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The partitioning of nanoparticles to endothelium or interstitium during ultrasound-microbubble-targeted delivery depends on peak-negative pressure

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Abstract

Patients diagnosed with advanced peripheral arterial disease often face poor prognoses and have limited treatment options. For some patient populations, the therapeutic growth of collateral arteries (i.e. arteriogenesis) that bypass regions affected by vascular disease may become a viable treatment option. Our group and others are developing therapeutic approaches centered on the ability of ultrasound-activated microbubbles to permeabilize skeletal muscle capillaries and facilitate the targeted delivery of pro-arteriogenic growth factor-bearing nanoparticles. The development of such approaches would benefit significantly from a better understanding of how nanoparticle diameter and ultrasound peak-negative pressure affect both total nanoparticle delivery and the partitioning of nanoparticles to endothelial or interstitial compartments. Toward this goal, using Balb/C mice that had undergone unilateral femoral artery ligation, we intra-arterially co-injected nanoparticles (50 and 100 nm) with microbubbles, applied 1 MHz ultrasound to the gracilis adductor muscle at peak-negative pressures of 0.7, 0.55, 0.4, and 0.2 MPa, and analyzed nanoparticle delivery and distribution. As expected, total nanoparticle (50 and 100 nm) delivery increased with increasing peak-negative pressure, with 50 nm nanoparticles exhibiting greater tissue coverage than 100 nm nanoparticles. Of particular interest, increasing peak-negative pressure resulted in increased delivery to the interstitium for both nanoparticle sizes, but had little influence on nanoparticle delivery to the endothelium. Thus, we conclude that alterations to peak-negative pressure may be used to adjust the fraction of nanoparticles delivered to the interstitial compartment. This information will be useful when designing ultrasound protocols for delivering pro-arteriogenic nanoparticles to skeletal muscle.

Keywords

Nanoparticles; Ultrasound; Microbubbles; Peripheral arterial disease; Skeletal muscle; Nanomedicine

Introduction

Peripheral arterial disease (PAD) is a major healthcare problem worldwide, affecting approximately 8 million in the United States alone (Roger et al. 2011). Unfortunately, many PAD patients are poor candidates for transluminal angioplasty or surgical revascularization (Bobek et al. 2006), leading many to explore alternative approaches. These include revascularization through a combination of therapeutic arteriogenesis and angiogenesis (Meisner and Price 2010), which ultimately may be used to restore distal limb perfusion and ameliorate skeletal muscle degeneration (Meisner et al. 2013a, 2015; Heuslein et al. 2015).

While the most common proposed avenue for growth factor delivery to skeletal muscle is via direct intramuscular injection, many investigators have developed strategies for therapeutic arteriogenesis and/or angiogenesis that are centered on the delivery of growth factor and gene delivery via the activation of contrast agent microbubbles (MBs) with ultrasound (US). Such approaches are appealing because they have the potential to generate markedly improved growth factor distribution in tissue (Leong-Poi et al. 2007) and enhanced gene expression (Burke et al. 2012). The underlying mechanism for drug and/or gene delivery is the enhancement of permeability of surrounding microvessels which occurs when systemically administered MBs are activated with US (Chappell and Price 2006). In essence, this process creates paths for the delivery of drugs or genes from the systemic circulation to the ultrasound-targeted tissue. US-MB-based approaches have been used to promote the delivery of various growth factors and growth factor genes to muscle and myocardium (Zhang et al. 2006; Mayer and Bekeredjian 2008). These factors include hepatocyte growth factor (Kondo et al. 2004; Taniyama et al. 2001), vascular endothelial growth factor (Leong-Poi et al. 2007; Kobulnik et al. 2009; Korpany et al. 2005), Angiopoietin-1 (Smith et al. 2012), and SDF-1 (Kuliszewski et al. 2011). Meanwhile, in studies conducted by our group, we have shown that the activation of microbubbles with ultrasound can facilitate the delivery of nanoparticles (NPs) as large as 100 nm diameter to skeletal muscle (Price et al. 1998; Song et al. 2002a; Burke et al. 2011; Price and Kaul 2002). Moreover, we have also shown that such approaches can be utilized for targeting the delivery of pro-arteriogenic fibroblast growth factor-2 (FGF-2) from controlled release depots (Chappell et al. 2008a) and the expression of reporter genes based on non-viral gene-bearing nanoparticle delivery (Burke et al. 2012).

One key observation made in our previous NP delivery studies is that NPs are delivered to both endothelial cells and the interstitial spaces between myofibers. Looking ahead, the approach could be refined by better controlling whether NPs partition primarily to the endothelium or interstitium. Indeed, if NPs could be partitioned more selectively to various tissue compartments, it would influence growth factor selection and growth factor release profile from NPs. When considering which US-MB parameters could be tuned to achieve

such partitioning, there are several options. Microvessel permeabilization caused by US–MB interactions can be selectively modulated by US center frequency (Miller and Chunyan 2004; Miller 2007; Nyborg 2007), US peak-negative pressure (PNP), pulse length (Tu et al. 2006), pulse interval (Samuel et al. 2009; Song et al. 2002a), MB concentration (Miller and Quddus 2000; Song et al. 2008), and MB size (Caskey et al. 2007). Here, we chose to test the hypothesis that, in the setting of ischemic mouse hindlimb model, PNP can be used to modulate the relative post-delivery partitioning of NPs between endothelial and interstitial compartments.

Materials and methods

Ultrasound transducer characterization

A 1 MHz (0.5 in. diameter) unfocused transducer (A303S, Olympus NDT Inc., Waltham, MA) was used for all in vivo studies. It was aligned in a degassed water bath and positioned 10 mm from a hydrophone. The 10-mm distance corresponded to the in vivo distance between the transducer face and the gracilis adductor muscle. The transducer emitted 5-cycle pulses at a pulse repetition frequency of 100 Hz as a LabVIEW program averaged the voltage measurements of 200 pulses collected by the hydrophone. This process was repeated for voltage inputs ranging from 0.05 to 0.8 V. The data acquired through LabVIEW were then analyzed in MATLAB to yield PNPs corresponding to each of the voltage inputs.

Microbubble injectate characterization

Albumin microbubbles (3.54×10^7 MB/ml concentration in 0.9 % saline, mean diameter of $3.53 \mu\text{m}$) were drawn into a 1-ml syringe. Special care was taken to slowly draw MBs into the syringe to lessen MB shearing and to replace the MB stock vial head space with high-molecular-weight perfluoropropane gas to minimize MB destabilization. The syringe was gently rocked as larger bubbles might have floated more to the edges and then attached to a cannula made of a 20-cm PE-10 tubing (Inner diameter: 0.28 mm, Outer Diameter: 0.61 mm, Becton–Dickinson & Co., Sparks, MD) pre-connected to a 30G needle (30G1/2, Precision Gluider, Becton–Dickinson & Co., Franklin Lakes, NJ). MBs were infused at $13.333 \mu\text{l}/\text{min}$ using an infusion pump (Standard infusion Only PHD 22/2000 Syringe Pump, Harvard Apparatus, Holliston, MA) after 0.04 ml of dead space of 1 % heparin saline (0.9 %) originally in the cannula was cleared with a manual infusion at a rate of 1 ml/min. MBs were sampled at 3 and 10 min after infusion began and analyzed using a MultisizerTM 3 Coulter Counter[®] (Beckman Coulter, Brea, CA).

Femoral artery ligation (FAL)

FAL procedures were performed on Balb/C mice that subsequently underwent either hindlimb laser Doppler perfusion (LDPI) measurements or NP delivery. We have used this same FAL model previously to elicit enhanced collateral flow in mouse gracilis adductor muscle (Meisner et al. 2013b). Mice were anesthetized by an I.P. injection of ketamine (0.12 mg/g mouse), xylazine (0.012 mg/g mouse), and atropine sulfate (0.00008 mg/g mouse). The inner thigh of each leg and the surrounding region were depilated and cleaned with a Betadine wipe followed by an ethanol swab. After depilation, the mouse was placed supine on a warmed surgical stage. A sterile drape covered the mouse almost entirely, the inner

thighs were exposed through openings in the drape, and the nose and mouth were exposed at the edge of the drape. Viewing through a Zeiss surgical dissecting microscope (Stemi 2000, Carl Zeiss, Inc., Thornwood, NY), a skin incision less than 5-mm long was made on one leg directly along the femoral artery immediately distal to the epigastric artery bifurcation. The fascia and fat between the skin and underlying muscle were gently cleared by blunt dissection to expose the artery–vein pair running distal to where the epigastric artery–vein pair bifurcates from the femoral artery–vein. A separation of less than 4 mm in length was made between the artery and vein, sufficient for ligature placement. Special care was taken to leave the nerve running along this vessel pair intact and minimally disturbed. Two sterile pieces of silk sutures (6-0, Deknatel, Inc., Fall River, MA) were then tied at less than 2 mm apart around the separated section of the artery. The artery segment between the two ligatures was then severed with micro dissecting scissors. The skin incision was closed with sutures (5-0 Prolene, Ethicon, Somerville, NJ), and the animal was left to recover on a heating pad. As the anesthesia wore off, an I.P. injection of buprenorphine (0.03 ml/animal, Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) was given, followed by a second dose 8–12 h later.

Hindlimb perfusion monitoring

For a subset of Balb/C mice, LDPI measurements were made pre-ligation, immediately post-ligation, and on days 2, 4, 7, 10, 12, 14, and 21. For LDPI pre- and post-ligation, the animal was anesthetized under the ketamine–xylazine–atropine mixture given in preparation for the femoral artery ligation. For LDPI measurements made days after FAL, the animal was anesthetized with ketamine (35 mg/kg mouse) and dexmedetomidine (0.25 mg/kg mouse). The feet were cleaned and inner thighs of both legs were depilated. The animal was placed supine on a non-reflective light-absorbing black rubber pad and its feet were adhered to the rubber surface with double-sided masking tape. The animal on the rubber pad was then placed onto a heating pad within a dark Plexiglas® box with a side opening and scanned using the LDPI system PeriScan (Perimed, Inc., Sweden) with accompanying software LDPIWin (Perimed Inc., Sweden). With the PeriScan scanner fixed at approximately 20 cm above the hindlimb region, scanning was performed with the specific settings of color scale at 0 Volt minimum and 10 Volt maximum, with amplification of 2× at the high-resolution setting. Upon completion of the scan, the animal was taken out of the box and removed from the rubber pad to recover on a heating pad. A subcutaneous injection of atipamezole (0.0006–0.001 mg per g of mouse) was then given to reverse the effect of dexmedetomidine. The LDPIWin software (Perimed, Inc., Sweden) was used to analyze the hindlimb laser Doppler images. Both feet were manually traced in each Doppler and measured for mean intensity. The perfusion ratio was calculated by dividing the mean intensity of the foot on the ligated side with that on the control side. This ratio served as a metric for the extent of perfusion or reperfusion in ligated hindlimbs.

Femoral artery cannulation

Balb/C ($n = 15$ total) mice that had received FAL 4 days earlier and were designated for NP delivery studies were anesthetized with an I.P injection of ketamine (0.12 mg/g mouse), xylazine (0.012 mg/g mouse), and atropine sulfate (0.00008 mg/g mouse), and the inner thighs of each leg were depilated. After being secured on a warmed surgical stage, a skin

incision approximately 1-cm long was made directly along the ligated femoral artery immediately after it branches away from the abdominal aorta. The fascia between the skin and the underlying muscle was gently cleared by blunt dissection to expose the femoral artery–vein pair. Using blunt-tip forceps, the artery and vein were gently separated from each other with the length of separated space limited to approximately 5 mm. Care was taken to leave the nerve running with the vessel pair intact and minimally disturbed. Two pieces of 6-0 black braided silk sutures (Ethicon, Somerville, NJ) were placed under the artery to provide tension as a 20 cm PE-10 tubing (Inner diameter: 0.28 mm, Outer Diameter: 0.61 mm, Becton–Dickinson & Co., Sparks, MD) pre-connected to a 30G needle (30G1/2, Precision Gluide, Becton–Dickinson & Co., Franklin Lakes, NJ) attached to a syringe of 1 % heparin saline was inserted through a small incision into the femoral artery and tied in place with the sutures.

Ultrasound-microbubble-mediated nanoparticle delivery

After the cannula was inserted into femoral artery, the feet were gently taped to the surgical stage such that the inner thighs were easily accessible. The 1-MHz unfocused transducer was positioned with a supporting metal arm over the hindlimb with an approximately 8 mm-thick layer of ultrasound transmission gel (Aquasonic® 100 ultrasound transmission gel, Parker Laboratories, Inc., Fairfield, NJ) between it and the skin surface. Albumin microbubbles (3.54×10^8 MB/ml stock concentration, mean diameter of $3.53 \mu\text{m}$) were diluted to 3.54×10^7 MB/ml in 0.9 % saline solution containing 1.53×10^{13} 50 nm red NPs (R50, Fluoro-Max Dyed Red Aqueous Fluorescent Particles, Thermo Scientific Inc., Waltham, MA) and 1.91×10^{12} 100 nm green NPs (G100, Fluoro-Max Dyed Green Aqueous Fluorescent Particles, Thermo Scientific Inc., Waltham, MA) with a 1 ml syringe (1 ml Sub-Q 26G5/8, Becton–Dickinson & Co., Franklin Lakes, NJ). MBs were drawn into the syringe slowly to lessen MB shearing and to replace the MB stock vial head space with high-molecular-weight perfluoropropane gas to minimize stock MB destabilization. The syringe was then connected to the cannula, and MBs were infused at a rate of $13.333 \mu\text{l}/\text{min}$ with an infusion pump (Standard infusion Only PHD 22/2000 Syringe Pump, Harvard Apparatus, Holliston, MA) after 0.04 ml of dead space of 1 % heparin saline (0.9 %) originally in the cannula was cleared with a manual infusion at around 1 ml/ min.

US pulsing was triggered with a LabView software program (National Instruments, Austin, TX) and applied for a total duration of 12 min. One pulse consisted of 10 bursts, each spaced 100 ms apart, where one burst consisted of 300 consecutive 1-MHz sinusoids of 0.8 ($n = 3$), 0.3 ($n = 4$), 0.2 ($n = 4$), or 0.1 ($n = 4$) volt peak-to-peak amplitude from a waveform generator (Tektronix AFG-310, Sony Tektronix, Beaverton, OR) corresponding to PNPs of 0.70, 0.55, 0.40, and 0.20 MPa, respectively. A total of 72 pulses were applied during the 12-minute treatment. The waveform signal was amplified by a 55-dB RF power amplifier (model 3100LA, ENI, Inc.) and fed into the transducer. In between each pulse, a 10-second interval allowed for MB reperfusion into the targeted region. A schematic diagram of the US pulsing protocol is shown in Fig. 1.

Gracilis muscle preparation

Immediately after completing the infusion of MBs and NPs, mice were euthanized with an injection of pentobarbital sodium solution (0.2 ml/mouse). The inferior vena cava was severed and, through the existing femoral artery cannula, 1 ml each of 2 % heparinized Tris-buffered saline with CaCl₂, Tris-buffered saline with CaCl₂, and 4 % paraformaldehyde (PFA) was infused at 0.09 ml/min. While the 2 % heparinized Tris-buffered saline was being infused, the region of the US-MB-NP-treated gracilis muscle between the saphenous artery and the proximal feeder on the treated leg was carefully dissected free from the underlying muscles. After the blood removal process was completed, 1 ml of 4 % PFA was suffused over the gracilis muscle, and the muscle was left to fix in situ for approximately 30 min. After fixation, the gracilis muscle was excised and stored in a 50/50 mixture of PBS and glycerol at -20 °C. Gracilis muscle fixation via perfusion was critical in this study to remove any NPs from the vessel lumen.

Gracilis muscle sectioning and staining

Excised gracilis muscles were dried and bisected about their midlines perpendicular to the longitudinal axes of muscle fibers. With the cut ends of the muscles oriented toward the bottom of sectioning dishes filled with Optimal Cutting Temperature (OCT) compound (Tissue-Tek®, Sakura Finetek U.S.A., Inc., Torrance, CA), the specimens in OCT were left in room temperature for approximately 20 min before being frozen in the -80 °C freezer. 8-micron-thick sections were cut using a cryotome (Leica CM3050 Cryostat, Leica Instruments GmbH, Nussloch, Germany), yielding sections from both the lateral and medial regions of the fixed muscles. The cryosections were incubated overnight with 1:140 AlexaFluor®647-conjugated isolectin GS-IB4 from Griffonia simplicifolia (Life Technologies, Inc., Carlsbad, CA) in 0.1 % saponin and 2 % BSA in PBS at 4 °C in a humid chamber. Afterwards the sections were washed in 0.1 % saponin and 2 % BSA in PBS 2 times 20 min each at room temperature, followed by a wash in PBS for 20 min in room temperature. The sections were then mounted on a microscopic slide with a mounting medium (VECTASHIELD® Hardset Mounting Medium with DAPI, Vector Laboratories Inc., Burlingame, CA) and a glass coverslip and left in 4 °C overnight for the mounting to set.

Gracilis muscle cross section imaging and analysis

Mounted gracilis muscle cross sections were imaged using a Nikon Eclipse C1si confocal microscope system (Nikon, Melville, NY) and the associated control software EZ-C1 (Nikon, Melville, NY) with a 20 × oil objective. Images of every field of view within each section were taken at a pixel dwell of 20.40 μs and gain levels at 6.60 for the red and green channels, and 6.40 for the blue channel. Using the photomerge function in Photoshop CS2 (Adobe Systems Inc., San Jose, CA), the fields of view for each section were combined into a montage to create a complete image.

The montaged images were analyzed using ImageJ. To determine total NP delivery per section, the images of NPs in cross sections were thresholded to zero out the background and autofluorescence from muscle fibers yielding only NPs within the image. The sections were then manually traced within the image to yield a pixel value per section, which is

representative of the total NP amount delivered per cross section. To calculate the amount of NPs delivered specifically to the endothelium and interstitium, the image calculator function in ImageJ was used. Specifically, thresholded images of endothelium in cross sections were subtracted from thresholded images of NPs in cross sections to give images showing NPs in the interstitium per cross section. Those images were then manually traced to get a pixel value presenting the amount of NPs delivered to the interstitium. To get the amount of NPs delivered to the endothelium, the pixel value representing NPs delivered to the interstitium was subtracted from the pixel value representing the total NPs delivered. Two representative sections were analyzed for each mouse.

Statistical analysis

Two-Way ANOVA was performed to analyze the statistical significance of differences between differently sized NPs delivered to different tissue spaces with different PNP levels. Pairwise multiple comparison procedures were performed using the Holm-Sidak method. Statistical significance was assessed at $P < 0.05$. All statistical procedures were performed using SigmaStat version 3.5 (Systat Software Inc., San Jose, CA).

Results

Ultrasound transducer characterization

The A303S 1-MHz unfocused transducer was characterized by acquiring the PNP profiles at voltage inputs ranging from 0.05 to 0.8 V at 10 mm away from the transducer. Figure 2 shows PNP as a function of voltage. For studies aimed at testing the effect of various PNP levels (i.e. 0.7, 0.55, 0.4, and 0.2 MPa), voltages of 0.8, 0.3, 0.2, and 0.1 V were chosen, respectively.

Microbubble delivery system characterization

To determine the actual MB concentration and size distribution delivered into the Balb/C mice, MBs outputted from the infusion system were analyzed. Figure 3 shows pre- and post-injection MB size distributions. During injection, there was a ~60 % decrease in MB concentration and 40 % decrease in mean diameter. There was a nearly complete loss of MBs with diameters above 4 μm , and a nearly 50 % loss of MBs in the 2–3 μm range.

Influence of femoral artery ligation on perfusion

Because we were interested in studying nanoparticle delivery to skeletal muscle that surrounds arteries running collateral to an arterial occlusion, all Balb/C mice underwent FAL. To verify that the FAL procedure significantly affected perfusion in the distal hindlimb, LDPI measurements were made on a subset of these FAL-operated Balb/C mice (Fig. 4). Representative LDPI scans are presented in Fig. 4a. Here, a marked drop in perfusion on the FAL-operated hindlimb immediately post-FAL was evident, followed by gradual perfusion recovery. LDPI scans were then quantified and presented as perfusion ratios (ligated hindlimb over control hindlimb; Fig. 4b). Perfusion ratio dropped to ~0.25 immediately post-FAL; however, due to endogenous arteriogenesis and angiogenesis, it recovered to ~0.65 by Day 12 and remained at this level through Day 21.

Nanoparticle delivery

To determine the influence of PNP and NP diameter on NP delivery to skeletal muscle with US and MBs, 50 nm and 100 nm fluorescent NPs were intra-arterially co-injected with MBs, while 1 MHz US was applied to the gracilis muscle of Balb/C mice that had undergone FAL 4 days earlier. Representative gracilis muscle sections from these experiments are provided in Fig. 5. An inspection of Fig. 5 reveals three key characteristics. First, for both the 50 nm and 100 nm NP groups, as PNP pressure increases from 0.2 to 0.7 MPa, total NP coverage also increases. Second, for both the 50 and 100 nm NP groups, NP is primarily colocalized with endothelium at the lowest PNP levels. Then, as PNP increases above 0.4 MPa, NP coverage becomes considerably more abundant in the interstitium. Third, at all PNP levels tested, in comparison to the 100 nm group, the delivery of 50 nm NPs is greater in both the endothelial and interstitial compartments.

Quantitative analyses of these confocal microscopy images (Fig. 6) confirm these observations. Figure 6a depicts the total NP coverage as a function of PNP. For both the 50 and 100 nm NP groups, total NP delivery increased more than 2-fold as PNP increased from the lower levels of 0.2 and 0.4 MPa to the higher levels of 0.55 and 0.7 MPa. Furthermore, as expected, given their smaller size, the 50 nm NP exhibited greater total coverage when compared to 100 nm NP at the 2 highest PNP levels tested (0.7 and 0.55 MPa). Figure 6b, c then illustrates how NPs were partitioned between interstitium and endothelium as a function of PNP. At the lowest PNP (0.2 MPa), NP delivery was split fairly evenly between the interstitial and endothelial compartments. However, the total fraction of NPs in the interstitium increased substantially with increasing PNP. Meanwhile, NP coverage in the endothelial compartment remained relatively steady as PNP increased for both the 50 and 100 nm NP groups. This last observation is further confirmed by Fig. 6d, wherein the total percentage of endothelial area with NP coverage is presented as a function of NP diameter and PNP. In Fig. 6d, increasing PNP had no statistically significant impact on NP delivery to the endothelium. Nonetheless, consistent with the data on interstitial delivery shown in Fig. 6b, smaller NPs (i.e. 50 nm) also exhibited greater delivery to the endothelial compartment when compared to 100 nm NP.

Discussion

The main objective of this study was to determine whether PNP could be used to adjust the relative partitioning of NPs to skeletal muscle interstitium after their delivery with US and MBs. To this end, fluorescent 50 and 100 nm NPs were intra-arterially co-injected immediately upstream of the gracilis adductor muscle of Balb/C mice (4 days post-FAL), while 1 MHz US was applied at 4 different PNP levels (0.20, 0.40, 0.55, and 0.70 MPa). Examination of cross-sectioned muscles showed that total NP delivery increased with increasing PNP. Most of this increase was due to enhanced delivery to the interstitial compartment, indicating that the relative partitioning of NPs to skeletal muscle interstitium may be tuned by increasing PNP. Going forward, our results will be useful for selecting US parameters in US-MB-mediated NP delivery experiments. For example, to direct maximum total transfer of 50 nm NPs to skeletal muscle or to the interstitial space in particular, PNP applied at 0.55 MPa may be preferred over 0.7 MPa as there are no significant differences in

the amount of NPs delivered to the tissue or the interstitium between the two, while there may be lower risk associated with the lower PNP (0.55 MPa) compared to the higher PNP of 0.7 MPa. On the other hand, if a relatively even distribution of 100 nm NPs across the interstitium and endothelium were preferred, a PNP of 0.2 MPa would perhaps be the better option.

Mechanisms of nanoparticle delivery

The biophysical mechanism(s) responsible for enhanced total and interstitial NP delivery with increasing PNP were likely the result of enhanced MB cavitation, which in turn elicited increased cell membrane and/or blood vessel wall permeability. Although cavitation detection was not used in these particular studies, it is probable that, at the higher PNP levels, MBs were undergoing inertial cavitation (Wu et al. 2014). During inertial cavitation, MBs undergo non-linear expansion and contraction, followed by asymmetric collapse. During such a violent collapse, the surrounding liquid rushes inward in a non-uniform manner that forms a liquid jet, which could pierce through endothelial cell membranes or across vessel walls to create transient pores that allow for agent distribution by convection (Caskey et al. 2007; Nyborg 2007; Prentice et al. 2005; Price and Kaul 2002; Price et al. 1998). This phenomenon could also possibly be generated from penetration by bubble shell fragments post bubble collapse (Chappell and Price 2006; Fan et al. 2010). Additionally, violent collapses of microbubbles are also known to produce shock waves, heat, and free radicals that may possibly alter cellular and vascular permeability (Miller et al. 2008; Mitragotri 2005). However, for microbubbles such as Optison® where the encapsulated perfluorocarbon gas has high heat capacity, the production of free radicals is greatly reduced, and the maximum temperature produced during MB collapse is also much less for such gases than air (Nyborg 2007). In fact, it has been shown that free radicals play an insignificant role in USMB-enhanced delivery of genes to vascular cells with Optison® as the choice for MBs (Lawrie et al. 2003). Ultimately, at the higher PNPs tested here, biasing toward interstitial delivery was likely caused by the creation of more numerous and/or larger pores in capillary walls which subsequently enabled greater NP transfer into the interstitium by convection and diffusion. Despite this physical disruption of the endothelium, previous studies performed by our group, wherein microbubbles were activated with 1 MHz ultrasound in skeletal muscle using peak-negative pressures in this range, have shown neither short-term perfusion changes (Song et al. 2004) nor long-term adverse effects (Burke et al. 2012; Chappell et al. 2008a). Moreover, long-term angiogenic (Chappell et al. 2005) and arteriogenic (Song et al. 2004, 2002b; Chappell et al. 2008b) responses, which may actually confer some benefit in the context of ischemic skeletal muscle, have actually been shown to occur. Nonetheless, it is still important to emphasize that, while higher peak-negative pressures provide greater NP delivery across the endothelial layer, exceeding the threshold for inertial cavitation is unlikely to be acceptable in some applications, including blood–brain barrier opening (Nance et al. 2014).

In contrast, at the lower PNPs, we believe at least some portion of the US-activated MBs were being driven in a stable cavitation regime. Because of this, fewer and/or smaller pores were most likely created in microvessel walls, resulting in diminished total and interstitial NP delivery. In the stable cavitation regime, US-activated MBs exert mechanical stresses on

cells and microvessels by either directly pushing or pulling against them (Caskey et al. 2007; van Wamel et al. 2004) or through microstreaming—small-scale steady circulatory flow in the immediate vicinity of a vibrating bubble—in proximity to the endothelium (Collis et al. 2010; Liu and Wu 2008). Such stresses seem to generate transient intercellular gaps and cell membrane pores (Juffermans et al. 2009; Meijering et al. 2009; van Wamel et al. 2006), and also stretch-activate cell membrane receptors and ion channels that trigger downstream signaling to promote endocytosis or alter membrane barrier properties (Meijering et al. 2009; Tran et al. 2008), which allow for greater delivery of therapeutic agents into targeted regions.

Limitations of the study

While our experimental design allowed us to demonstrate a relationship between peak-negative pressure and NP delivery to skeletal muscle interstitium, there are some limitations that should be considered. For example, as previously noted, cavitation was not monitored; therefore, we are only able to speculate on how the transition from stable to inertial cavitation correlated to NP delivery. In addition, because we sought a high concentration of NPs in the muscle to permit easy visualization with epifluorescence microscopy, the MBs and NPs were arterially injected immediately upstream of the gracilis muscle. Upon intravenous injection, which is a more common approach, some large MBs would likely be filtered in the lungs before reaching the muscle of interest. This altered MB diameter distribution would likely have some effect on endothelial barrier opening and subsequent NP delivery. Finally, the fluorescent tracer NPs used here were not made bioinert through the addition a polyethylene glycol coating. Such a coating would serve to increase both circulation time and penetration through tissue, which could affect the magnitude and dispersion of NP delivery to endothelium and interstitium.

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Fig. 1.
Schematic illustration of the ultrasound pulsing protocol used in the nanoparticle delivery experiments

Fig. 2.

Scatterplot of ultrasound peak-negative pressure (PNP) as a function of input voltage from the waveform generator. Measurements were made using a hydrophone located 10 mm from the face of the transducer, mimicking the distance between the transducer face and the gracilis adductor muscle during in vivo experiments

Fig. 3.
Pre- and post-injection microbubble diameter distributions

Fig. 4. Hindlimb perfusion recovery after femoral artery ligation. a Representative longitudinal Laser Doppler Perfusion scans from a Balb/ C mouse, taken before and after femoral artery ligation. b Line graph of Laser Doppler Perfusion ratio. Perfusion ratio refers to ligated over control

Fig. 5. Representative confocal microscopy images illustrating the influence of peak-negative pressure and nanoparticle diameter on nanoparticle delivery skeletal muscle. Images show the delivery of 50-nm (*red*) and 100-nm (*green*)-diameter nanoparticles with respect to skeletal muscle capillaries (*blue*). *Columns* denote peak-negative pressures of 0.70, 0.55, 0.40, and 0.20 MPa. (Color figure online)

Fig. 6.

Total nanoparticle (NP) delivery, as well as the relative partitioning of nanoparticles to endothelial or interstitial compartments, is dependent upon peak-negative pressure (PNP) and nanoparticle diameter. **a** Bar graph of total nanoparticle (50 and 100 nm diameter) delivery as a function of peak-negative pressure. $^+P < 0.05$ versus 100 nm NP at same PNP. $^*P < 0.05$ versus same sized NP at 0.4 and 0.2 MPa. $^{**}P < 0.05$ versus 100 nm NP at 0.2 MPa. **b** Bar graph of total NP delivery to endothelial (E) or interstitial (I) compartments as a function of NP diameter and PNP. $^{\#}P < 0.05$ versus E at same NP diameter and PNP. $^+P < 0.05$ versus 100 nm NP in I at same PNP. $^*P < 0.05$ versus same diameter NP in I at PNP of 0.4 and 0.2 MPa. $^{**}P < 0.05$ versus same size NP in same tissue space at 0.2 MPa. **c** Bar graph of fraction of NP delivered to E or I compartments as a function of NP diameter and PNP. $^{\#}P < 0.05$ versus same size NP in E at same PNP. $^*P < 0.05$ versus same size NP in I compartment at 0.4 and 0.2 MPa. $^{**}P < 0.05$ versus 50 nm NP in same compartment at 0.2 MPa. **d** Bar graph of percentage of total endothelial area containing NP. $^+P < 0.05$ versus 100 nm NP at same PNP.