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Peroxynitrite stimulates the activity of cytosolic phospholipase A_2 in U937 cells: the extent of arachidonic acid formation regulates the balance between cell survival or death

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Abstract

Peroxynitrite stimulates in U937 cells release of arachidonic acid (AA) sensitive to various phospholipase A_2 (PLA_2) inhibitors, including arachidonyl trifluoromethyl ketone ($AACOCF_3$), which specifically inhibits cytosolic PLA_2 ($cPLA_2$). This response linearly increases using non toxic concentrations of the oxidant, and reaches a plateau at levels at which toxicity becomes apparent. Three separate lines of evidence are consistent with the notion that AA generated by $cPLA_2$ promotes survival in cells exposed to peroxynitrite. Firstly, toxicity was suppressed by nanomolar levels of exogenous AA, or by AA generated by the direct PLA_2 activator melittin. Secondly $AACOCF_3$, or other PLA_2 inhibitors, promoted cell death after exposure to otherwise non toxic concentrations of peroxynitrite; exogenous AA abolished the enhancing effects mediated by the PLA_2 inhibitors. Finally, U937 cells transfected with $cPLA_2$ antisense oligonucleotides were killed by concentrations of peroxynitrite that were non-toxic for cells transfected with nonsense oligonucleotides. This lethal response was insensitive to $AACOCF_3$ and prevented by exogenous AA.
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Keywords: peroxynitrite; arachidonic acid; phospholipase A_2; cell death

Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonyl trifluoromethyl ketone; BSA, bovine serum albumin; $cPLA_2$, cytosolic phospholipase A_2; ETYA, 5,8,11,14-eicosatetraynoic acid; FBS, fetal bovine serum; PLA_2, phospholipase A_2; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

Introduction

The phospholipase A_2 (PLA_2) enzymes hydrolyze fatty acids from sn-2 position of phospholipids with the concomitant production of lysophospholipids and arachidonic acid (AA). Mammalian cells contain structurally diverse forms of PLA_2, e.g. secretory PLA_2, calcium-independent PLA_2 and calcium-dependent cytosolic PLA_2 ($cPLA_2$).[1–4] The ubiquitous nature of PLA_2s highlights their central role in various cellular physiological processes including the generation of AA, prostaglandins and leukotrienes and the regulation of both lipid metabolism and membrane remodelling.[5] A wealth of experimental evidence also suggests that PLA_2s are important modulators of drug-, chemical-, and ischemia/reperfusion-induced cell death. In particular, cellular perturbations typically induced by hydroperoxides, such as elevation of intracellular Ca^{2+} and lipid peroxidation, may serve as stimuli for a sustained PLA_2 activity. Indeed, PLA_2s, mostly the $cPLA_2$ isoform, have been identified as important mediators of cell death induced by hydrogen peroxide,[6] *tert*-butylhydroperoxide[7,8] and endogenous lipoperoxides.[9] Thus, although it was suggested that PLA_2s may also exert cytoprotective properties,[10] most of the literature in this field is consistent with the notion that stimulation of PLA_2s, in particular $cPLA_2$, plays a pivotal role in the lethal response evoked by various sources of reactive species.

Peroxynitrite, the coupling product of superoxides and nitric oxide, is a potent oxidant that mediates tissue injury in diverse pathological conditions including ischemia-reperfusion injury, immunocomplex-mediated pulmonary edema, acute endotoxemia, neurological disorders and atherosclerosis.[11,12] At the cellular level, peroxynitrite induces an array of deleterious events including peroxidation of membrane lipids,[13] depletion of glutathione,[14] DNA single strand breakage,[14–16] mitochondrial dysfunction,[12,17] alteration of calcium ion homeostasis[18] and nitration of protein and non protein sulphydryl residues.[19] All these events, or their combinations, can be a cause of important dysfunctions leading to cell death. In a recent study,[20] we reported that endogenous as well as exogenous peroxynitrite stimulates AA release, mediated by activation of PLA_2, in PC12 cells. This response was associated with delayed formation of reactive oxygen species that were in fact responsible for the peroxynitrite-dependent oxidation of the fluorescent probe dihydrorhodamine 123.[21] In addition, it was found that peroxynitrite caused activation of a low molecular weight PLA_2, most likely located at the mitochondrial level, via a mechanism involving peroxynitrite-dependent inhibition of complex III, followed by enforced formation of superoxides which, unlike hydrogen

peroxide, appeared to directly activate the enzyme.[22] The question therefore arises as to whether peroxynitrite-dependent activation of PLA_2 also takes place in cell types other than the PC12 cell line and, if this is the case, whether this response is involved in cytotoxicity. The present study was designed to provide an answer to these questions and, in particular, we investigated the effects of exogenous peroxynitrite in U937 cells using conditions resulting in a rapid necrotic response mediated by mitochondrial permeability transition.[23] We show that peroxynitrite stimulates the release of AA also in U937 cells and that this event is largely dependent on the activity of $cPLA_2$. Surprisingly, AA release was not found to contribute to cytotoxicity but, rather, appeared to mediate cytoprotection. Our results demonstrate that AA triggers signaling pathways promoting survival in U937 cells challenged with peroxynitrite and this may well represent a mechanism whereby some cell types (e.g. macrophages) cope with their own peroxynitrite.

Results

Peroxynitrite stimulates release of AA in U937 cells

The results illustrated in Figure 1A indicate that exposure (5 min) to authentic peroxynitrite, followed by a 10 min post-treatment incubation in fresh saline A, promotes release of AA in U937 cells; this response linearly increases at concentrations in the 50–200 μM range and reaches a plateau thereafter. Two hundred μM peroxynitrite caused a release of AA comparable to that mediated by 5 μM A23187 (not shown). Three lines of evidence support the notion that the observed effects were entirely mediated by peroxynitrite: (1) decomposed peroxynitrite did not cause release of AA (Figure 1A); (2) the peroxynitrite scavengers 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 1 mM) and L-methionine (20 mM) prevented the AA release stimulated by 200 μM peroxynitrite (Figure 1B); (3) AA release was not observed in vehicle-treated cells (Figure 1B). Additional studies revealed that the release of AA mediated by 200 μM peroxynitrite, while insensitive to a phospholipase C inhibitor (neomycin, 1 mM) or a diacylglycerol inhibitor 1,6-*bis*(cyclohexyloximonocarbonylamino)hexane (100 μM) (not shown), was effectively reduced by three different PLA_2 inhibitors, namely mepacrine, 5,8,11,14-eicosatetraynoic acid (ETYA) or arachidonyl trifluoromethyl ketone ($AACOCF_3$) (Figure 1C).

Exogenous, or endogenous, AA prevents U937 cell death induced by peroxynitrite

A short-term (5 min) exposure to concentrations of peroxynitrite higher than 200 μM, followed by a 55 min post-treatment incubation in fresh saline A, causes U937 cell death.[23] Under these conditions, the mode of cell death is necrosis and rapid cell lysis takes place so that very few trypan blue positive cells can be detected.[23] As a consequence toxicity is inferred by the decreased number of trypan blue negative cells. The results illustrated in Figure 2 confirm our previous observation that exposure to concentrations of peroxynitrite greater than 200 μM progressively reduces the number of viable cells. These results also indicate that exogenous AA (0.1 μM) affords cytoprotection when added during the 55 min of post-treatment incubation. The same effect was mediated by the direct PLA_2 activator melittin (30 nM, added 2 min before peroxynitrite and removed at the end of the 5 min of peroxynitrite exposure). The notion that endogenous AA mitigates the toxicity induced by peroxynitrite is further supported by the results from experiments using pharmacologic inhibitors of PLA_2, at the same concentrations at which they were previously shown to suppress the peroxynitrite-dependent AA release (Figure 1C). Indeed, $AACOCF_3$ (Figure 3A), ETYA (Figure 3B) or mepacrine (Figure 3C), added during the 55 min post-incubation, markedly enhanced the toxicity induced by low concentrations (<200 μM) of peroxynitrite. Toxicity was also observed in cells treated with increasing concentrations of $AACOCF_3$ (Figure 3D), ETYA (Figure 3E) or mepacrine (Figure 3F) after challenge with an otherwise non-toxic concentration of peroxynitrite (100 μM). The inhibitors failed to induce toxicity in cells challenged with decomposed peroxynitrite. The results illustrated in Figure 4 indicate that the lethal response evoked by peroxynitrite (100 μM)/$AACOCF_3$ (50 μM) is abolished by very low levels of AA. Similar results were obtained in experiments in which $AACOCF_3$ was replaced with either 50 μM ETYA or 50 μM mepacrine (not shown).

It was next established that the mechanism whereby low concentrations of peroxynitrite cause toxicity in PLA_2 inhibitor-supplemented cells is identical to that involved in cell killing induced by high concentrations of peroxynitrite. Indeed, as we previously observed in cells treated with 1.2 mM peroxynitrite,[23] toxicity mediated by the combined exposure to 100 μM peroxynitrite and a 50 μM concentration of each of the above PLA_2 inhibitors was both preceed by mitochondrial depolarization and prevented by cyclosporin A (0.5 μM), an inhibitor of mitochondrial permeability transition (not shown). The fact that cell lysis occurs soon after exposure to low concentrations of peroxynitrite associated with the PLA_2 inhibitors, as it was previously observed in cells challenged with toxic levels of peroxynitrite,[23] strongly suggests that also under these conditions the mode of cell death is necrosis and this notion was confirmed using biochemical and morphological analyses (not shown).

Finally, we asked the question of whether the early necrosis was followed by delayed apoptosis of the surviving cells. No evidence of secondary DNA fragmentation was found after 24 h of post-challenge growth and microscopic analysis revealed that virtually all the cells displayed normal morphology (not shown). In addition, these cells were able to proliferate with rates comparable with those observed in untreated cells (Figure 5). This notion was further established by showing that superimposable curves describing the time-dependent increase in cell number were obtained after seeding equal numbers of untreated cells or cells surviving the 60 min exposure to 100 μM peroxynitrite associated with each of the three different PLA_2 inhibitors (not shown).

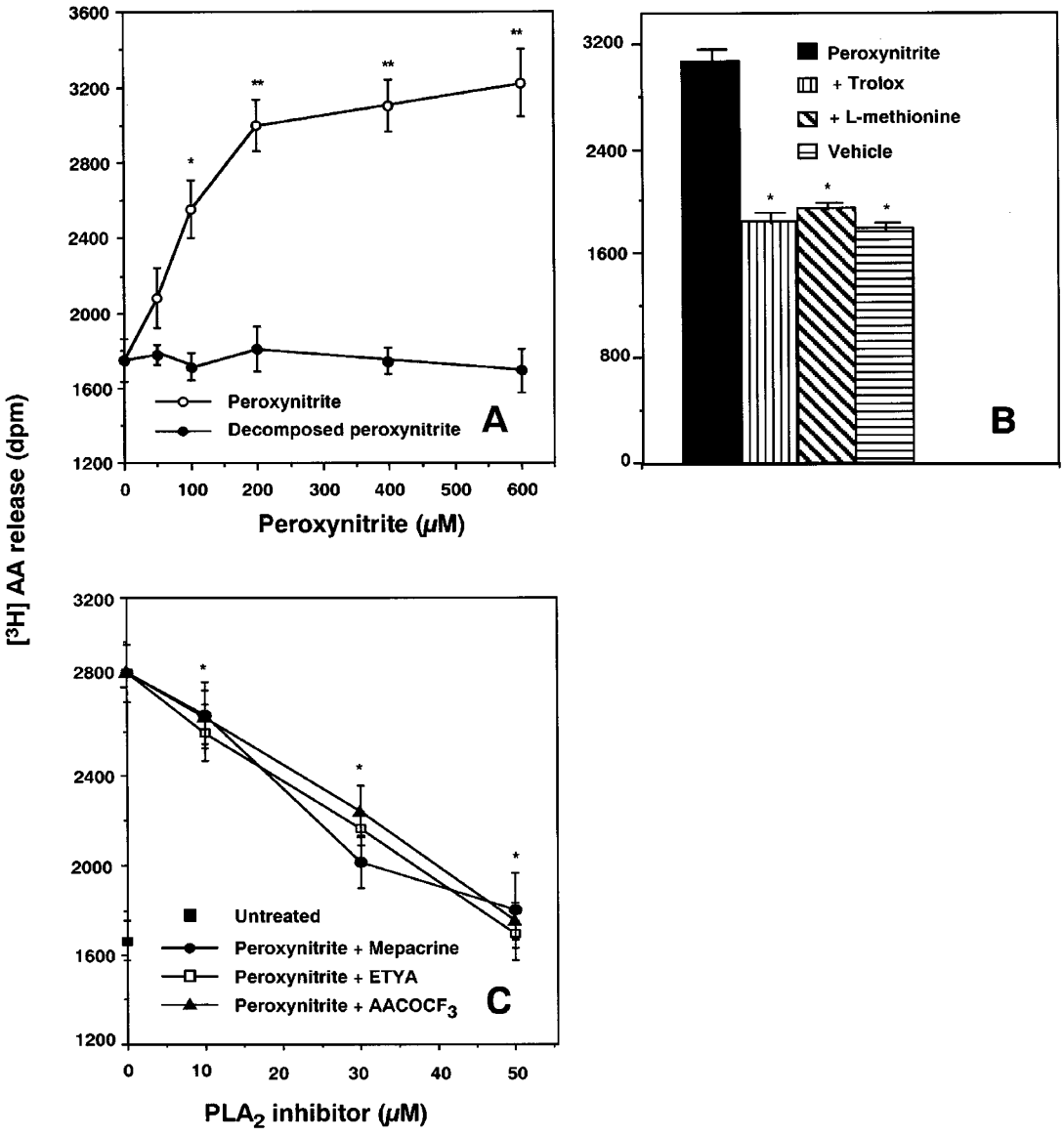

Figure 1 Peroxynitrite stimulates PLA_2 activity in U937 cells. (**A**) [^{3}H]-AA-labeled cells were exposed for 5 min to increasing concentrations of peroxynitrite or decomposed peroxynitrite, centrifuged and then post-incubated for a further 10 min in fresh saline A. After treatments, [^{4}H]-AA release was quantified as described in the Materials and Methods section. Results represent the means $\pm$ S.E.M. from 3–5 separate experiments, each performed in duplicate. (*)$P<0.001$ and (**)$P<0.0001$ as compared to untreated cells (unpaired *t*-test). (**B**) [^{3}H]-AA-labeled cells were exposed to 1 mM Trolox, 20 mM L-methionine or vehicle (e.g. an amount of NaOH identical to that contained in peroxynitrite stock solutions, immediately buffered at pH 7.4 with an appropriate volume of 1 N HCl). After 5 min, 200 μM peroxynitrite was added to the cultures that were incubated for a further 10 min at 37°C. [^{3}H]-AA release was determined immediately after treatments. Results represent the means $\pm$ S.E.M. from 3–5 separate experiments, each performed in duplicate. (*)$P<0.0001$ as compared to cells exposed to peroxynitrite alone (unpaired *t*-test). (**C**) [^{3}H]-AA-labeled cells were exposed for 5 min to 200 μM peroxynitrite, centrifuged and then post-incubated for a further 10 min in fresh saline A in the absence or presence of increasing concentrations of mepacrine, ETYA or $AACOCF_3$. [^{3}H]-AA release was determined immediately after treatments. Results represent the means $\pm$ S.E.M. from 3–5 separate experiments, each performed in duplicate. (*)$P<0.0001$ as compared to cells exposed to peroxynitrite alone (unpaired *t*-test)

$cPLA_2$ antisense oligonucleotides promote toxicity after exposure to otherwise non-toxic concentrations of peroxynitrite

U937 cells were transfected with phosphorothioate-modified antisense oligonucleotides (10 μM) designed to bind specifically to $cPLA_2$ mRNA initiation codon and therefore prevent the translation and synthesis of the enzyme protein.[24] Complementary nonsense oligonucleotides were used as a negative control. Immunocytochemical analysis using a monoclonal antibody to human $cPLA_2$ showed that the level of $cPLA_2$ protein was indeed significantly lower (77% decrease) in cells treated with antisense oligonucleotides (Figure 6B), as compared to cells treated with nonsense oligonucleotides (Figure 6A). Consistently, the $AACOCF_3$ (50 μM)-sensitive release of AA stimulated by 200 μM

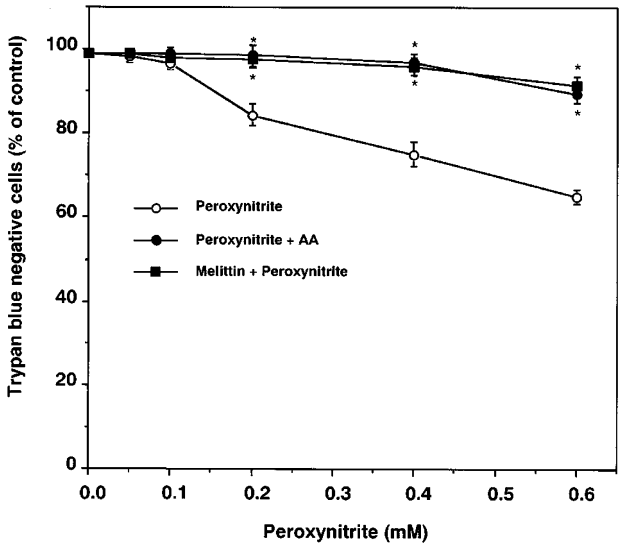

Figure 2 AA, or melittin, mitigates the lethal response evoked by peroxynitrite in U937 cells. Cells were exposed for 5 min to increasing concentrations of peroxynitrite, centrifuged and then post-incubated for a further 55 min in fresh saline A in the absence or presence of AA (0.1 μM). Also shown is the effect of melittin (30 nM) on peroxynitrite-induced cytotoxicity. Melittin was added 2 min before peroxynitrite and removed at the and of the 5 min treatment with the oxidant. Cytotoxicity was determined with the trypan blue exclusion assay. Results represent the means $\pm$ S.E.M. from 3–5 separate experiments, each performed in duplicate. (*)$P<0.0001$ as compared to cells exposed to peroxynitrite alone (unpaired *t*-test)

peroxynitrite was remarkably lower in cells transfected with antisense oligonucleotides, as compared to that observed in nonsense oligonucleotide transfected cells (Figure 6C). Interestingly 100 μM peroxynitrite, while not being toxic for nonsense oligonucleotide transfected cells, caused a lethal response in $cPLA_2$-depleted cells that was both insensitive to $AACOCF_3$ (50 μM) and prevented by exogenous AA (0.1 μM) (Figure 6D). The $cPLA_2$ inhibitor, however, promoted toxicity in peroxynitrite-treated cells transfected with nonsense oligonucleotides via a mechanism sensitive to exogenous AA (0.1 μM).

Discussion

The results reported in this study indicate that, as we previously observed in PC12 cells,[20,21] peroxynitrite promotes release of AA in U937 cells via a mechanism involving activation of PLA_2 (Figure 1A–C). In particular, this response linearly increases at concentrations in the 50–200 μM range and reaches a plateau thereafter. It was next shown that, using similar experimental conditions, toxicity became detectable after exposure to 200 μM peroxynitrite and that this response progressively increased at higher concentrations (Figure 2). We reasoned that it may not be just circumstantial that a lethal response becomes apparent only over the same concentration range in which peroxynitrite fails to promote additional release of AA (plateau phase of the dose-response curve, Figure 1A). Using various experimental approaches we were indeed able to make the following observations: (1) exogenous AA, or AA generated by direct PLA_2 activation mediated by melittin, prevent the lethal response evoked by peroxynitrite (Figure 2). The fact that AA was added during the 55 min post-treatment incubation rules out the possibility that the observed effects were causally linked to scavenging of peroxynitrite. (2) three different PLA_2 inhibitors, at concentrations at which they were previously shown to suppress the peroxynitrite-dependent AA release (Figure 1C), markedly enhanced the toxicity induced by peroxynitrite, in particular under conditions in which the latter was used at low concentrations (e.g. <200 μM) (Figure 3A–C). This notion is further demonstrated by the observation that toxicity occurs in cells supplemented with increasing concentrations of PLA_2 inhibitors (Figure 3D–F) after challenge with an otherwise non-toxic concentration of peroxynitrite (100 μM). The fact that the PLA_2 inhibitors were given to the cells 5 min after addition of peroxynitrite, a time at which the oxidant is no more in the culture medium,[23] emphasizes the specificity of the effects of the PLA_2 inhibitors and this notion is further supported by the observation that exogenous AA affords cytoprotection in PLA_2-inhibited cells exposed to peroxynitrite (Figure 4). It is important to note that the mechanism whereby peroxynitrite induces toxicity in PLA_2 inhibitor-supplemented cells may involve mitochondrial permeability transition since cell death was sensitive to cyclosporin A (not shown). One potential caveat with this conclusion is that cyclosporin A can have other actions in addition to inhibition of mitochondrial permeability transition; it is therefore possible that other mechanisms, besides inhibition of mitochondrial permeability transition, could account for its effects. It is important to note, however, that a mitochondrial permeability transition-dependent mechanism of peroxynitrite-induced U937 cell death was described in a recent study from our laboratory[23] and that the present investigation provides evidence for remarkable similarities in the toxicity paradigms using a toxic concentration of peroxynitrite or an otherwise non toxic concentration of peroxynitrite causing toxicity in PLA_2 inhibitor-supplemented cells (see above and below). Among these similarities is the rapid induction of necrosis associated with immediated cell lysis. Although mitochondrial permeability transition is generally agreed upon to be an apoptotic mechanism, there are gathering indications that the same mechanism can lead to necrosis, especially under conditions of oxidative stress and impaired energy metabolism.[25]

An additional observation presented in this study is that the cells which survived the treatment with 100 μM peroxynitrite associated with the PLA_2 inhibitors did not undergo delayed cell death but rather proliferated as untreated cells (Figure 5). This would indicate that under these conditions, as we previously observed in cells exposed to intrinsically toxic concentrations of peroxynitrite,[23] the oxidant either induces immediate necrosis or inflicts a reversible damage which does not result in delayed cell death.

Thus, the above results lead to the important conclusion that products of the PLA_2 pathway play a cytoprotective role in cells exposed to peroxynitrite. In particular, our

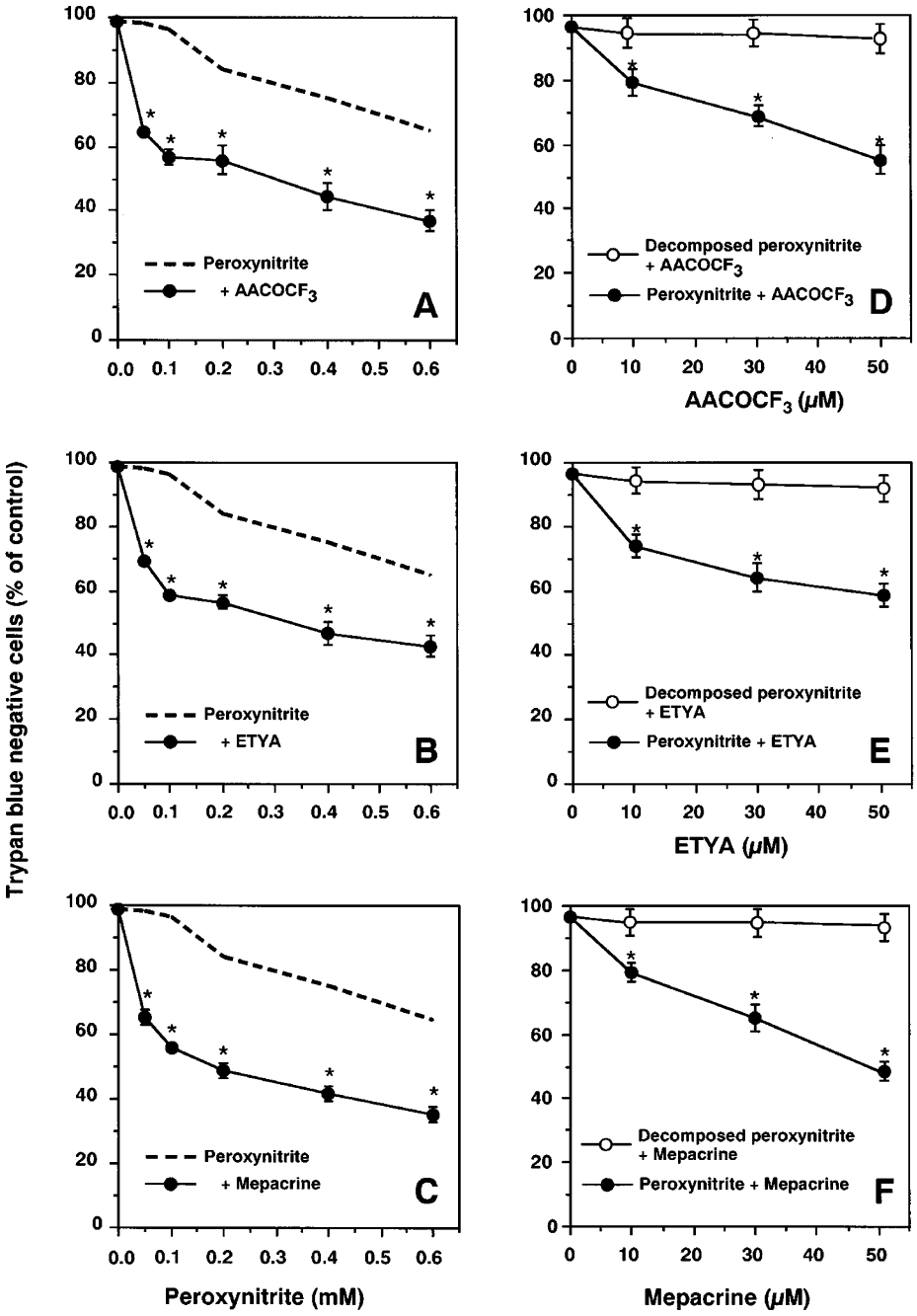

Figure 3 PLA_2 inhibitors enhance the toxicity mediated by peroxynitrite. Cells were exposed for 5 min to increasing concentrations of peroxynitrite, centrifuged and then post-incubated for a further 55 min in fresh saline A in the absence or presence of a 50 μM concentration of $AACOCF_3$ (**A**), ETYA (**B**) or mepacrine (**C**). In other experiments, the cells were exposed for 5 min to 100 μM peroxynitrite or 100 μM decomposed peroxynitrite, centrifuged and then post-incubated for a further 55 min in fresh saline A in the absence or presence of increasing concentrations of $AACOCF_3$ (**D**), ETYA (**E**) or mepacrine (**F**). Cytotoxicity was determined using the trypan blue exclusion assay. Results represent the means $\pm$ S.E.M. from 3–5 separate experiments. (*)$P<0.0001$ as compared to cells exposed to peroxynitrite alone (unpaired *t*-test)

results demonstrate that concentrations of peroxynitrite in the 50–200 μM range are either non-toxic, or produce very little toxicity, (Figure 2) because they effectively stimulate PLA_2 (Figure 1A). If the activity of PLA_2 is pharmacologically inhibited then cell death occurs via a mechanism that can be prevented by exogenous AA (Figure 4). Concentrations of peroxynitrite higher than 200 μM, while appearing in a plateau phase in terms of PLA_2 activation (Figure 1A), effectively kill cells, which can however be rescued by addition of exogenous AA (Figure 2).

The results presented above allow us to make some additional consideration on the identity of the specific PLA_2 isoform involved in the lethal response evoked by peroxynitrite. Indeed, while mepacrine and ETYA are

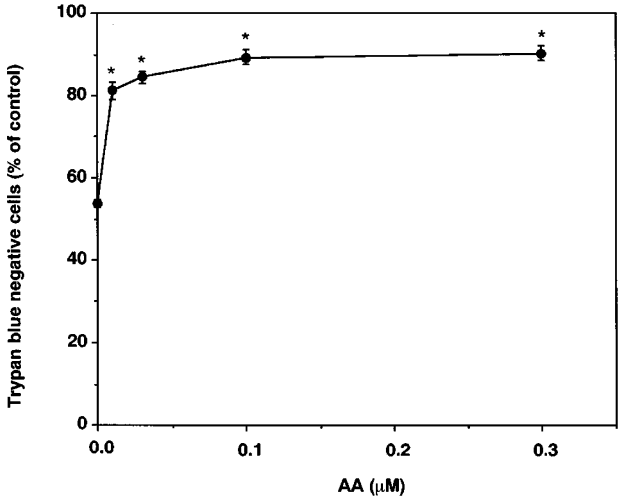

Figure 4 AA prevents the enhancing effects of $AACOCF_3$ on peroxynitrite-induced cytotoxicity. Cells were exposed for 5 min to 100 μM peroxynitrite, centrifuged and then post -incubated for a further 55 min in fresh saline A containing 50 μM $AACOCF_3$ in the absence or presence of increasing concentrations of AA. Cytotoxicity was then determined with the trypan blue exclusion assay. Results represent the means $\pm$ S.E.M. from 3–5 separate experiments, each performed in duplicate. (*)$P<0.0001$ as compared to cells exposed to peroxynitrite associated with $AACOCF_3$ (unpaired *t*-test)

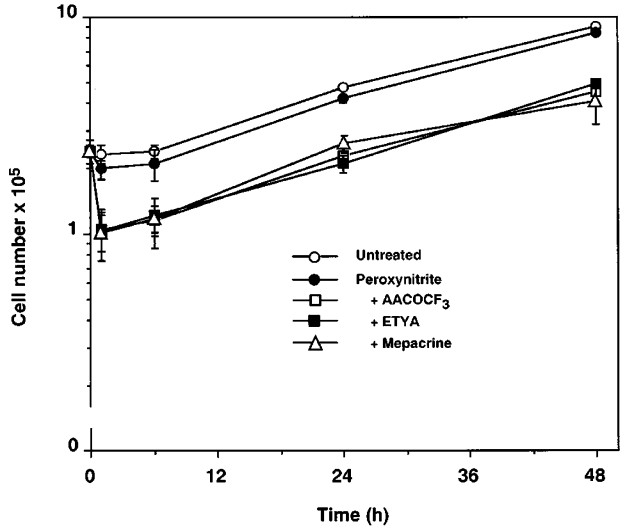

Figure 5 Cell counts of U937 cells at various time intervals after treatment with peroxynitrite and PLA_2 inhibitors. Cells were exposed for 5 min to 100 μM peroxynitrite, centrifuged and then post-incubated for a further 55 min in fresh saline A in the absence or presence of 50 μM $AACOCF_3$, 50 μM ETYA or 50 μM mepacrine. Also shown are the results obtained using cells that were not exposed to peroxynitrite and that did not receive additional treatments. Identical results were obtained using cells exposed to each of the three PLA_2 inhibitors during the last 55 min of incubation in saline A (not shown). After treatments, the cells were washed, resuspended in fresh culture medium and then grown for up to 48 h. The cells number was determined with a hemocytometer. Results represent the means $\pm$ S.E.M. from five separate experiments

thought to be general PLA_2 inhibitors, an extensive literature documents the ability of $AACOCF_3$ to specifically suppress the activity of the $cPLA_2$.[26,27] Thus, the fact that $AACOCF_3$ mimicked all the effects (Figures 1C and 3) generated by ETYA, or mepacrine, strongly suggests that $cPLA_2$ is activated by peroxynitrite and then promotes formation of species counteracting the ensuing lethal response.

The final series of experiments, utilizing U937 cells transfected with $cPLA_2$ antisense oligonucleotides, provides results consistent with this notion. These cells, while showing decreases in $cPLA_2$ protein immunoreactivity (Figure 6A,B) and release of AA in response to peroxynitrite (Figure 6C), displayed remarkable sensitivity to the ensuing lethal response evoked by peroxynitrite concentrations that were non toxic for nonsense oligonucleotide-transfected cells (Figure 6D). That this increased sensitivity was due to $cPLA_2$ depletion is demonstrated by the observed lack of effects of $AACOCF_3$ and by the fact that exogenous AA abolished toxicity. Interestingly, the $cPLA_2$ inhibitor promoted toxicity in peroxynitrite-treated cells transfected with nonsense oligonucleotides and the extent of this lethal response was comparable with that mediated by peroxynitrite in cells transfected with $cPLA_2$ antisense oligonucleotides. These results therefore confirm the notion that peroxynitrite-dependent activation of PLA_2 counteracts the ensuing cytotoxicity and provide an additional indication that $cPLA_2$ is the specific isoform involved in this response.

The results obtained in the present study are somewhat surprising since previous investigations have shown that stimulation of $cPLA_2$ is often associated with toxicity in cells exposed to a variety of noxious stimuli. In particular, the lethal responses evoked by hydrogen peroxide,[6] or *tert*-butylhydroperoxide,[7,8] were significantly reduced by various treatments resulting in inhibition of PLA_2 activity. More recently, toxicity was specifically associated with activation of $cPLA_2$ in cells exposed to either oxalate[28] or hydrogen peroxide.[29] Different mechanisms may explain the observed effects and, in particular, Sevanian[30] proposed that prolonged activation of PLA_2 may cause extensive hydrolysis of membrane phospholipids thereby decreasing membrane integrity. As pointed out by Cummings,[31] however, a different scenario may exist in which PLA_2 serves to remove membrane phospholipids damaged by free radical species thus allowing return to normal function upon reacylation of phospholipids. This repair process is therefore expected to promote survival.

Our results allow the formulation of a third possible role of PLA_2 in cells exposed to an oxidative insult. Indeed, the observation that nanomolar levels of AA are cytoprotective in cells supplemented with either inhibitors of PLA_2, or $cPLA_2$ antisense oligonucleotides, leads to the univocal conclusion that activation of $cPLA_2$ does not afford protection by removing damaged phospholipids. Rather, AA itself -or its metabolites generated by the cyclooxygenase and lipoxygenase pathways- is most likely responsible for the activation of a signaling pathway leading to survival. Since the toxicity paradigm utilized in the present study was associated with a necrotic mode of cell death, the involvement of some signaling event in this response can be inferred. While this notion is in obvious

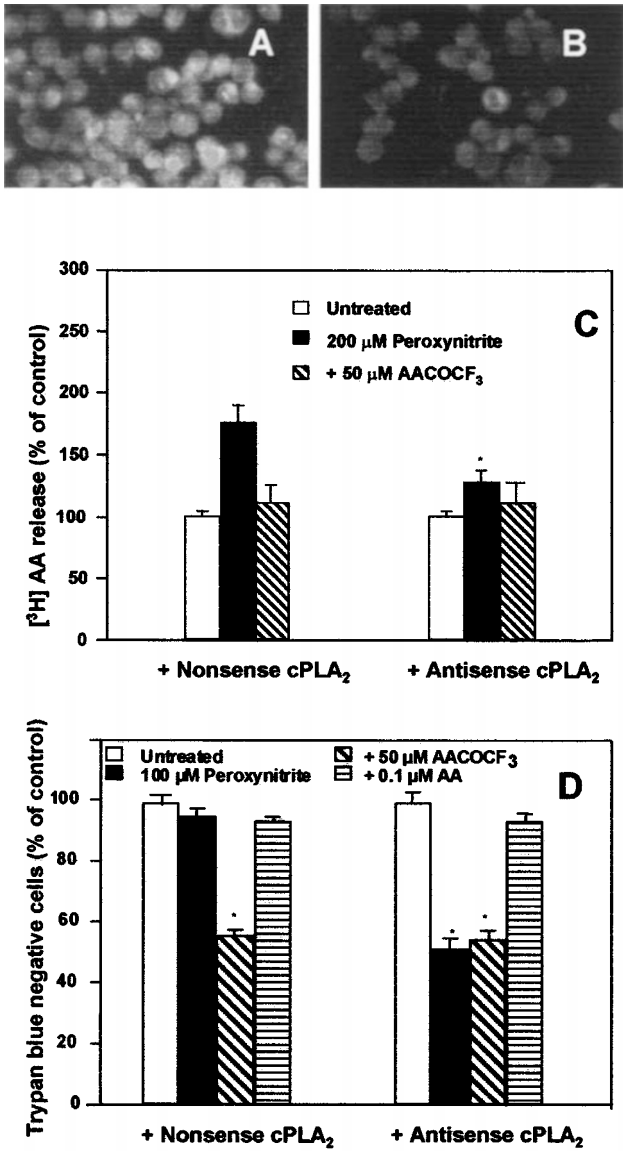

Figure 6 Transfection with $cPLA_2$ antisense oligonucleotides sensitizes U937 cells to peroxynitrite-induced cytotoxicity. U937 cells were treated with 10 μM phosphorothioate-modified nonsense or antisense oligonucleotides for 6 h in FBS-free medium, allowed to incubate for 24 h in 5% FBS containing medium and then stained with monoclonal antibodies against human $cPLA_2$, and visualized with a confocal microscope, as indicated in the Materials and Methods section. Representative confocal micrographs of nonsense- and antisense-transfected cells, are shown in (**A**) and (**B**), respectively. (**C**) Shows the results of experiments in which [^{3}H]AA- labeled nonsense or antisense-transfected cells were exposed for 5 min to 200 μM peroxynitrite, centrifuged and then post-incubated for a further 10 min in fresh saline A in the absence or presence of 50 μM $AACOCF_3$. After treatments, [^{3}H]AA release was quantified as described in the Materials and Methods section. Results represent the means $\pm$ S.E.M. from 3–5 separate experiments each performed in duplicate. (*)$P<0.001$ as compared to nonsense $cPLA_2$ transfected cells. (**D**) Shows the results of experiments in which oligonucleotide-transfected cells were exposed for 5 min to 100 μM peroxynitrite, post-incubated for a further 55 min in fresh saline A in the absence or presence of 50 μM $AACOCF_3$, or 0.1 μM AA, and then analyzed for cytotoxicity. Results represent the means $\pm$ S.E.M. from 3–4 separate experiments. (*)$P<0.0001$ as compared to untreated cells (unpaired *t*-test)

contrast with the hypothesis that necrosis is the result of a passive and obligatory response of the cells to overwhelming damage, further studies are needed to identify the signaling cascades triggered by peroxynitrite (leading to toxicity) and by AA (leading to cytoprotection). An involvement of cell signaling in necrotic cell death was recently proposed by Kumar *et al*.[32] that showed that c-Abl tyrosine kinase targets to mitochondria in response to hydrogen peroxide and thereby mediates mitochondrial dysfunction and U937 cell death. Importantly, toxicity induced by peroxynitrite was also mediated by a mitochondrial permeability transition-dependent mechanism[23] and we recently demonstrated that delayed formation of hydrogen peroxide plays a pivotal role in this toxicity mechanism.[33] Thus, the possibility exists that hydrogen peroxide produced its effects by acting at the level of the c-Abl tyrosine kinase pathway and that AA interferes with this response.

In conclusion, our results demonstrate that peroxynitrite activates $cPLA_2$ in U937 cells and that AA triggers downstream events counteracting the ensuing lethal response. Since AA and peroxynitrite are concomitantly produced in the inflammatory response, autocrine or paracrine generation of AA may allow survival of peroxynitrite-producing cells.

Materials and Methods

Chemicals

Mepacrine, ETYA, melittin, AA, Trolox, L-methionine, A23187, neomycin, 1,6-bis(cyclohexyloximonocarbonylamino)hexane, as well as most of reagent grade chemicals, were obtained from Sigma-Aldrich (Milan, Italy). $AACOCF_3$ was from Calbiochem (San Diego, CA, USA). Cyclosporin A was obtained from Sandoz A.G. (Bern, Switzerland). [^{3}H] AA was obtained from Amersham Pharmacia Bioetich (Buckinghamshire, UK). The oligonucleotides were phosphorothioate modified and synthesized by Oswel (University of Southampton, Southampton, UK). Mouse anti-human-$cPLA_2$ monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. Rhodamine-labeled anti-mouse IgG antibody was from Molecular Probes Europe (Leiden, The Netherlands).

Cell culture

U937 cells were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (50 units/ml) and streptomycin (50 μg/ml) (Sera-Lab Ltd., Crawley Down, UK), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air-5% CO_2.

Synthesis of peroxynitrite and treatment conditions

Peroxynitrite was synthesized by the reaction of nitrite with acidified H_2O_2, as described by Radi *et al*.,[34] and MnO_2 (1 mg/ml) was added to the mixture for 30 min at 4°C 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°C for 24 h. 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 (ε_{302}=1670 M^{-1} cm^{-1}).

Treatments were performed in 2 ml of saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose, pH 7.4) 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 its 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.

Cytotoxicity assay

Cells were analyzed for cytotoxicity using 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.

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

The cells were labeled 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 bovine serum albumin (BSA) resuspended in a final volume of 1 ml of saline A. The solution was then separated and centrifugated 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).

cPLA$_2$ antisense oligonucleotides

The human cPLA$_2$ antisense oligonucleotide (5′-TAC AGT AAA TAT CTA GGA ATG-3′) was directed against the initiation site. The nonsense oligonucleotide (5′-CCT ACT GAG GGT ACG GTA CAT-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 h in the absence or presence of oligonucleotides (10 μM). A final concentration of 5% FBS was then added, the cells were cultured for additional 24 h and finally utilized for experiments.

Immunocytochemical determination of cPLA$_2$ in U937 cells

Cells treated with antisense or nonsense oligonucleotide to cPLA$_2$ (1×10^6/ml) were mounted on slides by cytospinning, fixed with 4% paraformaldehyde and then permeabilized with ethanol:acetic acid (95 : 5) for 1 min at room temperature. The permeabilized cells were blocked with 1% BSA 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). The slide glass was then incubated over night (4°C) with anti-human-cPLA$_2$ antibody diluited 1 : 50 in phosphate-buffered saline containing 1% BSA. Excess antibody binding was removed by washing the slide glass with phosphate-buffered saline. Exposure to the secondary antibody (rhodamine-labeled anti-mouse IgG), diluted 1 : 50 in phosphate-buffered saline, was for 3 h at 37°C. After washing with phosphate-buffered saline, the cells were observed with a Bio Rad DVC 250 confocal laser microscope and the resulting images were taken with a Hamamatsu 5985 CCD camera and digitally recorded on a Macintosh computer.

Statistical analysis

All data in figures are expressed as mean $\pm$ S.E.M. For comparison between two groups the Student's unpaired *t*-test was used.

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