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A downstream role for protein kinase Cα in the cytosolic phospholipase A_2-dependent protective signalling mediated by peroxynitrite in U937 cells

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Abstract

Exposure to an otherwise non-toxic concentration of peroxynitrite ($ONOO^-$) promotes toxicity in U937 cells supplemented with pharmacological inhibitors of protein kinase C (PKC). This effect is not associated with enhanced formation of H_2O_2 and is in fact causally linked to inhibition of the cytoprotective signalling driven by endogenous arachidonic acid (AA). The outcome of various approaches using PKC or phospholipase A_2 inhibitors, cytosolic phospholipase A_2 or PKCα antisense-oligonucleotide-transfected cells and supplementation with exogenous AA or tetradecanoylphorbol acetate, as well as PKC down-regulated cells, indicated that $ONOO^-$ promotes AA-dependent cytosol to membrane translocation of PKCα, an event critical for the cytoprotective signalling under investigation. Evidence for a similar mechanism regulating survival of human monocytes exposed to $ONOO^-$ is also presented. These results, along with our previous work on this topic, contribute to the definition of the mechanism whereby monocytes survive to $ONOO^-$ in inflamed tissues.

JEL classification: Toxicology

Keywords: Peroxynitrite; Protein kinase Cα; Cytosolic phospholipase A_2; Arachidonic acid; Mitochondrial permeability transition; Cell death

1. Introduction

Peroxynitrite ($ONOO^-$), the coupling product of nitric oxide and superoxide, is a highly reactive nitrogen species that mediates toxicity in inflamed tissues and plays a pivotal role in diverse pathological conditions [1]. Since this potent biological oxidant produces lesions on an array of biomolecules [2], both directly or via delayed formation of reactive oxygen species [3], $ONOO^-$-producing cells must have developed adaptive mechanisms to cope with their own $ONOO^-$. As an example, astrocytes are remarkably resistant to $ONOO^-$ because of high levels of GSH [4] and with activation of glucose-6-phosphate dehydrogenase in response to $ONOO^-$ [5], a strategy allowing NADPH production and GSH regeneration from oxidised glutathione.

Cells belonging to the monocyte/macrophage lineage produce large amounts of $ONOO^-$ and our own work has led to the identification of a defensive pathway involving activation of cytosolic phospholipase A_2 ($cPLA_2$) with the formation of cytoprotective levels of arachidonic acid (AA) [6–9]. It is important to point out that the toxicity paradigm employed in these studies involved exposure to authentic $ONOO^-$ and was associated with a mitochondrial permeability transition (MPT)-dependent necrosis, taking place within minutes after treatment with $ONOO^-$ and rapidly evolving in cell lysis [7,10]. We were able to

Abbreviations: AS-ONs, antisense-oligonucleotides; AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; calcein-AM, calcein acetoxymethyl ester; CsA, cyclosporin A; $cPLA_2$, cytosolic phospholipase A_2; DHR, dihydrorhodamine 123; ETYA, 5,8,11,14-eicosatetraynoic acid; MPT, mitochondrial permeability transition; NS-ONs, nonsense-oligonucleotides; PLA_2, phospholipase A_2; PKC, protein kinase C; TPA, tetradecanoylphorbol acetate

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demonstrate that, during this short time, the fate of the cells was the consequence of the balance between two parallel events, namely the AA-dependent cytoprotective signalling and formation of H_2O_2 [9], arising as a consequence of $ONOO^-$-dependent inhibition of complex III [3]. Interestingly, the mechanism whereby delayed formation of H_2O_2 promotes toxicity in cells exposed to $ONOO^-$ does not involve formation of additional damage but, rather, inhibition of the $cPLA_2$-dependent cytoprotective signalling. The fine regulation of the balance between cell survival and death therefore indicates that necrosis caused by $ONOO^-$ in monocyte/macrophage cells is not the consequence of a stochastic process of cell damage, but rather depends on the inhibition of a signalling cascade leading to cytoprotection.

Having established a role for AA in this protective pathway the downstream events leading to survival need to be investigated. The present study addressed the possibility that members of the protein kinase C (PKC) family might be involved in this survival response. $ONOO^-$ was indeed shown to promote activation of PKC [11] and AA itself, or its metabolites, might play a critical role in this response. An indication in this direction is based on the well-established notion that AA enhances the diacylglycerol-dependent activation of PKC [12]. In addition, activation of PKC was shown to promote cytoprotection in variety of toxicity paradigms [13–15].

We herein report that a phorbol ester-sensitive PKC isoform, most likely PKCα, is activated by non-toxic concentrations of $ONOO^-$ via a AA-dependent mechanism, and plays a critical role in the $cPLA_2$-dependent cytoprotective signalling.

2. Materials and methods

2.1. Chemicals

5,8,11,14-Eicosatetraynoic acid (ETYA), AA, mepacrine, rotenone, catalase, tetradecanoylphorbol acetate (TPA), chelerythrine and antimycin A as well as most of reagent grade chemicals, were obtained from Sigma-Aldrich (Milan, Italy). Gö6850, arachidonyl trifluoromethyl ketone ($AACOCF_3$) and FK506 were from Calbiochem (San Diego, CA, USA). Cyclosporin A (CsA) was purchased from Sandoz A.G. (Bern, Switzerland). Rhodamine 123, dihydrorhodamine 123 (DHR) and calcein acetoxymethyl ester (calcein-AM) were from Molecular Probes Europe (Leiden, The Netherlands). [^{3}H]AA was obtained from Amersham Pharmacia Biotech (Buckinghamshire, England).

2.2. Antibodies

The antibodies against $cPLA_2$, actin and horseradish peroxidase-conjugated monoclonal secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The PKCα monoclonal antibody was obtained from BD Transduction Laboratories (Lexington, KY). Rhodamine-labeled anti-mouse IgG antibody was from Molecular Probes Europe (Leiden, The Netherlands).

2.3. Cell culture and treatment conditions

U937 cells were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% foetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (50 units/mL) and streptomycin (50 μg/mL) (Sera-Lab Ltd., Crawley Down, England), at 37 °C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air–5% CO_2. Human peripheral mononuclear cells were isolated by Ficoll gradient centrifugation, and monocytes were separated as previously described [8]. $ONOO^-$ was synthesised by the reaction of nitrite with acidified H_2O_2, as described previously [16] with minor modification [3]. Treatments were performed in 2 mL of pre-warmed saline A (8.182 g/L NaCl, 0.372 g/L KCl, 0.336 g/L $NaHCO_3$ and 0.9 g/L glucose) containing 5×10^5 cells.

2.4. Cytotoxicity assay

Cytotoxicity was determined with the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue and the cells were counted with a hemocytometer.

2.5. Measurement of extracellular release of [^{3}H]AA

The cells were labelled with [^{3}H]AA (0.5 μCi/mL) and grown for 18 h. Before treatments the cells (2×10^5) were washed twice with saline A supplemented with 1 mg/mL fatty acid-free BSA and re-suspended in a final volume of 1 mL of saline A. The solution was then separated and centrifuged at $5000 \times g$ for 1.5 min; 500 μL of the resulting supernatant were removed and radioactivity was determined in a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland).

2.6. Transfection with $cPLA_2$ or PKCα antisense- or nonsense-oligonucleotides

The human antisense-oligonucleotides (AS-ONs) for $cPLA_2$ (5′-GTA AGG ATC TAT AAA TGA CAT-3′) and for PKCα (5′-GTT CTC GCT GGT GAG TTT CA-3′) were directed against the initiation site or the 3′ region of the mRNA, respectively. The nonsense-oligonucleotides (NS-ONs) for $cPLA_2$ (5′-AGT AGA TTG AAT AGA CAC TAT-3′) and for PKCα (5′-TGG GCC GGG CAA TTT TTT TC-3′) were a random sequence of the antisense bases. The oligonucleotides were phosphorothioate-modified and

synthesised by MWG Biotech (Florence, Italy). The phosphorothioate backbone provides resistance to exonuclease and increases the stability of the oligonucleotide in serum and within the cell. U937 cells were transfected with the above oligonucleotides as follows: cells were washed twice with serum-free medium and seeded (1×10^6 mL^{-1}) in serum-free RPMI 1640 for 6 h in the absence or presence of oligonucleotides (10 μM). A final concentration of 5% foetal bovine serum was then added, the cells were cultured for additional 24 h and finally utilised for experiments. Using these conditions, we obtained a good transfection efficiency (see below) and thus avoided the use of transfection agents, as lipofectamine.

2.7. DHR oxidation, rhodamine 123 mitochondrial uptake, calcein staining and imaging

$ONOO^-$ pre-treated cells (5 min) were post-incubated in the presence of either 10 μM DHR (10 min) or fresh saline A (15 min) in which 11 μM rhodamine 123, calcein-AM and 1 mM $CoCl_2$ (15 min), as previously described [17], were added during the last 5 min of incubation. After treatments, the cells were washed, re-suspended in 20 μL of saline A and stratified on a slide. Cellular fluorescence were captured with a BX-51 microscope (Olympus), equipped with an SPOT-RT camera unit (Diagnostic Instruments). The excitation and emission wavelengths were 488 and 515 nm, respectively, with a 5 nm slit width for both emission and excitation. Images were collected with exposure times of 100–400 ms, digitally acquired and processed for fluorescence determination at the single cell level on a personal computer using Scion Image software (Scion Corp., Frederick, MD). Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition/experiment.

2.8. Western blot analysis

The cells were washed twice with phosphate-buffered saline, and incubated on ice for 1 h with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium vanadate and 1 mM sodium fluoride, pH 8.0). Cells were then lysed with a Sonicator Ultrasonic Liquid Processor XL (Heat System-Ultrasonics, Inc., NY, USA) and centrifuged at 21,500 × *g* for 10 min at 4 °C to remove detergent-insoluble material. Supernatants were assayed for protein concentration using the Bio Rad protein assay reagent. In some experiments, the plasma membrane-enriched fraction was separated from cytosol as described in ref. [11]. Protein samples (25 μg) were resolved in 7.5% or 10% sodium dodecyl sulfate polyacrylamide gel and electrotransferred to polyvinyldiene difluoride membranes. The blots were blocked for 1 h at room temperature with 5% milk powder in Tris-buffered saline (140 mM NaCl, 50 mM Tris HCl, pH 7.2) containing 0.06% Tween-20 and probed with a primary antibody against $cPLA_2$ or PKCα (1:500) overnight at 4 °C. Horseradish peroxidase-conjugated monoclonal antibodies (1:2000) was used for enhanced chemiluminescence detection.

2.9. Immunocytochemistry

After treatments, the cells were mounted on slides by cytospinning, fixed with 4% paraformaldehyde and then permeabilised with ethanol:acetic acid (95:5) for 1 min at room temperature. The permeabilised cells were blocked with 1% BSA in phosphate-buffered saline. The slide glass was then incubated overnight (4 °C) with anti-human-PKCα antibodies diluted 1:100 in phosphate-buffered saline containing 1% bovine serum albumin. Excess antibody binding was removed by washing the slide glass with phosphate-buffered saline. After exposure to the secondary antibody (rhodamine-labeled anti-mouse IgG), diluted 1:500 in phosphate-buffered saline, the cells were observed with a BX-51 microscope (Olympus) and the resulting images were processed as detailed above.

3. Statistical analysis

Experimental values reported in fugures are expressed as means ± S.E.M. For comparison between two groups, the Student's unpaired *t*-test was used.

4. Results

4.1. Inhibitors of PKC promote toxicity in U937 cells exposed to an otherwise non-toxic concentration of $ONOO^-$

A short-term exposure (5 min) to 100 μM $ONOO^-$, followed by a 55 min incubation in fresh saline A, does not promote toxicity in U937 cells. As previously reported [6,7,9], however, a lethal response is observed when pharmacological inhibitors of either phospholipase A_2 (PLA_2) or complex III of the respiratory chain are added during post-treatment incubation. The results reported in Fig. 1A confirm and extend these findings since $ONOO^-$-dependent toxicity was observed with the $cPLA_2$ inhibitor $AACOCF_3$, the complex III inhibitor antimycin A, as well as using two different PKC inhibitors, chelerythrine and Gö6850. None of the above inhibitors was toxic in the absence of $ONOO^-$ (not shown). The concentration-dependence of the effects mediated by the PKC inhibitors is shown in Fig. 1B. The observation that the lethal response mediated by antimycin A, unlike that elicited by the PLA_2 and PKC inhibitors (Fig. 1A), was abolished by rotenone (0.5 μM) or catalase (10 U/mL) suggests that, as we previously found with inhibitors of PLA_2 [9],

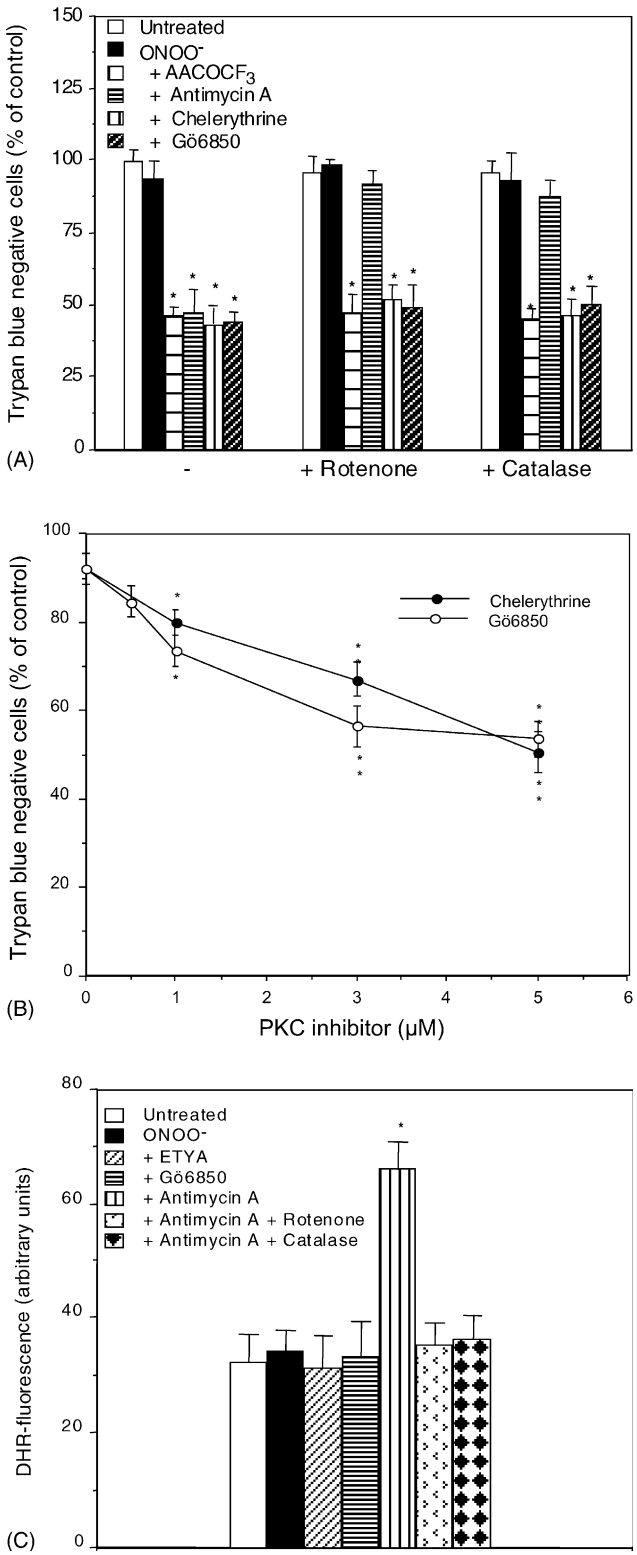


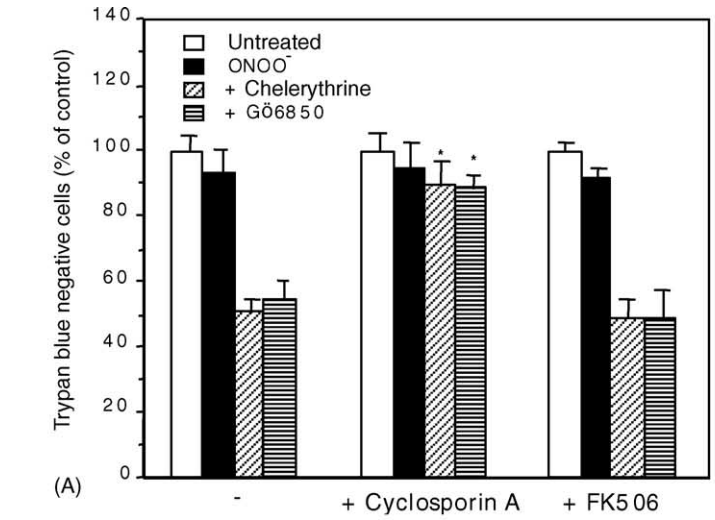
Fig. 1. PKC inhibitors cause toxicity in cells exposed to an otherwise non-toxic concentration of $ONOO^-$. (A) Cells were exposed for 5 min to 100 μM $ONOO^-$, centrifuged and then post-incubated for a further 55 min in fresh saline A containing 50 μM $AACOCF_3$, 1 μM antimycin A, 5 μM chelerythrine or 3 μM Gö6850, both in the absence and presence of 0.5 μM rotenone or 10 U/mL catalase. Cytotoxicity was determined using the trypan blue exclusion assay. Results represent the means ± S.E.M. from 3 to 5 separate experiments. $^*P < 0.001$ as compared to cells exposed to $ONOO^-$ alone (unpaired *t*-test). (B) Cells were exposed for 5 min to 100 μM $ONOO^-$ and then post-incubated for 55 min in the absence or presence of increasing concentrations of chelerythrine or Gö6850. Cytotoxicity was determined using the trypan blue exclusion assay. Results represent the means ± S.E.M. from 3 to 5 separate experiments. $^*P < 0.01$; $^{**}P < 0.001$, compared to cells exposed to $ONOO^-$ alone (unpaired *t*-test). (C) Cells were exposed for 5 min to 0 or 100 μM $ONOO^-$ centrifuged and then post-incubated for a further 10 min in fresh saline A containing 10 μM DHR in the absence or presence of 50 μM ETYA or 3 μM Gö6850. In other experiments, treatment with $ONOO^-$ was followed by exposure to 1 μM antimycin A, with or without 0.5 μM rotenone or 10 U/mL catalase. The DHR fluorescence was then quantified as detailed in Section 2. Results represent the means ± S.E.M. from at least four separate experiments. $^*P < 0.001$, compared to cells exposed to $ONOO^-$ alone (unpaired *t*-test).

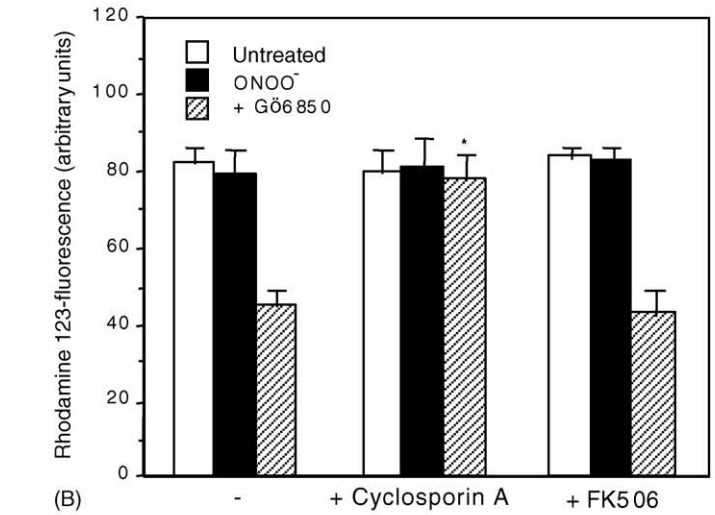
delayed formation of H_2O_2 is not involved in cell death caused by the PKC inhibitors. Consistent with this notion were the results obtained in experiments measuring formation of H_2O_2 with a fluorescent probe. DHR (10 μM) was given to the cultures after the 5 min treatment with $ONOO^-$, a condition that does not allow $ONOO^-$ to directly oxidise the probe.

The results illustrated in Fig. 1C indicate that $ONOO^-$ alone, or associated with either the PLA_2 inhibitor ETYA (50 μM) or Gö6850 (3 μM), did not increase fluorescence. Replacing ETYA with another PLA_2 inhibitor, mepacrine, led to identical outcomes (not shown). The intrinsic fluorescence of chelerythrine and $AACOCF_3$ did not allow us to perform experiments with these inhibitors. Antimycin A, however, caused a remarkable DHR fluorescence response sensitive to rotenone or catalase. Control experiments revealed that rotenone, or catalase, was not toxic and/or able to promote formation of H_2O_2, when given alone to the cultures (not shown).

4.2. A role for PKC in the AA-dependent cytoprotective signalling triggered by $ONOO^-$

The above results are consistent with the possibility that inhibition of PKC, as previously shown for inhibition of PLA_2 [6,7,9], prevents the cytoprotective signalling triggered by $ONOO^-$. This hypothesis would then be strengthened by the demonstration that the same mechanism mediates toxicity induced by otherwise non-toxic concentrations of $ONOO^-$ in cells supplemented with either inhibitors of PKC or PLA_2. Exposure of U937 cells to 100 μM $ONOO^-$, while not causing toxicity (Fig. 2A) or loss of mitochondrial membrane potential (Fig. 2B), produced dramatic effects in PKC-inhibited cells and these responses were prevented by CsA (0.5 μM). FK506 (1 μM), which shares with CsA the ability to inhibit calcineurin [18], but fails to affect formation of MPT pores [19], was inactive. The PKC inhibitors did not induce any effect in the absence of additional treatments. These results strongly suggest that MPT is the cause of $ONOO^-$ toxicity in PKC-inhibited cells. This inference is further supported by results in which MPT pore opening was assessed by monitoring chances in mitochondrial calcein fluorescence





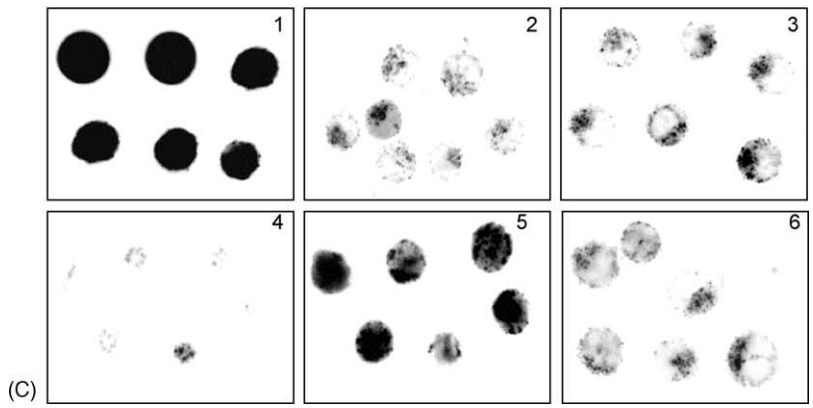


Fig. 2. CsA prevents toxicity and MPT induced by $ONOO^-$ in PKC-inhibited cells. (A) Cells were treated as detailed in the legend to Fig. 1. 0.5 μM CsA, or 1 μM FK506, was added 5 min prior to $ONOO^-$. Cytotoxicity was determined with the trypan blue exclusion assay. Results represent the means ± S.E.M. from 3 to 5 separate experiments, each performed in duplicate. $^*P < 0.001$, compared to cells exposed to $ONOO^-$ and the PKC inhibitor (unpaired *t*-test). (B) Cells were loaded with rhodamine 123 (11 μM), washed, incubated in fresh saline A for 15 min and then treated as described above. Rhodamine 123 fluorescence was quantified as detailed in Section 2. Results represent the means ± S.E.M. from at least four separate experiments. $^*P < 0.001$, compared to cells exposed to $ONOO^-$/Gö6850 (unpaired *t*-test). (C) Representative micrographs of cells loaded for 15 min with 1 μM calcein-AM alone (1) or associated with 1 mM $CoCl_2$ (2), washed and then post-incubated in fresh saline A for a further 15 min. Panel 3 shows that 100 μM $ONOO^-$ did not induce loss of mitochondrial calcein. This effect was however observed when 3 μM Gö6850 was added to the cultures 5 min after $ONOO^-$ (4). Panel 5 shows the same image in which the presence of the cells is demonstrated by darkening the digital image at the expenses of a loss of brightness. Loss of mitochondrial calcein induced by $ONOO^-$/Gö6850 was prevented by 0.5 μM CsA added 5 min prior to $ONOO^-$ (6). The micrographs are representative of at least three separate experiments with similar outcomes.

after quenching of the cytosolic and nuclear signals with Co^{2+} [17]. As illustrated in Fig. 2C, cells exposed to calcein-AM display a uniform, bright fluorescence (1). Addition of $CoCl_2$ during calcein-AM exposure provoked a punctate fluorescence (2) unaffected by exposure to $ONOO^-$ alone (3). Loss of mitochondrial calcein was however observed in cells treated with $ONOO^-$/Gö6850 (4) and this response was prevented by CsA (6), but not by FK506 (not shown). The image shown in (5) should be compared with that in (4), in which the presence of the cells is demonstrated by darkening the digital image at the expenses of a loss of brightness. These results provide an additional indication that a non-toxic concentration of $ONOO^-$ promotes MPT-dependent toxicity in PKC-inhibited cells. An identical outcome was previously obtained using inhibitors of PLA_2 [7].

The results illustrated in Fig. 3A indicate that toxicity mediated by $ONOO^-$ in cells supplemented with $AACOCF_3$ was abolished by AA (100 nM), as we previously reported [6,7,9], and by TPA (100 ng/mL), a potent PKC activator. Under identical experimental conditions, TPA caused maximal translocation of PKCα to the membrane fraction (not shown). Interestingly, chelerythrine, or Gö6850, did not further increase toxicity mediated by the $cPLA_2$ inhibitor and the observed lethal response, as well as that elicited by the PKC inhibitors in the absence of $AACOCF_3$, was not affected by AA or TPA.

These results therefore suggest a role for PKC downstream to $cPLA_2$ in the cytoprotective signalling triggered by $ONOO^-$ and this notion was further substantiated using cells transfected with $cPLA_2$ AS-ONs. U937 cells were transfected with phosphorothioate-modified AS-ONs, previously employed in our [6,7,9] and other [20] laboratories to successfully prevent the translation and synthesis of the enzyme protein. Complementary NS-ONs were used as a negative control. Western blot analysis using a monoclonal antibody against human $cPLA_2$ showed that the level of the protein was indeed significantly lower in cells treated with AS-ONs, as compared to cells treated with NS-ONs (Fig. 3B). The expression of the protein was identical in non-transfected cells and NS-ON-transfected cells.

Toxicity studies were next performed. $ONOO^-$, while not being toxic for NS-ON-transfected cells (Fig. 3C), caused an AA- or TPA-sensitive and Gö6850-insenstive lethal response in $cPLA_2$ AS-ON-transfected cells (Fig. 3D). Gö6850, however, promoted toxicity in $ONOO^-$-treated cells transfected with $cPLA_2$ NS-ONs. Finally, addition or either AA or TPA failed to prevent toxicity in $cPLA_2$ AS- or NS-ON-transfected cells supplemented with the PKC inhibitor.

4.3. PKCα is involved in the AA-dependent cytoprotective signalling triggered by $ONOO^-$

Prolonged exposure to high concentrations of TPA is known to down-regulate conventional and novel PKC isozymes [21]. We therefore used this approach to further establish the involvement of a TPA-sensitive PKC isoform in the AA-dependent cytoprotective signalling. U937 cells were exposed to a high concentration of TPA (1 μg/mL) for increasing time intervals and assayed for PKCα expression. As shown in Fig. 4A, Western blot analysis using a monoclonal antibody against human PKCα showed that the level of the protein was significantly reduced after only 4 h of exposure to TPA and barely detectable after 6 or 8 h. Visual inspection of these cells did not provide evidence of toxicity. This notion was confirmed by analysis of the ability of the cells to exclude trypan blue as well as by performing three different assays measuring apoptotic DNA fragmentation, namely agarose gel electrophoresis and the filter binding or comet assays (not shown). Thus, treatment with TPA caused PKCα down-regulation and failed to affect cell viability.

We therefore used these cells to investigate the sensitivity to toxicity induced by $ONOO^-$. We found that the progressive loss of PKCα expression was accompanied by an increased susceptibility of the cells to killing mediated by $ONOO^-$ via an AA-insensitive mechanism (Fig. 4B). Parallel experiments revealed that the ability of $AACOCF_3$ to increase $ONOO^-$ toxicity was independent on the extent of PKCα down-regulation. Under the same conditions, however, the cytoprotective effects of AA were remarkably affected by PKCα expression. AA was found to prevent the enhancing effects of $AACOCF_3$ in a way that the resulting toxicity curve was superimposable on the one obtained with $ONOO^-$ alone.

The above results confirm the notion that PKC plays a pivotal role in the AA-dependent cytoprotective signalling triggered by $ONOO^-$ and suggest an involvement of the isoform α in this response. Activation of PKCα by $ONOO^-$ was also reported in a recent study [11]. Furthermore the PKCε isoform, previously shown to afford cytoprotection in various toxicity paradigms [22,23], was not expressed in the specific U937 cell clone utilised in this study (not shown). We therefore investigated whether $ONOO^-$ does indeed stimulate PKCα activity under the specific conditions utilised in this study, a response that can be inferred by the observation of its translocation to the cell membrane fraction. Western blot analysis of cytosol and membrane fractions from untreated cells indicated that a large proportion of PKCα is in the cytosol (Fig. 5A). $ONOO^-$ promoted its translocation to the membrane fraction via a Gö6850 or $AACOCF_3$-sensitive mechanism. AA prevented the effects of $AACOCF_3$ with no apparent impact on the response mediated by Gö6850. $AACOCF_3$ or Gö6850, both in the absence or presence of AA, or AA alone, did not cause effects on PKCα compartmentalisation (not shown). These results provide a strong indication that PKCα is activated by $ONOO^-$ via an AA-dependent mechanism.

We next performed experiments using cells in which the expression of PKCα was suppressed using AS-ONs, pre-

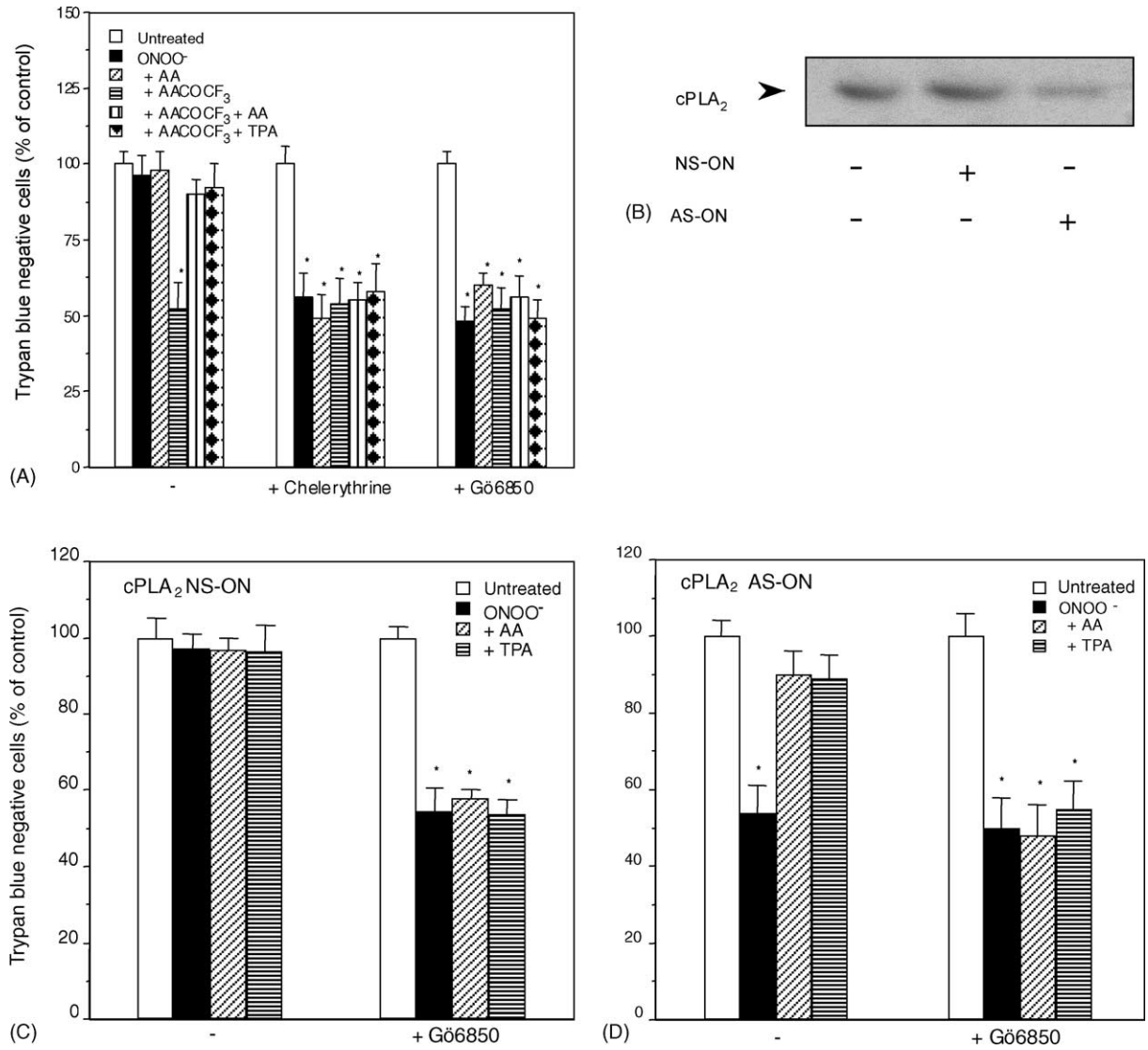


Fig. 3. AA, or TPA, prevents the toxicity mediated by $ONOO^-$ in cells in which $cPLA_2$ is pharmacologically inhibited or genetically depleted but does not affect the lethal response mediated by the PKC inhibitor. (A) Cells were exposed for 5 min to 100 μM $ONOO^-$, centrifuged and then post-incubated for a further 55 min in fresh saline A containing 50 μM $AACOCF_3$, 5 μM chelerythrine or 3 μM Gö6850, both in the absence and presence of 0.1 μM AA or 100 ng/mL TPA. Cytotoxicity was then determined with the trypan blue exclusion assay. Results represent the means ± S.E.M. from 3 to 5 separate experiments, each performed in duplicate. $^*P < 0.001$, compared to untreated cells (unpaired *t*-test). (B) Non-transfected cells or cells transfected with $cPLA_2$ NS- or AS-ONs were assayed for $cPLA_2$ protein expression by Western blot analysis. Cells transfected with $cPLA_2$ (C) NS- or (D) AS-ONs were treated as detailed in panel A and analysed for cytotoxicity. Results represent the means ± S.E.M. from 3 to 4 separate experiments. $^*P < 0.001$, compared to untreated cells (unpaired *t*-test).

viously shown to selectively down-regulate PKCα expression with no apparent modification in the expression of other PKC isoforms [24,25]. The cells were transfected with phosphorothioate-modified AS-ONs and complementary NS-ONs were used as a negative control. As shown in Fig. 6A, immunocytochemical (1 and 2) and Western blot (3) analyses using a monoclonal antibody to human PKCα showed that the level of the protein was indeed significantly lower in cells treated with AS-ONs, as compared to cells treated with NS-ONs. The expression of the protein was identical in non-transfected cells and NS-ON-transfected cells (3).

$ONOO^-$, while not being toxic for NS-ON-transfected cells (Fig. 6B), caused an AA-, TPA- or Gö6850-insensitive lethal response in PKCα AS-ON-transfected cells (Fig. 6C). Gö6850, however, promoted toxicity in cells transfected with PKCα NS-ONs. Finally, addition or either AA or TPA failed to prevent toxicity in PKCα AS- or NS-ON-transfected cells supplemented with the PKC inhibitor.

It therefore appears that PKCα is activated by $ONOO^-$ via an AA-dependent mechanism, and that this response plays a pivotal role in the survival signalling. Reports in the literature, however, indicate that PKCα may also serve as an upstream signal leading to $cPLA_2$ activation [26,27]. In order to provide more information in this direction, the release of AA stimulated by $ONOO^-$ was determined in cells supplemented with pharmacological inhibitors of PKC or with PKCα AS- or NS-ONs. The results illustrated

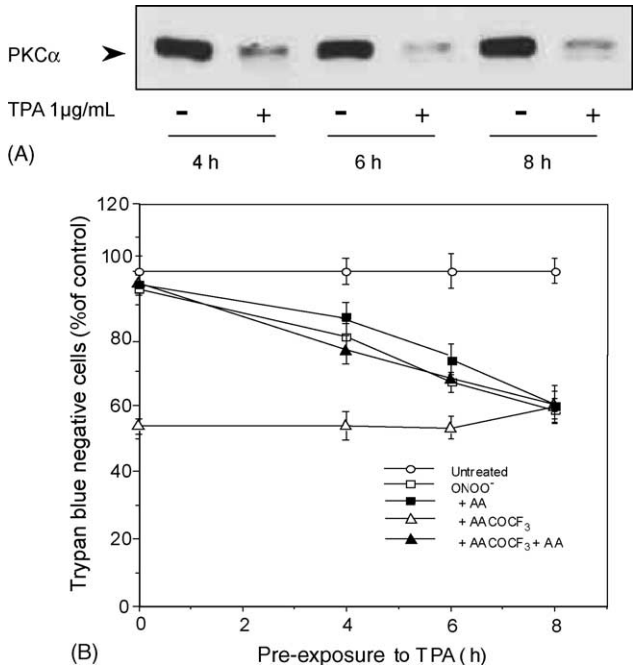


Fig. 4. PKC down-regulation promotes toxicity in cells exposed to an otherwise non-toxic concentration of $ONOO^-$ and AA supplementation fails to induce cytoprotection. Cells were exposed for increasing time intervals to 0 or 1 μg/mL TPA and then analysed for PKCα expression (A) or treated as detailed in the legend to Fig. 3A and then analysed for cytotoxicity (B). Results represent the means ± S.E.M. from 3 to 5 separate experiments, each performed in duplicate.

in Fig. 6D indicate that exposure to 100 μM $ONOO^-$, followed by a 10 min post-treatment incubation in saline A, promotes release of AA. This response was abolished by $AACOCF_3$ and unaffected by either chelerythrine or Gö6850. In addition, identical levels of $AACOCF_3$-sensitive AA release were detected in cells transfected with PKCα AS- or NS-ONs (Fig. 6D).

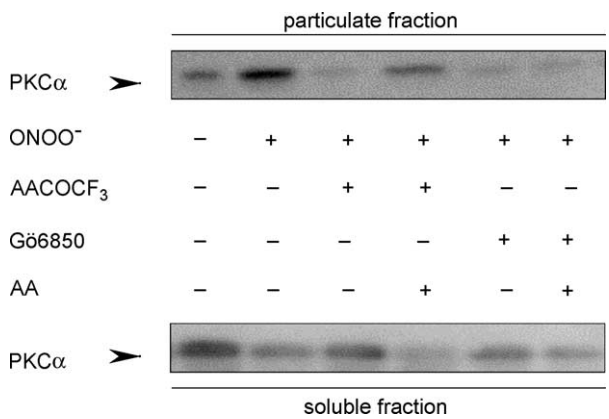


Fig. 5. $ONOO^-$ promotes translocation of PKCα to the membrane fraction via an AA-dependent mechanism. Cells were treated for 10 min, as indicated in the figure with $ONOO^-$ (100 μM), $AACOCF_3$ (50 μM), AA (0.1 μM) or Gö6850 (3 μM). Western blot analysis for PKCα was then performed on the cytosolic (soluble) and membrane (particulate) fractions. The blots shown are representative of three separate experiments with similar outcomes.

These findings therefore rule out the possibility that PKC in general, and in particular PKCα mediate the $ONOO^-$-dependent activation of $cPLA_2$. As a consequence, a unique role for PKCα downstream to $cPLA_2$ in the $ONOO^-$-dependent cytoprotective signalling is further established.

4.4. A role of PKC downstream to $cPLA_2$ is also observed in human monocytes exposed to $ONOO^-$

We recently reported that the AA-dependent protective signalling described in U937 cells exposed to $ONOO^-$ is a general response of cells belonging to the monocyte/macrophage lineage [8]. The results illustrated in Fig. 7 indicate that PKC is also likely to be involved downstream to AA in the survival response of human monocytes treated with a non-toxic dose of $ONOO^-$. Indeed, we found that the death-promoting effects mediated by $AACOCF_3$ were prevented by either AA or TPA and mimicked by chelerythrine or Gö6850. The lethal response observed in PKC-inhibited cells, however, was insensitive to both AA and TPA.

5. Discussion

Previous studies from our laboratory demonstrated that exposure to non-toxic concentrations of $ONOO^-$ nevertheless commit U937 cells to MPT-dependent toxicity, that is however prevented by the parallel activation of a survival pathway in which $cPLA_2$-released AA plays a critical role [6,7,9]. The results presented in this study point to PKC as a critical downstream player in this AA-dependent protective signalling.

The first clue in this direction was given by experiments showing that two structurally unrelated PKC inhibitors, chelerythrine and Gö6850, promote toxicity in cells exposed to an otherwise non-toxic concentration of $ONOO^-$. As we previously observed using PLA_2 inhibitors or $cPLA_2$ AS-ONs [6,7,9], this response was mediated by MPT and was not the consequence of enforced formation of H_2O_2, which promotes toxicity by inhibiting AA release [9]. The observation that the lethal response that takes place in cells supplemented with PLA_2 inhibitors or $cPLA_2$ AS-ONs was not further enhanced by PKC inhibitors, and was prevented by either exogenous AA or TPA, would rather indicate that PKC is involved in the AA-dependent survival signalling downstream to $cPLA_2$. This notion is also consistent with the inability of AA to rescue PKC-inhibited cells treated with $ONOO^-$. Additional evidence for a role of a TPA-sensitive PKC isoform downstream to AA derives from experiments in which PKC was down-regulated by prolonged exposure to a high concentration of the phorbol ester. Reports in the literature document the ability of low concentrations of TPA to induce, after 12 h, a small

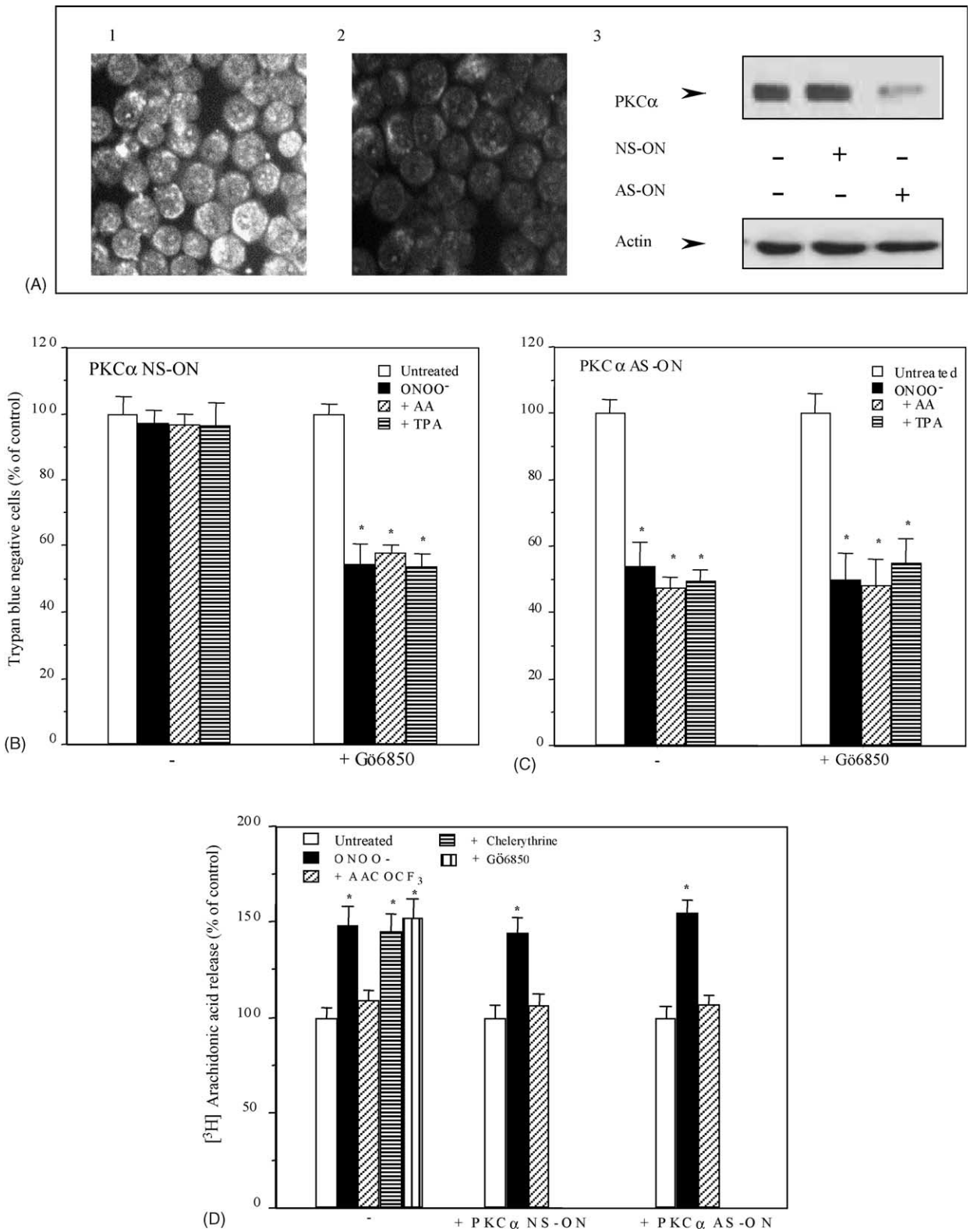


Fig. 6. Transfection with PKCα AS-ONs promotes toxicity in cells exposed to an otherwise non-toxic concentration of $ONOO^-$ and AA, or TPA, fails to induce cytoprotection. (A) Immunocytochemical analysis for PKCα in PKCα (1) NS- and (2) AS-ON-transfected cells. Western blot analysis was performed in sister cultures as well as in non-transfected cells (3). Blots were re-probed with an antibody directed against actin to ensure equal loading and transfer. NS- (B) and AS- (C) ON-transfected cells were treated as detailed in the legend to Fig. 3A and analysed for cytotoxicity using the trypan blue exclusion assay. Results represent the means ± S.E.M. from 3 to 4 separate experiments. $^*P < 0.001$, compared to untreated cells (unpaired *t*-test). (D) [^{3}H]AA-labelled non-transfected cells or NS- and AS-ON-transfected cells were exposed for 5 min to 100 μM $ONOO^-$, centrifuged and then post-incubated for a further 10 min in fresh saline A, both in the absence and presence of 50 μM $AACOCF_3$, 5 μM chelerythrine or 3 μM Gö6850. [^{3}H]AA release was determined immediately after treatments. Results represent the means ± S.E.M. from 3 to 5 separate experiments, each performed in duplicate. $^*P < 0.001$, compared to cells exposed to $ONOO^-$ alone (unpaired *t*-test).

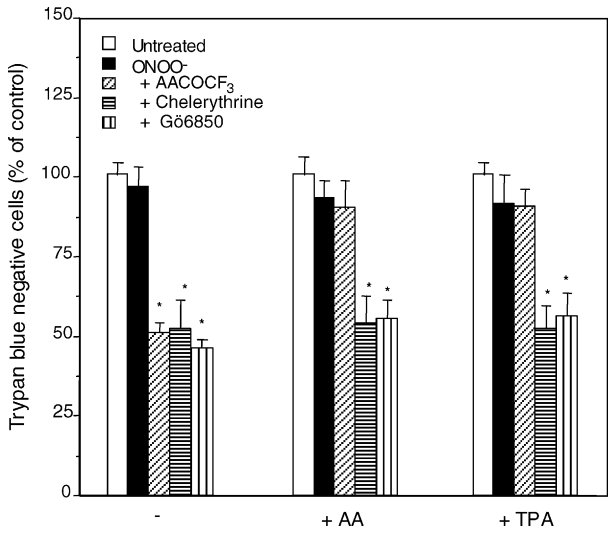


Fig. 7. Evidence for a role of a TPA-sensitive PKC isoform in the AA-dependent protective signalling observed in human monocytes exposed to $ONOO^-$. Human monocytes were treated as detailed in the legend to Fig. 3A and analysed for cytotoxicity using the trypan blue exclusion assay. Results represent the means ± S.E.M. from 3 to 5 separate experiments, each performed in duplicate. $^*P < 0.001$, compared to control cells (unpaired *t*-test).

apoptotic response in U937 cells [28] via a mechanism dependent on PKCδ translocation to the mitochondria. There was no evidence of apoptosis in our cells and this may be due to the fact that the high dose of TPA very rapidly down-regulates PKCδ (not shown). An alternative explanation is that in different laboratories, different U937 cell clones may be selected that respond differently to TPA. In any case, we carefully excluded the option that TPA per se causes toxicity in our cells and found that reduced expression of PKC was accompanied by enhanced sensitivity to $ONOO^-$, as well as by the progressive loss of the ability of exogenous AA to rescue cells supplemented with a PLA_2 inhibitor.

The results thus far discussed suggest that $ONOO^-$ stimulates $cPLA_2$ activity with concomitant AA release promoting activation of a TPA-sensitive PKC isoform that in turn leads to cytoprotection. PKC down-regulation was assessed by monitoring PKCα expression and this isoform is a likely candidate for the protective signalling since: (i) there was a good temporal correlation between PKCα down-regulation and the toxic effects of $ONOO^-$ or protective effects of AA; (ii) PKCα was previously shown to mediate cytoprotection in other toxicity paradigms [13,14]; (iii) an additional possible candidate, namely PKCε, is not expressed in the U937 cell line utilised in the present study; (iv) activation of PKCα by $ONOO^-$ was previously reported [11]; (v) AA-dependent activation of PKCα was described by other investigators [12,29] and found to take place under conditions utilised in this study. Indeed, PKCα translocation to the membrane fraction was elicited by a non-toxic dose of $ONOO^-$ and this response was prevented by a PLA_2 inhibitor and once again re-established upon additional supplementation of exogenous AA.

PKCα is directly activated by AA in cultured cells [30], albeit at levels unlikely to occur physiologically. In our experiments, AA alone was unable to promote PKCα translocation and did not further increase this response mediated by $ONOO^-$. It therefore appears that both endogenous AA (cells exposed to $ONOO^-$ alone) and exogenous AA (added to PLA_2-inhibited cells exposed to $ONOO^-$) are not directly responsible for PKCα translocation but are rather involved in the regulation of this process. As an example, AA may serve to direct PKCα to proper membrane targets even in the absence of an appropriate Ca^{2+} signal and eventually operate synergistically with diacylglycerol for optimal PKCα activation [31].

The involvement of PKCα in the cytoprotective signalling was finally inferred by experiments using AS-ONs previously shown to specifically down-regulate PKCα without affecting the expression of other PKC isoforms [24,25]. These cells were indeed killed by an otherwise non-toxic concentration of $ONOO^-$. Furthermore, the observation that TPA was unable to afford cytoprotection would indicate that PKCα is the sole TPA-sensitive PKC isoform involved in the survival signalling.

Thus, the above results lead to the important conclusion that a TPA-sensitive isoform, most likely PKCα, is activated through an AA-dependent mechanism, and that this event is associated with prevention of MPT-dependent U937 cell necrosis induced by $ONOO^-$. Future studies should establish whether this response is directly mediated by AA itself, or by its metabolites of the cyclooxygenase or lipoxygenase pathways. Our results also provide an indication that the same mechanism is involved in the regulation of resistance to $ONOO^-$ in human monocytes.

It therefore appears that U937 cells, and most-likely cells belonging to the monocyte/macrophage lineage, while committed by $ONOO^-$ to MPT can nevertheless survive by triggering an AA-dependent translocation and activation of PKCα. An attractive possibility is that PKCα translocates to the mitochondrial membranes, thereby preventing MPT via phosphorylation of some critical target. In this regard, a regulatory role for mitochondrial PKCα as a potential Bcl2 kinase involved in the direct phosphorylation of Bcl2 and prevention of apoptosis, was recently identified [13,14]. Since Bcl2 effectively prevents MPT [32,33], it is also expected to prevent necrotic death mediated by opening of MPT pores [34,35]. An additional mechanism whereby mitochondrial PKCα may cause cytoprotection is represented by phosphorylation of BAD [36]. It is well established that this process promotes translocation of BAD from the mitochondria to the cytosol and prevents its dimerization with Bcl2 or Bcl-X_L, thus favouring cell survival [37]. Recent experimental evidence indicates that TPA prevents MPT-dependent apoptosis via a PKC-Rsk-dependent pathway that leads to inactivation of BAD [38]. These possibilities are currently being tested.

In conclusion, the results presented in this study provide further details on the mechanism whereby monocytic cells cope with $ONOO^-$. It is important to keep in mind that these are $ONOO^-$-producing cells and, not surprisingly, are also highly resistant to $ONOO^-$ toxicity. Stimulation of these cells to produce $ONOO^-$ is normally observed under inflammatory conditions in which large amounts of the inflammation product AA are concomitantly released. Thus, the ability to these cells to use both endogenous and exogenous AA to stimulate a signalling pathway leading to survival appears to be an ideal strategy to promote monocyte survival at inflammatory sites. Our previous studies identified AA as a critical component of this signalling pathway and suggested a novel mechanism for the antiinflammatory effect of PLA_2 inhibitors [6,7,9] and glucocorticoids [39], that is promoting death of monocytes in response to otherwise non-toxic concentrations of $ONOO^-$. The present study furthers our knowledge on the AA-dependent cytoprotective signalling and provides the additional indication of a potential use of selective inhibitors of the PKCα signal transduction pathway in antiinflammatory therapy. This, may represent an effective strategy to promote monocyte death at inflammatory sites, associated with, and causally-linked to an effective antiinflammatory response, in the absence of the well known side effects often encountered in all of the conventional therapies.

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