the arteriole, could induce dilation to a remote site, over 1000 μ m upstream of the site of stimulation, and that the dilation at the remote site could be inhibited by two non-specific gap junction uncouplers, β GA and halothane. In the microcirculation, gap junctions are primarily composed of connexin (Cx) proteins, specifically CX37 (GJA4), CX40 (GJA5) and CX43 (GJA1) (Little *et al.* 1995, Gustafsson *et al.* 2003, Looft-Wilson *et al.* 2004a,b, Hakim *et al.* 2008). Currently, there are no tools to block gap junctions of a specific Cx isoform, only non-specific gap junction uncouplers such as the ones used in this study. The vasodilatory signal conducted through the gap junction connections is thought to be hyperpolarisation, although there are reports of various small molecules being able to traverse gap junctions (such as Ca^{++} and IP_3 (Evans & Martin 2002, Saez *et al.* 2003)). We have shown that relaxin vasodilates through K_{ATP} and K_{Ca} channels, therefore exhibiting the potential to initiate hyperpolarisation that may be transmitted longitudinally throughout the vascular network.

Conducted responses play an important physiological role in the coordinated distribution of blood flow to tissues and within tissues, ensuring adequate perfusion to tissues during times of increased metabolic demand. Conducted responses have been well characterised in skeletal muscle in response to both application of pharmacological dilatory substances (e.g. acetylcholine) (for example, Segal & Duling (1986, 1989)) and during skeletal muscle contraction (Berg *et al.* 1997, Murrant & Sarelius 2000). During skeletal muscle contraction, dilatory substances are released by active skeletal muscle, which stimulate the

vasculature and initiate conducted responses and signal upstream arterioles to vasodilate to increase tissue perfusion to the active skeletal muscle cells. Therefore, locally produced signals have the capacity to alter remote sites of the tissue vascular network to enhance tissue blood flow. It is possible that local production of relaxin within a tissue may act to enhance the response of the tissue vascular network and alter tissue blood flow. For example, during pregnancy in the myometrium, following implantation, locally produced relaxin has been demonstrated to be a critical step to implantation events and the stimulation of angiogenesis (Einspanier 2001, Goldsmith *et al.* 2004). Relaxin may enhance blood flow to this tissue during pregnancy through conducted responses. Finally, the propagation of vasodilation along arterioles may occur in tumours where relaxin has been demonstrated to be up-regulated (e.g. prostate and breast, Binder *et al.* (2002) and Silvertown *et al.* (2006)). The enhancement of blood flow to tumours is a critical step in the growth of cancer, and given that relaxin is locally produced in some tumours (Silvertown *et al.* 2006), relaxin-induced conducted vasodilation may act to enhance tumour blood flow. Given that the functional concentration of relaxin is very low, it is now critical to understand the local concentrations and release sites of relaxin in order to fully understand its potential for affecting tissue blood flow. This is especially important as the list of sources of relaxin continues to grow from the ovaries, decidua, placenta and prostate (Bani 1997) to include the left ventricle and atria (Dschiertzig *et al.* 2001).

Summary

Our data show that low concentrations of relaxin exhibit a differential ability to induce transient vasodilations in various levels of the terminal microvasculature of skeletal muscle *in situ* through NO- and potassium channel-dependent mechanisms. The transient nature of the arteriolar vasodilation in skeletal muscle in response to relaxin raises questions as to the role of relaxin in affecting sustained changes in total peripheral resistance and its contribution to sustained systemic haemodynamic changes (Conrad *et al.* 2004) and suggests that the locus of control of sustained haemodynamic changes due to relaxin resides elsewhere.

Conducted responses initiated by relaxin at both the capillary and the arteriolar level of the microvasculature introduces a new potential mechanism by which relaxin may alter blood flow to a tissue and alter blood flow distribution within a tissue. The ability of relaxin to

induce conducted responses indicates that small, localised release of relaxin within a tissue will be able to affect remote regions of the tissue microvasculature and change blood flow distribution within a tissue, and if the vasodilation is large enough, it will even change blood flow to the tissue itself.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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