Free Rad. Res., Vol. 22, No. 1, pp. 23-37
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OXIDANT-INDUCED ACTIVATION OF PROTEIN KINASE C IN UC11MG CELLS

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(Received May 15th, 1994; in revised form May 31st, 1994)

Free radical formation and subsequent lipid peroxidation may participate in the pathogenesis of tissue injury, including the brain injury induced by hypoxia or trauma and cardiac injury arising from ischemia and reperfusion. However, the exact cellular mechanisms by which the initial oxidative insult leads to the ultimate tissue damage are not known. A number of reports have indicated that protein kinase C (PKC) may be activated following oxidative stress and that this enzyme may play an important role in the steps leading to cellular damage. In this work, we have examined in a cell model whether PKC is activated following oxidative exposure. UC11MG cells, a human astrocytoma cell line, were treated with H_2O_2. Incubation with 0.5 mM H_2O_2 increased malondialdehyde levels by as early as 15 minutes. To assess the effects of H_2O_2 treatment on PKC activation, we measured phosphorylation of an endogenous PKC substrate, the MARCKS (*m*yristoylated *a*lanine-*r*ich *C k*inase *s*ubstrate) protein. Treatment of cells with 0.2-1.0 mM H_2O_2 resulted in a rapid increase in MARCKS phosphorylation. Phosphorylation was stimulated approximately 2.5-fold following treatment with 0.5 mM H_2O_2 for ten minutes. Treatment with phorbol 12-myristate 13-acetate, a PKC activator, increased MARCKS phosphorylation approximately 4-fold. The H_2O_2-induced MARCKS phosphorylation was inhibited by the addition of the kinase inhibitors H-7 and staurosporine. Furthermore, specific down-regulation of PKC by phorbol ester also inhibited H_2O_2-induced MARCKS phosphorylation. These results indicate that PKC is rapidly activated in cells following an oxidative exposure and that this cell system may be a good model to further investigate the role of PKC in regulating oxidative damage in the cell.

KEY WORDS: Oxidative injury, protein kinase C, hydrogen peroxide, UC11MG astrocytoma, MARCKS.

INTRODUCTION

Oxygen radical formation and subsequent lipid peroxidation may participate in the pathogenesis of tissue injury, including the brain injury induced by hypoxia or trauma and cardiac injury arising from ischemia and reperfusion.[1-3] However, the mechanism(s) by which oxidative exposure leads to tissue damage and ultimately to cell death are not well understood. A number of cellular responses may be initiated following an oxidative insult, including increased calcium influx, release of excitatory amino acids, alterations in energy metabolism, increased gene transcription of specific proteins and changes in lipid metabolism.[4-6] Studies addressing the question of the mechanism of oxidative injury have typically been done using whole tissues, but these responses would be better understood if probed

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at the cellular level. Therefore, we have established a cellular model for oxidative injury using UC11MG cells, a human astrocytoma cell line which expresses many of the properties associated with differentiated astrocytes.[7]

Protein kinase C (PKC) plays an important role in many signaling pathways, involving functions such as cell growth and differentiation, gene expression, secretion of hormones and neurotransmitters, and membrane functions.[8] The enzyme is activated by interaction with membranes and diacylglycerols. Activation is initiated through specific binding of ligands to cell-surface receptors which are functionally linked to phospholipase C, resulting in the generation of diacylglycerols from inositol lipids, particularly phosphatidylinositol 4,5-bisphosphate (PIP_2). In addition to diacylglycerol, hydrolysis of PIP_2 generates inositol 1,4,5-trisphosphate which causes an increase in cytosolic calcium. There is also evidence for activation of PKC through alternative routes involving hydrolysis of phosphatidylcholine by phospholipase D and by phospholipase A_2.[9]

PKC actually refers to a family of highly homologous enzymic isoforms which exhibit differential tissue-specific expression and intracellular localization. To date, at least ten types have been identified by molecular cloning; they are PKC-α, -β, -γ, -δ, -ϵ, -ζ, -η, -θ, -λ and -ι.[8,10-12] PKC can be divided structurally and functionally into a regulatory and a catalytic domain. The PKC isoforms are all highly homologous in their catalytic domains, but they can be divided into two major classes based on structural differences in their regulatory domains. The group of so-called classical PKC's, -α, -β and -γ, have a C_1 region which appears to mediate phospholipid-dependent diacylglycerol binding via two zinc finger-like domains. They also have a C_2 region which contributes to the calcium-dependence of kinase activity. In contrast, the remaining group of "novel" isozymes lacks the C_2 region, and, thus, the activity is calcium-independent. Furthermore, PKC's-ζ and -λ are unique in that they only have a single copy of the zinc finger domain in the C_1 region. This difference apparently accounts for the observation that these isoforms do not bind phorbol esters. Phorbol esters, such as phorbol 12-myristate 13-acetate, or PMA, bind to the diacylglycerol site of the other PKC's and activate the enzyme directly.

There is evidence that PKC may be one of the pathways activated within cells in response to oxidative stress. *In vitro* studies have shown that hydroperoxy fatty acids are potent activators of PKC.[13] As oxidation of unsaturated fatty acids to hydroperoxides is a primary event in lipid peroxidation, this suggests that PKC activation may be an early response to oxidative stress. Other studies have shown PKC activation by low levels of H_2O_2, both *in vitro* with purified PKC and *in vivo* in C6 glioma cells, lending support to this hypothesis.[14,15] Prolonged oxidation with H_2O_2 leads to PKC inactivation, suggesting an on/off mechanism for cellular signaling. Investigations of the fate of PKC in cultured cells following exposure to oxidants have demonstrated increased PKC membrane association, also an indication of PKC activation.[16-18]

Other results have not only implicated PKC in a cellular oxidative response, they have suggested a role for PKC in the pathway(s) to injury. In several animal models of cerebral ischemia, PKC levels were altered in the hippocampus of animals undergoing ischemia.[19-23] Short (5–30 minute) ischemic events were accompanied by redistribution of PKC from a predominantly soluble localization to the particulate fraction. Further evidence of a role for PKC in oxidative injury has been provided by studies utilizing kinase inhibitors. Treatment of rat hepatocytes with tert-butyl hydroperoxide resulted in oxidative injury as evidenced by bleb forma-

tion, lactate dehydrogenase release and lipid peroxidation.[16] As expected, radical scavengers inhibited these events, but the injury was also effectively inhibited by the protein kinase C inhibitors, H-7 and phloretin. In both gerbil and rat ischemia models, injection of staurosporine 30 minutes before ischemia protected against pyramidal cell loss in the CA1 region.[24] Administration of staurosporine to rats prior to five minutes of ischemia significantly reduced behavioral impairment of the rats, measured 24 hours after ischemia.[25]

Taken together, these results indicate that activation of PKC may be an early event following oxidative exposure. Furthermore, the inhibitor studies suggest that PKC may play a crucial role in the pathway to cellular injury. In the work reported here, we establish a cellular model for oxidative injury in which a human astrocytoma cell line is treated with H_2O_2. Activation of PKC was assessed by monitoring the phosphorylation of an endogenous PKC substrate, the MARCKS (*m*yristoylated *a*lanine-*r*ich *C k*inase *s*ubstrate) protein.[26,27] We show that hydrogen peroxide treatment results in a rapid stimulation of MARCKS phosphorylation which is inhibited by kinase inhibitors and by down-regulation of PKC by the phorbol ester, PMA.

MATERIALS AND METHODS

Materials

Hydrogen peroxide (30% solution) was from Mallinkrodt. A fresh bottle was obtained for every experiment. Ferrous ammonium sulfate, desferal, 2-thiobarbituric acid, leupeptin, soybean trypsin inhibitor and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Digitonin came from Calbiochem. Staurosporine was from Kyowa Medex Co., Ltd., and H-7 was from Seikagaku Kogyo Co. [γ-^{32}P]ATP, 500 Ci/mmol, was purchased from Amersham Corp. The PKC-α M4 monoclonal antibody used for probing nitrocellulose blots was from ammonium sulfate-precipitated hybridoma culture medium (1:200).[28] The PKC-δ and PKC-ϵ antibodies were a gift from D. Fabbro (Basel, Switzerland). The PKC-β and PKC-ζ antibodies were purchased from Gibco. Alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse IgG and the color development kit were from Promega. Nitroplus 2000 was from Integrated Separation Systems. X-OMAT film was purchased from Kodak.

Cell Culture

Human astrocytoma cells (UC11MG) were kindly provided by Dr. Frank Sun (Upjohn Labs). They were maintained as monolayers in tissue culture flasks in RPMI 1640 medium containing 2 mM L-glutamine, 10% fetal calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. Cells were serum-starved by incubating them for 48 hours at 37°C in growth medium without fetal calf serum.

MDA Assay

UC11MG astrocytes were grown to confluence in 150 mm dishes and incubated at 37°C with 0.5 mM ferrous ammonium sulfate and/or 0.5 mM hydrogen peroxide. The cells were washed twice with 10 ml ice-cold phosphate-buffered saline (PBS) and

scraped in 10 ml PBS/dish. The detached cells were pelleted by centrifugation at 600 × g, 4°C for 7 minutes, resuspended in 350 μl of 50 mM Tris buffer (pH 7.5) and sonicated. Cell homogenate (100 μl) was combined with trichloroacetic acid (250 μl, 12.5% trichloroacetic acid), and the mixture was vortexed. Desferal (5 μl of 5 mM) and thiobarbituric acid (50 μl of 0.23 M) were added and the volume was adjusted to 600 μl with water. The resulting mixture was vortexed and then incubated at 100°C for 20 minutes. Samples were centrifuged at 13,000 × g for 5 minutes. The absorbance at 532 nm of the supernatant was measured in a spectrophotometer. The concentration of malondialdehyde (MDA) in the sample was then calculated based on the absorbence and extinction coefficient at 532 nm for the TBA-MDA complex ($1.56 \times 10^5\ M^{-1}$). The absolute value of the TBA-MDA complex was normalized relative to the protein content in the sample. Protein content was measured by the Bradford method.[29]

MARCKS Phosphorylation

UC11MG cells, grown on 35 mm dishes to 50% confluence, were serum-starved for two days. They were washed twice with a 37°C KCl solution (10 mM PIPES (pH 7), 120 mM KCl, 30 mM NaCl, 1 mM $MgCl_2$, 1 mM K_2HPO_4, 1 mM ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) and 37 μM $CaCl_2$). The cells were incubated at 37°C with the additions indicated in the figures. During the last five minutes of the incubation, fresh KCl solution (500 μl), containing [γ-^{32}P]ATP and 40 μM digitonin and the indicated additions, was added to the cells. This solution was removed to a tube, and the cells were scraped with 200 μl Buffer B (10 mM Tris (pH 7.5), 100 mM NaF, 5 mM dithiothreitol, 0.25 M sucrose, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor). The scraped cells were added to the digitonin-soluble mixture. The resulting sample was incubated at 100°C for 15 minutes and then centrifuged for 15 minutes at 13,000 × g. The supernatant was precipitated with 10% trichloroacetic acid, and the precipitate was dissolved in Laemmli buffer and separated by SDS-PAGE on a 7.5% gel. The gel was fixed and dued and exposed to x-ray film. Densitometry was performed using a Molecular Dynamics Computing Densitometer.

Western Blots

Samples were run on SDS-PAGE, transferred to Nitroplus 2000 and incubated with the various PKC antibodies for 1 hour at room temperature. The blots were washed sequentially in 25 mM Tris, pH 7.5, containing 500 mM NaCl (Buffer A), and in Buffer A containing 0.05% NP-40, followed by incubation in alkaline phosphatase-conjugated second antibody. After further washing, blots were developed using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (NBT/BCIP).

RESULTS

Initially, it was important to characterize the PKC isozyme expression profile of the UC11MG astrocytoma cells. Cell lysates were prepared and run on SDS-PAGE, then immunoblotted and probed with isotype-specific PKC antibodies (Figure 1).

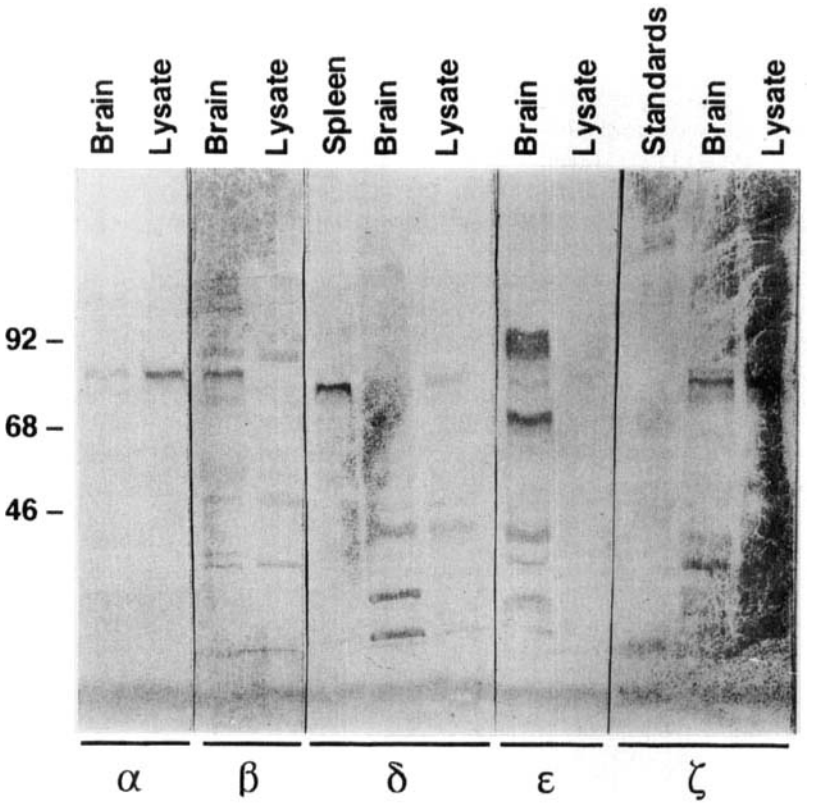

FIGURE 1 PKC isozyme expression in UC11MG cells. UC11MG cell lysate (100 μg), rat brain homogenate (200 μg) or pig spleen (130 μg) was run on 10% SDS-PAGE, transferred to a nitrocellulose membrane and blotted with antibodies to PKC isozymes, as indicated. Molecular weight standards are shown on the left.

Rat brain homogenate (or pig spleen, for δ) was included on the blots as a positive control. The PKC-α-, PKC-δ- and PKC-ζ-specific antibodies hybridized to proteins of about 80,000 daltons, demonstrating that UC11MG cells express the α, δ and ζ isoforms of PKC. There was no reactivity with the PKC-β or PKC-ε antibody, indicating that the PKC-β and PKC-ε isoforms were not present in the UC11MG lysate.

UC11MG cells were treated with ferrous ammonium sulfate and/or hydrogen peroxide to induce oxidative injury. According to the Fenton reaction, hydrogen peroxide will react with ferrous iron to form the hydroxide ion and the highly reactive hydroxyl radical:

$$FE^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$

In order to establish that oxidative damage to the cells had occurred under these conditions, malondialdehyde (MDA) levels were measured. In this assay MDA, formed from lipid peroxides, reacts with thiobarbituric acid (TBA), resulting in the formation of a pink chromogen.[30] Thus, MDA is a thiobarbituric acid-reactive

material, or TBAR. In cells treated with 0.5 mM H_2O_2, TBAR levels increased approximately 70–100% within 30 minutes (Figure 2). When ferrous ammoniun sulfate was added together with H_2O_2 to the cells, there was an augmentation of TBAR formation relative to that induced by H_2O_2 alone, presumably due to increased hydroxyl radical production via the Fenton reaction. However, these results demonstrate that treatment with H_2O_2 alone resulted in significant oxidative injury to the cells presumably by reaction of H_2O_2 with endogenous iron or transition metals. For this reason, subsequent experiments were performed without the addition of exogenous iron.

To examine whether H_2O_2 treatment resulted in PKC activation, we measured *m*yristoylated *a*lanine-*r*ich *C k*inase *s*ubstrate (MARCKS) protein phosphorylation. The MARCKS protein is a 32 kDa endogenous substrate of PKC that is widely expressed in cells and tissues.[26] Its apparent molecular mass on SDS-PAGE is 80–87 kDA, presumably due to its rod-like helical structure and general hydrophilicity. Following PKC activation, the MARCKS protein undergoes rapid phosphorylation; thus, it has been used as an indicator of the functional state of

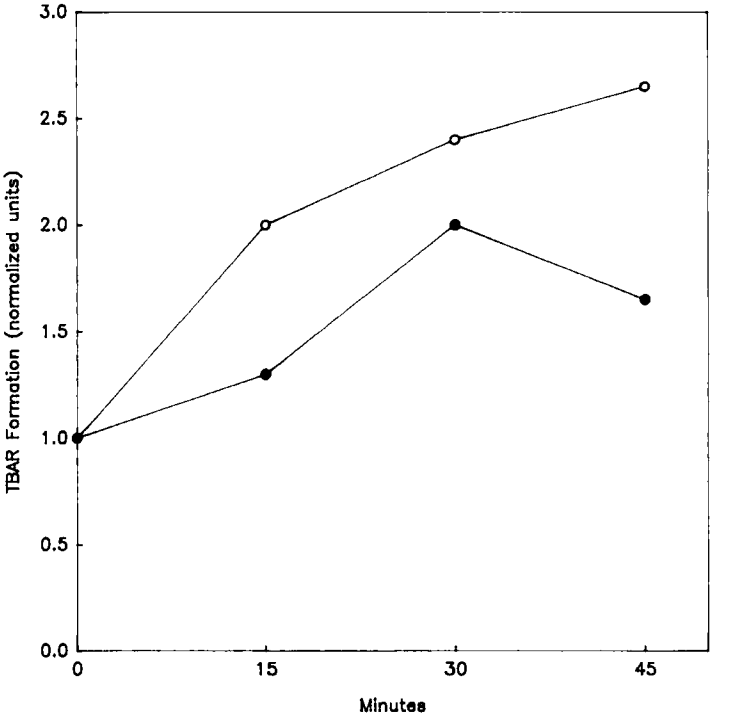

FIGURE 2 Treatment of UC11MG astrocytes with H_2O_2 leads to increased malondialdehyde (MDA) levels. UC11MG cells were treated at 37°C for the indicated time in medium containing 0.5 mM H_2O_2 (closed circles) or 0.5 mM H_2O_2 and 0.5 mM ferrous ammonium sulfate (open circles). The cells were harvested and then sonicated and assayed for MDA as described in Materials and Methods. Each point represents the average results of triplicate assays. Data was normalized relative to control levels at the zero time point because the curves were generated in separate experiments. TBAR, thiobarbituric acid-reactive material.

PKC. In addition, the MARCKS protein remains soluble during a 100°C incubation; therefore, the extent of its phosphorylation can be very conveniently assessed.

The concentration-dependence of H_2O_2-induced MARCKS phosphorylation was determined. As shown in Figure 3a, the phorbol ester PMA, a potent PKC activator, stimulated MARCKS phosphorylation approximately 4-fold (Lane 1). There is a low level of MARCKS phosphorylation under unstimulated conditions in serum-starved cells (Lane 2). Addition of 0.25 and 1.0 mM H_2O_2 for 10 minutes increased MARCKS phosphorylation above the unstimulated level 1.6 and 2.5 fold, respectively (Lanes 5–7). These results demonstrate that PKC in UC11MG cells is activated in a dose-dependent manner in response to exposure to H_2O_2. The addition of 0.5 mM ferrous ammonium sulfate with hydrogen peroxide did not increase the H_2O_2-induced phosphorylation of MARCKS (Figure 3b). Ferrous ammonium sulfate and 0.5 mM hydrogen peroxide (Lane 11) caused a 2 fold stimulation of MARCKS phosphorylation, in close agreement with the H_2O_2 dose response. Ferrous ammonium sulfate alone did not stimulate MARCKS phosphorylation (Lanes 3–7).

Incubation of UC11MG cells with 0.2 mM H_2O_2 resulted in an increase in the phosphorylation of the MARCKS protein that was apparent at five minutes, the earliest time point tested (Figure 4). Phosphorylation was further increased at the 10 and 20 minute time points to 2.0- and 2.7-fold over the control value, respectively. Therefore, PKC is rapidly activated in cells as a result of H_2O_2 treatment.

To establish that the H_2O_2-stimulated phosphorylation of MARCKS was mediated by PKC, the experiments were conducted in the presence of the kinase inhibitors, H-7 and staurosporine. Addition of 0.5 mM H_2O_2 to the cells stimulated phosphorylation, and this was inhibited by addition of either H-7 or staurosporine (Figure 5). Complete inhibition of phosphorylation was observed when cells were incubated in the presence of 100 μM H-7 or 0.1–1.0 μM staurosporine. This relative order of potency is consistent with the inhibitory potencies of these two compounds in *in vitro* experiments using purified protein kinase C.[31] Although neither H-7 nor staurosporine is a specific inhibitor of PKC, the K_i for inhibition of PKC by staurosporine is approximately 1 nM, a value 10-fold lower than that for inhibition of the cAMP-dependent kinase. As shown in Figure 5, 10 nM staurosporine completely inhibited the H_2O_2-stimulated phosphorylation of MARCKS, which supports the hypothesis that PKC mediates the H_2O_2-induced MARCKS phosphorylation.

In addition to the inhibitor studies, PKC down-regulation studies were carried out. Prolonged incubation of cells with PMA down-regulates PKC due to increased proteolysis.[10] Incubation of cells with 500 nM PMA for 48 hours resulted in the loss of PKC-α protein, as demonstrated by Western blot analysis (data not shown). In these PKC-down-regulated cells, stimulation of MARCKS phosphorylation by a subsequent dose of PMA was eliminated (Figure 6, compare Lanes 3 and 6). Similarly, H_2O_2-stimulated phosphorylation of MARCKS was eliminated (compare Lanes 2 and 5). This result provides further support for the proposed role of PKC in H_2O_2-induced MARCKS phosphorylation. It also suggests that either PKC-α or PKC-δ is involved in the signaling pathway initiated by treatment with H_2O_2, since PKC-ζ is not activated by phorbol esters.

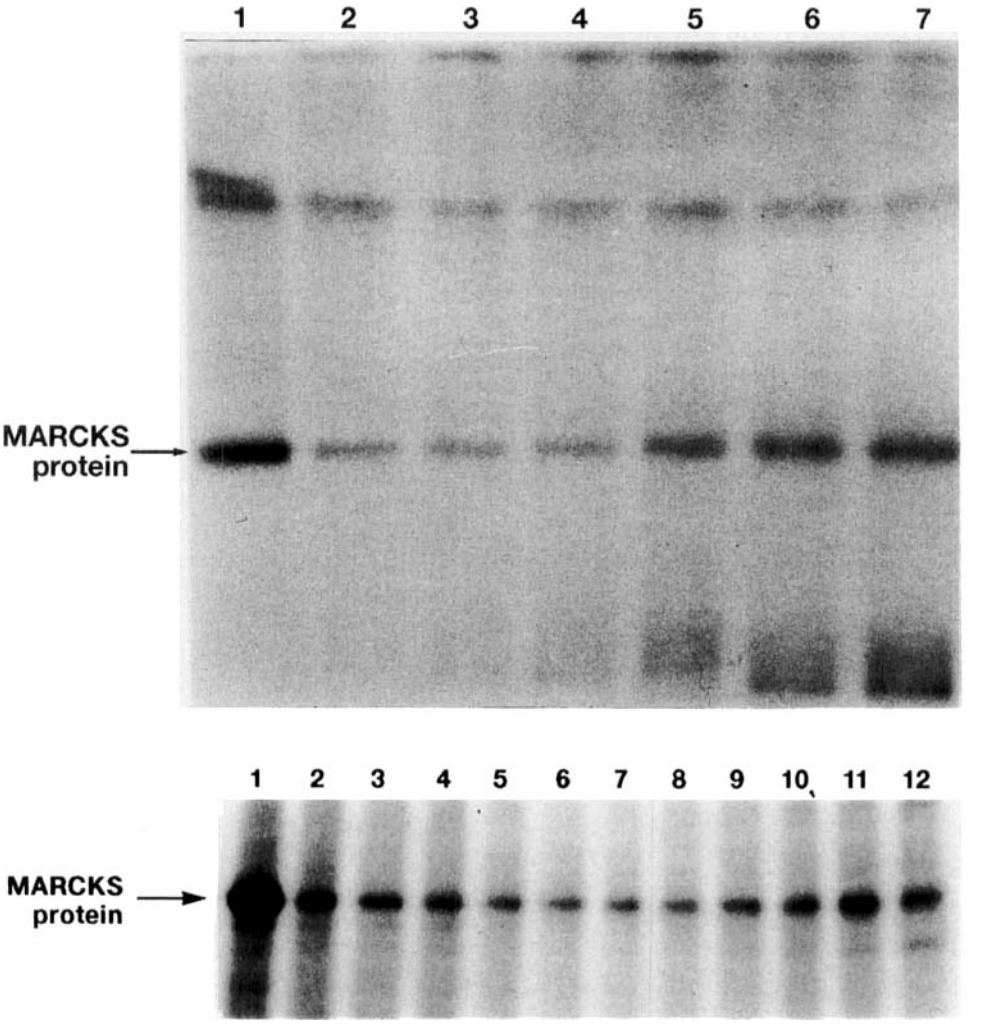

FIGURE 3 H_2O_2 treatment leads to a dose-dependent increase in MARCKS protein phosphorylation. A) UC11MG cells were incubated for ten minutes at 37°C in PIPES buffer containing H_2O_2, 40 μM digitonin and [γ-^{32}P]ATP. The cells were collected and processed as described in Materials and Methods. Cells were treated with: 1, 0.5 μM phorbol 12-myristate 13-acetate (PMA) in PIPES buffer containing digitonin and [γ-^{32}P]ATP (positive control); 2, digitonin and [γ-^{32}P]ATP only; 3, 50 μM H_2O_2; 4, 100 μM H_2O_2; 5, 250 μM H_2O_2; 6, 500 μM H_2O_2; 7, 1.0 mM H_2O_2. B) The cells were incubated and processed as in A) with: 1, 0.5 μM phorbol 12-myristate 13-acetate; 2, digitonin and [γ-^{32}P]ATP only; 3, 42 μM ferrous ammonium sulfate (FAS); 4, 125 μM FAS; 5, 250 μM FAS; 6, 500 μM FAS; 7, 1.0 mM FAS; 8, 500 μM FAS and 50 μM H_2O_2; 9, 500 μM FAS and 100 μM H_2O_2; 10, 500 μM FAS and 250 μM H_2O_2; 11, 500 μM FAS and 500 μM H_2O_2; 12, 500 μM FAS and 1.0 mM H_2O_2.

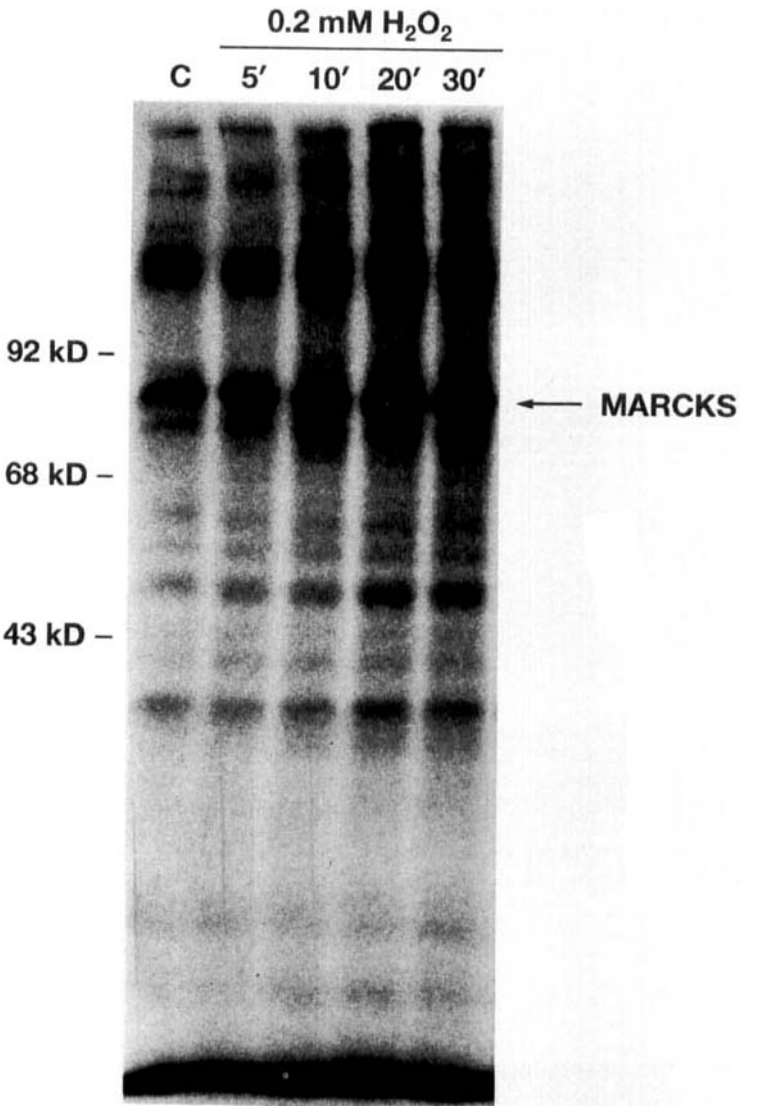

FIGURE 4 H_2O_2 treatment leads to a time-dependent increase in MARCKS protein phosphorylation. UC11MG cells were incubated at 37°C in PIPES buffer containing 0.2 mM H_2O_2 for the indicated time. During the last five minutes of the incubation, the buffer was replaced with PIPES buffer containing 40 μM digitonin, [γ-^{32}P]ATP and 0.2 mM H_2O_2. The cells were collected and processed as described in Materials and Methods. C, control cells were incubated in PIPES buffer containing [γ-^{32}P]ATP and digitonin for five minutes.

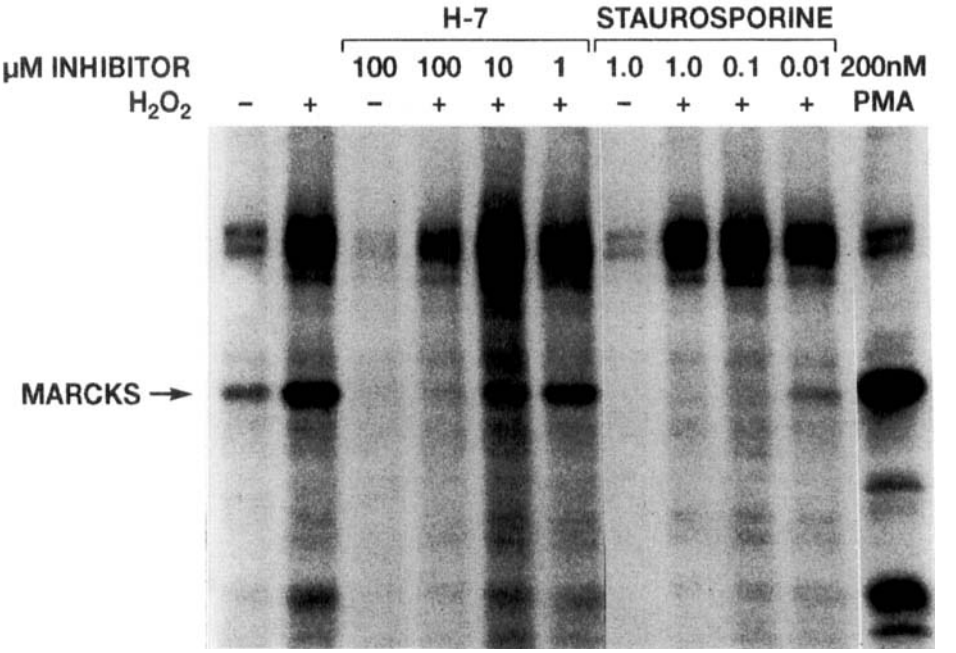

FIGURE 5 PKC inhibitors prevent H_2O_2-induced MARCKS phosphorylation. UC11MG cells were incubated for 30 minutes at 37°C in PIPES buffer plus or minus 0.5 mM H_2O_2. The inhibitors H-7 or staurosporine were included as indicated in the figure. During the last five minutes of the incubation, [γ-^{32}P]ATP and digitonin were added to the cells. Negative control cells were incubated in PIPES buffer containing [γ-^{32}P]ATP and digitonin for five minutes as shown in the far left lane and positive control cells were incubated in PIPES buffer containing [γ-^{32}P]ATP, digitonin and 200 nM PMA as shown in the far right lane. Cells were collected and processed as described in Materials and Methods.

DISCUSSION

The results of these experiments demonstrate that treatment of UC11MG cells with H_2O_2 results in increased lipid peroxidation, a measure of cellular oxidative injury. Hydrogen peroxide treatment also led to a rapid stimulation of MARCKS protein phosphorylation. Addition of the kinase inhibitors H-7 and staurosporine completely prevented the H_2O_2-induced MARCKS phosphorylation at 10 μM and 0.01 μM, respectively. Furthermore, in cells in which PKC was down-regulated by prolonged phorbol ester treatment, H_2O_2-induced MARCKS phosphorylation was inhibited. These results indicate that the increase in MARCKS phosphorylation resulting from H_2O_2 treatment was mediated by PKC.

The mechanism(s) by which H_2O_2 activates PKC is not known. Furthermore, it is not known if H_2O_2 itself mediates PKC activation or if the mechanism involves an oxidant generated by the reaction of H_2O_2 in a Fenton-like reaction. As shown in Figure 2, H_2O_2 alone stimulates TBAR formation, although to a lesser extent than H_2O_2 plus ferrous ammonium sulfate. This suggests that there are endo-

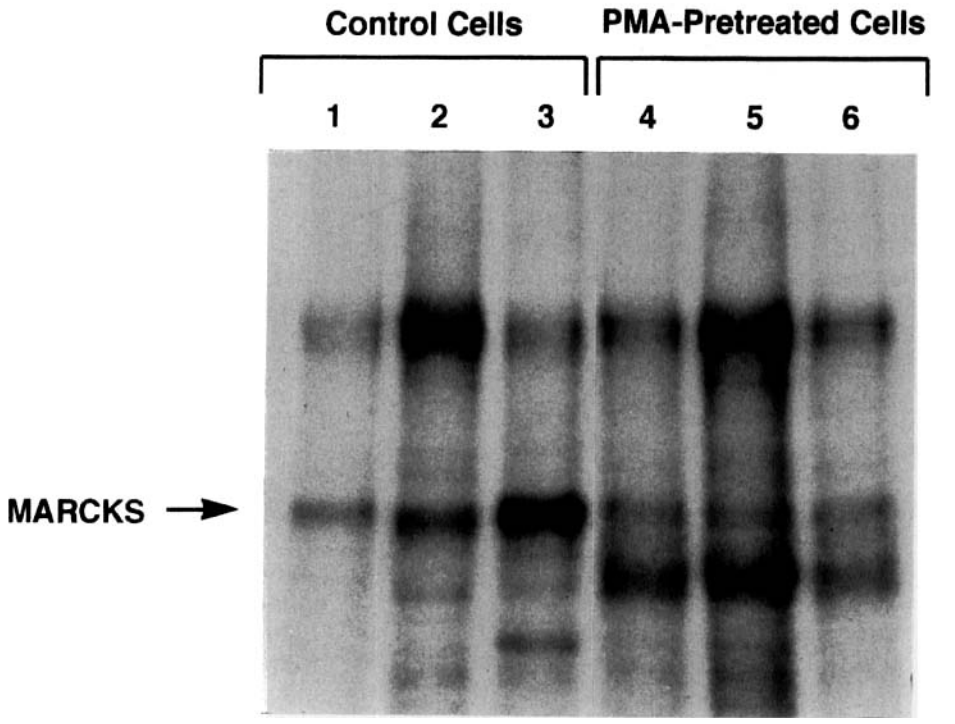

FIGURE 6 PMA down-regulation of PKC inhibits H_2O_2-induced MARCKS phosphorylation. UC11MG cells were pretreated for two days with 0.5 μM PMA (Lanes 4–6) or with vehicle (Lanes 1–3). The cells were washed and incubated with vehicle (Lanes 1 and 4), 0.5 mM H_2O_2 for 30 minutes (Lanes 2 and 5) or 0.2 μ PMA for 15 minutes (Lanes 3 and 6). During the last five minutes of the incubation, [γ-^{32}P]ATP and digitonin were added to the cells. Cells were collected and processed as described in Materials and Methods.

genous transition metals which participate in a Fenton-like reaction, leading to the production of the hydroxyl radical or an oxidant species of similar reactivity which mediates PKC activation. However, as shown in Figure 3, MARCKS phosphorylation, a readout of PKC activation, is induced to the same extent by H_2O_2 alone and H_2O_2 plus ferrous ammonium sulfate and not at all by ferrous ammonium sulfate alone. These data, together with the data in Figure 2, suggest that either H_2O_2 is itself sufficient to initiate activation of PKC or that a Fenton-derived oxidant is responsible and MARCKS phosphorylation is not as sensitive a readout as TBAR formation for the detection of differences in the levels of such an oxidant.

Whether via H_2O_2 itself or some other oxidant, PKC activation may occur through an affect on phospholipid metabolism and membrane enzymes, which thereby elevates diacylglycerol (DAG) levels. DAG has been shown in a number of studies to activate PKC, and agonists that increase DAG levels via receptor-coupled phospholipase C activation also stimulate PKC.[32–34] UC11MG cells were shown here to express the α, δ also ζ isoforms of PKC. Both the α and δ isoforms of PKC are activated by DAG; therefore, an oxidant-mediated increase in DAG levels could theoretically activate either isozyme. Our PKC down-regulation results, obtained

under conditions in which the α and δ, but not the ζ, isoforms are down-regulated, are consistent with this. Alternatively, PKC activity may be increased by a rise in intracellular calcium, though only PKC-α in UC11MG cells contains the C_2 region which contributes to calcium-dependence. In other experiments, we have demonstrated that H_2O_2 treatment of UC11MG cells increases intracellular calcium levels.[a] It would be interesting to examine which PKC isozymes are activated in UC11MG cells in response to oxidative injury.

The mechanism(s) by which PKC may be involved in intracellular oxidative injury is also unknown. One possibility is that PKC is involved in the pathway that leads to the membrane changes and blebbing which occur during cellular injury. Cytoskeletal rearrangements could be mediated, in part, by the MARCKS protein. Like PKC, MARCKS represents a large class of related proteins which are widely distributed.[35] Dephosphorylated MARCKS binds to the actin cytoskeleton and crosslinks the actin filaments. Hartwig *et al.*[36] have shown that the crosslinking activity of MARCKS is inhibited by PKC-mediated phosphorylation.

PKC may also play a role in influencing the ion fluxes and changes in calcium homeostasis that accompany oxidative injury. Modulation of ion channel activity, especially calcium and potassium channel activities, by PKC has been well documented in a variety of cell lines.[37] For example, in rat hippocampal CA1 pyramidal cells, PKC activation leads to inhibition of a Ca^{2+}-activated K^+ current.[38] In contrast, whole cell voltage-clamp analysis of cultured fetal rat hippocampal cells showed that PKC activation inhibits neuronal calcium currents.

PKC might further mediate cellular injury due to oxidative stress through changes in gene expression. Transcription of a wide variety of genes is responsive to treatment of cells with phorbol esters and activation of PKC. One mechanism for this transcriptional regulation is via the AP-1 complex. The AP-1 complex is actually a group of related complexes of transcriptional activators which are all homo- and heterodimers of the products of the *jun* and *fos* protooncogene families.[40] The dimeric AP-1 complex activates transcription of responsive genes by binding to a phorbol ester response element (TRE) present in their 5′ control region. AP-1 activity is itself activated by phorbol esters, as well as by agents that induce oxidative stress, including H_2O_2.[41-49] Furthermore, induction of AP-1 activity by H_2O_2 has, in some cases, been shown to be PKC-dependent. For instance, H_2O_2-induced expression of c-*jun* in HL-525 cells was inhibited by pretreatment of the cells with bryostatin or TPA to down-regulate PKC.[43] Similarly, in rat aortic smooth muscle cells, stimulation by H_2O_2 of both c-*fos* and c-*jun* mRNA expression was significantly reduced by down-regulation of PKC with phorbol ester.[48,49] An AP-1 recognition site has also been identified in the 5′ control regions of the genes for rat glutathione *S*-transferase and NAD(P)H: quinone reductase, which have been shown to be activated following oxidative injury.[50-52] Overall, these results suggest that changes in AP-1 activity are part of the pathway to H_2O_2-induced alterations in gene activity, thereby suggesting a mechanism for PKC involvement in injury.

In this paper we have demonstrated activation of protein kinase C by an oxidant derived from hydrogen peroxide using UC11MG cells, a human astrocytoma cell line. As discussed, the role of PKC in the cellular response to an oxidative challenge

[a]P.L. Munns and K.L. Leach (1994) Two novel antioxidants, U74006F and U78517F, inhibit hydrogen peroxide-stimulated calcium influx. Submitted for publication.

is not understood and may be quite complex. This study suggests an appropriate and convenient system with which to pursue the question of PKC's role in oxidative injury.

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Accepted by Dr M.B. Grisham