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EVIDENCE FOR THE ACTIVATION OF THE SIGNAL-RESPONSIVE PHOSPHOLIPASE A_2 BY EXOGENOUS HYDROGEN PEROXIDE

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Abstract—The intracellular events that lead to arachidonic acid release from bovine endothelial cells in culture treated with hydrogen peroxide were characterized. The hydrogen peroxide-stimulated release of arachidonic acid was time- and dose-dependent, with maximal release achieved at 15 minutes after the addition of 100 μM hydrogen peroxide. Hydrogen peroxide-stimulated release of arachidonic acid was blocked with the phospholipase A_2 inhibitor quinacrine. Treatment of the cells with hydrogen peroxide did not result in liberation of oleic acid, indicating that hydrogen peroxide exercised its effect on an arachidonate-specific phospholipase. Pretreatment of the cells with antioxidants, transition metal chelators, and hydroxyl radical scavengers did not affect the hydrogen peroxide-stimulated arachidonic acid release, indicating that the response to hydrogen peroxide is not oxygen radical-mediated. The response to hydrogen peroxide does not appear to be calcium-dependent, due to the following two observations: (a) No increase in intracellular calcium was seen upon exposure of the FURA2-loaded cells to hydrogen peroxide at concentrations sufficient to release arachidonic acid, and (b) no change in the release response was detected in cells loaded with the intracellular calcium chelator BAPTA. Significant inhibition of arachidonic acid release was seen when the cells were pretreated with inhibitors of protein kinase C, but not with inhibitors of tyrosine kinase. The results of these studies indicate that hydrogen peroxide-stimulated arachidonic acid release is mediated by a specific signal-responsive phospholipase A_2, and that this process is not mediated via the actions of either lipid peroxidation or calcium but, rather, that a stimulation of intracellular kinase activity is necessary for this response.

Key words: phospholipase A_2; endothelium; hydrogen peroxide; arachidonic acid; inflammation

The actions of arachidonic acid metabolites in inflammatory disease is well established. From the standpoint of the cell, the production of these mediators must be under very strict control. It is generally thought that the rate-limiting step in the production of eicosanoids is the intracellular liberation of arachidonic acid from the *sn*-2 position of phospholipids [1]. Thus, the control of the intracellular levels of arachidonate and, ultimately, the production of arachidonate-derived mediators is controlled by the phospholipase A_2 system and the associated enzymes involved in the reacylation of free fatty acids: the arachidonoyl-CoA:1-palmitoyl-*sn*-glycero-3-phosphocholine acyl transferase and the arachidonyl-CoA synthetase [1].

It has been known for some time that the release of free arachidonate from cellular phospholipids is mediated via the action of intracellular phospholipase A_2, and that this release was under the control of cellular second message systems (ref. 2 and references therein). Until recently, details of the mechanism(s) of that control were unknown. With the isolation and subsequent cloning of the $cPLA_2$† a signal responsive PLA_2 activity was identified [3–8]. Previous work with the low molecular weight (12–15 KDa) $sPLA_2$ has established that, while it is specific for the *sn*-2 position of phospholipids, its function was primarily digestive, and it thereby acted rather nonspecifically at the *sn*-2 position [9, 10]. Additionally, the $sPLA_2$ was found to be responsive to calcium only in the millimolar range, and essentially inactive in reducing environments (such as the cytoplasm) [9]. Thus, with the identification of the $cPLA_2$ (MW = 85 KDa), it was found that mammalian cells did indeed contain a PLA_2 that was responsive to physiologically relevant calcium concentrations [3, 5, 8] and G-protein activation [5, 11–13]. The $cPLA_2$ was also found to be a substrate for protein kinase C (PKC) [4, 7] and the mitogen-activated protein (MAP) kinase [6, 7] (i.e. a signal-responsive PLA_2 activity). Finally, the $cPLA_2$ has been found to be specific for arachidonic acid at the *sn*-2 position [3], thereby establishing it as the primary rate-limiting step in signal-mediated eicosanoid biosynthesis.

Recently we have characterized a eicosanoid-dependent vaso- and bronchoconstriction in the isolated and perfused rat lung in the presence of an oxidant, specifically hydrogen peroxide [14–17]. Oxidants, hydrogen peroxide in particular, have been shown to be important mediators in inflammatory lung diseases [18]. At sites of inflammation, the production of activated oxygen species is enhanced and can reach relatively high levels. One of the primary activated oxygen species is hydrogen peroxide, which can reach relatively high [0.10–1.0 mM) local concentrations in the presence of activated poly-

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† Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $cPLA_2$, cytosolic phospholipase A_2; CPAE, bovine (calf) pulmonary arterial endothelial cells; DPPD *N*,*N*-diphenyl-1,4-phenylenediamine; TPA, phorbol 12-myristate acetate; $sPLA_2$, secreted phospholipase A_2.

morphonuclear leukocytes (PMNs) [19–22]. The effects of hydrogen peroxide on cellular function are well-known: They provoke morphological and biochemical changes consistent with necrosis at relatively high (>1.0 mM) concentrations. Since the concentrations of hydrogen peroxide reached *in vivo* are probably somewhat lower that those used in most studies of its intracellular effects, it may, at times, act more as an intracellular signal than a cytotolytic or cytotoxic oxidant.

The present study is concerned with the mobilization of arachidonic acid in the presence of sublethal hydrogen peroxide concentrations, and addresses the very early events in the cellular response to exogenous hydrogen peroxide. Since one of the primary targets for activated PMNs is the endothelium [23], the release of arachidonic acid was assessed in cultured bovine endothelium after hydrogen peroxide exposure. Previous studies of oxidant-mediated arachidonic acid release have found that if the oxidant concentrations were high enough, arachidonic acid was released [24–28]. The variety of doses, time courses, and oxidant species used, however, has resulted in an unclear picture of the possible mechanisms of oxidant-mediated arachidonic acid release, because three main questions have frequently gone unanswered:

1. Is lipid peroxidation and subsequent activation of 'membrane maintenance' phospholipases involved in the observed arachidonic acid liberation?
2. Do the concentrations of the oxidant species result in a rise in intracellular calcium during the time course of the experiment?
3. Does the addition of oxidant result in a stimulation of the activity of the arachidonate-specific, signal-responsive $cPLA_2$ or the nonspecific $sPLA_2$?

The present study, using the hydrogen peroxide-treated bovine pulmonary endothelium model system, addresses these three questions. Additionally, some characterization of the possible intracellular signaling systems that could contribute to the release of arachidonic acid is presented.

METHODS

Materials

[^{3}H]arachidonic acid (approximately 180–230 Ci/mmol) and [^{3}H]oleic acid (10 Ci/mmol) were obtained from Dupont-New England Nuclear, Wilmington, DE. T-2 was a kind gift from Astra-Draco, Lund, Sweden. Ro-31 8220 was a kind gift from Roche Ltd., Welwyn Garden City, Herfordshire, U.K. Genistein was obtained from Biomol, Hamburg, Germany. Fatty acid-free bovine serum albumin, Hepes, FURA2/AM, and BAPTA/AM were obtained from Boehringer-Mannheim, Mannheim, Germany. Phorbol 12-myristate acetate (TPA), bradykinin, *N*-ethyl maleimide, azodicarboxylic acid bis[dimethylamide] (diamide), melittin, A23187, 2,2-dipyridyl, quinacrine, and *N,N*-diphenyl-1,4-phenylenediamine (DPPD) were obtained from Sigma. All other reagents were obtained from local sources, and were of reagent grade or better.

Cell culture

Bovine pulmonary endothelial cells (CPAE) were obtained from the American Type Culture Collection and maintained in Dulbecco's Minimal Essential Medium (Gibco) supplemented with 10% fetal calf serum at 37°C in an atmosphere of 95% air, 5% CO_2. For arachidonic acid release experiments, cells are grown to confluence in 2 cm^2 wells on 24-well multiwell plates. For the measurement of intracellular calcium using FURA2, the cells were grown on glass coverslips as described below.

Arachidonic acid release

The cells were loaded into the 24-well plates, and grown to confluence (approximately 5×10^4 cells/well). They were then loaded with 0.1 μ Ci [^{3}H]arachidonic acid (approximately 500 nmoles) per well for 24 hours in growth medium. Over the 24-hour period, the cells incorporated an average of 85% of the added [^{3}H]arachidonic acid. Prior to treatment, they were washed twice with Hepes-buffered Hank's Buffered Salt Solution, pH 7.4 (HHBSS), containing 1% fatty acid-free bovine serum albumin, and subsequently washed once with BSA-free HHBSS. Cells were treated in a final volume of 500 μl of HHBSS. Pretreatments were carried out in the incubation medium and the arachidonic acid-releasing agents were added directly to the pretreatment medium. The various pretreatments were as follows: DPPD, 50 μM for 10 minutes; T-2, 10 μM for 10 minutes; desferal, 1.0 mM for 60 minutes; dipyridyl, 100 μM for 20 minutes; mannitol, 20 mM for 30 minutes; quinacrine, 50 μM for 10 minutes; and TPA, 200 nM for 24 hours in growth medium. The released [^{3}H]arachidonic acid was determined by liquid scintillation counting of 425 μl of the extracellular medium. All treatments and pretreatments were checked for cytotoxicity by measuring LDH release and lipid peroxidation using the thiobarbituric acid (TBA) assay. Under none of the experimental conditions did either parameter increase over control values, except in the case of TBA, which showed significant elevations at 15 minutes after addition of 5.0 mM H_2O_2. The cells released approximately 5–8% of the tritium label when stimulated with calcium ionophore, and approximately 0.8–1% when maximally stimulated with H_2O_2. All [^{3}H]arachidonic acid release results are given as percent control of that particular experimental plate and as mean ± SEM of quadruplicate determinations performed at least three times.

HPLC analysis of released arachidonic acid

The purity of the released arachidonic acid in the extracellular medium after cell stimulation was assessed using a modification of the HPLC methods outlined by Peters *et al.* [29]. Briefly, 200 μl of the extracellular medium was removed from the plate and injected directly onto the HPLC. The chromatographic conditions were as follows: isocratic mobile phase of 30% acetonitrile, 70% H_2O with 0.05% acetic acid at 1.0 ml/min using a CT-sil C18, 250 × 4.6 mm, 5 μm particle size column (ChromTech, Norsborg, Sweden). Under these conditions, arachidonic acid had a retention time of approximately 20 minutes. Radioactivity in the effluent was measured by collection of 1 minute fractions.

Measurement of intracellular calcium

The measurement of intracellular calcium fluxes using the fluorescent calcium probe FURA2 was essentially according to the methods of Wickham *et al.* [30]. Briefly, CPAE cells, after trypsinization, were diluted to approximately 5×10^4 cells/ml, and 0.1 ml was carefully applied to a glass slide (10 mm × 40 mm). The cells were

allowed to attach to the glass for 4 to 8 hours, at which time a sufficient volume of growth medium was added to completely cover the glass slides. The cells were then allowed to grow to confluence. To load the cells with FURA2, each slide was immersed in a volume of 3.5 ml of growth medium containing 5 μM FURA2/AM for 20–30 minutes at 37°C. The glass slide was then removed from the medium, washed four times in HHBSS, and used immediately. The intracellular fluorescence of FURA2 was determined with a Sigma ZFP22 fluorometer. The exposure medium in all experiments was HHBSS (1.3 mM Ca^{2+}). For the intracellular chelation of calcium using BAPTA, BAPTA/AM (in 1 μl of DMSO) was added to the growth medium to a final concentration of 20 μM 30 min prior to washing and treatment of the cells. Cells were kept at 25°C in room air during BAPTA/AM loading. All experiments using BAPTA were carried out in 24 well plates.

Statistical analysis

Comparisons between treatment groups were carried out by the analysis of variance, followed by a *post hoc* Student's *t*-test. When converted to percent control, the data were checked to ensure that they were normally distributed around the mean. All values are presented as the mean ± SEM.

RESULTS

Dose-response and time course of arachidonate release

The time course of arachidonate release is given in Fig. 1. From the data it is apparent that extracellular arachidonic acid concentrations increase in response to 100 μM H_2O_2 during the first 10 minutes after exposure. Maximal release of arachidonic acid after treatment of the cells with calcium ionophore A23187 results in a somewhat more rapid release, which reaches a plateau after approximately 5 minutes. Treatment of the cells with 500 nM TPA results in a time course that appears both qualitatively and quantitatively very similar to that produced by hydrogen peroxide. Treatment of the cells with H_2O_2 and TPA resulted in a release of 0.8–1.0% of the incorporated arachidonate label at 15 minutes, whereas treatment with A23187 resulted in a release of 5–8% of the incorporated label. In all experiments, greater than 98% of the radioactivity in the extracellular fluid was associated with intact arachidonic acid. This was assessed by direct HPLC analysis of the extracellular medium after 15 minutes in either control, hydrogen peroxide-treated, or A23187-treated wells (Fig. 2). Significant elevations in the extracellular arachidonic acid concentrations were produced by the addition of as little as 50 μM hydrogen peroxide after 15 minutes, with a maximal response produced by 100 μM (Fig. 3). Equivalent release of arachidonic acid was seen between 100 and 500 μM, with a slight decrease appearing at 1.0 mM. Thus, unless otherwise specified, all subsequent treatments with hydrogen peroxide were at 100 μM.

Involvement of activated oxygen species in the release of arachidonic acid from endothelium

Figure 4 gives the arachidonic acid release response to 100 μM hydrogen peroxide in the presence of a variety of agents that interfere with various aspects of the generation of hydroxyl radical and/or lipid peroxidation. DPPD and T-2, two potent antioxidants that partition to cellular membranes and have been shown to prevent lipid peroxidation at or below the concentrations used in this study [31], failed to prevent hydrogen peroxide-stimulated arachidonic acid release. Chelation of Fenton-reactive transition metals either by the use of desferal (1.0 mM for one hour) or the more cell-permeable chelator dipyridyl (100 μM for 20 minutes) also failed to inhibit hydrogen peroxide-mediated arachidonate release. Finally, pretreatment of the cells with 20 mM mannitol for 30 minutes to scavenge hydroxyl radical also proved ineffective in preventing arachidonic acid release. Also shown in Fig. 4 are the results of pretreating the cells with the phospholipase inhibitor quinacrine [32]. Pretreatment of the cells with 50 μM quinacrine both reduced the control levels of arachidonic acid release and completely eliminated the stimulation of release after hydrogen peroxide treatment. Interestingly, treating the cells with the nonenzymatic thiol oxidant diamide (1–500 μM) did not result in arachidonic acid release. Although this result suggests that thiol oxidation is not involved in hydrogen peroxide-mediated arachidonic acid release, it has recently been shown that dia-

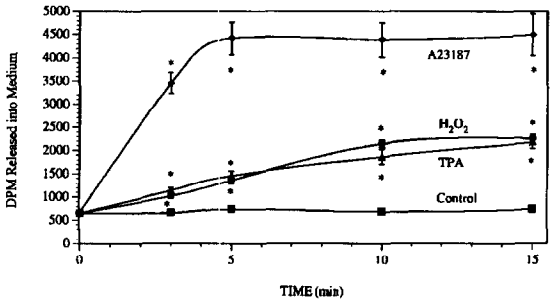

Fig. 1. Time course of arachidonic acid release from CPAE cells stimulated with 100 μM hydrogen peroxide (closed circles), TPA (closed triangles), or calcium ionophore A23187 (closed diamonds). Control release over the 15-minute time course is also shown (closed squares) and did not change significantly from the zero time point. Values represent the mean ± SEM for 4–6 determinations done on separate CPAE cultures. Values significantly different ($p < 0.01$) from those of the controls at that particular time point are marked with an asterisk.

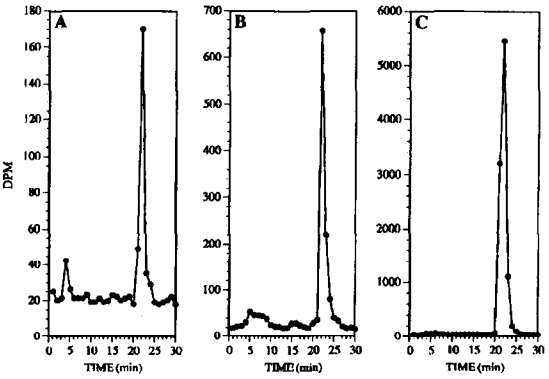

Fig. 2. HPLC analysis of the extracellular medium of CPAE cells loaded with [^{3}H]arachidonic acid. Medium was (A) removed from control, (B) hydrogen peroxide-exposed (500 μM), and (C) calcium ionophore A23187-exposed 15 minutes after addition, and immediately injected onto the HPLC. Chromatographic conditions are given in the Methods section.

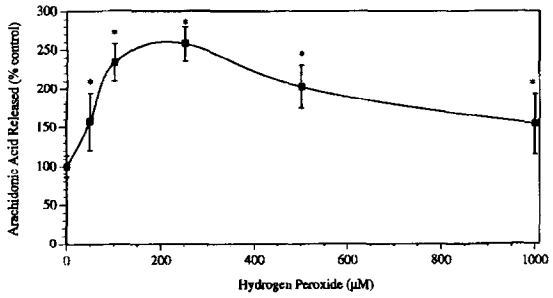

Fig. 3. Dose-response relationship of hydrogen peroxide concentration and arachidonic acid release from CPAE cells. Arachidonic acid release was assessed at 15 min after addition of stimulant. Values are expressed as the mean ± SEM of the percent control (n = 4–6). Control values were determined in quadruplicate for each 24-well plate and used as the control reference only for the treatment wells from that plate. Values significantly different ($p < 0.01$) from the untreated controls are marked with an asterisk.

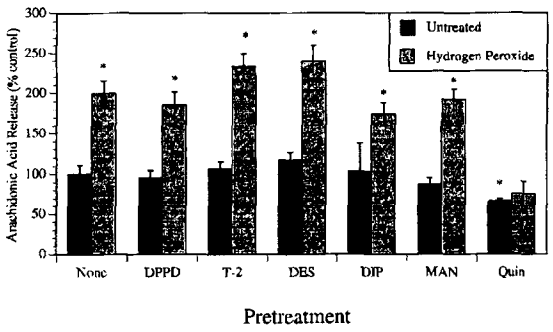

Fig. 4. Effect of antioxidant, metal chelator, oxygen radical scavenger, and PLA_2 inhibitor pretreatment on the hydrogen peroxide-stimulated release of arachidonic acid from CPAE cells. The dependence of arachidonic acid release on radical-mediated processes in CPAE cells stimulated with 100 μM hydrogen peroxide was assessed by pretreatment with the antioxidants DPPD (50 μM for 10 min) and T-2 (10 μM for 10 min), the metal chelators desferal (DES; 1.0 mM for 60 min) and dipyridal (DIP; 100 μM for 20 min) and 20 mM mannitol (MAN) for 30 min. No significant effect on arachidonic acid release was seen with the pretreatments listed above. Pretreatment of the cells with the PLA_2 inhibitor quinacrine (Quin; 50 μM for 10 min) completely inhibited hydrogen peroxide-stimulated arachidonic acid release. Arachidonic acid release was assessed at 15 min after addition of stimulant. Values are expressed as the mean ± SEM of the percent control (non-pretreated, non-hydrogen peroxide-treated) (n = 4–6). Non-hydrogen peroxide-treated control values were determined in quadruplicate for each 24-well plate and used as the control reference only for the treatment wells from that plate. Values marked with an asterisk are significantly different ($p < 0.01$) from the non-hydrogen peroxide-treated controls within that treatment, except in the case of the quinacrine-pretreated, non-hydrogen peroxide-treated controls, which were compared to the non-pretreated, non-hydrogen peroxide-treated controls and were significantly different only at the $p < 0.05$ level. None of the pretreatment regimens resulted in significant elevations in medium LDH activity, and no significant differences were observed between the non-hydrogen peroxide-treated control values from the various pretreatments and the non-pretreated, non-hydrogen peroxide-treated control value.

mide and hydrogen peroxide do not result in mixed disulfide formation in exactly the same proteins [33], and thus, the role of thiol oxidation in PLA_2 activation remains unclear.

Specificity of the release of sn-2 *fatty acids by hydrogen peroxide*

The inhibition of hydrogen peroxide-stimulated release of arachidonic acid in the CPAE cell by the phospholipase inhibitor quinacrine indicates that this release is indeed mediated by a phospholipase A_2. Some cells contain both a low molecular weight (12–14 KDa) secreted phospholipase ($sPLA_2$) and a high molecular weight (85 KDa) intracellular phospholipase ($cPLA_2$). Since it is thought that the only phospholipase A_2 form in the cell responsible for signal-mediated arachidonic acid mobilization is the $cPLA_2$ [7, 8], it would be of interest to determine the identity of the phospholipase species activated by hydrogen peroxide. To investigate the identity of the phospholipase A_2 species responsible for hydrogen peroxide-stimulated arachidonic acid release, the property of fatty acid specificity at the *sn*-2 position was utilized. The $sPLA_2$ is rather nonspecific at the *sn*-2 position of phospholipids, cleaving essentially any species of fatty acyl side chain, whereas the $cPLA_2$ is somewhat specific for arachidonic acid [3, 9, 10]. Thus, if hydrogen peroxide treatment of the cells resulted in the release of another fatty acid species, such as oleic acid, it could then be said that hydrogen peroxide activates the $sPLA_2$ and possibly the $cPLA_2$. From the data shown in Fig. 5, hydrogen peroxide treatment of the CPAE cells does not result in the release of preloaded oleate, whereas the $sPLA_2$ stimulatory peptide melittin [34, 35] stimulates the release of either arachidonate or oleate. These results indicate that it is a stimulation of the activity of the $cPLA_2$ form that is responsible for hydrogen peroxide-mediated arachidonic acid mobilization, thereby raising the possibility that hydrogen peroxide activates the $cPLA_2$ by interacting with some component of the signal transduction system that is responsible for the control of $cPLA_2$ activity.

Hydrogen peroxide treatment and intracellular calcium levels

Oxidants have been shown to increase intracellular calcium concentrations in a number of systems (re-

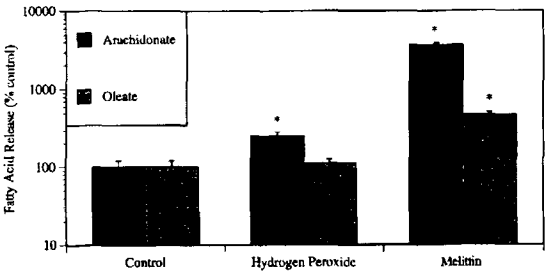

Fig. 5. Release of arachidonic acid or oleic acid from CPAE cells after hydrogen peroxide and melittin treatment. The identity of the PLA_2 species stimulated by hydrogen peroxide in CPAE cells was investigated by loading the cells with 0.1 μCi of either [^{3}H]arachidonate or [^{3}H]oleate for 20–24 hours and treating them with either 100 μM hydrogen peroxide or 200 ng of melittin, a venom-derived $sPLA_2$ stimulatory peptide. Arachidonic acid release was assessed at 15 min after addition of stimulant. Values are expressed as the mean ± SEM of the percent control (n = 4–6). Control values were determined in quadruplicate for each 24-well plate, and used as the control reference only for the treatment wells from that plate. Values significantly different ($p < 0.01$) from the control value for that particular fatty acid are marked with an asterisk.

viewed in ref. 36). Since the $cPLA_2$ is responsive to physiologically relevant calcium concentrations, and since enhancement of intracellular calcium concentration by receptor-mediated pathways or by ionophore treatment results in the release of arachidonic acid, a potential mechanism of hydrogen peroxide stimulation of the $cPLA_2$ is by enhancement of intracellular calcium levels. Direct measurement of intracellular calcium levels using FURA2-loaded CPAE cells grown on glass coverslips indicates that even after treatment with 500 μM hydrogen peroxide, no enhancement in intracellular calcium levels could be observed during the first 15 minutes following exposure to the oxidant (Fig. 6). Finally, hydrogen peroxide-stimulated release of arachidonic acid was not affected by preloading the cells with the intracellular calcium chelator BAPTA. Percent control arachidonic acid release values in the presence of 100 μM H_2O_2 in BAPTA-loaded cells were 220 ± 23 (n = 3); compared to 263 ± 12 (n = 3) (not significantly different) in nonpretreated cells exposed to 100 μM H_2O_2 on the same plates. Thus, from these observations it appears that hydrogen peroxide does not stimulate the activity of the $cPLA_2$ in CPAE cells by increasing intracellular calcium concentrations.

Activation of protein kinases by hydrogen peroxide

$cPLA_2$ can be activated by phosphorylation either by protein kinase C or by MAP2 kinase. Oxidants such as hydrogen peroxide have been shown to have stimulatory effects on the protein kinase C system [37–39]. Thus, activation of $cPLA_2$ indirectly by stimulation of intracellular kinase activity was investigated in the CPAE cells. Hydrogen peroxide-stimulated release of arachidonic acid was inhibited in a dose-dependent fashion by the PKC inhibitors staurosporine and Ro-31 8220 [40] (Fig 7.) As shown in Fig. 8, down regulation of PKC by chronic TPA treatment also significantly affected hydrogen peroxide-stimulated arachidonic acid release. The effect of chronic TPA treatment, while apparently complete for TPA-stimulated arachidonic acid release, only partially blocks hydrogen peroxide-stimulated release. This result suggests the possibility of the involvement of another kinase system in the hydrogen peroxide response. The involvement of cyclic AMP-dependent ki-

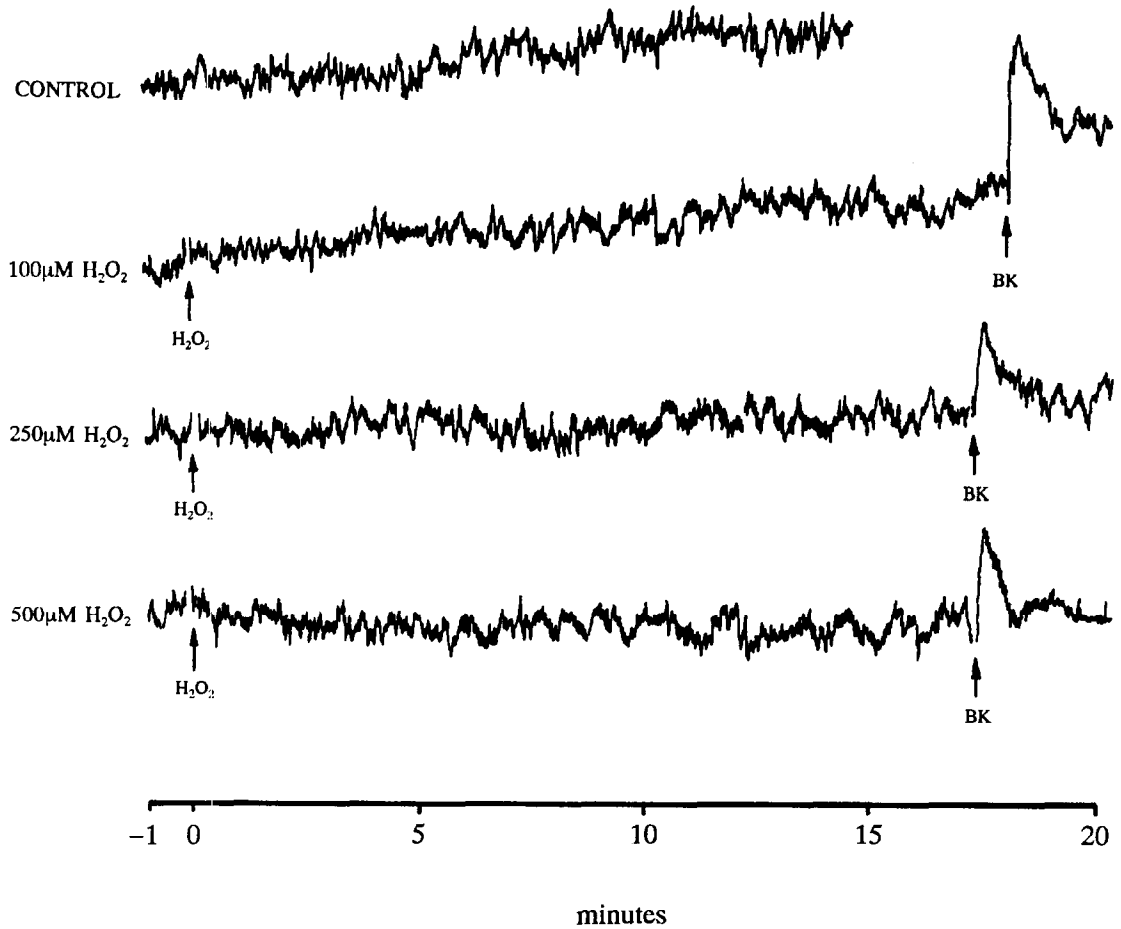

Fig. 6. Intracellular calcium levels in CPAE cells in the presence of hydrogen peroxide. Traces are representative of the typical response. Cells were grown to confluence on glass coverslips and loaded with the calcium indicator FURA2/AM, washed three times in HHBSS, and placed diagonally in a 3 ml quartz cuvette with constant stirring. At the indicated times, the indicated concentrations of hydrogen peroxide and 1 μM bradykinin (BK) were added. The resting $[Ca^{2+}]_i$ was 110 nM ± 20 (n = 5–10 for each concentration) and did not change significantly over the 15 min time course in cells treated with ≤500 μM hydrogen peroxide. The mean $[Ca^{2+}]_i$ after BK addition was 340 nM ± 50 (n = 25). The bradykinin response did not change significantly after hydrogen peroxide treatment at any of the concentrations used in this study.

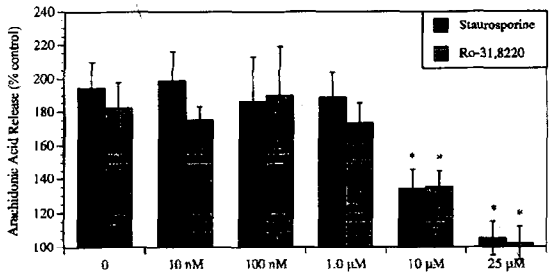

Fig. 7. Effect of the protein kinase C inhibitors staurosporine and Ro-31 8220 on hydrogen peroxide-stimulated arachidonic acid release from CPAE cells. [^{3}H]arachidonate-loaded cells were washed and incubated for 30 minutes in the presence of the inhibitor prior to the addition of 100 μM hydrogen peroxide. The data show a dose-dependent inhibition of AA release in the presence of either PKC inhibitor. Arachidonic acid release was assessed at 15 min after addition of stimulant. Values are expressed as the mean ± SEM of the percent control (n = 4–6). Control values were determined in quadruplicate for each 24-well plate and used as the control reference only for the treatment wells from that plate. Values significantly different (p < 0.01) from the nonpretreated control values within that pretreatment category are marked with an asterisk.

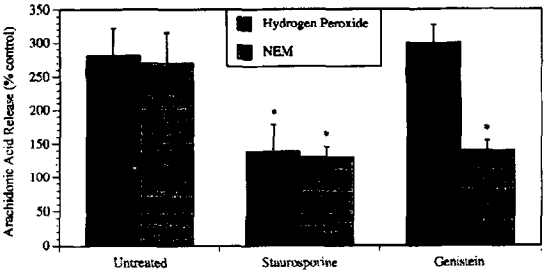

Fig. 9. Effect of the tyrosine kinase inhibitor genistein on hydrogen peroxide- and N-ethylmaleimide (NEM)-stimulated arachidonic acid release. Pretreatment of the cells with 10 μM staurosporine for 30 min inhibited AA release by either hydrogen peroxide or NEM, whereas a 30-min pretreatment with 10 μM of the tyrosine kinase inhibitor genistein inhibited only NEM-stimulated release. Arachidonic acid release was assessed at 15 min after addition of stimulant. Values are expressed as the mean ± SEM of the percent control (n = 4–6). Control values were determined in quadruplicate for each 24-well plate, and used as the control reference only for the treatment wells from that plate. Values significantly different (p < 0.01) from the nonpretreated control values within that pretreatment category are marked with an asterisk.

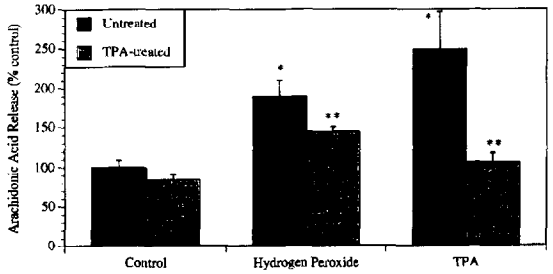

Fig. 8. Effect of protein kinase C down-regulation by chronic TPA treatment on hydrogen peroxide- and TPA-stimulated arachidonic acid release from CPAE cells. PKC activity was down-regulated by treatment of the cells with 200 nM TPA for 24 hours prior to hydrogen peroxide (100 μM) or TPA (1 μM). Arachidonic acid release was assessed at 15 min after addition of stimulant. Values are expressed as the mean ± SEM of the percent control (n = 4–6). Control values were determined in quadruplicate for each 24-well plate and used as the control reference only for the treatment wells from that plate. Values significantly different (p < 0.01) from the control values for that particular pretreatment category are marked with an asterisk, and values significantly different from those within the particular treatment category are marked with a double asterisk.

nase arachidonic acid release was investigated by the addition of the cell-permeable cAMP analog dibutyryl cAMP to the cells. Treatment of the cells with 1–100 μM dibutyryl-cyclic AMP did not result in arachidonic acid release in these cells (data not shown). Thus, it appears unlikely that hydrogen peroxide could enhance intracellular cPLA$_2$ activity by increasing intracellular cAMP levels. Figure 9 shows that treatment of the cells with tyrosine kinase inhibitor genistein did not affect the hydrogen peroxide-mediated release of arachidonic acid, but interestingly, when the thiol alkylator N-ethyl maleimide (NEM) was applied to the cells, they responded by releasing arachidonic acid with this response being completely abolished by pretreatment of the cells with genistein. While these data suggest that hydrogen peroxide does not act through a tyrosine kinase, it is interesting that the response to a thiol alkylator can be blocked by a tyrosine kinase inhibitor. Stimulatory effects of NEM on tyrosine kinase activity have been reported recently [41], and characterization of this effect in the endothelial cell suggests that NEM-stimulated arachidonic acid mobilization does not require calcium mobilization, and that arachidonic acid release can be stimulated with thiol reagents that do not pass the plasma membrane [42].

DISCUSSION

It is clear from previous reports that exposure of cells to a variety of extracellular oxidants can result in the release of arachidonic acid [24–28]. Since the production of free arachidonic acid is the rate-limiting step in the production of eicosanoids [1], the initial intracellular mobilization of arachidonic acid in response to exogenous oxidants is an important factor in the development of inflammatory loci. An excessive production of eicosanoids at an inflammatory site could result in the recruitment of additional inflammatory cells to the original site and the further enhancement of local oxidative damage. The present investigation has attempted to address in a somewhat systematic manner the various mechanisms that could be involved in the release of arachidonic acid from the cell under oxidative stress: oxidant-mediated lipid peroxidation of cellular membranes followed by the stimulation of a nonspecific 'maintenance' phospholipase (sPLA$_2$) [9, 43]; an oxidant-mediated rise in intracellular calcium [35, 36]; or oxidants acting as intracellular signals that could stimulate the activity of the signal-sensitive phospholipase A$_2$.

The recent characterization of the high molecular weight cytosolic phospholipase A$_2$ (cPLA$_2$) has raised the possibility of a stimulation of its activity by oxidants either directly or by the interaction of oxidant with other components of the intracellular signaling system. Thus, the basic determination to be made about an oxidant is whether it stimulates the sPLA$_2$ or the cPLA$_2$. Since it is a form of sPLA$_2$ that has been shown to hydrolyze per-

oxidized lipids [44], one important issue is the contribution of oxidant-mediated lipid peroxidation in the observed liberation of arachidonic acid from cellular phospholipids. From the data presented in Fig. 4, the use of a variety of agents that interfere with radical-mediated lipid peroxidation either by acting as electron acceptors, metal chelators (which remove Fenton-active transition metals), or direct hydroxyl radical scavengers is ineffective in inhibiting hydrogen peroxide-mediated arachidonic acid release. Thus, in this system it appears that radical-mediated processes are not involved in the release of arachidonic acid from endothelium exposed to hydrogen peroxide.

It has been suggested that once the polyunsaturated fatty acyl side chains of phospholipids are peroxidized, they assume a conformation that would 'flag' them for removal by phospholipases and further degradation or repair [43, 44]. Although it is not certain which form of phospholipase would carry out this function in the intact cell, it has been shown that forms of the $sPLA_2$ are capable of removing peroxidized fatty acyl side chains; this function is thought of as a general 'housekeeping' function [44]. If this process were taking place in the endothelial cells treated with hydrogen peroxide used in this study, one would expect to be able to make two observations. First, not only would free arachidonic acid be found in the extracellular medium, but also some more polar oxidation products would appear. Since peroxidized fatty acids would be more polar than arachidonic acid, these species would be seen at retention times below that of arachidonate. From the RP-HPLC analysis of the extracellular medium after hydrogen peroxide exposure, it is apparent that no other fatty acid species are released, thereby indicating that liberation of peroxidized fatty acids is not the cause of the appearance of tritium label in the extracellular medium. Second, if $sPLA_2$ played a significant role in the maintenance of a peroxidized membrane, it would undoubtedly release some unmodified fatty acids from the *sn*-2 position as well [44]. By loading the cells with oleic acid incorporated into phospholipids at the *sn*-2 position, the activation of the nonspecific $sPLA_2$ can be tested. From the data in Fig. 5, it is apparent that hydrogen peroxide does not stimulate the release of oleic acid, suggesting that activation of the $sPLA_2$ is not necessary to liberate arachidonic acid from the endothelium. These results taken together would indicate that arachidonic acid is liberated intact from endothelial cells, and that this liberation is not mediated by the nonspecific $sPLA_2$, suggesting a role for the $cPLA_2$ in the hydrogen peroxide-mediated arachidonic acid release from these cells.

The exposure of cells to a variety of oxidant species eventually leads to increases in intracellular calcium levels in a number of cell types. This is probably due to a number of phenomena, including direct damage to the barrier capacity of the membrane, inhibition of plasma membrane and endoplasmic reticulum calcium pumps, and possibly damage to the calcium retention properties of the mitochondria as well [36]. Recent investigations have reported transient calcium increases with relatively low oxidant concentrations [45]. The transient rises in calcium provoked in certain cell types appear to be sufficient to mimic a receptor-mediated calcium mobilization which, in turn, could result in the stimulation of $cPLA_2$ activity. Thus, it was important in the context of the present study to determine whether calcium could contribute to the observed hydrogen peroxide-mediated arachidonic acid mobilization. From measurements of calcium influx and intracellular calcium concentrations after hydrogen peroxide, it appears that the hydrogen peroxide concentrations used with this cell type were not adequate to result in rises in intracellular calcium in the time course of these experiments (15 minutes). Additionally, when these cells were loaded with the intracellular calcium chelator BAPTA, no effect was seen on the time course (data not shown) or on the absolute amount of arachidonic acid released (data summarized in Results section). Hence, in the context of the present system, oxidant-mediated intracellular calcium increases are not necessary to cause arachidonic acid release from endothelium.

Characterization of the signaling systems responsible for the regulation of intracellular $cPLA_2$ activity indicate several overlapping pathways that can result in differing degrees of arachidonic acid release. Recent investigations have focused on the activation of $cPLA_2$ by phosphorylation catalyzed by either protein kinase C or by the MAP2 kinase [4–7]. A stimulation of PKC and MAP2 kinase activity via a number of pathways including phospholipase C, G-proteins, and stimulation of tyrosine kinase activity appears to be possible [46]. A variety of oxidant species have been shown to stimulate intracellular kinase activity [37–39, 47–52], and a functional link between oxidant-mediated kinase stimulation and stimulation of intracellular PLA_2 activity has been suggested previously [26, 27, 49]. Due to the observed inhibition of hydrogen peroxide-mediated arachidonic acid release by the PKC inhibitors staurosporine and Ro-31 8220 and by chronic TPA pretreatment, our results would support the role of kinase activation and subsequent activation of $cPLA_2$ in the endothelium after hydrogen peroxide exposure. Finally, activation of phospholipase C by agents that stimulate lipid peroxidation has been reported [51, 53, 54] and could account for some of the stimulatory effects on either PKC.

In contrast to stimulation of PLA_2, oxidants and thiol-reactive agents have been implicated in the induction of an imbalance in the acylation/deacylation cycle of arachidonic acid by inhibiting the reacylation step [55–59]. Convincing evidence has recently been presented indicating that the mechanism of hydrogen peroxide-mediated arachidonic acid release is through an inhibition in the reacylation pathway of free arachidonate [59]. It was suggested that this effect was not due to a direct inhibition of the enzymes involved in reacylation, but to an intracellular depletion of ATP, which is required in the synthesis of the arachidonate reacylation intermediate, arachidonyl CoA [59]. No increase in PLA_2 activity was seen in a whole-cell homogenate 30 minutes after hydrogen peroxide treatment. This, combined with the authors' previous findings that glucocorticoid pretreatment of the cells does not inhibit hydrogen peroxide-mediated arachidonic acid release [25], leads them to conclude that activation of PLA_2 is not involved in this process. Our findings using the PLA_2 inhibitor quinacrine are clearly inconsistent with this view. Besides differences in cell types between these studies [25, 59] and the present study, previous characterization of the agonist-stimulation of intracellular PLA_2 activity indicated that stimulation is relatively short-lived, with the stimulation in activity returning to control levels by 10 minutes [60]; therefore, at 30 minutes, little stimulation

would be expected. Additionally, the lack of an inhibition of the hydrogen peroxide response by glucocorticoid pretreatment is not definitive proof of a lack of PLA_2 stimulation, because the exact form(s) of PLA_2 that are inhibited by glucocorticoid pretreatment are not known [61]. It is possible that both mechanisms could be operating in hydrogen peroxide-exposed cells: early (0–15 minutes) stimulation of $cPLA_2$ (present study) followed by depression of the reacylation pathways.

In summary, the present study has investigated the mechanism of hydrogen peroxide-stimulated arachidonic acid mobilization in the endothelial cell. Arachidonic acid release is mediated by phospholipase A_2 and, based on the oleic acid versus arachidonic acid release results, it appears that hydrogen peroxide specifically stimulates the activity of the high molecular weight, signal-responsive PLA_2 rather than the low molecular weight secreted form. It was found that manipulations of the antioxidant and radical scavenging capacity of the cells as well as removal of free iron by chelators did not affect the response to hydrogen peroxide. Additionally, it appears that at the doses of hydrogen peroxide used in these studies, enhancement in intracellular calcium levels does not take place. Inhibition of the serine/threonine kinases by either staurosporine or Ro-31 8220 resulted in a dose-dependent decrease in the activation of PLA_2 by hydrogen peroxide in these cells. Thus, it appears that hydrogen peroxide acts as a specific messenger in this system, acting not through the more general mechanisms of lipid peroxidation or calcium disturbances, but rather via a stimulation of intracellular kinase activity. The results of this study would indicate, however, that PKC is not exclusively responsible for the activation of intracellular phospholipase activity. Clearly, the direct determination of the phosphorylation state of the PLA_2 before and after hydrogen peroxide addition and a more definitive identification of the kinase(s) activated by hydrogen peroxide are critical questions in the resolution of the mechanism of this effect.

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