Peroxynitrite activates mitogen-activated protein kinase (MAPK) via a MEK-independent pathway: a role for protein kinase C

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Abstract In this study we show that phosphorylation of extracellular signal-regulated kinase (ERK1/2; also known as p44/42MAPK) following peroxynitrite ($ONOO^-$) exposure occurs via a MAPK kinase (MEK)-independent but PKC-dependent pathway in rat-1 fibroblasts. $ONOO^-$-mediated ERK1/2 phosphorylation was not blocked by MEK inhibitors PD98059 and U0126. Furthermore, no increase in MEK phosphorylation was detected upon $ONOO^-$ treatment. Staurosporine was used to investigate whether protein kinase C (PKC) is involved. This was confirmed by down-regulation of PKC by phorbol-12,13-dibutyrate, which resulted in significant reduction of ERK1/2 phosphorylation by $ONOO^-$, implying that activation of ERK by $ONOO^-$ depends on activation of PKC. Indeed, PKCα and ε were activated upon $ONOO^-$ exposure. When cells were treated with $ONOO^-$ in a calcium-free buffer, no activation of PKCα was detected. Concomitantly, a reduction of ERK1/2 phosphorylation was observed suggesting that calcium was required for translocation of PKCα and ERK phosphorylation by $ONOO^-$. Indeed, $ONOO^-$ exposure resulted in increased cytosolic calcium, which depended on the presence of extracellular calcium. Finally, data using Gö6976, an inhibitor of calcium-dependent PKC activation, implied that $ONOO^-$-mediated ERK1/2 phosphorylation depends on activation of a calcium-dependent PKC.

Key words: Peroxynitrite; Mitogen-activated protein kinase; Protein kinase C; Mitogen-activated protein kinase kinase; Oxidative stress; Signal transduction

1. Introduction

Signal transduction pathways respond to a variety of extracellular signals such as mitogenic or environmental stimuli, and oxidative stress. One such component of the signal transduction pathway is mitogen-activated protein kinase (MAPK), whose activation or inactivation plays a key role in transducing extracellular signals into intracellular events and leads ultimately, to modulation of gene expression. The MAPK family constitutes three major classes, namely: stress-activated protein kinases (SAPK or JNK), the p38MAPK and the extracellular signal-regulated kinases (ERK). While JNK and p38MAPK mainly respond to environmental stress and cytokine stimulation, the ERKs serve mainly as signaling molecules involved in regulation of cell proliferation. In mammalian cells two forms of ERK namely, ERK1 and ERK2 have been identified.

There is increasing evidence suggesting that reactive oxygen species activate or inactivate signaling pathways involving tyrosine and serine/threonine kinases and thereby, regulate phosphorylation of MAPKs. Indeed, MAPKs are reported to be activated in response to various forms of oxidative stress such as UV irradiation [1], hydrogen peroxide [2], nitric oxide [3], and peroxynitrite ($ONOO^-$) [4–7]. Of particular interest is the highly reactive $ONOO^-$, which is involved in oxidative damage detected in several pathological conditions such as inflammation, ischemia/reperfusion injury, atherosclerosis and neurological disorders (reviewed in [8,9]). In vivo, $ONOO^-$ is formed from a diffusion-limited reaction of nitric oxide and superoxide. As stated above, $ONOO^-$ activates MAPK. Recently, Zhang et al. have suggested that activation of MAPK kinase (MEK) is necessary for MAPK phosphorylation in rat lung fibroblasts [7]. However, the exact mechanism by which $ONOO^-$ induces MAPK phosphorylation remains unclear.

The present work was conducted to investigate the mechanism by which $ONOO^-$ activates ERK1/2 in rat-1 fibroblasts. We report in this paper that $ONOO^-$ can activate ERK independent of MEK activation. Further, for the first time, we present evidence that activation of ERK by $ONOO^-$ is dependent on protein kinase C (PKC), most likely, a calcium-dependent PKC.

2. Materials and methods

2.1. Cells

Rat-1 fibroblasts [10] were obtained from Hubrecht laboratories, Utrecht, The Netherlands. The cells were grown in Dulbecco's modified Eagle's medium (Gibco, UK) with 10% fetal calf serum (Gibco, UK) and cultured at 37°C in a 5% CO_2 humidified atmosphere. The cells were allowed to grow in 35-mm dishes to a final density of 60 000 cells/cm^2.

2.2. Antibodies

The antibodies against phosphorylated ERK1/2, MEK1/2, and phosphorylated MEK1/2 were purchased from New England Biolabs, Beverly, MA, USA. The ERK2 monoclonal antibody was obtained from Upstate Biotechnologies, Lake Placid, NY, USA. Antibodies against various PKC isoforms were from Transduction labs, Lexing-

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; $ONOO^-$, peroxynitrite; MEK, MAPK kinase; PBS_{glc}, phosphate-buffered saline; PKC, protein kinase C; PDBU, phorbol-12,13-dibutyrate; EGF, epidermal growth factor; EGFR, EGF receptor

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ton, KY, USA. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA.

2.3. $ONOO^-$ treatments

$ONOO^-$ was obtained as a 75 mM stock in 10 mM KOH (stored at −80°C) and was a gift from Prof. W.H. Koppenol (Laboratorium für Anorganische Chemie ETHZ, Zurich, Switzerland). Prior to $ONOO^-$ treatment, cells were washed with phosphate-buffered saline (PBS_{glc}) (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.2 with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5 mM glucose added freshly on day of use) and incubated in the PBS_{glc} for 10–15 min at 37°C. In some experiments, $CaCl_2$ was excluded from PBS_{glc} (PBS_{-Ca}). $ONOO^-$ was added directly to the dishes, using shaking for 10 s, to a final concentration of 200 μM (unless otherwise mentioned) and subsequently incubated for 2–180 min at 37°C. To adjust for the possible effect of a change in pH, controls received equivalent amounts of 10 mM KOH. A parallel set of plates received decomposed $ONOO^-$, to exclude effects of decomposition products. Decomposed $ONOO^-$ was prepared by incubating $ONOO^-$ in PBS_{glc} (to a final concentration of 200 μM) at 37°C for 3 h.

Following treatment, cells were placed on ice and washed with ice cold PBS_{glc} and samples prepared for Western blotting.

2.4. MEK inhibitors

Cells were pre-incubated with MEK inhibitors PD98059 (50 μM of 20 mM stock; Calbiochem, La Jolla, CA, USA) for 40 min or U0126 (40 μM of 10 mM stock; Promega, Madison, WI, USA) for 30 min prior to treatment with $ONOO^-$. The inhibitors were present in the PBS_{glc} throughout $ONOO^-$ treatment.

2.5. PKC inhibitors/down-regulators

PKC inhibitors and down-regulators were from Calbiochem, La Jolla, CA, USA. Staurosporine was added to cells for 15 min prior to and during treatment with $ONOO^-$ at a final concentration of 1 μM. Down-regulation of PKC was obtained by pre-incubating cells for 24 h with 200 nM phorbol-12,13-dibutyrate (PDBU; 200 μM stock in sterile distilled water). To activate PKC cells were incubated for 10 min with 200 nM PDBU. At short incubation times PDBU activates all PKC (except atypical PKC). Gö6976, a specific inhibitor of calcium-dependent PKC was added to the cells for 40 min before $ONOO^-$ exposure.

2.6. Cell fractionation

The plasma membrane-enriched fraction of cells was separated from cytosol using centrifugation as described by Evans, [11] with minor modifications. Briefly, following treatment with $ONOO^-$ or PDBU, cells were scraped on ice in homogenization buffer (250 mM Sucrose, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 μg/ml aprotinin and 5 μg/ml leupeptin). Cells were homogenized by passing 20 times through a 24-G syringe. Whole cells and nuclei were removed by centrifuging for 5 min at 1000×*g*, 4°C. The supernatant was subsequently centrifuged for 15 min at 10 000×*g*, 4°C. The supernatant was considered as cytosolic fraction. The pellet (plasma membrane-enriched fraction) washed twice with homogenization buffer. The protein content of each of the fractions was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) as per the manufacturer's instructions. Samples were then prepared for Western blotting.

2.7. Preparation of samples and Western blotting

Following treatment, the rat-1 cells or cell fractions were taken up in sample buffer (8.3% glycerol, 75 mM dithiothreitol, 1.7% sodium dodecyl sulfate, 0.0025% bromophenol blue and 20 mM Tris–HCl pH 8.8), boiled for 10 min and stored at −20°C until use. Proteins (20–25 μg) were separated by SDS–PAGE on a 10% polyacrylamide gel and blotted on to PVDF membranes. The blots were blocked overnight at 4°C using 2% milk powder (Protifar, Nutricia, The Netherlands) in PBS–Tween (0.05% v/v Tween-20 in PBS). Following incubation with primary antibodies (diluted in 0.5% milk powder in PBS–Tween) for 60 min at room temperature, the blots were washed for 40 min and incubated for 45 min with secondary horseradish peroxidase-coupled antibodies (diluted in the 0.5% milk powder). After washing for 30 min, the proteins of interest were detected using a chemiluminescence detection kit (Life Sciences Products, Boston, MA, USA).

2.8. Measurement of calcium

Cytosolic calcium in cells was measured using the fluorescent dye INDO-1 AM (Molecular Probes Inc., Eugene, OR, USA). Cells were seeded on 25-mm coverslips to a density of 60 000 cells/cm2. Cells were washed with PBS_{glc} or PBS_{-Ca} coverslips were transferred to a dish containing 5 μg INDO-1 AM/ml PBS_{glc} or PBS_{-Ca} and incubated for further 30 min, protected from light. The intensity of INDO-1 bound to calcium was observed using a confocal scanning laser microscope (Nikon RCM8000 connected to a Nikon Diaphot 300 inverted microscope; Objective water immersion 40×, NA1.15 excitation ∼351 nm; emission PMT1∼400 nm; emission PMT2∼490 nm). Results presented are a ratio of emission 1 and emission 2 using 'false colors' to visualize changes in fluorescence intensities. Changes in calcium levels were recorded continuously for up to 10 min after $ONOO^-$ exposure.

All results presented are a fair representation of results obtained from at least three independent experiments.

3. Results

3.1. $ONOO^-$ activates ERK1/2

Incubation of cells with 50 μM or more of $ONOO^-$ for 10 min resulted in increased ERK phosphorylation (Fig. 1A). The activation was also time-dependent (Fig. 1B; panel 1). Maximum phosphorylation was observed between 10 and 30 min after $ONOO^-$ supplementation followed by a transient decrease in ERK phosphorylation. Further, treatment with decomposed $ONOO^-$ did not affect the phosphorylation of MAPK indicating that the observed activation is not the effect of a degraded product of $ONOO^-$ (data not shown).

3.2. ERK1/2 phosphorylation by $ONOO^-$ occurs via a MEK-independent pathway

Inhibitors of MEK (PD98059 and U0126) were used to investigate if $ONOO^-$ activated ERK via MEK. PD98059 exerts its inhibitory effects by binding directly to the inactive (non-phosphorylated) form of MEK after blocking its activation by Raf [12,13]. U0126 on the other hand, does not affect MEK phosphorylation but directly inhibits activation of MAPK by MEK1 [13,14].

Cells were incubated with the inhibitors and were subsequently, treated with 200 μM $ONOO^-$ for 2–30 min at 37°C. Incubation of rat-1 cells with $ONOO^-$ alone resulted in maximum phosphorylation of ERK1/2 at 10–30 min (Fig. 1B; panel 1). Surprisingly, both inhibitors did not block ERK activation by $ONOO^-$ (Fig. 1B; panels 2 and 3) implying that MEK activation is not required for ERK phosphorylation by $ONOO^-$. In contrast, both inhibitors completely blocked ERK phosphorylation in serum-deprived cells incubated with complete medium for 10 min (used as positive control; data not shown) indicating that serum activation of ERK occurs via the MEK pathway. This result also confirmed the effectiveness of both the inhibitors in blocking activation of ERK via MEK.

MEK-independent ERK1/2 phosphorylation was further verified by detecting phosphorylated MEK in cells following $ONOO^-$ treatment (Fig. 1C). Incubating cells with $ONOO^-$ for 2–30 min did not significantly increase MEK phosphorylation. On the other hand, serum-starved cells incubated for 10 min in complete medium exhibited elevated MEK1/2 phosphorylation (data not shown). This result confirms that indeed, ERK phosphorylation in rat-1 cells by $ONOO^-$ occurs via a MEK-independent pathway, in contrast to the MEK-dependent, MAPK activation by serum.

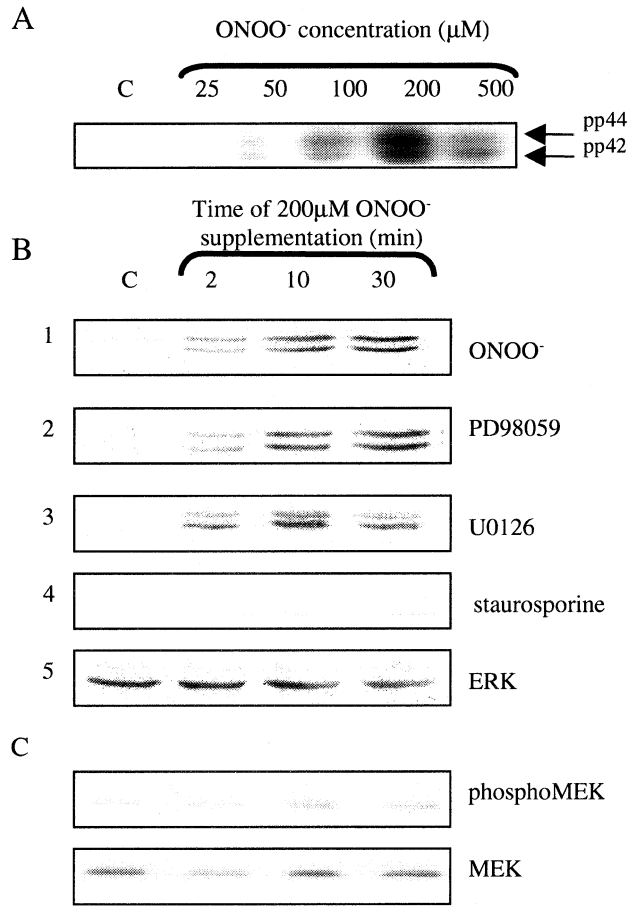

Fig. 1. $ONOO^-$ exposure results in phosphorylation of ERK1/2 via a MEK-independent but PKC-dependent pathway. A: Cells were incubated with 25–500 μM $ONOO^-$ for 10 min. Controls (C) were incubated with an equivalent amount of 10 mM KOH. B: Detection of phosphorylated ERK1/2 in cells exposed to 200 μM $ONOO^-$ for 2–30 min (Panel 1). Panels 2 and 3 present data on $ONOO^-$-induced ERK1/2 activation when cells are pre-treated with MEK inhibitors PD98059 and U0126, respectively. The effect of PKC inhibitor staurosporine on phosphorylation of ERK1/2 by $ONOO^-$ is depicted in panel 4. ERK2 is presented as a loading control (panel 5) and is a fair representation of the proteins loaded on to each lane. C: Phosphorylation of MEK following treatment of cells with 200 μM $ONOO^-$ for 2–30 min. For this experiment anti-MEK was used as a loading control. Control cells (C) received no $ONOO^-$.

3.3. Effect of PKC inhibition/down-regulation

To elucidate the mechanism by which ERK1/2 is activated by $ONOO^-$, PKC inhibitors and down-regulators were used.

Panel 4 of Fig. 1B shows the effect of staurosporine, an inhibitor of PKC activity, on $ONOO^-$-induced phosphorylation of MAPK. Pre-incubation of cells with Staurosporine resulted in a significant inhibition of $ONOO^-$ activation of ERK (compare panels 1 and 4) suggesting that in rat-1 fibroblasts, $ONOO^-$ activates MAPK via a PKC-dependent pathway. Furthermore, ERK phosphorylation on serum activation was not affected by staurosporine suggesting once again, that different pathways are involved in the activation of ERK by serum and $ONOO^-$ (data not shown).

To validate the role of PKC in $ONOO^-$ activation of ERK, we down-regulated PKC in the cells by incubating them with PDBU for 24 h. The PKC down-regulated cells were treated with $ONOO^-$ and subsequently, PKCα and phosphorylated ERK detected. The results are presented in Fig. 2. A complete down-regulation of PKCα protein, being one of the PKC isoforms known to be down-regulated by PDBU, was indeed observed in all cell samples incubated with PDBU (Fig. 2; top panel). Interestingly, PDBU-treated cells exhibited significant reduction in ERK1/2 phosphorylation (Fig. 2; middle panel). These results support and give more specific evidence for our proposition that PKC is involved in $ONOO^-$-induced MAPK activation.

3.4. $ONOO^-$ translocates PKC to the membrane

The results using PKC inhibition/down-regulation clearly indicate that activation of PKC is involved in the phosphorylation of ERK by $ONOO^-$. PKC is known to translocate to the plasma membrane upon activation. Therefore, plasma membrane and cytosol fractions of rat-1 cells were separated after exposure to $ONOO^-$ and total PKC protein detected in the fractions. To date 12 different PKC isoforms have been identified in mammalian cells. The isoforms α, ε, δ, λ, and ζ could be identified in rat-1 fibroblasts (data not shown; Western blot on total cell lysates using specific antibodies directed against the various PKC isoforms). In order to identify which of these isoforms were activated by $ONOO^-$, membrane and cytosolic fractions of the cells were probed with antibodies directed against each of these isoforms. We found only PKCα and ε to be activated upon $ONOO^-$ treatment (Fig. 3A,B, respectively). As expected, PDBU substantially activated PKCα and PKCε. Exposure of cells to $ONOO^-$ resulted in a reduction of PKCα in the cytosol fraction concomitant with a marked increase (~2.4-fold; measured using densitometry) in membrane-bound PKCα (Fig. 3A). Similarly, a >2-fold increase in membrane-associated PKCε was also observed (Fig. 3B). PKCα belongs to the conventional PKC group and is therefore, dependent on calcium for its activation. On the other hand, PKCε is a novel PKC and is not dependent on calcium for its activation. In order to determine the PKC isoform that might be involved in ERK1/2 phosphorylation, we studied the role of calcium in PKC and ERK activation by $ONOO^-$.

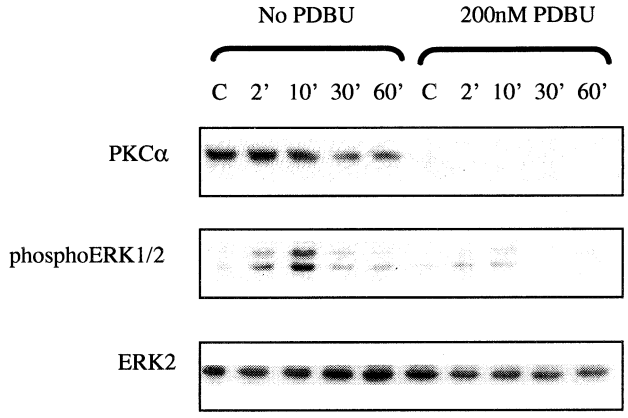

Fig. 2. PKC down-regulation with PDBU results in an inhibition of ERK1/2 activation by $ONOO^-$. Detection of PKCα (top panel) or phosphorylated ERK1/2 (middle panel) in cells incubated overnight with (lanes 6–10) or without (lanes 1–5) 200 nM PDBU. Subsequently, cells were washed with PBS_{glc} and exposed to 200 μM $ONOO^-$ for 2–60 min. Anti-ERK2 was used to check protein loading (bottom panel). Controls (C) were not treated with $ONOO^-$.

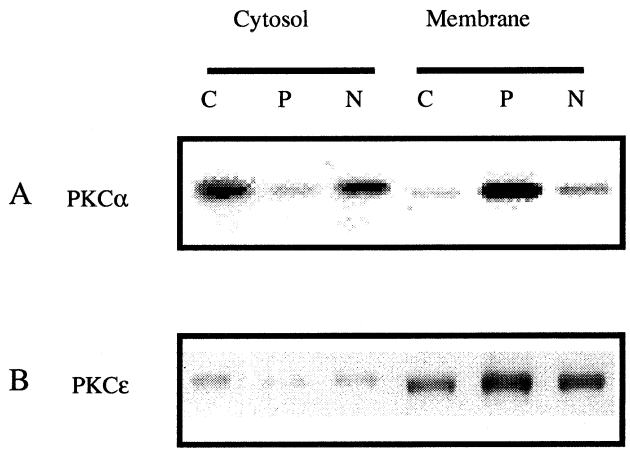

Fig. 3. $ONOO^-$ treatment results in PKC translocation to the plasma membrane. Detection of (A) PKCα or (B) PKCε in cytosol (lanes 1–3) or membrane (lanes 4–6) fractions of rat-1 fibroblasts. Cells were incubated with 200 nM PDBU (P) or 200 μM $ONOO^-$ (N) or left untreated (C) for 10 min in PBS_{glc}. After fractionation equal amounts of protein (20 μg) were loaded on the gel.

3.5. The role of calcium

There have been reports that $ONOO^-$ supplementation results in increased cytosolic calcium [15]. An increase in cytoplasmic calcium can occur via an influx of extracellular calcium following $ONOO^-$ exposure. Such an increase in cytosolic calcium might result in activation of Ca^{2+}-dependent PKC and subsequent phosphorylation of ERK. In order to test this hypothesis we treated cells with $ONOO^-$ in PBS_{-Ca}. The level of intracellular calcium after $ONOO^-$ treatment in PBS_{glc} or PBS_{-Ca} was visualized by loading the cells with INDO-1 AM. The results are presented in Fig. 4. Within 2 min of treatment with $ONOO^-$ in PBS_{glc} a significant rise in cytosolic calcium was observed (Fig. 4A). The calcium levels remained elevated throughout the 10 min tested. In contrast, the calcium levels did not change when the cells were exposed to $ONOO^-$ in PBS_{-Ca} (Fig. 4B). These results clearly show that treatment of rat-1 fibroblasts with $ONOO^-$ results in an increase in cytosolic calcium. Further, the results suggest that an influx of calcium from the extracellular medium is responsible for the rise in cytosolic calcium after $ONOO^-$ incubation in PBS_{glc}.

To determine whether such increases in calcium levels coincide with, and might play a role in the activation of PKC by $ONOO^-$, we studied the translocation of PKCα (the only calcium-dependent PKC detected in rat-1 fibroblasts) to the membrane following $ONOO^-$ treatment in PBS_{glc} or PBS_{-Ca} (Fig. 5A). When rat-1 fibroblasts were exposed to $ONOO^-$ in PBS_{glc} containing calcium an increase in the amount of PKCα protein was detected in the membrane fraction. In contrast, when the cells were treated with $ONOO^-$ in PBS_{-Ca}, no translocation of PKCα occurred indicating that an influx of extracellular calcium was indeed, required for activation of PKCα by $ONOO^-$. As a control for PKC translocation some cells were treated with PDBU for 10 min (Fig. 5A). A substantial translocation of PKCα was observed when the cells were stimulated with PDBU in PBS_{glc} as well as in PBS_{-Ca}. This indicates, as expected, that upon PDBU stimulation PKCα translocates to the plasma membrane independent of the calcium level in cells.

Since our results with PKC inhibition/down-regulation also suggested that PKC activation was involved in ERK phosphorylation by $ONOO^-$, we investigated whether extracellular calcium influenced ERK1/2 phosphorylation by $ONOO^-$. Rat-1 fibroblasts were exposed to $ONOO^-$ in PBS_{glc} or in PBS_{-Ca}. The results are presented in Fig. 5B. We observed

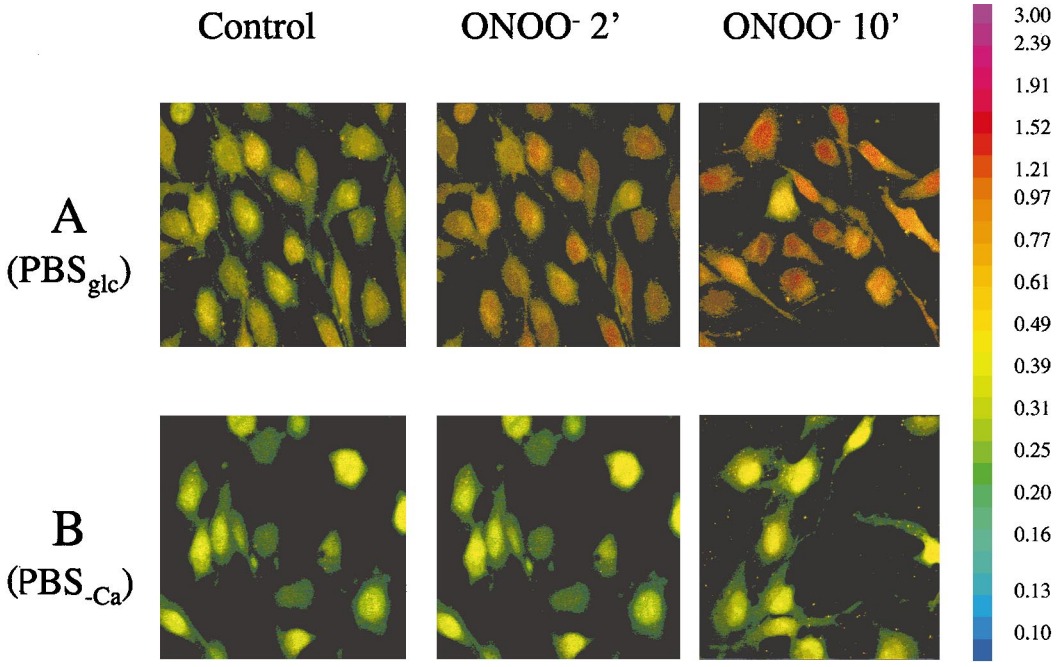

Fig. 4. $ONOO^-$ exposure in PBS_{glc} results in increased cytosolic calcium. Cells were loaded with INDO-1 AM for 30 min before they were treated with 200 μM $ONOO^-$ for 2 or 10 min in (A) PBS_{glc} or (B) PBS_{-Ca}. Calcium in cells was observed using confocal scanning laser microscopy. The color bar is a measure of the ratio of bound INDO-1/unbound INDO-1 indicating increasing calcium concentration in cells. Controls indicate the calcium measured in the cells before $ONOO^-$ exposure.

that ERK phosphorylation after $ONOO^-$ incubation was significantly diminished in cells that had been incubated in PBS_{-Ca} as compared to those treated in PBS_{glc} (Fig. 5B; compare panel 2 with panel 1). The results show that extracellular calcium is involved, at least partly, in the activation of ERK by $ONOO^-$.

Since calcium levels were also involved in the activation of PKCα by $ONOO^-$ we hypothesized that the phosphorylation of ERK by $ONOO^-$ may require the activation of a calcium-dependent PKC. To find evidence for this hypothesis, a selective inhibitor of Ca^{2+}-dependent PKC, Gö6976, was employed. Rat-1 fibroblasts were incubated with 10 μM Gö6976 for 30 min prior to treatment with $ONOO^-$. The inhibitor was present throughout $ONOO^-$ exposure. Treatment of cells with $ONOO^-$ in the absence of the inhibitor resulted in a transient increase in ERK phosphorylation (Fig. 5B; panel 1). In contrast, a marked reduction in ERK phosphorylation was observed in cells that had been incubated with Gö6976 prior to treatment with $ONOO^-$ (Fig. 5B; compare panel 3 with panel 1). The result suggests involvement of a Ca^{2+}-dependent PKC in ERK1/2 activation by $ONOO^-$.

4. Discussion

The present results show that $ONOO^-$ induces the phosphorylation of ERK1/2 in rat-1 fibroblasts. For the first time, to our knowledge, it is reported that in rat-1 fibroblasts, the activation of ERK by $ONOO^-$ occurs via a MEK-independent, but PKC- and calcium-dependent pathway. Further, we present evidence that a Ca^{2+}-dependent PKC might be involved in ERK phosphorylation by $ONOO^-$.

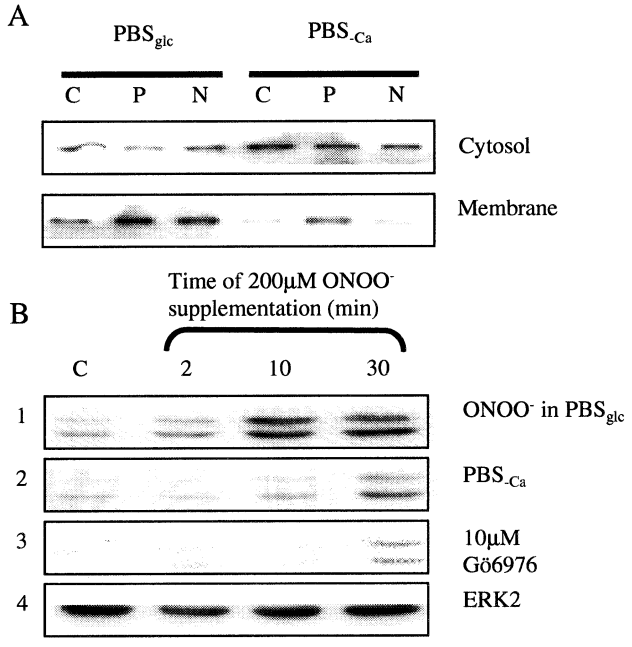

Fig. 5. Phosphorylation of ERK1/2 by $ONOO^-$ involves a calcium-dependent PKC. A: Detection of PKCα in cytosol or membrane fraction of cells treated with 200 nM PDBU (P) or 200 μM $ONOO^-$ (N) or left untreated (C) for 10 min in PBS_{glc} (lanes 1–3) or PBS_{-Ca} (lanes 4–6). After cell fractionation equal amounts of protein (20 μg) were loaded on gel. B: Cells were treated with 200 μM $ONOO^-$ for 2–60 min or left untreated (C; 10 min) in PBS_{glc} (panel 1), PBS_{-Ca} (panel 2) and phosphorylated ERK was detected. In panel 3 cells were pre-treated for 30 min with PKC inhibitor Gö6976 prior to 200 μM $ONOO^-$ exposure. Controls (C) were left untreated for 10 min. ERK2 was used as the loading control (panel 4).

Phosphorylation of ERK1/2 following $ONOO^-$ exposure has been previously demonstrated by other researchers [4–7]. These studies have reported that maximum activation of ERK1/2 by $ONOO^-$ occurred at approximately 15 min of $ONOO^-$ exposure. Our results indicate that phosphorylation of ERK1/2 after $ONOO^-$ treatment is highest at 10–30 min and is therefore, in agreement with the findings of others.

In order to investigate if ERK1/2 is activated via the Raf–MEK–MAPK pathway we employed two MEK inhibitors namely, PD98059 and U0126. Both these inhibitors failed to inhibit phosphorylation of ERK suggesting that $ONOO^-$-mediated MAPK activation occurs independent of MEK. This hypothesis was strengthened by the observation that no significant phosphorylation of MEK occurred upon $ONOO^-$ exposure. Jope et al. [6] recently showed that in PC12 cells, ERK1/2 activation by $ONOO^-$ is mediated via the epidermal growth factor (EGF) receptor. On the other hand, in a separate study, Zhang et al. [7] have reported that $ONOO^-$-mediated phosphorylation of ERK1/2 in rat lung fibroblasts occurs independent of EGF receptor (EGFR) or Raf-1 but is dependent on MEK activation. In contrast to the findings of these researchers, we have observed that in rat-1 fibroblasts, MEK phosphorylation is not required for ERK1/2 activation by $ONOO^-$. Thus, while these groups observed ERK activation 15 min after $ONOO^-$ exposure, there are differences regarding the proposed mechanisms. This disparity might be attributed to differences in cell type, concentrations of $ONOO^-$, mode of administration etc.

In rat-1 fibroblasts we observed that the PKC inhibitor staurosporine, significantly blocked $ONOO^-$-induced ERK1/2 phosphorylation. This observation implies that the activation of ERK by $ONOO^-$ depends on PKC. PDBU down-regulation of PKC resulted in substantial inhibition of $ONOO^-$-mediated ERK phosphorylation providing further evidence that PKC activation is required for ERK1/2 activation in response to $ONOO^-$.

The MEK inhibitor U0126 failed to block $ONOO^-$-induced ERK1/2 activation. U0126 directly inhibits activated MEK1 and prevents endogenously active MEK1/2 from phosphorylating and activating ERK1/2 [13,14]. Therefore, the inability of U0126 to block the effect of $ONOO^-$ suggests that PKC can activate ERK1/2 independent of MEK activation. Furthermore, the lack of MEK phosphorylation upon $ONOO^-$ stimulation also points to a MEK-independent pathway for ERK activation. MEK-independent ERK1/2 activation by PKC has also been reported by other researchers [16]. PKC is a multigene family comprising at least 12 distinct isoforms and is a pivotal constituent of a cascade of biochemical events that can be triggered by mitogens through cell surface receptors. Oxidative stress such as hydrogen peroxide has been reported to activate PKC [17,18]. We find that $ONOO^-$ exposure specifically activates PKCα and ε as demonstrated by their translocation to the plasma membrane. It is possible that $ONOO^-$ stimulates cell surface receptors initiating a signal transduction pathway involving PKC and leading ultimately, to phosphorylation of ERK1/2.

PKCα and ε belong to different classes of PKC isoforms. PKCα is a conventional PKC and is dependent on calcium for

its activation. On the other hand, activation of PKCε (a novel PKC) is not dependent on calcium. To determine which PKC isoform might play a role in ERK1/2 phosphorylation by $ONOO^-$ we investigated the role of calcium in the activation of PKC and ERK. Exposure of cells to $ONOO^-$ led to a substantial rise in intracellular calcium levels. Increased cytosolic calcium resulting in calcium overload following $ONOO^-$ exposure has also been reported by other researchers [15,19]. Such an increase could occur through enhanced influx of extracellular calcium and/or increased efflux from endogenous calcium stores. We observed that in the absence of calcium in the buffer, $ONOO^-$ caused no change in calcium levels suggesting that treatment of cells with $ONOO^-$ resulted in influx of extracellular calcium rather than an efflux of calcium from cellular stores.

Elevated intracellular calcium levels have been reported to be capable of activating ERK1/2 in B- and T-lymphocytes [20,21]. We hypothesized similarly, that in the rat-1 fibroblasts, exposure to $ONOO^-$ might elevate levels of intracellular calcium leading to activation of ERK via PKC. Indeed, we found that extracellular calcium was required for $ONOO^-$-induced translocation of PKCα. Thus, when cells were treated with $ONOO^-$ in PBS_{-Ca} (calcium-deficient PBS_{glc}), PKCα did not translocate to the plasma membrane. Interestingly, when the cells were treated with $ONOO^-$ in the absence of calcium (PBS_{-Ca}), the activation of ERK was also markedly reduced. This result demonstrates a clear link between rise in cellular calcium levels, translocation of PKCα and phosphorylation of ERK following $ONOO^-$ exposure. Further evidence for involvement of a calcium-dependent PKC in ERK phosphorylation was obtained when we observed that pre-incubating cells with Gö6976 (a specific inhibitor of Ca^{2+}-dependent PKC) significantly diminished ERK1/2 activation by $ONOO^-$. This confirms that $ONOO^-$-mediated phosphorylation of ERK depends on PKC and furthermore, it implies that a Ca^{2+}-dependent PKC such as α, β or γ might be involved. However, PKCα was the only calcium-dependent PKC detected in rat-1 fibroblasts. Therefore, our results suggest a role for PKCα in $ONOO^-$-induced ERK phosphorylation in these cells. Clearly, additional investigations are necessary to determine the PKC isoform involved in ERK phosphorylation by $ONOO^-$.

In summary, the present results provide evidence for involvement of a calcium- and PKC-dependent pathway in the activation of ERK by $ONOO^-$ in rat-1 fibroblasts. To our knowledge, this is also the first report demonstrating the translocation of PKC upon $ONOO^-$ stimulation. We hypothesize that exposure of rat-1 fibroblasts to $ONOO^-$ results in a rise in calcium levels and an activation of PKC which ultimately results in ERK1/2 phosphorylation in a MEK-independent manner. Whether the translocation of PKC by $ONOO^-$ occurs directly or involves the activation of phospholipase C and subsequent generation of diacylglycerol is not known and needs to be investigated.

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References

[1] Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J. and Davis, R.J. (1995) J. Biol. Chem. 270, 7420–7426.
[2] de Wit, R., Boonstra, J., Verkleij, A.J. and Post, J.A. (1998) J. Biomol. Screen. 3, 277–284.
[3] Lander, H.M., Jocovina, A.T., Davis, R.J. and Tauras, J.M. (1996) J. Biol. Chem. 271, 19705–19709.
[4] Schieke, S.M., Briviba, K., Klotz, L.O. and Sies, H. (1999) FEBS Lett. 448, 301–303.
[5] Oh-hashi, K., Maruyama, W., Yi, H., Takahashi, T., Naoi, M. and Isobe, K. (1999) Biochem. Biophys. Res. Commun. 263, 504–509.
[6] Jope, R.S., Zhang, L. and Song, L. (2000) Arch. Biochem. Biophys. 376, 365–370.
[7] Zhang, P., Wang, Y., Kagen, E. and Bonner, J. (2000) J. Biol. Chem. 275, 22479–22486.
[8] Ducrocq, C., Blanchard, B., Pignatelli, B. and Ohshima, H. (1999) Cell. Mol. Life Sci. 55, 1068–1077.
[9] Muisers, R., Folkerts, G., Henricks, P., Sadeghi-Hashjin, G. and Nijkamp, F. (1997) Life Sci. 60, 1833–1845.
[10] Topp, W.C. (1981) Virology 113, 408–411.
[11] Evans, W.H. (1992) Preparative Centrifugation: A Practical Approach, Oxford University, Oxford.
[12] Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J. and Saltiel, A.R. (1995) Proc. Natl. Acad. Sci. USA 92, 7686–7689.
[13] Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., Copeland, R.A., Magolda, R.L., Scherle, P.A. and Trzaskos, J.M. (1998) J. Biol. Chem. 273, 18623–18632.
[14] DeSilva, D.R., Jones, E.A., Favata, M.F., Jaffee, B.D., Magolda, R.L., Trzaskos, J.M. and Scherle, P.A. (1998) J. Immunol. 160, 4175–4181.
[15] Brown, A.S., Moro, M.A., Masse, J.M., Cramer, E.M., Radomski, M. and Darler-Usmar, V. (1998) Cardiovasc. Res. 40, 380–388.
[16] Grammer, T.C. and Blenis, J. (1997) Oncogene 14, 1635–1642.
[17] Taher, M.M., Garcia, J.G.N. and Natarajan, V. (1993) Arch. Biochem. Biophys. 303, 260–266.
[18] Chen, C., Liau, C.S. and Lee, Y.T. (1996) J. Cardiovasc. Pharmacol. 28, 240–244.
[19] Packer, M.A. and Murphy, M.P. (1995) Eur. J. Biochem. 234, 231–239.
[20] Franklin, R., Tordai, A., Mazer, B., Terada, N., Lucas, J. and Gelfand, E. (1994) J. Immunol. 153, 4890–4898.
[21] Atherford, P.A., Norris, M.S., Robinson, P.J., Gelfand, E.W. and Franklin, R.A. (1999) Mol. Immunol. 36, 543–549.