Hydrogen peroxide-induced c-fos expression is mediated by arachidonic acid release: role of protein kinase C

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ABSTRACT
We found previously that stimulation of c-fos and c-myc mRNA expression are early events in hydrogen peroxide-induced growth in rat aortic smooth muscle (RASM) cells. In the present study, we investigated the role of phospholipase A₂ (PLA₂) and protein kinase C (PKC) in mediating hydrogen peroxide-induced c-fos mRNA expression in RASM cells. Mepacrine and p-bromophenacylbromide, potent inhibitors of PLA₂ activity, blocked hydrogen peroxide-induced c-fos mRNA expression. Arachidonic acid, a product of PLA₂ activity, stimulated the expression of c-fos mRNA with a time course similar to that of hydrogen peroxide. PKC down-regulation attenuated both hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression by 50%. Nordihydroguaiaretic acid (a lipoxygenase-cytochrome P450 monooxygenase inhibitor) significantly inhibited both hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression, whereas Indomethacin (a cyclooxygenase Inhibitor) had no effect. Together, these findings indicate that 1) hydrogen peroxide-induced c-fos mRNA expression is mediated by PLA₂-dependent arachidonic acid release, 2) both PKC-dependent and independent mechanisms are involved in hydrogen peroxide-induced expression of c-fos mRNA and 3) arachidonic acid metabolism via the lipoxygenase-cytochrome P450 monooxygenase pathway appears to be required for hydrogen peroxide-induced expression of c-fos mRNA.

INTRODUCTION
Work from other laboratories suggested that oxidants stimulate growth in several cell types (1). For example, Ellem and Kay (2) have demonstrated that ferricyanide stimulates the growth of melanoma cells. Ferric sulfate has also been shown to stimulate growth of 3T3 cells (3). In addition, Murrell et al. (4) have reported that active oxygen species stimulate proliferation of human skin fibroblasts. It has become clear that active oxygen species stimulate early growth signals typical of mitogens including induction of c-fos and c-myc mRNA expression and Na⁺/H⁺ antiporter activity (5—8). These oxidants also induce phosphorylation of a major ribosomal protein S6, whose phosphorylation has been shown to be required for growth competence in response to a variety of growth factors (9—12). In addition, the cellular redox state regulates the activity of ion channels (13), nuclear factors (14—17) and protein tyrosine kinases (18). These observations, in combination with the findings that many anticancer drugs inhibit the plasma membrane redox system (19,20), suggest that the cellular redox system may be an important element in the control of cell growth.

A role for oxidative stress in tumor promotion and atherogenesis has been proposed previously (21—23). Increased concentrations of active oxygen species (O₂⁻, H₂O₂, ·OH) have been reported in blood vessels and myocardium in response to a variety of injury-related conditions such as ischemia, thrombosis and angioplasty (24—25). These same circumstances are associated with vascular smooth muscle hyperplasia and accelerated atherosclerosis. In our studies of the relationship between oxidative stress and atherogenesis, we reported earlier that hydrogen peroxide, a cellular oxidant and an active oxygen species, induces protooncogene expression and replicative DNA synthesis in rat aortic smooth muscle (RASM) cells (26). In the present work, we have extended our studies to the mechanisms by which hydrogen peroxide induces the expression of c-fos mRNA, an early growth responsive gene whose steady-state mRNA levels have been shown to be increased acutely in response to a variety of growth stimuli (27—30). Here, we demonstrate for the first time that hydrogen peroxide induces c-fos mRNA expression via phospholipase A₂-dependent activation of protein kinase C in RASM cells.

MATERIALS AND METHODS
Materials
Arachidonic acid, indomethacin, mepacrine, p-bromophenacylbromide (pBPB), phorbol 12,13-dibutyrate (PDBu) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St Louis, MO). Nordihydroguaiaretic acid (NDGA) was purchased from Aldrich (Milwaukee, WI). [³H]-arachidonic acid was from NEN (Boston, MA).
Cell culture

Vascular smooth muscle cells were isolated from the thoracic aortae of 200-250 g male Sprague-Dawley rats by enzymatic dissociation as described elsewhere (31). Cells were grown in Dulbecco's Modified Eagle's (DME) medium, supplemented with 10% (v/v) heat-inactivated calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in humidified 95% air-5% CO2 at 37°C. For all experiments, cells at 70-80% confluence were made quiescent by incubation in fresh DME medium containing 0.4% calf serum for 72 hours. Throughout the course of these experiments, cells were used at passage numbers 8-15.

RNA blot analysis

Total cellular RNA from quiescent and agonist-treated RASM cells was isolated by the guanidine isothiocyanate-cesium chloride protocol of Chirgwin et al. (32). Equal amounts of total RNA (20 μg) from quiescent and agonist-treated cells were size-fractionated on 1% (w/v) agarose gel in 25 mM MOPS buffer (pH 7.8), containing 1 mM EDTA and 2% (w/v) formaldehyde. RNA was transferred to a Nytran membrane (Schleicher & Schuell, Inc., Keene, N.H.), according to the method of Thomas (33). RNA was cross-linked to the membrane using UV irradiation (Stratalinker, Stratagene, La Jolla, CA). The cDNAs (an EcoRl and SstI fragment of 1.2 kb mouse c-fos cDNA and a full length rat glyceraldehyde 3-phosphatedehydrogenase (GAPDH) cDNA) were labeled with [α-32P]dCTP using a BRL random primer labeling kit per the manufacturer's protocol. After a 4 hour prehybridization in 50% (v/v) formamide, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's (1× Denhardt's = 0.02% (w/v) each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 50 mM sodium phosphate (pH 6.5) and 250 μg/ml of sheared salmon sperm DNA at 42°C, the Nytran membrane was hybridized in the above buffer containing 10% (w/v) dextran sulfate and 1×106 cpm/ml of probe for 16 hours at 42°C. The membrane was washed three times in 2× SSC, 0.2% SDS (15 min at 25°C) and twice in 0.1× SSC, 0.1% SDS (15 min at 60°C). The membrane was then exposed to Kodak X-Omat AR X-Ray film with an intensifying screen at -70°C for 4 to 6 hours. Densitometric analysis of the autoradiograms exposed in the linear range of film density was made on a Pharmacia Ultrascan XL laser densitometer.

RESULTS

Hydrogen peroxide (200 μM) stimulated c-fos mRNA expression in a time-dependent manner (Fig. 1). Maximal increases (40-fold) in the levels of c-fos mRNA occurred 40 min after the addition of hydrogen peroxide and by 2 hours of treatment, the steady-state c-fos mRNA levels returned to baseline (Fig. 1). Hydrogen peroxide had no effect on GAPDH mRNA levels.

c-fos expression can be modulated by several intracellular second messengers. In other systems (35) H2O2 has been shown to activate PLA2, resulting in the release of arachidonic acid, an important second messenger. To determine whether H2O2 also activates PLA2 in RASM cells, we measured the release of [3H]-arachidonic acid from pre-labeled cells stimulated with H2O2 (200 μM, 1h). As shown in Figure 2, hydrogen peroxide stimulated the release of [3H]-arachidonic acid over control by 8-fold. Since activation of several phospholipases, including phospholipase A2 and C, may result in the release of [3H]-arachidonic acid (35), we studied the effect of mepacrine,
a potent inhibitor of phospholipase A2 activity (36,37), on hydrogen peroxide-stimulated release of [3H]-arachidonic acid. Mepacrine (5 µM) inhibited the hydrogen peroxide-induced release of [3H]-arachidonic acid by 80% (Fig. 2). This result indicated that the hydrogen peroxide-stimulated release of [3H]-arachidonic acid was primarily due to activation of phospholipase A2.

To determine whether hydrogen peroxide-induced c-fos mRNA expression is due to activation of PLA2, PLA2 activity was blocked with mepacrine, and c-fos mRNA expression was measured in quiescent RASM cells stimulated with hydrogen peroxide for 40 min. Equal amounts (20 µg) of total RNA from each condition were then analyzed by Northern blotting for c-fos mRNA levels. As shown in Figure 3A, mepacrine completely abolished the hydrogen peroxide-induced c-fos mRNA expression, a result which indicates that hydrogen peroxide-stimulated c-fos mRNA expression was due to activation of phospholipase A2.

To prove that the effect of mepacrine on hydrogen peroxide-induced c-fos mRNA expression is specific, we studied its effect on platelet-derived growth factor (PDGF)-stimulated expression of c-fos mRNA levels in growth-arrested RASM cells. As is shown in Figure 3B, mepacrine did not block the PDGF-induced c-fos mRNA expression. To gain further evidence that hydrogen peroxide-stimulated expression of c-fos mRNA in growth-arrested RASM cells was mediated by PLA2 activation, we also studied the effect of another potent PLA2 inhibitor, namely p-bromophenacylbromide (pBPB) (38) on hydrogen peroxide-induced c-fos mRNA. pBPB (5 µM) also significantly inhibited hydrogen peroxide-induced expression of c-fos mRNA in growth-arrested RASM cells (Figure 3C). None of the above treatments changed the mRNA levels for GAPDH. Together, these results clearly indicate that hydrogen peroxide-induced c-fos mRNA expression is PLA2-dependent.

Because arachidonic acid is one of the principal products of PLA2 activity, we examined the effect of arachidonic acid (25 µM) on c-fos mRNA expression. As shown in Fig. 4, arachidonic acid induced c-fos mRNA with a time course and magnitude similar to that of hydrogen peroxide (compare with Fig. 1). We have previously shown that hydrogen peroxide-induced c-fos mRNA expression was in part due to protein kinase C activation (26). To prove that arachidonic acid-induced c-fos mRNA expression was also mediated by PKC, cells were growth-arrested and PKC was down-regulated by treatment for 48 hours with 200 nM phorbol 12,13-dibutyrate (PDBu) prior to stimulation with hydrogen peroxide or arachidonic acid. Prolonged PDBu treatment has been shown to significantly down-regulate PKC in RASM cells (39), as well as in other cell types (40,41). Northern blot analysis revealed that both hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression was blocked by ~50% in the PDBu-pretreated cells (Fig 5). These findings indicate that both hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression is in part mediated by PKC.

Figure 3. Mepacrine and p-bromophenacylbromide (pBPB) inhibition of hydrogen peroxide-induced c-fos mRNA expression. Growth-arrested RASM cells were treated with 200 µM H2O2 or PDGF-AB (10 ng/ml) for 40 min with or without 5 µM mepacrine or pBPB and total cellular RNA was isolated. Equal amounts of RNA (20 µg/lane) were analyzed for c-fos transcripts as described in Figure legend 1.

Figure 4. Northern blot analysis showing the time course of arachidonic acid-induced c-fos and GAPDH mRNA expression in RASM cells. Conditions were the same as in Figure legend 1, except that the cells were treated with 25 µM arachidonic acid (AA) for the indicated times.

Figure 5. Effect of protein kinase C down regulation on hydrogen peroxide and arachidonic acid induction of c-fos mRNA. Growth-arrested RASM cells were treated as follows (lanes 1–6, from left to right): lane 1: quiescent, no additions; lane 2, hydrogen peroxide (200µM) alone for 40 min; lane 3, AA (25 µM) alone for 40 min; lane 4, phorbol 12,13-dibutyrate (PDBu, 200 nM) for 48 h, followed by hydrogen peroxide (200 µM) for 40 min; lane 5, PDBu (200 nM) for 48 h, followed by AA (25 µM) for 40 min; lane 6, PDBu (200 nM) for 48 h. Total cellular RNA was isolated and equal amounts (20 µg/lane) were analyzed for c-fos and GAPDH transcripts as described in Figure legend 1.
Haliday et al (42) have demonstrated that lipoxygenase-dependent metabolism of arachidonic acid is required for tumor necrosis factor (TNF)-induced c-fos mRNA expression in adipogenic TA1 cells. To determine whether hydrogen peroxide-induced c-fos mRNA expression in growth-arrested RASM cells also requires arachidonic acid metabolism, cells were treated with either hydrogen peroxide or arachidonic acid for 40 min in the presence or absence of NDGA (10 µM) and/or indo- methacin (10 µM), potent inhibitors of lipoxygenase-cytochrome P450 monooxygenase and cyclooxygenase activities, respectively (43). RNA blot analysis of total cellular RNA showed that NDGA, but not indomethacin, inhibited both hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression significantly (Fig. 6). This result suggests that hydrogen peroxide induced expression of c-fos mRNA requires lipoxygenase-cytochrome P450 monooxygenase-dependent metabolism of arachidonic acid.

DISCUSSION

The major finding of the present study is that hydrogen peroxide-induced c-fos expression is mediated by phospholipase A2-dependent release of arachidonic acid. Results in support of this conclusion are: 1) hydrogen peroxide stimulated PLA2 activity as shown by the release of [3H]-arachidonic acid and its inhibition by mepacrine, 2) mepacrine and pBPB, two inhibitors of PLA2, blocked hydrogen peroxide-induced c-fos mRNA expression, and 3) arachidonic acid, a product of PLA2, stimulated c-fos mRNA expression with a time-course and magnitude similar to that of hydrogen peroxide.

The finding that PKC may be involved in transduction of H2O2 and arachidonate signals is intriguing, because there are several mechanisms by which it could be involved. Earlier studies by others (44–47) have demonstrated that cis-unsaturated free fatty acids stimulate PKC in several tissues including aorta. Furthermore, Gopalakrishna and Anderson (48), Larsson and Cerutti (49) and Palumbo et al (50) have shown that oxidants, in particular hydrogen peroxide, cause activation of PKC. The present findings provide a physiological example of PKC stimulation by hydrogen peroxide and arachidonic acid, because down regulation of PKC by PDBu pre-treatment attenuated both hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression by 50%. Down regulation of PKC by PDBu pretreatment, however, blocked PMA-induced c-fos mRNA by 90% (26). These differences in the responsiveness of hydrogen peroxide, arachidonic acid and PKC-dependent induction of c-fos mRNA expression to PKC down regulation may be explained as follows: One interpretation for the failure of PKC down regulation by PDBu treatment to completely abolish the hydrogen peroxide and arachidonic acid-induced c-fos mRNA may be that both PKC-dependent and independent mechanisms are involved in c-fos mRNA induction by these agents. A second possible scenario is preferential loss of one PKC isofrom compared to the others. For example, if PDBu treatment reduces PKC-α more than PKCβ, and hydrogen peroxide and arachidonic acid stimulate c-fos mRNA expression by both PKC-α and -β-dependent mechanisms, then PDBu pretreatment may not block the hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression completely while it abrogates the PMA effect on c-fos mRNA expression. In fact, Cooper et al. (51) have demonstrated that treatment of myocytes with phorbol ester for 24 hours resulted in a preferential loss of PKC-α with little or no effect on PKC-β-dependent vinculin phosphorylation. Messina et al (40) have shown that the insulin induction of c-fos mRNA in hepatoma cells is decreased by only 50% after PKC down regulation, while PMA-induced c-fos transcription in these cells is abolished completely. Although the present findings do not address which isofrom of PKC mediates the hydrogen peroxide and arachidonic acid effect on c-fos mRNA expression in RASM cells, these results do suggest a role for PKC in the signal transduction, most likely via the TRE element (PKC-responsive element) in the c-fos gene. This result also suggests transcriptional activation of c-fos by these agents, although some role for post-transcriptional mechanisms, particularly message stabilization cannot be ruled out. In fact, recently, Nose et al (52) have demonstrated transcriptional activation of c-fos gene in mouse osteoblastic cell line, MC3T3, by hydrogen peroxide.

Arachidonic acid has been shown to mediate TNF-induced c-fos mRNA expression in adipogenic TA1 cells (42). It was further demonstrated that metabolism of arachidonic acid via the lipoxygenase system is required for TNF-induced expression of c-for mRNA (42). In the present study, we show that hydrogen peroxide induces c-fos mRNA expression and that this effect is mediated by arachidonic acid release. In accordance with the findings of Haliday et al (42) our results also suggest that further metabolism of arachidonic acid via lipoxygenase-cytochrome P450 monooxygenase pathway(s) is required for hydrogen peroxide-induced expression of c-fos mRNA in RASM cells because NDGA, an inhibitor of this pathway, blocked hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression. Further studies are, however, required to identify the products of lipoxygenase-cytochrome P450 monooxygenase-dependent metabolism of arachidonic acid which are responsible for hydrogen peroxide and arachidonic acid-induced c-fos mRNA expression in RASM cells.

The c-fos and c-jun proteins dimerize non-covalently to form a transcriptional factor, AP-1 (activator protein 1). Studies from several laboratories (53–56) have shown that AP-1 plays an important role in serum-, growth factor- (e.g. epidermal growth factor (EGF), fibroblast growth-factor (FGF) and PDGF) and phorbol ester-stimulated growth in various cell types. We have reported earlier that hydrogen peroxide induces early growth competence genes c-fos and c-myc and DNA synthesis in growth-
arrested RASM cells. The present findings show that hydrogen peroxide-induced expression of c-fos mRNA requires arachidonic acid release and its metabolism by lipoxygenase-cytchrome P450 monoxygenase system. This is consistent with several reports indicating that arachidonic acid metabolism by lipoxygenase-cytochrome P450 monoxygenase system is required for mitogenesis induced by serum, EGF and FGF in various cell types (57–59). In addition, Setty et al (60) and Brinkman et al (61) have shown that arachidonic acid stimulated growth of bovine aortic endothelial and human vascular smooth muscle cells, respectively. Since hydrogen peroxide stimulates the release of arachidonic acid and the latter induces c-fos mRNA with a time course and magnitude similar to that of hydrogen peroxide, it is likely that arachidonic acid mediates the growth competence signals of hydrogen peroxide in growth-arrested RASM cells. Studies are in progress to determine whether hydrogen peroxide and arachidonic acid induce c-jun, the protein product of which is a functionally required component of the AP-1 complex, and to observe whether these agents also stimulate the AP-1 activity in growth-arrested RASM cells.

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