Pulsed ultrasound attenuates the hyperglycemic exacerbation of myocardial ischemia–reperfusion injury

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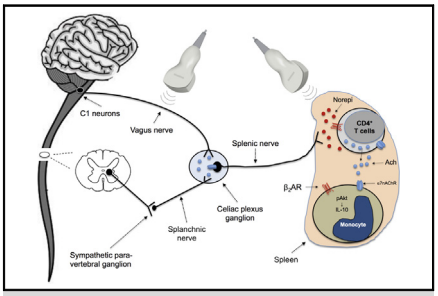
ABSTRACT

Objective: Acute hyperglycemia during myocardial infarction worsens outcomes in part by inflammatory mechanisms. Pulsed ultrasound has anti-inflammatory potential in bone healing and neuromodulation. We hypothesized that pulsed ultrasound would attenuate the hyperglycemic exacerbation of myocardial ischemia–reperfusion injury via the cholinergic anti-inflammatory pathway.

Methods: Acute hyperglycemia was induced in wild-type C57BL6 or acetylcholine-receptor knockout (α7nAChR$^{-/-}$) mice by intraperitoneal injection of glucose. Pulsed ultrasound (frequency 7 MHz, bursting mechanical index 1.2, duration 1 second, repeated every 6 seconds for 2 minutes, 20-second total exposure) was performed at the spleen or neck after glucose injection. Separate mice underwent vagotomy before treatment. The left coronary artery was occluded for 20 minutes, followed by 60 minutes of reperfusion. The primary end point was infarct size in explanted hearts.

Results: Splenic pulsed ultrasound significantly decreased infarct size in wild-type C57BL6 mice exposed to acute hyperglycemia and myocardial ischemia–reperfusion injury (5.2% ± 4.4% vs 16.9% ± 12.5% of risk region, $P = .013$). Knockout of α7nAChR abrogated the beneficial effect of splenic pulsed ultrasound (22.2% ± 12.1%, $P = .79$ vs control). Neck pulsed ultrasound attenuated the hyperglycemic exacerbation of myocardial infarct size (3.5% ± 4.8%, $P = .004$ vs control); however, the cardioprotective effect disappeared in mice that underwent vagotomy. Plasma acetylcholine, β2 adrenergic receptor, and phosphorylated Akt levels were increased after splenic pulsed ultrasound treatment.

Conclusions: Pulsed ultrasound treatment of the spleen or neck attenuated the hyperglycemic exacerbation of myocardial ischemia–reperfusion injury leading to a 3-fold decrease in infarct size. Pulsed ultrasound may provide cardioprotection via the cholinergic anti-inflammatory pathway and could be a promising new nonpharmacologic, noninvasive therapy to reduce infarct size during acute myocardial infarction and improve patient outcomes. (J Thorac Cardiovasc Surg 2021;161:e297-306)



pUS provides cardioprotection via cholinergic anti-inflammatory pathway.

CENTRAL MESSAGE

pUS treatment at the spleen or neck attenuates myocardial infarct size in a murine model of HG-exacerbated myocardial IRI.

PERSPECTIVE

In a murine model of myocardial IRI, pUS treatment at the spleen or neck led to a 3-fold decrease in infarct size. pUS may provide cardioprotection via the CAP and could be a promising new nonpharmacologic, noninvasive therapy to reduce infarct size during acute MI and improve patient outcomes.

See Commentaries on pages e307 and e308.

Heart disease continues to be the leading cause of death in the United States, with a rate of 165 deaths per 100,000 people based on 2017 data from the Centers for Disease Control and Prevention.[1] Ischemic heart disease, specifically myocardial infarction (MI), is responsible for the majority of those deaths. Acute hyperglycemia (HG) is common in patients with MI and independently associated with larger infarct size, impaired left ventricular function, and higher

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Abbreviations and Acronyms
BUS = B-mode ultrasound
CAP = cholinergic anti-inflammatory pathway
GEJ = gastroesophageal junction
HG = hyperglycemia
IRI = ischemia–reperfusion injury
LCA = left coronary artery
MI = myocardial infarction
pUS = pulsed ultrasound
WT = wild-type

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mortality.[2-6] Unfortunately, correction of acute, stress-induced HG by insulin fails to abrogate the increase in myocardial infarct size.[7-9] Thus, research is needed to further define the molecular mechanisms underlying the hyperglycemic exacerbation of MI and to identify effective therapies for reducing infarct size and mortality.[3]

Research in our laboratory has demonstrated that splenic leukocytes are activated by acute HG and play a pivotal role in leukocyte-mediated postischemic myocardial reperfusion injury.[10-12] Furthermore, in a murine model of acute kidney injury, we found that pulsed ultrasound (pUS) modulates splenic leukocytes into an anti-inflammatory phenotype.[13] pUS is a low-intensity mode of ultrasound that delivers pulsed waves rather than continuous delivery of acoustic energy and has been shown to have potential therapeutic benefits in humans in orthopedic fracture healing, inhibition of inflammation, and neuromodulation.[14,15] Ultrasound is a ubiquitous technology in modern health care with a low risk for off-target complications, making it an attractive treatment modality to protect the heart against hyperglycemic exacerbation of ischemia–reperfusion injury (IRI).[16]

The objective of the current study was to assess the ability of pUS to inhibit the splenic inflammatory response and reduce the hyperglycemic exacerbation of MI in a murine model of cardiac IRI. We hypothesized that pUS treatment would decrease myocardial infarct size. Additionally, we sought to investigate the relationship between pUS and acetylcholine and vagus nerve signaling. We hypothesized that splenic pUS would directly activate acetylcholine receptors on splenic leukocytes and that neck pUS would have an indirect but similar effect through upstream vagus nerve neuromodulation; therefore, application at these 2 anatomic locations was investigated. The ability to directly or indirectly reduce infarct size during conditions of HG using a nonpharmacologic and noninvasive treatment could lead to improved outcomes and lower mortality for patients with acute MI.

MATERIALS AND METHODS

This study complied with the 2011 Guide for the Care and Use of Laboratory Animals, 8th edition as recommended by the US National Institutes of Health ensuring that all animals received humane care. The University of Virginia Animal Care and Use Committee reviewed and approved the study protocol.

Animals and Experimental Protocols

C57BL/6 wild-type (WT) mice and α7-nicotinic acetylcholine receptor knockout (α7nAChR$^{-/-}$) mice (both 9-11 weeks, purchased from The Jackson Laboratory, Bar Harbor, Me) were used in the study. For myocardial IRI experiments, all mice underwent the IRI procedure described next (n = 5-10 per group). Acute HG was induced by intraperitoneal injection of 20% glucose (10 μL/g body weight) 15 minutes before occlusion of the left coronary artery (LCA). Blood glucose levels were monitored with a conventional glucometer (Auto Control Med, Inc, Mississauga, Canada) via tail venipuncture. Groups were further differentiated by treatment with or without pUS or control B-mode ultrasound (BUS) at the spleen or the neck, or the addition of concomitant vagotomy at the neck or the gastroesophageal junction (GEJ). Separate groups of naïve mice underwent splenic or neck pUS followed by splenectomy for plasma and splenic molecular analyses.

Myocardial Ischemia–Reperfusion Injury Procedure

Myocardial IRI was induced in mice by ligation of the LCA for 20 minutes, followed by 60 minutes of reperfusion. In previous work, we established that 60 minutes of reperfusion attains 95% of myocardial infarct size at 24 hours postreperfusion, making the first hour after ischemic insult the optimal time to investigate mechanisms underlying reperfusion injury.[8,10,17] A video of the procedure is available online.[10] Briefly, mice were anesthetized with sodium pentobarbital (80 mg/kg intraperitoneally), placed supine, and orally intubated. Mechanical ventilation was maintained (120 strokes/minute, tidal volume of 10 μL/g body weight). The heart was exposed through a left thoracotomy by dividing the third and fourth ribs. An 8-0 Prolene suture (Ethicon Inc, Somerville, NJ) was passed underneath the LCA 1 mm inferior to the lower edge of the left atrium and secured down over a short piece of PE-60 tubing (Becton Dickinson, Franklin Lakes, NJ). Occlusion of the LCA was confirmed by color change of the epicardium from pink to gray in the ischemic zone and electrocardiogram changes (QRS widening and ST-segment elevation) visualized on a PowerLab monitor (ADInstruments, Colorado Springs, Colo). After 20 minutes of occlusion, the suture was untied and the PE-60 tubing was removed, allowing the myocardium to reperfuse for 60 minutes. Analgesia was provided with ketoprofen (4 mg/kg subcutaneous injection). All animals received 1 mL of normal saline solution intraperitoneal injection to replace fluids lost during the operation. Core body temperature was monitored with a rectal thermometer (Barnant Co, Barrington, Ill) and maintained between 36.5°C and 37.5°C with a heating lamp.

Measurement of Infarct Size

Mice were euthanized under deep anesthesia after the 60-minute reperfusion period. The heart was explanted, cannulated through the ascending aorta with a blunted 23-gauge needle, and perfused with 3 mL of 37°C phosphate-buffered saline and 3 mL 1% 2,3,5-triphenyltetrazolium (Sigma-Aldrich, St Louis, Mo). The previously placed 8-0 Prolene suture was then used to reocclude the LCA, followed by perfusion with 1 mL of 10% Phthalo blue (Heucotech, Fairless Hill, Pa) to delineate the nonischemic tissue. The left ventricle was then divided transversely (5-7 slices per heart), fixed in 10% neutral buffered formalin, weighed, and digitally photographed. The infarct size, risk region, and nonischemic area were

then measured manually for each slice using ImageJ software (National Institutes of Health, Bethesda, Md) and multiplied by the weight of the slice. Total infarct size for each heart is presented as a percentage of the myocardial risk region.[18,19] The timeline of experimental interventions is shown in Figure 1.

Pulsed Ultrasound Protocol

A clinical Sequoia 512 US machine (Siemens Healthcare Diagnostics, Inc, Tarrytown, NY) with a 15L8 transducer (Acuson, Malvern, Pa) was used for all treated groups. After glucose injection and 10 minutes before LCA occlusion, ultrasound was applied at the appropriate anatomic location (spleen or neck) depending on treatment group. For splenic pUS, the hair over the dorsal aspect of the mouse was removed. The probe was placed just left of midline and the spleen localized in real-time using standard B-mode continuous ultrasound (frequency 14 MHz, mechanical index 0.99). pUS was then applied with a frequency of 7 MHz and bursting mechanical index of 1.2 for a duration of 1 second, repeated every 6 seconds for 2 minutes, providing a total exposure time of 20 seconds. For neck pUS, the ventral neck hair was removed and the probe was placed transverse over the neck. pUS treatment was applied with the same parameters as previously stated (20-second total exposure). BUS-treated mice underwent B-mode continuous ultrasound at the appropriate location for the same duration.

Two additional groups of mice were used to assess the duration of ultrasound effect. These mice underwent neck pUS or BUS 24 hours before undergoing the myocardial IRI procedure.

Cervical and Gastroesophageal Junction Vagotomy

For cervical vagotomy, a midline cervical incision was made after induction of anesthesia and intubation. The left paratracheal muscle was divided to expose the carotid artery and internal jugular vein. The left vagus nerve was identified and divided. The procedure was repeated on the right side. The skin incision was then closed with a 5-0 Prolene suture. For GEJ vagotomy, a vertical midline incision was made in the upper abdomen. The GEJ was exposed by retracting the left liver lobe cephalad and the stomach caudally. Both anterior and posterior vagus nerve trunks along the esophagus were divided. The incision was then closed in 2 layers with 4-0 Vicryl suture (Ethicon Inc, Somerville, NJ).

Splenectomy

Separate groups of naïve mice underwent pUS or BUS treatment followed by splenectomy 10 minutes later to allow for plasma and splenic molecular analyses. After anesthesia and intubation, a vertical midline incision was made to enter the peritoneal cavity. The spleen was brought to the incision and the hilum was clamped, ligated with a 3-0 silk suture, and divided. The spleen was removed, and the laparotomy was closed with 2 layers of 4-0 Vicryl suture.

Molecular Analyses

Acetylcholine in plasma and splenic tissue were measured with EnzyChrom Acetylcholine Assay kit (BioAssay Systems, Hayward, Calif). Protein expression of $\beta 2$ adrenergic receptor ($\beta 2$AR), Akt, and phosphorylated Akt (pAkt) were evaluated via Western blot using corresponding antibodies (Sigma-Aldrich, St Louis, Mo). Molecular analyses were performed in separate groups of normoglycemic mice.

Statistical Analysis

Comparisons between multiple groups were performed with 1-way analysis of variance with Bonferroni's correction for multiple comparisons (adjusted P values reported). Unpaired Student t test was used for comparisons between 2 groups. Prism 7 (GraphPad Software Inc, La Jolla, Calif) was used to perform statistical calculations. Data are presented as mean ± standard deviation.

RESULTS

Splenic Pulsed Ultrasound Attenuates Hyperglycemic Exacerbation of Myocardial Infarct Size

Blood glucose levels were significantly higher in WT mice exposed to intraperitoneal glucose injection before

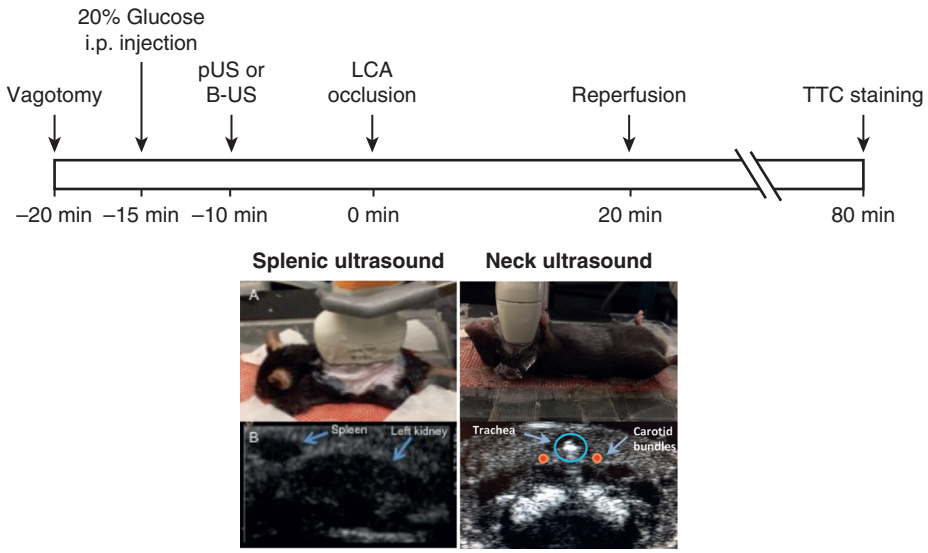

FIGURE 1. *Top*: Experimental protocol and temporal manipulations of the mice. *Bottom*: Ultrasound probe positions and locations of the targets (spleen or neck structures) before application of pulsed or BUS. *i.p.*, Intraperitoneal; *pUS*, pulsed ultrasound; *BUS*, B-mode ultrasound; *LCA*, left coronary artery; *TTC*, 1% 2,3,5-triphenyltetrazolium.

TABLE 1. Mean blood glucose levels for control and ultrasound-treated mice

Group	Mean blood glucose (g/dL)
WT control	180.8 ± 22.6
HG	342.4 ± 41.1
HG + splenic pUS	339.5 ± 49.9
HG + splenic BUS	347.3 ± 49.6
α7nAChR$^{-/-}$ control	183.6 ± 21.5
α7nAChR$^{-/-}$ + HG	315.2 ± 53.3
α7nAChR$^{-/-}$ + HG + splenic pUS	331.6 ± 26.7

No significant difference in mean blood glucose levels between groups exposed to acute hyperglycemia. *WT control*, Wild-type control mice; *HG*, WT mice with acute hyperglycemia; *HG + splenic pUS*, WT mice with acute hyperglycemia treated with pulsed ultrasound at the spleen; *HG + splenic BUS*, WT mice with acute hyperglycemia treated with control B-mode ultrasound at the spleen; *α7nAChR$^{-/-}$ control*, α7 Nicotinic acetylcholine receptor knockout control mice; *α7nAChR$^{-/-}$ + HG*, α7 nicotinic acetylcholine receptor knockout mice with acute hyperglycemia; *α7nAChR$^{-/-}$ + HG + splenic pUS*, 7 nicotinic acetylcholine receptor knockout mice with acute hyperglycemia treated with pulsed ultrasound at the spleen.

myocardial IRI (HG: 342.4 ± 41.1 vs control: 180.8 ± 22.6 g/dL, $P < .0001$; n = 5-6). HG refers to the reference group of mice who had acute HG and underwent myocardial IRI but were not treated with any form of ultrasound. There was no significant difference in mean blood glucose levels among any of the groups exposed to HG ($P = .75$; n = 5-7, Table 1). Acute HG significantly increased infarct size in WT mice (HG: 16.9% ± 12.5% vs WT control: 3.9% ± 1.4% of risk region, $P = .007$; n = 8-9, Figure 2). This effect was attenuated by pUS treatment of the spleen (5.2% ± 4.4% of risk region, $P = .013$ vs HG; n = 9) but not by BUS (19.8% ± 8.6% of risk region, $P = .99$ vs HG; n = 8).

Knockout of α7nAChR Abrogates the Beneficial Effect of Splenic Pulsed Ultrasound

Acute HG significantly increased infarct size in α7nAChR$^{-/-}$ mice (19.2 ± 11.9 vs α7nAChR$^{-/-}$ control: 2% ± 1.9% of risk region, $P = .004$; n = 8). However, splenic pUS failed to decrease the infarct size (22.2% ± 12.1% of risk region, $P = .79$ vs α7nAChR$^{-/-}$ + HG; n = 9, Figure 2).

Neck Pulsed Ultrasound Attenuates Hyperglycemia Exacerbation of Myocardial Infarct Size

Hyperglycemic exacerbation of myocardial infarct size was attenuated by pUS treatment at the neck (3.5% ± 4.8% of risk region, $P = .004$ vs HG; n = 10, Figure 3). BUS treatment at the neck demonstrated no effect on infarct size (18.7% ± 11% of risk region, $P = .9$ vs HG; n = 10).

Cervical and Gastroesophageal Junction Vagotomy Abrogate the Beneficial Effect of Neck Pulsed Ultrasound

The cardioprotective effect of neck pUS disappeared in mice that underwent cervical (17% ± 7.6% of risk region, $P = .99$ vs HG; n = 7) or GEJ vagotomy (17.9% ± 9.3% of risk region, $P = .99$ vs HG; n = 7, Figure 3). Vagotomy alone, without pUS treatment, did not affect infarct size compared with acute HG (14.4% ± 10.3% of risk region, $P = .95$ vs HG; n = 8).

Cardioprotective Effect of Neck Pulsed Ultrasound Persists for Up to 24 Hours

Mice treated with neck pUS 24 hours before undergoing myocardial IRI were protected from the hyperglycemic exacerbation of myocardial IRI compared with BUS treatment 24 hours prior (pUS: 4.8% ± 7.5% vs BUS: 22.1% ± 13.4% of risk region, $P = .009$; n = 6-7, Figure 3). The beneficial effect was similar to neck pUS treatment just before LCA occlusion ($P = .7$). On the contrary, neck pUS performed 10 minutes after reperfusion was not protective (20.7% ± 10.1% of risk region, $P = .5$ vs HG; n = 8).

Acetylcholine Levels Affected by Splenic and Neck pUS

Both splenic and neck pUS significantly decreased the acetylcholine level in the spleen compared with splenic BUS (splenic pUS: 10.1 ± 4.1 vs splenic BUS: 17.3 ± 4.2 μg/100 mg tissue, $P = .03$; neck pUS: 11.4 ± 2.7 μg/100 mg tissue, $P = .03$ vs splenic BUS; n = 5, Figure 4) and increased the level in plasma (splenic pUS: 13.3 ± 1 vs splenic BUS: 11.4 ± 0.4 ng/mL, $P = .02$; neck pUS: 15.7 ± 1.2 ng/mL, $P = .001$ vs splenic BUS; n = 5, Figure 4).

Protein Expression of β2 Adrenergic Receptors and Phosphorylated Akt

Both splenic and neck pUS significantly increased β2AR protein expression in the spleen as measured by photometric density (splenic pUS: 4697 ± 1247 vs splenic BUS 1575 ± 1277, $P = .008$; neck pUS: 5309 ± 1236, $P = .003$ vs splenic BUS; n = 5, Figure 5, *A*). pAkt levels (as a percentage of total Akt) increased after splenic treatment with pUS (67.1 ± 8.7 vs splenic BUS: 42.2 ± 6%, $P = .002$; n = 5, Figure 5, *B*) and decreased after neck pUS treatment (31.1% ± 4.4%, $P = .015$ vs splenic BUS; n = 5, Figure 5, *B*).

DISCUSSION

By using an established murine model of myocardial IRI, the present study sought to evaluate the effect of splenic and neck pUS on the hyperglycemic exacerbation of myocardial

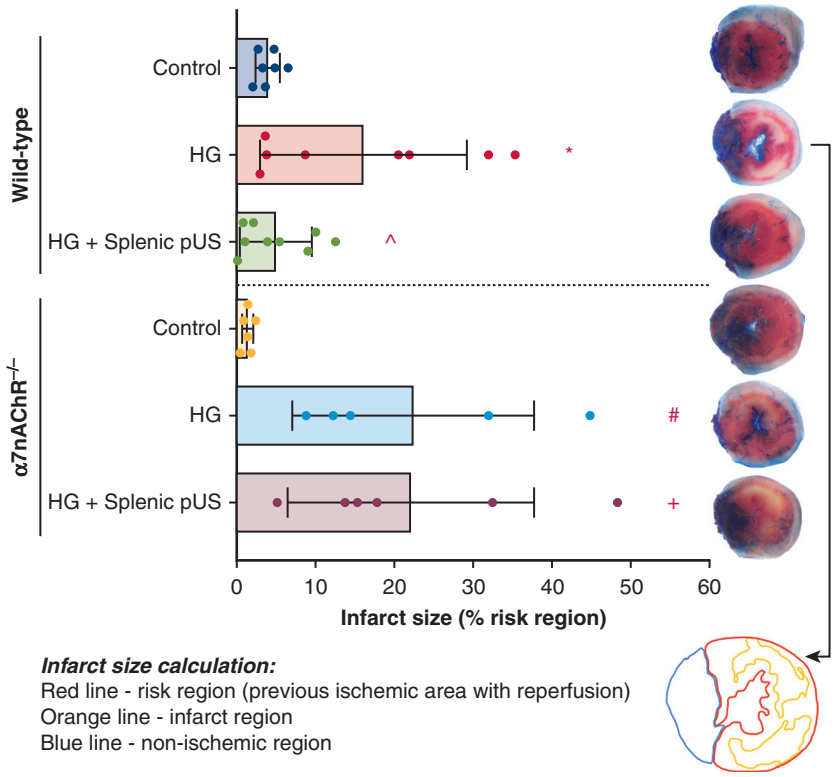

FIGURE 2. Splenic pUS attenuated hyperglycemic exacerbation of myocardial infarct size in a mouse model of IRI, but no cardioprotective effect was seen in α7nAChR$^{-/-}$ mice. Representative *heart slices* shown are from approximately the same level of the left ventricle in an area perfused primarily by the LCA. *$P = .007$ versus WT control, $^{\wedge}P = .013$ versus WT HG, #$P = .004$ versus α7nAChR$^{-/-}$ control, + $P = .79$ versus α7nAChR$^{-/-}$ + HG. *Control*, No hyperglycemia; *HG*, mice with acute hyperglycemia; *HG + splenic pUS*, mice with acute hyperglycemia treated with pulsed ultrasound at the spleen; *pUS*, pulsed ultrasound; *α7nAChR$^{-/-}$*, *α7*, nicotinic acetylcholine receptor knockout mice.

infarct size and to investigate the impact of pUS on modulation of the splenic inflammatory response via acetylcholine and vagus nerve signaling. Both splenic and neck pUS significantly attenuated the hyperglycemic exacerbation of MI, leading to a 3-fold decrease in infarct size. Knockout of the α7nAChR abrogated the beneficial effect of splenic pUS. Additionally, the cardioprotective effect demonstrated with neck pUS disappeared in mice who underwent cervical or GEJ vagotomy. When neck pUS was performed 24 hours before myocardial IRI, the beneficial decrease in infarct size was similar to that seen when the same treatment was performed just before LCA occlusion. Molecular analyses of plasma and spleen tissue demonstrated that both splenic and neck pUS modulate acetylcholine levels and β2AR and pAkt protein expression. Collectively, both splenic and neck pUS appear to dampen the splenic inflammatory response associated with hyperglycemic exacerbation of MI through vagus nerve and acetylcholine signaling.

Acute HG in patients presenting with MI is exceedingly common, even in patients without a history of diabetes.[20,21] In-hospital mortality rates are 3.9-fold higher in nondiabetic patients who present with HG during acute MI compared with euglycemic patients.[22] Given the negative impact of HG, normalizing blood glucose levels in patients who present with MI is recommended in the American College of Cardiology/American Heart Association guidelines.[23] Unfortunately, clinical trials have failed to show that intensive insulin therapy reduces the mortality of MI.[24,25] Acute HG activates the inflammatory cascade including NADPH oxidase. Once this cascade is initiated, simple reversal of HG with insulin fails to block this downstream signal pathway. Alternative therapies are needed that target the mechanisms behind HG-induced infarct exacerbation, which our group hypothesizes is mainly inhibition of Akt phosphorylation leading to alterations of the PI3K/Akt pathway, as well as disruption of signaling pathways downstream of adenosine A1 receptor activation.[9]

Previous work by our group has shown that acute HG stimulates splenic leukocytes and leads to enhancement of the inflammatory response during reperfusion of ischemic tissue.[10-12] Splenic-resident leukocytes are potent suppliers

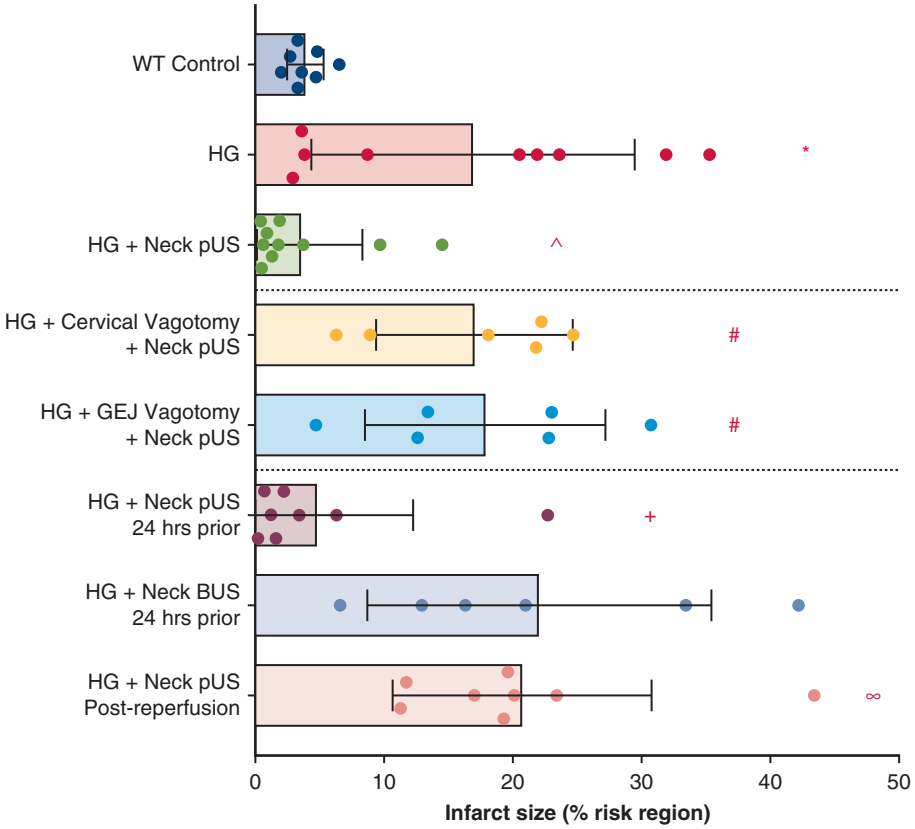

FIGURE 3. Neck pUS attenuated hyperglycemic exacerbation of myocardial infarct size in a mouse model of IRI, but no cardioprotective effect was seen in mice who underwent cervical or GEJ vagotomy. The cardioprotective effect of neck pUS persisted for up to 24 hours, but treatment with neck pUS after initiation of reperfusion was not protective. *$P = .007$ versus WT control, ^$P = .004$ versus HG, #$P = .99$ versus HG, + $P = .7$ versus HG + neck pUS and $P = .02$ versus HG + neck BUS 24 hours prior. ∞ $P = .5$ versus HG. *WT control*, WT control mice; *HG*, WT mice with acute hyperglycemia; *HG + neck pUS*, WT mice with acute hyperglycemia treated with pulsed ultrasound at the neck; *HG + cervical vagotomy + neck pUS*, WT mice with acute HG who underwent cervical vagotomy and were treated with pUS at the neck; *HG + GEJ vagotomy + neck pUS*, WT mice with acute HG who underwent GEJ vagotomy and were treated with pUS at the neck; *HG + neck pUS 24 hours prior*, WT mice with acute hyperglycemia treated with pUS at the neck 24 hours before LCA occlusion; *HG + neck BUS 24 hours prior*, WT mice with acute hyperglycemia treated with control B-mode ultrasound at the neck 24 hours before LCA occlusion; *HG + neck pUS postreperfusion*, WT mice with acute hyperglycemia treated with pUS at the neck 10 minutes after the start of reperfusion; *pUS*, pulsed ultrasound.

of cytokines and chemokines and play an important role in mediating the postischemic reperfusion injury associated with MI. Acute HG appears to prime splenic leukocytes through activation of the NADPH oxidase pathway, which are then released into circulation during reperfusion and contribute to worsening myocardial injury.[8,11]

In the present study, we used pUS as a noninvasive therapy to modulate the leukocyte-mediated hyperglycemic exacerbation of myocardial IRI. Gigliotti and colleagues[13,26] have shown that splenic pUS attenuates acute kidney injury by activating sympathetic innervation of splenocytes and thus inhibiting detrimental inflammatory responses. We found that splenic pUS attenuated postischemic myocardial reperfusion injury, leading to a 3-fold decrease in infarct size. However, pUS failed to reduce infarct size in α7nAChR$^{-/-}$ mice. These data suggest that activation of α7nAChR, a ligand-gated ion channel expressed on many cell types including CD4 + T lymphocytes, is involved in the cardioprotective mechanism of splenic pUS.[27] We chose to focus on the α7nAChR due to its documented role in the "cholinergic anti-inflammatory pathway (CAP)."[28,29]

Tracey[30,31] describes CAP as an "efferent neural signaling pathway" that "via an inflammatory reflex of the vagus nerve, can inhibit cytokine release and thereby prevent tissue injury and death." Regulation of cytokine production through CAP occurs via α7nAChR-dependent signaling.[29,31,32] In an experimental rat model of

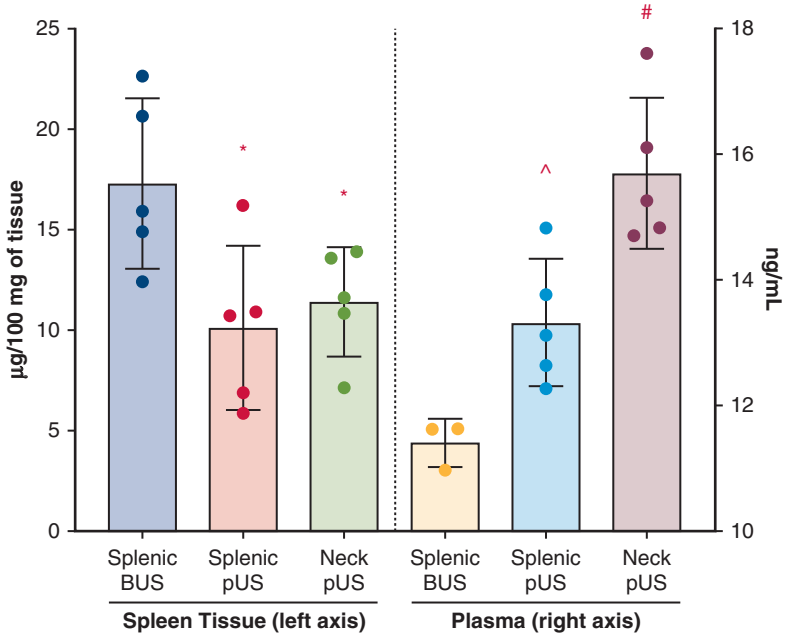

FIGURE 4. Splenic and neck pUS decreased splenic levels of acetylcholine and increased plasma levels in mice. Spleen tissue: *$P = .03$ versus splenic BUS. Plasma: ^$P = .02$ versus splenic BUS, #$P = .001$ versus splenic BUS. *Splenic BUS*, WT mice treated with B-mode ultrasound at the spleen; *splenic pUS*, WT mice treated with pUS at the spleen; *neck pUS*, WT mice treated with pUS at the neck; *pUS*, pulsed ultrasound.

endotoxemia, electrical stimulation of the vagus nerve inhibited systemic proinflammatory cytokine levels, as did acetylcholine treatment of cultured human macrophages.[33]

To further test our hypothesis that acetylcholine and vagus nerve signaling may be involved in the splenic-leukocyte mediated hyperglycemic exacerbation of

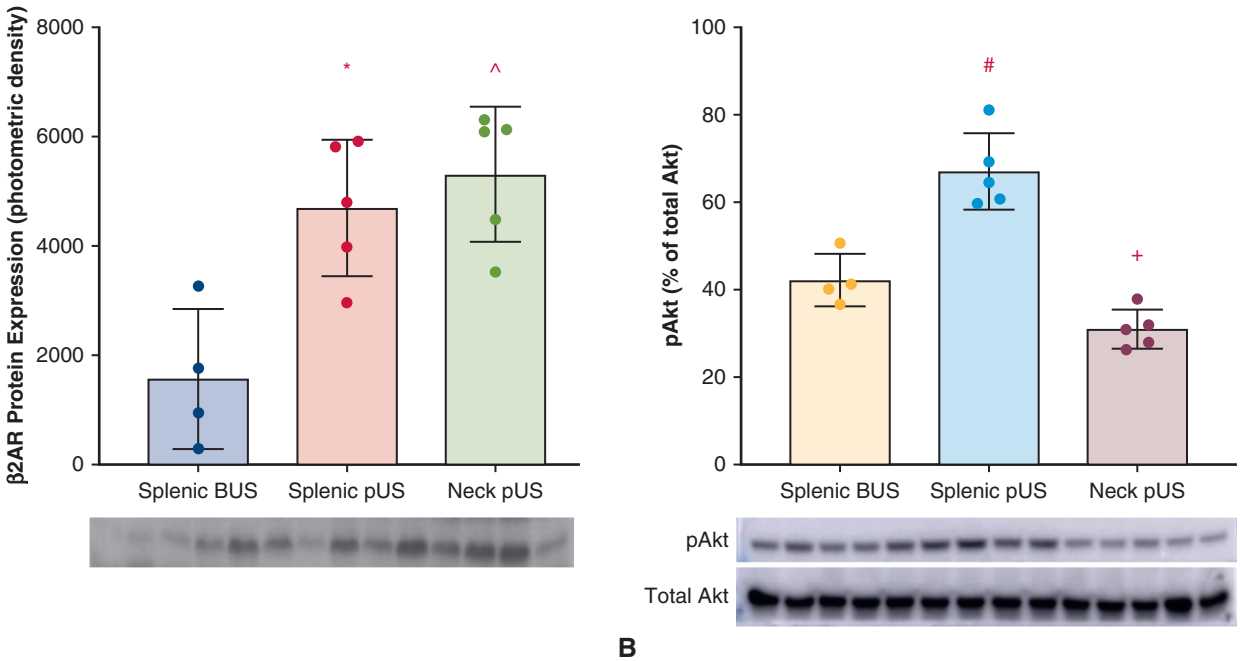

FIGURE 5. A, Splenic and neck pUS increased β2AR protein expression in splenic tissue of mice. *$P = .008$ versus splenic BUS, ^$P = .003$ versus splenic BUS. B, pAkt protein expression in splenic tissue increased after treatment with pUS at the spleen and decreased after treatment at the neck in mice. #$P = .002$ versus splenic BUS, + $P = .015$ versus splenic BUS. *β2AR*, β2 Adrenergic receptor; *splenic BUS*, WT mice treated with B-mode ultrasound at the spleen; *splenic pUS*, WT mice treated with pUS at the spleen; *neck pUS*, WT mice treated with pulsed ultrasound at the neck; *pUS*, pulsed ultrasound; *pAkt*, phosphorylated Akt.

myocardial IRI, we compared myocardial infarct size in mice treated with and without neck pUS. Wasilczuk and colleagues[34] demonstrated that pUS stimulates the vagus nerve leading to modulation of the inflammatory reflex. We found that neck pUS significantly attenuated myocardial infarct size, with similar cardioprotection to that seen with splenic pUS. However, the infarct-sparing effect of neck pUS disappeared in mice who underwent cervical or GEJ vagotomy. These data suggest that the protective mechanism of neck pUS occurs through efferent vagus nerve activity, which is further supported by the finding of increased plasma levels of acetylcholine, the primary vagal neurotransmitter, which were elevated in mice treated with splenic or neck pUS.

Previous work in our laboratory demonstrated that splenic leukocytes were inhibited by selective β2AR agonist administration via a pAkt-IL-10 pathway leading to attenuation of myocardial IR injury.[17] Activation of β-ARs by sympathetic neurotransmitters, as opposed to α-ARs, promotes the release of anti-inflammatory cytokines.[35] The present study found that β2AR protein expression and phosphorylation of Akt were enhanced after splenic treatment with pUS. Neck pUS increased β2AR protein expression similarly, but did not increase phosphorylation of Akt, which may highlight slight differences in the downstream effects of neck versus direct splenic pUS. These changes in protein levels occurred rapidly, which warrants further evaluation in subsequent studies. Collectively, our data support the vagus nerve-splenic CAP as an important mechanism involved in the cardioprotective effect of neck and splenic pUS via a β2AR-α7nAChR-pAkt mechanism (Figure 6).

Study Limitations

Although the findings in the present study reveal a critical role for the spleen in myocardial infarct exacerbation by acute HG and demonstrate a cardioprotective effect of nonpharmacologic treatment with neck or splenic pUS, there are some important limitations that warrant discussion. This study was performed in a small animal model of acute MI. The findings are limited by the low sample size and inherent variability in animal models, both of which reduce the precision of the estimated effects. The pUS signal potentially has an effect on other organs in addition to the target organs. Additionally, researchers were not blinded when performing myocardial infarct measurements, which is a potential source of bias. Given the nature of this exploratory study, causal relationships defining the mechanism by which splenic pUS stimulation or vagus nerve signaling attenuates myocardial infarct size cannot be determined at this time. However, the present study provides data necessary for designing further studies

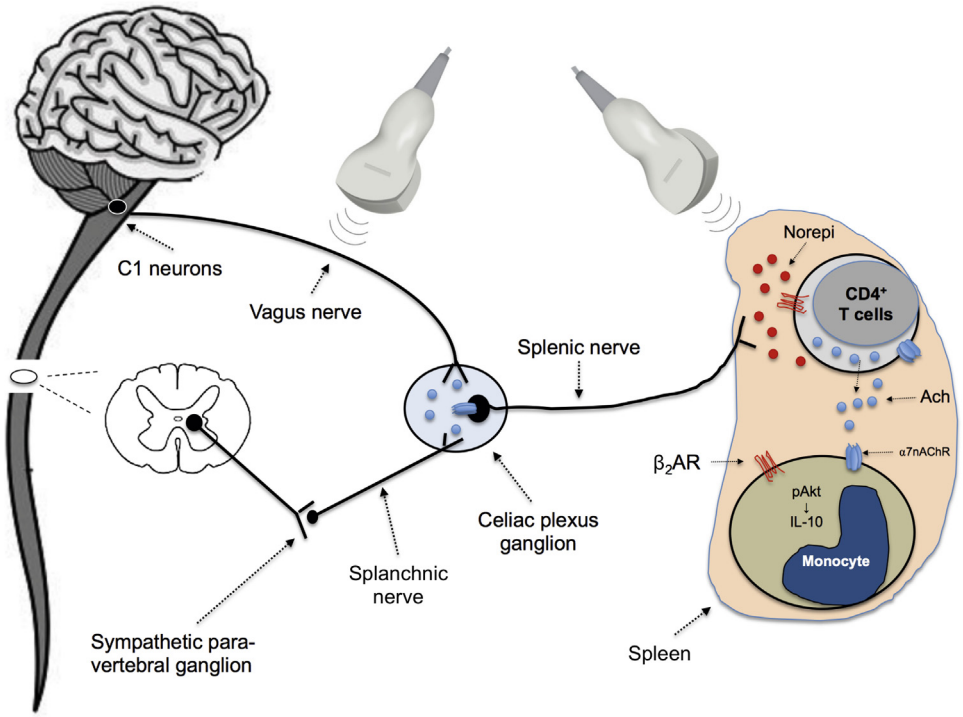

FIGURE 6. Proposed CAP involved in the cardioprotective effect of neck and splenic pUS via β2AR-α7nAChR-pAkt mechanism. *Norepi*, Norepinephrine; *ACh*, acetylcholine; *α7nAChR*, α7 nicotinic acetylcholine receptor; *β2AR*, β2 adrenergic receptor; *pAkt*, phosphorylated Akt; *IL-10*, interleukin 10.

that will delineate the mechanisms involved. Our future directions include focusing on the role of splenic CD4 + T lymphocytes that we hypothesize are the interface between neural signals and downstream immunologic effects. We will further evaluate the role of splenic monocytes in the proposed β2AR-α7nAChR-pAkt pathway and the mechanisms by which pUS generates neuronal signal transmission on a cellular level. Because 70% of the vagus nerve is afferent fibers connecting C1 neurons, the role of afferent vagus nerve signaling and C1 neurons in pUS-induced cardioprotection will be further evaluated (Figure 6). Although our current model of applying the pUS treatment before the ischemic insult may have clinical applicability for patients undergoing cardiac surgery or when ischemia can be predicted, we are planning future studies using a longer ischemia time that will accommodate delivery of the pUS treatment during the ischemic period rather than before it.

CONCLUSIONS

The present study demonstrates a cardioprotective effect of both splenic and neck pUS in a murine model of HG-exacerbated myocardial IRI. Treatment with pUS significantly attenuated myocardial injury, leading to a 3-fold decrease in infarct size. Knockout of the α7nAChR and vagotomy both abrogated the beneficial effects of pUS, highlighting a likely connection between CAP and the protective effects of pUS. Given changes in acetylcholine, β2AR, and pAkt levels, a proposed β2AR-α7nAChR-pAkt mechanism in the spleen is involved. With a better understanding of the molecular mechanisms involved, pUS could be a promising new nonpharmacologic, noninvasive therapy to reduce infarct size during acute MI and improve patient outcomes.

Webcast

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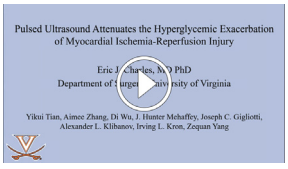

Conflict of Interest Statement

Dr Gigliotti discloses a patent for systems, methods, and computer-readable media for ischemic injury protective ultrasound. All other authors have nothing to disclose with regard to commercial support.

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Key Words: myocardial ischemia-reperfusion injury, pulsed ultrasound, cholinergic anti-inflammatory pathway

Discussion

Dr Friedhelm Beyersdorf *(Freiburg, Germany)*. You have clearly shown that HG increases infarct size 3-fold, which poses a major problem. Your hypothesis is that insulin doesn't work? So that means you believe that HG will damage the tissue, but if you treat HG, it still doesn't work. Why is this so?

Dr Eric J. Charles *(Charlottesville, Va)*. We think HG affects the splenic leukocytes and primes them for their inflammatory response. Once that priming has happened, even if you correct HG with insulin, those cells are already on a trajectory to release a lot more cytokines and produce a greater inflammatory response than otherwise would have happened.

Dr Beyersdorf. Do you think that pUS might also work, first of all, after ischemia without reperfusion, and thereafter if you think it might also work after reperfusion has already occurred?

Dr Charles. Those are both great questions, which we are currently working on answering in the lab. I doubt there will be much effect in the scenario of ischemia without reperfusion, because the detrimental inflammatory response that we are modulating in our model occurs with the onset of reperfusion. However, applying pUS after ischemia but before reperfusion will likely be beneficial. Additionally, applying it after reperfusion has already occurred may show a benefit in reducing further injury, but the overall response may be less. To translate this clinically, it would be valuable if we can demonstrate a beneficial effect with application of pUS at these other time points.