Requirement for Protein Kinase C in Reactive Oxygen Species–Induced Apoptosis of Vascular Smooth Muscle Cells

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Background—Vascular smooth muscle cell (VSMC) apoptosis is a component of a variety of cardiovascular diseases and may be related to reactive oxygen species (ROS). This study was designed to determine the role of protein kinase C (PKC) in ROS-induced VSMC apoptosis.

Methods and Results—Rat aortic VSMCs were exposed to \( \text{H}_2\text{O}_2 \), and the nature of cell death was characterized in the absence or presence of different PKC inhibitors. The results demonstrate that exposure of VSMCs to \( \text{H}_2\text{O}_2 \) led to a dose-dependent (25 to 100 \( \mu \text{mol/L} \)) and time-dependent (peak at 2 minutes) activation of PKC. Among the PKC isoforms \( \alpha, \beta, \delta, \epsilon, \) and \( \zeta \), only PKC-\( \alpha \) and PKC-\( \epsilon \) were found to change their intracellular distribution on \( \text{H}_2\text{O}_2 \) treatment. Apoptosis was the predominant form of cell death when PKC had been activated by \( \text{H}_2\text{O}_2 \) alone or by \( \text{H}_2\text{O}_2 \) in the presence of 50 nmol/L phorbol 12-myristate 13-acetate. In contrast, necrosis became the predominant form of cell death when PKC had been downregulated by prolonged exposure to 200 nmol/L phorbol 12,13-dibutyrate or inhibited by 50 nmol/L staurosporine, 100 nmol/L calphostin C, or 30 \( \mu \text{mol/L} \) H-7. In addition, caspase-3 was activated in \( \text{H}_2\text{O}_2 \)-induced VSMC apoptosis but not when PKC was downregulated or inhibited. Inhibition of caspase-3 by 50 \( \mu \text{mol/L} \) Ac-DEVD-CHO could significantly attenuate \( \text{H}_2\text{O}_2 \)-induced apoptosis and was not associated with the induction of necrosis.

Conclusions—We conclude that in VSMCs, PKC converts the ROS-induced signals from necrotic cell death to the activation of an apoptotic cell death program. These data imply a novel and important role of PKC for the pathogenesis of such vascular diseases as atherosclerosis, restenosis, and hypertension. (Circulation. 1999;100:967-973.)

Key Words: muscle, smooth \( \square \) cells \( \square \) free radicals \( \square \) apoptosis \( \square \) kinase \( \square \) caspase

Reactive oxygen species (ROS) comprise a group of molecules such as singlet oxygen (\( O_2^* \)), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), superoxide anion (\( O_2^- \)), and hydroxyl radical (\( \cdot \text{OH} \)). An increasing body of evidence indicates an important role for ROS in the pathogenesis of such vascular diseases as hypertension, restenosis after balloon angioplasty, and atherosclerosis (see References 1 and 2 for recent reviews). For example, exposure to superoxide anion leads to vascular smooth muscle cell (VSMC) proliferation, a prominent component of atherosclerosis and hypertension.\(^3\) Furthermore, \( \text{H}_2\text{O}_2 \) has been demonstrated to induce VSMC death,\(^4\) which may occur by apoptosis.\(^5\)

For many years, necrotic VSMCs have been recognized to be present in atherosclerotic plaques.\(^6,7\) Recently, it has become evident that apoptosis of VSMCs is involved in the pathogenesis of atherosclerosis and restenosis.\(^8-10\) In this regard, it is important to clearly distinguish between necrotic and apoptotic cell death, because either form of cell death elicits distinct reactions within the afflicted organ system and thus may have opposite consequences on the pathophysiology of these diseases. Necrosis and apoptosis differ in their mechanisms, morphology, and biochemistry. Necrosis is characterized by cell and mitochondrial swelling, disruption of internal and external membranes, and preservation or swelling of the nuclear structure. In addition, necrosis is accompanied by the liberation of cellular contents, cytokine production, and inflammation. In contrast, apoptosis is a genetically controlled form of cell death that is activated by specific cell death signals during organ development, tumorigenesis, and certain diseases. Apoptotic cells exhibit shrinkage, intact membrane in the early stage, chromatin condensation, and internucleosomal DNA fragmentation known as “DNA laddering.”

Protein kinase (PK) C plays a critical role in signal transduction pathways leading to a variety of cellular functions, such as cell growth and differentiation. Previous studies have shown that \( \text{H}_2\text{O}_2 \) is capable of activating PKC in a variety of cell types, including vascular cells.\(^4,12\) Our present study was designed to determine the role of PKC in \( \text{H}_2\text{O}_2 \)-induced death of VSMCs.
Methods

Cell Culture
VSMCs were prepared and cultured as previously described. Cells were made quiescent by incubation for 48 hours in a culture medium containing 0.2% FCS. Subsequently, they were divided into different groups, each of which was cultured for another 48 hours, including the various preincubation times of PKC activator/inhibitors: phorbol 12-myristate 13-acetate (PMA) for 30 minutes, phorbol 12,13-dibutyrate (PDBu) for 48 hours, staurosporine, H-7, and calphostin C for 2 hours each, before treatment with H\textsubscript{2}O\textsubscript{2}. For treatment, the cells were incubated at 37°C for 1 hour in HBSS containing various concentrations of H\textsubscript{2}O\textsubscript{2} and 100 μmol/L ferrous sulfate. The reaction was stopped by removal of the HBSS containing H\textsubscript{2}O\textsubscript{2}/ferrous sulfate. The cells were further cultured in freshly prepared culture medium. The control cells were incubated under the same conditions without the addition of PKC activator/inhibitors or H\textsubscript{2}O\textsubscript{2}. Under these conditions, neither 100 μmol/L ferrous sulfate nor DMSO, which was used to solubilize PKC inhibitors at a final concentration of <0.1%, caused death of VSMCs as assessed by MTT assay and cell death ELISA or influenced PKC activity.

Assay of PKC and PKA Activity
VSMCs were washed with PBS (without Ca\textsuperscript{2+} or Mg\textsuperscript{2+}), sonicated for 1 minute in TE buffer (20 mmol/L Tris pH 7.4, 2 mmol/L EDTA) containing 3 mmol/L EGTA, 2 mmol/L DTT, 330 mmol/L sucrose, 0.1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin, 50 μg/mL pepstatin, 100 μg/mL aprotinin, and 0.5% Triton-X 100, and gently rocked for 40 minutes at 4°C. The homogenates were centrifuged at 100 000g for 30 minutes at 4°C. PKC activity was assayed with the Amersham PKC enzyme assay system. PKA activity was assayed with the Promega assay system. PKA activity was determined with a Bio-Rad protein assay kit.

Cell Viability Assay, In Situ Nick End-Labeling and Propidium Iodide Staining, Cell Death Detection ELISA
Cell viability was assessed by MTT assay (Boehringer Mannheim). Kit instructions were followed for assay procedures. The terminal deoxynucleotidyl transferase (TDT)–mediated dUTP–digoxigenin nick end-labeling (TUNEL) assay (Oncor) was used to detect DNA fragmentation in situ. The detection procedures were performed according to the kit instructions. Fixed and permeabilized samples were stained with propidium iodide (PI) as described. To quantify the percentage of apoptotic cells, cells attached to culture dishes were counted. Fewer than 3% of the cells detached from culture dishes and were not counted. Cell death detection ELISA was performed according to the manufacturer’s instructions (Boehringer Mannheim) as described before.

LDH Detection
Lactate dehydrogenase (LDH) was assessed by use of an LDH detection kit (Boehringer Mannheim). The assay procedures were followed according to the kit instructions. Optical density was read at 490 nm.

Flow Cytometry Analysis of Cell Death
Cells were labeled with annexin V (R&D Systems) and PI according to the manufacturer’s instructions. Samples (10\textsuperscript{5} events) were analyzed with a flow cytometer (Coulter Epics XL/XL-MCL System II). Cells labeled annexin V\textsuperscript{−}/PI\textsuperscript{−} were considered apoptotic cells, and those labeled annexin V\textsuperscript{−}/PI\textsuperscript{+} were considered necrotic cells.

Detection of Caspase-3 Activity
Caspase-3 activity was detected with an assay kit (Biomol). The assay procedures were followed according to the kit instructions. Protein concentration was determined with a Bio-Rad protein assay kit.

Immunocytochemistry
The techniques for confocal microscopy were as described previously. Primary PKC antibodies were diluted in PBS with 0.1% BSA (1:80 to 1:200), washed twice with PBS, and then exposed to the secondary antibody (Cy2/Cy3-conjugated anti-rabbit or anti-mouse IgG, at 1:100, 1% BSA/PBS, Dianova) for 1 hour. Highly specific antibodies directed against peptide sequences of PKC were used that reacted specifically with the α- (monoclonal, UBI) or β-, δ-, ε-, and ξ-subspecies of PKC (polyclonal, Gibco). The preparation was mounted with 50% glycerol under a glass coverslip on a Nikon-Diaphot microscope. A Biorad MRC 600 confocal imaging system (Bio-Rad Laboratories) with an argon/krypton laser was used. At least 10 to 18 cells were examined from a minimum of 5 experiments under each experimental condition. The results were reproduced by 2 separate investigators, and multiple experiments were done. The observers were unaware of the technical design.

Statistical Analysis
The results are expressed as mean±SEM of at least 3 independent experiments, unless stated otherwise. Paired data were evaluated by Student’s t test. A 1-way ANOVA was used for multiple comparisons. A value of P<0.05 was considered significant.

Results

\textbf{H}_{2}\textsubscript{O}_{2} Induces PKC Activation and Apoptosis in VSMCs
\textbf{H}_{2}\textsubscript{O}_{2} caused a time-dependent increase in PKC activity, reaching a maximum at 2 minutes, followed by a gradual decline to baseline values at 10 minutes (Figure 1A). To investigate whether \textit{H}_{2}\textsubscript{O}_{2}–induced activation of PKC can be influenced by preincubation of VSMCs with PMA, PDBu, staurosporine, H-7, or calphostin C, PKC activity was determined 2 minutes after \textit{H}_{2}\textsubscript{O}_{2} treatment (Figure 1B). A 20-minute preincubation with 50 mmol/L PMA led to a significant increase in PKC activity. Forty-eight hours of preincubation with 200 nmol/L PDBu induced a significant reduction in PKC activity. Pretreatment with PMA could not potentiate the effect of \textit{H}_{2}\textsubscript{O}_{2} on PKC activity, probably because PKC activity already had reached the maximum of its saturation kinetics. Preincubation with PDBu for 48 hours or staurosporine, H-7, or calphostin C for 2 hours completely abrogated \textit{H}_{2}\textsubscript{O}_{2}–induced activation of PKC in VSMCs.

To determine whether \textit{H}_{2}\textsubscript{O}_{2} leads to apoptosis, VSMCs were labeled with annexin V and PI and were subsequently analyzed by flow cytometry. This method allows quantification of apoptotic cells and also differentiation between apoptotic (annexin V\textsuperscript{−}/PI\textsuperscript{−}) and necrotic (annexin V\textsuperscript{−}/PI\textsuperscript{+}) cells. Figure 1C shows that there was a dose-dependent increase in apoptotic VSMCs treated with \textit{H}_{2}\textsubscript{O}_{2} at 25 to 100 μmol/L, whereas the number of necrotic cells was low and remained unaffected by the increased doses. This is in agreement with our previous study, which demonstrated that \textit{H}_{2}\textsubscript{O}_{2} can cause VSMC apoptosis.

\textbf{PKC-α and PKC-ε Are Involved in \textit{H}_{2}\textsubscript{O}_{2}–Induced Apoptosis of VSMCs
To identify specific PKC isoforms involved in \textit{H}_{2}\textsubscript{O}_{2}–induced apoptosis in VSMCs, we used confocal microscopy and
assessed the intracellular distribution of PKC isoforms. There was no detectable change in distribution for PKC isoforms β, δ, and ζ between 30 seconds and 60 minutes of exposure to H_{2}O_{2} (data not shown). As depicted in Figure 2, under resting conditions PKC-α was evenly distributed throughout the cytoplasm and the perinuclear region and PKC-ε showed a punctate pattern, mainly in the perinuclear region. After 2 minutes of exposure to H_{2}O_{2}, a time at which we had detected the highest activation of PKC by H_{2}O_{2} in VSMCs (see Figure 1A), we observed a rapid translocation of PKC-α and PKC-ε to the nucleus (Figure 2), which was confirmed by vertical section via confocal microscopy (data not shown). In addition, the cytoplasmic pattern of distribution of both PKC-α and PKC-ε changed, resulting in a fibrillary pattern for both isoforms. The time course of redistribution for both isoforms correlated well with that detected by the PKC kinase assay as depicted in Figure 1A (data not shown). These results indicate that PKC-α and PKC-ε may be involved in H_{2}O_{2}-induced apoptosis of VSMCs and that their specific change of intracellular distribution could reflect their role in the disassembly of VSMCs during apoptotic death.

**PKC Switches H_{2}O_{2}-Induced Death From Necrosis to Apoptosis**

To investigate whether PKC activation is important for H_{2}O_{2}-induced death of VSMCs, we first detected cell viability by MTT assay in the absence or presence of PMA, PDBu, staurosporin, H-7, or calphostin C. As depicted in Figure 3A, pretreatment with PMA alone did not influence the viability of VSMCs. Also, the reduction of VSMC viability induced by H_{2}O_{2} was not altered by preincubation with PMA. A 48-hour pretreatment of VSMCs with PDBu had no influence on H_{2}O_{2}-induced cell death, and 2 hours of preincubation with staurosporin, H-7, or calphostin C also did not affect H_{2}O_{2}-induced death of VSMCs.

In light of the lack of specificity of the MTT assay to differentiate between necrotic and apoptotic cell death, we reasoned that the inhibition of PKC activity might reduce...
H$_2$O$_2$-induced apoptosis but simultaneously leads to an increase in H$_2$O$_2$-induced necrosis. To test this hypothesis, several methods were used to evaluate the type of cell death when PKC was downregulated or inhibited. With those cells regarded as apoptotic cells that simultaneously exhibited condensed nuclei and positive labeling by TUNEL (Figure 3B), the percentage of apoptotic cells was quantified by cell counting and summarized in Figure 3C. There were <10% apoptotic cells in VSMC cultures not exposed to H$_2$O$_2$. However, stimulation with 100 $\mu$mol/L H$_2$O$_2$ led to cell death characteristic of apoptosis in 54% of VSMCs. Activation of PKC by pretreatment with 50 nmol/L PMA did not affect apoptotic death in H$_2$O$_2$-exposed VSMCs. In contrast, downregulation or inhibition of PKC markedly decreased the percentage of H$_2$O$_2$-induced apoptotic cells compared with H$_2$O$_2$ alone. Next, we performed simultaneous quantitative detection of cytoplasmic histone-associated DNA fragments characteristic for apoptosis, with the assessment of LDH release occurring during necrosis. Whereas pretreatment with 50 nmol/L PMA had no effect on histone-associated DNA fragments or LDH release, exposure to 100 $\mu$mol/L H$_2$O$_2$ led to a marked increase in cytoplasmic histone-associated DNA fragments without a parallel increase in LDH leakage. When PKC was downregulated or inhibited, a marked decrease in histone-associated DNA fragments paralleled by an increase in LDH leakage could be observed.

To test whether PKA also contributes to H$_2$O$_2$-induced apoptosis and necrosis, H$_2$O$_2$-treated cells were used to inhibit PKA by preincubation of VSMCs for 1 hour before exposure to H$_2$O$_2$. At low doses (1 to 3 $\mu$mol/L), H-8 inhibited neither the activation of PKC induced by 100 $\mu$mol/L H$_2$O$_2$ nor the subsequent induction of apoptosis as assessed by TUNEL and cell death ELISA (data not shown). Also, 100 $\mu$mol/L H$_2$O$_2$ did not lead to significant changes of PKA activity (data not shown). However, at higher doses (50 to 100 $\mu$mol/L), H-8 led to a decreased activity of both PKA and PKC, under which circumstances H$_2$O$_2$-induced VSMC death occurred mainly by necrosis, as revealed by TUNEL and cell death ELISA with LDH release (data not shown). Thus, it appears that PKA is not involved in H$_2$O$_2$-induced VSMC apoptosis.

An alternative method to differentiate between apoptosis and necrosis is the double labeling of cells with annexin V (binds to phosphatidylserine, being located at the cytoplasmic layer of the plasma membrane and at the extracellular layer at early stages of apoptosis) and PI and subsequent analysis by flow cytometry. As shown in Figure 4, the majority of cells in the control were healthy cells (annexin V$^{-}$/PI$^{-}$, left lower quadrant). When cells were treated with 100 $\mu$mol/L H$_2$O$_2$, a significant number of apoptotic cells appeared (V$^{+}$/PI$^{-}$, right lower quadrant). When PKC was downregulated or inhibited, the pattern of distribution of cells changed tremendously, resulting in an increase in population of necrotic cells (V$^{+}$/PI$^{+}$, right upper quadrant).

To further specify the role of PKC in regulating cell death in VSMCs, we decided to inhibit apoptosis farther downstream in the signaling cascade. Caspase-3, a key regulatory protease at which many signaling pathways merge for the execution of apoptosis, was shown to be activated in VSMCs treated with 100 $\mu$mol/L H$_2$O$_2$ (Figure 5A). Inhibition of caspase-3 by Ac-DEVD-CHO, a relatively specific inhibitor for caspase-3, could dose-dependently reduce H$_2$O$_2$-induced caspase activation (Figure 5A). The activation of PKC by
pretreatment with 50 nmol/L PMA did not alter the activation of caspase-3 by H$_2$O$_2$. In contrast, the activation of caspase-3 by H$_2$O$_2$ was markedly reduced when PKC was downregulated or inhibited, indicating that PKC acts upstream of caspase-3 in H$_2$O$_2$-induced VSMC apoptosis (Figure 5A). Neither apoptosis nor necrosis occurred when VSMCs were treated with H$_2$O$_2$ in the presence of Ac-DEVD-CHO (Figure 5B). Moreover, Ac-DEVD-CHO was unable to prevent cell death induced by H$_2$O$_2$ when PKC was downregulated or inhibited (Figure 5B). Thus, although both PKC and caspase-3 appear to be important for the apoptotic signal transduction induced in VSMCs exposed to H$_2$O$_2$, it is PKC that by virtue of the prevention of necrotic cell death allows the execution of the apoptotic program.

Mitochondria participate in the initiation of apoptotic programs either by releasing cytochrome c or by opening mitochondrial membrane transitions. These 2 events are upstream of caspase-3 activation. In mitochondria, MTT is cleaved to formazan by the succinate–tetrazolium reductase system. Thus, the loss of the ability of mitochondria to cleave MTT appears to be downstream of caspase-3 activation, because inhibition of caspase-3 by Ac-DEVD-CHO can prevent H$_2$O$_2$-induced decrease of MTT cleavage.

**Discussion**

Our data indicate a central role for PKC in VSMCs exposed to oxidative stress. Here it not only functions as a key component of the apoptotic signaling cascade but also proves to be indispensable for the prevention of the otherwise unavoidable and deleterious induction of necrotic cell death. This is an important finding, because apoptosis and necrosis represent 2 distinct histopathological entities that subsequently lead to the activation of divergent cytochemical cascades within the surrounding tissue and thus may have different effects on organ function and organ integrity.11

Up to now, numerous studies have been undertaken to investigate the role of PKC in apoptosis, many of which found PKC to inhibit rather than to induce apoptosis.15–19 From most of these studies, it is not clear whether the different PKC activators or inhibitors exerted their effects on apoptosis directly by themselves or through modulation of PKC activity. A few cases, most of which were obtained from immortalized cells, describe an induction of apoptosis due to activation of PKC.20 –22 However, to the best of our knowledge, the indispensability of PKC for the coordinated execution of the apoptotic program by virtue of its prevention of necrosis has never before been described, and we must address whether this is unique to VSMCs and/or to H$_2$O$_2$-induced cell death.

Certainly, our data do not exclude the possibility that other regulators of the apoptotic signaling cascade may exert a role similar to that of PKC for the execution of the apoptotic program in VSMCs exposed to oxidative stress. In this regard, it is of interest that a comparable function was recently determined for intracellular ATP.23 Energy depletion of cells triggered to die converts an apoptotic death signal into necrosis. Thus, at least for ROS-induced death in VSMCs, PKC appears to play a similarly important role.

At first, it seems reasonable that the inhibition of the signal transduction pathway of H$_2$O$_2$, which leads to an active form of cell death, does not prevent the ultimate passive form of cell death. However, our data show that the blockage of the
H₂O₂-induced signaling cascade on the level of caspase activity not only results in the prevention of apoptosis but also in the subsequent survival of these cells, whereas the inhibition of PKC activity is absolutely incompatible with the viability of VSMCs exposed to oxidative stress. Our data are insufficient to explain which of the plethora of signaling pathways controlled by PKC are responsible for the suppression of necrosis, and further studies are needed to identify specifically those pathways that keep the cells alive. However, I explanation for this observation may be the fact that PKC is not a single entity but rather is composed of a family of at least 11 isoenzymes, of which at least 5 were shown to be expressed in VSMCs.¹⁴ Of those, we found a rapidly changing distribution pattern exclusively for PKC-α and PKC-ε on stimulation with H₂O₂. Recent reports have described a role of PKC-α or PKC-ε in apoptosis, indicating that the function of these PKC isoforms is anything but consistent and depends largely on the apoptotic stimulus and cellular target.¹⁴ ¹² ²⁵ This also holds true for the other 3 isoforms shown to be expressed in VSMCs, namely PKC-β, PKC-δ, and PKC-γ, which in our experiments were not shown to translocate. Thus, one might speculate that H₂O₂ leads to the activation of PKC-α and PKC-ε, initiating their participation in the apoptotic program, while other members of the PKC family may be engaged in processes essential for the prevention of necrosis. Although this idea still lacks experimental evidence, it is supported by the known complexity of action of different PKC isoforms.²⁶ ²⁹ Also, our observation that both H₂O₂ and PMA were able to induce PKC activation to nearly the same extent (Figure 1B) but only PKC activation by H₂O₂ was followed by VSMC apoptosis could be related to the complex nature of activity of different PKC isoforms. PMA has been shown to activate a variety of PKC isoforms, each of which appears to have specific roles in the regulation of apoptosis.²¹ Thus, the diversity of functions exerted by the individual PKC isoforms could explain the difference in the effect of H₂O₂ and PMA and also provide a theoretical model for the complex role of PKC in H₂O₂-induced VSMC death. Nevertheless, future studies are needed to address the specific role of other PKC isoforms for the regulation of apoptosis in VSMCs. Although our results provide intriguing evidence for a role of PKC-α and PKC-ε in apoptotic death of VSMCs exposed to H₂O₂, they do not explain their specific role for the apoptotic disassembly of the cell. Therefore, it is important to identify the cellular targets of PKC-α and PKC-ε in H₂O₂-induced apoptosis of VSMCs to gain more insight into the execution of cell death in these cells.
The results in our present study demonstrate that H$_2$O$_2$ only transiently stimulates PKC in VSMCs; the ensuing decrease of PKC activity may be due to the depletion of PKC as a consequence of death of VSMCs exposed to H$_2$O$_2$. Staurosporine and calphostin C have been shown to induce apoptosis in many cell types; in particular, calphostin C is able to trigger VSMC apoptosis. In that study, however, cells were exposed to calphostin C continuously for up to 6 days, whereas in our study, only a 2-hour incubation was used. This may explain why we could not observe calphostin C–induced apoptosis under our experimental design.

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