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● *Original Contribution*

ENHANCED CAVEOLAE-MEDIATED ENDOCYTOSIS BY DIAGNOSTIC ULTRASOUND *IN VITRO*

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Abtract—The modulation of cellular endothelial permeability is a desirable goal for targeted delivery of labels and therapeutic macromolecules; the underlying mechanisms, however, remains poorly understood. Here, we hypothesize that a higher endothelial permeability may result as an outcome of selective enhancement of caveolar endocytosis by ultrasound (US), in the frequency and intensity range of current clinical diagnostic use. To assess the role of free radicals in this phenomenon, we exposed confluent human endothelial cells to pulsed diagnostic US for 30 min, with a mechanical index (MI) of 0.5 and 1.2, using a 1.6-MHz cardiac US scan, and endothelial cells not exposed to US were used as control. Here we show that pulsed diagnostic US with a MI of 1.2 (high mechanical index ultrasound [HMIUS]) were able to selectively enhance endothelial caveolar internalization of recombinant glutathione-S-transferase (GST)-Tat11-EGFP fusion protein (26 ± 1 *vs.* 11.6 ± 1 A.U, $p < 0.001$ *vs.* control), without disruption of plasma membrane integrity. Moreover, pulsed diagnostic US with a MI of 0.5 (low mechanical index ultrasound) did not increase caveolar endocytosis compared with control (11.4 ± 1.2 *vs.* 11.6 ± 1). Free-radical generation inhibitors, such as catalase and superoxide dismutase, reduced the HMIUS-induced caveolar internalization by a 49.29% factor; finally, HMIUS-induced caveolar endocytosis was found to be associated with a significant increase in the phosphorylation of tyr-14-caveolin1, ser1177-eNOS and Thr202/Tyr204-ERK½ compared with control. These findings show how HMIUS irradiation of human endothelial cells cause a selective enhancement of caveolar-dependent permeability, partially mediated by free radicals generation, inducing a marked increase of phosphorylation of caveolar-related proteins. Thus, the use of diagnostic US could potentially be used as an adjuvant to drive caveolar traffic of extracellular peptides by using a higher level of US energy. (E-mail: enzolionetti@hotmail.com)

Key Words: **Caveolae, Endothelial cells, Endocytosis, Ultrasound, Tat11, Free radicals.**

INTRODUCTION

Human endothelial cells use several endocytic pathways to internalize a large variety of molecules, the most prominent pathways being clathrin-mediated endocytosis and caveolar internalization/endocytosis (Le Roy et al. 2005). In vascular endothelial cells, caveolae are a highly-expressed subset of lipid raft domains (Predescu and Palade 1993) exploiting multiple functions ranging from serving as endocytic carriers (Pelkmans et al. 2004) to organizing and regulating signalling cascades (Minshall et al. 2003). Furthermore, caveolae transcytosis plays an important role in the continuous exchange of molecules across the endothelium (Predescu et al. 2007); caveolin-1 is the major caveolar protein required for the integrity of such caveolae functions (Liu et al. 2002).

Specific physical or chemical stimuli, such as mechanical forces or free radicals, can affect endocytic pathways (Langer 1998) and membrane permeability and if not properly modulated, can limit therapeutic strategies based on targeted delivery of peptides, DNA and other biotherapeutic compounds across cell membranes. The issues regarding plasma

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membrane permeability to drugs, peptides and DNA in mammalian cells have been addressed through the use of diagnostic ultrasound (US) (Mukherjee et al.2000). Despite these proofs of principle that diagnostic US can enhance some therapies by modulating drug internalization in cells, the progress on this topic has been limited by an insufficient understanding of US mechanisms of action (Andreassi et al. 2007). Most likely, during US exposure several biological effects are mediated by intracellular reactive oxygen species generation (Basta et al. 2003), which subsequent formation to US treatment is strongly dependent on threshold acoustic pressure at specific frequencies (Riesz and Kondo 1992). Therefore, we wanted to explore and assess the role of US, in the frequency and intensity range of current clinical diagnostic use, in modulating cellular functions of key biological relevance, such as vesicular endocytosis in endothelial cells.

So far, no study has been carried out to separate chemical effects (*i.e.*, free radicals generation) from mechanical effects on membrane permeability induced by US in endothelial cells.

In our study, we hypothesized that US irradiation in pulsed mode, in the frequency and intensity range of current clinical diagnostic use, could potentially be used for regulation of the endothelial permeability through enhancement of caveolae-dependent endocytic pathways at different US energy intensity levels.

One of the major aims of the present study was to evaluate the *in vitro* effects of transient US exposure (30 min) at different MI on a caveolae-dependent endocytic pathway, in a monolayer of endothelial cells treated with inhibitors of free radical generation. To achieve this goal, antioxidant catalase and superoxide dismutase (SOD) were used during low mechanical index ultrasound (LMIUS) and/or high mechanical index ultrasound (HMIUS) in the presence of specific probes for cellular permeability.

Although the levels of protein internalization in cells treated with LMIUS were comparable with those observed in untreated cells, HMIUS significantly increased cellular permeability through a caveolae-dependent pathway. The use of free radicals (ROS) generation inhibitors on cells treated with HMIUS reduced the caveolae-dependent internalization of our protein probe of a 49.29% factor; nonetheless, the levels of protein delivery remained significantly higher than those detected in cells not treated with HMIUS, thus proving that HMIUS effectively stimulates caveolae-mediated endocytosis in endothelial cells, with a mechanism only partially related to free radicals generation.

MATERIALS AND METHODS

Diagnostic US irradiation

Pulsed US irradiation was performed with an echocardiograph (MyLab 30, Esaote, Genoa, Italy) equipped with a transducer emitting at 1.6 MHz, with a mechanical index (MI) of 0.5 or 1.2. The parameters of the US equipment were set up by an expert operator. The tip of the transducer was carefully positioned at 5 cm from the cell surface, corresponding to the focal distance of the transducer in the z axis (Fig. 1a). The MI indicates the potential for mechanical bioeffects and is the default display with 2-D/B-mode imaging. In these conditions, the duty cycle and the pulse repetition frequency were equal to 2.9% and 15.4KHz, respectively. The MI is related to the peak rarefactional pressure, which in turn is related to the intensity that it is always below the 720 mW/cm^2 threshold set by Food and Drug Administration restrictions for human diagnostic devices (Feigenbaum 2004). In our experiments, the *in situ* MI was calculated using acoustic pressure measurements in the free field using a polyvinylidene fluoride membrane ultrasonic hydrophone (0.5 mm nominal diameter, S/N EW304, GEC-Marconi, Chelmsford, UK). The peak negative pressure at the position of the cell surface was in the range of 0.6–1.515 MPa, which corresponds to a MI of 0.47–1.197. We cannot, however, definitively exclude the possibility of interferences because of ultrasonic waves reflection from the solid-fluid interface of culture dish.

Cell culture and viability

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in degassed Medium 199 (Life Sciences, Grand Island, NY, USA) supplemented with 20% of heat-inactivated fetal bovine serum, heparin (50 UI/mL), growth factors (endothelial cell growth factor, 50 μg/mL) and antibiotics (200 U/mL penicillin, 100 mg/mL streptomycin) and then incubated at 37° C in a humidified atmosphere with 5% CO_2. Cultured endothelial cells were characterized as endothelium as described previously (Basta et al. 2003). Temperature was measured using a copper–constantin thermocouple and recorded with a laboratory thermometer (Pouiley Instruments, Saddle Brook, NJ, USA) sensitive to temperature changes of 0.1° C. The nonadherent cells were removed by replacing the medium. Once grown to confluence, cells were exposed to US. Cell viability was assessed by exclusion of 0.2% Trypan blue solution (Medzihradsky and Marks 1975). Human cells were obtained after informed consent was given, in accordance with the policy approved by the local ethical committee.

Fluorescent probes

Enhanced green fluorescent protein (EGFP) or tetramethylrhodamine isothiocyanate (TRITC) were used

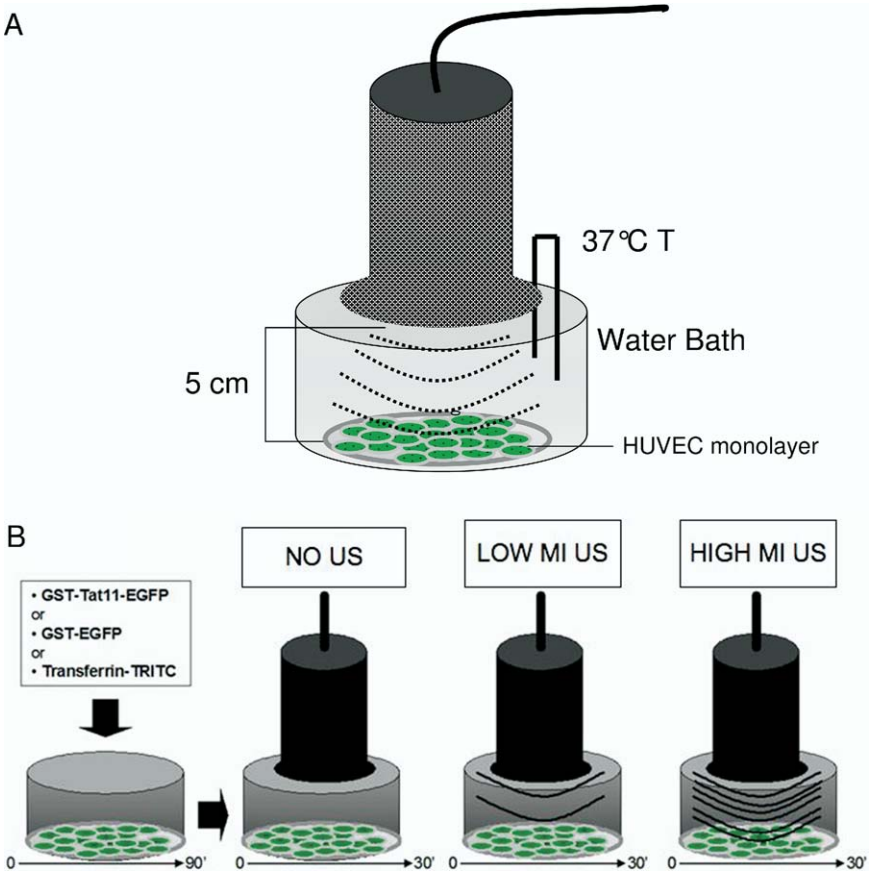

Fig. 1. Figure of the experimental setup (a) and protocol (b).

as fluorescent molecular tags for specific protein probes of cellular permeability: (i) glutathione-S-transferase (GST)-Tat11-EGFP, as a probe for caveolae-mediated endocytosis (Fittipaldi et al. 2003); (ii) transferrin-TRITC, as a probe for clathrin-mediated endocytosis (Fittipaldi et al. 2003); and (iii) GST-EGFP, as a probe for plasmalemmal integrity (Niswender et al. 1995). A plasmid expressing the GST-Tat11-EGFP fusion protein was obtained by replacing the Tat86-coding region in the GST-Tat86-EGFP plasmid (Tyagi et al. 2001), with the sequence encoding for amino acids $_{47}$YGRKKRRQRRR$_{57}$ of the basic domain of Tat86, the transactivator protein of HXB2 strain immunodeficiency virus type 1.

Experimental design

Confluent HUVEC were incubated in 60-mm Petri dishes at 37° C for 90 min with fresh degassed M199 medium serum free containing either (GST)-Tat11-EGFP (2 μg/mL) or (GST)-EGFP (2 μg/mL) or transferrin-TRITC (10 μg/mL) and were then exposed to one of two different US irradiation for 30 min at a constant temperature of 37° C (Fittipaldi et al. 2003): (i) low mechanical index US (LMIUS, MI = 0.5) or (ii) high mechanical index US (HMIUS, MI = 1.2) (Fig. 1b). As a control, we used cells not stimulated for the same period of time and in the same experimental conditions. Cells were collected and immediately subjected to fluorescence-activated cell sorting (FACS) analysis at the end of US irradiation. In a second set of experiments, we wanted to suppress the effects on endothelial permeability mediated by free radicals; for this purpose, human endothelial cells were exposed to one of two different US irradiation for 30' in the presence of antioxidants as catalase (Sigma-Aldrich, Milan, Italy) (1000 U/mL) (Basta et al. 2003) and SOD (Sigma-Aldrich) (200U/mL) (Dreher and Junod 1995) added in the extracellular medium.

Flow cytometry

Quantification of internalized recombinant fluorescent proteins was performed as already described (Fittipaldi et al. 2003). Briefly, to assess the effects of diagnostic US on endothelial permeability, HUVEC were plated in 6-well plates to about 60% confluence and incubated with fluorescent probes for the points indicated

in the experimental protocol. Cells were then washed twice with sterile phosphate-buffered saline (PBS), trypsinized, again washed with PBS, washed with 2 M NaCl to completely take off surface-bound, noninternalized proteins, again washed twice with PBS and, finally, analyzed by flow cytometry (FACScan™ Flow Cytometer, BD Biosciences, Milan, Italy) by collecting 10,000 events. The cells were washed before flow cytometry to remove fluorescent noninternalized probes.

Fluorescence and confocal microscopy

To assess localization of caveolae, confluent HUVEC grown on chamber slides (Labtech International, Woodside, UK) from different experimental conditions as described previously were analyzed by confocal microscopy using a laser-scanning microscope (Leica TCS-SP, Leica Microsystems S.p.A, Milan, Italy) (Fittipaldi et al. 2003). Confocal fluorescent images were acquired digitally from five random fields for each sample.

Western blotting

A total protein count was extracted from HUVEC cellular fraction and Western blot analysis was performed using standard laboratory procedures. The following primary antibodies were used in this study: rabbit polyclonal antibodies against phospho-Ser1177-eNOS, phosphoThr202/Tyr204 extracellular-related kinase ½ (ERK½) and phospho-Tyr14-Cav1 (1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA); mouse monoclonal antibody against eNOS (1:1000 dilution, BD, Franklin Lakes, NJ, USA); rabbit polyclonal antibody against ERK½ (1:1000 dilution, Santa Cruz Biotechnology Inc., CA, USA); and rabbit polyclonal against caveolin-1 (1:500 dilution, Transduction Laboratories, San Diego, CA, USA). The identities of the bands visualized in the Western blots were confirmed by comparison with molecular mass standards.

Statistical analysis

Data are presented as mean ± SD. Statistical significance was tested by analysis of variance and p-values were obtained by Bonferroni's test. Statistical significance was established at a value of $p < 0.05$.

RESULTS

Diagnostic US enhances caveolae-mediated endocytosis

Endothelial caveolae-mediated endocytosis, assessed as internalization of extracellular GST-Tat11-EFGP, is shown in Fig. 2a. Time control (30' of no US exposure) did not result in any change compared with the baseline measurements made after the preincubation period.

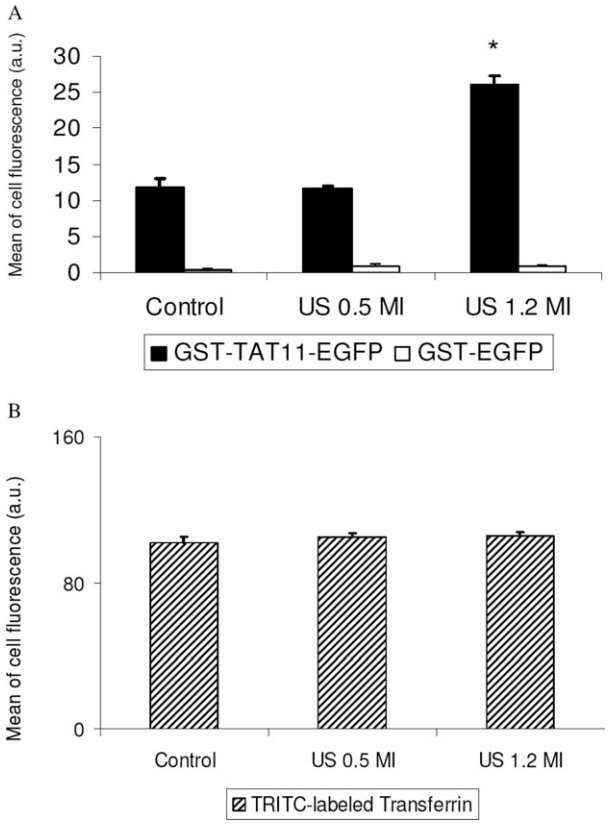

Fig. 2. Transient high-stress pulsed US increased endothelial caveolae-mediated endocytosis, but not clathrin-dependent endocytosis. Endothelial internalization of (GST)-Tat11-EGFP and (GST)-EGFP (a) or extracellular TRITC-labeled transferrin (b) at control (transducer off) and at 30 min after US irradiation (LMIUS 0.5 or HMIUS 1.2 MI). All measurement are mean ± SD, n = 5. *$p < 0.001$ *vs.* control and LSPUS.

After 30 min of HMIUS, transport of GST-Tat11-EFGP had significantly increased (by more than twofold) compared with LMIUS, as confirmed by green fluorescence localization inside the cytoplasm (Fig. 3a); there was no statistically significant difference between LMIUS and control. Diagnostic US irradiation did not produce alterations of plasmalemmal integrity, as shown in Figs. 2a and 3b, as assessed by the lack of fluorescence in the cytoplasm of cells incubated with recombinant GST-EGFP, and did not reduce cellular viability (Trypan blue exclusion was >95% in all experiments).

Antioxidants partially reduce caveolae-mediated endocytosis

To directly assess the role of ROS generation on the US-mediated effect on caveolar internalization, we exposed HUVEC to HMIUS in the presence of antioxidants. As shown in Fig. 4, the GST-Tat11-EFGP intracellular delivery during HMIUS exposure was significantly reduced by catalase and SOD treatment compared

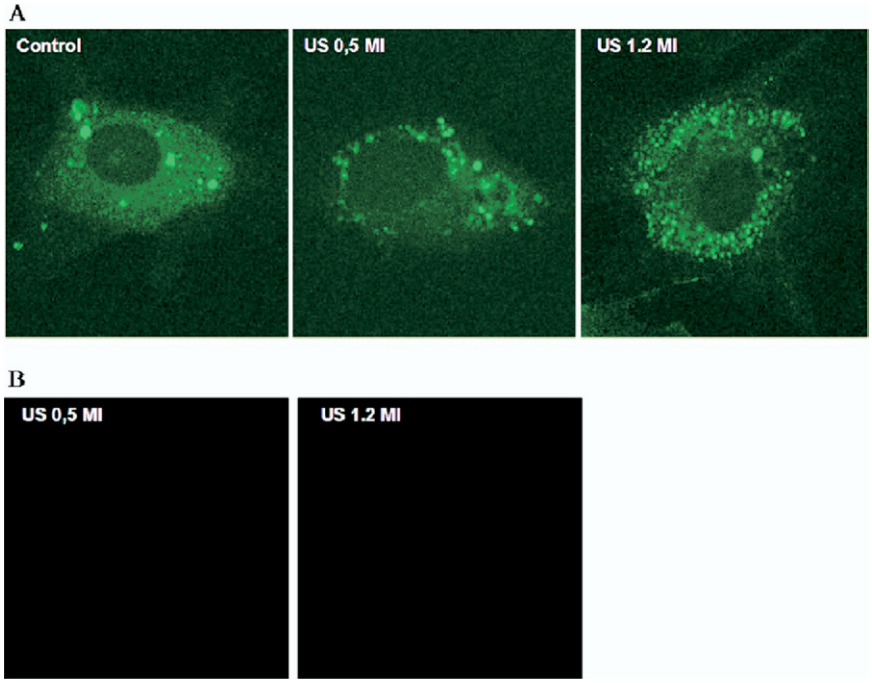

Fig. 3. Transient high-stress pulsed US irradiation produced an intracitoplasmatic localization of Tat11-EGFP without alterations of plasmalemmal integrity. Fluorescence analysis by confocal microscopy of (GST)-Tat11-EGFP (green) (a) and GST-EGFP intracellular localization in fixed HUVEC (b). Objective Leica HCX PL APO CS 63.0x 1.40 Oil (Pinhole [A.U] 1.00 ary).

with nontreated cells (18.2 ± 1.02 *vs.* 26 ± 1.1); nonetheless it was still significantly higher than the delivery level observed in control—non-HMIUS–treated—cells (18.2 ± 1.02 *vs.* 11 ± 0.9, $p < 0.05$).

Diagnostic US does not affect clathrin-mediated endocytosis

Figure 2b shows the clathrin-mediated endocytosis assessed as internalization of extracellular TRITC-labeled transferrin. Analysis of the mean cellular fluorescence values by flow cytometry shows that internalization of TRITC- labeled transferrin was 102 ± 2.44 A.U in control cells and did not change significantly after exposure to low (105 ± 1.63 A.U) or HMIUS (105.66 ± 1.69 A.U).

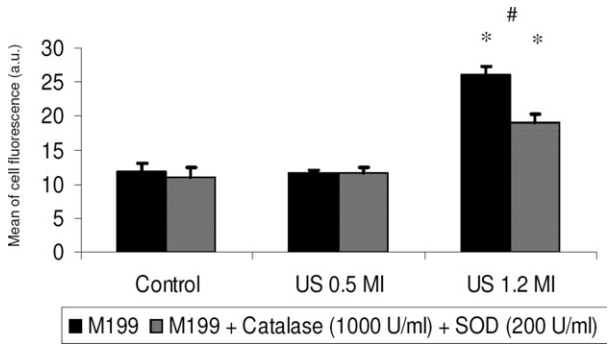

Fig. 4. Catalase and SOD reduce endothelial internalization of Tat11-EGFP. Endothelial internalization of (GST)-Tat11-EGFP in the presence or absence of free radicals scavengers. All measurements are mean ± SD, $n = 5$. *$p < 0.05$ *vs.* control counterpart. #$p < 0.05$ *vs.* absence of antioxidants.

Diagnostic US–induced phosphorylation of Cav-1, eNOS and ERK½

It is known that caveolae-mediated intracellular transport in endothelial cells is functionally coupled to eNOS activation by phosphorylation of caveolin-1 (Maniatis et al. 2006). The densitometric analysis of the discrete Tyr14-Cav1, Ser1177-eNOS and Thr202/Tyr204-ERK½ phosphorylated bands (Fig. 5) showed that HMIUS produced, respectively, a 14, 15 and 12-fold increase over baseline (no US) ($p < 0.05$ *vs.* no US). The LMIUS exposure did not produce any significant phosphorylation of the following proteins compared with control: (i) p-Cav1/Cav1 (0.1 ± 0.01 *vs.* 0.08 ± 0.01); (ii) p-eNOS/eNOS (0.08 ± 0.01 *vs.* 0.02 ± 0.01); and (iii) p-ERK½ /ERK½ (0.09 ± 0.01 *vs.* 0.07 ± 0.01).

DISCUSSION/SUMMARY

Our results demonstrate that transient exposure of confluent HUVEC to diagnostic HMIUS increases endo-

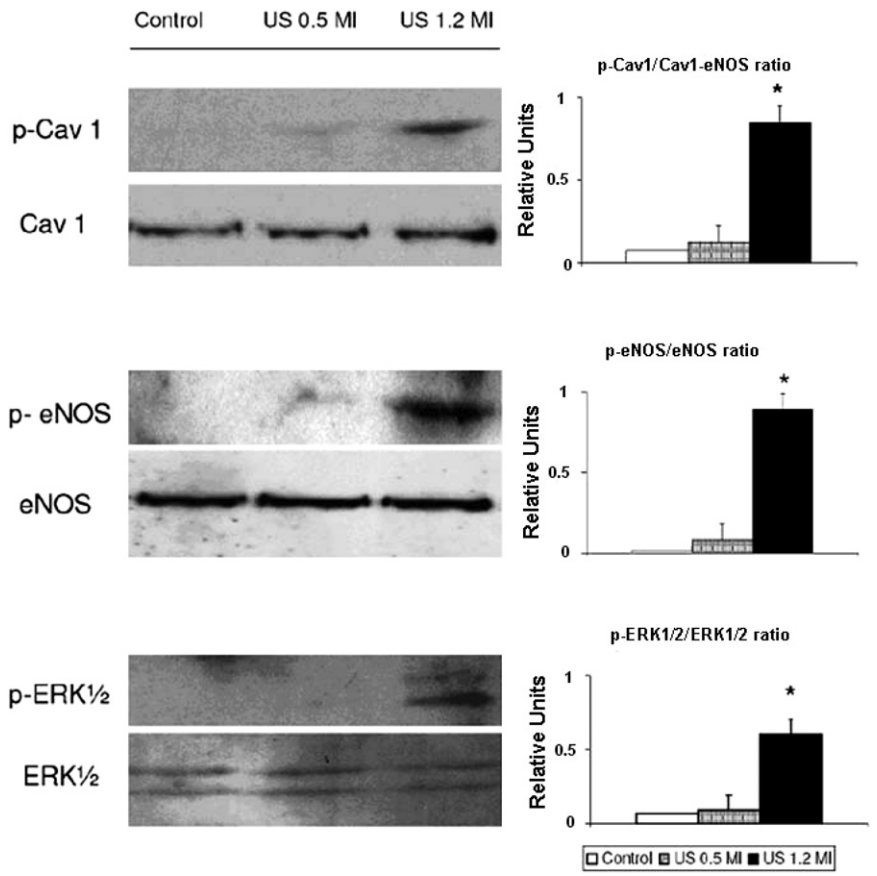

Fig. 5. Transient high-stress pulsed US irradiation increased phosphorylation of Cav-1, eNOS and ERK½. Cav-1, eNOS and ERK½ phosphorylation in HUVEC at control and at 30′ after US irradiation (LMIUS 0.5 or HMIUS 1.2 MI). The intensity of the bands from three independent experiments was measured and relative intensity was calculated. Level of phospho-Cav-1/Cav-1 or phospho-eNOS/eNOS or phospho- ERK½ / ERK½ is shown as an arbitrary unit. All measurements are mean ± SD, $n = 3$. *$p < 0.01$ *vs.* control and LSPUS.

thelial permeability through a selective enhancement of caveolae-dependent endocytosis while leaving clathrin-dependent endocytosis unaltered. This effect is partially mediated by ROS and does not involve alterations in cell viability and membrane integrity, and also results in activation of caveolin-1, eNOS and ERK½ by phosphorylation, with a rapid on–off response time. Our *in vitro* results further suggest that the combination of Trojan peptide Tat11 and diagnostic pulsed US exposure could potentially be used for an efficient and selective noninvasive approach to deliver complex therapeutic molecules into human endothelial cells.

Previous studies have provided several conflicting hypotheses on the mechanisms underlying the enhancement of endothelial permeability by US treatment (Andreassi et al. 2007). It is still unknown whether diagnostic US at different MI could modulate endothelial permeability *via* selective mechanisms such as caveolar trafficking.

Our findings show that transient pulsed US irradiation significantly enhances the rate of internalization of GST-Tat11-EGFP at a higher level of MI, as demonstrated by the cytoplasmic distribution of Tat11-containing caveolae (Fig. 3a). This conclusion is supported by our previous work demonstrating how Tat11-containing caveolae at an earlier time point (30 min) are still close to the plasma membrane of cells not exposed to mechanical forces, temperature variations or ROS overload (Fittipaldi et al. 2003).

To further corroborate these findings, we pretreated HUVEC cells for 30' with 5mM methyl-β-cyclodextrin, a drug that disrupts lipid rafts by extracting cholesterol from the phospholipidic bilayer (Ferraro 2004; Fittipaldi 2003). As expected, after 30' of HMIUS exposure in cells treated with this drug, internalization of Tat11–GFP was not evident (<1.0 A.U).

The mechanisms driving US-induced caveolar activation are still unclear. One of the most important prod-

ucts of US exposure are the ROS and, accordingly in a previous study, we found that diagnostic-pulsed US increases intracellular oxidative stress on HUVEC *in vitro* in a time and dose-dependent manner (Basta et al. 2003). Parat et al. (2002) showed that a ROS overload induced caveolin-1 phosphorylation in HUVEC, while inhibiting the trafficking of newly synthesized caveolin-1 to membrane raft domains. Because in our study, US exposure resulted in an increase of caveolae-mediated endocytosis, we could hypothesize that ultrasonic waves may increase the rate of internalization of preformed caveolae through caveolin-1 phosphorylation.

In accordance with previous studies (Johns 2002; Naota and Koori 2005), it is possible that several different US-generated mechanical forces could combine to produce effects that cause signifcant perturbations in cells' membrane and molecular structures. Based on our experimental approach, however, we were not able to discern among the types of mechanical forces involved (*i.e.*, mechanical energy and/or shear forces).

To better understand the effect of US exposure on caveolar traffic, we first wanted to rule out the possible effect on caveolar internalization of ROS production induced by HMIUS. The presence of antioxidants in the culture medium, in the same experimental conditions, significantly reduced the GST-Tat11-EFGP delivery during HMIUS irradiation—by a 49.29% factor (Fig. 4). Thus, caveolar trafficking activation in HUVEC exposed to HMIUS may be mediated by a ROS-dependent mechanism likely triggered by cell surface shearing. Because protein delivery remained significantly higher than the one observed in cells not treated with HMIUS (Fig. 4), the rapid US-induced transient permeability cannot be easily explained through a selective ROS-dependent mechanism alone.

As demonstrated by the Trypan blue exclusion and the lack of GST-GFP internalization, the enhancement in protein uptake induced by exposure to HMIUS occurred in the absence of endothelial cell damage. Thus, the mechanical bioeffects seem to not be related to the occurrence of acoustic cavitation, a well known source of extracellular ROS production (Honda et al. 2004). The absence of microbubbles and dissolved gases in the medium further corroborate this finding, even if we could not entirely rule out a contribution of undetected inertial cavitation to the observed effects.

External mechanical stimuli can enhance membrane endocytosis in endothelial cells (Apodaca 2002), and it was proposed that phosphorylation could mediate the rapid intracellular signal transduction triggered by mechanical forces. A very recent study has shown direct evidence for the role of caveolin-1, the main structural protein of caveolae, as a sensor for external mechanical forces and as a signal transducer in endothelial cells (Yu et al. 2006). External mechanical forces can be transmitted along the cytoskeleton, and the interaction between cytoskeletal-associated proteins and enzymes related to signal transduction (*i.e.*, kinases) may convert physical forces into biochemical reactions (Han et al. 2004), such as ROS generation. This is a strikingly relevant feature in the context of mitochondria, because these organelles constitute the main source of ROS in the endothelial cells (Zhang and Gutterman 2007), also during US exposure (Honda et al. 2004). The enhancement of caveolar membrane trafficking induced by HMIUS might depend on membrane and cytoskeleton deformation (Mundy et al. 2002), and accordingly it is well established that the ultrasonic wave impact generates a cytoskeleton deformation (Statnikov et al. 2006). However, we could not clearly define the nature of such alteration.

In a previous study, acoustic pulsed energy generated during low-intensity pulsed US selectively induced, in human cells, a transient cell activation by phosphorylation of intracellular kinases, such as ERK½ (Zhou et al. 2004). In our study, HMIUS irradiation induced a marked phosphorylation of Thr202/Tyr204-ERK½, tyr14-caveolin1 and ser1177-eNOS (Fig. 5), whereas ERK½ is a well-known mechanical stress-dose–dependent protein kinase (Azuma et al. 2000), phosphorylation of caveolin-1 and eNOS on the aforementioned residues was found to be activated in a novel pathway started by caveolae internalization (Maniatis et al. 2006). Because the phosphorylation was very low or not detectable after LMIUS irradiation, we hypothesize that in accord to previous findings (Azuma 2000; Hsu 2004), the thresholds of activation by mechanical stress might be a fundamental property of the model system. Finally, we suggest that US may prime endothelial permeability through activation of caveolar membrane trafficking, with a mechanism partially relying on free radicals.

Our findings on US-dependent protein delivery are consistent with recent *in vivo* observations on enhanced endothelial delivery of extracellular biocompounds (Stieger 2007; Taniyama 2002) and nitric oxyde synthase activity (Bertuglia et al. 2004) induced by low-intensity US. However, *in vivo* attenuation phenomena must be taken into account, and longer periods of imaging with higher MI (as high as 1.9) and continuous (Doppler) mode should be studied. Our study, however, could provide an important basis on which to develop innovative selective therapeutical strategies, mediated by diagnostic pulsed US for targeted endothelial delivery of hydrophilic drugs (*i.e.*, insulin) and labels through a lipohilic endocytotic pathway (lipid rafts or caveolae). Recently, it has been demonstrated that caveolar proteins cross-talk drives the effects of insulin and oral antidiabetic drug (Müller et al. 2005). Our biophysical approach might be useful to allow an effect targeted to the

area irradiated by US, thus avoiding deleterious systemic side effects.

Further investigations, however, will be needed to better assess the role of mechanical effects induced by diagnostic pulsed US on cell permeability at the level of the apical endothelial cell surface.

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