Original Paper

Bubbles Induce Endothelial Microparticle Formation via a Calcium-Dependent Pathway Involving Flippase Inactivation and Rho Kinase Activation

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Key Words

Bubble • Endothelial microparticles • Calcium • Rho kinase • Flippase

Abstract

Background/Aims: Intravascular bubbles can exert pleiotropic detrimental effects, partly by inducing endothelial microparticles (EMPs) production, which play critical roles in cell communication and vascular inflammation cascades. However, the underlying mechanisms remain unclear. This study aimed to delineate the possible mechanisms involving bubble-induced EMPs formation. **Methods:** Human umbilical vein endothelial cells (HUVECs) were contacted by bubbles and EMPs level in supernatant were quantified by flow cytometry. Cytoplasmic calcium (Ca^{2+}) was measured by the Ca^{2+} binding dyes Fluo-3 AM and flippase activity was assessed by translocation rate of fluorescent phosphatidylserine (PS) analogue NBD-PS. Protein levels of phospho-myosin light chain (MLC, a Rho kinase substrate) and phospho-extracellular signal-regulated kinase 1 or 2 (ERK1/2) were determined by western blotting. The score of actin colocalization was assessed by phalloidin-FITC using an immunofluorescent microscopy. **Results:** EMPs level markedly increased after bubble stimulus. Cytoplasmic calcium (Ca^{2+}) significantly elevated ($P < 0.05$), flippase activity decreased ($P < 0.05$), protein levels of phospho-MLC and phospho-ERK1/2 significantly increased ($P < 0.05$, $P < 0.05$), and the score of actin colocalization markedly reduced ($P < 0.05$) in bubble-stimulated HUVECs. All the above changes except the increase in phospho-ERK1/2 can be reversed by Ca^{2+} channel blocker LaCl_3 ($P < 0.05$). Additionally, MLC phosphorylation was significantly inhibited and actin colocalization markedly increased by Rho kinase inhibitor pretreatment and more importantly, bubble-induced EMPs markedly decreased. **Conclusions:** These results demonstrate that bubble stimulates EMPs formation by cytoplasmic Ca^{2+} elevation and subsequently activating Rho kinase pathway and cytoskeleton reorganization. Simultaneously, cytoplasmic Ca^{2+} inhibits the flippase activity and subsequently increases phosphatidylserine exposure, which also contributes to EMPs formation.

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Introduction

Microparticles (MPs) are submicron vesicles (0.1–1 μm) generated from membranes of activated or apoptotic cells via an exocytosis process [1]. Harboring cell surface proteins, cytoplasmic and nuclear constituents from parent cells, MPs can transfer these contents to recipient cells and alter the development of several diseases [2–4]. Believed to be formed by all cell types, MPs can be detected in the peripheral blood, mainly from platelets, leukocytes, erythrocytes and endothelial cells [5, 6]. MPs harboring endothelial-associated molecules were identified both *in vitro* and *in vivo* and therefore, were called endothelial MPs (EMPs) [7]. Increasing evidence suggests that EMPs not only constitute an emerging marker of endothelial injury, but can also be considered as important mediators capable of regulating a variety of biological functions, including inflammation, coagulation, angiogenesis and thrombosis [8, 9]. Thus EMPs appear to serve as both markers and hazards in vascular pathology [10–14].

Mounting evidence suggests that plasminogen activator inhibitor-1 [15], tumor necrosis factor α [16] and thrombin [17] are potent agonists for membrane blebbing and MP release [18–20]. Bubble formation can occur in vasculature and tissue as a result of rapid reduction in ambient pressure [21]. Besides, it can occur when gas enters into the vasculature during surgical procedures and catheterization operations, which likely contributes to the incidence of cognitive deficit [22]. In the vasculature, bubbles can exert pleiotropic detrimental effects, such as influencing hemodynamics, altering vasomotion and facilitating coagulation [23, 24]. The vascular sequelae of bubbles include the activation of platelet and other blood cells, leading to MPs formation and subsequent endothelial injury [25–27]. Bubbles may contact with the endothelium, leading to endothelial activation and/or dysfunction and subsequently EMPs formation. Entering into the circulation, EMPs may promote coagulation, scavenge nitric oxide, directly produce reactive oxygen species, and regulate extracellular matrix turnover. Additionally, EMPs may physically interact with the surface of a target cell or fuse with a target cell and transfer the contents [28, 29].

Given that bubbles act as a causal factor in membrane blebbing and EMPs formation, little is known about the underlying mechanisms. Elucidation of the molecular mechanisms will bring new insights into understanding bubble-induced endothelial injury. And more importantly, it can provide potential therapeutic applications for bubble related disorders based on the inhibition of EMP generation. To this end, an *in vitro* model was applied and the importance of calcium in bubble-induced EMPs formation was identified. Besides, activation of Rho kinase and inhibition of flippase activity were revealed participating in this process.

Materials and Methods

Endothelial microparticles generation and characterization

Human umbilical vein endothelial cells (HUVECs) were grown to confluence on 35 mm diameter dishes (Corning, USA) in a humidified 5% CO_2 incubator at 37 °C. Cells were cultured in endothelium cell basal medium (ECM, Science Cell) containing vascular endothelial growth factors and 2.5% fetal bovine serum (Gibco), supplemented with the antibiotics streptomycin (100 U/mL) and penicillin (100 U/mL). ECM was exchanged to CO_2 -independent medium (Gibco, USA) before bubble stimulus to sustain the pH. HUVECs were then subjected to 30 min of bubble contact as previously described and non-treated cells were used as controls. In brief, half of one milliliter nitrogen (N_2) was injected to CO_2 -independent medium and the device was put into a 37 °C shaking table so that bubbles were allowed to contact with the confluent cells [30]. Six hours after bubble stimulus, the cultural medium was collected and cleared of cells and cell debris by centrifugation at 1500 g for 15 min at 4°C. The supernatant was then centrifuged at 20500 g for 1 h at 4°C to pellet EMPs. EMPs pellet were resuspended in 1×Binding Buffer and the number of EMPs was analyzed as previously described.

Identification of Ca^{2+} in bubble-induced EMPs formation

To identify the importance of Ca^{2+} in bubble-induced EMPs generation, intracellular Ca^{2+} was measured by using the Ca^{2+} binding dyes Fluo-3 AM (Sigma). In brief, cells were loaded with Fluo-3 AM at 5 μM and

incubated for 30 min at 37°C in dark before bubble stimulus. Cells were washed twice with phosphate buffered saline and incubated for a further 30 minutes at 37°C. Subsequently, cells were subjected to the same stimulus. After that, cells were obtained and washed twice with 1×Binding Buffer and Fluo-3 AM signal was measured by flow cytometry. Fluorescence was measured with excitation at 488 nm and emission at 525 nm. Besides, intracellular Ca^{2+} was determined by fluorescent microscope (Leica). The Ca^{2+} channel blocker lanthanum chloride (LaCl_3 , Sigma) was applied to verify the importance of Ca^{2+} in bubble-induced EMPs formation. Cells were subjected to the same stimulus as mentioned before with LaCl_3 (100 μM) in the medium and the number of EMPs was analyzed.

Measurement of flippase activity and phosphatidylserine exposure

Flippase activity was measured as the rate of translocation of the fluorescent phosphatidylserine (PS) analogue NBD-PS, from the outer to the inner leaflet of the plasma membrane [31]. Cells were loaded with 0.5 μM NBD-PS and inward movement of NBD-PS was measured using the bovine serum albumin back-exchange. Aliquots from the HUVECs were removed at the indicated time intervals and placed on ice for 20 min in the presence of 1% BSA or not, respectively. Pellets were obtained and suspended in 1% Triton X-100 and the fluorescence intensities were measured (λ_{ex} 485 nm, λ_{em} 535 nm). The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after back-exchange.

Six hours after bubble stimulus, HUVECs were washed twice with the PBS and trypsinized to obtain cell suspension. Collected cells were resuspended in 1×Binding Buffer and stained with Annexin V FITC and PI dyes. Subsequently, cells were analyzed with flow cytometry (Beckman Coulter, USA) and Annexin V⁺/PI⁺ were regarded as the PS exposure.

Western Blotting

HUVECs were washed with cold PBS and scraped off in RIPA buffer (Sigma). The lysates were sonicated and protein content was quantified using BCA method. Equal amounts of proteins were separated by SDS-PAGE followed by western blotting. Membranes were blocked with 5% (w/v) non-fat milk and incubated at 4 °C for 12 h with human monoclonal primary antibodies against ERK, phospho-ERK, p38/MAPK, phospho-p38/MAPK, MLC, phospho-MLC or GAPDH (Cell Signaling Technology). Proteins were visualized by using HRP-conjugated goat anti-human IgG (Cell Signaling Technology) and the intensity of each band was measured by Image J software (National Institutes of Health, USA).

Immunofluorescent microscopy of actin co-localization

Cells were fixed in 4% paraformaldehyde for 10 minutes, washed twice with PBS. F-actin localization was assessed using phalloidin-FITC (Sigma) and DAPI (Sigma) by co-staining for polymerized actin. Fluorescent labeling was analyzed using a Leica fluorescent microscope. Actin localization was quantified by a score system for each image (0: no localization to 3: full localization) by three different blinded operators [32]. The experiment protocol was approved by the Ethics Committee of the Second Military Medical University and all of the procedures were carried out in compliance with related guidelines and regulations.

Statistical analysis

Data are shown as mean $\pm$ SD. The one way ANOVA test was applied in the case of multiple parameters. Student's t-test or Mann Whitney-U test was used to determine statistical significance between two comparable parameters. $P < 0.05$ was considered significant.

Results

Role of Ca^{2+} in regulating bubble-induced EMPs release

Both immunofluorescent microscopy assay and flow cytometric detection identified that fluorescence intensity of cytoplasmic Ca^{2+} markedly increased after bubble stimulus (Fig. 1A). Consistent with our previous study, EMPs level increased about three fold in the bubble stimulus group. Next, we examined whether Ca^{2+} participated in regulating bubble-induced EMPs formation. Bubble stimulated EMPs formation was diminished by LaCl_3 pre-

treatment (Fig. 1B) and the number of EMPs increased in accordance with intracellular Ca^{2+} elevation (Fig. 1C, $r=0.687$, $P<0.05$). All together, these results indicated that Ca^{2+} may play a critical role in regulating bubble-induced EMPs formation.

Flippase activity and phosphatidylserine exposure

To explore the putative mechanisms responsible for phosphatidylserine (PS) exposure governing MPs formation, flippase activity was examined, which is a critical regulator for PS translocation. It has been reported that flippase activity can be inhibited by Ca^{2+} and elevated Ca^{2+} could result in PS externalization [33]. As shown in Fig. 2A and 2B, bubble stimulus resulted in a rapid decrease of flippase activity and simultaneously an increase of PS exposure, which was diminished by LaCl_3 pretreatment. The correlation between PS exposure and flippase activity was analyzed and we observed that the increase of PS exposure

was accompanied by flippase activity decline (Fig. 2C, $r=-0.894$, $P<0.01$). Given that the decline of flippase activity can be diminished by LaCl_3 pretreatment, the correlation between Ca^{2+} and flippase activity was determined, that flippase activity decreased in accordance with intracellular Ca^{2+} augmentation (Fig. 2D, $r=-0.862$, $P<0.01$), which suggests that intracellular Ca^{2+} is involved in regulating flippase activity and PS exposure.

Actin location

Actin depolymerization at the plasma membrane is a key factor in circulating EMPs formation [34, 35]. Cytoplasmic Ca^{2+} is a regulator of actin cytoskeleton dynamics in B lymphocytes and the Rho/ROCK pathway participates in this process [36]. Bubble stimulus resulted in F-actin depolymerization at the plasma membrane, which can be restored by the LaCl_3 or the ROCK inhibitor Y27632 (Fig. 3A). Given that the effect of F-actin depolymerization can be inhibited by LaCl_3 , the correlation between Ca^{2+} and F-actin location was further determined. The amount of F-actin localization at the membrane decreased in accordance with cytoplasmic Ca^{2+} elevation (Fig. 3B, $r=-0.728$, $P<0.05$).

Pathways involved in bubble-induced EMPs formation

Different mechanisms have been demonstrated in regulating EMPs formation according to different agonists. ROCK has been identified as an important signal molecule in cytoskeleton

Fig. 1. Role of Ca^{2+} in bubble-induced EMPs formation. A, Representative images of cytoplasmic Ca^{2+} by immunofluorescent microscopy and mean fluorescence intensity by flow cytometry. B, Flow cytometric detection of EMPs number in different groups. * $P<0.05$ vs. normal control groups, # $P<0.05$ vs. bubble stimulus groups, $n=4$. C, Fold changes of Ca^{2+} and EMPs number vs. normal control groups, $r=0.687$, $n=4$.

Fig. 2. Changes of flippase activity and PS exposure after bubble stimulus. A, Flippase activity assay: Non-extractable NBD-PS (%) represented internalized NBD-PS, which indicated the flippase activity. B, Flow cytometric detection of PS exposure in different groups. * $P < 0.05$ vs. normal control groups, # $P < 0.05$ vs. bubble stimulus groups, $n = 4$. C, Fold changes of flippase activity and PS exposure vs. normal control groups, $n = 4$. D, Fold changes of Ca^{2+} and flippase activity vs. normal control groups, $n = 4$.

organization and EMPs formation, which can be activated by Ca^{2+} [37]. The contributions of MAPK pathways have also been identified in different pathophysiological conditions [38, 39]. In this regard, we examined the potential role of ROCK and MAPK signals in bubble-induced EMPs formation. As shown in Fig. 4A, phospho-MLC (a ROCK substrate) significantly increased from 2 h to 4 h and phospho-ERK1/2 significantly increased from 0 h to 2 h after bubble stimulus, but not phospho-p38/MAPK (results not shown). Given that phospho-MLC and phospho-ERK1/2 expressions have time lag, we examined whether these molecules crosstalked during bubble-induced EMPs generation. The ROCK inhibitor Y27632 and the ERK1/2 inhibitor PD98059 reduced only the levels of phospho-MLC and phospho-ERK1/2, respectively. Interestingly, the level of phospho-MLC was significantly suppressed by $LaCl_3$ pretreatment, which suggested that intracellular Ca^{2+} elevation was involved in ROCK regulation (Fig. 4B). Finally, the ROCK inhibitor Y27632, the ERK1/2 inhibitor PD98059 and Ca^{2+} channel blocker $LaCl_3$ were applied to determine their effects in bubble-induced EMPs formation. As demonstrated in Fig. 5, the number of EMPs markedly decreased by the Y27632 or $LaCl_3$ pretreatment, but not PD98059 pretreatment, which further evidenced the critical role of Ca^{2+} and ROCK in regulating bubble-induced EMPs formation.

Discussion

The presented results demonstrate that bubble stimulus acts as an important trigger factor in EMPs formation and cytoplasmic Ca^{2+} elevation serves as an initiator in this process and facilitates EMPs formation by flippase inactivation and actin reorganization through the Rho/ROCK pathway.

Fig. 3. Role of actin colocalization in bubble-induced EMPs formation. A, Score of actin colocalization. HUVECs pretreated with or without $LaCl_3$ or Y27632 were subjected to bubble stimulus or not. Actin localization was determined by fluorescence microscope. * $P < 0.05$ vs. normal control groups, # $P < 0.05$ vs. bubble stimulus groups, $n = 4$. B, Fold changes of Ca^{2+} and actin localization vs. normal control groups, $r = -0.728$, $n = 4$.

Fig. 4. Intracellular mechanisms involved in bubble-induced EMPs formation. A, Representative blot of total ERK1/2 and MLC, ERK1/2 and MLC phosphorylation at different time points after bubble stimulus. * $P < 0.05$ vs. normal control groups, $n = 3$. B, ERK1/2 (0 h) and MLC (2 h) phosphorylation under normal or bubble stimulus groups treated with or without PD98059, Y27632, or LaCl_3 , normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). * $P < 0.05$ vs. normal control groups, # $P < 0.05$ vs. bubble stimulus groups, $n = 3$.

The endothelium operates a crucial role in maintaining cardiovascular homeostasis by secreting endothelium-derived relaxing and contracting factors [40-42]. As a "target organ" of intravascular bubbles, the endothelium receives considerable attention in decompression sickness and other bubble related vascular disease [43, 44]. The endothelium plays a key role in response to bubble contact, which convert it from inactivated to activated condition, resulting in endothelial dysfunction and mediating deleterious effects upon the surrounding tissues. Previous studies demonstrated that bubble contact promoted MPs formation, which further facilitated endothelial dysfunction [25-27, 30]. An illustration of the mechanisms involved in bubble-induced EMPs formation is meaningful because it may provide an innovative perspective for preventing adverse effects of bubble-induced EMPs.

Ca^{2+} is regarded as an intrinsic pathogenic factor in many vascular diseases and has been observed to contribute to bubble-induced injury [23, 45]. Ca^{2+} influx and subsequent activation of Ca^{2+} -dependent protease calpain stimulated platelet MPs formation and release [46-48]. In contrast, the inhibition of Ca^{2+} influx abolishes agonist-induced PS externalization and MPs formation. As a hazard and marker after endothelial injury, we speculated that cytoplasmic Ca^{2+} elevation may engage in bubble-induced EMPs formation. The number of EMPs significantly increased in accordance with cytoplasmic Ca^{2+} elevation and this effect can be diminished by Ca^{2+} channel blocker (LaCl_3) pretreatment, indicating the pivotal role of intracellular Ca^{2+} in modulating bubble-induced EMPs formation.

Under the physiological conditions, cell membrane phospholipids are asymmetrically

Fig. 5. Effects of different inhibitors on bubble-induced EMPs formation. * $P < 0.05$ vs. normal control groups, # $P < 0.05$ vs. bubble stimulus groups, $n = 4$.

distributed; the choline-containing phospholipids and sphingolipids are enriched on the outer surface, while the amine-containing phospholipids are sequestered on the inner leaflet of the plasma membrane [49]. This distribution is the result of an active process dependent upon phospholipid transporters governing either the inward or the outward translocation by flippase or floppase. Flippase is highly selective for PS translocation and keeping this lipid sequestered from the cell surface [4]. It is generally assumed that MPs form when asymmetrical distribution is lost and PS externalization is a common underlying feature of MP release [33, 50, 51]. Significant differences in flippase activity were observed; low surface PS exposure of the normal groups was generally associated with high flippase activity and high surface PS exposure of bubble stimulus groups was accompanied by low flippase activity. The same relationship applied to the number of EMPs. These results reinforce the importance of flippase activity in bubble-induced EMPs formation.

Given that both Ca^{2+} and flippase activity was associated with MPs formation, their relationship was further analyzed [31, 52-56]. A negative correlation between Ca^{2+} and flippase activity was observed. Low cytoplasmic Ca^{2+} of the normal control groups was generally associated with high flippase activity, whereas high cytoplasmic Ca^{2+} of bubble stimulus groups was accompanied by the low flippase activity. This effect was significantly attenuated by LaCl_3 pretreatment. Together, our results demonstrate that elevation of PS exposure during bubble-induced EMPs formation is likely regulated by Ca^{2+} dependent decline in flippase activity.

Different signaling pathways have been reported in regulating EMP formation, depending on the agonist initiating membrane vesiculation. For example, p38 mitogen-activated protein kinase (MAPK) was identified as a critical molecule during tumor necrosis factor α induced EMPs formation [38], whereas thrombin or angiotensin II-dependent EMP generation was controlled by the Rho-ROCK pathway [28, 57]. Few studies referred to the molecular mechanisms of EMPs formation induced by mechanical stimulus and it still remains uncertain whether intracellular signaling participates in bubble-induced EMP generation. However, there is no question that MPs formation and shedding necessitate modifications in cell structure involving cytoskeleton rearrangement [58]. Vesiculation formation depends on actin cytoskeleton and actin-myosin contraction, which is regulated by caspase-3-induced ROCK I and II activation [34, 35]. In this study, LaCl_3 pretreatment can reverse actin depolymerization facilitated by bubbles, which suggested that Ca^{2+} was involved in actin reorganization. ERK1/2, p38/MAPK and ROCK are common signal molecules involved in cytoskeleton organization and MPs generation. To evaluate whether these signals participated in regulating bubble-induced EMPs formation, we examined the levels of phospho-ERK, phospho-p38/MAPK and phospho-MLC (a ROCK substrate). In agreement with a previous study, the levels of phospho-ERK and phospho-MLC significantly increased after bubble stimulus. The level of phospho-MLC, but not phospho-ERK1/2, could be significantly eliminated by LaCl_3 pretreatment, indicating that the Ca^{2+} may involve in regulating ROCK. In order to obtain explicit evidence of these molecules in bubble-induced EMPs formation, each of their inhibitors was applied. The contribution of ROCK in modulating bubble-induced EMPs formation was evidenced by the increase of actin colocalization and the decrease of EMPs number by ROCK inhibitor Y27632 pretreatment. In this regard, bubble-mediated cytoplasmic Ca^{2+} elevation may promote EMPs formation through activating the Rho/ROCK signal pathway and subsequent actin rearrangement.

Conclusion

This study identified Ca^{2+} as a major determinant in bubble-mediated EMPs formation. Cytoplasmic Ca^{2+} elevation promoted EMPs generation by down-regulating flippase activity, activating the Rho-ROCK pathway and cytoskeleton reorganization. This provides a potential therapy for bubble related disorders by precisely inhibiting EMPs generation through intracellular Ca^{2+} reduction.

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Disclosure Statement

No conflict of interests exists.

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