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Reactive oxygen species mediate shear stress-induced fluid-phase endocytosis in vascular endothelial cells

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Abstract

To elucidate the role of shear stress in fluid-phase endocytosis of vascular endothelial cells (EC), we used a rotating-disk shearing apparatus to investigate the effects of shear stress on the uptake of lucifer yellow (LY) by cultured bovine aortic endothelial cells (BAEC). Exposure of EC to shear stress (area-mean value of 10 dynes/cm^2) caused an increase in LY uptake that was abrogated by the antioxidant, *N*-acetyl-L-cysteine (NAC), the NADPH oxidase inhibitor, acetovanillone, and two inhibitors of protein kinase C (PKC), calphostin C and GF109203X. These results suggest that fluid-phase endocytosis is regulated by both reactive oxygen species (ROS) and PKC. Shear stress increased both ROS production and PKC activity in EC, and the increase in ROS was unaffected by calphostin C or GF109203X, whereas the activation of PKC was reduced by NAC and acetovanillone. We conclude that shear stress-induced increase in fluid-phase endocytosis is mediated via ROS generation followed by PKC activation in EC.

Keywords: *Endothelial cells, shear stress, reactive oxygen species, fluid-phase endocytosis, protein kinase C*

Introduction

Vascular endothelium not only acts as a physical barrier between blood and tissues but also regulates the transport of nutritional substances from blood plasma to the underlying tissues. Nutritional substances in the plasma are believed to pass through the endothelium via two pathways: (1) paracellular pathways that are present at cellular junctions or transcellular channels, and (2) vesicle-mediated transcellular pathways [1,2]. It is thought that impaired regulation of these pathways causes an accumulation of low-density lipoproteins (LDL) in vessel walls, which leads to intimal hyperplasia and atherosclerosis [3,4]. Therefore, investigation of the mechanisms of molecular transport via the endothelium is important to understand both the physiological functions of EC and the pathogenesis of vascular diseases.

Endocytosis is an important function of all cell types. The primary role of this process is thought to be the regulation of cellular metabolism. In addition, it is also thought that the surface volume of the cell membrane is regulated by endocytosis. Thus, an increase in endocytosis reduces the surface volume of the cell membrane [5]. In cases of polarized cells, such as vascular EC, endocytosis and subsequent transcytosis play a pivotal role in the passage of macromolecules from lumen to the underlying tissues. A variety of signaling molecules such as Ca^{2+}[6],

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protein kinase C (PKC) [7,8], and small G proteins [9,10] have been shown to contribute to the regulation of endocytosis. However, the signal transduction cascade involved in endocytosis is largely unknown. Furthermore, the regulatory mechanisms and signaling molecules responsible for endocytosis seem to vary depending on cell type, agonist, and type of endocytosis (i.e. receptor-mediated endocytosis and non-specific endocytosis). For example, Guillot and Audus have shown that fluid-phase endocytosis induced by angiotensin II was mediated by prostaglandin, whereas bradykinin-induced endocytosis did not require prostaglandin in brain microvessels EC [11].

Shear stress, which is induced by blood flow, affects several functions of EC such as cell growth [12], shape change [13], and genetic expression of nitric oxide synthase [14]. The intracellular signaling cascade responsible for shear stress-induced activation of endothelial functions is called "mechanotransduction". Extensive studies in the past decade have revealed several signaling molecules involved in mechanotransduction, and it appears that some of them are identical to those involved in humoral factor-triggered intracellular reactions [15]. Previous studies have reported that shear stress regulates the activity of endocytosis in EC. Sprague et al. showed that incubation of cultured bovine aortic endothelial cells (BAEC) in the presence of shear stress increased binding, internalization and degradation of LDL [16]. Recently, we also demonstrated that shear stress augmented the uptake of LDL by BAEC using a rotating-disk shearing apparatus [17]. It is likely that shear stress-induced alterations of endothelial function including endocytosis are closely associated with the localized development of atherosclerosis, since atherosclerotic lesions preferentially occur at areas exposed to slow blood flow (low shear stress) [18]. Therefore, investigation of the cellular mechanisms of endocytosis in response to shear stress is very important to elucidate the pathogenesis of atherosclerosis.

An early study by Sundqvist and Liu showed that a low dose of hydrogen peroxide stimulated an increase in fluid-phase uptake of macromolecules by cultured BAEC, suggesting that reactive oxygen species (ROS) are involved in the regulation of endocytosis [7]. Recently, it was reported that ROS were increased by exposure of EC to shear stress [19]. Based on these results, we postulated that ROS act as a signal for endothelial endocytosis induced by shear stress. In the present study, we investigated the effects of several inhibitors of ROS on endocytosis, and demonstrated that ROS mediate shear stress-induced activation of fluid-phase endocytosis. In addition, we obtained evidence that PKC contributes to shear stress-induced fluid-phase endocytosis downstream from ROS generation.

Materials and methods

Materials

BAEC were purchased from Cell Systems (WA, USA). Iscov's modified Dulbecco's medium (IMDM), calphostin C, GF109203X, *N*-acetyl-L-cysteine (NAC), lucifer yellow (LY) CH and antibodies to PKC were from SIGMA (MO, USA). Acetovanillone (apocynin) was from Acros Organics (Belgium). 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CH_2DCF-DA) was from Molecular Probes (OR, USA). BCA protein assay reagent was from Pierce (IL, USA). Protein A/Gagarose was from Santa Cruz Biotechnology (CA, USA). PepTag non-radioactive PKC assay kit was from Promega (WI, USA).

Cell culture

BAEC were grown in IMDM supplemented with fetal bovine serum (FBS; 10%), penicillin (100 IU/ml) and streptomycin (100 μg/ml) in a CO_2 incubator. Once the cells were grown to confluence, they were detached from the dishes with trypsin-EDTA solution and re-seeded at a ratio of 1:4. Culture medium was renewed every 3–4 days. For all experiments, confluent BAEC were used at passages 6–12.

Imposition of shear stress on BAEC

To impose laminar shear stress on cells, we employed a rotating-disc shearing apparatus as described elsewhere [17,20]. The apparatus consisted of a power supply, DC motor and a stainless-steel disc (32 mm in diameter). By rotating the stainless-steel disc immersed in culture medium, a constant level of shear stress was imposed on cells grown at the bottom of the dish (35 mm in diameter). The shear stress imposed on EC can be expressed with the following equation:

$$\tau = \frac{\mu r \omega}{h}$$

where τ is the wall shear stress, μ is the viscosity of culture medium, r is the distance from the center of the dish, ω is the angle velocity of disc rotation (rad/s), and h is the distance between the dish and disc. The conditions of present study were: μ, 0.73 mPa s (0.0073 poise); h, 1 mm; speed of rotation, 1220 rpm. Thus, the estimated shear stress imposed on cells varied from 0 dynes/cm^2 (0 Pa) at the center of the culture dish to 15 dynes/cm^2 (1.5 Pa) at the periphery, establishing an area-mean wall shear stress of 10 dynes/cm^2.

Fluid-phase endocytosis

Activity of fluid-phase endocytosis in EC was measured with LY according to methods described

elsewhere [21,22]. Culture dishes (35 mm in diameter) with confluent BAEC were filled with 2 ml of IMDM containing FBS (10%) and LY (100 μg/ml). The cells were incubated for various periods of time in a CO_2 incubator. In the shear stress studies, cells were incubated with or without shear stress (area-mean value of 10 dynes/cm^2) for 2 h. After incubation, cells were washed four times with cold phosphate-buffered saline (PBS) and lysed with 2 ml of PBS containing triton X-100 (0.2%). Fluorescence intensity of the cell lysates was then measured with a spectrofluorometer (FP-750; Jasco, Japan) with excitation and emission wavelengths of 430 and 540 nm, respectively. Protein content of cell lysates was determined with a commercial kit (BCA protein assay reagent).

Visualization of fluid-phase endocytosis was also performed as follows. Cells were incubated with LY for 2 h. After rinsing with Hanks' balanced salt solution (HBSS), cells were cleared in glycerol. The stained cells were imaged using a confocal imaging system (FV-300; Olympus, Japan). The LY-stained cells were examined with 458 nm excitation and a band-pass emission filter (505–550 nm), and all images were stored as TIF format files.

Measurement of intracellular ROS

Intracellular ROS production was measured using CH_2DCF-DA, an ROS sensitive fluorescent dye, as previously described [23]. CH_2DCF-DA penetrating the cells is converted to CH_2DCF by esterase. Once oxidized by cellular ROS, CH_2DCF is converted to CDCF, a fluorescent molecule. BAEC grown in 35 mm culture dishes were preincubated in culture medium containing 30 μM CH_2DCF-DA for 30 min. Then the cells were incubated for various periods of time in the presence or absence of shear stress. CH_2DCF-DA was continuously present in the culture medium throughout the incubation. After that, cells were washed four times with PBS and immersed in 2 ml of HBSS. The dishes were set into a microplate fluorometer (Fluoroskan Ascent; Thermo Labsystems, Finland) and the fluorescence intensity of CDCF from the cells was measured with excitation and emission wavelengths of 485 and 538 nm, respectively. In each measurement, the fluorescence of 66 points all around the dish was detected within 30 s, and the mean value of fluorescent intensity was calculated. The calculated value was normalized by the CDCF signal obtained from cells that were incubated during the same period without shear stress.

Immunoprecipitation and kinase assay for PKC

PKC activity was assessed by immunoprecipitation and non-radioactive kinase assay with a commercial kit. After the cells were exposed to shear stress, they were washed with PBS, suspended in 1 ml of a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA and EDTA, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 μg/ml leupeptin, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride and 1% Triton X-100) and kept on ice for 30 min. Cell lysates were centrifuged at 10,000 g for 15 min at 4°C, then the supernatants were collected and incubated with an anti-PKC monoclonal antibody on a rotating wheel for 1 h at 4°C. After the addition of 20 μl of a 50% slurry of protein A/G-agarose, the lysates were further incubated overnight at 4°C. Samples were washed three times with PBS and the precipitates were suspended in 9 μl of PKC buffer. Kinase assay for PKC was performed according to manufacturer's recommendation as follows. The samples were added to a reaction buffer (20 mM HEPES, 1.3 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP) containing a fluorescent peptide substrate with an amino acid sequence of PLSRTLSVAAK. The mixture was incubated for 30 min at 30°C, and the reaction was terminated by boiling the samples for 10 min. The net charge of the fluorescent substrate was changed from +1 to −1 when the peptide was phosphorylated by PKC. Thus, this charge change allowed the phosphorylated and non-phosphorylated forms of the substrate to be separated on agarose gel. After the addition of 1 μl of 80% glycerol, samples were subjected to electrophoresis with 1% agarose gel. The gel was then photographed on a transilluminator.

Statistical analysis

Results were expressed as the mean ± SEM. Differences in means between groups were determined by Student's *t*-test.

Results

Effects of shear stress on fluid-phase endocytosis

Figure 1(A) shows photographs of BAEC observed under a light (left panel) or confocal (right panel) microscope after incubation with LY. As evident from the figures, LY taken up by cells could be observed as bright spots within the cells. Figure 1(B) shows time-dependent changes in the uptake of LY by BAEC. The LY uptake showed a rapid increase in the initial 30 min followed by a phase of constant uptake throughout the duration of the experiment. To examine the effects of shear stress on fluid-phase endocytosis, BAEC were exposed to shear stress (area-mean value of 10 dynes/cm^2) for 2 h with a rotating-disk apparatus in the presence of LY. As shown in Figure 1(C), the uptake of LY was increased 40% by shear stress.

Roles of ROS and PKC in fluid-phase endocytosis

We next tested whether ROS and PKC are involved in the shear stress-induced increase of fluid-phase

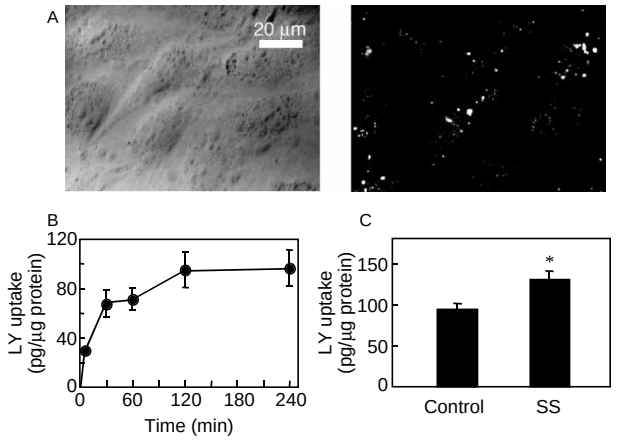

Figure 1. Uptake of LY by BAEC. (A) BAEC observed under light (left panel) and confocal (right panel) microscopy. Cells were incubated with LY (100 μg/ml) for 2 h. (B) Time-dependent changes in the uptake of LY (100 μg/ml) by BAEC. After the cells were incubated for the indicated time periods, the fluorescence intensity of cell lysates was determined with a spectrofluorometer. Data are mean ± SEM for $n = 3$. (C) Effect of shear stress (SS) on the uptake of LY by BAEC. After the cells were incubated for 2 h with or without shear stress (area-mean value of 10 dynes/cm^2), fluorescence intensity of cell lysates was determined with a spectrofluorometer. Data are mean + SEM for $n = 6$. *, $P < 0.05$.

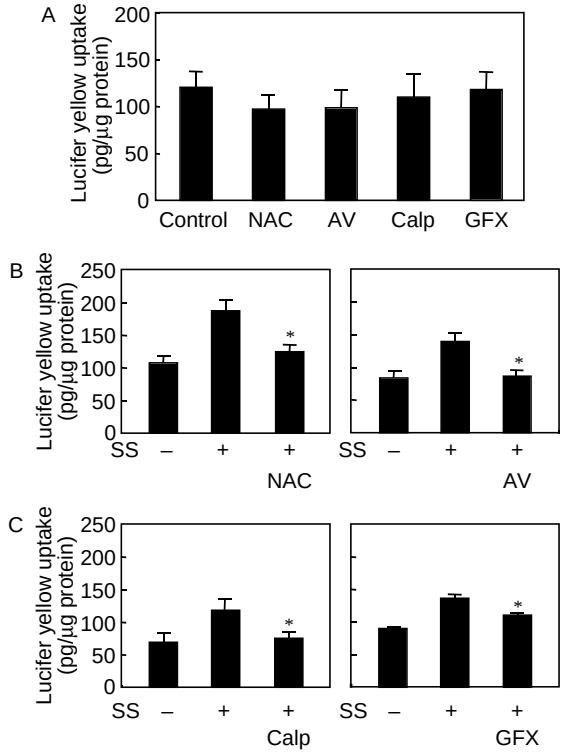

Figure 2. Uptake of LY by BAEC. (A) Effects of an antioxidant (*N*-acetyl-L-cysteine, NAC), a NADPH inhibitor (acetovanillone, AV) and PKC inhibitors (Calphostin C, Calp and GF109203X, GFX) on the uptake of LY by BAEC without shear stress. Cells were incubated with LY and NAC (15 mM), AV (200 μg/ml), Calp (1 μM) or GFX (20 μM) for 2 h. Inhibitors were added to culture medium 30 min before the addition of LY. Data are mean + SEM for $n = 6$. (B) Effects of *N*-acetyl-L-cysteine (NAC) and acetovanillone (AV) on the response to shear stress (SS; area-mean value of 10 dynes/cm^2). *N*-acetyl-L-cysteine (15 mM) or acetovanillone (200 μg/ml) was added to the culture medium 30 min before shear stress. Data are mean + SEM for $n = 3$–5. (C) Effects of PKC inhibitors on the response to shear stress (area-mean value of 10 dynes/cm^2). Calphostin C (Calp, 1 μM) or GF109203X (GFX, 20 μM) was added to culture medium 30 min before shear stress. Data are mean + SEM for $n = 4$–6. *, significantly different from the value obtained without the inhibitors ($P < 0.05$).

endocytosis using several drugs such as NAC (an antioxidant), acetovanillone (a NADPH oxidase inhibitor), calphostin C and GF109203X (PKC inhibitors). At first, we examined whether these drugs interfere the endocytotic pathway without shear stress. As shown in Figure 2(A), NAC, acetovanillone, calphostin C and GF109203X had slight and insignificant effects on the basal uptake of LY by EC. Therefore, we next examined the effects of these drugs on shear stress-induced endocytosis. Figure 2(B) shows the effects of NAC and acetovanillone on LY uptake by EC. shear stress-induced increase in LY uptake was abrogated by both NAC and acetovanillone. These results suggest that intrinsic ROS production via the activation of NADPH oxidase contributes to the increase in fluid-phase endocytosis in response to shear stress. Experiments were also carried out to investigate whether shear stress-induced activation of fluid-phase endocytosis is mediated by PKC. The PKC inhibitor, calphostin C, significantly inhibited shear stress-induced increase in LY uptake as shown in Figure 2(C). GF109203X, another PKC inhibitor, also reduced LY uptake. These results suggest that PKC is responsible for the increase in shear stress-induced fluid-phase endocytosis.

Relationship between ROS generation and PKC activation

Because it was suggested that both ROS and PKC were involved in shear stress activated fluid-phase endocytosis, we next investigated their relationship in signal transduction. Figure 3(A) shows the electrophoresis pattern of substrates phosphorylated by PKC extracted from the shear stress-stimulated EC. PKC activity was enhanced by shear stress in a time-dependent manner. We next assessed the involvement of ROS in shear stress-induced PKC activation. As shown in Figure 3(B), phosphorylation of the PKC substrate was increased by shear stress, and this increase was abrogated by NAC, suggesting that ROS mediate PKC activation in response to shear stress.

Finally, we assessed whether PKC is involved in shear stress-induced generation of ROS in EC. To probe the production of ROS by shear stress in EC, the ROS sensitive dye, CH_2DCF-DA, was applied. Figure 4(A) shows time-dependent changes in endothelial ROS production in response to shear stress. Exposure of EC to shear stress caused an

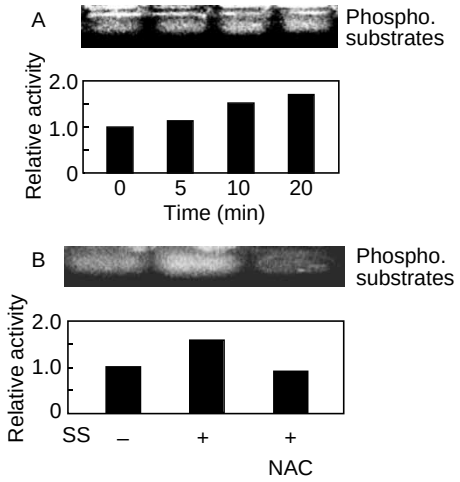

Figure 3. Activation of PKC induced by shear stress (area-mean value of 10 dynes/cm^2) in BAEC. (A) PKC activity of EC exposed to shear stress. After the imposition of shear stress, total PKC in the cells was collected with immunoprecipitaion. PKC activity was measured with a kinase assay using a fluorescent substrate for PKC. Representative data from three independent experiments are shown. (B) Effects of *N*-acetyl-L-cysteine (NAC) on PKC activation induced by shear stress in BAEC. Shear stress was imposed on cells for 30 min. NAC (15 mM) was added to the culture medium 30 min before the shear stress treatment. Representative data from three independent experiments are shown.

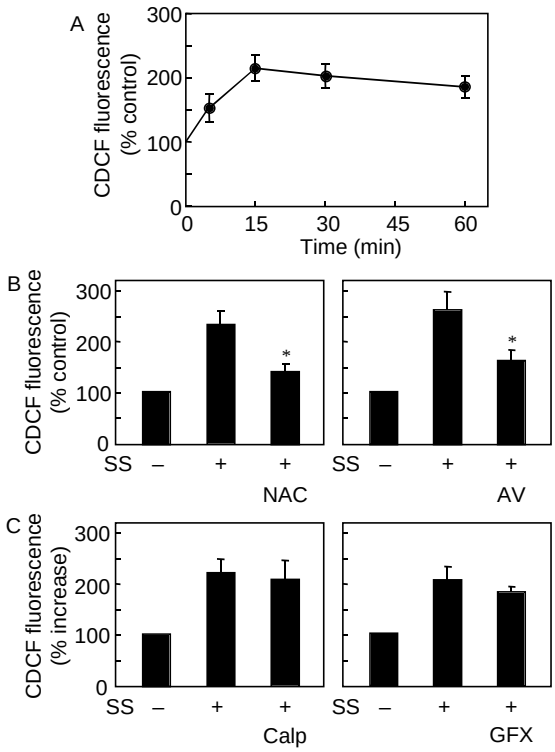

Figure 4. ROS generation induced by shear stress (area-mean value of 10 dynes/cm^2) in EC. ROS generation was measured using the ROS-sensitive fluorescent dye, CH_2DCFDA. (A) Time-dependent changes in ROS generation in EC exposed to shear stress. Data are mean + SEM for $n = 4–11$. (B) Effects of antioxidant (left panel) and NADPH oxidase inhibition (right panel) on ROS generation in EC exposed to shear stress for 30 min. *N*-acetyl-L-cysteine (NAC, 15 mM) or acetovanillone (200 μg/ml) was added to culture medium 30 min before shear stress (SS). Data are the mean + SEM for $n = 5–7$. (B) Effects of PKC inhibitors on ROS generation in EC exposed to shear stress for 30 min. Calphostin C (Calp, 1 μM) or GF109203X (GFX, 20 μM) was added to culture medium 30 min before shear stress. Data are mean + SEM for $n = 10–20$. *, significantly different from the value obtained without the inhibitor ($P < 0.05$).

increase of ROS production, followed by a gradual decrease. As expected, NAC and acetovanillone inhibited ROS production (Figure 4(B)). However, as shown in Figure 4(C), inhibition of PKC activation by calphostin C or GF109203X had no effect on the ROS production. These results suggest that PKC is not required for shear stress-induced ROS production.

Discussion

Since atherosclerosis preferentially occurs at areas exposed to low shear flow in vessel walls [18], it is thought that shear stress is closely associated with atherogenesis. In fact, studies in the last few decades have revealed that shear stress affects a variety of endothelial functions related to atherogenesis [24]. It is generally accepted that accumulation of LDL in the subendothelial space of vessel walls is the initial phenomenon leading to atherosclerosis [25]. One mechanism whereby vascular LDL accumulation occurs in the presence of an intact endothelial monolayer is increased endocytotic transport [26], and several studies including our study have shown that shear stress modifies the endocytotic activity of EC [16,17,27]. Nevertheless, little is known about the intracellular signal transduction cascade responsible for shear stress-induced activation of endocytosis. In the present study, we demonstrated that shear stress augmented the uptake of LY by EC and that this augmentation was inhibited by an antioxidant and NADPH oxidase inhibitor. To our knowledge, this is the first evidence showing that intracellular ROS generation is responsible for shear stress-induced activation of fluid-phase endocytosis in EC.

It is well recognized that under some conditions ROS can be harmful to living cells [28,29]. In contrast, it is also known that ROS act as important intracellular messengers that promote cell proliferation [30,31]. For instance, it has been shown that exposure of EC to ROS such as H_2O_2 and superoxide results in necrosis or apoptosis via expression of p53 and activation of caspases, while ROS had anti-apoptotic effects by activating phosphatidylinositol 3-kinase kinase and Akt [29,32–34]. These variable effects of ROS on cell growth may depend on their concentration as well as the cell type. Furthermore, the role of ROS may not be restricted to regulation of cell growth. Yeh et al. showed that intrinsic ROS generation induced by shear stress was responsible for tyrosine phosphorylation of many

proteins, including mitogen activated protein kinase in EC [19]. From this observation, it is inferred that shear stress-induced ROS generation contributes to many functions of EC. In fact, recent studies have shown that ROS generation induced by mechanical forces, including shear stress and cyclic strain, is responsible for several endothelial functions such as genetic expression of monocyte chemotactic protein-1 [35] and c-fos [36], and protein expression of intercellular adhesion molecule-1 [37] and plasminogen activator inhibitor-1 [38]. These previous observations and our present study suggest that intrinsic generation of ROS plays an important role in the regulation of physiological and/or pathophysiological functions of EC. The present study also showed that shear stress-induced ROS generation was inhibited by acetovanillone (apocynin), suggesting that ROS was generated at least in part via the activation of NADPH oxidase. These results are consistent with a previous report by Yeh et al. [19], who demonstrated that shear stress-induced tyrosine phosphorylation was blocked in EC expressing a dominant negative Rac 1, which is an integral part of the NADPH oxidase complex. Although it has been shown that EC possess membrane-associated NADPH oxidase with a structure similar to that expressed in neutrophils [39,40], the mechanism of NADPH oxidase regulation by shear stress is unknown. Several membrane-binding molecules, such as integrin, caveolae, G protein and ion channels, have been proposed as "mechanosensors" that recognize shear stress [15]. Hence, it may be valuable to assess the involvement of these plausible mechanosensors in the activation of NADPH oxidase.

In the present study, we showed that the shear stress-induced increase in LY uptake was inhibited by PKC inhibitors, indicating that PKC activation is responsible for endothelial fluid-phase endocytosis induced by shear stress. We also demonstrated that PKC was activated downstream of ROS generation in shear stress-stimulated EC. PKC activation by ROS generation has been demonstrated in other tissues such as rat hippocampus [41] and guinea pig heart [42]. The PKC family is a ubiquitous serine-threonine kinase and contributes to a wide range of cellular functions. Twelve isozymes of PKC have been described so far [43,44]. Several studies have shown that PKC contributes to endocytosis in EC [7,45] whereas others have failed to demonstrate a role for PKC in it. [6,11]. Thus, the contribution of PKC to endocytosis may differ depending on the cell type and the stimulus. In addition, the fact that the PKC family consists of twelve subtypes makes it difficult to elucidate the regulatory mechanisms of endocytosis by PKC. We could not determine the type of PKC responsible for shear stress-induced fluid-phase endocytosis in the present study, while we observed that GF109203X inhibited the increase in fluid-phase endocytosis. Although GF109203X is generally used as a broad range inhibitor of PKC, it was reported that GF109203X specifically inhibited classical PKC but not novel PKC in human monocytes [46]. Thus, it is possible that classical PKC including PKC -α, -β and -γ, but not novel PKC, contributes to shear stress-induced fluid-phase endocytosis via the generation of ROS. In fact, it was reported that shear stress of 25 dynes/cm^2 enhanced phosphorylation of PKC α in human umbilical vein EC [47] and that PKC α activation was required for EGF receptor endocytosis in fibroblast cell line [48]. In addition, PKC α in vascular smooth muscle cells was reported to be redox sensitive [49]. To elucidate the precise regulatory mechanisms of endocytosis by PKC, further studies using subtype-specific PKC inhibitors or molecular biological methods, such as dominant-negative PKC expression, may be required.

It has been shown that several proteins such as endophilin, dynamin [50,51], and small G proteins [9,10,52] are implicated in the regulation of membrane invagination, trafficking, and sorting. Although the interaction of these molecules with cell membranes or endosomes has been studied extensively, the regulatory mechanisms of these molecules by second messengers such as Ca^{2+} and cAMP are still unclear. Furthermore, as pointed out by Apodaca, very little is known about signal transduction of endocytosis stimulated by mechanical forces [53]. Since the development of vascular diseases such as atherosclerosis and aneurysm is closely related to mechanical forces derived from blood flow, insights into the mechanotransduction of vascular cells may provide important information for the prevention and treatment of vascular diseases.

In summary, the present study demonstrated that shear stress-induced increase in fluid-phase endocytosis in EC is mediated by NADPH oxidase-derived ROS generation and PKC activation. The present results further indicated that in shear stress-stimulated EC, PKC is activated downstream of ROS generation. Further studies on the regulatory mechanisms of NADPH oxidase by shear stress and the PKC subtypes contributing to fluid-phase endocytosis must be performed.

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