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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Continuous Diagnostic Frequency Ultrasound and the Microcirculation

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Bioengineering

by

C. Makena Hightower

Committee in charge:

Professor Marcos Intaglietta, Chair Professor Roland Blantz Professor Shu Chien Professor Paul Johnson Professor Juan Lasheras

2007

The Dissertation of C. Makena Hightower is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2007

DEDICATION

I dedicate this dissertation to my grandmother, Evelyn Dixon, and my mother Joyce Hightower, for their super awesome strength, fortitude, resilience, and faith, which they were able to imparted to me. I also dedicate this dissertation to my older brother Wilbur Hightower, III and younger brother George Hightower, for being the indestructible supports reinforcing me on both sides throughout my journey to here. I would like to thank the rest of my family and also my friends for being the great teachers and at times stumbling blocks that forced me to learn and adapt, in order to excel.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

D	diameter

- V flow velocity
- V_a average flow velocity
- Q flow

Abbreviations

1400W	N-(3-aminomethyl) benzylacetamidine dihydrochloride
chamber	dorsal skin fold window chamber
eNOS	endothelial nitric oxide synthase
FCD	functional capillary density
FITC-dextran	fluorescein isothiocyanate, bound to dextran
iNOS	inducible nitric oxide synthase
I/R or IR	ischemia reperfusion
L-NAME	N^{ω} -nitro-l-arginine methyl ester
NO	nitric oxide
NOS	nitric oxide synthase
RBC	red blood cell
ROS	reactive oxygen species
TPX	PolyMethylPentene
WB	western blot analysis

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Chapter 2, in full, is a reprint of the material as it appears in Microcirculation 2007, Hightower C M and Intaglietta M., Taylor & Francis, 2007. The dissertation author was the primary investigator and single author of this paper.

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ABSTRACT OF THE DISSERTATION

Continuous Diagnostic Frequency Ultrasound and the Microcirculation

by

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Doctor of Philosophy in Bioengineering

University of California, San Diego, 2007

Professor Marcos Intaglietta, Chair

Vital organ tissue in victims of strokes and heart attacks and patients undergoing cardiac and transplant surgeries present damage resulting from ischemia reperfusion injury in the form of impaired vascular function and tissue perfusion, and inflammatory conditions. Patient studied by diagnostic ultrasound have been found to present significantly attenuated indications of reperfusion injury, leading to the hypothesis that the use of ultrasound could be a therapeutic treatment for ischemia reperfusion injury. These studies were carried out to elucidate the biochemical mechanism underlying

ultrasound irradiation's efficacy in the prevention of ischemia reperfusion injury with particular emphasis on the affects on nitric oxide (NO) production.

Studies were carried out in the microcirculation using the methods of intravital microscopy and the dorsal skin fold window chamber hamster preparation. *In vivo* real-time analysis of the microcirculation was performed in the tissue of conscious animal subjects. The principal data assessed was functional capillary density and microvascular diameter, blood flow velocity and flow, in order to assess tissue injury and vessel function. We compare tissue injury with and without ultrasound irradiation as well as ultrasound irradiation during ischemia or reperfusion. We also compare the influence of NO synthase (NOS) inhibition on the effects of ultrasound irradiation of animals. We carried out molecular comparative analysis of irradiated and unexposed tissue by Western Blotting. The effects of ultrasound exposure on inflammatory conditions were determined by assessing venular leukocyte endothelial cell interaction with ultrasound irradiation.

Improved microcirculatory function of all vessels following ultrasound irradiation was seen as a long-term effect (24 hours), supporting the hypothesis of injury moderation by diagnostic ultrasound stimulation. Arterioles showed increased flow; capillaries had enhanced perfusion and venules showed decreased leukocyte endothelial cell interaction. Molecular analysis and inhibition of endothelial and inducible NOS clarified the difference between stimulated NO production pathways in causing beneficial effects in the damaged microcirculation. The mechanism underlying the efficacy of ultrasound irradiation in the prevention of ischemia reperfusion injury is evidently mediated by NO production.

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Introduction

ISCHEMIA REPERFUSION INJURY

Ischemia and subsequent reperfusion of blood flow is a common event occurring at least once in the average life span of all human beings. Minor ischemic events take place in instances where prolonged pressure is put on a part of the body, restricting or preventing the flow of blood to that area. Ischemia, the interruption of blood flow, allows the accumulation of metabolic waste products, regularly removed by flowing blood. Damage to the surrounding soft tissue takes place as a result of the increased exposure to the toxic metabolites (25). Depending on the length of the ischemic period damage may also result from a lack of oxygen. Removal of the pressure, and therefore terminating the ischemic period, reduces ischemic injury to the tissue. Blood once again allowed to perfuse the tissue removes waste products and replenishes the area's oxygen levels. However, detrimental effects are also associated with reperfusion's flow velocity and oxygen influx, especially in the microvasculature (4, 18, 25). The term ischemia reperfusion (IR) injury in this current study describes both the damage associated with ischemia and the further increase in damages after the onset of reperfusion.

Concern regarding this topic relate to the major clinical occurrence of IR injury evidenced in, heart attacks, strokes, hemorrhagic shock, and during cardiac and transplant surgeries. Mitigation of the resulting damages would improve recovery and recovery time as well as enhance the patient's quality of life. Recent investigation into the attenuation and prevention of IR injury have resulted in developmental treatment

Ι

methods relating to preconditioning (32), postconditioning (40) and the use of antioxidants (33, 54). However, these methods cannot be used in general due to their limited effectiveness in treating specific IR injuries.

ULTRASOUND IRRADIATION AND BLOOD FLOW

Ultrasound application may be a general treatment for IR injury (2, 12, 59). This assertion originates from the observation of patients who were studied by echocardiography after experiencing a heart attack showing a lower incidence and level of cardiac damage (personal communication, S. Bertuglia, (12). Subsequent studies showed that *in vivo* ultrasound irradiation of coronary and peripheral blood vessels results in vasodilation in healthy tissue (52, 69).

The systemic and microvascular regulation of blood flow is regulated through neural and myogenic controls and endogenous mediators produced locally or transported through the circulating blood. A release of neurotransmitters and stimulation of receptors generally results in vasoconstriction of arterioles and decrease in local flow. Stimulation of vascular smooth muscle receptors causes relaxation increasing blood flow. The myogenic mechanism reacts to intravascular changes in blood pressure, regulating vascular constriction. The endogenous mediators nitric oxide (NO), prostaglandins, and endothelins are the major effectors in vascular dynamics. NO, the more potent mediator that determines the basal level of blood flow regulating arteriolar diameter is produced by endothelium and partially counteracts effects of myogenic and neuronal control.

Influence of ultrasound irradiation on any of the 3 regulatory mechanism, during vascular occlusion or IR can result in the reported vasodilation (52, 69). Ultrasound

irradiation of cell cultures resulted in stimulated temporary cellular alterations (31, 59) and increased production of the NO conversion product nitrite (2, 61), which was significantly reduced with NO production inhibitory treatment (61). Recent studies of IR (12) and vessel occlusion (71, 72) reported enhanced microcirculatory and soft tissue function related to NO production after irradiation. Notably to the present, all *in vivo* investigations aimed at developing ultrasound irradiation as a therapy for IR injury have relied on data form exposed tissue models and or anesthetized animals. However, surgical procedure to expose tissue is neither practical nor a customary clinical procedure in treating IR injuries.

OUR APPROACH

To further characterize and delineate the exact effects of ultrasound irradiation of soft tissue in IR injury attenuation and blood flow mediation we employed a microvascular function comparative analysis, with the use of intravital microscopy and irradiation with continuous mode diagnostic frequency ultrasound. The limitations in transferring previously published experimental setups (12, 71, 72) to general clinical practice, as stated above, are removed with the use of the hamster window chamber model (chamber). The chamber allows microvascular assessment in conscious animals, avoiding the effects of anesthesia (21), and the use of intravital microscopy enables direct real-time measurement and analysis of vascular parameters (16, 24). Use of continuous mode diagnostic frequency ultrasound irradiation allows uninterrupted stimulation, contrasting pulse mode stimulation, while allowing for tissue penetration depth similar to that of diagnostic procedures.

To investigate the efficacy of continuous diagnostic frequency ultrasound exposure in improving damage to the microcirculatory system resulting from IR injury, as well as investigating the relationship of these effects with NO production we carried out the following studies:

- Determine how the effects from IR injury on functional capillary density (FCD) and microvascular diameter and velocity are influenced by exposure to diagnostic frequency continuous ultrasound.
- Determine how ultrasound treatment affects the microcirculation during inhibition of NO production.
- Analysis of the activity of NO synthase (NOS) isoforms and interfering with NO synthesis during ultrasound irradiation after IR injury and determining the effects on microvascular function.
- Examine the effect of ultrasound exposure on the leukocyte endothelium interaction resulting from IR injury and in relation to NO production.

ORGANIZATION OF THE TEXT

This introductory chapter provides a brief description of ischemia and subsequent reperfusion, and their injurious effects. We further discussed clinical IR injury and the current developmental treatments. Our discussion of blood flow regulation, explains the need to further investigate the therapeutic use of ultrasound in IR injury mitigation. We end the chapter by addressing the limitations of previous investigation ultrasound irradiation procedures, following a brief overview of the most common and recently used experimental protocols.

Chapter 2 describes our methodology and experimental protocol used to investigate the influence of continuous diagnostic frequency ultrasound on microcirculatory function, during IR. Our methodology applies the use of fluorescent intravital microscopy, 2 differing irradiation protocols, as well as treatment with N^{oo}nitro-l-arginine methyl ester (L-NAME), a NOS inhibitor. We show comparative analysis results in enhanced microvascular function after ultrasound irradiation and negation of effects with L-NAME. The results of this study show that ultrasound treatment provides beneficial results evident 24 hours from the intervention.

In chapter 3, we demonstrate the differing influence of NO synthesis from eNOS and iNOS enzymes. We use the iNOS specific inhibitor N-(3-aminomethyl) benzylacetamidine dihydrochloride (1400W), in a similar IR injury and ultrasound irradiation model as described in chapter 2. In addition we used the western blot protocol for the analysis of eNOS and iNOS protein levels in irradiated injured tissue samples. Our analysis confirms the relation between NO production and ultrasound irradiation, and highlights the difference in effects due eNOS and iNOS isoforms.

Chapter 4 examines the effects of ultrasound treatment on venular injury. In this study we focused on the enhanced leukocyte endothelial cell interaction resulting from IR. Our comparative examination shows that enhanced NO production by ultrasound irradiation restores/preserves venular function, and may stimulate other therapeutic effects not related to NO production.

In chapter 5 we focus on the conclusions derived from our findings and relate these to previous studies of IR injury and treatment, specifically treatment with ultrasound irradiation. We discuss the strengths and weakness of our investigative approach as well as the study's limitations. We also discuss possible future work to optimize the affects of our current experimental ultrasound irradiation protocol. We end with concluding remarks and a brief comment on the clinical implications of our study. This chapter, in full, is a reprint of the material as it appears in Microcirculation 2007, Hightower C M and Intaglietta M., Taylor & Francis, 2007. The dissertation author was the primary investigator and single author of this paper.

The Use of Diagnostic Frequency Continuous Ultrasound to Improve Microcirculatory Function after Ischemia-Reperfusion Injury

ABSTRACT

Objective: Damage to the circulatory system resulting from ischemia-reperfusion injury (I/R injury) occurs during heart attacks and hemorrhagic shock. We report a method for mitigating microcirculatory injury, using diagnostic frequency continuous mode ultrasound and how effects are influenced by nitric oxide production impairment. **Methods:** Five groups of hamsters were studied using the dorsal skin fold window chamber: 1) I/R; 2) I/R+ ultrasound during ischemia; 3) I/R+ ultrasound after ischemia; 4) I/R+ N^Φ-nitro-L-arginine methyl ester (L-NAME); and 5) I/R+ L-NAME+ ultrasound. Functional capillary density (FCD) and microvascular diameter, flow velocity, and flow were monitored. 2.49 MHz continuous ultrasound was used during exposures. **Results**: Significant improvements in animals exposed to ultrasound after ischemia were

found at 24 h of reperfusion in FCD, arteriolar diameter, and arteriolar and venular flow velocity and flow. Animals exposed to ultrasound during ischemia showed significantly improved FCD. L-NAME treatment reduced the improvement of microvascular function, compared to animals exposed after ischemia.

Conclusion: The use of continuous mode diagnostic frequency ultrasound is beneficial in preventing long-term ischemia-reperfusion effects in the microcirculation as shown by the return of microvascular parameters to baseline values, an effect not attained in the

absence of ultrasound treatment. The effects may be in part due to the production of nitric oxide consequent to locally induced shear stress effects by ultrasound exposure. **Key words:** ischemia-reperfusion; diagnostic frequency ultrasound; microcirculation; L-NAME

INTRODUCTION

Ischemia-reperfusion injury (I/R injury) is a two step process that begins with the cessation of blood flow to a tissue or organ leading to an oxygen deficit and the build up of toxic metabolites normally removed by the flowing blood. Restoration of blood flow stops and reverses the ischemic damage, however gives rise to cellular injury due to the formation of oxygen radicals (4, 25, 51, 66, 73) resulting from the sudden influx of significant quantities of oxygen. I/R injury occurs in victims of heart attacks, strokes, and hemorrhagic shock. It is a process particularly evident in the microcirculation where arterioles show impaired vasoactivity (24, 51), blood flow in the capillary network is reduced (14, 25, 51) and, leukocyte sticking (4, 24, 25, 77) and migration from the lumen to the surrounding tissue (78) takes place in venules.

There is evidence that the damage consequent to I/R injury may be in part due to the impairment of nitric oxide (NO) production by the microvascular endothelium, a process partly due to shear stress and mechanotransduction (7, 23) resulting from the flowing blood. Mechanotransduction enables NO production (8) and has been proposed to be the underlying cause for the beneficial effects resulting from the exposed tissue (and organisms), recovering from sustained I/R injury, to ultrasound (72). Ultrasound exposure was shown to stimulate DNA and protein synthesis (61), NO production (60), and induce cellular alterations and enhance cellular proliferation (59) in cell cultures. It was also shown to increased capillary perfusion and microvascular diameter and velocity in animals recovering from sustained I/R injury (12).

Currently most experiments using ultrasound to obtain effects in tissues and cell cultures employ low range frequencies with continuous mode (60, 61, 71) or diagnostic

frequencies (1, 12) with pulse mode exposure. Low frequency continuous mode exposures allow for greater tissue penetration without heating. Pulse mode exposures used in diagnostic procedures have a shorter period of interaction with the system being exposed when compared to the continuous mode. In this context the use of the lower ranges of diagnostic frequencies (MHz) in combination with continuous mode exposure allows for greater depth in tissue penetration and a longer period of interaction.

The objective of this study was to investigate the efficacy of continuous mode (i.e. 100% duty cycle) diagnostic frequency ultrasound exposure in mitigating the damage to the microcirculation resulting from I/R injury. Specifically this study analyzes how functional capillary density (FCD) and microvascular diameter and velocity are influenced by exposure to continuous mode diagnostic frequency ultrasound after I/R injury. In view of the significant role that NO has in mediating I/R injury and the mechanical nature of the ultrasound intervention we also test the hypothesis that the effects of ultrasound in I/R injury are due to the reversal of NO production impairment in the endothelium.

MATERIALS AND METHODS

Animal model and preparation

The dorsal skin fold window chamber model (chamber) was used for investigation in Golden Syrian male hamsters (Charles River, Boston, MA), weight range of 50 to 75g. The Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) was followed for animal handling and provided care. Experiments are approved by the University of California, San Diego Animal Subjects Committee. The chamber, a widely used procedure, eliminates anesthetic influence during microcirculation examination. As described previously (21), chamber and carotid artery catheterization surgery was performed under general anesthesia, 50 mg/kg ip. injection of pentobarbital sodium (24). TPX (Westlake Plastics, Placentia, CA) was used in place of the usual glass cover slip. TPX incorporates the protective properties of glass with greater acoustical coupling (15). Initial microvascular observation carried out at least 3 days after chamber implantation, diminished post surgical complications.

Inclusion criteria

Animals used were free of edema, pre-observation injury to window area, and or infection to surgical sites.

Assessment of microcirculatory parameters

All microscopic observations were preformed using an upright microscope (BX51WI; Olympus, New Hyde Park, NY). Images in the microscopic field of view could also be viewed on a monitor, through the projection of the image onto a chargecoupled device camera (COHU 4815) connected to a videocassette recorder (AG-7355; JVC, Tokyo, Japan). A 40x (LUMPFL-WIR, numerical aperture 0.8; Olympus) water immersion objective was used in both transillumination and epiillumination observations. Contrast enhancement between flowing red blood cells (RBC) and tissue was accomplished using a BG12 (420 nm) band pass filter. Epiillumination microscopy used an additional mercury 100-W lamp and appropriate filters. Vessel diameter (D) was obtained online using a video shearing technique (37). Real time blood flow velocity (V) was acquired using the photodiode cross-correlation method (Fiber Optic Photo Diode and Velocity Tracker, model 102B; Vista Electronics, San Diego, CA) (36), using a correction factor average velocity (V_a) was calculated, V_a = V/1.6 (48). Blood flow (Q) was calculated using the measured and calculated parameters, Q = π V_a*(D/2)². FCD was determined in 13 to 25 stepwise vertically successive microscopic fields, (a region of about 1.7 to 1.9 mm²). The initial field chosen by an anatomical feature allowed quick recognition in repeated measurements. Functional capillaries had at least one RBC flowing during the observation period.

Only arterioles and venules with baseline diameters less than $60\mu m$ were included in the present study.

Ischemia-reperfusion injury

A pressure tourniquet, described previously (24, 50), was used to induce ischemia in the chamber. Flow of the area under ischemia, interrupted by tightening a screw connected to the chamber, was monitored until obtaining complete occlusion of all feeding and draining vessels (50). Ischemia was ensured through periodic inspection. The release of the tourniquet marked the end of ischemia and start of reperfusion.

Ultrasound exposure

During ultrasound exposure animals were suspended over the ultrasound transducer using a three-pronged clamp and support ring stand. The transducer (Valpey Fisher Corporation immersion transducer, part # IL0208HP, nominal frequency 2.25 MHz, element diameter 1 in.) was secured in a water bath. Animal exposure was accomplished using setup one or two. Setup one: A degassed water filled piece of latex glove covered the transducer head. A thin layer of acoustic gel connected the latex and cover slip. Setup two: An open ended plastic cone affixed to the face of the transducer, filled with degassed water. In each setup a piece of lightweight rubber absorbed waves continuing through the skin fold. Waves were generated using a function generator (30 MHz Synthesized Function Generator, model DS345; Stanford Research Systems, Sunnyvale, CA) set to produce a continuous sine wave at a frequency of 2.49 MHz with a 10 V_{p-p} amplitude. Resultant intensity and pressure produced is 8.33 W/cm² and about 0.5 MPa, respectively. (The transducer was calibrated using a hydrophone in the Ferrara Laboratory, Department of Biomedical Engineering, University of California, Davis).

Experimental groups

Animals were randomly assigned into one of 5 groups as follows: I/R group (n=5), I/R+ ultrasound during ischemia group (n=5), I/R+ ultrasound after ischemia group (n=5), I/R+ N^{ω}-nitro-L-arginine methyl ester (L-NAME) group (n=5), and I/R+ L-NAME+ ultrasound group (n=5).

Experimental setup and protocol

Conscious animals were placed in plexi-glass restraining tubes, allowing for protrusion of chamber and minimized movement during observation and ultrasound exposure. Tubes containing hamsters were further secured to custom-made frames and placed on the microscope stage. Similar microcirculatory assessment procedures were preformed in all groups. Transillumination was used for baseline vascular architecture

documentation and vessel site selection. Vessels were investigated in terms of diameter, RBC velocity, and blood flow. The same measurement sites were used throughout each experiment to allow direct comparison with baseline values. FCD was also determined during this time. Animals were then subjected to a 4 h period of ischemia and measurements were repeated at 0.5, 2, and 24 h, from the start of reperfusion. Vessel diameter and FCD were assessed using epiillumination during repeated measurements, while transillumination was used for vessel flow velocity. An injection of fluorescein isothiocyanate, bound to dextran (MW 150,000; FITC-Dextran 150 Sigma Chemical, St. Louis, MO; 0.1 ml of a 12.5 mg/ml saline) was given 5 to 10 min before each individual repeated measurement period. Epiillumination and FITC-Dextran normally used for enhanced visualization of vessels was used here to augment damage sustained from I/R (24). The volume for infusion of L-NAME (Sigma) solution in saline was less than 5% of systemic blood volume, an estimate of 7% of hamster total body weight on day one of the experiment. Injections where dosed at 10 mg/kg in each group. The sample solution of 5 ml/g (0.1 to 0.15 ml) was infused through the carotid artery by hand and the catheter was flushed with heparinized saline (Figure 2.1).

Ischemia-reperfusion and ultrasound exposure groups

Experimental procedure of the I/R and I/R+ ultrasound groups followed the general protocol as mentioned above. In addition the I/R+ ultrasound during ischemia group was exposed to ultrasound for a 10 min period, during the last 10 min of ischemia. The I/R+ ultrasound after ischemia group was exposed to ultrasound for a 20 min period, 5 min following the onset of reperfusion.

Ischemia-reperfusion L-NAME and ultrasound exposure groups

Experimental procedure for the I/R+ L-NAME and I/R+ L-NAME+ ultrasound groups followed the general protocol for baseline and repeated microcirculation assessments. L-NAME was administered to both groups as a bolus injection 5 min before the release of the tourniquet. Animals in the I/R+ L-NAME+ ultrasound group were exposed to ultrasound for a period of 20 min, 5 min following the onset of reperfusion.

Statistical analysis

Results are presented as means \pm standard deviation. Data graphs are presented normalized to baseline values. Data comparisons made within groups and between group controls and treatments were analyzed using the Kruskal-Wallis nonparametric test with Dunn's post test or the Mann-Whitney nonparametric test. Differences were considered significant for p < 0.05. All statistics were calculated using GraphPad Prism 4.01 (GraphPad Software; San Diego, CA).

RESULTS

Ischemia-reperfusion and ultrasound exposure groups

In this study we focus on the changes due to ultrasound exposure not those due to ischemia-reperfusion. Ultrasound treatment significantly increased FCD 24 h after release of the tourniquet in both I/R+ ultrasound during ischemia and after ischemia groups relative to unexposed animals; FCD was below baseline for all groups at all time points with the exception of one animal exposed to ultrasound after ischemia (p < 0.01, **Figure 2.2**).

Vessel diameters increased for most time points during reperfusion in all animal groups. At 24 h, arteriolar diameters of animals exposed to ultrasound after ischemia were significantly increased above those of both the I/R and I/R+ ultrasound during ischemia groups (p < 0.001, **Figure 2.3**).

A common trend was seen in the arteriolar flow velocity of all groups. Flow velocity values recorded at 0.5 h into reperfusion were observed to decrease at 2 h and then to increase at 24 h. All groups showed significance in the decrease at 2 h, with the greatest significance seen in the I/R group (p < 0.001). A significant increase at 24 h was only seen in the I/R+ ultrasound after ischemia group. At 24 h both arteriolar and venular flow velocity of the I/R+ ultrasound after ischemia group increased significantly over that of the I/R group (p < 0.05). Arteriolar flow velocity of the I/R+ ultrasound after ischemia group increased significantly over that of the I/R group (p < 0.05). Arteriolar flow velocity of the I/R+ ultrasound after ischemia group increased significantly over that ultrasound ultrasound ultrasound ultrased at 24 h compared with the group exposed to ultrasound during ischemia (p < 0.001, **Figure 2.4**).

Blood flow was maintained in all vessels of all groups throughout the reperfusion period. Calculated flows showed substantial increases in the arteriolar and venular flow of the group exposed to ultrasound after ischemia compared to the I/R group only at 24 h. A similar trend seen in the arteriolar flow velocity of each group was evident in the calculated blood flow. Blood flow values 0.5 h into reperfusion were observed to decrease at 2 h and then to increase at 24 h (**Figure 2.5**). All groups were significantly decreased at 2 h, with a statistically significant increase at 24 h, above both 0.5 and 2 h flows, seen in the I/R + ultrasound after ischemia group (**Figure 2.5**).

Ischemia-reperfusion L-NAME and ultrasound exposure groups

The degree of I/R injury impacted by NO production inhibition was determined by changes in FCD and vessel diameter, flow velocity, and flow, relative to baseline at each measurement. The effect of the L-NAME bolus injection was seen in arteriolar and venular flow velocity and flow, at 0.5 h of reperfusion, being statistically different from I/R animal arteriolar flow velocity (**Figure 2.8**). **Figure 2.6** shows how NO inhibition affects FCD at each repeated measurement. FCD was decreased in all animals, compared to baseline values. It was higher in I/R+ L-NAME group animals at the 0.5 and 24 h measurements; however this observation did not reach statistical significance.

Figure 2.7 shows the changes in arteriolar and venular diameter during reperfusion, comparing the I/R+ L-NAME and I/R+ L-NAME+ ultrasound groups. The majority of arteriolar and venular values were increased compared to baseline. Changes in arteriolar and venular diameter between L-NAME groups were not significant.

Vessel flow velocity decreased compared to baseline, for both arterioles and venules up to 24 h after termination of ischemia (**Figure 2. 8**). Values increased at 24 h in each L-NAME group for both arterioles and venules, although significant increases compared to I/R+L-NAME were seen only in arterioles, exposed to ultrasound (p < 0.05). A significant increase was seen from the 0.5 h to the 24 h measurement in the animals treated with L-NAME and exposed to ultrasound (**Figure 2.8**).

Blood flow was maintained in all animals after receiving L-NAME treatment and exposure to ultrasound. A statistically significant difference in arteriolar flow was seen at 24 h compared to the I/R+ L-NAME group (**Figure 2.9**). The arteriolar flow of the I/R+ L-NAME+ ultrasound group showed a significant increase from the 0.5 h to the 24 h

measurement. Both arteriolar and venular flow also showed statistical significance in the increased flow from the 2 h to the 24 h measurement (**Figure 2.9**). Flow of one arteriole and one venule did not return in the I/R+ L-NAME group after the ischemic period. Arteriolar flow did not return through 24 h of reperfusion; while venular flow returned at 24 h. Vessels lacking flow at any time point were excluded from all graphs.

I/R + ultrasound after ischemia and I/R + L-NAME+ ultrasound exposure groups

We compared the changes in flow in the two groups (**Figures 2.5 and 2.9**). Flow was decreased at 0.5 and 24 h in both groups treated with L-NAME compared to the group I/R+ ultrasound after ischemia. Decreased flow in the I/R+ L-NAME+ ultrasound group at the 0.5 h assessment was statistically significantly different (p < 0.001) from the I/R+ ultrasound after ischemia group at the 0.5 h point, while there was no significant difference at 24 h (p = 0.1).

DISCUSSION

The principal finding of this study is that exposure to diagnostic frequency continuous ultrasound after 4 h of ischemia improves FCD and microvascular flow 24 h after reperfusion. This result was obtained by exposing the tissue to continuous wave ultrasound for 20 min, 5 min from the onset of reperfusion. Exposure of the tissue to the same ultrasound settings for 10 min, during the last 10 min of the ischemic period, resulted in significant improvement of FCD seen 24 h into reperfusion. These improvements were negated by the inhibition of NO production by treatment with L-NAME prior to reperfusion. Although ultrasound did improve L-NAME treated animals at 24 h after reperfusion, the degree of recovery was significantly reduced by comparison to the untreated group.

Twenty min of ultrasound exposure resulted in microcirculatory improvements in all microvessels, while 10 min exposure only improved FCD. The difference in effects may be due to several factors. It is hypothesized that vessel blood flow may be necessary to produce beneficial effects through the production of mediators. This hypothesis is contradicted in part by the observations of Suchkova et al. (71) who found that tissue perfusion increased and acidosis decreased during thrombosis throughout the application of ultrasound (40 kHz, continuous wave mode). However, the increased perfusion was thought to result from collateral vessel flow, which is not a possibility in our experimental setup using complete ischemic isolation of blood flow to the area under investigation. A second possibility for explaining the differences in ultrasound exposure effects is based on the existence of a threshold time limit. The 10 min exposure did not reach the time needed to successfully influence the damaged microcirculation. This result could be related to the existence of a threshold of total acoustic energy exposure that determines positive effects in endothelial cell cultures proposed by Raz et al. (59) that is dependent on the amount of energy transferred.

Experimental studies examining the influence of preventative treatments or treatment administered after sustained injury, followed over extended observation periods report attainment of statistical significance when assessed after an extended period with little or no significance in short term assessments. Significant differences between control and endothelial cell cultures exposed to different parameters of ultrasound irradiation became evident days after the first 24 h observation (59). Hangai-Hoger et al. (27) observed a significant improvement of FCD in animals resuscitated from septic shock with polyethylene glycol conjugated bovine albumin (PEG-BSA-24). This result was only apparent from systemic and microvascular measurements 24 h after initial treatment.

The efficacy of the dosage of L-NAME in inhibiting NO bioavailability was previously established in other investigations in the same preparation and tissue. In these studies of vasoconstriction and arterial flow reduction was found with the dosage of 10 mg/kg (65).

Observations of L-NAME treated animals suggest the existence of both fast and slow acting responses to ultrasound exposure. L-NAME treatment may only influence the earlier stimulation effect, leaving the slower response to continue its action, resulting in the decrease of significant microvascular improvements. L-NAME being a nitric oxide synthase (NOS) inhibitor directly affects the arterioles because of NO's affects on smooth muscle. The majority of microcirculatory improvements, observed in animals not treated with L-NAME were in the arterioles suggesting improvements previously seen in venules and capillaries were indirect effects of ultrasound stimulation. The moderate improvements in the down stream vessels. It may also suggest that decreased improvements of venules may be due to other factors not investigated in this study, such as the accumulation of leukocytes in venules, which may be reduced with the presence of NO (15, 16). This hypothesis may also partially explain the reduction in FCD.

Decreased initial reperfusion flow would lessen vessel exposure to oxygen radicals, while still rapidly flushing away built-up metabolites. Animals exposed to
ultrasound after ischemia showed decreased initial arteriolar flow compared to control animals 0.5 h into reperfusion. Two hours into reperfusion the decrease in arteriolar flow is hypothesized to be evidence of the amount of damage caused by ischemia and initial reperfusion. This effect may be in part responsible for lowering reperfusion damage, since it inherently limits the availability of oxygen, directly implicating the formation of ROS.

The animal groups treated with L-NAME also show evidence of the benefit of reduced initial arteriolar flow. The 0.5 and 24 h flows of the I/R+ L-NAME+ ultrasound group are decreased compared to I/R+ ultrasound after ischemia group, further supporting the hypothesis that an intermediate initial flow is best for long-term recovery. The increase in arteriolar flow at 2 h for each L-NAME group from 0.5 h, as compared to the decrease seen in the groups not treated with L-NAME is thought to be an artifact of L-NAME treatment. The use of L-NAME as the only NOS inhibitor in the study does not allow for a definitive distinction between the three forms of NOS, endothelial, inducible, and neuronal, although it does have a greater capacity of preventing eNOS and nNOS production over that of iNOS (14, 68).

In summary this study provides evidence of a new approach in the therapeutic use of ultrasound. It also suggests further improvements may be obtained without increased ultrasound exposure by including delivery of naturally occurring beneficial mediators of vascular health, such as L-arginine. Evidence for the benefits of diagnostic cardiac (12), high frequency (1, 60, 61), and low frequency (60, 61, 71) ultrasound have been previously demonstrated. Our recent observations expand the range of beneficial ultrasound applications, showing its applicability in conditions of microvascular I/R injury.

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APPENDIX



Figure 2.1 Schematic outline of the experimental protocol for each group. US, ultrasound.



Changes in functional capillary density (FCD) due to ischemia-reperfusion injury and exposure to ultrasound. FCD of groups receiving ultrasound exposure, for 10 min during ischemia and for 20 min after ischemia showed a significant increase from I/R at 24 h. Data are presented as mean \pm standard deviation. I/R, I/R group; D, I/R+ ultrasound during ischemia group; A, I/R+ ultrasound after ischemia group. *p < 0.05 vs. I/R.



Changes in arteriolar and venular diameters due to ischemia-reperfusion injury and exposure to ultrasound. *Top Panel:* I/R+ ultrasound after ischemia animals showed significant increase of arteriolar diameters at 24 h, compared to both I/R and I/R+ ultrasound during ischemia animals. *Bottom Panel:* No significant venular diameter difference from I/R was found for either of the groups exposed to ultrasound. Values are presented as mean \pm standard deviation. I/R, I/R group; D, I/R+ ultrasound during ischemia; A, I/R+ ultrasound after ischemia group. ***p < 0.001 vs. I/R. ^^^p < 0.001 vs. I/R.



Changes in arteriolar and venular flow velocity due to ischemia-reperfusion injury and ultrasound exposure. *Top Panel:* Arteriolar flow velocity of I/R+ ultrasound after ischemia animals significantly increased at 24 h, compared to both I/R and I/R+ ultrasound during ischemia animals. *Bottom Panel:* I/R+ ultrasound after ischemia animals showed significant increases in venular flow velocity at 24 h, compared to the I/R group. Values are presented as mean ± standard deviation. I/R, I/R group; D, I/R+ ultrasound during ischemia group; A, I/R+ ultrasound after ischemia group. +++p < 0.001 vs. I/R 0.5 h assessment. $\beta\beta p < 0.01$ vs. I/R+ ultrasound during ischemia 0.5 h assessment. $\Phi p < 0.05$ vs. I/R+ ultrasound after ischemia 0.5 h assessment. $\gamma p < 0.001$ vs. I/R+ ultrasound during ischemia 0.5 h.



Calculated changes in arteriolar and venular flow due to ischemia-reperfusion injury and ultrasound exposure. *Top Panel:* In the I/R+ ultrasound after ischemia group arteriolar flow significantly increased at 24 h, compared to both the I/R and the I/R+ ultrasound during ischemia groups. *Bottom Panel:* Venular flow significantly increased at 24 h, for the I/R+ ultrasound after ischemia group compared to the I/R group. Values are presented as mean \pm standard deviation. I/R, I/R group; D, I/R+ ultrasound-during ischemia group; A, I/R+ ultrasound after ischemia group. +++p < 0.001 vs. I/R 0.5 h assessment. $\beta p < 0.05$ vs. I/R+ ultrasound during ischemia 0.5 h assessment. $\beta p < 0.05$ vs. I/R+ ultrasound after ischemia. $\nabla \nabla p < 0.01$ vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.001 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.05 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.05 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.05 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.05 vs. I/R+ ultrasound after ischemia 0.5 h assessment. *p < 0.05 vs. I/R.



Changes in functional capillary density (FCD) due to ischemia-reperfusion injury, exposure to ultrasound, and NO production inhibition using L-NAME. FCD of the I/R+ L-NAME group is greater than that of the I/R+ L-NAME+ ultrasound group, at 0.5 and 24 h, without statistical significance. Data are presented as mean ± standard deviation. I/R, I/R group; I/RL, I/R+ L-NAME group; UL, I/R+ L-NAME+ ultrasound group.



Changes in arteriolar and venular diameters due to ischemia-reperfusion injury, exposure to ultrasound, and NO production inhibition using L-NAME. *Top Panel:* Most arteriolar diameters dilated compared to baseline values. *Bottom Panel:* Venular diameters also show mostly dilation at all observation time points compared to baseline values, without statistical significance. Values are presented as mean ± standard deviation. I/R, I/R group; I/RL, I/R+L-NAME group; UL, I/R+L-NAME+ ultrasound group.



Changes in arteriolar and venular flow velocity due to ischemia-reperfusion injury, exposure to ultrasound, and NO production inhibition using L -NAME. *Top Panel:* The influence of L-NAME is seen in the 0.5 h measurement, with a significant decrease compared to I/R. Arteriolar velocity exhibited an increase from 0.5 h through the 24 h time point. A significant increase was seen at 24 h in I/R+ L-NAME+ ultrasound animals, compared to I/R+ L-NAME. *Bottom Panel:* Venular velocity of both groups was highest at the 24 h time point but not significantly different. Values are presented as mean \pm standard deviation. I/R, I/R group; I/RL, I/R+ L-NAME group; UL, I/R+ L-NAME+ ultrasound group. #p < 0.05 vs. I/R. *p < 0.05 vs. I/R+ L-NAME. $\nabla \nabla p < 0.01$ vs. I/R+ L-NAME+ ultrasound 2 h assessment.



Changes in arteriolar and venular flow due to ischemia-reperfusion injury, exposure to ultrasound, and NO production inhibition using L-NAME. *Top Panel:* Arteriolar flow increased in I/R+ L-NAME+ ultrasound animals, while the increase at 2 h was not maintained through 24 h, in I/R+ L-NAME animals. Statistical significance was seen at 24 h for I/R+ L-NAME+ ultrasound compared to I/R+ L-NAME animals. *Bottom Panel:* Venular flow exhibited a stepwise increase for I/R+ L-NAME animals, while the decrease at 2 h increased at 24 h for ultrasound exposed animals. No significant differences were seen between the groups. Values are presented as mean ± standard deviation. I/R, I/R group; I/RL, I/R+ L-NAME group; UL, I/R+ L-NAME+ ultrasound group. **p < 0.01 vs. I/R+ L-NAME. $\nabla \nabla p < 0.01$ vs. I/R+ L-NAME+ ultrasound 2 h assessment.

Early iNOS Impairment and Late eNOS Enhancement during Reperfusion following 2.49 MHz Continuous Ultrasound Exposure after Ischemia ABSTRACT

Objective: Ischemia reperfusion (IR) injury, occurring during heart attacks, hemorrhagic shock, and bypass and transplant surgeries, impairs microcirculatory function and nitric oxide (NO) synthesis. We report a regulation of endothelial and inducible NO synthase (eNOS and iNOS) proteins, with continuous mode diagnostic frequency ultrasound irradiation during IR injury.

Methods: Animals were assigned to one of 5 groups for microcirculatory assessment or western blot analysis (WB) as follows: 1) IR+1400W; and 2) IR+1400W+ultrasound for microcirculatory assessment, 3) Control; 4) IR; and 5) IR+ultrasound for WB. Functional capillary density and microvascular diameter, flow velocity, and flow were monitored for microcirculatory assessment. Skin tissue samples were harvested for WB. 2.49 MHz continuous ultrasound was used for irradiation.

Results: Both the inhibition of iNOS alone and iNOS inhibition with ultrasound irradiation positively influenced the microcirculation of observed animals, however results were not significantly different; Ultrasound exposure resulted in a significant production of eNOS protein in skin tissue harvested 24 h into reperfusion (p < 0.01). iNOS levels from the same tissue of irradiated animals were found to be significantly decrease 0.5 h into reperfusion (p < 0.05).

Conclusion: Protection from lasting IR injury effects in the microcirculation, with continuous mode diagnostic frequency ultrasound, results from augmented eNOS levels

during late reperfusion. Ultrasound inhibited iNOS production during early reperfusion may also confer damage preventative influences during IR injury.

Key words: ischemia reperfusion; ultrasound; microcirculation; eNOS; iNOS

INTRODUCTION

The beneficial effects of nitric oxide (NO) in the circulation and microcirculation have been widely documented (4, 41, 44, 55, 70). Reports demonstrate its vasodilatory properties and its role in maintaining and regulating blood flow in healthy tissues (34, 35). Increase of tissue damage following initial injury, through microvascular impairment and leukocyte endothelial cell interaction, has been attributed to the decreased production of NO (11, 17, 44, 56). Injury in diseased or abnormal tissue leading to microcirculatory dysfunction has also been associated with inhibited NO synthesis (45, 74). Analysis of the increased impairment of microcirculatory function and tissue injury following NO inhibitory treatment (53, 55) led to the identification of the NO synthase (NOS) enzyme and its endothelial and inducible isoforms, eNOS and iNOS respectively.

Studies on the prevention and treatment of ischemia reperfusion (IR) injury (9, 11, 56, 58, 67) have shown that eNOS and iNOS activity leads to differences in NO production encompassing both negative and positive effects. eNOS activity is most commonly reported as beneficial (17, 29), contrastingly, a majority of findings showing that iNOS derived NO has a detrimental influence (26, 58) on damaged tissue.

The usual protocol for analysis of the influence of NO on IR involves the use of exposed tissue from anesthetized animals, and the local manipulation of NO production thus avoiding responses due to systemic effects (42). These investigations are therefore clinically relevant in the context of cardiac angioplasty or bypass and transplantations, involving the ligation of vasculature or the removal of a vascular block.

IR injury seen clinically in victims of heart attack, stroke, hemorrhagic shock, and transplant recipients, does not always afford the possibility of exposing the damaged tissue for treatment. Furthermore, such invasive actions may increase tissue damage through the procedure itself or possible infection. The noninvasive use of ultrasound irradiation, during circulatory occlusion and IR injury, should be advantageous in mitigating tissue damage in exposed (12, 72) and unexposed (13, 28) tissue.

The objective of this present study was to differentiate between eNOS and iNOS activity during IR injury with ultrasound exposure. Specifically this study examines the production and regulation of eNOS and iNOS protein due to the application of continuous mode diagnostic frequency ultrasound during IR injury. Our previous work reported significant improvement with ultrasound irradiation after an extended period of reperfusion, which was partially negated with N^{ω}-nitro-L-arginine methyl ester (L-NAME) treatment (28) therefore we will also investigate the hypothesis that effects of ultrasound in IR injury are due to the specific reversal of iNOS NO production impairment in the endothelium.

MATERIALS AND METHODS

Animal preparation

Investigations conducted in Golden Syrian male hamsters (Charles River, Boston, MA), weight range of 50 to 75g, employed the hamster window chamber model (chamber), allowing the elimination of anesthetic influence and exposed tissue during investigation. The complete technique has been previously described (16, 21). Animal surgeries, chamber and carotid artery catheterization, were performed under general

anesthesia, 50 mg/kg ip injection of pentobarbital sodium (24). TPX used here in place of the glass cover slip incorporates the protective properties of glass with greater acoustical coupling (15). The Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) was followed for animal handling and provided care, with all experiments approved by the local Animal Subjects Committee.

Inclusion criteria

Animals used were free of edema, pre-observation injury to window area, and or infection to surgical sites. Initial observation carried out at least 3 days after chamber implantation, diminished postsurgical complications.

Ischemia reperfusion injury

A pressure tourniquet, described previously (24, 50), was used to induce complete ischemia in the chamber. Flow of the area studied was interrupted by compressing a rubber ring against the chamber window tissue. The degree of compression was controlled by tightening a screw connected to the chamber, and the tissue within the ring was monitored until obtaining complete occlusion (blood low cessation) of all vessels (50). Continuous ischemia was ensured through periodic inspection. The release of the tourniquet marked the end of ischemia and initiation of reperfusion.

iNOS inhibition

N-(3-aminomethyl) benzylacetamidine dihydrochloride (1400W, Sigma) treatment was administered to appropriate animal groups as a bolus injection 1 min before the release of the tourniquet. The volume for infusion of 1400W solution was less than 5% of systemic blood volume, an estimate of 7% of hamster total body weight on day one of the experiment. Injections where dosed at 5 mg/kg in each group, a dose previously shown to inhibit iNOS in mice IR models (67). The sample solution of 2.5 ml/g (0.1 to 0.15 ml) was infused through the carotid artery by hand and the catheter flushed with heparinized saline.

Ultrasound exposure

All animals exposed to ultrasound were suspended over the transducer using a three-pronged clamp and support ring stand. Exposure to ultrasound, for a 20 min period, began 5 min following the onset of reperfusion. The transducer (Valpey Fisher Corporation immersion transducer, part # IL0208HP, nominal frequency 2.25, element diameter 1 in.) was secured in a water bath, acting as a heat sink for the transducer head.

Animal exposure was accomplished using an open ended plastic cone affixed to the face of the transducer, filled with degassed water. Wave production was stimulated using a function generator (30 MHz Synthesized Function Generator, model DS345; Stanford Research Systems, Sunnyvale, CA) with continuous sine wave, 2.49 MHz frequency, and 10 V_{p-p} amplitude settings. Resultant pressure produced is about 0.5 MPa. (The transducer was calibrated using a hydrophone in the Ferrara Laboratory, Department of Biomedical Engineering, University of California, Davis).

Experimental groups

Animals were randomly assigned into one of 5 groups for microcirculatory assessment or Western Blot analysis (WB) as follows: IR+1400W group (n=5), IR+1400W+ultrasound group (n=5), control group (n=3), IR group (n=3), or IR+ultrasound group (n=3).

Microvascular experimental setup

Conscious animals were placed in plexi-glass restraining tubes, allowing for protrusion of chamber and minimized movement. Tubes containing hamsters were then fixed to the stage of an upright transillumination intravital microscopic (BX51WI; Olympus, New Hyde Park, NY). Projection onto a charge-coupled device camera (COHU 4815) connected to a videocassette recorder (AG-7355; JVC, Tokyo, Japan) enabled viewing of microscopic images on a monitor. Baseline vessel site selection used a 40x (LUMPFL-WIR, numerical aperture 0.8; Olympus) water immersion objective. The same measurement sites were followed throughout each experiment to allow direct comparison with baseline levels (**Figure 3.1**).

Functional capillary density

Contrast enhancement between flowing red blood cells (RBCs) and tissue was accomplished using a BG12 (420 nm) band pass filter. Functional capillary density (FCD), functioning capillaries contained the flow of at least one RBC during the observation period, was determined in 13 to 25 stepwise vertically successive microscopic fields, (a region of about 1.7 to 1.9 mm²). The initial field chosen was documented by an anatomical feature to allowed recognition in repeated measurements.

Fluorescent microscopy

Vessel diameter and FCD were assessed using epiillumination and fluorescein isothiocyanate, bound to dextran (MW 150,000; FITC-Dextran 150 Sigma Chemical, St. Louis, MO; 0.1 ml of a 12.5 mg/ml saline), during repeated measurements. FITC-Dextran was injection 5 to 10 min before individual repeated assessment periods. Intravital fluorescent microscopy used an additional mercury 100 W lamp and appropriate filters.

Microhemodynamics

Vessel diameter (*D*) was obtained online using a video image-shearing technique (37). Real-time arteriolar and venular blood flow velocity measurements were achieved using the photodiode cross-correlation method (36) (Photo Diode/Velocity Tracker model 102B; Vista Electronics, San Diego, CA). Measured microvascular centerline velocity (*V*) was corrected using the corresponding vessel size correction factor to obtain the mean RBC velocity (*V_a*) (48). Blood flow (*Q*) was calculated using the measured and calculated parameters as $Q = \pi V_a^* (D/2)^2$. The use of this calculation has been found appropriate for tubes of 15 to 80 µm internal diameters (48), assuming a parabolic velocity profile.

Western blot analysis experimental setup

Skin samples from animals in IR and IR+ultrasound groups were harvested in place of microcirculatory assessment at the proper assessment periods; 0.5, 2, or 24 h after the on set of reperfusion, following a 4 h period of ischemia. Samples from control group animals were harvested prior to IR. All samples of animals were harvested under anesthesia then quickly weighed, placed into individual tubes, and snap frozen.

Preparation of lysates from hamster skin tissue

The frozen samples, cut into smaller pieces, and homogenization buffer (50 mM Tris, pH 7.5, 150 mM NaCl and Complete protease inhibitor cocktail (Roche)) added at 10ml/g tissue were added to and homogenized with a Dounce homogenizer. Samples were solubilized by addition of $1/10^{\text{th}}$ volume of 1% TritonX-100 and 600 mM octylglucoside. After incubating on ice for 20 min, the samples were centrifuged at 14000 *g* for 20 minutes. Supernatants were collected and protein amounts were determined by BCA protein assay (Pierce) using bovine serum albumin as standard. Equal amount of proteins were separated on NuPAGE® Bis-Tris Gels (Invitrogen) using MOPS running buffer and transferred to polyvinylidene fluoride (PVDF) membranes.

Immunoblot

After blocking with 5% BSA in Tris-buffered saline (TBS), membranes were incubated with primary antibodies against eNOS (monoclonal, BD Biosciences) or iNOS (monoclonal, BD Biosciences) and β -Tubulin (polyclonal, Santa Cruz Biotechnology, Inc.) at 4°C overnight in TBS 0.1% Tween 20 (TBST). Bound primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies: goat–anti-mouse IgG (Pierce, 1/5000) and goat–anti-rabbit IgG (Pierce, 1/5000), respectively, followed by chemiluminescent SuperSignal substrate (Pierce). Band intensity was quantified on unsaturated X-ray film by a digital image analyzer (Quantity-One; BioRad). All comparisons were made relative to individual band intensities of the quantified β -Tubulin of each sample.

Data analysis

Results are presented as means \pm standard deviation. Data comparisons between groups were analyzed using the unpaired student's t-test. Microhemodynamic measurements were compared to baseline levels obtained before the experimental procedure and data are presented as ratios relative to baseline values. As stated above the same baseline vessels and capillary fields were assessed through repeated measurements for direct comparisons to be completed, allowing for more robust statistics in small sample populations. Differences were considered significant for p < 0.05. All statistics were calculated using GraphPad Prism 4.01 (GraphPad Software, Inc.; San Diego, CA).

RESULTS

Microcirculation: IR+1400W and IR+ultrasound+1400W groups

Both the inhibition of iNOS alone and ultrasound exposure with iNOS inhibition positively influenced the microcirculation of animals under study. Animals exposed to ultrasound had decreased FCD at the 0.5 h observation, below that of unexposed animals after IR and 1400W treatment. However, at both the 2 and 24 h assessments FCD of ultrasound irradiated animals exceeded baseline and unexposed animal values. Differences in FCD did not reach significance at anytime during reperfusion (**Figure 3.2**).

Vessel diameters where measured to be at or above baseline for all repeated observations during reperfusion in both microcirculatory assessment groups. Arteriolar diameters of unexposed animal were increased at 2 and 24 h during reperfusion, compared to exposed animals, without reaching significance. Venules of exposed animals showed increased diameters compared to unexposed animals at all assessments; however, no significance was reached (**Figure 3.3**).

A similar trend was seen in arteriolar and venular flow velocity for all animals treated with 1400W. Flow velocity levels at 0.5 h of reperfusion where increased at 2 h but returned to decreased levels at 24 h into reperfusion. Decreased levels of flow velocity in exposed animals were found throughout reperfusion, relative to the unexposed group. The measured arteriolar flow velocities of ultrasound exposed animals were elevated, above baseline values, compared to those of unexposed animals. The reverse response was true for venular flow velocities. Animals without ultrasound exposure had raised values compared to exposed animals; with both groups resulting in values above baseline. No significance was found in the changes between the groups (**Figure 3.4**).

Calculated microvascular flow (**Figure 3.5**), for arterioles and venules, resulted in increased levels throughout reperfusion compared to baseline levels for both animal groups. As with flow velocity there was a trend of increased microvascular flow levels at 2 h, compared to 0.5 h, returning to decreased levels at 24 h in both animals groups.

Presence of eNOS and iNOS

Figure 3.6 shows WB of eNOS and iNOS protein, in three individual hamster chamber skin samples; in controls and in animals subjected to IR injury with or without ultrasound exposure. Ultrasound exposure resulted in a significant production of eNOS protein, in the chamber skin samples. Skin samples harvested from ultrasound exposed animals 24 h into reperfusion showed a significant production of eNOS protein (p < 0.01), compared to unexposed animals. eNOS protein levels in exposed animals decreased slightly from 0.5 to 2 h of reperfusion. Increased eNOS presence during 24 h of reperfusion after ultrasound exposure was significant compared to amounts produced during 0.5 and 2 h of reperfusion (p < 0.05). Skin samples harvested from unexposed animals showed a continuous decrease in eNOS production from 0.5 through 24 h of reperfusion, without reaching significance (**Figure 3.7**).

Levels of iNOS protein analyzed from the same harvested skin samples of animals exposed to ultrasound were found to be decreased at all time points, compared to unexposed animals. A significant decrease between chamber tissue iNOS levels was seen at 0.5 h into reperfusion in animals exposed to ultrasound, compared to those that were not. iNOS levels in unexposed animal skin increased slightly from 0.5 to 2 h of reperfusion and then decreased below 0.5 h levels in the 24 h sample. Ultrasound exposed animals had a slight steady increase in iNOS level through 24 h of reperfusion (**Figure 3.7**).

DISCUSSION

The principle finding of this study is that exposure to diagnostic frequency continuous ultrasound after 4 h of complete ischemia decreased the production of iNOS at 0.5 h while increasing the production of eNOS at 24 h of reperfusion. Also the beneficial effect of iNOS inhibition, during ischemia reperfusion, was confirmed. These outcomes were obtained by irradiating the tissue for a 20 min period with continuous wave ultrasound, starting 5 min after the onset of reperfusion. Inhibition of iNOS resulted in a lasting early recovery of microvascular function of both animals groups, often surpassing baseline values from 0.5 through 24 h of reperfusion.

The mitigation of IR injury with ultrasound exposure has been previously reported in a variety of tissues (12, 28, 72). We hypothesize that similar findings did not result in this current study of microvascular function because of the simultaneously occurring positive effect of iNOS inhibition. It is likely that a maximal decrease in microcirculatory injury was the result of iNOS inhibition alone, therefore the added beneficial effects due to ultrasound irradiation would not provide any further improvement of microvascular function. The prevention of iNOS production through genetic manipulation (58) and treatment with iNOS specific inhibitors (38, 80) during ischemia reperfusion results in decreased injury and improved recovery. We also show that IR injury in the chamber model (28) is eliminated by treatment with the iNOS specific inhibitor 1400W. Although there is evidence supporting the benefit of iNOS inhibition (3, 47, 75), our results specifically show that inhibition of iNOS activity attenuates microvascular injury during IR. WB of chamber tissue samples using an anti-iNOS antibody showed increased, above baseline, iNOS concentration during early reperfusion in both animal groups. We hypothesize that attenuated long-term IR damage is partially attributed to the decreased presence of iNOS in animals exposed to ultrasound, compared to unexposed animals. The upregulation of iNOS and or NO synthesis through the iNOS pathway induced apoptosis during *in vitro* (39) and *in vivo* ischemia reperfusion studies (76). The build up of toxic metabolites during ischemia in addition to the influx of oxygen and subsequent reactive oxygen species (ROS) formation upon reperfusion leads to microvascular dysfunction in IR studies (25, 66). The increased production and presence of iNOS resulting from both ischemia and reperfusion enhances the early injury of vessel endothelial and surrounding tissue cells, of animals not exposed to ultrasound. Microvascular iNOS levels, increased from baseline intensify all IR injurious effects. Whether damage is temporary or permanent, however, depends on the severity of the injury as well as the effect of any protective and or preventative mechanisms.

The microvascular protective influences related to eNOS activity in the production of NO have been well documented. Inhibition of eNOS and or the production of NO through the eNOS pathway while subjecting tissue to ischemia and subsequent reperfusion (10, 17, 56), results in increased circulatory dysfunction and tissue injury, compared to uninhibited groups. Ultrasound exposed chamber samples, compared to unexposed samples, demonstrated increased eNOS production with WB utilizing an anti-eNOS antibody. Increased eNOS production currently seen during late reperfusion (24 h) confirms our previous work showing improved microcirculatory function of ultrasound exposed animals 24 h into reperfusion (28). The enhanced production of eNOS with

ultrasound exposure extends our hypothesis for the attenuation of long-term IR damage. Twenty-four hours after the onset of reperfusion is sufficient time for the subsistence of IR effects and the establishment of a new level of homeostasis. A surplus in NO production at this later time point would greatly benefit microvessel function.

WB of both NOS isoforms eNOS and iNOS supports our hypothesis relating to the efficacy of ultrasound exposure in the reduction of IR injury and the existence of both fast and slow acting responses to stimulation (28). Ultrasound exposure during IR downregulates the quantity of iNOS produced at the 0.5 h assessment point, a rapid response to stimulation during early reperfusion, while upregulating eNOS production 24 h into reperfusion, a delayed stimulus response. However, contrasting our previous conclusion on the use of L-NAME treatment with ultrasound exposure, we now propose that L-NAME treatment negatively influences the delayed, slow acting stimulation effect. Negatively affecting the rapid response to ultrasound stimulation should produce results similarly to those obtained with iNOS inhibition (**Figures 3.2-3.5**).

In summary the relationship between attenuated IR damaged to tissue, ultrasound exposure and NO production *in vivo* has previously been reported (1, 12, 71, 72). This present study extends our understanding of the mechanism by which irradiation with continuous wave ultrasound using diagnostic frequency affects the levels of iNOS and eNOS leading, respectively, to rapid and protracted effects. Our findings provide further evidence that the beneficial effects of ultrasound exposure are related to NO and suggest that the effects of ultrasound exposure are beneficial in the treatment of IR injured tissue.

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APPENDIX



Figure 3.1

Schematic outline of the microvascular and chamber tissue harvest experimental protocol for each group, not including control group. US, ultrasound.



Changes in functional capillary density due to ischemia reperfusion injury and 1400W treatment, with and without 20 min of ultrasound exposure. Data are presented as mean \pm standard deviation. IR14, IR+1400W group; US14, IR+1400W+ultrasound group.



Changes in arteriolar and venular diameters due to ischemia reperfusion injury and 1400W treatment, with and without 20 min of ultrasound exposure. *Top Panel:* Arteriolar diameters relative to baseline levels. *Bottom Panel:* Venular diameters relative to baseline levels. Data are presented as mean ± standard deviation. IR14, IR+1400W group; US14, IR+1400W+ultrasound group.



Figure 3.4.

Changes in arteriolar and venular flow velocity due to ischemia reperfusion injury and 1400W treatment, with and without 20 min of ultrasound exposure. *Top Panel:* Arteriolar flow velocities relative to baseline values. *Bottom Panel:* Venular flow velocities relative to baseline values. Data are presented as mean ± standard deviation IR14, IR+1400W group; US14, IR+1400W+ultrasound group.



Calculated changes in arteriolar and venular flow due to ischemia reperfusion injury and 1400W treatment, with and without 20 min of ultrasound exposure. *Top Panel:* Arteriolar flows relative to baseline levels. *Bottom Panel:* Venular flows relative to baseline levels. Data are presented as mean ± standard deviation. IR+1400W, IR+1400W group; US14, IR+1400W+ultrasound group.



Western blot analysis of NOS protein content in 3 individual hamster chamber tissue samples of animals subjected to ischemia reperfusion injury with or without ultrasound exposure. Tissue was harvested at 0.5, 2, or 24 h of reperfusion. β -Tubulin band intensities of each sample are not shown. *Left Panel:* eNOS protein content. *Right Panel:* iNOS protein content. IR, IR group; IR&US, IR+ultrasound group.



A graphical representation of the change in levels of nitric oxide synthase enzymes, relative to individual band intensities of quantified β -Tubulin of each sample. *Top Panel:* Detected protein levels of the eNOS enzyme during assessment periods. *Bottom Panel:* Detected protein levels of the iNOS enzyme during assessment periods. Data are presented as mean \pm standard deviation. Control, control group; IR, IR group; IR&US, IR+ultrasound group. *p < 0.05 vs. IR. **p < 0.01 vs. IR. ++p < 0.01 vs. 0.5 h IR+ultrasound. ^^p < 0.01 vs. 2 h IR+ultrasound.

Diagnostic Frequency Continuous Ultrasound Directly Mitigates Venular Ischemia Reperfusion Damage

ABSTRACT

Objective: Our objective was to determine the effects of ischemia reperfusion (IR) treatment with continuous mode diagnostic frequency ultrasound irradiation by assessing leukocyte endothelial cell interactions, in terms of the frequency and relative proportions of rolling and firmly attached leukocytes.

Methods: Studies were carried out in the awake hamster chamber window preparation. Tourniquet ischemia was implemented by compressing a circular ring on the chamber window tissue for 4 hours, followed by reperfusion. Animals were randomly assigned into one of 4 groups as follows: 1) IR; 2) IR+ultrasound; 3) IR+L-NAME; and 4) IR+L-NAME+ultrasound. Venules were exposed to epiillumination, video recorded, and leukocytes were categorized as "rolling," flowing with endothelial contact, or "immobilized" cells and counted during digital video payback in 100 µm length segments.

Results: Leukocyte interaction with venular endothelium significantly decreased, during long-term reperfusion (p < 0.05) with ultrasound irradiation. NO production inhibition, following L-NAME treatment, and ultrasound irradiation resulted in additional earlier significant decreased leukocyte endothelial cell interactions (p < 0.05).

Conclusion: Venular function improvement, after IR damage, is a primary benefit derived from continuous mode diagnostic frequency ultrasound irradiation. Although decreased interaction of adherent leukocytes may also be attributed to enhanced arteriolar

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flow, reduced interaction of rolling leukocytes is an immediate consequence of ultrasound irradiation.

Key words: ischemia reperfusion; ultrasound; leukocyte endothelial cell interaction
INTRODUCTION

Ischemia reperfusion (IR) injury occurs in untreated tissues subjected to the interruption and re-establishment of blood flow, an occurrence common to victims of heart attack, stroke, hemorrhagic shock, and transplant recipients. Its effects are primarily radicated in the microcirculation and treatment aimed at preventing or reducing IR injury includes pre and post conditioning (81), the use of antioxidants (33, 54, 57) and antibodies (6, 49, 64, 79), and exposure to ultrasound (12, 71, 72). These therapeutic approaches yield beneficial outcomes, significantly reducing microcirculatory injury when compared to controls. The majority of these treatments are clinically effective in cases where IR is foreseen, such as coronary bypass surgery, transplantation and revascularization, or their influence is reduced through systemic involvement, as with ingestion or injection of antioxidant and antibodies. Ultrasound, which is also effective in IR treatment, can be directed to the tissue at risk and used in unplanned IR occurrences.

Presently, varying ultrasonic frequencies and wave formats have been implemented for therapeutic use. The use of ultrasound irradiation in treatment and prevention of IR injury has also been implemented using microbubbles (5). Mitigation of IR microcirculatory injury with ultrasound exposure has been found to be related to the stimulation of nitric oxide (NO) production and NO related effects (12, 71, 72). *In vitro* studies, using endothelial cell cultures (2, 31) have also shown increased NO production.

We recently reported that diagnostic frequency continuous ultrasound exposure improved microcirculatory function during IR injury in the hamster window chamber model (28). However, venular effects were undistinguishable from the consequences of improved arteriolar flow. The increased emigration and capture of leukocytes by the endothelium during IR injury (4, 19, 77, 78) results in the breakdown and increased permeability of affected vessels. Attenuation of the leukocyte endothelial cell interaction after diagnostic frequency continuous ultrasound exposure would present evidence of direct effects in the venules.

The objective of this present study was to determine whether exposure of tissue to diagnostic frequency continuous ultrasound affected leukocyte endothelial cell interaction in venules differentiating between rolling and firmly attached leukocytes. We also assessed the incidence of rolling and firmly attached leukocytes in a reduced NO environment through N^{ω} -nitro-L-arginine methyl ester (L-NAME) treatment. The fluorescent dye acridine orange was used to monitor the degree of endothelium leukocyte interaction.

MATERIALS AND METHODS

Animal model and preparation

The chamber was used for investigation in Golden Syrian males (Charles River, Boston, MA), weight range of 50 to 75g. The Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) was followed for animal handling and provided care. Experiments are approved by the University of California, San Diego Animal Subjects Committee. The widely used chamber procedure eliminates anesthetic influences during microcirculatory examination. The complete technique is described previously (16, 21). TPX (Westlake Plastics, Placentia, CA) used as the cover slip, incorporates the protective properties of glass with greater acoustical coupling (15). Initial microvascular observation carried out at least 3 d after chamber implantation, diminished postsurgical complications.

Inclusion criteria

Animals used were free of edema, pre-observation injury to window area, and or infection to surgical sites.

Assessment of microcirculatory parameters

All microscopic observations were preformed using an upright microscope (BX51WI; Olympus, New Hyde Park, NY). Images in the microscopic field of view could also be viewed on a monitor, through the projection of the image onto a low light camera (ORCA 9247, Hamamatsu) connected to a videocassette recorder (AG-7355; JVC, Tokyo, Japan). A 40x (LUMPFL-WIR, numerical aperture 0.8; Olympus) water immersion objective was used in both transillumination and low light fluorescent microscopy. Contrast enhancement between flowing red blood cells (RBC) and tissue was accomplished using a BG12 (420 nm) band pass filter.

Leukocyte endothelium interaction

Acridine orange (5mg/kg solution in saline, Sigma) injected intraarterioly was used for contrast augmentation of microvascular leukocytes in venules. Low light fluorescent microscopy was used for leukocyte endothelium interaction assessment, as described elsewhere (16). Briefly, the straight portion of venules (4 to 6 venules per animal) was exposed to epiillumination for 30 sec and video recorded. Leukocytes categorized according to their flow behavior as "rolling", flowing with endothelial contact, or "immobilized" cells (18), were counted during digital video playback in 100 µm length segments.

Ischemia reperfusion injury

A pressure tourniquet, described previously (24, 50), was used to induce ischemia in the chamber. Flow of the area under ischemia, interrupted by pressing a rubber ring to the tissue in the window chamber, was monitored until achieving global occlusion of feeding and draining vessels (50). Ischemia was ensured through periodic inspection. The release of the tourniquet marked the end of ischemia and start of reperfusion.

Ultrasound exposure

During ultrasound exposure animals were suspended over the ultrasound transducer using a three-pronged clamp and support ring stand. The transducer (Valpey Fisher Corporation immersion transducer, part # IL0208HP, nominal frequency 2.25, element diameter 1 in.) was secured in a water bath. Animal exposure was accomplished using an open ended plastic cone affixed to the face of the transducer, filled with degassed water. Pressure waves were created using a function generator (30 MHz Synthesized Function Generator, model DS345; Stanford Research Systems, Sunnyvale, CA) with continuous sine wave, 2.49 MHz frequency, and 10 V_{p-p} amplitude settings. Resultant intensity and pressure produced is 8.33 W/cm² and about 0.5 MPa, respectively. (The transducer was calibrated using a hydrophone in the Ferrara Laboratory, Department of Biomedical Engineering, University of California, Davis).

Experimental groups

Animals were randomly assigned into one of 4 groups as follows: IR group (n=5), IR+ultrasound group (n=5), IR+L-NAME group (n=5), or IR+L-NAME+ultrasound group (n=5).

Experimental setup and protocol

Conscious animals were placed in plexi-glass restraining tubes, allowing for protrusion of chamber and minimized movement during experiment. Tubes containing hamster were further secured to custom-made frames and placed on the microscope stage, during observation. Similar microcirculatory assessment procedures were preformed in each group. Transillumination was used in baseline vascular architecture documentation and vessel site selection. The same sites were evaluated throughout individual experiments to allow direct comparison with baseline values. Animals were then subjected to a 4 h period of ischemia and assessments repeated at 0.5, 2, and 24 h, from the start of reperfusion. The volume for infusion of L-NAME (Sigma) solution in saline was an estimate of 7% of hamster total body weight on day one of the experiment. Injection dose, 10 mg/kg, was previously shown effective in nitric oxide (NO) inhibition in hamsters (28, 65) and the prevention of NO protective effects in mice (38). The sample solution of 5 ml/g (0.1 to 0.15 ml) was infused through the carotid artery by hand and the catheter flushed with heparinized saline (**Figure 4.1**).

Ischemia reperfusion and ultrasound exposure groups

Experimental procedure of I/R and I/R+ ultrasound groups followed the general protocol as mentioned above. In addition the I/R+ ultrasound group was exposed to ultrasound for a 20 min period, 5 min following the onset of reperfusion.

Ischemia reperfusion L-NAME and ultrasound exposure groups

Experimental procedure for I/R+ L-NAME and I/R+ L-NAME+ ultrasound groups followed the general protocol for baseline and repeated microcirculatory assessment. L-NAME was administered to both groups as a bolus injection 5 min before the release of the tourniquet. Additionally animals in the I/R+ L-NAME+ ultrasound group were exposed to ultrasound for a period of 20 min, 5 min following the onset of reperfusion.

Statistical analysis

Results are presented as means \pm standard deviation. Data graphs are presented normalized to baseline values, unless otherwise specified. Data comparisons made between groups and group controls and treatments were analyzed using the unpaired student t-Test with differences considered significant for p < 0.05. All statistics were calculated using GraphPad Prism 4.01 (GraphPad Software; San Diego, CA).

RESULTS

Ischemia reperfusion and ultrasound exposure groups

Ultrasound treatment resulted in reduced leukocyte endothelial cell interaction in all post-ischemia assessments relative to unexposed animals. Leukocyte presence and interaction with the endothelium during reperfusion was increased compared to baseline for all animal groups and time points. However, at 24 h of reperfusion the number of rolling leukocytes in venules of animals exposed to ultrasound was significantly decreased compared to unexposed animals (p < 0.05, **Figure 4.2**).

The number of immobilized leukocytes in venules of unexposed animals also increased 0.5 h into reperfusion, compared to baseline measurements. At 2 h of reperfusion immobilized cells remained unchanged from 0.5 h, decreasing slightly at 24 h. At the 24 h time point immobilized cell numbers were significantly decreased in animals exposed to ultrasound compared to those that were not (p < 0.05, **Figure 4.3**).

Ischemia reperfusion L-NAME and ultrasound exposure groups

The relationship between the production of NO, ultrasound irradiation, and the leukocyte endothelial cell interaction following IR injury, examined using L-NAME, resulted in decreased interaction during the earlier reperfusion time points and the 24 h time point.

Figure 4.4 shows the changes in rolling leukocytes from baseline values through 24 h of reperfusion, comparing I/R+L-NAME and I/R+L-NAME+Ultrasound groups. Venules exposed to ultrasound show a slightly increased amount of rolling cells compared to unexposed animals 0.5 h into reperfusion. However, at 2 and 24 h of reperfusion rolling leukocytes of ultrasound exposed venules were significantly reduced (p < 0.05). During reperfusion immobilized leukocytes of animals exposed to ultrasound were decreased at all time points, compared to unexposed animals, with significance at 0.5 and 24 h of reperfusion (p < 0.5, **Figure 4.5**).

Ultrasound exposure with and without L-NAME

A comparison of the data from animal groups receiving ultrasound irradiation, normalized to baseline (graph not show), revealed a significant increase in leukocyte rolling cells at 0.5 h, with L-NAME treatment, relative to untreated animals (p < 0.01). Two and 24 h measurements in the same evaluation also found increased interaction, without significance. Review of the ultrasound exposure data of immobilized leukocytes (**Figures 4.3 and 4.5**) showed a significant difference at 24 h of reperfusion (p < 0.05). The immobilized leukocytes of L-NAME treated animals were significantly increased compared to untreated animals.

DISCUSSION

The principle finding of this study is that treatment of IR injury with diagnostic frequency continuous ultrasound results in a significant reduction in leukocyte endothelial cell interaction in venules 24 h after reperfusion. This result was obtained by exposing the tissue to continuous wave ultrasound for 20 min, 5 min after the onset of reperfusion. This effect was present at earlier time points when NO production was inhibited by treatment with L-NAME prior to reperfusion. Ultrasound and L-NAME treated animals also showed improvements at the 0.5 and 2 h time points.

Ultrasound, propagating through the skin tissue is hypothesized to stimulate the production of NO by the endothelium. Vessel walls inundated with oxygen free radicals form lipid peroxidase, one of many reactive oxygen species (ROS) (44) produced during reperfusion, which damages the endothelium. In this scenario increased NO availability

improves venular function and integrity as a consequence of NO's ability to quench the activity of ROS (44, 70). These protective effects should be related to the reduced leukocyte endothelial cell interaction in tissues exposed to ultrasound. Thus NO mitigates IR venular endothelial reperfusion damage by attenuating ROS damages which signals the start of the leukocyte adhesion cascade.

Decreased immobilized and rolling venular leukocytes with ultrasound exposure at 24 h of reperfusion correlate with our previously reported increased blood flow velocity and flow during the corresponding time point. Lower flow rates in venules promote the increase of adhering leucocytes. Kubes et al. and Ritter et al. (43, 62) reported lower venular flow rates following ischemia resulted in augmented immobilized leukocytes, while rolling cell numbers were unaffected (43). However, the presence of leukocytes, rolling and or free flowing, increases the possibility for cellular interaction and adhesion with the endothelium. The emigration and adherence of leukocytes to damaged vessels and subsequent extravasasion and disruption of intercellular junctions (66) provokes continued leukocyte endothelial cell interaction, additional adhesion and or rolling of leukocytes not having previous endothelial interaction.

Although less likely, it is also hypothesized that decreased interactions result from ultrasound stimulation through a downregulation of cytokine production and or endothelial cell P-selectin and or E-selectin activation. Interaction of leukocyte cells with the endothelium was reported to be adhesion molecule dependent (46), with activation of both necessary for firm venular adhesion(63). Ultrasound exposure may specifically moderate endothelial P-selectin activation. Uninhibited leukocyte activation permits the continuation of occasional endothelium interaction in the form of rolling cells; however, cell adhesion to vessels is prevented. As reperfusion persist through 24 h, decreased overall interaction results from downregulation of adhesion molecules in both leukocyte and endothelial cells.

Treatment with L-NAME, reducing flow and flow rates, significantly increased amounts of immobilized leukocytes during early reperfusion. Reduced flow may influence increased leukocyte adherence to venules by decreasing the exposure of circulating NO as well as preventing the washout of toxic metabolites formed during ischemia. A lower flow also affords a longer exposure period between activated leukocytes and endothelial cells. The significance seen at 0.5 h into reperfusion may have resulted from a combination of several affects. A lower flow would lead to increased adhesion, however, the protective affects of reduced ROS production and ultrasound stimulation of NO and downregulation of endothelial adhesion molecules would result in the reduction of adhering leukocytes. Low flow at the onset of reperfusion, inhibiting the amount of oxygen influx and the production of ROS, affects both untreated and exposed groups; thus the beneficial effects of ultrasound seem to outweigh the common detrimental influences to both groups.

The effects found with L-NAME treatment and exposure to ultrasound (increase in both rolling and immobilized leukocytes), suggest that decreasing eNOS production of NO negates the protective effects of ultrasound. It is hypothesized that protective effects initiated during early reperfusion continue to act throughout reperfusion, limiting the firm adhesion of leukocytes. However, ultrasound exposure after inhibited NO production does not seem to limit early reperfusion rolling leukocytes assessed at 0.5 h. The level of NO present, perhaps also produced by other NO synthase (NOS) isoforms, may be sufficient to hinder only firm adhesion of leukocytes during early reperfusion. Endothelial signals received through interaction with rolling leukocytes at 0.5 h of reperfusion are proposed to aid in the subsequent increase of immobilized cells at 2 h. A continuous simulation of endothelial cells by leukocyte interaction in combination with reduced venular flow during continued reperfusion results in significantly increased immobilized leukocytes at 24 h.

In summary previous *in vivo* studies demonstrate improved microvascular function with exposure to pulsed (1, 12) and continuous (28, 71, 72)wave ultrasound, during ischemia reperfusion. The present study shows that continuous diagnostic ultrasound exposure of tissue undergoing IR injury reduces venular endothelial impairment through the reduction of leukocyte endothelium interaction. The mitigation of IR injury by ultrasound exposure is counteracted by the initial inhibition of NO production, an effect that persists 24 h after reperfusion.

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APPENDIX



Figure 4.1 Schematic outline of the experimental protocol for each group. US, ultrasound.



Number of rolling leukocyte/ 100 μ m of venules before ischemia and 0.5, 2, and 24 h after onset of reperfusion in the hamster chamber, with and without 20 min of ultrasound exposure. Data are presented as means ± standard deviation. IR, IR group; IR+US, IR+ultrasound group. *p < 0.05 vs. IR 24 h assessment.



Number of immobilized leukocytes/ 100 μ m of venules before ischemia and 0.5, 2, and 24 h after onset of reperfusion in the hamster chamber, with and without 20 min of ultrasound exposure. Data are presented as means ± standard deviation. IR, IR group; IR+US, IR+ultrasound group. *p < 0.05 vs. IR 24 h assessment.



Number of rolling leukocytes/ 100 μ m of venules before ischemia and 0.5, 2, and 24 h after L-NAME treatment and onset of reperfusion in the hamster chamber, with and without 20 min of ultrasound exposure. Data are presented as means ± standard deviation. IR+L, IR+L-NAME group; IR+L+US, IR+L-NAME+ultrasound group. **p < 0.05 vs. IR+L 2 h assessment. *p < 0.05 vs. IR+L 24 h assessment.



Number of immobilized leukocytes/ 100 μ m of venules before ischemia and 0.5, 2, and 24 h after L-NAME treatment and onset of reperfusion in the hamster chamber, with and without 20 min of ultrasound exposure. Data are presented as means ± standard deviation. IR+L, IR+L-NAME group; IR+L+US, IR+L-NAME+ultrasound group. **p < 0.05 vs. IR+L 0.5 h assessment. *p < 0.05 vs. IR+L 24 h assessment.

Discussion and Conclusions

DISCUSSION

The principal finding from these investigations is that continuous mode diagnostic frequency ultrasound is indeed effective in preventing damage to the microcirculation resulting from IR injury. Effects are related to NO production through the regulation of eNOS and iNOS protein levels. Ultrasound irradiation resulted in improved FCD and microvascular diameter, flow velocity and flow, 24 h from the termination of ischemia. Irradiation following L-NAME treatment resulted in a moderation of significant effects, while treatment with 1400W was found to be therapeutic for IR injury improving all animal groups. Molecular analysis enabled the elucidation of eNOS and iNOS activity timelines, demonstrating that early reperfusion inhibited iNOS activity and late reperfusion enhanced eNOS activity in ultrasound irradiated animals. Analysis of venular function showed that ultrasound irradiation decreased leukocyte endothelial cell interaction, with additional earlier improvements seen with L-NAME treatment.

Ultrasound irradiation affects on the microcirculation, reached significance 24 h from the onset of reperfusion (and time of application). The correlation of these effects, with the currently shown increased eNOS protein presence in chamber tissue samples, confirms the direct involvement of ultrasound irradiation in NO production and the mitigation of IR damages.

The dual effect of ultrasound irradiation indicated by western blot analysis, which showed inhibition of iNOS and stimulation of eNOS production, suggests that it may also

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be used as a preventative intervention in addition to its protective role. Our hypothesized fast and slow acting stimulated response, proposed as an explanation of L-NAME treatment results, was supported but amended following molecular analysis and iNOS inhibition treatment outcomes. The application of L-NAME should have produced results similar to those found with iNOS inhibition, if affecting the early response. However, L-NAME treatment solely caused significant (and expected) changes in late reperfusion. The dissimilarities in results with NOS inhibition treatment, L-NAME and 1400W, also indicate that L-NAME related effects to be closely related to the inhibition of eNOS activity rather than the general NOS activity.

Improvement of microcirculatory function, after IR injury in differing experimental models, has been widely related to enhanced microvascular function, NO production, and NO related effects (5, 7, 9, 11, 20, 24, 29, 30, 41, 43, 50, 51). Ultrasound irradiation as a method of microvascular IR injury therapy has also been directly related to the stimulation of NO production (2, 5, 12). The differences in response and timing of response to ultrasound stimulation in our current study compared to other studies (12, 52, 69, 72), may be related to the differing experimental models, ultrasound irradiation setting, and or exposure protocols. Although we hypothesize an endothelium influence in NO production with ultrasound irradiation, reported endothelium independent vasodilation (22) does not contradict our hypothesis of ultrasound provoked production of NO.

STUDY STRENGTHS

Our use of the chamber model allowed us to assess the effect of microcirculatory function following IR damage and ultrasound irradiation in conscious animal subjects, without contributions from anesthesia. This same model facilitated the investigation of tissue in physiologic homeostasis over an extensive observation period. Real-time analysis enabled determination of changes in microcirculatory parameters at several different time points. Real-time analysis also eliminates artifacts that my skew results from histological examination of preserved tissue, by allowing comparison of changing tissue function in the same animal.

STUDY WEAKNESSES AND LIMITATIONS

While we did measure improvements to microvascular function through changes in FCD, microvascular diameter and flow velocity, and leukocyte endothelial cell interaction, we did not measure the effect of our current settings of ultrasound irradiation on the reactive oxygen species (ROS) levels resulting from IR injury. In addition our assessment of NO production through inhibition and molecular analysis did not incorporate direct detection of NO levels. The perpendicular placement of the transducer during the exposure period as well as the use of a plastic cone affixed to the transducer head, did not allow for visualization of effects during ultrasounds treatment. This experimental configuration may have also caused reflected waves to produce secondary affects not present in the usual application of ultrasound diagnostics in humans, where the original effects were noted. Nonetheless, we believe the findings of our current study are novel and supportive of the use of ultrasound as therapeutic in relieving IR injury.

FUTURE DIRECTIONS

Our investigation of irradiation with ultrasound resulted in enhanced tissue function following IR injury, however, tissue function did not return to baseline levels. Our study demonstrates a direct link between ultrasound effects and NO production. Future studies should attempt to maximize the therapeutic benefit by establishing a biomechanical model that links the ultrasound excitation with mechanical events within the components of endothelial cells. A likely area of investigation of the interaction of ultrasound excitation with the expression or activation of G proteins, which are prominent in the field of mechanotransduction as mediators of NO production as a consequence of altered mechanical stimuli on the endothelium. This kind of study will eventually indicate how to optimize the characteristics of the ultrasound application, presently circumscribed to ultrasound treatment accepted for human intervention.

CONCLUSION

Noninvasive, continuous mode diagnostic frequency ultrasound attenuates IR microvascular damage in the hamster window chamber model, through the production of NO by the eNOS enzyme and inhibition of iNOS enzyme NO synthesis. The significant effect of microscopically assessed mitigation is present during extended reperfusion (\geq 24 h). Molecular protein analysis confirms this finding showing beneficial effects occurring during both early and late reperfusion (\leq 0.5 and \geq 24 h). The beneficial effects of ultrasound treatment were further confirmed by the reduction of leukocyte endothelial cell interaction, which paralleled results with other positive microvascular findings. Results where not related to the thermal effects of ultrasound irradiation or increased

shear stress due to hyperemia following the onset of reperfusion. The improvement to microcirculatory function caused by externally applied continuous mode ultrasound may have the potential to reduce myocardial injury following an occlusion in conjunction with diagnostic frequencies and could provide supportive therapy to the administration of NO mediators such as nitroglycerin and L-arginine.

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