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● *Original Contribution*

LOW-FREQUENCY (20 KHZ) ULTRASONIC MODULATION OF DRUG ACTION

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Abstract—We tested the effect of low-frequency ultrasound (LUS, 20 kHz, 4 W/cm^2) on the function of rat mesentery and human pulmonary arteries with wire myography. The vessels were induced to contract with either noradrenaline or physiologic saline solution (PSS) with a high potassium concentration (KPSS) and then incubated with capsaicin (2.1×10^{-7} M, TRPV1 [transient receptor potential vanilloid 1] activator), dopamine (1×10^{-4} M, dopamine and α_2-receptor activator), or fenoldopam (dopamine$_{A1}$ receptor agonist, 1×10^{-4} M) with and without glibenclamide (1 μM, KATP [adenosine triphosphate {sensitive potassium channel (ATP)}-sensitive potassium channel] inhibitor and α_2-receptor modulator), and insonated. Vessels were incubated in Ca^{2+}-free PSS and induced to contract with added extracellular Ca^{2+} and noradrenaline. Pulmonary arteries were induced to contract with KPSS and dopamine. Then the vessels were insonated. LUS inhibited the influx of external Ca^{2+}, inhibited the dopamine-induced vasoconstriction in the KPSS (glibenclamide reversible), reduced the capsaicin-induced vasorelaxation, increased the gentamicin-induced vasorelaxation and increased the dopamine-induced contraction in the KPSS in human pulmonary arteries. (E-mail: Silvijus.Abramavicius@lsmu.lt) **© 2020 The Author(s). Published by Elsevier Inc. on behalf of World Federation for Ultrasound in Medicine & Biology. This is an open access article under the CC BY-NC-ND license. (http://creativecommons.org/licenses/by-nc-nd/4.0/).**

Key Words: **Low-frequency ultrasound, Calcium signaling, Ultrasonic modulation, Drug action, Insonation.**

INTRODUCTION

Low-frequency ultrasound (LUS) induces endothelium-independent vasodilation *in vivo* (Fischell et al. 1991; Steffen et al. 1994). LUS induces vascular relaxation in the canine coronary arteries (Miyamoto et al. 2003), human superficial femoral arteries (Siegel et al. 1992) and brachial arteries (Iida et al. 2006). Contradictory findings indicate that LUS-induced vasodilation is endothelium dependent and is abolished by nitric oxide synthase inhibition (Suchkova et al. 2002). Some authors have proposed that LUS-induced vascular relaxation is mediated *via* changes in prostacyclin release (Maruo et al. 2004). Recently, it was reported that insonation before contraction promotes vascular contraction in human thoracic arteries, and calcium is involved in the contraction mechanism (Bubulis et al. 2017). Very recently, high-frequency ultrasound (27.38 MHz) was reported as a means to modulate the voltage-gated potassium currents (in pyramidal neurons) (Lin et al. 2019); thus, it is more likely than not that potassium channels (K^+) are involved in the vascular LUS effects.

Various cellular mechanisms have been described to explain the biological effects of insonation, including the upregulation of vascular endothelial growth factor, endothelial nitric oxide synthase (eNOS), proliferating cell nuclear antigen and chemoattractant factors and activation of stem cells (Liu et al. 2019). Sonoporation, which is an ultrasound-induced increase in the permeability of biological barriers, may explain how ultrasound may alter pharmacologic drug action (Hu et al. 2013; Bouakaz et al. 2016). A recent article incorporating the aforementioned concepts reported that insonation may increase the response to phosphodiesterase type 5 inhibitors in patients with erectile dysfunction, thus laying the ground for the hypothesis that insonation may alter and potentiate the pharmacologic drug action (Tsai et al. 2017) or be effective as a standalone intervention (Cui et al. 2019). Low-intensity ultrasound has

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found various other potential applications in medicine: oncological sonodynamic therapy, ultrasound-mediated chemotherapy, gene delivery and vascular ablation (Wood and Sehgal 2015).

The aim of the work described here was to determine whether LUS can alter the pharmacologic effects of drugs on isolated rat mesentery and human pulmonary vessels.

METHODS

Ethics statement

The investigation was carried out in accordance with the *Guide for the Care and the Use of Laboratory Animals* published by the U.S. National Institutes of Health (2011), and the Animal Research: Reporting of In Vivo Experiments guidelines (Kilkenny et al. 2010) were carefully followed. Also, this research was approved by a local Institutional Care and Animal Use Committee. Adult Wistar rats (12−14 wk) were euthanized with CO_2 and decapitated. The study with human pulmonary arteries was conducted according to the principles defined by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH 1996) and the Declaration of Helsinki (Carlson et al. 2004). Permission to perform this study was obtained from the local institutional review board of the Kaunas Regional Biomedical Research Ethics Committee. Furthermore, written informed consent was obtained from all patients that allowed use of their lung tissue for pulmonary artery harvesting.

Chemicals and materials

The drugs used were noradrenaline (NA), acetylcholine, glibenclamide, gentamicin, fenoldopam, dopamine, capsaicin and ethylene glycol-bis(*β*-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) from Sigma-Aldrich (St. Louis, MO, USA). The physiologic salt solution (PSS) comprised NaCl 119 mM, $NaHCO_3$ 25 mM, glucose 5.5 mM, $CaCl_2$ 1.6 mM, KH_2 O_4 1.18 mM, $MgSO_4$ 1.17 mM and EDTA 0.027 mM. The Ca^{2+}-free PSS solution was identical to the PSS solution except for the exclusion of $CaCl_2$. The 119 mM K^+ solution stands for physiological saline solution with high potassium concentration (KPSS) had the same composition as the PSS, but with NaCl replaced by KCl on an equimolar basis to reach the final 119 mM K^+ concentration.

LUS

LUS with a previously described ultrasound tube-shaped waveguide wire (Figs. 1 and 2) (Bubulis et al. 2018). The ultrasonic device was immersed in the tissue organ bath, so that the vessel would be insonated externally, as the current hypothesis is that LUS can produce identifiable biological effects in vessel tissues without the need for intravascular access. The ultrasound generator VT-400 has a supply voltage of 200−240 V and output power up to 400 W, operates in the output frequency between 15 and 60 kHz (a 20-kHz frequency was used during the experimental procedures) and has dimensions of 300 × 425 × 135 mm (Fig. 3). The waveguide wire is 260 mm in length and 1.5 mm in diameter and is considered to be an interventional medical device (Figs. 1 and 2). The ultrasound transducer comprises the conical concentrator (**1**) and four piezo ceramic rings PZT-4 (**2**) with a diameter of 25 mm and thickness of 5 mm that are fitted on the conical concentrator and spaced by 0.5-mm-diameter copper rings and paper insulation. The entire system is reinforced with a 16-mm-thick, stainless steel fastening component smaller by 1 mm in diameter (Bubulis et al. 2018). The power density used during the

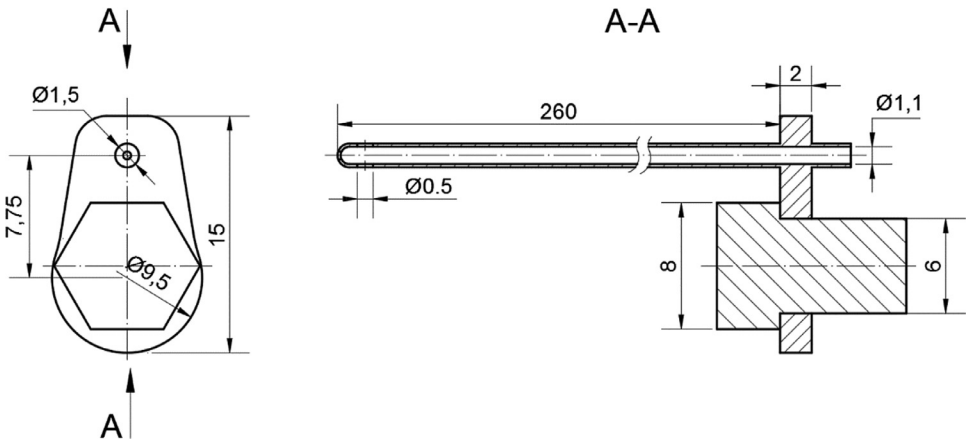


Fig. 1. Ultrasonic waveguide wire device (cross-section).

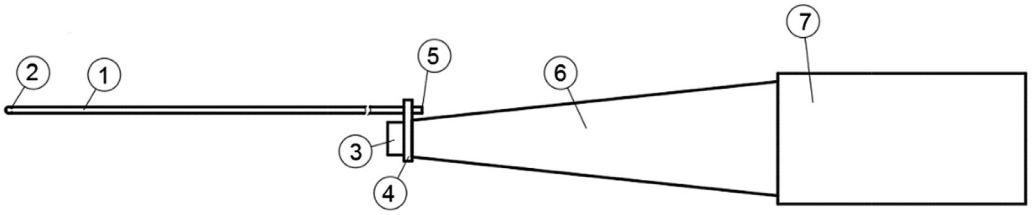


Fig. 2. Ultrasonic waveguide device: **1** = tube-shaped waveguide; **2** = waveguide hole; **3** = fixing screw; **4** = lug; **5** = intake; **6** = concentrator; **7** = transducer.

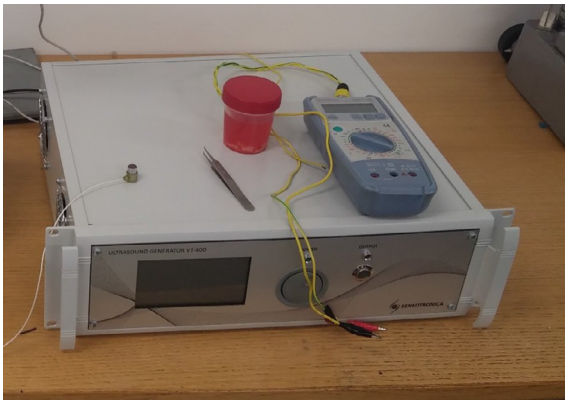


Fig. 3. Ultrasound generator VT-400.

experiments was 4 W/cm^2, 20 kHz. Waveguide displacement measurements were made using a Polytec PSV 3D laser vibrometer (Polytec, Waldbronn, Germany). Analysis of the results revealed a resonance for the system of 20.33 kHz. Maximum displacements along the z-axis at the frequency of 20.33 kHz were 29 nm. Because the principal functionality of the ultrasonic system is to trespass the thrombus in the frontal direction (along the z-axis), this particular frequency is hereinafter considered to be the operational frequency of the system. Meanwhile, maximum displacements in the directions of the x-axis (with an amplitude of 69 nm) and y-axis (with an amplitude of 21 nm) mean that the operational end of the waveguide is drawing an ellipse of the maximum radius, which in turn enables us to assume that the cavitation process is the most vigorous at this frequency. Results obtained through investigation on waveguide filled with water and placed in a tube that was also filled with water revealed a maximum displacement of 2.1 μm. The wave period was observed to decrease more than 1.5 times (Navickas 2018). The acoustic power density was 4 W/cm^2 at 20.33 kHz. This corresponds to a mechanical index (MI) of 2.43, which is above what the safe threshold would be if it were administered intraluminally (Barnett 1998). The waveguide was placed about 10 mm away from the blood vessel and immersed in the same suspension, which also serves as a damper to reduce the influence of MI on the wall of the blood vessel. Every insonation iteration lasted no longer than 10 s; the temperature in the tissue bath in which the vessel was immersed was monitored throughout the experiment and was kept within physiologic temperature (37°C) limits. No damage to the vessel tissue was observed during the experiments.

Data gathered in a previous experiment revealed that it takes as little as 5 s to reach the temperature of 42°C (from a starting point of 36°C) but does not exceed 45 °C in a few minutes of continuous work.

Experimental protocol

To investigate LUS-induced relaxation, arterial segments with functioning endothelium were mounted (Hedegaard et al. 2016). Only vessels with endothelium were used in our study: when acetylcholine (10 μM) (or the KPSS in human pulmonary arteries) induced at least 50% relaxation in the vessels pre-contracted with 5 μM NA, the vessels were considered as having a functioning endothelium.

Functional studies in mesenteric arteries. Rat mesenteric and human pulmonary arteries were dissected from the vascular bed and mounted on the 40-μm steel wires in the myographs (Danish Myotechnology, Aarhus, Denmark) for isometric tension recording as previously described (Mulvany and Halpern 1977) (Fig. 4). The vessels were equilibrated in oxygenated (5% CO_2, 20% O_2, 75% N_2) PSS at 37°C for 30 min and, by stretching, normalized to a lumen diameter (d100) equivalent to 100 mm Hg (23 mm Hg in human pulmonary arteries), after which tension was set to 90% × d100 (Mulvany and Halpern 1977). After normalization, the arterial segments were stimulated with KPSS, washed in PSS and stimulated with 10 μM NA. Arteries were included only if they developed an active force corresponding to a transmural pressure of 100 mm Hg (or 23 mm Hg in human pulmonary arteries). The PowerLab data system and Chart 5.5 (ADInstruments, Oxfordshire, UK) were used to record the data. Mechanical responses of the vessel segments were measured as active wall tension (ΔT), which is the change in force (ΔF) divided by twice the segment length (Mulvany and Halpern 1977).

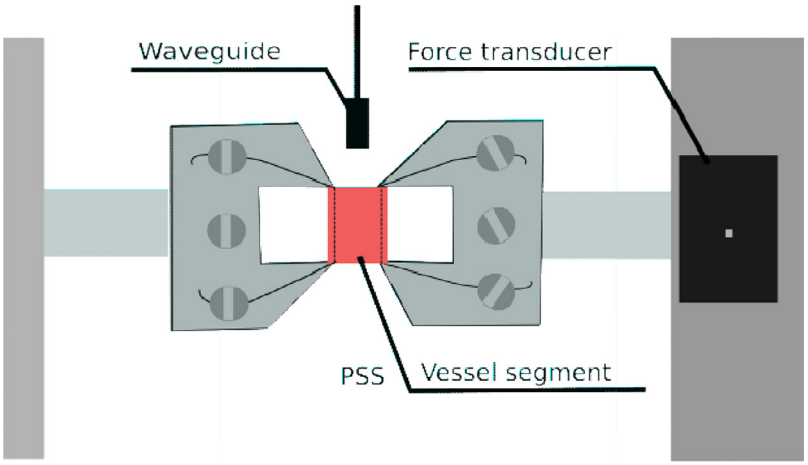


Fig. 4. Small-vessel wire myography. When an isometric contraction occurs in a mounted vessel segment, it is registered by a force transducer and transmitted to a computer.

LUS effect on dopamine and fenoldopam vasomodulation via potassium channels. The high extracellular potassium concentration activates the potassium channels and induces endothelium-independent vascular contraction by depolarizing the muscle cell membrane, resulting in opening of the voltage-dependent Ca^{2+} channels and a surge of calcium ions from the extracellular space inward (Karaki et al. 1984; Arvola et al. 1992). KATP channels (a subtype of potassium channels, activated by the KPSS, composed of KIR6 × [6.1 or 6.2] and SUR subunits) modulate the vascular tone by controlling membrane potential and, subsequently, the influx of Ca^{2+} through the L-type voltage-dependent Ca^{2+} channels (Tinker et al. 2014). The opening of the KATP channels makes a major contribution to vascular smooth muscle cell hyperpolarization (Tinker et al. 2014) (Fig. 5).

Dopamine acts on several dopamine (D_1−D_5) receptors (Pyne-Geithman et al. 2009; Beaulieu et al. 2015), α_2-receptors, causes vascular contraction (Dai et al. 1989) and activates the sensitive potassium channel (ATP)-sensitive K^+ currents, *via* the D_1 dopamine receptors, adenylate cyclase and protein kinase A (Kawano et al. 2008; Wu et al. 2001). The KATP inhibitor glibenclamide (Wu et al. 2001) modulates α_2-receptor signaling (Fagerholm et al. 2011) and the effects the KATP channels (functionally intertwined with the α_2-adrenoreceptors

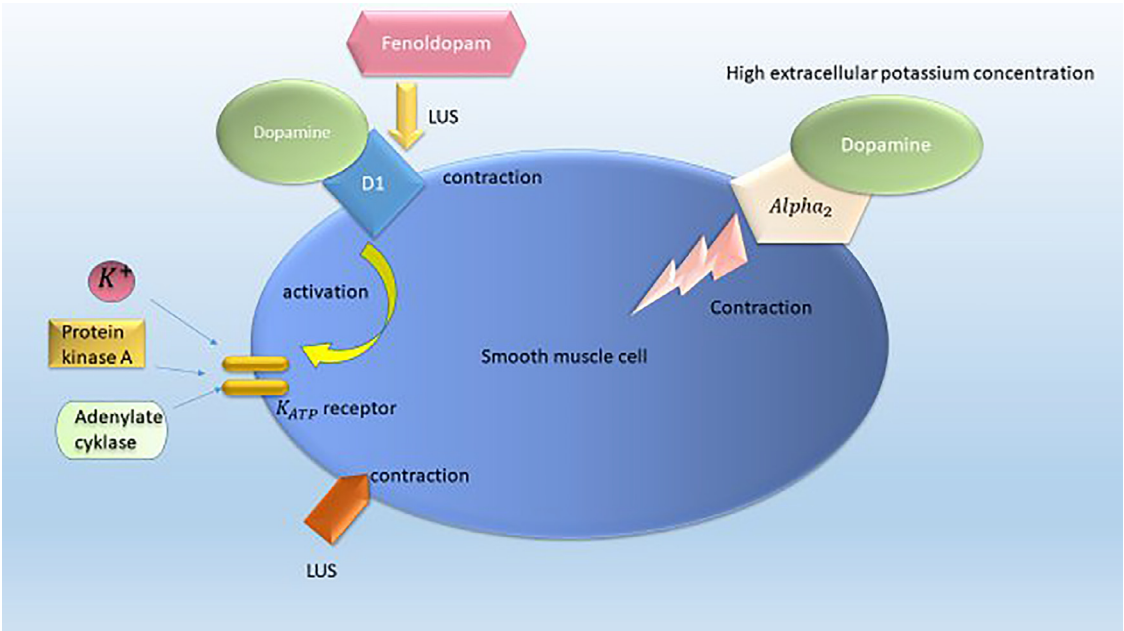


Fig. 5. Effect of low-frequency ultrasound (LUS) on dopamine and fenoldopam vasomodulation *via* potassium channels.

[Jonas et al. 1992; Thomas et al. 1997]). Thus, we aimed to test if the potassium channels, especially KATP and α_2-receptors, could be modulated with LUS. Therefore, the LUS effect was tested in the KPSS solution with the addition of dopamine alone or in combination with glibenclamide (Fig. 5).

Fenoldopam is a selective dopamine receptor (D_{A1}) agonist (Han et al. 1999; Truskey and Fernandez 2015; Szymanski and Richards 2019). As a vascular dopamine (D_{A1}) receptor agonist it produces vascular relaxation in a dose-dependent manner in human brachial, cerebral, cervical, colic, coronary, lumbar, pulmonary, renal, splenic arteries (Hughes and Sever 1989), subcutaneous and omental small arteries (Hughes and Sever 1989). The converse effect is observed in human umbilical arteries, where fenoldopam and dopamine induce vasoconstriction (Sato et al. 2003). Because dopamine is active at several dopamine (D_1−D_5) receptors (Pyne-Geithman et al. 2009; Beaulieu et al. 2015), we aimed to compare the dopamine effect on blood vessels in a high-potassium environment with that of fenoldopam, under similar conditions, to elucidate if dopaminergic signaling can be modulated with LUS in rat mesenteric arteries, where the D_1 and D_2 receptors are abundant (Kim et al. 1999) (Fig. 5).

Calcium signaling in the vascular contraction and the effect of LUS on $CaCl_2$- induced vascular contraction. The increased Ca^{2+} cytosolic concentration activates vascular contraction *via* the calcium/calmodulin complex with the myosin light chain, by phosphorylation of the light chain of myosin (Goulopoulou and Webb 2014). Cytosolic Ca^{2+} levels rise as a result of calcium mobilization from the sarcoplasmic reticulum, calcium entry from the extracellular space through the plasma membrane calcium channels and other mechanisms (voltage-operated channels, *e.g.,* L-type, the receptor-operated channels, the store-operated calcium entry mechanism [by which reduced calcium concentration in the endoplasmic/sarcoplasmic reticulum promotes calcium entry *via* the activation of plasma membrane calcium channels], purinergic receptors, transient receptor membrane potential [TRP] channels and the Na^+/Ca^{2+} exchanger) (Goulopoulou and Webb 2014). The reduction of extracellular $CaCl_2$ concentration results in vessel contractions of smaller magnitude (Taggart et al. 1995) and it was used to illustrate the effects of Ca^{2+} entry blockers (*e.g.,* nicardipine) (Salom et al. 1990). LUS was used to insonate the vessels during $CaCl_2$-induced contraction to see if LUS can alter the entrance of extracellular Ca^{2+} into vascular smooth muscle cells. EGTA was used as a chelator to selectively bind Ca^{2+} before the addition of extracellular $CaCl_2$ (Kähönen et al. 1994) (Fig. 6).

Amlodipine incubation. Amlodipine is an L-type (and T-type [Cerbai and Mugelli 2018]) calcium channel blocker (Striessnig et al. 2015). Voltage-gated calcium channels (*e.g.,* L-type and T-type Ca^{2+} channels) mediate calcium ion flow through the cell membrane from the extracellular space and mediate vascular contraction (Borysova et al. 2018; Cerbai and Mugelli 2018). Amlodipine was used to block L-type Ca^{2+} channels during $CaCl_2$-induced contraction (in Ca^{2+}-free PSS).

Aminoglycoside (gentamicin)-induced vascular relaxation modulation with LUS. The aminoglycosides (Gergawy et al. 1998) seem to interfere with intracellular Ca^{2+} accumulation; inhibit phospholipase C (PLC), protein kinase C, transmembrane calcium channels and

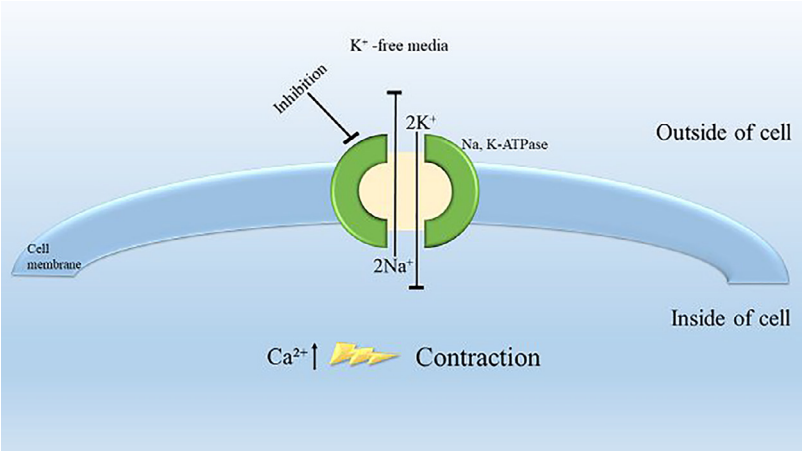


Fig. 6. Calcium signaling in the vascular contraction and the effect of low-frequency ultrasound on $CaCl_2$-induced vascular contraction.

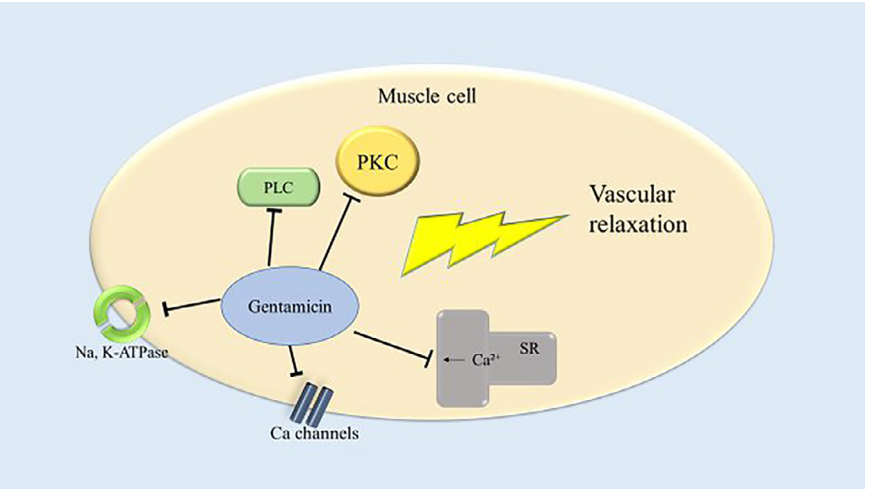


Fig. 7. Aminoglycoside (gentamicin)-induced vascular relaxation modulation with low-frequency ultrasound. PKC = protein kinase C; PLC = phospholipase C; SR = sarcoplasmic reticulum.

calcium release from the sarcoplasmic reticulum of the skeletal muscle (Gergawy et al. 1998); alter ^{45}Ca movement and inhibit the contractile smooth muscle response (Adams et al. 1974; Goodman and Adams 1976); and inhibit the basolateral calcium ATPase or Na^+-K^+ ATPase (Elliott and Patchin 1992). The effect of LUS on gentamicin-induced vascular relaxation was used as a supportive finding to explore the LUS effect on the calcium signaling-dependent vascular relaxation (Fig. 7).

Sodium/potassium ATPase inhibition and LUS. The distribution of ions between the intracellular and extracellular spaces is mediated by Na^+, K^+-ATPase, inhibition of which increases the calcium concentration in the vascular cells and causes vascular contraction (Aperia 2001). Na^+, K^+-ATPase can be inhibited with ouabain (Nguyen et al. 2007) or addition of K^+-free medium (Arvola et al. 1992). We immersed the vessels in K^+ free PSS and added KCl to contract the vessels (the KCl concentration was gradually increased: $C01 = 3 \times 10^{-6}$ M, $C0_2 = 1 \times 10^{-5}$ M, $C03 = 3 \times 10^{-5}$ M and $C04 = 1 \times 10^{-4}$ M) and insonated them to determine if LUS has an effect on the action of the Na^+, K^+-ATPase. This effect was compared with the contraction of the control vessels, which were induced to contract only with the KCl without insonation. The K^+-free buffer solution was prepared by substituting the KH_2PO_4 and KCI with NaH_2PO_4 and NaCl, respectively, on an equimolar basis (Arvola et al. 1992) (Fig. 8).

LUS effect on transient receptor potential vanilloid 1 receptors. Transient receptor potential vanilloid 1 (TRPV1) is expressed by vascular endothelial cells, hepatocytes, adipocytes, smooth muscle and other cells

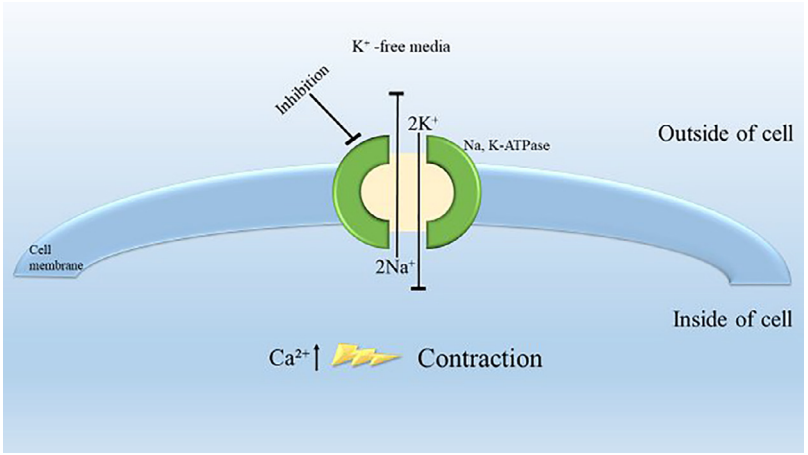


Fig. 8. Sodium/potassium ATPase inhibition and low-frequency ultrasound.

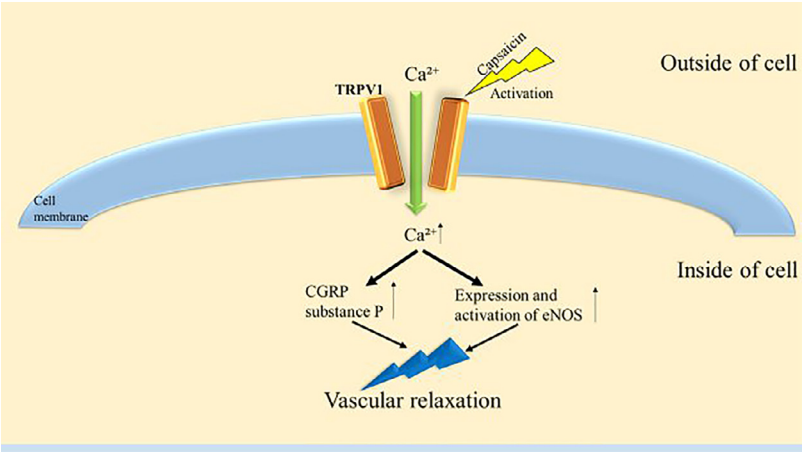


Fig. 9. Effect of low-frequency ultrasound on transient receptor potential vanilloid 1 (TRPV1) receptors. CGRP = calcitonin gene-related peptide.

(Gunthorpe and Szallasi 2008). TRPV1 is a membrane receptor, acting as a non-specific cation channel, which, on activation by capsaicin (or physiologic activators: heat, low pH or some lipid metabolites), allows inward calcium flow and increases intracellular free calcium levels (McCarty et al. 2015); this, in turn, activates calcitonin gene-related peptide and substance P release from the perivascular sensory nerve terminals (Yang et al. 2010) or increases the expression and activation of eNOS, in endothelial cells) and causes vascular relaxation (McCarty et al. 2015). LUS was used to modulate the effect of capsaicin (a TRPV1 receptor activator [McCarty et al. 2015]) in the endothelium intact vessels (Fig. 9).

Data and statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM) with a significance level of $p < 0.05$; n represents the number of individual animals. A two-way analysis of variance was used to compare the means of functional studies observations. Graphs were created and statistical analyses performed using the SAS University Edition.

Group size

Each experiment was performed five times on rat mesentery vessels harvested from different animals. Three vessels were harvested from different humans undergoing pneumectomy.

Selection criteria

Adult Wistar rats (12−14 wk) were used in this study to harvest mesentery vessels. Pulmonary arteries were harvested from patients undergoing pneumectomy.

RESULTS

LUS failed to induce vascular relaxation in the vessels induced to contract with 10 μM NA; the same lack of effect was observed in the vessels pre-contracted with 10 μM NA and incubated with 10 μM indomethacin for 20 min. In KPSS-induced vascular contraction, LUS also exhibited no discernable effect (the experiment was repeated on vessels from three different animals).

LUS effect on influx of external Ca^{2+} ions in vascular contraction

LUS inhibits the influx of external Ca^{2+} ions and thus inhibits vascular contraction (Fig. 10a−d). This effect is mostly visible in $CaCl_2$-induced vascular contraction in Ca^{2+}-free PSS with 1×10^{-4} M EGTA and L-type Ca^{2+} blockade with 3×10^{-5} M amlodipine and 10 μM NA (Fig. 10). In addition, LUS seems to potentiate the gentamicin-induced vascular relaxation (pre-contracted with 10 μM NA): 6×10^{-5} mol gentamicin + LUS versus 6×10^{-5} mol gentamicin alone produces 59.28% and 39.47% vascular relaxation, respectively (two animals, data not shown).

LUS and the TRPV1 channel blockade

LUS significantly decreased vascular relaxation in the vessels immersed in standard PSS, pre-contracted with 10 μM NA and treated with increasing concentrations of capsaicin, a TRPV1 receptor activator (Fig. 11).

Sodium/potassium ATPase inhibition

LUS had no discernable effect on the action of sodium/potassium ATPase (Fig. 12).

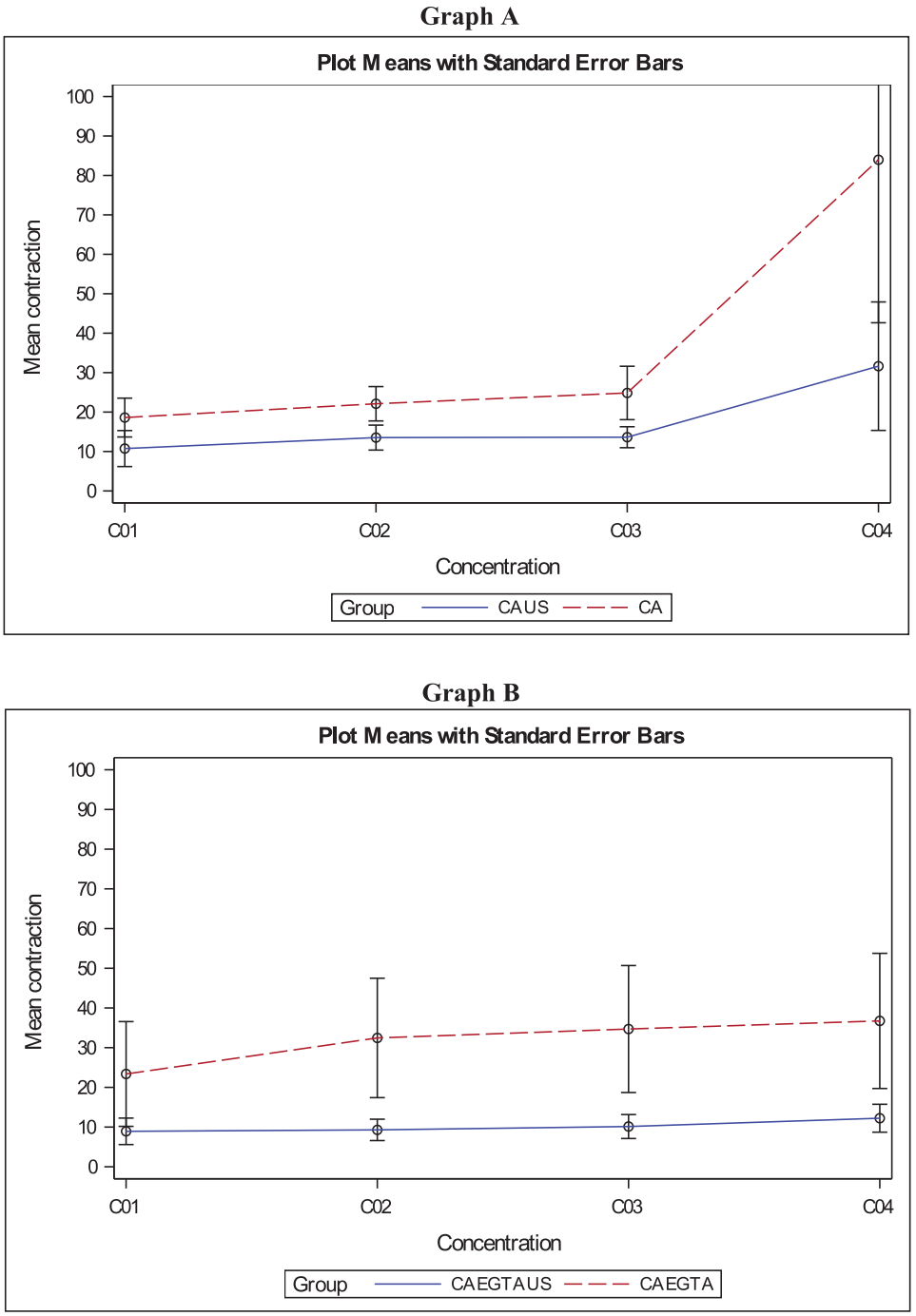
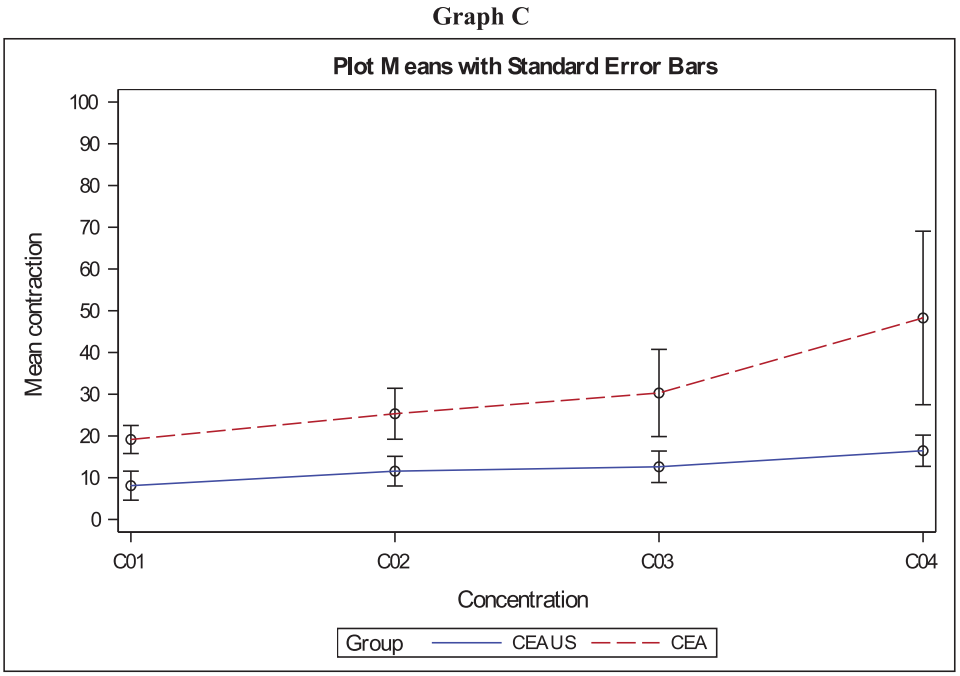


Fig. 10. Effect of low-frequency ultrasound on the influx of external Ca^{2+} ions in vascular contraction. Graph A: External $CaCl_2$-induced contraction (represented as the mean and standard error). Two-way (class factors: concentration and groups) analysis of variance (ANOVA): $F(7) = [2.51, 0.68]$, $p = [0.06, 0.44]$. Graph B: External $CaCl_2$-induced contraction with EGTA (represented as mean and standard error). Two-way (class factors: concentration and groups) ANOVA: $F(7) = [3.36,0.01\ ]$, $p = [0.08,0.94]$. Significant contraction ($p = 0.03$) at C02. Graph C: External $CaCl_2$-induced contraction (represented as mean and standard error) in EGTA and L-type calcium channel blockade with amlodipine in low-frequency ultrasound (CEAUS) and control group (EA); n = 5. Two-way ANOVA: $F(7) = [3.72, 4.56]$, $p = [0.07, 0.07]$. Significant contraction occurs at C02, $p = 0.0176$. Graph D: Low-frequency ultrasound versus control (the experimental data from graphs A–C were merged) group (EA), n = 15. Two-way ANOVA: $F(25) = [4.39,6.14\ ]$, $p = [0.01,0.02]$. Graphs A–D represent the vascular response to increasing concentrations of extracellular $CaCl_2$: C01 = 3×10^{-6} M, C02 = 1×10^{-5} M, C03 = 3×10^{-5} M, C04 = 1×10^{-4} M.



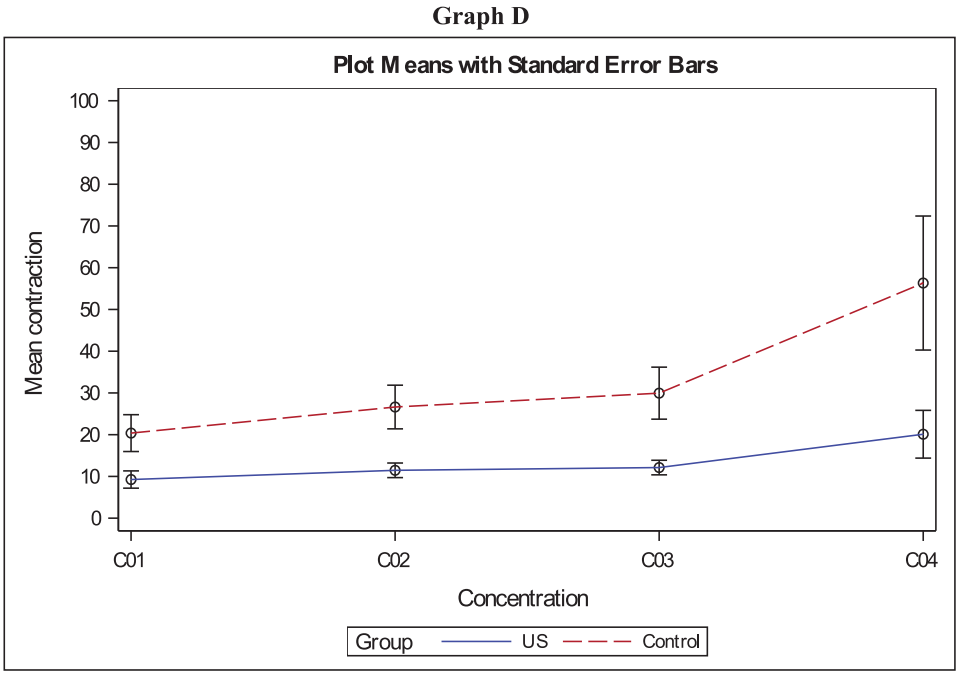


Fig. 10. Continued

LUS effect on potassium channels

LUS seems to inhibit dopamine-induced vascular contraction in mesenteric arteries pre-contracted with KPSS. This effect was abolished with 1 μM glibenclamide (Fig. 13a, 13b). The contraction induced by 1×10^{-4} M dopamine + KPSS was repeated in human pulmonary arteries, where dopamine alone induced a mean contraction (n = 3) of 143.03% contraction and dopamine + LUS produced a mean contraction (n = 3) of 166.38 % (in comparison to the maximum KPSS contraction). The Fenoldopam (a selective dopamine [D_{A1}] agonist) failed to produce vascular relaxation (in KPSS-contracted vessels) in both LUS-insonated and control vessels (Fig. 13c, 13d).

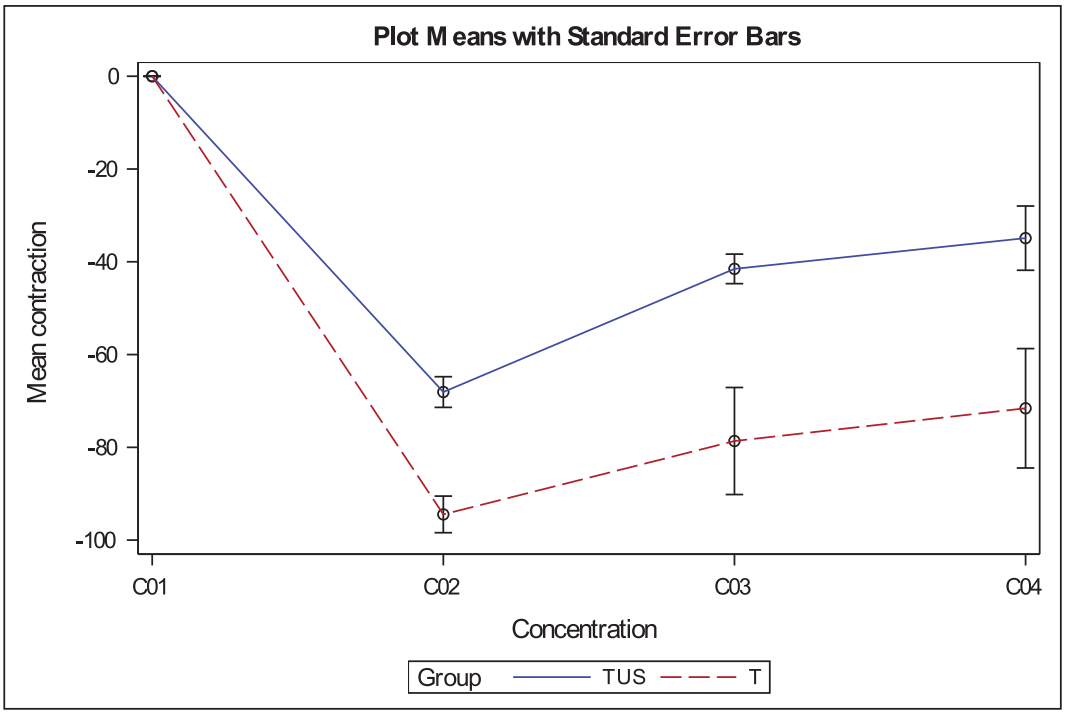


Fig. 11. Low-frequency ultrasound (LUS) and transient receptor potential vanilloid 1 (TRPV1) channel blockade. TRPV1 channel blockade in LUS (TUS) and control group (T), n = 5. Two-way analysis of variance (ANOVA): $F(7) = [14.14, 38.41]$, $p = 0.0035$ (concentration), $p(\text{group}) = 0.0004$. C01 = 10 μM noradrenaline (or 0 M capsaicin) contraction; C02 = 7×10^{-8} M capsaicin; C03 = 1.4×10^{-8} M capsaicin; C04 = 2.1×10^{-7} M capsaicin.

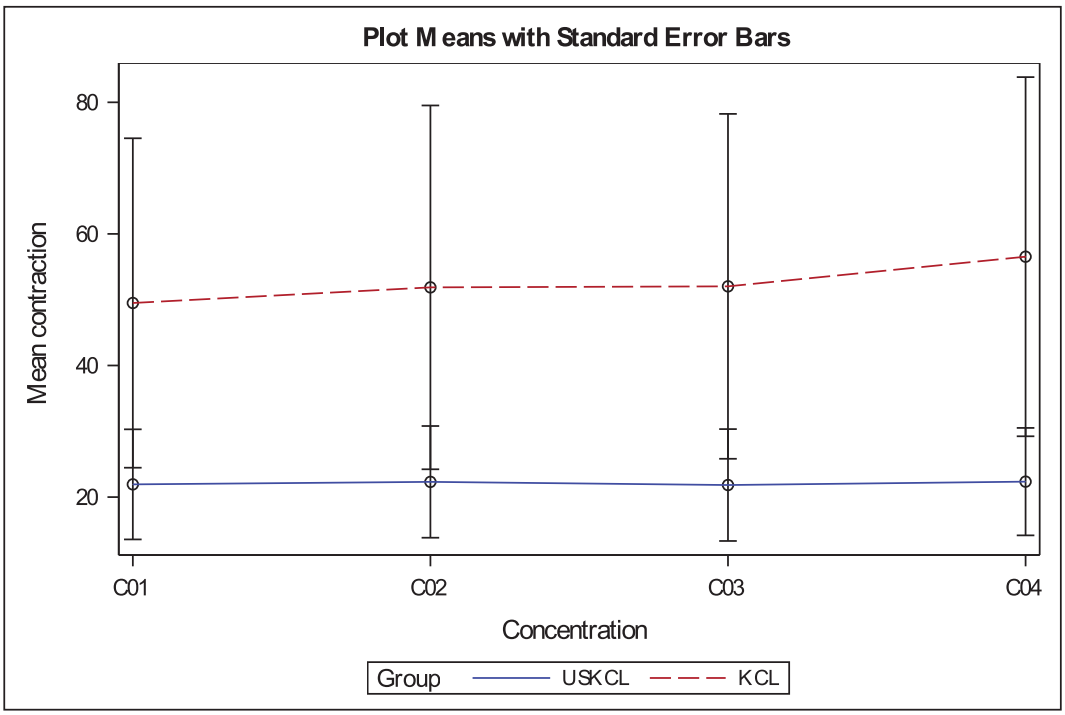
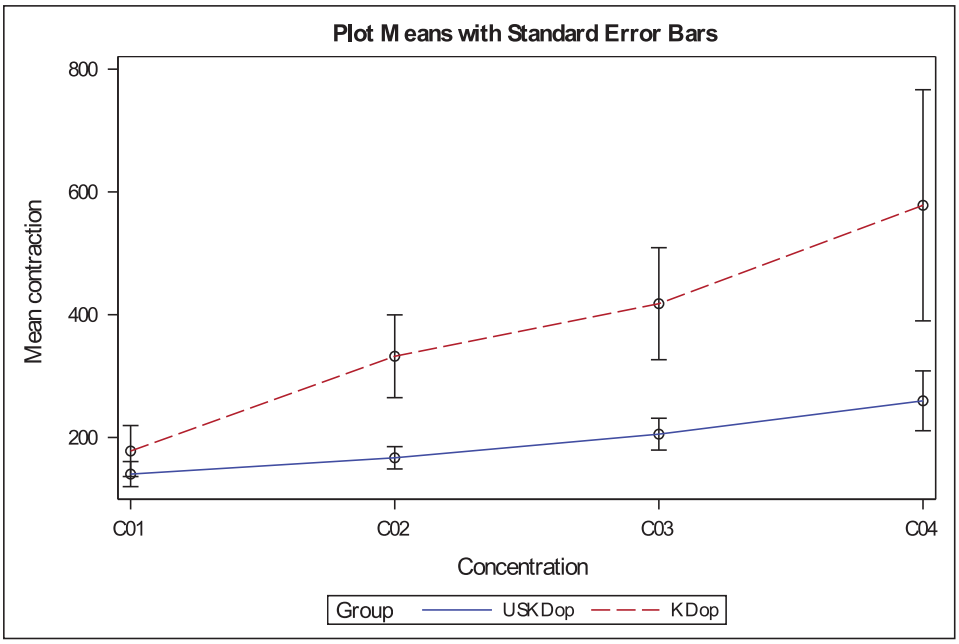


Fig. 12. Low-frequency ultrasound (LUS) and sodium/potassium ATPase inhibition. KCl induced vascular contraction in LUS (USKCL) and control (KCL) groups. ANOVA: $F(8) = [2.87, 0.88]$, $p = [0.10, 0.37]$, n = 5. The KCl concentration was gradually increased: C01 = 3×10^{-6} M, C02 = 1×10^{-5} M, C03 = 3×10^{-5} M and C04 = 1×10^{-4} M.

Graph A



Graph B

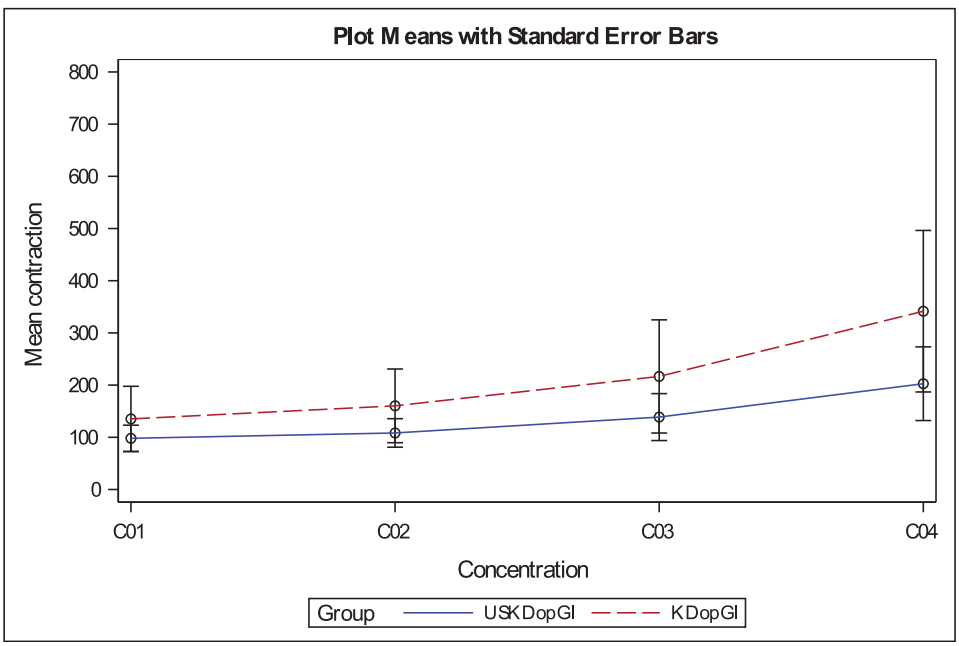
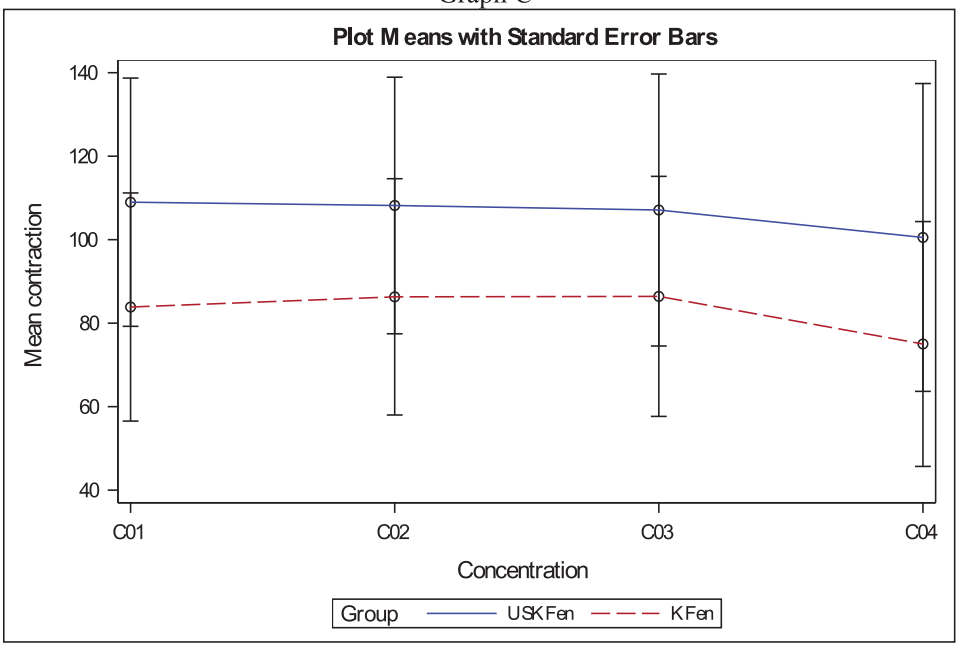


Fig. 13. Effect of low-frequency ultrasound on dopamine and fenoldopam vasomodulation in high-potassium environment. The experiment was conducted with increasing concentrations of either dopamine or fenoldopam: C01 = 1×10^{-5} M, C01 = 3×10^{-5} M, C03 = 1×10^{-4} M. Graph A: LUS effect on dopamine-induced vascular contraction in KPSS (USKDop) and control (KDop) groups. Two-way analysis of variance (ANOVA): $F(8) = [7.55,5.88]$, $p = [0.01, 0.04]$, n = 5. Graph B: LUS effect on dopamine-induced vascular contraction in KPSS (USKDopGl) and control (KDopGl) groups in KATP channel blockade with glibenclamide (1 μM). Two-way ANOVA: $F(8) = [6.20, 0.29]$, $p = [0.02, 0.6]$, n = 5. Graph C: LUS effect on fenoldopam reduced vascular contraction in vessels pre-contracted with KPSS. Two-way ANOVA: $F(8) = [5.28, 0.16]$, $p = [0.03, 0.7]$, n = 5. Graph D: LUS effect on fenoldopam induced vascular relaxation in KPSS (USKFenGl) and control KFenGl) groups in KATP channel blockade with glibenclamide (1 μM). Two-way ANOVA: $F(8) = [3.94, 0.27]$, $p = [0.06, 0.62]$, n = 5.

Graph C



Graph D

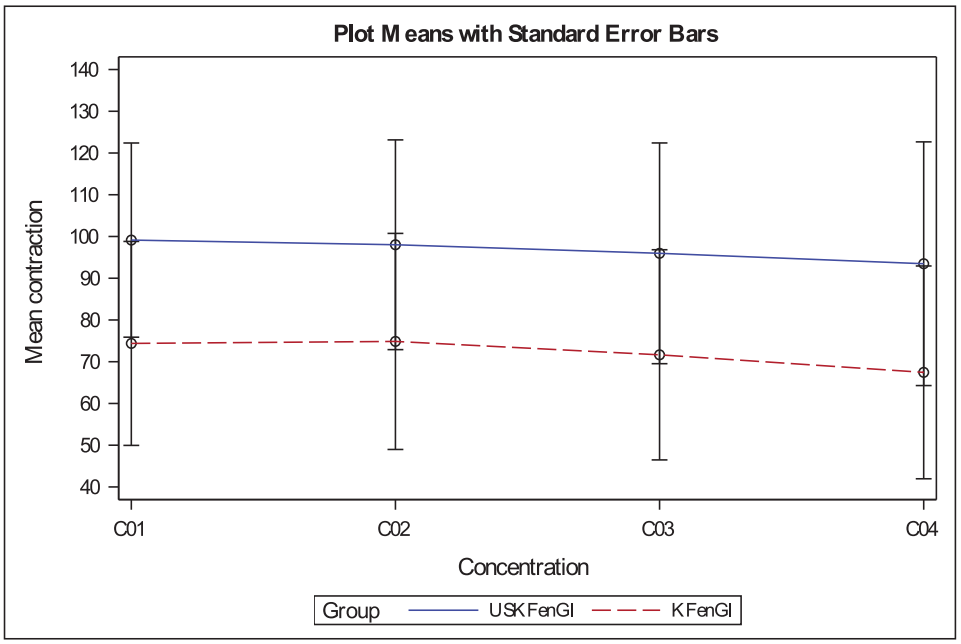


Fig. 13. Continued

DISCUSSION

The various Ca^{2+} channels (including L-type, N-type, T-type and P-type) can be modulated with insonation at a frequency of 0.44 – 0.67 MHz (Tyler et al. 2008; Kubanek et al. 2016). We found that even lower ultrasonic frequencies of 20−100 kHz have an inhibitory effect on the influx of external Ca^{2+} and seemingly potentiate the L-type Ca^{2+} channel blockade (with amlodipine incubation). These findings are also corroborated by the finding that LUS also inhibits capsaicin-induced vascular relaxation; LUS seems to interfere with the calcium influx in endothelial cells and thus decreases eNOS activity, ultimately inhibiting vascular

relaxation (Hopps et al. 2012; McCarty et al. 2015). Interestingly, LUS inhibited dopamine-induced vascular contraction in KPSS (which activates non-specifically various potassium channels)-induced vascular contraction; this effect was blocked with glibenclamide, while similar effects were not observed with fenoldopam. Dopamine (a drug with multiple effects and also a KATP activator [Kawano et al. 2008; Wu et al. 2001])-induced vascular contraction in KPSS (reversible with glibenclamide, a KATP blocker) does not seem to be mediated by KATP channels, as decreased activation of KATP channels leads to vasoconstriction and vice versa (Tykocki et al. 2017), while we observed an opposite effect. This effect may be explained by the LUS modulation of the actions of dopamine and glibenclamide on α_1-receptor (Özkan et al. 2017) or α_2-receptor signaling. Dopamine produces vascular contraction *via* activation of α_1- and α_2-receptors (Segawa et al. 1998). Glibenclamide has a mixed vasorelaxant effect not explained by KATP inhibition (Dai et al. 1989; Jonas et al. 1992; Tykocki et al. 2017) and likely modulates α-adrenergic transmission (Fagerholm et al. 2008). We think that dopamine induced significant vascular contraction *via* α_1- and α_2-adrenoreceptors, glibenclamide blocked this action in a KATP-independent manner and these effects can be modulated with LUS. This is even more likely because dopaminergic pathways are inhibited in our experiment by a high extracellular potassium concentration, as it has been reported that potassium channel activity (G protein-gated inwardly rectifying K^+ [GIRK/Kir3]) may inhibit dopaminergic transmission (Hibino et al. 2010; Lüscher and Slesinger 2010; McCall et al. 2017). Ca^{2+} signaling may also be involved in such an effect. It was found that glibenclamide decreases L-type calcium currents (Khatib and Boyett 2003) and opens voltage-dependent Ca^{2+} channels, and this, in turn, modulates vascular muscle cell excitability, vascular contraction and relaxation (Khatib and Boyett 2003; Ling et al. 2006; Ko et al. 2013). Dopamine may also promote Ca^{2+} signaling *via* the interplay of the D_1 dopamine receptor (D_{1R}) by forming a hetero-oligomer with the D_2 dopamine receptor (D_{2R}) (Chun et al. 2013) or by activating voltage-gated L-type Ca^{2+} channels (Cameron et al. 2015). LUS also potentiates gentamicin-induced vascular relaxation, mediated *via* PLC, protein kinase C and transmembrane calcium channels. In addition, LUS had no effect on fenoldopam, a selective dopamine (D_{A1}) agonist (Han et al. 1999; Truskey and Fernandez 2015; Szymanski and Richards 2019), lacking an obvious action on the influx of extracellular Ca^{2+}.

Dopamine-induced human pulmonary artery contraction was enhanced with LUS in KPSS. This finding is different from what was observed in rat mesentery arteries. This can be explained by the differences in the α_1-, α_2− and β_2−adrenoceptor-mediated vasoconstrictor components (Ohgushi et al. 1993; Priest et al. 1997). On the other hand, it was also reported that vascular contractions exhibit significant variability in response to adrenergic drugs in different vascular beds, even in the same species (Ohgushi et al. 1993). Dopamine D_1, D_2, D_4 and D_5 receptor subtypes are also identified in pulmonary arteries and likely mediate endothelium-dependent vasorelaxation (Ricci et al. 2006). However, the observed effect is not attributed to the activation of dopamine receptors, as KPSS inhibits dopaminergic transmission (Hibino et al. 2010; Lüscher and Slesinger 2010; McCall et al. 2017); thus, this effect was likely mediated *via* α_1- or α_2-receptor signaling, as discussed previously.

Our data indicate that LUS modulates drug action *via* modulation of extracellular Ca^{2+} entry through L-type calcium channels and modulates activation of α_1- and α_2-receptors in rat mesentery and human pulmonary arteries. LUS modulation of local drug action, as observed in human pulmonary arteries, has a potential application in the treatment of pulmonary hypertension, for which one of the major goals is to prescribe drugs that induce local vascular relaxation, while ultrasonic modulation could improve the action of such drugs (Vonk Noordegraaf et al. 2016).

CONCLUSIONS

We found that low-frequency (20,71 kHz) insonation inhibits extracellular Ca^{2+} entry and modulates the action of drugs that have an effect on extracellular Ca^{2+} entry (amlodipine and capsaicin) or adrenergic effects (dopamine, which causes vascular contraction *via* α_1- and α_2-adrenoreceptor activation). We also found that insonation modulates the action of dopamine in human pulmonary arteries and, thus, has a potential role in local modulation of vasoactive drug action in lungs, a beneficial property with potential clinical use in pulmonary hypertension or primary arterial hypertension. such ultrasound can be applied externally in the clinical setting (*e.g.,* as a vest or a belt).

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