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Thermally-mediated ultrasound-induced contraction of equine muscular arteries in vitro and an investigation of the associated cellular mechanisms

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Abstract

We have previously shown that MHz frequency ultrasound causes contraction of the carotid artery *in vitro*. We now extend this investigation to equine mesenteric arteries and investigate the cellular mechanisms. *In vitro* exposure of the large lateral cecal mesenteric artery to 4 minute periods of 3.2 MHz continuous wave ultrasound at acoustic powers up to 145 mW induced reversible repeatable contraction. The magnitude of the response was linearly dependent on acoustic power, and at 145 mW the mean increase in wall stress was 0.020 ± 0.017 mN/mm^2 (n=34). These results are consistent with our previous study and the effect was hypothesised to be thermally mediated. A 2 ˚C temperature rise produced an increase in intracellular calcium, measured by Fluo-4 fluorescence. Inhibition of the inward-rectifier potassium ion channel with BaCl$_2$ (4μM) increased the response to ultrasound by 55 ± 49 %, indicating a similar electrophysiological basis to the response to mild hyperthermia. In small mesenteric arteries (0.5-1.0 mm diameter) mounted in a perfusion myograph, neither ultrasound exposure nor heating produced measureable vasoconstriction or a rise in intracellular calcium and we conclude that temperature-sensitive channels are absent or inactive in these small vessels. It therefore appears that response of blood vessels to ultrasound depends not only on the thermal properties of the vessels and surrounding tissues but also on the electrophysiology of the smooth muscle cells.

Introduction

Ultrasound is widely accepted as a safe medical imaging modality and is finding increasing applications as a therapeutic tool in bone, soft tissue and tendon healing and nerve regeneration. Understanding of the bioeffects of ultrasound is important in assessing the safety of medical diagnostic imaging and therapeutic ultrasound and in clarifying the mechanisms underlying the latter applications.

Ultrasound has been shown to alter metabolic and proliferative activity in a wide range of cells grown in culture, including Schwann cells (Crisci and Ferreira 2002; Raso et al. 2005; da Costa Gonçalves et al. 2007; Zhang et al. 2009), aortic endothelial cells (Raz et al. 2005; Mizrahi et al. 2007), fibroblasts (Ramirez et al. 1997; Zhou et al. 2004), chondrocytes (Nishikori et al. 2002; Korstjens et al. 2008; Takeuchi et al. 2008), and bone model cultures (Harle et al. 2001; Rutten et al. 2008). Revealing as these results are, cells in culture are in many respects abnormal and such preparations do not lend themselves to investigation of interactions between cell types and many other behaviours important at the level of tissue physiology. A concern which is particularly pertinent to the present investigation is that whilst some studies do not report the ultrasound exposure conditions in sufficient detail to be certain, their design makes it likely that cellular heating will occur, but this is generally discounted as a causative mechanism under conditions for which the expected temperature rise is less than 1-2 ˚C.

Experiments on the effects of ultrasound at the whole-tissue level are relatively few. Reflecting the potential importance of the vascular system in mediating whole-body effects of ultrasound, several groups have investigated effects on blood vessels. We recently reported ultrasound-induced reversible contraction of the equine carotid artery *in vitro* (Martin et al. 2010).

The increase in wall tension from both continuous wave and pulsed ultrasound at 3.2 MHz was linearly dependent on acoustic power, but the response was independent of endothelial integrity. The first aim of the present research was to compare the responses in other muscular arteries, particularly those of small calibre that play a major role in flow regulation. We are now able to confirm that the response is indeed similar in large mesenteric arteries, but entirely absent in mesenteric vessels of sub millimetre diameter.

The latter unexpected observation raises questions concerning the cellular mechanism underlying vasoconstriction. A number of studies on the effects of ultrasound on cultured cells have reported changes in intracellular calcium e.g. (Mortimer and Dyson 1988) which mediate muscle cell contraction and many other processes. In our previous paper, we hypothesised that a similar process occurs in the vascular wall. Further, we suggested that this change was a result of very slight heating of the smooth muscle cell membrane, which has previously been reported to induce changes in vascular tone (Mustafa et al. 2004), through changes in potassium ion channel activity (Mustafa and Thulesius 2005). We now directly confirm this hypothesis by experiments on the effects of specific channel inhibitors on the response to ultrasound and by fluorescence-probe measurements of thermally-induced changes. We also demonstrate that the absence of a response to ultrasound in the small vessels is due, not to differences in ultrasound absorption, but to the distribution of temperature-sensitive ion channels.

Methods

Tissue preparation

Equine carotid arteries from animals less than 15 years of age were collected at a local abattoir (Potters, Taunton, UK) immediately after death and placed in cold (4 °C) Krebs-Ringer buffer for transport to the laboratory. Samples of mesentery containing the lateral cecal mesenteric

arteries were collected approximately 10 minutes after death and the tissue remained warm and blood filled. The tissue was placed in warm Krebs-Ringer buffer for transport back to the laboratory in order to prevent the surrounding adipose tissue from solidifying and inhibiting dissection.

Dissection of vessels was performed in a dissecting bath under Krebs-Ringer buffer at 4 °C to minimise shock and endothelial damage. The surrounding muscle, fat and loose adventitia was gently removed from the large arteries and the vessels were cut with a scalpel into segments approximately 1.5 cm in length, avoiding side branches. Branches of the lateral cecal mesenteric artery 0.5 to 1 mm in diameter were located, dissected free of surrounding tissue and cut into sections approximately 4 to 5 mm in length, again avoiding side branches, under a dissecting microscope. All samples were stored in fresh Krebs-Ringer buffer at 4 °C until needed.

Tissue characterisation

Transverse frozen sections 20 μm in thickness were stained with haematoxylin and eosin to show the smooth muscle nuclei and cytoplasm. The large vessels exhibited an intact endothelial layer, and the media contained circumferentially aligned smooth muscle cells with a density of 2830 ± 40 nuclei per mm^2 in the carotid artery (CA) and 4080 ± 340 nuclei per mm^2 in the large mesenteric artery (LM). The 1st order branches of the mesenteric artery (SM) had a thin media, again containing circumferentially aligned cells at a density of 4810 ± 130 nuclei per mm^2.

Force-extension curves were produced for the large vessels using the wire myograph described below and pressure radius curves were produced for the mesenteric artery branches using the pressure myograph system. These data were used to calculate stress-strain curves, based on microscopic measurements of vessel dimensions for the three vessel types (Figure 1). The SM had the highest incremental elastic modulus and the CA the lowest. At the mean wall stress applied

during exposure to ultrasound it was 14.1 ± 3.9 mN/mm² for CA, 18.1 ± 2.9 mN/mm² for LM and 260 ± 46 mN/mm² for the SM.

Ultrasound system

The ultrasound system has been described previously (Martin et al. 2010). In brief, the source was a weakly focussed (amplitude focussing gain of about 5) nominal 3 MHz transducer, with a single circular damped lead zirconate titanate (PZT) element (MD3483 Diagnostic Sonar, Livingston, UK). The radiating area was 3.8 cm² and the diameter was 2.2 cm. The input signal was generated by a signal generator (Agilent 33220A, Agilent, Wokingham, UK) and amplified by a 150A100B radio frequency power amplifier (Amplifier Research, Souderton, PA, USA), in line with the transducer. For measurement of the acoustic field and positioning of tissue samples, a 0.2 mm PVDF (polyvinylidene fluoride) needle hydrophone with preamplifier was used (Precision Acoustics Ltd., Dorchester, UK). The signal from the hydrophone was visualised on an oscilloscope (HM504-2, Hameg, Mainhausen, Germany) for positioning and peak acoustic pressure measurement. Acoustic power in the beam was measured before each set of experiments using a radiation force balance (Perkins 1989). The peak positive and negative acoustic pressures, acoustic power, calculated intensities and $\frac{p_r}{\sqrt{f}}$ (used as an analogue to MI) were as quoted in our previous paper.

Wire myograph

The wire myograph system was as described in our previous work, modified to enable exchange of fluid in which the tissue is mounted (Figure 2a). Sections of large artery were mounted in a thin walled (62.5 μm) polythene container (manufactured from medium duty layflat polythene tubing, Key Industrial Equipment Ltd., Dorset, UK) filled with Krebs-Ringer buffer, and placed in an acoustic absorber-lined (Aptflex 28, Precision Acoustic Ltd., Dorchester, UK) water bath at the focus of the acoustic field. The presence of the polythene container caused a reduction in acoustic peak

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negative pressure of approximately 2%. The ultrasound transducer was coupled with ultrasound gel

to a TPx (polymethylpentene) membrane (Goodfellow, Huntingdon UK) in one end of the bath. The

bath was heated to 37 ˚C by polythene heat exchange tubes placed in the bath with hot water

circulating from an external water bath. A positioning system used micrometer screw gauges to

enable careful positioning of the tissue in the acoustic field at the position of spatial peak negative

acoustic pressure. The hydrophone was used to locate this position, it was then removed and the

polythene container was inserted and the tissue mounted in this position. A high sensitivity force

transducer (FORT 25, with TBM4M bridge amplifier, World Precision Instruments Inc., Sarasota, FL,

USA) was used to make isometric force measurements. Artery rings were mounted between a

stainless steel L-shaped rod attached to the transducer arm and a similar one attached to a

micrometer that could be adjusted to apply strain to the ring of tissue. The rods were cushioned

with PVC tubing to reduce damage to the endothelium. The force transducer output was detected

by an ADC-20 High Resolution Data Logger connected to a computer and displayed in real time using

PicoLog Data Acquisition software (Pico Technology). The force transducer signal was sampled at 1

second intervals.

Pressure myograph

A pressure myograph was constructed in which arteries of sub-millimetre diameter could be

perfused at constant pressure and exposed to ultrasound whilst the vessel diameter was

continuously recorded. The system (Figure 2b) consisted of a small, two-compartment bath with the

ultrasound transducer mounted in a fixed position with the radiating area immersed in Krebs-Ringer

buffer. A TPx membrane separated this section of the bath from the shallower section in which

vessels were mounted on glass pipettes, tied in place with suture thread and perfused by flow

between an upstream and downstream reservoir filled with Krebs Ringer buffer, whose height could

be adjusted to alter luminal pressure. The vessel was positioned above an optical window in the

base of the bath, and the bath was mounted on an inverted microscope (Leica DM IL, Leica Microsystems GmbH, Wetzlar, Germany) with camera (KY-F55B, JVC, NJ, USA) and video capture card (194003-USB Live, Hauppauge Computer Works UK Inc., London, UK) connected to a computer running Ulead DVD Movie Factory (Ulead Systems, Torrance, CA, USA) for image capture at a rate of 1 frame per second. Both sections of the bath were lined with acoustic absorber (Aptflex, Precisions Acoustics Ltd., as previously). Perspex heat exchangers (made in house) were immersed in the bath to maintain a temperature of 37˚C.

Video capture was continuous during the experiments and the files were decompressed, cropped and rotated using VirtualDub software (released under GNU General Public License (GPL)) then converted to structural arrays in Matlab (The Mathworks Ltd., Cambridge, UK) before processing and analysis was performed in Octave (GNU Octave, released under GNU GPL). An algorithm was written which traced the vessel edges frame by frame using edge based segmentation, and then plotted the time course of changes in the diameter of the vessel.

Exposure of large arteries to ultrasound

The mounting and exposure to ultrasound of the vessels in these experiments was as in our earlier study. CA or LM rings were placed over the wire supports and stretched to a wall stress of 4.1 ± 0.7 mN/mm^2 and left to equilibrate under constant strain for 45 minutes before the vessel function was tested. Vasoconstriction was induced with a 1 µM dose of noradrenaline (Sigma Aldrich Ltd., Dorset, UK). When the tension had reached a maximum level, approximately 1 minute later, a 1 µM dose of acetylcholine (Sigma Aldrich Ltd., as previously) was added to the bath. In artery sections with intact endothelium this induced vasodilatation; in artery sections where the endothelium was not intact an increase in tension was induced (Furchgott and Zawadzki 1980). Once the function test had been performed, the buffer contained within the polythene container was refreshed. Vessels which did not show results consistent with intact endothelium were discarded. Following this

procedure, vessels were left to equilibrate until wall stress had returned to a stable level. Before ultrasound exposure, a small syringe was used to gently flush fluid across the outer surface of the vessel to remove bubbles which may have collected and would have acted to screen the tissue from the beam. The TPx membrane was carefully wiped with a cotton bud for the same reason.

Rings of LM artery were first exposed to continuous wave ultrasound at an acoustic power of 145 mW for 4 minute periods. Following each exposure there was a gap of at least 15 minutes before the next. In a second set of experiments, the acoustic power was alternated from one exposure to the next between 145 mW and 100 mW in half of the experiments, and 145 mW and 72 mW in the others. The responses at these three powers were then compared.

Exposure of small arteries to ultrasound

The small arteries were mounted on the pressure myograph under cold Krebs-Ringer buffer, then brought up to temperature and luminal pressure and allowed to equilibrate for 30 minutes. The contractile response was tested by the addition of noradrenaline (1µM) and acetylcholine (1µM). The buffer was then refreshed and the vessel was left to equilibrate for a further 30 minutes before exposures were begun. Video capture commenced 25 minutes before exposure of the vessels to ultrasound to provide baseline data. Vessels were exposed to ultrasound under two different conditions. The first group was exposed for 4 minute periods at an acoustic power of 145 mW with a gap of at least 15 minutes between exposures to mimic the regime used in large vessel experiments (open field configuration). The second group was mounted as described but an additional section of absorber of dimensions 25mm x 22 mm x 5 mm (Aptflex, Precision Acoustics Ltd., Dorchester, UK) was placed within a few millimetres of the tissue in the beam path. This was intended to induce heating of the vessels by convection during exposure, to replicate the temperature rise measured in the large vessel experiments. The temperature rise during exposure was measured both with and

without the absorber section in place using a thermocouple (RS components Ltd., Northants, UK) placed on the vessel wall and coupled to a data logger (Picolog ADC-20).

In order to measure the response of SM to changes in temperature, a further group of vessels was then exposed to a rapid increase of temperature of approximately 1 °C above the baseline temperature of 37.5 °C, followed by natural cooling. This procedure was repeated four times on each vessel while temperature and vessel radius were recorded continually.

Measurements of ion channel activity

Barium chloride was used as a potassium channel blocker specific to potassium inward rectifier channels at low concentrations. Experiments were performed on 12 CA samples and 4 LM samples. After equilibration, each artery section was subject to a minimum of 3 periods of continuous wave ultrasound exposure at an acoustic power of 145 mW lasting 4 minutes each, with a recovery period of at least 15 minutes between exposures. Following this set of exposures, a 4 µM dose of $BaCl_2$ (Sigma Aldrich Ltd., Dorset, UK) was added to the bath. This concentration had no effect on the basal tone of the vessels. A further set of at least three exposures to ultrasound was then made and increases in tension during ultrasound exposure before and after the addition of $BaCl_2$ were compared.

The calcium sensitive fluorescent dye, Fluo-4 (Fluo-4 AM form, Invitrogen Ltd. Paisley, UK) was used to examine changes in intracellular calcium levels during thermal stimulation. Sections of CA and LM were incubated for 3.5 hours at room temperature in a 5µM Fluo-4 solution in Krebs-Ringer buffer, then transferred to fresh buffer and left for 30 minutes before examination. SM were incubated for 1.5 hours at room temperature in the Fluo-4 solution then transferred to fresh buffer and left for 30 minutes before examination (based on the procedure described by Lamont et al. (2003; 2006)). Rings of CA and LM approximately 2mm in thickness or 4mm lengths of SM

were placed in a Petri dish containing buffer at 37 ˚C under a fluorescent microscope (DM LFS, Leica Microsystems Wetzlar GmbH). Imaging was performed under x20 magnification (excitation at 450 - 490 nm, emission 515 nm). Images were recorded every 5 seconds with a Moticam 2000 digital microscopy camera and Motic Images Plus digital microscopy software suite (Motic, Barcelona, Spain).

Image capture continued for 10 minutes before the bath was rapidly heated by 2-2.5 °C and allowed to cool slowly. Temperature was recorded throughout. Image capture was continued until the temperature returned to baseline over a period of approximately 15 minutes. Noradrenaline (1µM) was then added to check contractility of the vessels and provide a reference change in fluorescence related to maximal smooth muscle stimulation. Image capture continued for a further 5 minutes.

Images were post processed using Octave (GNU Octave, released under GNU GPL). Five square regions of interest were defined across the tissue. The time course of fluorescence intensity throughout the experiments was plotted for each region of interest and for the whole image. Measurements were made on 8 CA, 8 LM and 6 SM. Measurements were also made on samples not exposed to the fluorophore to determine the contribution of intrinsic tissue fluorescence.

Results

Large mesenteric artery

Data were obtained from 14 specimens from 13 animals. 12 further specimens were discarded because of unsatisfactory responses to the vasoactive agents, presumably because of cellular damage during preparation.

After stretching to an initial wall stress of 4.1 ± 0.7 mN/mm^2, wall stress decreased by approximately 80% over 60 minutes at constant strain. Having reached a minimum, strain then began to increase steadily at a rate of approximately 0.01 mN mm^{-2} min^{-1}. It was during this period that all exposures to ultrasound were performed. The steady tension increase was fitted with a polynomial function and subtracted from the data set to provide a zero baseline. Results are expressed as the change in wall stress above the baseline.

Exposure to ultrasound induced reversible vasoconstriction (Figure 3). The response was qualitatively similar to that of the carotid artery. The initial tension increase was rapid, then after approximately 45 s, the rate of vasoconstriction decreased but continued until the end of the exposure period. After this point, the tension began to decay, rapidly at first then more slowly until it reached baseline level after approximately 10 minutes. The response was reproducible over a period of several hours, as long as a recovery period of at least 15 minutes was allowed between exposures.

The mean peak increase in wall stress across all exposures of all samples was 0.020 ± 0.017 mN/mm^2 (mean ± sd, n = 34), approximately 5% of the mean increase in wall stress induced by a 1 μM dose of noradrenaline. The large standard deviation reflects the large inter sample variation: the peak magnitude varied by approximately 80% between vessels, by approximately 60% between samples from the same vessel and by 30 % between repeat exposures.

The relationship between contraction and acoustic power was investigated in 8 samples. The time course of the response was independent of power level, but the amplitude decreased with decreasing intensity, as shown in Figure 4. Figure 5 shows the mean change in wall stress at each acoustic power level for each of the samples. For all except one sample, the change in wall stress due to the lower power level was significantly smaller than for the 100% power level. The ratios of

the mean changes in wall stress at the two power levels were calculated for each sample (n = 3 to 4). The mean of these means was then calculated and these ratios of the change in wall stress at acoustic powers of 100 mW and 72 mW to those at 145 mW are shown in Table 1 (n = 5, and n = 3 respectively), together with data for CA obtained in our previous study (Martin et al. 2010). Both vessels show a very similar, approximately linear relationship. The standard deviations of the ratios are small compared to the standard deviation of the overall result as the effects of inter sample variability have been minimised by the ratiometric analysis.

Small mesenteric artery

Data were obtained from 11 vessels showing satisfactory vasoactive responses. Eight vessels were exposed to ultrasound for four minute periods at an acoustic power of 145 mW (exposure parameters as previously) in the open field configuration. The vessel diameter change during exposure was less than 1%, which was not significant. Three vessels were positioned close to an absorbing panel during ultrasound exposure and also showed no significant changes in diameter. A typical trace of vessel diameter with time is shown in Figure 6. The temperature rise at the vessel surface during ultrasound exposure was less than 0.1 °C in the open field and 0.2 °C with the absorber in place, which is comparable to the temperature rise in the large arteries.

Three vessels were mounted in the myograph and exposed to rapid temperature changes of approximately 1 ˚C. Again, as shown in Figure 6, no significant changes in vessels diameter correlating with periods of increased temperature were observed.

Ion channel analysis

Ion channel inhibition

The effects of blocking potassium channels with barium chloride were investigated in 11 CA rings from 5 animals, and 4 LM rings from 2 animals. The addition of barium chloride had no effect on basal vessel tone. In both types of vessel the time course of the response to ultrasound and recovery after exposure were not affected by the addition of barium chloride, but the magnitude of the response was increased, as can be seen from the set of typical responses shown in Figure 7. *The mean of the change in wall stress during exposures before the addition of $BaCl_2$ was compared to the mean of the change in wall stress after the addition of $BaCl_2$ (n = 3 or 4 for each set for each sample). The mean of these changes was then calculated.* The mean increase in magnitude was 55 ± 49 %, but as the standard deviation indicates, there was considerable variation between samples.

Intracellular calcium temperature dependence

The effects of bath temperature on intracellular calcium fluorescence were investigated in 8 CA and 8 LM preparations. Calcium fluorescence fell as the tracer diffused out of the tissue; this falling baseline level was fitted to an exponential curve and responses to changes in temperature or noradrenaline administration were measured as the increase of intensity relative to the fitted baseline fluorescence. After a step increase in bath temperature of 2.4 ± 0.9 ˚C, the fluorescence increased after a delay of approximately 1 minute and continued to rise for at least 20 minutes while the bath temperature cooled back to the initial conditions (Figure 8). The magnitude of the increase was approximately 35% in both CA and LM, generally smaller than the increase in the fluorescence following administration of noradrenaline.

Experiments in SM arteries showed no change in vessel diameter in response to changes in bath temperature. There was also no increase in calcium fluorescence following a temperature

increase of 2.6 ± 1.3 ˚C, but noradrenaline administration produced a rapid increase in fluorescence similar to that seen in the larger vessels (Figure 9).

Discussion

Mesenteric arteries were selected for this study because the tone of these vessels varies considerably under different physiological conditions and they might therefore be expected to be particularly susceptible to effects of ultrasound. The response in the larger arteries was very similar to that we previously reported in the carotid artery. Quantitative comparison is difficult because there was considerable variation in response between samples, which may reflect uncontrolled variations in metabolic state and initial tone between vessels, as well as experimental factors such as small variations in positioning of the ultrasound beam. The two vessels were similar in terms of smooth muscle cell orientation, though the LM had slightly greater cell density and a 25% higher incremental elastic modulus. These two factors may compensate for each other to give a similar response.

One of the aims of this study was to investigate the involvement of thermal mechanisms in the observed responses. The results from our previous study and from the experiments on large mesenteric arteries in this study showed that the response of these arteries to ultrasound was dependent on acoustic power, pointing to a thermal mechanism, and it was shown that heating of the surrounding medium induced vasoconstriction. In our previous study we also investigated the possibility of mechanical effects in the response. The peak negative acoustic pressure was 0.2 MPa at the highest power level used and the quantity $\dfrac{p_r}{\sqrt{f}}$, calculated as an analogue to Mechanical Index (MI) was 0.1 MPa MHz$^{-1/2}$. As these values are low – the FDA limit for MI = 1.9 and the peak negative pressure threshold for spontaneous cavitation in tissue is of the order of 4 MPa - the potential for mechanical effects is low. Fluid streaming around the tissue during ultrasound exposure

was also investigated and found to be small and to have little effect on vascular tone. Thermal mechanisms therefore seem to be the dominant determinant of the response.

We anticipated that as the mesenteric vessels became smaller and more responsive to changes in smooth muscle tone, the effect of ultrasound would be larger. This proved not to be the case and we were concerned that in vessels less than 1mm in diameter with walls less than 100µm in thickness, ultrasound absorption would be too low to produce thermal effects. The large arteries had a greater area than the -6dB beam width which was approximately 3mm, so the whole beam was incident on the tissue. The spatial average intensity over this area was 0.76 W cm^{-2}, assuming an attenuation coefficient of 0.3 nepers cm^{-1}, the rate of energy deposition per unit volume in the tissue was 0.46 W cm^{-3}. Assuming the density of the tissue is equivalent to that of water, the acoustic dose rate, i.e. the rate of energy absorption per unit mass of the medium (Duck 2009) was 460 W kg^{-1}. The beam would have passed through both walls of the large vessels, and taking into account attenuation in the artery wall upon which the beam was first incident, the energy deposited in the arteries during one four minute exposure period was approximately 10.3 J in the carotid artery and 8.8 J in the mesenteric artery. In the small arteries, the whole cross section of the artery was within the beam area. The volume of tissue within the beam was therefore equal to the cross sectional area of the tissue times a length equal to the beam width. The energy deposited during one four minute exposure to ultrasound was approximately 0.4 J. Assuming the same specific heat capacity for all three tissues, the initial temperature rise would therefore be greater in the large arteries. We therefore undertook a further series of experiments on small mesenteric arteries with an absorber in close proximity to the vessel surface to simulate the effects of surrounding tissue in vivo. Although this procedure produced a temperature rise comparable to that in the larger arteries there was still no contraction.

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A further hypothesis was that lack of response in small arteries was because at the strains employed in the experiments, the incremental elastic modulus was an order of magnitude greater than that of the larger arteries and the two-fold increase in cellularity might be insufficient to generate the requisite contractile force. However, the experiments on channel activation suggested an alternative explanation and provided additional support for our original hypothesis that the effect in large arteries is mediated by temperature-sensitive potassium channels.

In LM and CA, inhibition of potassium inward rectifier channels increased contraction, presumably due to increased flow of calcium into the cell during depolarisation. The inward rectifier channels help to maintain resting membrane potential. By blocking them with $BaCl_2$, the cell was prevented from returning to the rest state by a greater extent than during ultrasound exposure alone. These findings are consistent with evidence presented by Mustafa and Thulesius (2005) who observed that vasoconstriction induced by mild hyperthermia was mediated by potassium channel activity in the smooth muscle cell membrane. Several types of potassium ion channel were implicated in vasoconstriction due to hyperthermia (Mustafa and Thulesius 2005) and further work is required to establish exactly which are involved in this response.

It is well known that there are variations in the ion channels present in different blood vessels. The lack of response in SM would suggest that these channels are absent in these vessels. Calcium fluorescence measurements provided an illustration of the integrated effects of the whole channel population and whilst an increase in temperature caused changes in intracellular calcium in CA and LM, no such change was evident in SM. This suggests that calcium channels or other channels which are involved in pathways leading to calcium channel activity in these vessels are not influenced by thermal changes, or that their activity does not lead to calcium channel activity.

An important and unexpected conclusion of the present study was therefore that temperature-sensitive channels are not present in the small mesenteric arteries.

Caution is clearly required in drawing general conclusions about the effects of ultrasound on blood vessels, but ion channels are almost universally involved in cellular function and channel-mediated effects may also be important in other tissues. For example, studies on isolated cells and frog skin have both shown that the activity of calcium ion channels is affected by ultrasound (Mortimer and Dyson 1988; Dinno et al. 1989; Al-Karmi et al. 1994). If thermal effects on the membrane channels are indeed a ubiquitous phenomenon, physiological responses will depend not only on the electrophysiology of the cells but also on the ultrasound absorption and thermal properties of the tissue and of surrounding structures.

The mechanism by which temperature influences ion channel activity is not yet known. One possibility is that the effect arises indirectly through changes in the physical state of the lipid bilayer rather than directly by action of ultrasound on the protein molecule itself. The membrane lipids exist close to phase transition between liquid and solid under normal conditions and so a small change of temperature could radically alter the physical state of the lipid bilayer and its interaction with embedded proteins. Such changes could be similarly induced by heating from sources other than ultrasound, such as microwave and RF radiation and these issues require further investigation.

Conclusion

In the first part of this study we showed that exposure to 3.2 MHz ultrasound at powers within the diagnostic range induced reversible contraction of the equine lateral cecal mesenteric artery in vitro which was linearly dependent on acoustic power and was likely to be thermally mediated. A similar response was demonstrated in our previous study for equine common carotid artery in vitro, these new results show that this result is not limited to that particular vessel.

However, no such effect was observed in first order branches of the lateral cecal mesenteric artery. The response to ultrasound was shown to be mediated by inhibition of inward rectifier potassium channels in the smooth muscle cell membrane. In carotid and large mesenteric arteries it was also found that increases in temperature caused changes in ion channel activity that led to increases in intracellular calcium concentration. In small mesenteric arteries, no such change in intracellular calcium was observed.

Acknowledgements

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Figure Captions

Figure 1 Typical stress-strain curves for carotid artery and large mesenteric artery rings obtained from force extension curves, and for sections of 1st order braches of the mesenteric artery obtained from pressure radius curves.

Figure 2 a) Schematic diagram of wire myograph for large-vessel measurements; b) pressure myograph for small vessels.

Figure 3 A typical response of a mesenteric artery section during a 4 minute exposure to ultrasound at 145 mW (black line), a typical response of a carotid artery is also shown for comparison (grey line) (data taken from previous study (Martin et al. 2010)); the dotted lines indicate the beginning and end of the exposure. Wall stress increases during the period of insonation, rapidly at first, then at a decreasing rate, before slowly returning to baseline after the exposure.

Figure 4 Typical responses to 4 minute ultrasound exposures at different acoustic power levels; a)— exposure to ultrasound at 145 mW, — exposure to ultrasound at 100 mW; b) —exposure to ultrasound at 145 mW, — exposure to ultrasound at 72 mW; the dotted lines show the times at which the exposure began and ended.

Figure 5 a) Results from experiments on 5 artery rings (numbered 1 – 5 on horizontal axis) showing the mean change in wall stress due to exposure at the 100% acoustic power level ■ and the 69% acoustic power level □ (*n = 3, 4, 3, 3, 4 for sets of exposures in each group respectively)*; b) results showing the mean change in wall stress due to exposure of three artery rings (1-3 on axis) to ultrasound at the 100% acoustic power level ■ and the 50% acoustic power level □ *(n = 4 for each set of exposures in each group)*. Error bars show ± 1 standard deviation of the mean.❖ shows a P-value of ≤0.1, ◆ shows a P-value of ≤0.05.

Figure 6 Typical time course of vessel diameter during: (black line) exposure to ultrasound for a period of 4 minutes denoted by the dotted lines with a section of absorbing material placed close to the vessel to increase heating; (grey line) a period of rapid temperature increase of approximately 1 ˚C beginning at time a (T = 37.4 ˚C), reaching maximum at time b (T = 38.5˚C), then returning to baseline temperature. The vertical black line illustrates the pixel size in the images of the vessel.

Figure 7 Typical responses to 4 minute ultrasound exposures before (▬) and after (▬) the addition of $BaCl_2$ to a concentration of 4 μM in the bath for a) a carotid artery ring and b) a mesenteric artery ring. The dotted lines show the beginning and end of the period of ultrasound exposure.

Figure 8 Typical time course of changes in fluorescence intensity in Fluo-4 loaded mesenteric artery ring during temperature elevation by approximately 1.5 ˚C and noradrenaline induced contraction. The time course of the ratio of measured fluorescence intensity to predicted baseline intensity is shown. Temperature increase of approximately 1.5 ˚C was initiated at time a, noradrenaline (1 µM) was added at time b. The time course of the bath temperature is also shown (lower graph).

Figure 9 Typical time course of changes in fluorescence intensity in Fluo-4 loaded length of small mesenteric artery during temperature elevation by approximately 1.5 ˚C and noradrenaline induced contraction. The time course of the ratio of measured fluorescence intensity to predicted baseline intensity is shown. Temperature increase of approximately 1.5 ˚C was initiated at time **a**, noradrenaline (1 µM) was added at time **b**. The time course of the bath temperature is also shown (lower graph).

Tables

Table 1 Magnitude of change in wall stress due to ultrasound at 145 mW and proportional changes at powers of 100 mW and 72 mW for the lateral cecal mesenteric artery and the carotid artery (* shown for comparison – data taken from Martin et al. (2010)).

Power (mW)	Change in wall stress	
	Mesenteric artery	Carotid artery *
145 (100%)	0.020 ± 0.017 mN/mm^2	0.020 ± 0.018 mN/mm^2
100 (69%)	0.71 ± 0.14 of max	0.68 ± 0.07 of max
72 (50%)	0.48 ± 0.11 of max	0.53 ± 0.1 of max