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Non-toxic concentrations of peroxynitrite commit U937 cells to mitochondrial permeability transition-dependent necrosis that is however prevented by endogenous arachidonic acid

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Abstract

The present study was aimed at examining the mechanism whereby an otherwise non-toxic concentration of peroxynitrite promotes a rapid necrotic response in U937 cells in which cytosolic phospholipase A_2 is pharmacologically inhibited or genetically depleted. We found that loss of viable cells is appreciable 15 min after addition of peroxynitrite, does not further increase at 30 min and is mediated by mitochondrial permeability transition (MPT). Both MPT and toxicity were prevented by exogenous arachidonic acid (AA). Various experimental approaches produced results consistent with the notion that the AA-dependent protective mechanism takes place 10–15 min after addition of peroxynitrite. The observation that the extent of DNA strand scission induced by peroxynitrite did not vary under conditions of different AA availability suggests that this event is either upstream to mitochondrial dysfunction or irrelevant for cytotoxicity. Collectively, these data indicate that a non-toxic concentration of peroxynitrite commits U937 cells to MTP-dependent necrosis that is however prevented by endogenous AA. Thus, mitochondria are a likely target of the cytoprotective signalling triggered by AA.

Keywords: Peroxynitrite; Mitochondrial permeability transition; DNA single-strand breakage; Arachidonic acid; Cytosolic phospholipase A_2; Cell death

1. Introduction

Peroxynitrite, a highly reactive nitrogen species generated by the reaction of nitric oxide and superoxide, produces extensive damage at the level of various biomolecules [1]. Its reported activities include DNA strand scission [2–4], peroxidation of membrane lipids [5,6], oxidation of sulphydryls [7], etc. As observed with other toxic agents, high concentrations of peroxynitrite lead to a rapid necrotic response whereas lower concentrations promote an apoptotic death taking place within several hours [8,9]. We however reported different results using U937 cells exposed to a bolus of peroxynitrite. Under these conditions, cell death occurred very rapidly via a MPT-dependent necrosis and the surviving cells did not undergo delayed cell death, but actually proliferated as untreated cells [10]. Remarkably, lower concentrations of peroxynitrite did not promote immediate or delayed toxicity. Thus, an all or nothing mechanism appears to regulate the fate of U937 cells challenged with toxic levels of peroxynitrite: some cells undergo an extremely fast necrotic response, whereas the remaining cells are fully viable and capable of performing energy-demanding functions such as proliferation. In addition, concentrations of peroxynitrite not immediately cytotoxic fail to elicit a delayed lethal response.

Previously, we showed that peroxynitrite promotes an early activation of the $cPLA_2$ isoform and that the ensuing release of AA mediates cytoprotection [11]. In particular, we found that concentrations of peroxynitrite in the range 50–200 μM are non-toxic, or produce little toxicity, because they effectively stimulate $cPLA_2$. If the activity of $cPLA_2$ was pharmacologically inhibited or genetically depleted, then cell death occurred and could be prevented by exogenous AA. Concentrations of peroxynitrite higher

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Abbreviations: AA, arachidonic acid; BA, bongkrekic acid; Calcein-AM, calcein acetoxymethyl ester; $cPLA_2$, cytosolic phospholipase A_2; CsA, cyclosporin A; ETYA, 5,8,11,14-eicosatetraynoic acid; PLA_2, phospholipase A_2; MPT, mitochondrial permeability transition.

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than 200 μM, while appearing in a plateau phase in terms of $cPLA_2$ activation, effectively killed cells via an AA-sensitive mechanism.

Thus, the availability of AA is critical for U937 cell survival after challenge with peroxynitrite. Additional studies revealed that delayed formation of H_2O_2 plays a pivotal role in the lethal response evoked by peroxynitrite [12] and that the mechanism underlying this event does not involve the accumulation of additional damage but, rather, the inhibition of $cPLA_2$,[1] a condition which obviously prevents the AA-dependent cytoprotection.

All together, these findings lead us to hypothesise that peroxynitrite triggers parallel events resulting in a potentially lethal damage and activation of a survival pathway and that the peroxynitrite-induced U937 cell necrosis occurs as a consequence of the inhibition of the cytoprotective mechanism. This is a particularly important concept that would be further strengthened by the demonstration that the mechanism whereby otherwise non-toxic concentrations of peroxynitrite kill cells with an impaired $cPLA_2$-dependent AA release is identical to that involved in cell death elicited by intrinsically toxic concentrations of the oxidant.

The aim of the present study was to gather insights in this direction. It was found that cell death arises as a consequence of MPT and that the protective effects of AA are independent on scavenging of peroxynitrite or H_2O_2. In addition, the results obtained allowed us to identify a very narrow time-window in which U937 cells exposed to non-toxic concentrations of peroxynitrite are nevertheless committed to MPT-dependent necrosis, sensitive to endogenous AA. Thus, mitochondria are the most likely target of the AA-induced cytoprotective mechanism that, in order to prevent MPT-dependent necrosis, must take place in a specific time-frame. We interpret these findings as an indication that AA triggers signalling pathways opposing MPT.

2. Materials and methods

2.1. Chemicals

ETYA, AA as well as most of reagent grade chemicals, were obtained from Sigma-Aldrich. FK506 and BA were from Calbiochem. Rhodamine 123 and calcein acetoxymethyl ester (calcein-AM) were from Molecular Probes Europe. CsA was purchased from Sandoz A.G. The oligonucleotides were phosphorothioate modified and synthesised by MWG Biotech. Mouse anti-human-$cPLA_2$ monoclonal antibody and horseradish peroxidase-conjugated monoclonal antibodies were obtained from Santa Cruz Biotechnology, Inc.

[1] I. Tommasini, P. Sestili, A. Guidarelli, O. Cantoni, submitted for publication.

2.2. Cell culture

U937 cells were cultured in suspension in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Biological Industries), penicillin (50 units/mL) and streptomycin (50 μg/mL) (Sera-Lab Ltd.), at 37° in T-75 tissue culture flasks (Corning) gassed with an atmosphere of 95% air–5% CO_2.

2.3. Synthesis of peroxynitrite and treatment conditions

Peroxynitrite was synthesised by the reaction of nitrite with acidified H_2O_2, as described previously [7]. MnO_2 (1 mg/mL) was added to the mixture for 30 min at 4° to eliminate the excess of H_2O_2. MnO_2 was removed by centrifugation and filtration through 0.45 μm pore microfilters. The solution was frozen at −80° for 24 hr. The concentration of peroxynitrite, which forms a yellow top layer due to freeze fractionation, was determined spectrophotometrically by measuring the absorbance at 302 nm in 1.5 M NaOH $\varepsilon_{302} = 1670\ M^{-1}$ cm. Stock solutions of peroxynitrite were routinely checked for the presence of H_2O_2 as described previously [13], using an oxygen electrode (YSI mod. 5300 Oxygen Monitor, Yellow Springs Instruments Co) which monitors the amount of O_2 released from the decomposition of the oxidant in the presence of an excess of catalase. H_2O_2 concentration was always below the detection limit of the assay (<1 μM).

Treatments were performed in 2 mL of prewarmed 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. The cell suspension was inoculated into 15 mL tubes before addition of peroxynitrite. Peroxynitrite was rapidly added on the wall of plastic tubes and mixed for few seconds to equilibrate the peroxynitrite concentration on the cell suspension; to avoid changes in pH due to the high alkalinity of the peroxynitrite stock solution, an appropriate amount of 1 N HCl was also added.

2.4. $cPLA_2$ antisense oligonucleotides

The human $cPLA_2$ antisense oligonucleotide (5′-GTA AGG ATC TAT AAA TGA CAT-3′) was directed against the initiation site. The nonsense oligonucleotide (5′-AGT AGA TTG AAT AGA CAC TAT-3′) was a random sequence of the antisense bases. U937 cells were washed twice with serum-free medium and seeded (1×10^6/mL) in serum-free RPMI 1640 for 6 hr in the absence or presence of oligonucleotides (10 μM). A final concentration of 5% fetal bovine serum was then added, the cells were cultured for additional 24 hr and finally utilized for experiments.

2.5. Western blot analysis

The cells were washed twice with phosphate buffered saline, and incubated on ice for 1 hr 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 sonicated with a Sonicator Ultrasonic Liquid Processor XL (Heat System-Ultrasonics, Inc.) and centrifuged at 21,500 *g* for 10 min at 4° to remove detergent-insoluble material. Supernatants were assayed for protein concentration using the Bio Rad protein assay reagent. Samples (25 μg) were subjected to gel electrophoresis on 7.5% SDS–PAGE and blotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 hr 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 $cPLA_2$ (1:500) overnight at 4°. Horseradish peroxidase-conjugated monoclonal antibodies (1:2000) was used for enhanced chemiluminescence detection. Densitometric anaysis of blots was performed using the electrophoresis documentation and the public domain NIH Image 1.63 program.

2.6. 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.

2.7. Measurement of DNA single-strand breakage

DNA single-strand breakage was determined using the alkaline halo assay, as described previously [14] with minor modifications. After the treatments, the cells were resuspended at 2.0×10^4 cells/100 μL in 1.5% low-melting agarose in phosphate-buffered saline (8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl) containing 5 mM ethylenediaminetetraacetic acid and immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling, the coverslips were removed and the slides were immersed in an alkaline buffer (0.1 M NaOH/1 mM ethylenediaminetetraacetic acid [pH 12.5]), washed and stained for 5 min with 10 μg/mL ethidium bromide.

The ethidium bromide-labelled DNA was visualised using a Bio Rad DVC 250 confocal laser microscope (Bio Rad) and the resulting images were taken and processed with a Hamamatsu chilled CCD 5985 camera (Hamamatsu Italy S.p.a.) coupled with an Apple Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

The level of DNA single-strand breakage was quantitated by calculating the nuclear spreading factor values, which represent the ratio between the area of the halo (obtained by subtracting the area of the nucleus from the total area, nucleus + halo) and that of the nucleus, from 50 to 75 randomly selected cells/experiment/treatment condition.

2.8. Rhodamine 123 mitochondrial uptake and calcein staining and imaging

Cells (2.5×10^5/mL) were incubated at 37° for 15 min in saline A with 11 μM rhodamine 123 or 1 μM calcein-AM and 1 mM $CoCl_2$, as previously described [15]. After treatments, the cells were washed, re-suspended in 20 μL of saline A and stratified on a slide. Fluorescence images 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.).

Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition/experiment.

2.9. Statistical analysis

Experimental values reported in tables and figures are expressed as means ± SEM. For comparison between two groups, the Student's unpaired *t*-test was used.

3. Results

3.1. Kinetics of cell death and cytoprotection

As previously shown [11,12], treatment with 100 μM peroxynitrite, followed by a 60 min incubation in the same medium, does not cause toxicity in U937 cells (Fig. 1A). However, if this treatment is combined with exposure to the PLA_2 inhibitor ETYA (50 μM), a remarkable lethal response can be observed. Loss of viable cells is detectable after 15 min and gradually increases up to 30 min, a time at which about 45% of the cells had died by necrosis. Decomposed peroxynitrite failed to promote toxicity also in the presence of ETYA (Fig. 1A). Control experiments were performed throughout this study and revealed that decomposed peroxynitrite, or the vehicle, was devoid of any intrinsic effect (not shown). Interestingly, ETYA was equally effective in eliciting toxicity when added to the cultures 5 or 10 min after peroxynitrite (Fig. 1B). Identical outcomes were provided by experiments in which ETYA was replaced with the selective $cPLA_2$ inhibitor arachidonyl trifluoromethyl ketone (50 μM, not shown). Importantly, under identical experimental conditions, ETYA or arachidonyl trifluoromethyl ketone abolished AA release

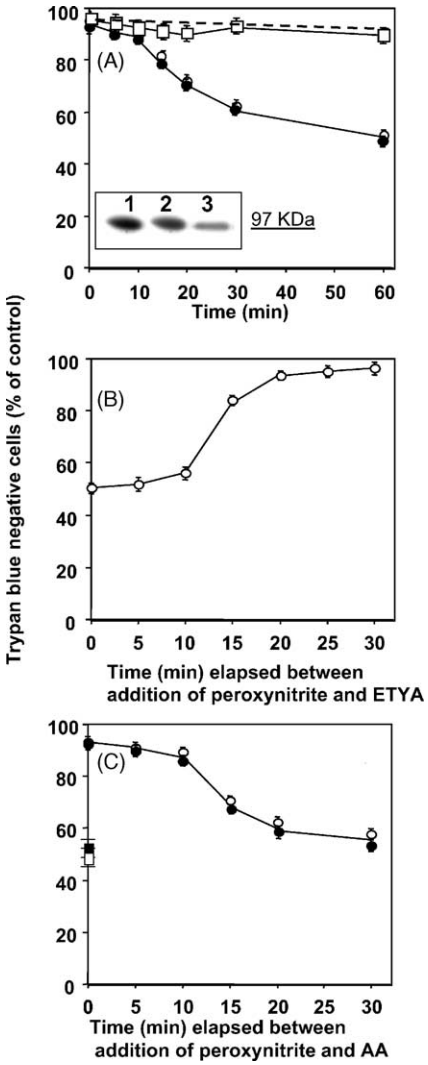

Fig. 1. Kinetics of cell death induced by peroxynitrite under conditions of different AA availability. (A) Non-transfected cells (open squares) or $cPLA_2$ antisense oligonucleotide-transfected cells (closed circles) were treated with peroxynitrite (100 μM) and, after increasing time intervals, analysed for cytotoxicity. Also shown (open circles) are the results obtained using non-transfected cells treated with either peroxynitrite/50 μM ETYA or (dashed line) decomposed peroxynitrite/ETYA. The outcome of experiments using $cPLA_2$ nonsense oligonucleotide-transfected cells was identical to that obtained with non-transfected cells (not shown). Results represent the means ± SEM from four separate experiments. Inset: Western blot analysis of $cPLA_2$ in non-transfected cells (lane 1), $cPLA_2$ nonsense oligonucleotide-transfected cells (lane 2) or $cPLA_2$ antisense oligonucleotide-transfected cells (lane 3). (B) Cells were exposed to peroxynitrite and ETYA was added to the cultures either concomitantly with peroxynitrite or after increasing time-intervals. At the end of the 60 min exposure, the cells were analysed for cytotoxicity. Results represent the means ± SEM from five separate experiments. (C) Non-transfected cells were treated with peroxynitrite/ETYA and analysed for cytotoxicity after 60 min (open square). In some experiments, AA (0.1 μM) was added to the cultures at various times intervals after peroxynitrite and processed for the assessment of viability at the end of the 60 min incubation (open circles). $cPLA_2$ antisense oligonucleotide-transfected cells were treated with peroxynitrite in the absence of ETYA. Cell viability observed with no addition of exogenous AA is indicated by the closed square whereas the closed circles indicates the results obtained after addition of AA. The outcome of experiments using or $cPLA_2$ nonsense oligonucleotide-transfected cells was identical to that obtained using non-transfected cells (not shown). Results represent the means ± SEM from five separate experiments.

stimulated by peroxynitrite [11]. The results illustrated in Fig. 1C show that the PLA_2 product AA (0.1 μM) prevents toxicity mediated by peroxynitrite in PLA_2-inhibited cells only when added within the same 10 min time frame window. These results confirm our previous findings [11] indicating that peroxynitrite-dependent activation of $cPLA_2$ represents a critical event for cell survival and suggest that this response, in order to mediate cytoprotection, must take place within 10–15 min after addition of peroxynitrite.

This inference finds experimental support in studies using cells transfected with $cPLA_2$ antisense oligonucleotides. These cells display a robust decline in $cPLA_2$ protein levels (inset to Fig. 1A) and, unlike the $cPLA_2$ nonsense oligonucleotide-transfected cells or non-transfected cells, that were characterised by similar levels of $cPLA_2$ protein, are efficiently killed by 100 μM peroxynitrite (Fig. 1A). We found that the time-dependence of viability loss in $cPLA_2$ antisense oligonucleotide-transfected cells treated with 100 μM peroxynitrite was identical to that observed in PLA_2-inhibited non-transfected cells exposed to peroxynitrite (Fig. 1A). Furthermore, the time-dependence of the AA-induced cytoprotection observed in $cPLA_2$ antisense oligonucleotide-transfected cells (Fig. 1C) is superimposable on that observed in PLA_2-inhibited non-transfected cells. Finally, $cPLA_2$ nonsense oligonucleotide-transfected cells exhibited responses identical to those observed in non-transfected cells (not shown).

3.2. Impact on cell proliferation

We recently reported that PLA_2-inhibitor-supplemented U937 cells surviving treatment with 100 μM peroxynitrite do not undergo delayed apoptosis and in fact proliferate with kinetics superimposable on those of untreated cells [11]. The results reported in Fig. 2 confirm and extend these findings by showing that cells rescued with AA also proliferate with similar kinetics. AA was equally effective when added 5 or 10 min after peroxynitrite. No effect on cell proliferation was observed after exposure to ETYA, AA or the two agents combined.

3.3. Mechanism of cytotoxicity

Our recent findings indicate that delayed formation of H_2O_2 is responsible for the peroxynitrite-dependent DNA single-strand breakage [4]. We therefore utilised this approach to investigate the extent of DNA strand scission under conditions of different AA availability. The results reported in Table 1 clearly show that the extent of DNA cleavage detected 15 min after addition of 100 μM peroxynitrite is not affected by ETYA, AA or a combination of the two agents. Similar results were obtained under conditions in which ETYA and/or AA were added to the cultures 5 or 10 min after peroxynitrite (not shown). ETYA, AA or a combination of the two agents did not

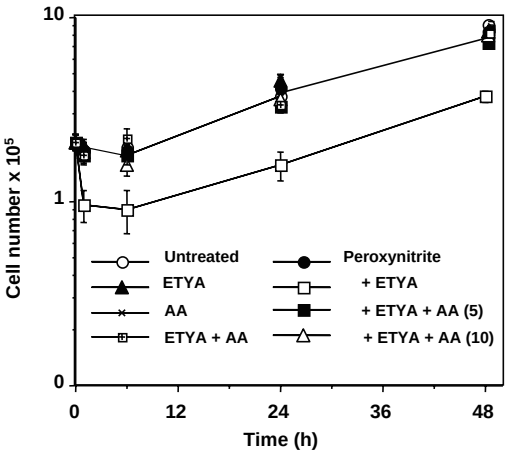

Fig. 2. Kinetics of cell proliferation after exposure to peroxynitrite under conditions of different AA availability. The cells were treated with peroxynitrite (100 μM) in the absence or presence of either ETYA (50 μM) or ETYA and AA (0.1 μM). ETYA was added 5 min after peroxynitrite and AA 5 or 10 min after peroxynitrite. Also shown are the results obtained using cells that were not exposed to peroxynitrite and that did not receive additional treatments or cells treated with ETYA, AA or the two agents combined. After treatments, the cells were washed, suspended in fresh culture medium and grown for up to 48 hr. The cells number was determined with a hemocytometer. Results represent the means ± SEM from five separate experiments.

Table 1
DNA single-strand breakage induced by peroxynitrite under conditions of different AA availability

Treatment	Nuclear spreading factor
Untreated cells	
No addition	0.95 ± 0.08
AA	1.03 ± 0.09
ETYA	0.84 ± 0.07
ETYA + AA	0.91 ± 0.05
Peroxynitrite	2.78 ± 0.20*
+AA	2.85 ± 0.25*
+ETYA	2.74 ± 0.29*
+ETYA + AA	2.93 ± 0.30*
$cPLA_2$ nonsense oligonucleotide-transfected cells	
No addition	1.01 ± 0.06
AA	0.86 ± 0.09
Peroxynitrite	2.65 ± 0.13*
+AA	2.73 ± 0.24*
$cPLA_2$ antisense oligonucleotide-transfected cells	
No addition	0.80 ± 0.07
AA	0.99 ± 0.09
Peroxynitrite	2.80 ± 0.28*
+AA	2.77 ± 0.19*

Non-transfected cells or $cPLA_2$ nonsense or antisense oligonucleotide-transfected cells were treated with peroxynitrite (100 μM) and allowed to incubate for 15 min. ETYA (50 μM), AA (0.1 μM) or a combination of the two agents was added to the cultures 5 min after peroxynitrite. The level of DNA single-strand breaks was measured immediately after the treatments using the alkaline halo assay. Results represent the means ± SEM from three to four separate experiments.

* $P < 0.001$ as compared to untreated cells (unpaired t-test).

produce DNA cleavage in the absence of additional treatments. Finally, peroxynitrite produced similar levels of AA-insensitive DNA strand scission in $cPLA_2$ antisense or nonsense oligonucleotide-transfected cells (Table 1). Under all of the above experimental conditions, the extent of DNA strand scission was uniformly distributed in target cells.

It therefore appears that similar levels of DNA strand scission accumulate in cells committed to death (e.g. cells supplemented with the PLA_2 inhibitor or transfected with $cPLA_2$ antisense oligonucleotides) or cells committed to death but rescued with AA and non-transfected cells exposed to peroxynitrite alone. As a consequence, changes in AA availability do not impact significantly on the peroxynitrite-induced DNA cleavage. These results are also consistent with the notion that AA does not act as a scavenger of reactive oxygen species.

Our previous studies demonstrated that the extent of cell death elicited by 100 μM peroxynitrite in U937 cells in which $cPLA_2$ is pharmacologically inhibited, or genetically depleted, is identical to that mediated by 1.2 mM peroxynitrite in naïve U937 cells [11]. Since the high concentration of peroxynitrite kills cells by inducing MPT [10], the possibility exists that the same mechanism is operative in $cPLA_2$-manipulated cells exposed to the low concentration of peroxynitrite.

The uptake of rhodamine 123 was therefore investigated with the aim of assessing the effects of a low concentration of peroxynitrite, under conditions of different AA availability, on mitochondrial transmembrane potential. The results from these experiments are reported in Table 2 and representative images are shown in Fig. 3. Exposure to 100 μM peroxynitrite (Fig. 3B) did not significantly reduce mitochondrial rhodamine uptake measured after 15 min of incubation (untreated cells are shown in Fig. 3A). This observation is consistent with the inability of this concentration of peroxynitrite to elicit toxicity. Addition of ETYA 5 (Fig. 3C) or 10 min (not shown) after peroxynitrite, however, caused reduction in mitochondrial membrane potential (Table 2). Visual inspection of these cultures revealed the presence of two distinct sub-populations of cells, one population showing a fluorescence identical to that of untreated cells and the other one characterised by a virtually complete loss of membrane potential. Some of the affected cells also displayed significant swelling. Importantly, exogenous AA (0.1 μM) added either 5 min (Fig. 3D) or 10 min (Fig. 3E) after peroxynitrite prevented the effects observed in the sensitive cell sub-population and did not affect mitochondrial membrane potential of resistant cells. AA, or ETYA, failed to produce effects in the absence of additional treatments (not shown). Thus, a decline in mitochondrial membrane potential was found to precede the loss of viability, both responses being prevented by AA. Interestingly, an identical outcome was obtained using CsA, (0.5 μM, Fig. 3F). FK506 (1 μM), which shares with CsA the ability to

Table 2
Mitochondrial depolarisation and cytotoxicity induced by peroxynitrite under conditions of different AA availability and modulation of these responses by MPT inhibitors

Treatment	Rhodamine 123-derived fluorescence cells (arbitrary units)	Trypan blue negative cells (% of control)
Untreated		
No addition	84.3 ± 2.2	–
Peroxynitrite	82.5 ± 3.5	98 ± 3.5
+ETYA	43.2 ± 2.3*	54 ± 4.1*
+ETYA + AA[a]	82.1 ± 2.8	90 ± 3.8
+ETYA + AA[b]	80.4 ± 3.3	92 ± 4.2
+ETYA + CsA	83.5 ± 2.4	94 ± 3.6
+ETYA + BA	81.4 ± 3.5	92 ± 2.9
+ETYA + FK506	44.6 ± 3.8*	57 ± 4.5*
$cPLA_2$ nonsense oligonucleotide-transfected cells		
No addition	83.5 ± 2.4	–
Peroxynitrite	81.9 ± 2.7	96 ± 2.8
+ETYA	46.5 ± 3.5*	49 ± 3.7*
+ETYA + AA[a]	80.9 ± 1.9	92 ± 2.8
+ETYA + AA[b]	82.6 ± 2.8	93 ± 3.2
$cPLA_2$ antisense oligonucleotide-transfected cells		
No addition	81.4 ± 2.2	–
Peroxynitrite	45.5 ± 3.5*	55 ± 2.5*
+ETYA	44.2 ± 2.3*	52 ± 3.7*
+AA[a]	79.8 ± 1.8	92 ± 3.8
+AA[b]	80.7 ± 2.8	89 ± 3.6

The cells were loaded with rhodamine 123 (11 μM), washed, and then incubated in fresh saline A for 15 min in the absence or presence of peroxynitrite (100 μM). CsA (0.5 μM), FK506 (1 μM), or BA (50 μM), was added to the cultures 5 min prior to peroxynitrite. ETYA (50 μM) was added to the cultures 5 min after peroxynitrite and the time of AA (0.1 μM) addition is indicated here. After accurate washing, the cells were observed with a microscope and the rhodamine 123 fluorescence quantified as detailed in Section 2. Results represent the means ± SEM from at least four separate experiments. Cells that had not been pre-loaded with rhodamine 123 were treated as detailed above and analysed for cytotoxicity 60 min after the addition of peroxynitrite. Results represent the means ± SEM from five separate experiments.

* $P < 0.001$ as compared to untreated cells (unpaired t test).

[a] AA was added 5 min after peroxynitrite.

[b] AA was added 10 min after peroxynitrite.

inhibit calcineurin [16], but fails to affect formation of MPT pores [17] did not modify the effects of peroxynitrite (Table 2). BA, a ligand of the adenine nucleotide translocator of the inner mitochondrial membrane [18] also prevented the effects mediated by peroxynitrite/ETYA on mitochondrial membrane potential. The results reported in Table 2 indicate that the cytoprotective effects mediated by AA were mimicked by those treatments resulting in inhibition of mitochondrial membrane depolarisation (e.g. CsA and BA) but not by FK506, which failed to prevent these effects. Finally, peroxynitrite alone produced AA-sensitive mitochondrial depolarisation and toxicity in $cPLA_2$ antisense oligonucleotide-transfected cells and the extent of these responses was not affected by ETYA and was similar to that observed in $cPLA_2$ nonsense oligonucleotide-transfected cells treated with peroxynitrite/ETYA (Table 2).

These results strongly suggest that MPT is the cause of peroxynitrite toxicity in $cPLA_2$-inhibited and/or depleted cells. This inference is further supported by results of experiments in which MPT pore opening was assessed by monitoring the changes in mitochondrial calcein fluorescence after quenching of the cytosolic signal with Co^{2+}. As illustrated in Fig. 4, cells exposed to calcein-AM display a uniform, bright fluorescence (Fig. 4A). Addition of $CoCl_2$ during calcein-AM exposure provoked remarkable changes in the fluorescence pattern and the resulting images (Fig. 4B) are consistent with the well-established notion that Co^{2+} quenches cytosolic and nuclear calcein [15]. As a consequence, the resulting punctate fluorescence is caused by the calcein localised in the mitochondrial compartment since Co^{2+} fails to penetrate the mitochondrial membrane. Loss of mitochondrial calcein therefore represents a strong indication of MPT pore opening. This event was not observed in untreated cells (Fig. 4B) or in cells incubated for 15 min after addition of 100 μM peroxynitrite alone (Fig. 4C) but was clearly detected under conditions in which ETYA was given to the cultures 5 min (Fig. 4D) or 10 min (not shown) after peroxynitrite. The image in "D" should be compared with that in "E" in which the presence of the cells is demonstrated by darkening the digital image at the expenses of a loss of brightness. AA added 5 min (Fig. 4F) or 10 min (Fig. 4G) after peroxynitrite prevented the loss of fluorescence. CsA (Fig. 4H) and BA (not shown), unlike FK506 (not shown), also prevented the decline in mitochondrial calcein fluorescence. Furthermore, peroxynitrite alone produced AA- or CsA-sensitive loss of mitochondrial calcein fluorescence in $cPLA_2$ antisense oligonucleotide-transfected cells (not shown). This response was not observed in $cPLA_2$ nonsense oligonucleotide-transfected cells treated with peroxynitrite but became readily apparent upon addition of ETYA (not shown).

Thus, it appears that exposure to otherwise non-toxic concentrations of peroxynitrite promotes MPT-dependent toxicity in cells in which $cPLA_2$ is pharmacologically inhibited or genetically depleted. This process is prevented by exogenous AA.

4. Discussion

Consistently with our previous findings [11], we report that treatment with 100 μM peroxynitrite, followed by a 60 min incubation in the same medium, does not cause toxicity in U937 cells, unless $cPLA_2$ is pharmacologically inhibited or genetically depleted (Fig. 1). The loss of viable cells was appreciable after 15 min and their number gradually increased up to 30 min. The observations that addition of the PLA_2 inhibitor 10 min after peroxynitrite promoted equal levels of toxicity, and that addition of AA at the same time point to either ETYA-supplemented cells or to cells transfected with $cPLA_2$ antisense oligonucleotides, afforded

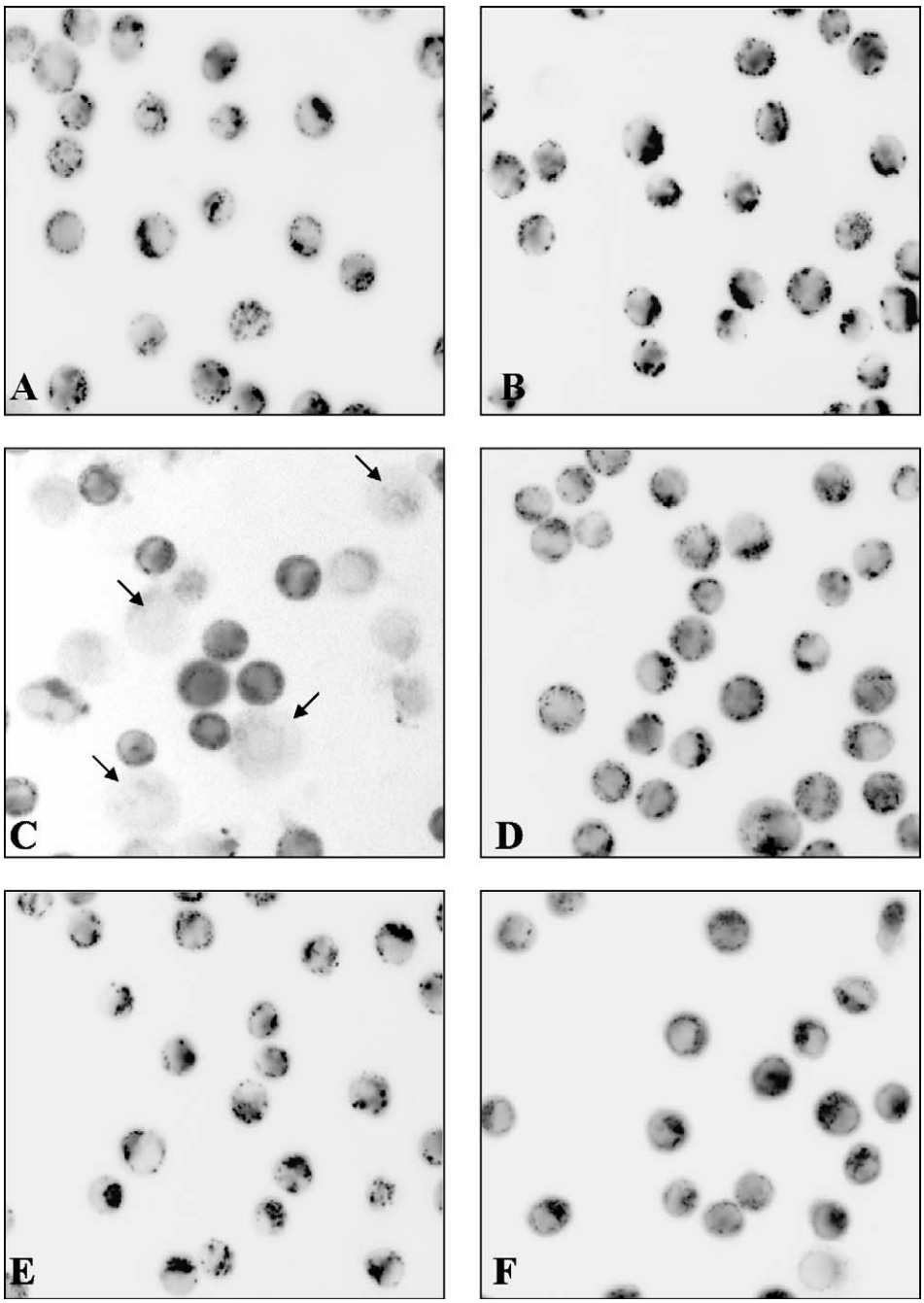

Fig. 3. AA, or CsA, prevents the peroxynitrite-induced mitochondrial depolarisation in PLA_2-inhibited cells. Representative micrographs of U937 cells loaded for 15 min with 11 μM rhodamine 123, washed and then post-incubated in fresh saline A for a further 15 min in the absence (A) or presence of peroxynitrite (100 μM, B). Also shown is the effect of ETYA (50 μM) added to the cultures 5 min after peroxynitrite (C) or AA (0.1 μM) added 5 min (D) or 10 min (E) after peroxynitrite to ETYA-supplemented cells. Panel F shows the rhodamine 123 fluorescence in cells in which CsA (0.5 μM) was added 5 min prior to peroxynitrite. The arrows indicate cells, with depolarised mitochondria, characterised by an extensive swelling. The micrographs are representative of at least three separate experiments with similar outcomes.

complete cytoprotection, lead to the identification of a very narrow time window in which AA exerts its effects.

It therefore appears (see also the results discussed below) that about 50% of the cells exposed to a low concentration of peroxynitrite are committed to death, which however is normally prevented by the activation of parallel pathways resulting in $cPLA_2$ activation and release of AA. In order to allow survival of the entire cell population critical levels of AA must be generated within 10–15 min after addition of peroxynitrite and, if this process is prevented, exogenous AA can still abolish toxicity. These findings are intriguing for at least two different reasons. Firstly, they demonstrate that nanomolar levels of AA prevent a lethal response, that would have otherwise taken place in a very short time interval, even when added 10 min after peroxynitrite. Secondly, they

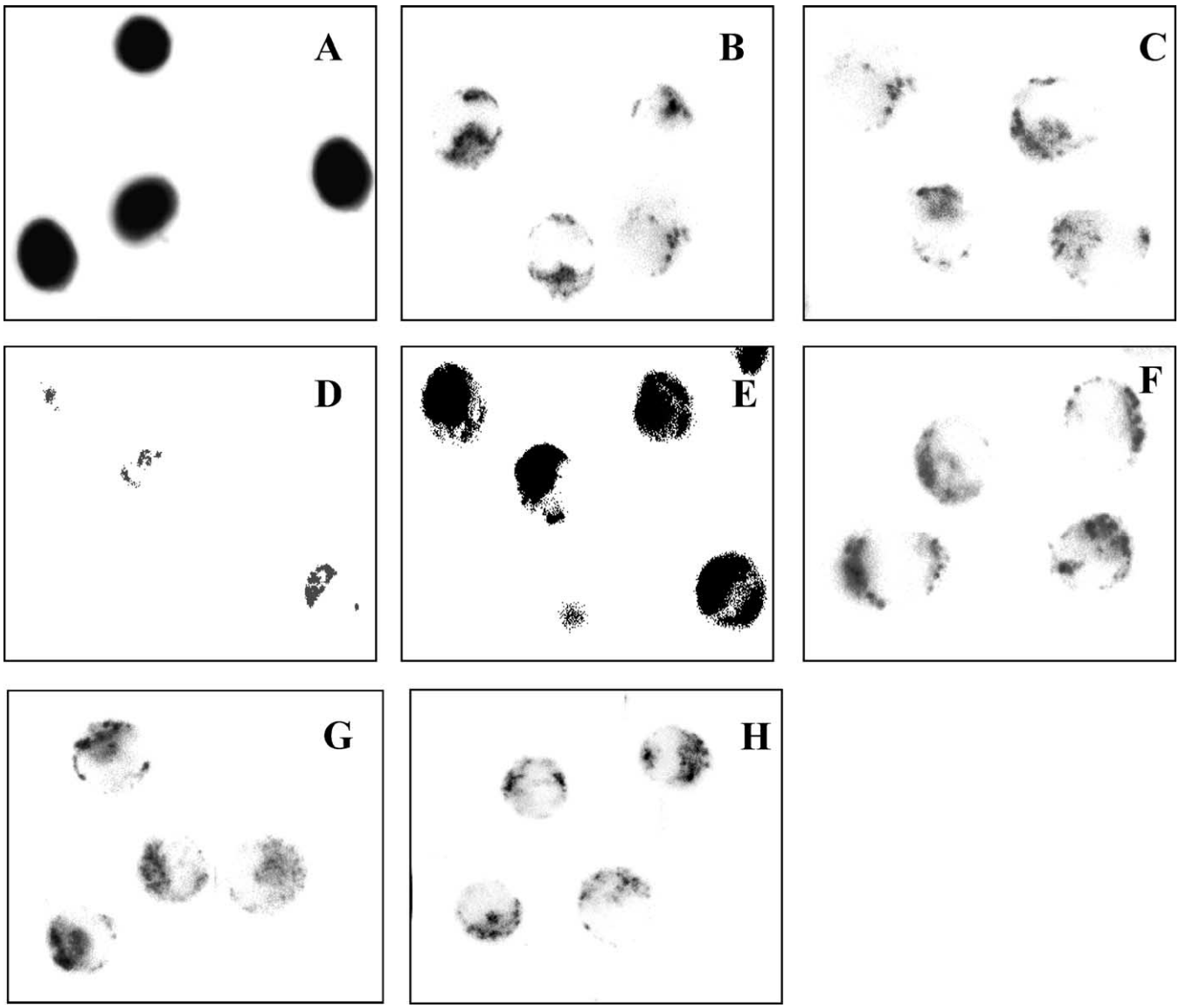

Fig. 4. AA, or CsA, prevents the peroxynitrite-induced MPT in PLA_2-inhibited cells. Representative micrographs of U937 cells loaded for 15 min with 1 μM calcein-AM alone (A), or associated with 1 mM $CoCl_2$ (B), washed and then post-incubated in fresh saline A for a further 15 min. Panel C shows that peroxynitrite (100 μM) did not induce loss of mitochondrial calcein. This effect was however observed when 50 μM ETYA was added to the cultures 5 min after peroxynitrite (D). Panel E 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 peroxynitrite/ETYA was prevented by AA (0.1 μM), added 5 min (F) or 10 min (G) after peroxynitrite or by 0.5 μM CsA added 5 min prior to peroxynitrite (H). The micrographs are representative of at least three separate experiments with similar outcomes.

imply that the effects that AA mediates in this narrow window are extremely efficacious in preventing necrosis, the mode of cell death occurring under these conditions. An additional, important information provided by the present study is that cells rescued with AA do not undergo delayed cell death but, rather, proliferate as untreated cells (Fig. 2). This is consistent with the above notion that non-toxic levels of peroxynitrite inflict a potentially lethal damage sensitive to AA. In the absence of this lipid messenger affected cells die whereas in its presence cells survive and proliferate as the non-affected cells.

In principle cytoprotection mediated by AA could be explained by a peroxynitrite scavenging effect. AA, however, cannot act as a peroxynitrite scavenger because it affords cytoprotection when added to the cells 10 min after peroxynitrite, a time at which the oxidant is already decomposed [10,19]. An alternative possibility is that AA acts as a scavenger of reactive oxygen species, generated as a consequence of peroxynitrite-dependent inhibition of complex III. We indeed reported that this event takes place after exposure to toxic concentrations of peroxynitrite and that delayed formation of hydrogen peroxide plays a pivotal role in the ensuing lethal response [12]. Various lines of evidence indicate that this second possibility is also unlikely. Firstly, this mechanism is in contrast with the observed ‘‘window effect’’ of the lipid messenger.

Secondly, AA was cytoprotective at a very low concentration (100 nM), and our previous studies demonstrated remarkable protective effects at 30 or even 10 nM [11]. Thirdly, we showed that respiration-deficient cells intrinsically resistant to peroxynitrite, because unable to generate reactive oxygen species, loose their resistance phenotype upon pharmacological or genetic depletion of $cPLA_2$.[1] Finally, inhibiting AA release does not appear to cause a parallel increase in H_2O_2 formation. This response was below the detection limits of the assay after exposure to 100 μM peroxynitrite and an identical outcome was observed in cells supplemented with a PLA_2 inhibitor or in $cPLA_2$ antisense oligonucleotide-transfected cells, both in the absence or presence of exogenous AA. Formation of H_2O_2 was however detectable if peroxynitrite was combined with antimycin A and this response was not affected by ETYA and/or AA.[1]

It is important to note that formation of H_2O_2 nevertheless takes place, and is generated in a time-dependent fashion, after exposure to 100 μM peroxynitrite. This inference is based on our previous findings indicating that delayed formation of H_2O_2 is responsible for the peroxynitrite-dependent DNA single-strand breakage [4]. We found that AA availability had hardly any effect on the extent of DNA strand scission induced by 100 μM peroxynitrite (Table 1).

This inference is based on the use of PLA_2 inhibitors, cells transfected with in $cPLA_2$ antisense and nonsense oligonucleotides as well as on AA add-back experiments. Since these conditions are associated with either survival or toxicity, it may be inferred that DNA cleavage does not play a pivotal role in peroxynitrite-induced U937 cells death. This concept is in apparent conflict with the paradigm according to which DNA single-strand breaks trigger a suicide mechanism response by activating the enzyme poly(ADP-ribose) polymerase-1, a process followed by consumption of NAD^+ and subsequent ATP depletion leading to necrosis [20]. Importantly, however, poly(-ADP-ribose) polymerase-1-mediated necrotic death was shown to be accompanied by mitochondrial alterations [21]; thus, the possibility exists that also under our conditions DNA strand scission represents an event upstream to mitochondrial damage. Although this notion needs further investigation in order to be established, we can conclude that, regardless of the specific relevance of DNA damage in the peroxynitrite-induced U937 cell death, the cytoprotective effects of AA are downstream to DNA strand scission. As a corollary, the above results also confirm that AA does not act as a scavenger of peroxynitrite or reactive oxygen species. This is not an unexpected finding since peroxynitrite and reactive oxygen species co-exist with AA in the inflammation process and, to our best knowledge, no evidence in the literature supports the notion that AA prevents the deleterious effects mediated by free radicals on target tissues. The available evidence actually suggests the opposite since products of the PLA_2 pathway and reactive nitrogen/oxygen species co-operate in eliciting inflammation-dependent tissue damage. In addition, stimulation of PLA_2 is often associated with toxicity in cells exposed to various sources of reactive species [22,23].

All together, the above results provide a clear cut indication that AA promotes downstream events counteracting U937 cell necrosis once the initial damage has been produced.

These considerations lead to the conclusion that the mechanism of cell death under investigation presents features which cannot be explained by the conventional theory that necrosis is a passive response to overwhelming damage. The most likely explanation of the above results is that toxicity is not the results of a stochastic process of cell damage but, rather, the consequence of a finely tuned mechanism regulating the balance between survival and death. The lethal event takes place abruptly only under conditions in which the AA-dependent protective mechanisms are impaired. This concept may be re-phrased by stating that the peroxynitrite-induced U937 cell necrosis occurs as a consequence of the inhibition of survival pathways.

Cell death elicited by 100 μM peroxynitrite in U937 cells with an impaired $cPLA_2$-dependent signalling was mediated by a MPT dependent mechanism. This inference is based on various lines of evidence. We observed that a treatment with 100 μM peroxynitrite, followed by a 15 min incubation in the same medium, had hardly any effect on mitochondrial membrane potential of cells with a proficient $cPLA_2$ signalling but produced dramatic changes in cells in which $cPLA_2$ is pharmacologically inhibited or genetically depleted (Table 2). This response was observed in a specific cell sub-population (Fig. 3). Indeed, while resistant cells displayed a normal mitochondrial membrane potential, the affected cells showed a robust mitochondrial membrane depolarisation associated with cell swelling, a typical feature of necrosis. The observation that loss of mitochondrial membrane potential (Fig. 3 and Table 2) and toxicity (Table 2) are prevented by CsA or BA, but not by FK506, is consistent with the notion that MPT is the cause of peroxynitrite toxicity in PLA_2-inhibited cells. Coherent with this inference are the results of experiments in which permeability transition pore opening was assessed by monitoring the changes in mitochondrial calcein fluorescence after quenching of the cytosolic signal with Co^{2+} (Fig. 4), an event once again sensitive to CsA and BA (not shown), but not to FK506 (not shown).

If MPT is the cause of U937 cell death induced by otherwise non-toxic concentrations of peroxynitrite, then AA should prevent both the loss of mitochondrial calcein and mitochondrial membrane potential. The results shown in Table 2 and Figs. 3 and 4 are consistent with this premise. Indeed, AA was protective also when added 10 min after peroxynitrite. Thus, it appears that a sub-population of cells

is resistant to peroxynitrite and that the remaining cells can survive, provided that AA is available in the specific timeframe in which critical events leading to MPT and immediate cell death take place.

In conclusion, the results presented in this study indicate that sub-toxic concentrations of peroxynitrite inflict a potentially lethal damage that would lead U937 cells to MPT-dependent necrosis in the absence of the AA-dependent cytoprotective signalling. The effects of AA are restricted to a very narrow time frame (10–15 min after addition of peroxynitrite) and in the absence of AA cell death takes place immediately. In addition, the AA-driven cytoprotective signalling is most likely targeted to the mitochondria and expected to promote downstream events opposing the opening of permeability transition pores. Various kinases, including protein-tyrosine kinase [24], Ca^{2+}/calmodulin-dependent protein kinase A [25], protein kinase C [26], as well as extracellular signal-regulated kinases 1/2 [27], are stimulated by AA and are therefore potential candidates for the downstream events involved in the AA-dependent cytoprotective signalling.

As a final note, it is important to emphasise that the above results were obtained using U937 cells, a promonocytic cell line, and are therefore of potential importance in the regulation of survival mechanisms triggered by peroxynitrite in peroxynitrite-producing cells, like monocytes or macrophages. More generally, these results provide additional support to the emerging notion that oxidized lipoproteins, lipids and phospholipid oxidation products have the ability of promoting diverse cytoprotective signalling pathways [28,29].

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