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RENAL RETENTION OF LIPID MICROBUBBLES: A POTENTIAL MECHANISM FOR FLANK DISCOMFORT DURING ULTRASOUND CONTRAST ADMINISTRATION

Ya Ni Liu, M.D.^{*,†}, Jaspreet Khangura, M.D.^{*}, Aris Xie, M.S.^{*}, J. Todd Belcik, R.D.C.S.^{*}, Yue Qi, M.D.^{*}, Brian P. Davidson, M.D.^{*}, Yan Zhao, M.D.^{*}, Sajeevani Kim, M.D.^{*}, Yoichi Inaba, M.D.^{*}, and Jonathan R. Lindner, M.D.^{*}

^{*}Knight Cardiovascular Institute, Oregon Health & Science University, Portland, OR

[†]Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China

Abstract

Background—The etiology for flank pain sometimes experienced during administration of ultrasound contrast agents is unknown. We investigated whether microbubble ultrasound contrast agents are retained within the renal microcirculation which could lead to either flow disturbance or local release of vasoactive and pain mediators downstream from complement activation.

Methods—Retention of lipid-shelled microbubbles in the renal microcirculation of mice was assessed by confocal fluorescent microscopy and contrast-enhanced ultrasound (CEU) imaging with dose-escalating intravenous injection. Studies were performed with size-segregated microbubbles to investigate physical entrapment, after glycocalyx degradation, and in wild-type and C3-deficient mice to investigate complement-mediated retention. Urinary bradykinin was measured before and after microbubbles. Renal CEU in human subjects (n=13) was performed 7–10 min after completion of lipid microbubble administration.

Results—In both mice and humans, microbubble retention was detected in the renal cortex by persistent CEU signal enhancement. Microbubble retention in mice was linearly related to dose and occurred almost exclusively in cortical glomerular microvessels. Microbubble retention did not affect microsphere-derived renal blood flow. Microbubble retention was not influenced by glycocalyx degradation nor by microbubble size, thereby excluding lodging, but was reduced by 90% (p<0.01) in C3-deficient mice. Urinary bradykinin increased by 65% five minutes after microbubble injection.

Conclusion—Lipid-shelled microbubbles are retained in the renal cortex due to complement-mediated interactions with glomerular microvascular endothelium. Microbubble retention does not adversely affect renal perfusion but does generate complement-related intermediates that are known to mediate nociception and could be responsible for flank pain.

Contrast-enhanced ultrasound (CEU) imaging including myocardial contrast echocardiography relies on the acoustic detection of gas-filled microbubble contrast agents.

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Address correspondence to: Jonathan R. Lindner, MD, Cardiovascular Division, UHN-62, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239, Tel. (503) 494-8750, Fax (503) 494-8550, lindnerj@ohsu.edu.

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Microbubbles that are approved for clinical use are encapsulated by albumin or lipid surfactants and behave similar to erythrocytes in the microcirculation of the heart and skeletal muscle.^{1, 2} Although the safety of commercially-produced microbubbles has been established by trials that evaluated serious reactions,^{3, 4} post-marketing surveys and retrospective studies have revealed that flank pain is one of the most frequent non-serious adverse reactions that can necessitate termination of lipid microbubble administration.^{5, 6}

Although the etiology for flank pain with ultrasound contrast agents is unknown. Similar symptoms have been reported with high doses of liposomal drug preparations.^{7, 8} It has been proposed that liposome-related flank pain is caused by complement (C') activation which occurs in proportion to total lipid dose.⁷⁻⁹ Lipid microbubbles can also promote C' activation resulting in deposition of human C3b on the microbubble surface, particularly when highly-charged lipids are present in the shell.¹⁰ Complement proteins on the shell surface can mediate microbubble adhesion to activated leukocytes and vascular endothelium.¹⁰⁻¹² Hence, it is possible that C' may mediate renovascular retention of microbubbles and produce flank discomfort through either physical obstruction to flow or local production of vasoconstrictors and/or nociceptive peptides produced downstream from C' activation.

Our aim was to evaluate whether microbubble retention occurs within the renal microcirculation in mice and humans, and to determine the histologic location of retention by confocal microscopy. To evaluate the potential mechanisms of retention, we assessed for size-dependent lodging and evaluated the effects of microbubble charge, competence of the C' system, and integrity of the glycocalyx which influences cell-microbubble interaction.

METHODS

Microbubbles for Murine Experiments

Lipid-stabilized decafluorobutane microbubbles were prepared by sonication of a gas-saturated aqueous dispersion of lipids. Microbubbles with a near-neutral charge (MB) were prepared with a dispersion of 2.0 mg/mL distearoylphosphatidylcholine and 0.5 mg/mL polyoxyethylene-40-stearate. Microbubbles with a net negative (MB⁻) or positive (MB⁺) surface charge were prepared by adding either distearoyl phospho-L-serine or distearoyl trimethylammonium propane (0.1 mg/mL), respectively. Microbubble zeta-potential was determined by laser Doppler velocimetry (Zetaplus, Brookhaven Instruments, Holtsville, NY) in 1 mM KCl. Results of three separate measurements were averaged. Separation of microbubbles into small size population was performed by flotation-centrifugation at 400g for 1 min and isolation of the turbid supernatant.¹³ For confocal microscopy, lipid microbubble shells were fluorescently labeled by adding dioctadecylindocarbocyanine (DiI) to the suspension before sonication. Studies with albumin-shelled octafluoropropane microbubbles (MB-A) were performed with Optison (GE Healthcare, Princeton, NJ). Microbubble concentration and size distribution were determined by electrozone sensing (Multisizer III, Beckman Coulter, Fullerton, CA).

Animal Preparation

The study was approved by the Animal Care and Use Committee of the Oregon Health & Science University. Male wild-type C57Bl/6 mice and C3-deficient (C3^{-/-}) mice (Jackson Laboratory) were studied at 8–12 wks of age. Mice were anesthetized with inhaled isoflurane (1.0–1.5%). Body temperature was maintained at 37°C with a heating platform. A jugular vein was cannulated for administration of microbubbles.

Contrast-Enhanced Ultrasound in Mice

Renal CEU was performed from a dorsal approach with a linear-array probe (Sequoia, Siemens Medical Systems) using phase-inversion amplitude-modulation imaging at 7 MHz and a mechanical index (MI) of 0.16. Microbubbles were injected as an intravenous bolus injection. Imaging for retained microbubbles was performed 8 min after injection. After initial image acquisition, several high-power (MI 1.0) frames were applied to destroy microbubbles in the sector. Signal from retained microbubbles was calculated by digitally subtracting several averaged frames obtained >5 s after the destructive pulse sequence from averaged frames obtained before the destructive pulse sequence. Intensity was measured from a region-of-interest placed on the entire kidney guided by fundamental 2-D imaging acquired after each CEU imaging sequence.

Murine CEU Protocols to Assess Retention

Kidney retention of lipid microbubbles was evaluated with bilateral renal CEU using the following five conditions: (**A**) injection of MB at escalating doses from 1×10^4 to 1×10^6 ($n=12$); (**B**) injection of 1×10^6 MB with either normal size dispersion or size-separated to obtain the small MB population ($n=5$); (**C**) injection of 1×10^6 MB in C3 $^{-/-}$ mice ($n=5$); (**D**) injection of 1×10^6 MB, MB $^{+}$, and MB $^{-}$ performed in random order ($n=5$); (**E**) injection of 1×10^6 MB, MB $^{+}$, and MB $^{-}$ in random order before and after degradation of the glomerular glycocalyx produced by intravenous administration of hyaluronidase (1.5×10^4 U/kg, 60 min prior) and heparinase III (8.2 U/kg, 15 min prior) ($n=5$).^{14, 15} To evaluate relative retention of albumin and lipid microbubbles, CEU was performed in 3 mice after injection of MB (5×10^5) and MB-A at medium and high dose (5×10^5 and 1×10^6).

Confocal Microscopy for Microbubble Retention

In five wild-type mice, 1×10^6 DiI-labeled MB microbubbles were injected intravenously. After 8 min, 100 μ g FITC-labeled lycopersicon esculentum lectin was injected to label the endothelium. Mice were euthanized and the blood pool was flushed by isothermic saline (5 mL) infused via the left ventricle (LV) at 80–100 mm Hg. Confocal fluorescent microscopy (FW1000, Olympus, Center Valley, PA) was performed using non-fixed thick sections (1 mm) of the kidney.

Renal Blood Flow

Changes in renal microvascular blood flow produced by microbubble injection in mice was evaluated by microsphere technique ($n=3$). An arterial cannula was placed in the carotid artery and advanced retrograde into the aorta. Fluorescently-labeled microspheres (5×10^4) with a diameter of 15 μ m (Dye Track-F, Triton Technology, San Diego, CA) were suspended in 20 μ L saline and injected over 1 min. A second injection of microspheres labeled with a different fluorophore was repeated 8 min after intravenous injection of 1×10^6 MB. Both kidneys were removed, cut into 1 mm thick sections, and the number of microspheres labeled with each fluorophore was quantified by fluorescent microscopy.

Urinary Bradykinin

Urine was sampled through a urethral microcatheter at baseline and 5–10 min after intravenous injection of 1×10^6 MB. Bradykinin concentration was measured by ELISA (Phoenix Pharmaceuticals, Burlingame, CA).

Histology

Renal tissue was obtained 24 hrs after intravenous injection of 1×10^6 MB, fixed, and stained with hematoxylin to evaluate for inflammatory cell infiltration ($n=3$). Complement

regulatory protein expression in the glomeruli on dual fluorescent confocal microscopy was assessed (n=2) by intravenous injection of 5 µg/g phycoerythrin-labeled mAb against murine CD55 (RIKO3, Biolegend) followed 30 min later by 100µg FITC-lectin.

Human CEU Imaging

The study was approved by the human Investigational Review Board at Oregon Health & Science University. Fourteen hemodynamically-stable outpatients who were undergoing contrast echocardiography for endocardial delineation were recruited. Exclusion criteria included severe LV systolic dysfunction, heart failure, renal insufficiency, active inflammatory or auto-immune disorder, or immunomodulatory therapy. Renal CEU was performed with a phased-array transducer (IE33, Philips Ultrasound) using amplitude-modulation imaging at 1.6 MHz and an MI of 0.12–0.14. Images were acquired 7–10 min after completion of an infusion of lipid-shelled octafluoropropane microbubbles (Definity, Lantheus Medical Imaging) as part of their clinically-indicated contrast echocardiographic study (dose range 0.8–1.2 mL). Images were acquired before and after several high-power (MI=1.0–1.1) frames and image analysis was performed similar to murine experiments.

Statistical Methods

Data are expressed as mean $\pm$ SD unless otherwise mentioned. Normally-distributed continuous variables were analyzed using the unpaired Student *t*-test (two-sided). Paired *t*-tests were used for microbubble retention data before and after glycocalyx disruption and for microsphere blood flow data. One-way ANOVA was used to compare the effects of microbubble charge and, when significant, post-hoc analysis with unpaired Students *t*-test and Bonferroni correction for multiple comparisons was performed. Urinary bradykinin levels were compared by Wilcoxon test. Dose-intensity relationship was evaluated with linear regression analysis.

RESULTS

Retention of Microbubbles in the Renal Microcirculation

In wild-type mice, signal enhancement on CEU from retained lipid MB was consistently detected eight minutes after their intravenous injection at a time when most microbubbles had cleared from the circulating blood pool. Signal enhancement from retained MB was linearly related to dose (Figure 1A). Signal was proportionally much greater for microbubbles with lipid shell (MB) than for albumin shell (MB-A) (Figure 1B). Signal enhancement for MB was observed primarily in the region of the renal cortex (Figure 1C). Confocal microscopy indicated that this signal was attributable primarily to MB retention within the cortical glomerular microvessels (Figure 1D). There was no evidence for extravascular transit of MB. Retention of MB in peritubular vessels was uncommon.

Mechanisms for Microbubble Retention

To investigate physical entrapment as a mechanism for MB retention, renal CEU was performed with MB preparations either with conventional size distribution or after size-segregation to eliminate large MBs. Size separation produced a modest reduction in the mean microbubble diameter, but the proportion of microbubbles >5 mm which represents the minimal glomerular capillary diameter¹⁶ was reduced by $>85\%$ (Table 1). *In vitro* CEU imaging experiments using dose-escalation in a water bath indicated a similar intensity for the conventional and size-segregated agent, thereby eliminating the need for dose adjustments for size-segregated MB to compensate for any reduction in microbubble signal in the *in vivo* studies (Figure 2). Signal enhancement from retained microbubbles in the

kidney was minimally reduced when using the small size-segregated population (Table 1), arguing against size-dependent lodging as a major mechanism.

For experiments investigating the effect of net microbubble surface charge, zeta potential was -6 mV for MB, -38 mV for MB⁻, and $+47$ mV for MB⁺. Altering the charge in either an anionic or cationic direction resulted in mild differences in microbubble retention under basal conditions (ANOVA $p=0.03$) with slightly greater retention for MB⁺ (Figure 3A). However, differences did not meet statistical significance after correcting for multiple comparisons. Signal for all three agents was almost identical after systemic treatment with hyaluronidase and heparinase to degrade the glycocalyx, arguing against any interactions with the glomerular glycocalyx. Renal retention of MB was reduced by almost 90% in C3-deficient compared to wild-type mice (Figure 3B). Confocal microscopy demonstrated that CD55 (decay-accelerating factor) which is an endothelial-associated complement regulatory protein, was highly expressed on the glomerular capillaries (Figure 3C). Urinary bradykinin which was used as a marker for glomerular complement activation that is also involved in nociception was significantly increased after microbubble injection (Figure 4A).

Renal Perfusion and Histology

Renal histopathology 24 hrs after contrast injection did not detect any evidence for renal injury, cellular inflammatory infiltrate, or cellular edema (Figure 4B). Microsphere-derived renal perfusion measured 10 min after MB injection was similar to that prior to injection (Figure 5), indicating that MB retention did not substantially alter renal perfusion.

Retention in Humans

In human subjects, a variable degree of signal from renal retention of MB was detected 7–10 min after completing an infusion of lipid microbubbles (Figure 6). Almost all patients demonstrated some degree of renal MB retention. The highest degree of signal from retained microbubbles encountered in this limited group of subjects were found in the only patient who was studied that experienced mild flank pain, and incidentally in a patient with active paroxysmal nocturnal hemoglobinuria, a disorder of complement dysregulation.

DISCUSSION

In this study, we undertook a series of steps to understand a potential mechanism for back or flank pain which is one of the most commonly reported adverse events especially for certain lipid-shelled ultrasound contrast agents. Post-marketing surveys and retrospective studies from patients undergoing rest or stress echocardiography have indicated that these symptoms will occur in approximately 0.7% of patients receiving perflutren lipid microbubbles (currently marketed in the U.S. as Definity); and in up to 1.4% (severe in 0.5%) of those receiving Sonovue (currently marketed in Europe) during stress.^{5, 6, 17} Although it has been reported, back pain is uncommon ($<0.5\%$) with albumin-shelled microbubbles (perflutren protein marketed as Optison in the U.S.)¹⁸ Our murine and human results suggest that lipid microbubbles can be retained within the renal microcirculation and that this phenomenon is most likely secondary to complement-mediated interaction with the glomerular endothelial surface.

There are several organs that are sites of preferential retention of microbubble contrast agents, the most notable of which are reticuloendothelial tissues such as the liver and spleen. Microbubble retention in these tissues is in part due to adhesion and even phagocytosis by Kupffer cells.^{19, 20} Our renal contrast-enhanced ultrasound studies using dose-response in mice and in patients without pre-existing renal disease confirmed our first hypothesis that lipid microbubbles are also retained within the kidney. These imaging studies were

performed after an 8 min delay after injection to allow clearance of microbubbles from the blood pool for both mice and humans,^{13, 21} and was performed using an algorithm that nulls signal from freely-circulating microbubble. In mice, retention for lipid MB was seen primarily in the cortex which was further confirmed by dual fluorescent confocal microscopy which showed preferential retention of microbubbles in the cortical glomerular capillaries. Microbubble egress through the fenestrated glomerular endothelium was not seen. Microbubble retention was also detected. Subsequent experiments were designed to test the mechanism responsible for retention.

Size-dependent lodging of microbubbles was considered as a potential mechanism since pulmonary arteriovenous shunts are relatively abundant in mice and can be responsible for up to 9% of transpulmonary flow.²² Although these arteriovenous connections are relatively small, they can allow passage of stable encapsulated microbubbles that are of sufficient size (>5 μ m) to become entrapped in systemic capillaries and cause delayed opacification of organs.¹³ The tortuosity and amount of flow through the glomerular vessels provide a reasonable justification for evaluating microbubble entrapment. We tested this hypothesis by comparing microbubble populations with sufficiently different size distributions that differences in lodging should be readily apparent.¹³ Our size-segregated microbubble preparations contained 10-fold lower percentage over 5 μ m in diameter, the minimal glomerular capillary dimension in mice,¹⁶ yet signal from these microbubbles was not substantially different from standard preparations. The fact that flank pain has also been reported to occur in a dose-dependent fashion during the administration of liposomal drugs which are much smaller than microbubbles but have a greater total lipid dose further also argues against lodging.^{7, 8}

The glycocalyx is a highly-charged barrier layer of glycoproteins and proteoglycans on the endothelial luminal surface and has been reported to be particularly prominent in glomerular vessels.²³ Drastic alterations in glycocalyx integrity post-ischemia have been shown to be associated with tissue retention of certain types of MBs.²⁴ Charge-mediated interactions between MBs and the glycocalyx were investigated and largely excluded by studies examining the effect of degrading the glycocalyx. It was interesting to note, however, that renal retention of strongly anionic MBs was less than that for other MBs, suggesting that charge repulsion with the glycocalyx may partially counteract microbubble-endothelial interaction.

The possibility of complement-mediated attachment was investigated on the basis that lipid-shelled microbubbles, like liposomes, can promote complement activation on their surface.^{10, 11} Surface activation of C' can result in opsonization by phagocytic cells and microbubble attachment to the vascular endothelium.^{11, 25-27} Complement-mediated retention is likely to be one of the mechanisms responsible for preferential retention by Kupffer cells in the liver. Complement activation on microbubbles is amplified by higher surface charge and lack of a protective polymeric (e.g. polyethyleneglycol) barrier; and for strongly anionic microbubbles can mediate attachment even to normal microvessels.¹⁰ In the current study, imaging of lipid MB retention in C3-deficient mice provided strong evidence that complement-mediated interactions are a major mechanism for microbubble retention in the kidney. Additionally, the mild increase in signal from retained microbubbles with strongly cationic charge could be consistent with a complement-mediated mechanism through alternative pathway acceleration.²⁸ The finding that retention was much greater for lipid than albumin microbubbles is consistent with a complement-dependent process since our previous data has suggested that microbubble attachment to phagocytic cells is much more complement-dependent for lipid-shelled agents.¹¹

The site of lipid MB retention was almost exclusively in the glomeruli which is the primary renal site for complement deposition, regulation and binding proteins.²⁹ Although we are not certain that complement-mediated retention is the primary reason for flank pain, we are reassured that retention does not cause substantial renal ischemia from MB obstruction or abnormal vasoregulation. Urinary bradykinin assays were elevated after microbubble injection. These studies were performed in order to demonstrate detectable increases in biologic mediators of nociceptive afferent nerve activation that are produced consequent to complement activation.^{30, 31}

There are several limitations and unsolved issues. First, although we are reasonably sure that renal retention is complement-related, we do not know the specific complement receptors that mediate retention. We know of no studies that demonstrate a particularly high density of complement receptors on the glomerular endothelium. For that reason we chose to study one endothelial membrane-associated complement regulatory protein, decay activating factor (CD55). Although there was dense expression of CD55 in the glomerular vessels, this does not necessarily indicate that CD55 was responsible for microbubble retention. We also cannot say with absolute certainty that glomerular retention of microbubbles is responsible for flank pain. Although signal from retained MB was quite high in the one patient who experienced flank pain, MB retention also occurred in subjects who were completely without symptoms. Accordingly, it is unlikely that that a clinical trial where renal CEU data are compared for patients with and without flank pain will be particularly revealing, especially since it is quite probable that patients with pain have a higher sensitivity to nociceptive triggers downstream from complement rather than greater complement consumption. In our clinical studies, we evaluated retention after contrast infusion rather than bolus injections which are used by many. Anecdotally, bolus injection and very high dose infusion are actually more likely to produce these non-serious adverse events. We are also unsure of the reason why flank pain for Sonovue has been reported in prospective although not retrospective safety trials,^{17, 32} although it should be mentioned that symptoms of back pain may not necessarily queried in these trials. Finally, we did not measure urinary bradykinin in our clinical trials.

In conclusion, we have taken several important steps towards understanding potential mechanisms for flank discomfort with ultrasound contrast agents. We provide the first report of retention of microbubbles in the renal cortex in mice and in humans, which is likely related to complement activation but does not appear to cause any significant renal ischemia or injury. These studies help understand the mechanism and implications of an uncommon and usually non-serious side effects of contrast agents.

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Figure 1. Microbubble retention in the mouse kidney

(A) Relation between intravenous lipid MB dose and CEU signal enhancement in the renal cortex from retained microbubbles after subtraction of signal from freely circulating component. (B) CEU signal enhancement for lipid MB and for albumin MB (MB-A).

* $p < 0.05$ vs MB. (C) Examples of background-subtracted color-coded renal CEU images reflecting only retained microbubbles at increasing MB doses. (D) Confocal microscopy at low- and high-magnification illustrating Di-I-labeled microbubbles within the glomerular microcirculation labeled with FITC-lectin.

Figure 2.

Concentration versus intensity relationship for conventional microbubble agents and size-segregated microbubbles agents after removal of large microbubbles (see Table 1).

Figure 3.

Effect of glycocalyx and complement system integrity on microbubble retention. **(A)** Mean $\pm$ SEM CEU intensity from microbubble retention according to surface charge. Data are shown at baseline and after degradation of the glycocalyx with hyaluronidase and heparinase (hyal/hepar). ANOVA $p=0.03$ for baseline conditions; * $p<0.05$ vs MB for single comparisons but not after correction for multiple comparisons; [†] $p=0.01$ vs baseline. **(B)** Mean $\pm$ SEM CEU intensity from MB retention in wild-type and complement-deficient (C3^{-/-}) mice. * $p=0.0001$ vs wild-type. **(C)** Examples of immunohistochemistry for microvascular endothelium with FITC-lectin, CD55, and a merged image demonstrating preferential CD55 staining on the glomerular microvascular endothelium.

Figure 4. Urinary bradykinin and renal histology

(A) Urinary bradykinin measured at baseline and 5–10 min after intravenous injection of MB. (B) Examples of renal histology at low (upper left) and high magnification with hematoxylin and eosin staining 24 hrs after MB injection illustrating normal tissue and absence of cellular inflammatory response or edema of the cellular components of the glomerulus.

Figure 5. Effects of microbubble injection on renal blood flow

(A) Renal microsphere counts per section for fluorescently-labeled microspheres injected before and 8 min after intravenous injection of 1×10^6 MB before. (B) Examples of microsphere lodging (red=pre-MB; blue=post MB) within the kidney illustrating localization primarily within the glomeruli which are identified by focal areas where background is dark.

Figure 6. Renal retention of microbubbles in humans

(A) Background-subtracted video intensity on renal CEU after correcting for signal from freely circulating microbubbles. Signal from the right and left kidney from a patient with flank pain (filled circles) and a patient with paroxysmal nocturnal hemoglobinuria (filled triangles) are differentiated from other patients (open circles). (B and C) Examples of CEU from two separate patients illustrating signal enhancement on the initial frame (*IF*) obtained 7–10 min after completing contrast administration, immediately post high-power destructive sequence (*PD*), 7 seconds post high-power destructive sequence representing freely circulating microbubble signal (*PD+7s*), and background-subtracted color-coded image representing signal attributable only to retained microbubbles (*BS-CC*).

Table 1

Characteristics of Size-separated Microbubbles

	Conventional	Size-segregated (small population)
Microbubble Diameter (μm)	2.2 $\pm$ 1.5	1.7 $\pm$ 0.7
Diameter >5 μm (%)	3.82	0.44
Signal Intensity (VIU)	25 $\pm$ 3	21 $\pm$ 2

VIU, video intensity units, corrected for signal from freely circulating population.