Enhancement of Acetyl-CoA: 1-*O*-Alkyl-2-lyso-*sn*-glycero-3-phosphocholine Acetyltransferase Activity by Hydrogen Peroxide

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The synthesis of platelet-activating factor (PAF) by human umbilical vein endothelial cell (HUVEC) in response to H_2O_2 was significantly increased in a concentration-dependent manner. When HUVEC were pretreated with diethyl maleate, which depletes intracellular glutathione, PAF synthesis was enhanced 3-fold upon 5 mM H_2O_2-treatment. Intracellular redox was involved in regulating PAF synthesis, since the addition of antioxidants such as *N*-acetylcysteine, pyrrolidinecarbodithioic acid (PDTC), and Trolox reduced PAF production in H_2O_2-treated HUVEC. The activity of acetyl-CoA: 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine acetyltransferase, which is involved in the last step of PAF synthesis, was also activated in H_2O_2-treated cells. However, exogenous lyso-PAF addition had not effected to acetyltransferase activity. The acetyltransferase activity responded quickly to H_2O_2-treatment, but the activation was transitory. A tyrosine kinase inhibitor and a calmodulin antagonist blocked acetyltransferase activity in H_2O_2-stimulated cells, suggesting that tyrosine kinase and calcium/calmodulin-dependent protein kinase are involved in regulating acetyltransferase activity. These observations suggest that H_2O_2 is one of the modulators of lyso-PAF acetyltransferase activity *via* a phosphorylation system and platelet-activating factor (PAF) synthesis.

Key words umbilical vein endothelial cell; platelet activating factor; hydrogen peroxide; lyso-platelet-activating factor (lyso-PAF) acetyltransferase

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a phospholipid that is synthesized by a variety of different cells and tissues in response to different stimuli, and is involved in numerous biological responses. Consequently, PAF is recognized as a major mediator that plays a central role in a variety of host defense system mechanisms and inflammatory diseases.[1—4)]

Two pathways for PAF synthesis have been identified. One route is a *de novo* pathway, which is thought to constitutively produce a small amount of PAF to serve some basal physiological roles.[5)] The second route is a remodeling pathway, which synthesizes PAF *via* two enzymatic reaction steps. In this pathway, phospholipase A_2 (PLA_2) hydrolyzes 1-alkyl-2-arachidonyl-*sn*-glycero-3-phosphocholine to 1-alkyl-2-lyso-glycero-3-phosphocholine (lyso-PAF), which is then acetylated by acetyl-CoA: lyso-PAF acetyltransferase to generate PAF. The remodeling pathway appears to be involved in synthesizing most of the PAF that is generated in response to agonist receptor binding.[6)] Since acetyl-CoA: lyso-PAF acetyltransferase is unstable, it is difficult to purify, and consequently, little is known about the enzyme. However, the use of rat spleens,[7—9)] human neutrophils,[10)] guinea pig parotid glands[11)] and mouse mast cells[12)] as sources of the enzyme for *in vitro* assays has shown that the enzymatic activity appears to be regulated by the intracellular calcium levels and involve a phosphorylation–dephosphorylation mechanism. The kinase responsible for the phosphorylation is unknown, but catalytic subunits of cyclic AMP-dependent protein kinase[10,11)] and calcium/calmodulin–dependent protein kinase[11)] are capable of activating the acetyltransferase *in vitro*. Furthermore, it has also been shown that p38 mitogen-activated protein kinase (MAPK) can lead to increased activation of the acetyltransferase.[13,14)]

It has previously been reported that hydrogen peroxide (H_2O_2) causes cytotoxic injury to endothelial cells,[15,16)] but, under some conditions, it initiates the synthesis of biologically active molecules such as PAF. The reactive oxygen species (ROS)-mediated damage to intracellular molecules is limited by cellular antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase, and catalase. Although direct evidence for the involvement of antioxidant enzymes in the production of PAF is still lacking, it has been shown that selenium deficiency, which causes a drop in GPx activity along with a corresponding rise in hydroperoxide levels,[17—19)] increases PAF production in cultured human umbilical vein endothelial cell (HUVEC).[20)] These observations suggest that oxidative stress could modulate PAF synthesis and/or that scavenger enzymes could regulate the activity of the enzymes involved in PAF biosynthesis by controlling intracellular redox. On the basis of these observations, we investigated whether intracellular redox could regulate PAF synthesis. To achieve this, we used HUVEC and show here that PAF synthesis in these cells was caused by H_2O_2 in a concentration- and time-dependent manner that was consequent to the activation of acetyl-CoA: lyso-PAF acetyltransferase. The PAF synthesis caused by H_2O_2 was reduced by antioxidants but intensified by the depletion of the endogenous antioxidant, glutathione. In addition, a tyrosine kinase inhibitor and a calmodulin antagonist suppressed the activity of lyso-PAF acetyltransferase. Thus, intracellular redox affects the signal transduction system, involving a tyrosine kinase and/or a calmodulin-dependent protein kinase, and thereby modulates PAF synthesis.

MATERIALS AND METHODS

Medium 199 (M199) and fetal bovine serum were purchased from Gibco BRL. [1-^{14}C]-Arachidonic acid (2.22 GBq/mmol) and [^{3}H]-acetic acid (5.55 GBq/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]-Acetyl coenzyme A was purchased from Amersham Pharmacia Biotech UK Ltd. Diethyl maleate

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(DEM), KN-93, sodium salt of acetyl coenzyme A and fatty acid-free bovine serum albumin were obtained from Sigma. PD98059 was obtained from Calbiochem. H-7 was obtained from Seikagaku Co. Ltd. (Tokyo, Japan). PAF and lyso-PAF were obtained from Funakoshi Co. Ltd. (Tokyo, Japan). The thin-layer chromatography plates were from Merck.

Cell Culture Human umbilical vein endothelial cells were grown on collagen-coated 35 mm dishes. Isolated cells were resuspended in M199 containing glutamax, 10% fetal bovine serum, 20 mg/ml endothelial growth supplement (ECGS), 100 μg/ml heparin, 50 μg/ml penicillin, and streptomycin. The medium was changed every 3—4 d until the cells were confluent. Cells were only used between passages 4 and 7.

Assay for Intracellular PAF Accumulation PAF accumulation was measured by the incorporation of [^{3}H]-acetic acid as described previously.[21] Briefly, the culture medium was removed from a confluent monolayer of HUVEC in a 3.5 cm diameter dish, and replaced with 1 ml of Hanks' balanced salt solution (containing 1.3 mM Ca^{2+}, 10 mM HEPES, pH 7.4; HBSS) containing 370 kBq/ml [^{3}H]-acetic acid. Following a 10 min preincubation at 37 °C, the cells were incubated with H_2O_2 for the indicated period. The concentration of H_2O_2 was 5 mM unless otherwise indicated. The incubation was terminated by adding 1.5 ml of methanol containing 2% acetic acid. The cells were harvested and total lipids were extracted by the methods of Bligh and Dyer. Each extract was evaporated to dryness under reduced pressure and the residues were then dissolved in a small amount of a 2 : 1 v/v mixture of chloroform and methanol, followed by application to a TLC plate (Silica Gel 60 F254). The plate was developed with a 65 : 35 : 6 v/v mixture of chloroform, methanol, and H_2O. Products and standards were visualized with primulin reagent and the products were identified by comparison with the chromatographic standards. PAF was then scraped from the TLC plate, and the radioactivity incorporated into it was determined by liquid scintillation counting.

Measurement of Acetyltransferase Activity Acetyltransferase activity was determined according to the method described by Nakagawa *et al.*[21] Briefly, confluent monolayer cells were stimulated with H_2O_2 in 2 ml HBSS, and the reaction was terminated by adding 0.5 ml of ice-cold 10 mM HEPES buffer. The cells were harvested and sonicated in HEPES buffer with a probe sonicator (two pulses of 25 watts for 30 s; Branson sonifier 250). The freshly isolated whole cell sonicates (200 μl, about 100 μg of total protein) were then incubated with 200 μM [^{3}H]-acetyl-CoA (0.25 GBq/mmol) and 350 μM lyso-PAF in a total volume of 1 ml. Reactions were carried out at 37 °C for 20 min and then terminated by adding 1.75 ml of ethanol containing 2% acetic acid. As described above, total lipids were extracted and dried, and the residues dissolved in a small amount of chloroform and methanol, before application to a TLC plate (Silica Gel 60 F254). The plate was developed, and PAF was scraped from the TLC plate and its radioactivity was determined by liquid scintillation counting. Enzyme activities were calculated from the radioactivity of PAF.

Liberation of [1-^{14}C]-Arachidonic Acid from H_2O_2-Stimulated Cells Cells (confluent monolayer in a 35 mm dish) were incubated with [1-^{14}C]-arachidonic acid (1.85 kBq) for 24 h at 37 °C, and then washed twice with M199 containing 1 mg/ml fatty acid-free bovine serum albumin. The cells were preincubated in HBSS for 10 min, and then stimulated with 5 mM H_2O_2 for 10 min. The release of radiolabeled arachidonic acid was determined as described previously.[22]

Quantification of Proteins Proteins concentrations were determined with Protein Assay Reagent (Bio-Rad), using bovine serum albumin as the standard.

Data Analysis Data are expressed as means±S.D. from three independent experiments. Statistical analysis was performed with Student's *t* test.

RESULTS

H_2O_2 Stimulates PAF Synthesis in HUVEC The production of PAF in HUVEC was examined by incubating the cells with radioactive acetic acid and then determining the radioactivity of the resulting PAF (Fig. 1). HUVEC produced PAF when treated with 5 mM H_2O_2 for 20 min, since the PAF levels were approximately four-fold higher in stimulated cells than in untreated cells. There was consistent accumulation of radioactive PAF at H_2O_2 concentrations ranging from 1 to 10 mM, with the maximal effect at 10 mM (Fig. 1). The PAF levels in HUVEC increased over time, the maximum being reached within 20 min after H_2O_2-treatment, after which the observed amount of PAF leveled off after 20 min (Fig. 2). The PAF accumulation was not accompanied by leakage of lactate dehydrogenase from the cells indicating that no cell injuries were caused up to 30 min. However, in 4 h 40% of lactate dehydrogenase was leaked from H_2O_2-stimulated cells, after which the leakage increased gradually to 16 h, reached 90% or more. To ascertain whether other oxidants induce PAF production in HUVEC, we determined the PAF levels in cells treated with amphiphilic *tert*-butyl hydroperoxide, hydrophobic cumen hydroperoxide or 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), which is a hydrophilic initiator of radical chain reactions inducing lipid peroxidation. *tert*-Butyl hydroperoxide had an effect on PAF synthesis, whereas cumen hydroperoxide and AAPH had no effects on PAF synthesis by HUVEC (Table 1). Thus membrane-permeable oxidant seems to be more effective to provoke the PAF synthesis.

To determine whether PAF synthesis was oxidative stress-

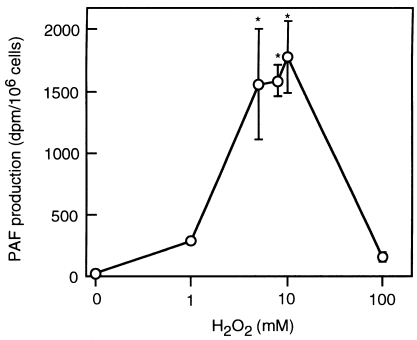

Fig. 1. Effect of H_2O_2 on PAF Synthesis in HUVEC

HUVEC were labeled with [^{3}H]-acetic acid for 10 min, and then stimulated with various concentrations of H_2O_2 for 20 min. The cells and culture media were collected. Total lipids were extracted, concentrated and applied to a TLC plate. The plate was developed, and the radioactivity of the products was determined by liquid scintillation counting. Values are the means±S.D. of three independent experiments. $*p<0.05$, by comparison to nil H_2O_2 addition.

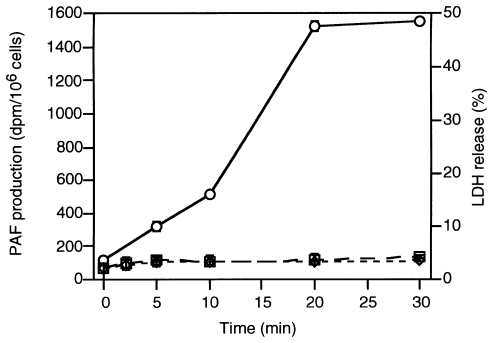

Fig. 2. Effect of H_2O_2-Stimulation on PAF Synthesis over Time in HUVEC

Cells were labeled with [^{3}H]-acetic acid for 10 min, and then stimulated with 5 mM H_2O_2 for the indicated periods (open circles). The radioactivity of the products was determined by liquid scintillation counting. The percentage of LDH released from HUVEC into the incubation medium was calculated by measuring the LDH activity in the incubation medium and in cell lysates prepared from the cells stimulated with 5 mM (open squares) or 10 mM (open triangles) H_2O_2. Values are the means±S.D. of three independent experiments.

Table 1. Effects of Oxidants on PAF Synthesis in HUVEC

Oxidants	PAF production (dpm/10^6 cells)
Control	114.97±87.62
5 mM H_2O_2	323.28±77.06*
1 mM *t*Bu-OOH	210.54±37.14*
5 mM Cume-OOH	106.41±86.33
10 mM AAPH	58.00±20.77

Cells were labeled with [^{3}H]-acetic acid, and then stimulated with 5 mM H_2O_2, 1 mM *tert*-butyl hydroperoxide, 5 mM cumen hydroperoxide, or 10 mM 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) for 10 min. The rate of PAF synthesis was then determined. Values are the means±S.D. of the three independent experiments. $*p<0.05$, by comparison to nil oxidant addition.

responsive, we examined the effect on PAF synthesis of pretreating HUVEC with antioxidants. Pretreatment with the antioxidants chosen restrained the PAF synthesis that occurred in HUVEC after H_2O_2-stimulation (Table 2). Thus, oxidative stress caused by H_2O_2 induces PAF synthesis.

Acetyl-CoA: Lyso-PAF Acetyltransferase Activity Is Activated by H_2O_2 Two enzymes, PLA_2 and lyso-PAF acetyltransferase, are involved in the remodeling system of PAF synthesis. To determine the effect of H_2O_2 on these enzyme activities, we measured arachidonic acid release and acetyltransferase activity in a cell-free system using cell lysates prepared from HUVEC after incubation with 5 mM H_2O_2 for various incubation periods (Fig. 3). The release of radiolabeled arachidonic acid from H_2O_2-stimulated cells to the incubation medium increased up to 20 min, and then leveled off. In the stimulated cell lysates, acetyltransferase activity increased over time, the maximum being reached within 10 min after H_2O_2-treatment, after which the activity declined. We examined the effect on PAF synthesis of pretreating HUVEC with lyso-PAF acetyltransferase inhibitor Sanguinarin before H_2O_2-treatment. The PAF synthesis that occurred after H_2O_2-treatment was significantly inhibited by Sanguinarin (data not shown). To clarify the effect of H_2O_2 on the acetyltransferase activity, we examined whether the acetyltransferase activity was modulated by the increased intracellular lyso-PAF levels resulting from up-regulation of arachidonic acid release by H_2O_2. Exogenous lyso-PAF had no effects on arachidonic acid release, PAF production and

Table 2. Effects of Antioxidants on PAF Synthesis in HUVEC

Condition	PAF production (dpm/10^6 cells)
Control	105.24±33.79
5 mM H_2O_2 10 min	426.29±59.01
+1 mM Deferoxamine 20 h	338.05±60.59*
+5 mM Deferoxamine 20 h	236.01±46.53*
+0.1 mM Trolox 2 h	402.77±130.40
+1 mM Trolox 2 h	150.10±49.24*
+3 mM Trolox 2 h	140.39±63.88*
+30 μM NAC 2 h	186.51±72.86*
+150 μM NAC 2 h	170.33±21.89*
+20 μM PDTC 2 h	211.46±30.10*
+50 μM PDTC 2 h	201.43±43.37*

Cell were labeled with [^{3}H]-acetic acid, and then stimulated with 5 mM H_2O_2 for 10 min after preincubation with the antioxidants deferoxamine, Trolox, *N*-acetylcysteine or PDTC as indicated. The rate of PAF synthesis was then determined. Values are the mean±S.D. of three independent experiments. $*p<0.05$, by comparison to antioxidant non-pretreated cell.

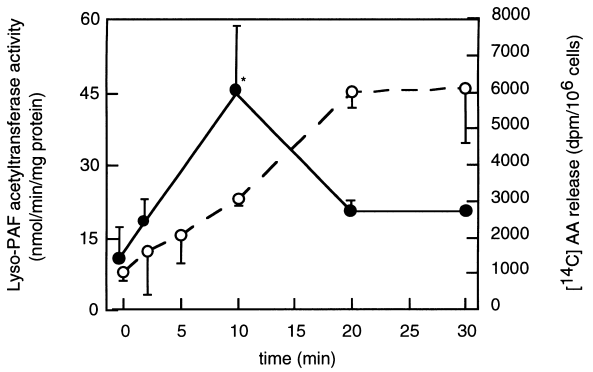

Fig. 3. Effect of H_2O_2-Stimulation on Lyso-PAF Acetyltransferase Activity over Time in HUVEC

Cells were stimulated with 5 mM H_2O_2 for the indicated periods before cell lysate preparation, after which the acetyltransferase activity was determined (closed circles). Cells, which had been labeled with [1-^{14}C] arachidonic acid for 24 h, were then stimulated with 5 mM H_2O_2 for the indicated periods. The rate of radiolabeled arachidonic acid release into the incubation medium was determined (open circles). Values are the means±S.D. of three independent experiments. $*p<0.05$, by comparison to the activity at 0 min.

acetyltransferase activity in either quiescent cells or H_2O_2-stimulated cells (Fig. 4). Furthermore, we examined the effect on acetyltransferase activity of pretreatment with antioxidants. Pretreatment with the antioxidant chosen restrained the acetyltransferase activity in HUVEC after H_2O_2 treatment (Table 3). Thus, acetyl-CoA: lyso-PAF acetyltransferase activity is activated by H_2O_2-treatment of HUVEC.

Depletion of Intracellular Glutathione Potentiates H_2O_2-Induced PAF Production To determine whether intracellular redox affected PAF synthesis induced by H_2O_2, we examined the effect on PAF synthesis of pretreating HUVEC with DEM. DEM diminishes intracellular glutathione levels resulting in a reduction in the activity of antioxidant enzymes such as glutathione peroxidase,[23] and increases in the activity of protein kinases such as extracellular-signal regulated kinase (ERK), p38 and protein kinase B.[24,25] PAF synthesis in H_2O_2-stimulated cells was markedly increased by treatment with DEM (Fig. 5A). DEM decreases the level of glutathione to about 10% of that in non-treated cells under our present condition (data not shown). Furthermore, when unstimulated cells were treated with DEM, the basal level of PAF synthesis, arachidonic acid release, and acetyltrans-

ferase activity were increased (Figs. 5A—C). In contrast, pyrrolidinecarbodithioic acid (PDTC) treatment along with DEM counteracted the effects of lowering the intracellular glutathione levels on PAF synthesis and arachidonic acid release. Thus, a change in the intracellular redox condition seems to be essential for both the activation of arachidonic acid release and acetyltransferase activity in stimulated cells and the expression of the basal activity in quiescent cells.

Table 3. Effects of Antioxidants on Lyso-PAF Acetytransferase Activity in HUVEC

Condition	Lyso-PAF acetyltransferase activity (nmol/min/mg)
Control	23.8±3.9
+1 mM Trolox 2 h	21.7±4.4
+30 μM NAC 2 h	20.1±3.3
5 mM H_2O_2 10 min	43.9±3.6
+1 mM Trolox 2 h	29.6±3.6*
+30 μM NAC 2 h	31.9±3.4*

Cell were stimulated with 5 mM H_2O_2 for 10 min after preincubation with the antioxidants Trolox or N-acetylcysteine as indicated before cell lysate preparation, after which the acetyltransferase activity was determined. Values are the mean±S.D. of three independent experiments. * $p<0.05$, by comparison to antioxidant non-pretreated cell.

Protein Kinase Inhibitors Block PAF Synthesis and Acetyltransferase Activation in H_2O_2-Stimulated HUVEC To determine the enzyme responsible for modulating acetyltransferase activity in H_2O_2-stimulated HUVEC, we examined the effect on PAF synthesis of pretreating HUVEC with kinase inhibitors before H_2O_2-stimulation. The inhibitors were chosen to selectively inhibit Ca^{2+}/calmodulin-dependent protein kinase II (KN-93), protein kinase C (H-7), and tyrosine kinase (genestein). We also used a calmodulin antagonist (W-7). The PAF synthesis that occurred in HUVEC after H_2O_2-stimulation was abolished by treatment with genestein, KN-93 or W-7, but H-7 had less effect (Fig. 6C). The activation of acetyltransferase by H_2O_2-treatment of HUVEC was also almost totally suppressed by treatment with the tyrosine kinase inhibitor or calmodulin antagonist, KN-93 and W-7 (Fig. 6A), but unaffected by the protein kinase C inhibitor. In contrast, only the protein kinase C inhibitor suppressed arachidonic acid release from the H_2O_2-stimulated cells. Thus, tyrosine kinase and Ca^{2+}/calmodulin kinase are responsible for the activation of acetyltransferase in H_2O_2-stimulated HUVEC and the signaling pathway for acetyltransferase activation is separate from that for PLA_2 activation.

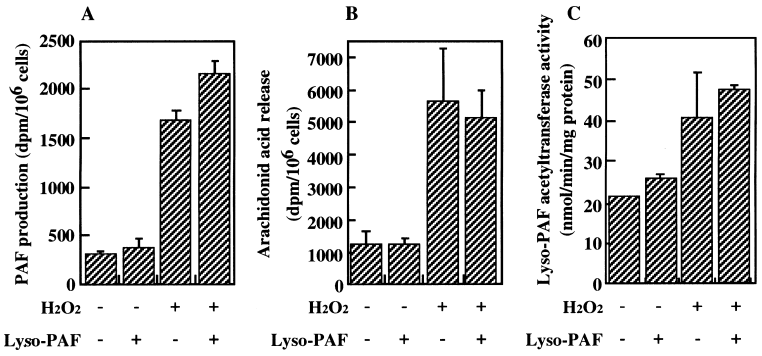

Fig. 4. Effect of Lyso-PAF on PAF Synthesis and Acetyltransferase Activity in HUVEC

(A) Effect of lyso-PAF on PAF synthesis. HUVEC were incubated with 0.2 μM lyso-PAF for 4 min and then labeled with [^{3}H]-acetic acid for 10 min. The labeled cells were stimulated with 5 mM H_2O_2 for 10 min, and then the PAF synthesis was determined. (B) Effects of lyso-PAF on the release of arachidonic acid. Labeled HUVEC were incubated with 0.2 μM lyso-PAF for 4 min, and then stimulated with 5 mM H_2O_2 for 10 min. Radioactive arachidonic acid released into the incubation medium was determined by liquid scintillation counting. (C) Effects of lyso-PAF on the activity of acetyltransferase. HUVEC were stimulated with 5 mM H_2O_2 for 5 min after incubation with 0.2 μM lyso-PAF. The cells were sonicated and subsequently assayed for acetyltransferase activity. Data represent the means±S.D. of three independent experiments.

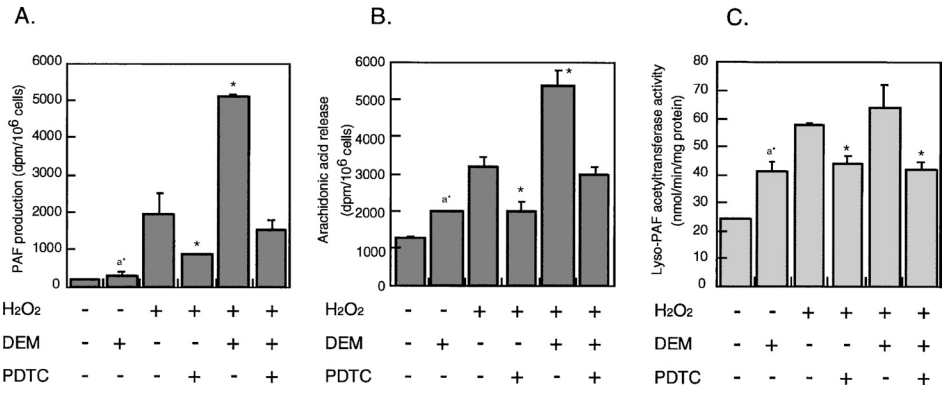

Fig. 5. Effects of Diethyl Maleate on PAF Synthesis in HUVEC

(A) Effect of DEM and/or PDTC on PAF synthesis. HUVEC were incubated for 2 h at 37 °C with or without 1 mM DEM and/or 50 μM PDTC, after which they were labeled with [^{3}H]-acetic acid for 10 min. Labeled cells were stimulated with H_2O_2 for 10 min, and then PAF synthesis was determined. (B) Effects of DEM and/or PDTC on the release of arachidonic acid. Labeled HUVEC were incubated with 1 mM DEM and/or 50 μM PDTC for 2 h, and then stimulated with H_2O_2 for 10 min. Radioactive arachidonic acid release into the incubation medium was determined by liquid scintillation counting. (C) Effects of DEM and/or PDTC on the activity of acetyltransferase. HUVEC were stimulated with H_2O_2 for 5 min after incubation with 1 mM DEM and/or 50 μM PDTC. The cells were sonicated and subsequently assayed for acetyltransferase activity. Data represent the means±S.D. of three independent experiments. * $p<0.05$, by comparison to antioxidant non-pretreatment. (a) $p<0.05$, by comparison to nil DEM addition.

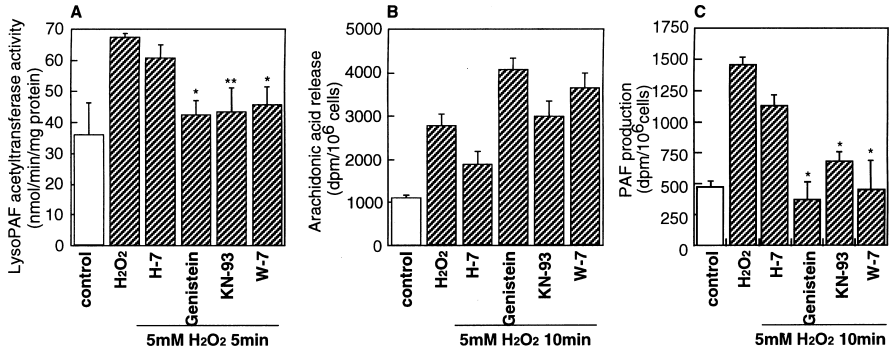

Fig. 6. Effect of Protein Kinase Inhibitors on PAF Synthesis and Acetyltransferase Activity in HUVEC

(A) Effect of protein kinase inhibitors on lyso-PAF acetyltransferase activity. HUVEC were incubated with 50 μM of KN-93, H-7, PD98059, genistein, or W-7 for 10 min, and then stimulated with H_2O_2 for 5 min. The cells were sonicated and subsequently assayed for acetyltransferase activity. (B) Effects of inhibitors on the release of arachidonic acid. Labeled HUVEC were incubated with each inhibitor for 10 min and then stimulated with H_2O_2 for 10 min. Radioactive arachidonic acid release into the incubation medium was determined by liquid scintillation counting. (C) Effect of inhibitors on PAF synthesis. HUVEC were incubated with 50 μM of H-7, genistein, KN-93 or W-7 for 10 min, and then labeled with [^{3}H]-acetic acid for 10 min. The labeled cells were stimulated with 5 mM H_2O_2 for 10 min, and then PAF synthesis was determined. Data represent the means±S.D. of three independent experiments. * $p<0.05$ and ** $p<0.1$, by comparison to no inhibitor.

DISCUSSION

It is known that PAF is synthesized in response to stimulants by a variety of cells, including endothelial cells, mast cells, basophils, neutrophils, eosinophils, macrophages, and platelets.[26] ROS, including H_2O_2, appear to be involved in regulating multiple cellular processes by their interactions with various protein and lipid species.[27] These observations have led to the hypothesis that ROS act as cofactors in the regulation of many intracellular signal transduction cascades. To investigate the regulatory mechanisms involved in PAF synthesis induced by oxidative stress, we examined the effect of H_2O_2 on PAF synthesis in HUVEC. HUVEC in primary culture synthesize and accumulate PAF when the cells are stimulated by H_2O_2, indicating that oxidative stress is involved in PAF synthesis. It is known that HUVEC synthesize PAF *via* a *de novo* pathway, and a remodeling pathway that involves PLA_2 and acetyltransferase.[28] However, PAF synthesis in the endothelium occurs predominantly *via* the remodeling pathway. The PAF accumulation induced by H_2O_2 was concentration- and time-dependent, and not accompanied by lytic cell injury (Figs. 1, 2). Lewis *et al.* demonstrated that brief exposure of endothelial cells to H_2O_2 stimulated PAF production and increased endothelial cell permeability to Ca^{2+}.[29] Exposing endothelial cells to membrane-permeable *tert*-butyl hydroperoxide increased PAF synthesis, whereas cumen hydroperoxide or AAPH, which are not membrane-permeable, had no effects on PAF synthesis (Table 1), suggesting that the cytosolic redox change is needed for the initiation of PAF synthesis *via* increment of the intracellular calcium levels. In our system, the peroxyl radical scavenger Trolox and NAC, which causes increment of the intracellular glutathione levels, suppressed PAF synthesis, whereas the iron chelator deferoxamine had less effect, suggesting that the generation of peroxyl radicals and their derivatives might trigger the PAF synthesis. H_2O_2 was shown to induce oxidation of omega-6 fatty acids and initiate hydrolysis of phospholipids in endothelial cells by the activation of PLA_2.[30] We found that pretreatment with DEM, which conjugates with glutathione and inactivates glutathione-dependent enzymes such as glutathione peroxidases, enhanced acetyltransferase activity, while PDTC counteracted its effect on the enzyme, indicating the involvement of intracellular redox change in regulating the acetyltransferase activity. Thus, H_2O_2 may have induced the activation of both PLA_2 and acetyltransferase.

We observed that the concentration–response relationship for PAF synthesis induced by H_2O_2 was narrow. PAF production was observed at 1 mM and maximal at 10 mM. Exposure to greater than 10 mM H_2O_2 decreased the PAF accumulation in HUVEC, suggesting that higher concentrations of H_2O_2 may inactivate the enzyme(s) involved in PAF synthesis. Although the concentration of H_2O_2 which induces PAF production is high and it is unknown whether such concentration reaches *in vivo*, neutrophils release H_2O_2 sufficient to cause high local concentration and millimolar concentration of H_2O_2 may be attained in inflammatory lesion.[31—33] Variable effects of H_2O_2 on biological events have been reported. Endothelial cells preexposed to H_2O_2 under sublytic conditions are unable to respond to thrombin and histamine, which cause enhancement of the intracellular calcium levels, inositol phosphate hydrolysis, and PAF production,[34] suggesting that preexposure to sublytic H_2O_2, which does not cause cell lysis or denudation, alters the signal transduction for the production of endothelial lipid mediators. Although the biochemical target(s) of H_2O_2 have not been defined in endothelial cells, the sequence of events in nonendothelial target cells after H_2O_2 exposure includes induction of glutathione oxidation,[16] reduction in cellular ATP concentration, and depletion of nicotinamide adenine dinucleotide,[35] followed by increases in intracellular calcium and sodium ions, cytoskeletal alterations, cell membrane bleb formation, and lysis.[36] Therefore, H_2O_2 levels that are within the degradation ability of endothelial cells might result in nonlethal variations of metabolic systems such as glutathione oxidation and cellular ATP reduction. More concentrated exposure to H_2O_2 might result in a calcium influx invoking PAF production, and furthermore, sublethal cellular alterations in DNA structure involved in subsequent effects on repair and cell death. With further intense exposure to H_2O_2, endothelial cells might lose the ability to respond to agonists such as thrombin *via* the appropriate signaling pathway.

Previous reports have indicated that exposure to H_2O_2 leads to an increase in arachidonic acid release by upregula-

tion of mitogen-activated protein kinases, and thereby, the phosphorylation of $cPLA_2$.[37,38] However, Weigel *et al.* reported that H_2O_2 did not activate $cPLA_2$ in either intact endothelial cells or cell membrane fractions, although H_2O_2 strongly enhanced eicosanoid synthesis derived from arachidonic acid.[39] They speculated that H_2O_2 activates $cPLA_2$ in calcium-independent manner, and that the increase in intracellular calcium ions leads to interfacial association of $cPLA_2$ with lipids, but does not enhance the hydrolytic activity of it. After stimulation of various cell types with agonists of receptors involving tyrosine kinase activity or PKC activators, $cPLA_2$ is phosphorylated on a serine residue, activated and translocated to the cell membrane,[40—42] where it produces lysophospholipids such as lysophosphatidylcholine (lyso-PC). Recent studies in endothelial cells have shown that lyso-PC induces the activation of several protein kinases, protein kinase C,[43] tyrosine kinases[44] and mitogen-activated protein kinase (MAPK) such as ERK1/2 and JNK.[45] In the results reported here, H_2O_2 led to a marked increase in arachidonic acid release in HUVEC, but pretreatment with a PKC inhibitor attenuated this effect of H_2O_2. Thus, it is suggested that our results demonstrate that H_2O_2 induces the activation of protein kinase C *via* some signaling pathways, and that of $cPLA_2$, resulting in the release of arachidonic acid and lyso-PAF. However, in the stimulation of HUVEC with H_2O_2, blockade of lyso-PAF acetyltransferase activation suppressed PAF synthesis though the release of arachidonic acid increased. Thus, the generation of lyso-PAF probably is not rate limiting step in PAF synthesis. Bernatchez *et al.* report that vascular endothelial growth factor (VEGF) enhanced PAF synthesis in HUVEC. They suggest that group V $sPLA_2$ and lyso-PAF acetyltransferase are responsible for the PAF synthesis mediated by VEGF. Thus H_2O_2 probably induces distinct signaling pathway from that in VEGF-exposed HUVEC.[46]

The rapid rise in PAF synthesis following exposure to H_2O_2 resulted from the activation of lyso-PAF acetyltransferase, and raised the question of how the enzyme was activated. It seems likely that covalent modifications, phosphorylation and dephosphorylation, are responsible for the rapid change in acetyltransferase activity. Lenihan and Lee have speculated that PKC might be responsible for the stimulation of acetyltransferase activity.[8] Domenech *et al.* suggested that the enzyme activity is regulated by phosphorylation and dephosphorylation by calcium/calmodulin-dependent protein kinase and type-2 protein phosphatase, respectively,[11] and contradict the participation of PKC and cAMP-dependent protein kinase in acetyltransferase activation. In the stimulation of HUVEC with H_2O_2, a calmodulin antagonist and a tyrosine kinase inhibitor blocked both PAF synthesis and acetyltransferase activity in the cells suggesting the participation of Ca^{2+}/calmodulin-dependent protein kinase and tyrosine kinase(s) in regulating acetyltransferase activity, and thereby, PAF synthesis. Unlike those inhibitors, the PKC inhibitor had less effect on acetyltransferase activity and PAF synthesis in stimulated cells. This is significant because there is some controversy about the roles of Ca^{2+}/calmodulin-dependent protein kinase and PKC in the steps that regulate PAF synthesis. When endothelial cells are exposed to H_2O_2, intracellular calcium,[47] inositol phosphate and diacylglycerol[48] levels are increased before PAF synthesis, indicating the activation of phospholipase C (PLC) by oxidants. The PLC family includes alpha, beta, gamma and delta isoforms. It is known that the PLC gamma isoform is activated by phosphorylation on a tyrosine residue. In the study reported here, we observed that a tyrosine kinase inhibitor suppressed acetyltransferase activity and PAF synthesis, suggesting that PLC gamma is involved in increasing the intracellular calcium levels *via* activation of the polyphosphoinositide cycle, and that Ca^{2+}/calmodulin-dependent protein kinase may be responsible for the activation of acetyltransferase in HUVEC stimulated by H_2O_2. On the other hand, PKC may be responsible for $cPLA_2$ activation. Thus, different phases of PAF biosynthesis *via* the remodeling pathway may be regulated by distinct protein kinases in H_2O_2-exposed HUVEC.

From the results reported here, we suggest that oxidative stress by H_2O_2 exposure invokes activation of lyso-PAF acetyltransferase, and thereby PAF synthesis, and that calcium/calmodulin-dependent protein kinase is a likely candidate for the enzyme responsible for phosphorylating lyso-PAF acetyltransferase in intact HUVEC.

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