Ultrasound stimulates formation and release of vasoactive compounds in brain endothelial cells

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Davis CM, Ammi AY, Alkayed NJ, Kaul S. Ultrasound stimulates formation and release of vasoactive compounds in brain endothelial cells. Am J Physiol Heart Circ Physiol 309: H583–H591, 2015. First published June 19, 2015; doi:10.1152/ajpheart.00690.2014.—Stroke outcome is improved by therapeutic ultrasound. This benefit is presumed to be principally from ultrasound-mediated thrombolysis. We hypothesized that the therapeutic benefit of ultrasound in stroke may, in part, be mediated by the release of beneficial vasoactive substances. Accordingly, we investigated the effect of ultrasound on levels of cytochrome P-450, lipooxygenase, and cyclooxygenase metabolites of arachidonic acid as well as adenosine release and endothelial nitric oxide synthase (eNOS) phosphorylation in primary brain endothelial cells in vitro. Brain endothelial cells were exposed to 1.05-MHz ultrasound at peak rarefractional acoustic pressure amplitudes of 0.35, 0.55, 0.90, and 1.30 MPa. Epoxideicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), PGE2, adenosine, nitrate/nitrite, and eNOS phosphorylation were measured after ultrasound exposure. Levels of 8,9-EET, 11,12-EET, and 14,15-EET increased by 230, 30%, and 246% (P < 0.05), respectively, whereas 5-HETE and 15-HETE levels were reduced to 24 ± 14% and 10 ± 3% (P < 0.05), respectively, compared with cells not exposed to ultrasound. PGE2 levels were reduced to 56 ± 14% of control. Adenosine increased more than sixfold after ultrasound exposure compared with unstimulated cells (1.36 ± 0.22 vs. 0.37 ± 0.10 ng/ml, P < 0.05), nitrate/nitrite was below levels of quantification, and eNOS phosphorylation was not altered significantly. Our results suggest that ultrasound may enhance tissue perfusion during stroke by augmenting the generation of vasodilator compounds and inhibiting that of vasoconstrictors. Such regulation supports a beneficial role for therapeutic ultrasound in stroke independent of its effect on the occlusive thrombus.

ultrasound; endothelial cell; adenosine; eicosanoids; endothelial nitric oxide synthase

NEW & NOTEWORTHY

Stroke outcome is improved by therapeutic ultrasound. This benefit is presumed to be from ultrasound-mediated thrombolysis. We show that cerebrovascular endothelial cells release vasodilator compounds when stimulated by ultrasound, suggesting that, in addition to physical clot lysis, ultrasound may also enhance tissue perfusion during stroke through vasodilation.

Clinical trials have demonstrated a benefit of therapeutic ultrasound during stroke (2, 13, 15, 16, 21), which is presumed to be mainly from ultrasound-mediated thrombolysis. Similar conclusions were drawn from therapeutic ultrasound after acute myocardial infarction (7, 10, 34, 35). However, beneficial effects of ultrasound in acute myocardial infarction have been demonstrated even when the infarct-related artery is not recannalized (36). Experimental studies have confirmed that ultrasound has an independent protective effect on the ischemic myocardium, which is thought to be mediated from the release of nitric oxide (NO) (36).

Therefore, we hypothesized that the beneficial effect of therapeutic ultrasound in stroke could, in part, also be mediated by the release of tissue-protective vasoactive substances. At least three groups of endothelium-derived vasoactive compounds have been identified in the brain: arachidonic acid metabolites, adenosine, and NO. Consequently, we investigated the effect of ultrasound on the production of cytochrome P-450 (CYP450), lipooxygenase (LOX), and cyclooxygenase (COX) metabolites of arachidonic acid: epoxideicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), and PGE2, respectively, as well as adenosine and nitrite/nitrate levels and eNOS phosphorylation as surrogates for NO in cerebrovascular endothelial cells (ECs).

METHODS

Experiments were performed according to the National Institutes of Health Guidelines for the Use and Care of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University.

Ultrasound Stimulation of ECs

Primary mouse brain ECs were isolated from 8-wk-old male C57BL6 mice (Charles River Laboratory, Wilmington, MA) as previously described (19) and plated into 12-well cell culture plates. Confluent ECs were washed with PBS, and wells were filled with phenol red-free high-glucose DMEM, sealed with optically/acoustically transparent MicroAmp film (Invitrogen, Life Technologies, Carlsbad, CA), and submerged in a tank filled with filtered and degassed water at 37.4°C. For some experiments, DMEM contained 10 μM 12-(3-adamantan-1-yl-ureido)-dodecanoic acid or N-(methylsulfonyl)-2-(2-propynylxoy)-benzenehexanamide (MS-PPOH; Cayman Chemical, Ann Arbor, MI); for measurements of COX metabolites, DMEM contained 10 μM indomethacin (Sigma-Aldrich, St. Louis, MO).

Cells were insonified using a 1.05-MHz focused (2 mm) single element transducer (H-171, Sonic Concepts, Bothell, WA) with a depth of field of 11 mm (Fig. 1) driven by a pulser receiver (RAM 5000, Ritec, Warwick, RI) connected to a duplexer (RA-32, Ritec) and mounted on a computer-controlled translation system (Velmax, Bloomfield, NY). First, the transducer was positioned above one well axially aligned by maximizing the pulse echo signal on an oscilloscope (WaveRunner-MXI-A, Teledyne LeCroy, Chesnut Ridge, NY). During treatment, the transducer was scanned twice over the center of each well, with each scan consisting of 10 back-and-forth 1-cm lines separated by 1 mm. Four different peak rarefractional acoustic pressure amplitudes (PRAPAs) were used: 0.35, 0.55, 0.90, and 1.30 MPa. The pulse repetition frequency was 50 Hz, and 50...
cycles were transmitted; therefore, each well received ultrasound exposure for 2 min at a given PRAPA.

After ultrasound exposure, cells were incubated at 37°C for 15 or 45 min. Medium was removed and stored on dry ice for PGE2, 6-keto-PGF1α, adenosine, and nitrate/nitrite analysis. Cells were washed with PBS, scraped, collected, and frozen on dry ice; this was used for eicosanoid profile analysis, normalization of eicosanoid concentrations, and phospho-eNOS immunoblot analysis.

Immunocytochemistry

Cells were fixed in culture plates using 4% paraformaldehyde and blocked for 1 h at room temperature in 5% normal donkey/goat serum + 1% BSA + 0.1% Triton X-100 solution. Anti-CD31, CD102 (both 1:1,000, BD Biosciences, San Jose, CA), CD34 (1:100, Cedarlane, Burlington, NC), von Willebrand factor (1:100, Santa Cruz Biotechnology, Dallas, TX), glial fibrillary acidic protein (1:1,000, EMD Millipore, Billerica, MA), or ionized Ca2+-binding adapter molecule 1 (1:500, Wako Chemicals, Richmond, VA) were diluted in blocking buffer, applied, and incubated overnight at 4°C. Cells were washed with PBS + 0.1% Tween 20 and secondary antibody, and Hoechst 33342 (1:1,000, Life Technologies) was applied in blocking buffer for 2 h at room temperature. Cells were then washed, mounted in AF1 (Citifluor, London, UK), and examined under an inverted fluorescence microscope (Nikon TE200, Nikon, Tokyo, Japan).

Preparation of Samples for Adenosine and Eicosanoids

Adenosine analysis was adapted from previously published methods (29, 32). Concentrated standard stocks were prepared to 10 mg/ml: adenosine (Sigma) was prepared in 1% formic acid, and 2-chloroadenosine (Sigma) was prepared in water. Further dilutions were made in mass spectrometry (MS)-grade water. Standard curves were prepared by spiking 5 µl of stocks into 40 µl high-glucose DMEM followed by spiking both samples and standards with the 2-chloroadenosine internal standard. Samples were 0.22 µm filtered for analysis by liquid chromatography-tandem MS (LC-MS/MS). Standards of adenosine from 0 to 12.5 ng/ml were prepared in medium.

For LOX and CYP450 metabolite analysis, 15% KOH (Fisher Scientific) was added to samples, which were base hydrolyzed at 40°C for 1 h and then acidified with 300 µl glacial acetic acid (T.J. Baker, Phillipsburg, NJ). Internal standard mix was added, and samples extracted with ethyl acetate, hexane-ethyl acetate (1:1), and hexane. Extracts were combined and dried under vacuum for 35 min at 35°C. Tubes were washed with hexane, and samples were stored at −80°C until LC-MS/MS analysis or immediately redried, solubilized in acetonitrile and water, and analyzed. Eicosanoid standard curves were prepared in PBS and extracted identically to samples as well as an unextracted standard curve run at the same time. Area ratios were plotted, and unknowns were determined using the slopes. The slopes of the unextracted and extracted curves were very similar, and unextracted standard curves were prepared and compared with a spiked quality control media extract.

For COX metabolites, media was diluted 1:1 with 1% formic acid, spiked with deuterated internal standards, and loaded onto 2-ml Biotage SLE+ cartridges followed by a 5-min wait and then eluted with 2 × 6 ml of t-butylmethylether. Samples were spiked with 20 µl of a trap solution consisting of 10% glycerol in methanol with 0.01 mg/ml butylated hydroxytoluene (BHT) and dried for 30 min in a speed vac concentrator. Large tubes were rinsed twice with 1 ml hexane, and the remainder was transferred to a smaller tube before being dried until a small aqueous residue remained. Fifty microliters of water-acetonitrile (80:20) with 0.1 mg/ml BHT were added and spin filtered with a 0.22-µm Millipore ultrafilter filter. Samples were transferred to vials, and 30 µl of sample were analyzed on the same column and buffer system as EETs/HETEs, with the only difference being the starting percentage of organic and gradient used. Prostaglandin standard curves were spiked into naïve media and prepared identically to samples. Area ratios were plotted, and unknowns were determined using the slopes.

LC-MS/MS for adenosine and eicosanoids. Samples were analyzed using a 5500 Q-TRAP hybrid/triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Carlsbad, CA) with electrospray ionization in the positive mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR autosampler followed by two LC-20AD XR LC pumps. Adenosine and internal standard were separated on an Agilent Zorbax Eclipse XDB-C18 narrow-bore RR 2.1 × 100-mm, 3.5-µm column held at 30°C using a Shimadzu CTO-20AC column oven using a gradient mobile phase delivered at a flow rate of 0.3 ml/min consisting of two solvents: 0.1% formic acid in water and 0.1% formic acid in methanol.
Scheduled multiple reaction monitoring transitions were monitored with a 2-min window for EETs/HETEs and a 1-min window for prostaglandins, as shown in Table 1. The gradient mobile phase was delivered at a flow rate of 0.5 ml/min and consisted of two solvents: 0.05% acetic acid in water and acetonitrile. The Betabasic-18 2 × 100-mm, 3-µm column was kept at 40°C using the Shimadzu column oven. Data were acquired and analyzed using Analyst 1.5.1 and Multiquant 3.0.1 (AB Sciex).

**Measurement of Nitrate/Nitrite**

As a surrogate of measuring NO release into the medium, the final products of NO, nitrate and nitrite, were measured using the Nitrate/Nitrite Fluorometric Assay (Cayman Chemical) according to the manufacturer’s protocol and measured on a Victor3 multilabel plate counter (Perkin-Elmer) using excitation/emission wavelengths of 355/405 nm.

**Western Blot Analysis for eNOS Phosphorylation**

Cells were lysed and centrifuged, and the supernatant was collected. Denatured protein samples were separated by gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Blots were blocked in 5% dry milk and incubated at 4°C overnight with antibodies (Pierce) and reimaged. Densitometry was quantified using Alpha Scan densitometry software (Bio-Rad). Rabbit polyclonal antibody against murine eNOS (Pierce Antibodies, Thermo Fisher Scientific, Waltham, MA), and rabbit polyclonal antibody against murine phospho-eNOS (phospho-Ser1177, Pierce Antibodies, Thermo Fisher Scientific, Waltham, MA). The signal was visualized using anti-rabbit horseradish peroxidase-conjugated antibody (GE Healthcare, Piscataway, NJ) linked (GE Healthcare, Piscataway, NJ) supersignal chemiluminescent reagents (Thermo Fisher Scientific) and a Fluorchem FC2 (Protein Simple, Santa Clara, CA). Bands were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and reprobed using rabbit polyclonal antibody against murine eNOS (Pierce Antibodies) and reimaged. Densitometry was quantified using Alpha Innotech FluorChem FC2 (R&D Systems); phospho-eNOS protein was normalized relative to eNOS.

**Statistical Methods**

Data are expressed as means ± SE. Groups were compared using one-way or two-way ANOVAs with a post hoc Dunnett multiple-comparison test. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

We characterized our primary brain ECs by immunocytochemical labeling for the endothelium-specific markers CD31, CD34, CD102, and von Willebrand factor to confirm endothelial identity as well as the astrocyte marker glial fibrillary acidic protein and microglial marker ionized Ca\(^{2+}\)-binding adapter molecule 1 to confirm purity (Fig. 2).

All results were normalized to the control, composed of cells not exposed to ultrasound, which also had their medium changed at the same time as cells undergoing ultrasound stimulation and harvested at the same time points.

**Eicosanoids**

**CYP450 metabolites.** All EET regioisomers (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) were detected, although 5,6-EET was below the level of quantification (Fig. 3). Ultrasound stimulation resulted in increased levels of 8,9-EET, 11,12-EET, and 14,15-EET at the 15-min time point and at the highest PRAPA of 1.30 MPa; levels increased to 230% and 246% at either time point (\( P < 0.05 \)). Dihydroxyeicosatetraenoic acid (10 µM), 8,9-EET and 11,12-EET levels were again observed 15 min after 0.90-MPa ultrasound stimulation (\( n = 4–6, P < 0.05 \); Fig. 3A). In the presence of the soluble epoxide hydrolase (sEH) inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (10 µM), 8,9-EET and 11,12-EET levels were again observed 15 min after 1.30-MPa exposure, increasing to 230% and 231% of control, respectively (\( n = 5, P < 0.05 \); Fig. 3B). No effect of ultrasound was observed at 45 min after stimulation (\( n = 6, P > 0.05 \)). In the presence of the CYP450 inhibitor MS-PPOH (10 µM), ultrasound was no longer able to increase EET levels at either time point (\( n = 5–7, P > 0.05 \); Fig. 3C). Dihydroxyeicosatetraenoic acid (DHET) regioisomers, metabolic byproducts of 8,9-EET, 11,12-EET, and 14,15-EET, i.e., 8,9-DHET, 11,12-DHET, and 14,15-DHET, were below the level of detection.

**Table 1. Multiple reaction monitoring parameters for the analysis of arachidonic acid metabolites**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time, min</th>
<th>Q1 Mass</th>
<th>Q3 Mass</th>
<th>DP</th>
<th>EP</th>
<th>CE</th>
<th>CXP</th>
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<tr>
<td>14,15-EET</td>
<td>7.45</td>
<td>319</td>
<td>219</td>
<td>−120</td>
<td>−10</td>
<td>−18</td>
<td>−11</td>
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<tr>
<td>11,12-EET</td>
<td>8.14</td>
<td>319</td>
<td>208</td>
<td>−80</td>
<td>−10</td>
<td>−18</td>
<td>−9</td>
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<tr>
<td>8,9-EET</td>
<td>8.50</td>
<td>319</td>
<td>68.9</td>
<td>−90</td>
<td>−10</td>
<td>−26</td>
<td>−11</td>
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<tr>
<td>5,6-EET</td>
<td>8.83</td>
<td>319</td>
<td>191</td>
<td>−85</td>
<td>−10</td>
<td>−16</td>
<td>−7</td>
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<tr>
<td>11,12-EET</td>
<td>6.90</td>
<td>319</td>
<td>115</td>
<td>−100</td>
<td>−10</td>
<td>−20</td>
<td>−9</td>
</tr>
<tr>
<td>8,9-HETE</td>
<td>6.08</td>
<td>319.2</td>
<td>167</td>
<td>−75</td>
<td>−10</td>
<td>−24</td>
<td>−3</td>
</tr>
<tr>
<td>11,12-HETE</td>
<td>6.31</td>
<td>319.2</td>
<td>179</td>
<td>−105</td>
<td>−10</td>
<td>−20</td>
<td>−9</td>
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<td>15,12-HETE</td>
<td>5.74</td>
<td>319.2</td>
<td>219</td>
<td>−115</td>
<td>−10</td>
<td>−20</td>
<td>−9</td>
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<tr>
<td>18-HETE</td>
<td>4.96</td>
<td>319.2</td>
<td>261</td>
<td>−65</td>
<td>−10</td>
<td>−22</td>
<td>−9</td>
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<tr>
<td>19-HETE</td>
<td>4.62</td>
<td>319.2</td>
<td>231</td>
<td>−100</td>
<td>−10</td>
<td>−22</td>
<td>−7</td>
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<tr>
<td>20-HETE</td>
<td>4.80</td>
<td>319.1</td>
<td>289</td>
<td>−130</td>
<td>−10</td>
<td>−26</td>
<td>−11</td>
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<tr>
<td>15-HETE</td>
<td>5.6</td>
<td>327</td>
<td>226</td>
<td>−85</td>
<td>−10</td>
<td>−20</td>
<td>−9</td>
</tr>
<tr>
<td>20-HETE</td>
<td>4.7</td>
<td>325</td>
<td>281</td>
<td>−75</td>
<td>−10</td>
<td>−24</td>
<td>−9</td>
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<tr>
<td>14,15-DHET</td>
<td>3.6</td>
<td>337</td>
<td>207</td>
<td>−100</td>
<td>−10</td>
<td>−26</td>
<td>−3</td>
</tr>
<tr>
<td>11,12-DHET</td>
<td>4.00</td>
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<td>167</td>
<td>−90</td>
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<td>−28</td>
<td>−11</td>
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<td>−75</td>
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<tr>
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<td>348</td>
<td>207</td>
<td>−120</td>
<td>−10</td>
<td>−20</td>
<td>−15</td>
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<td>6-keto-PGF1α</td>
<td>4.2</td>
<td>369.1</td>
<td>163.1</td>
<td>−120</td>
<td>−10</td>
<td>−38</td>
<td>−3</td>
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<tr>
<td>PGE2</td>
<td>5.9</td>
<td>351.0</td>
<td>189.1</td>
<td>−85</td>
<td>−10</td>
<td>−26</td>
<td>−5</td>
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<tr>
<td>PGF2α</td>
<td>5.7</td>
<td>353.1</td>
<td>291.0</td>
<td>−55</td>
<td>−10</td>
<td>−28</td>
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<td>Thromboxane B₂</td>
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<td>169.1</td>
<td>−75</td>
<td>−10</td>
<td>−28</td>
<td>−5</td>
</tr>
</tbody>
</table>

EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; DHET, dihydroxyeicosatrienoic acid.
LOX metabolites. Changes in HETEs after ultrasound stimulation were only observed at the 15-min time point and did not persist to 45 min (Fig. 4). Specifically, 5-HETE levels were reduced after stimulation at 0.90 MPa, to 24% of control \((n = 4, P < 0.05)\). 15-HETE levels were also decreased after ultrasound stimulation at 0.90 and 1.30 MPa to 10% of control at both pressures \((n = 4, P < 0.05)\). No differences were observed in 11-HETE or 12-HETE.

COX metabolites. Ultrasound had no effect on PGE2 levels at the 15-min time point \((n = 5, P > 0.05; \text{Fig. 5})\). However, 45 min after ultrasound exposure, PGE2 levels were reduced in response to every PRAPA. Levels were reduced to 56% \((14.03 \pm 2.17 \text{ ng/ml})\) of the unstimulated control \((30.19 \pm 7.12 \text{ ng/ml})\) at 1.30 MPa; similar reductions were seen with all other PRAPAs tested \((n = 4, P < 0.05)\). Levels of 6-keto-PGF1\(_\alpha\), the stable product of PGI2, were also measured. Similar to PGE2, ultrasound appeared to decrease 6-keto-PGF1\(_\alpha\) 45 min after stimulation from an average of 29.39 ± 7.32 ng/ml in the unstimulated control; however, many values fell below the level of quantification \((10 \text{ ng/ml})\), thus impeding statistical analysis (not shown). Other COX metabolites, including PGF2\(_\alpha\) and thromboxane B2, were also investigated; however, these were not detected in the samples.

Adenosine Levels

Adenosine release increased with increasing PRAPAs, reaching significance at 1.30 MPa \((P < 0.05)\) compared with the unstimulated control (Fig. 6). Adenosine increased more than sixfold, from 100% \((0.37 \pm 0.10 \text{ ng/ml})\) in unstimulated cells to 675 ± 48% \((1.36 \pm 0.22 \text{ ng/ml}, n = 12)\) after 1.30-MPa exposure. Sampling time, whether 15 or 45 min after exposure, was not a significant factor in this increase.

NO Levels

Measurement of NO is very challenging due to its very short half-life; therefore, as surrogates for NO, we measured eNOS phosphorylation within cells as well as the products of NO, nitrate and nitrite, in the culture medium. Phosphorylation of eNOS showed some increase, especially at 15 min after ultrasound exposure; however, this was not statistically significant at any PRAPA or time point \((n = 4, P > 0.05; \text{Fig. 7})\). Nitrate/nitrite release into the medium was detected in some of our samples; however, this was below the level of quantification \((<50 \text{ nM})\; \text{data not shown})

DISCUSSION

In the present study, we report, for the first time, that stimulation of primary cerebrovascular ECs with ultrasound leads to increased levels of vasoactive compounds that are tissue protective during ischemia (adenosine, 8,9-EET, 11,12-EET, and 14,15-EET) as well as a decrease in PGE2, which can be either protective or detrimental during ischemia, depending on downstream effectors (1, 25). We were unable to observe any appreciable effect on phosphorylation of eNOS or nitrate/nitrite levels.
Comparison with Previous Studies

The intriguing observation that ultrasound exposure results in tissue protection during acute coronary and femoral artery occlusions led to experiments to delineate the mechanism(s) of this finding. In one study (38) of femoral artery occlusion, ultrasound exposure increased NO production, which was blocked by N-nitro-L-arginine methyl ester. In another study (36) of coronary artery occlusion where NO was not directly measured, administration of N-nitro-L-arginine methyl ester abolished this protective effect. The conclusions of both studies were that ultrasound exposure directly increases NO levels, and through it tissue perfusion, despite proximal artery occlusion.

Fig. 3. A: 1.30-MPa ultrasound increased levels of 8,9-epoxyeicosatrienoic acid (EET), 11,12-EET, and 14,15-EET 15 min after exposure; at 45 min only, 11,12-EET was increased with 0.90-MPa ultrasound, with no effect on 8,9-EET or 14,15-EET (n=4–6, *P<0.05). B: in the presence of the soluble epoxide hydrolase inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA), 1.30-MPa ultrasound was again able to increase levels of 8,9-EET and 11,12-EET 15 min after exposure; ultrasound had no effect at 45 min (n=5–6, *P<0.05). C: in the presence of cytochrome P-450 inhibitor N-(methylsulfonyl)-2-(2-propynylxyloxy)-benzenexanamide (MS-PPOH), ultrasound was not able to increase levels 8,9-EET, 11,12-EET, or 14,15-EET at either time point (n=5–7, P>0.05).

Fig. 4. Ultrasound (0.90 MPa) decreased levels of 5-hydroxyeicosatetraenoic acid (HETE) and 15-HETE, and 1.30-MPa ultrasound also decreased levels of 15-HETE at 15 min with no effect on 11- or 12-HETE (n=4, *P<0.05); this reduction was not observed 45 min after exposure.
It has also been previously shown, using EC lines (human umbilical vein ECs and bovine aortic ECs), that low-intensity ultrasound is able to increase nitrate/nitrite levels, the end product of NO, indicating increased endothelial NO synthesis (4). This increase, however, was transient, reaching a maximum at 1 min after stimulation and returning to baseline by 30 min. We did not investigate earlier time points in our study. We were unable to measure nitrate/nitrite levels at sufficient concentrations in our study at either 15 or 45 min after ultrasound stimulation. This is due to a limitation of our experimental setup resulting in any nitrate/nitrite generated being too dilute to measure. Our study requires culture wells be filled to the top with medium and sealed to prevent any air bubbles before being placed in the water tank (Fig. 1). It is crucial to prevent any air bubbles as these would cause an impedance mismatch; since air is a perfect reflector of ultrasound, it would perturb ultrasound propagation and distort the acoustic field. To fill a well completely requires ~11 ml medium, resulting in very dilute samples, with nitrate/nitrite falling below the level of detection or, in cases where it was detected, below the level of quantification of the fluorometric kit of 50 nM. While not significant, we did observe a trend toward increased eNOS phosphorylation (eNOS phosphorylation is a surrogate of eNOS activation (5), although eNOS activity and NO production are affected by multiple factors) with increasing PRAPAs; it is therefore possible that eNOS may be phosphorylated significantly at earlier time points after ultrasound stimulation, transiently, decreasing again by 15 min. Alternatively, it is also possible that primary cerebral ECs respond differently than cell lines or even differently than ECs from other organs.

The differences between our and previous studies could also be explained by the type (continuous wave vs. pulsed) and intensity (low vs. high) of ultrasound as well as its frequency (kilohertz vs. megahertz). However, physiological principles can also explain the differences between the previous in vivo studies and our in vitro study. In vivo NO is released through increased shear stress on ECs, resulting in flow-mediated vasodilation. However, flow must first increase in smaller, more distal resistance vessels through other mechanisms. Adenosine and EETs are known to act specifically on resistance vessels, thereby increasing flow throughout the vascular unit. Previous studies did not measure the levels of these vasodilators after ultrasound exposure; in our study, we found that levels of both compounds increase, whereas that of HETEs decrease, which in vivo would result in vasodilation and increased blood flow. The higher tissue blood flow would in turn increase flow in feeding arteries resulting in NO release and flow-mediated vasodilation. Our results indicate that, unlike adenosine and EETs, NO may not be directly produced by ultrasound stimulation but may increase secondarily from enhanced small vessel blood flow in vivo. However, an increase in NO production in response to ultrasound should not be ruled out.

At both 15 and 45 min, adenosine release was increased after 1.30-MPa ultrasound stimulation ($n = 12$, *$P < 0.05$). No significant increase was observed with other peak rarefractional acoustic pressure amplitudes.

Ultrasound stimulation did not significantly alter endothelial nitric oxide synthase (eNOS) phosphorylation at any peak rarefractional acoustic pressure amplitude or time point ($n = 5$, $P > 0.05$). A representative Western blot from the 15-min time point is shown.

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**Fig. 5.** Ultrasound had no effect on PGE2 levels at 15 min after stimulation; 45 min after ultrasound exposure, 0.35, 0.55, 0.90, and 1.30 MPa reduced PGE2 levels ($n = 5$, *$P < 0.05$).

**Fig. 6.** At both 15 and 45 min, adenosine release was increased after 1.30-MPa ultrasound stimulation ($n = 12$, *$P < 0.05$). No significant increase was observed with other peak rarefractional acoustic pressure amplitudes.

**Fig. 7.** Ultrasound stimulation did not significantly alter endothelial nitric oxide synthase (eNOS) phosphorylation at any peak rarefractional acoustic pressure amplitude or time point ($n = 5$, $P > 0.05$). A representative Western blot from the 15-min time point is shown.
out due to lack of a statistically significant difference in the phosphorylation of eNOS at 15 and 45 min after ultrasound stimulation or lack of quantifiable nitrate/nitrite in our dilute medium samples.

**Effect of Ultrasound on Eicosanoid Levels**

Our results indicate that ultrasound stimulation of cerebral ECs changes eicosanoid concentration either within or released by ECs, increasing EETs while decreasing HETEs, PGE2, and 6-keto-PGF1α. EETs are potent vasodilators. We have previously shown that inhibition of the enzyme responsible for their production, CYP450 epoxygenase, results in reduced cerebral blood flow (CBF), whereas deletion of the enzyme responsible for their degradation, sEH, increases CBF (3, 41). EETs are also directly beneficial to ECs: treatment of cerebral ECs with 14,15-EET is protective against in vitro ischemia-induced cell death, whereas 11,12-EET has anti-inflammatory properties (19, 27). Ablation of sEH results in reduced infarct size after cerebral ischemia (41).

LOX metabolites of arachidonic acid, 5-HETE and 15-HETE, have differing effects on the vasculature compared with EETs. In the pulmonary circulation, both 5-HETE and 15-HETE have been shown to cause vasoconstriction and increase vascular permeability (9, 42). In addition, 15-HETE has been implicated in vasoconstriction of internal carotid arteries after ischemia and has proinflammatory actions on both pulmonary and pancreatic ECs (23, 24). Here, we show that ultrasound stimulation of cerebral ECs is able to acutely reduce 5-HETE and 15-HETE, results that have the potential to be beneficial to both ECs and brain vascular function.

PGE2, a COX metabolite of arachidonic acid, has been shown to be both protective and detrimental in the context of cerebral ischemia. Studies have shown that PGE2, acting upon EP2 receptors, is neuroprotective after stroke (25), whereas its actions mediated via EP1 receptors is neurotoxic and blockade of EP1 receptors has been shown to reduce infarct volume (1). COX2/PGE2 activity is also proinflammatory and has been implicated in vascular pathology (18). Here, we demonstrate that ultrasound decreases PGE2 levels; whether this decrease is protective or not during ischemia remains to be determined. Another COX metabolite, PG12, a vasodilator and inhibitor of platelet aggregation and has been shown to be protective after cerebral ischemia (17, 30). We found that 6-keto-PGF1α (stable product of PG12) is reduced 45 min after ultrasound but unaltered at 15 min. The protective role for PG12 was only observed when PG12 was elevated before ischemia but had no effect if elevated after insult. Whether the decrease observed by ultrasound will be detrimental in the context of ischemia needs to be established; however, the timing may prove to be inconsequential.

The observed ultrasound-induced change in endothelial EET levels may be brought about in either of two ways: alteration in their production or their degradation. The main pathway of EETs metabolism is conversion into DHETs by sEH (20). Since DHETs were below the limit of quantification in these experiments, we are unable to establish whether there is a change in EET metabolic products. However, our pharmacological experiments suggest that ultrasound does not influence EET degradation, as inhibition of sEH had no effect on the ability of ultrasound to increase EETs levels or the magnitude of this response. An alternative explanation of our observations after ultrasound stimulation may be an upregulation of the generation of EETs from arachidonic acid. Our experiments carried out in the presence of MS-PP0H support this mechanism; upon inhibition of CYP450, the enzyme responsible for EET generation from arachidonic acid (31), ultrasound was no longer able to stimulate an increase in EET levels in ECs. Our results therefore suggest that ultrasound increases the generation of EETs rather than decreasing their breakdown. These inhibitor experiments do not, however, inform us as to whether ultrasound increases the activity of CYP450 or whether arachidonic acid is increased within the cell, which, as a substrate for CYP450, may lead to increased EET levels.

Multiple eicosanoid mediators are formed from arachidonic acid via the LOX, COX, and CYP450 epoxygenase pathways. As already discussed, EETs are generated by CYP450, whereas the majority of HETEs are formed by LOXs and PGE2 is formed by COXs (8, 22, 37). Our results demonstrate opposing effects of ultrasound on these eicosanoids: an increase in EETs and decrease in HETEs and PGE2, indicating that the synthetic enzymes generating these species of eicosanoids are perhaps differentially regulated, upregulating the activity of CYP450 while downregulating LOX and COX activity, therefore preferentially generating EETs. Since EETs, HETEs, and PGE2 are all formed from arachidonic acid, this may indicate that the increase in EETs observed in response to ultrasound may be due to increased CYP450 activity rather than an increase in arachidonic acid. Interestingly, the increase of EETs was observed at 15 min after ultrasound; however, PGE2 and 6-keto-PGF1α were unaltered at 15 min but decreased at 45 min after stimulation. The latter may be the result of either substrate switch from COX to CYP450, as discussed, or due to arachidonic acid depletion at 15 min as a result of increased consumption by the CYP450 pathway.

**Adenosine Production by Ultrasound**

Adenosine is a potent vasodilator, playing an important role in the metabolic regulation of CBF and in the vascular response to ischemia. It acts upon, and causes relaxation of, smooth muscle cells, thus increasing CBF (11, 26). Adenosine is increased by cerebral ischemia, where it reduces ischemia-induced damage (6, 31, 40). Studies on ECs of vascular beds, other than the cerebral vasculature, have demonstrated that adenosine also enhances endothelial barrier integrity (33, 39). Decreased barrier has been shown to both contribute to and be caused by cerebral ischemic injury (14, 28). An increase in endothelial adenosine by ultrasound after cerebral ischemia may therefore improve endothelial barrier function in addition to improving CBF.

**Mechanism of the Ultrasound Effect**

Why do ECs produce vasoactive substances upon ultrasound exposure? Our results, and those of others, do not answer this important question. It is well known that ECs are sensitive to shear and that the myogenic response in resistance blood vessels is mediated by changes in intraluminal pressure. Ultrasound produces acoustic pressures, alternating positive and negative pressures on the cell surface, causing oscillation of the cell membrane. This may stimulate receptors or other proteins normally activated by shear stress, resulting in downstream
signaling causing the production and release of vasoactive substances. The finding that ultrasound exposure results in an increase in vasodilators and decrease in vasoconstrictors, as would occur with low-flow conditions in vivo, is very intriguing. It is not that ultrasound exposure results in a nonspecific increase in all metabolites. Therefore, the type of EC sensor that responds to ultrasound may be similar to that responding to low perfusion pressure in vitro. Although the exact nature of such a sensor is unknown, the recently described family of piezo mechanically activated ion channels are probable candidates (12).

Conclusions

We found that stimulation of primary cerebrovascular ECs with ultrasound leads to increased levels of vasointeractive compounds that are tissue protective during ischemia and decreased levels of compounds that are deleterious during ischemia. These findings indicate that ultrasound treatment should be attempted in preclinical models of cerebral ischemia to dissect out the beneficial effects of ultrasound on tissue perfusion versus its effect on thrombosis in patients with stroke.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


