

Dexamethasone Promotes Toxicity in U937 Cells Exposed to Otherwise Nontoxic Concentrations of Peroxynitrite: Pivotal Role for Lipocortin 1-Mediated Inhibition of Cytosolic Phospholipase A₂

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ABSTRACT

Pretreatment with dexamethasone (Dex) was not toxic for U937 cells but caused a rapid lethal response upon subsequent exposure to otherwise nontoxic concentrations of peroxynitrite. This effect was not associated with enhanced formation of hydrogen peroxide taking place after peroxynitrite and was shown previously to play a pivotal role in the ensuing lethal response. Further analyses revealed that although Dex did not affect cytosolic phospholipase A₂ (cPLA₂) expression, it markedly reduced the extent of arachidonic acid (AA) release mediated by peroxynitrite-dependent stimulation of cPLA₂. This event, as well as the enhanced toxicity, was abolished by mifepristone, a glucocorticoid receptor antagonist. The outcome of various approaches, using phospholipase A₂ inhibi-

tors, cPLA₂ antisense oligonucleotide-transfected cells, and supplementation with exogenous AA, led to the demonstration that inhibition of cPLA₂ activity is causally linked to the increased susceptibility to peroxynitrite caused by Dex. Finally, the effects of Dex were shown to be mediated by enhanced expression of lipocortin 1 (LC1), a cPLA₂ inhibitory protein. These results indicate that Dex promotes toxicity in U937 cells exposed to otherwise nontoxic concentrations of peroxynitrite and that this event is causally linked to enhanced expression of LC1 leading to inhibition of cPLA₂. Thus, the increased lethal response arises because of LC1-dependent impairment of the AA-induced cytoprotective mechanism triggered by peroxynitrite.

Peroxynitrite, the coupling product of nitric oxide and superoxide, is a highly reactive nitrogen species relevant in various pathological conditions (Moncada et al., 1991; Heales et al., 1999) and thought to play a pivotal role in eliciting tissue damage during inflammation (Szabó, 1996). This potent biological oxidant produces lesions at the level of an array of biomolecules (Salgo et al., 1995) both directly or via delayed formation of reactive oxygen species (Tommasini et al., 2002a). The latter mechanism seems to be particularly important because peroxynitrite has a very short half-life (Hughes, 1999) and, as we recently showed, delayed formation of H₂O₂, resulting from exposure of U937 cells to toxic levels of peroxynitrite, plays a pivotal role in the ensuing lethal response (Tommasini et al., 2002a). Notably, the toxicity paradigm adopted in this study was associated with a mitochondrial permeability transition-dependent necrosis,

taking place within minutes after treatment with peroxynitrite and rapidly evolving in cell lysis (Sestili et al., 2001). Although these results may suggest that H₂O₂ contributes to toxicity by inflicting molecular damage in addition to that directly produced by peroxynitrite, recent work indicates that H₂O₂ causes toxicity by impairing a cytoprotective signaling triggered by peroxynitrite (Tommasini et al., 2004). It is important to keep in mind that U937 cells are a promonocytic cell line, often used as a monocyte cellular system, and it is not surprising that these cells are provided with a machinery to defend themselves from their own peroxynitrite. This defensive mechanism is represented by activation of cytosolic phospholipase A₂ (cPLA₂) leading to formation of cytoprotective levels of arachidonic acid (AA) (Tommasini et al., 2002b). Under these conditions, pharmacological inhibition or genetic depletion of cPLA₂ caused cell death after exposure to otherwise nontoxic levels of peroxynitrite, and exogenous AA prevented this lethal response as well as that mediated by intrinsically toxic concentrations of peroxynitrite in the absence of additional manipulations.

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ABBREVIATIONS: cPLA₂, cytosolic phospholipase A₂; AA, arachidonic acid; PLA₂, phospholipase A₂; GC, glucocorticoid; LC1, lipocortin 1; Dex, dexamethasone; MAFP, methyl arachidonyl fluorophosphonate; RU 486, mifepristone; AACOCF₃, arachidonyl trifluoromethyl ketone; DHR, dihydrorhodamine 123; FBS, fetal bovine serum; BSA, bovine serum albumin.

The fine regulation of the balance between cell survival and death indicates that U937 cell necrosis caused by peroxynitrite is not the consequence of a stochastic process of cell damage; rather, it depends on the inhibition of a signaling cascade leading to cytoprotection. By extension of our results, it may be suggested that, during inflammation, monocyte survival in the presence of the inflammation product peroxynitrite is assured by an additional inflammation product (i.e., AA). These findings therefore imply that inhibitors of phospholipase A₂ (PLA₂) display their anti-inflammatory action both via inhibition of formation of toxic species generated by the cyclooxygenase or lipoxygenase pathways and by decreasing monocyte survival in inflamed tissues in which peroxynitrite is being formed. The question therefore arises as to whether a similar effect is mediated by glucocorticoids (GCs).

GCs are anti-inflammatory and immunosuppressive drugs commonly used in the therapy of many long- and short-term inflammatory illnesses (Barnes and Adcock, 1993; Boumpas et al., 1993). Their effects are mediated by induction/repression of gene transcription, resulting in impaired formation of important mediators of the inflammatory response and inhibition of AA release from cellular lipids (Hong and Levine, 1976). The latter event may result from decreased expression of cPLA₂ (Gewert and Sundler, 1995) or enhanced expression of cPLA₂ inhibitory proteins belonging to a family of structurally related, calcium-dependent phospholipid-binding proteins (Flower, 1988; Flower and Rothwell, 1994), generally referred to as lipocortins or annexins. In particular, lipocortin 1 (LC1), a 37-kDa member of this family of proteins, was previously shown to mediate the inhibition of cPLA₂ in response to GCs (Kim et al., 2001).

We therefore decided to extend our previous studies to investigate the effects of GCs on the response of U937 cells to peroxynitrite. It was found that dexamethasone (Dex), a potent synthetic GC, inhibits cPLA₂ activity via enhanced LC1 expression and that this event is associated with, and causally-linked to, U937 cell death mediated by exposure to otherwise nontoxic concentrations of peroxynitrite.

Materials and Methods

Chemicals and Antibodies. AA (sodium salt), methyl arachidonyl fluorophosphonate (MAFP), Dex, mifepristone (RU 486), and most of the reagent grade chemicals were obtained from Sigma-Aldrich (Milan, Italy). Arachidonyl trifluoromethyl ketone (AA-COCF₃) was from Calbiochem (San Diego, CA). Dihydrorhodamine 123 (DHR) and rhodamine-labeled anti-mouse IgG antibody were from Molecular Probes Europe (Leiden, The Netherlands). [³H]AA was obtained from Amersham Biosciences (Buckinghamshire, UK). Mouse anti-human-LC1, cPLA₂ and horseradish peroxidase-conjugated monoclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture and Dex Exposure. U937 cells were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA) 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, England), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY) gassed with an atmosphere of 95% air-5% CO₂. Cells were seeded at 0.3 × 10⁶ cells/ml in a 12-well plate and primed with Dex in RPMI 1640-complete, with or without the GC receptor antagonist RU 486. Cells were then harvested, centrifuged, and the cell pellets were suspended in fresh saline A for different studies.

Synthesis of Peroxynitrite and Treatment Conditions. Peroxynitrite was synthesized by the reaction of nitrite with acidified H₂O₂ as described by Radi et al. (1991) and MnO₂ (1 mg/ml) was added to the mixture for 30 min at 4°C to eliminate the excess of H₂O₂. MnO₂ 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 because of freeze fractionation, was determined spectrophotometrically by measuring the absorbance at 302 nm in 1.5 M NaOH; ε₃₀₂ = 1670 M/cm. Stock solutions of peroxynitrite were routinely checked for the presence of H₂O₂ by the method of Webster (1975). Treatments were performed in 2 ml of prewarmed saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l NaHCO₃, and 0.9 g/l glucose) containing 5 × 10⁵ 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 caused by the high alkalinity of the peroxynitrite stock solution, an appropriate amount of 1 N HCl was also added.

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.

Measurement of Extracellular Release of [³H]AA. The cells were labeled with [³H]AA (0.5 µCi/ml) and grown for 18 h. Before treatments, the cells (2 × 10⁵) were washed twice with saline A supplemented with 1 mg/ml fatty acid-free bovine serum albumin (BSA) and resuspended in a final volume of 1 ml of saline A. The solution was then separated and centrifuged at 5000g for 1.5 min; 500 µl of the resulting supernatant was removed, and radioactivity was determined in a liquid scintillation counter (1409; PerkinElmer Wallac, Turku, Finland).

cPLA₂ and LC1 Antisense Oligonucleotides. The human cPLA₂ antisense oligonucleotide (5'-GTA AGG ATC TAT AAA TGA CAT-3') was directed against the initiation site. The nonsense oligonucleotide (5'-GAT GAT CAG ATA TAC GAT AAT-3') was a random sequence of the antisense bases. The human LC1 antisense oligonucleotide (5'-CCG GGA CCA CCT TTG G-3') targeted to a region coding the unique N-terminal portion of LC1. The nonsense oligonucleotide (5'-GCG CCA GCA TCT GCT G-3') was a random sequence of the antisense bases. The oligonucleotides were phosphorothioate-modified and synthesized by MWG Biotech (Florence, Italy). U937 cells were washed twice with serum-free medium and seeded (1 × 10⁶/ml) in serum-free RPMI 1640 for 6 h in the absence or presence of oligonucleotides (10 or 20 µM for cPLA₂ and LC1 antisense/nonsense oligonucleotides, respectively). A final concentration of 5% FBS was then added and the cells were cultured for an additional 48 h and finally used for experiments.

DHR Oxidation and Confocal Imaging. Cells were preincubated with or without Dex (20 µM), treated with peroxynitrite (5 min) in saline A, centrifuged, and then postincubated in fresh saline A in the presence of 10 µM DHR (10 min). After accurate washings, the cells were resuspended in 100 µl of phosphate-buffered saline (0.121 M NaCl, 10 mM NaH₂PO₄, 1.5 mM KH₂PO₄, and 3 mM KCl); 20 µl (50,000 cells) of this cell suspension was stratified on a slide, and cellular fluorescence was then imaged using a confocal laser microscope (DVC 250; Bio-Rad, Hercules, CA) equipped with a chilled charge-coupled device camera (5985; Hamamatsu Italy, Milan, Italy). Cells were illuminated with the 488 nm line of the argon laser and the fluorescence emitted was monitored at λ > 515 nm. The laser intensity, the shutter aperture, and the exposure/integration settings were kept constant to allow quantitative comparisons of relative fluorescence intensity of cells between treatment groups. Laser exposure was limited to brief image acquisition intervals (≤ 5 s) to minimize photo-oxidation of DHR. Confocal images were digitally acquired and processed for fluorescence determination at the single cell level on a Macintosh G4 computer using the public domain NIH Image 1.63 program (developed at the United States National

Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/ni-image/>). Mean fluorescence values were determined by averaging the fluorescence of at least 50 cells/treatment condition/experiment.

Immunocytochemical Determination of cPLA₂ and LC1 in U937 Cells. After treatment, the cells 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. The slide glass was then incubated overnight (4°C) with anti-human-cPLA₂ or anti-human-LC1 antibodies diluted 1:100 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 1:500 in phosphate-buffered saline, was for 3 h at 37°C. After washing with phosphate-buffered saline, the cells were observed with a confocal microscope and the resulting images were processed as detailed above.

Western Blot Analysis. After treatment, medium was removed and 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 phenylmethylsulfonyl fluoride, 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, Farmingdale, NY) and centrifuged at 21,500g for 10 min at 4°C to remove detergent-insoluble material. Supernatants were assayed for protein concentration using a Bio-Rad protein assay reagent. Protein samples (25 µg) were resolved in 7.5% (cPLA₂) or 10% (LC1) sodium dodecyl sulfate polyacrylamide gel and electrotransferred to polyvinylidene difluoride membranes. The blots were blocked for 1 h at room temperature with 5% powdered milk in Tris-buffered saline (140 mM NaCl and 50 mM Tris-HCl, pH 7.2) containing 0.06% Tween 20 and probed with a primary antibody against cPLA₂ (1:500) or LC1 (1:500) overnight at 4°C. Horseradish peroxidase-conjugated monoclonal antibodies (1:2000) were used for enhanced chemiluminescence detection. Densitometric analysis of blots was performed using the electrophoresis documentation and the public domain NIH Image 1.63 program.

Statistical Analysis. All data in figures and table are expressed as mean ± S.E.M. Student's unpaired *t* test was used for comparisons between two groups.

Results

Dex Enhances U937 Cell Death Induced by Peroxynitrite. A short-term (5 min) exposure to 100 µM peroxynitrite, followed by 55-min post-treatment incubation in fresh saline A, was not toxic for U937 cells (Fig. 1A) but caused a significant lethal response in cells pre-exposed to Dex (Fig. 1, A and B). The synthetic GC (5–20 µM) was not toxic in the absence of additional treatments (Fig. 1B). The extent of the lethal response was a direct function of the duration of Dex exposure (Fig. 1A) and Dex concentration (Fig. 1B). Additional studies revealed that the enhancing effects of Dex were more pronounced using nontoxic or marginally toxic, rather than intrinsically toxic, concentrations of peroxynitrite (Fig. 1C).

In an attempt to determine whether the effects of Dex are mediated by GC receptor interaction, the cells were incubated for 48 h with both Dex (20 µM) and RU 486 (1–10 µM), a GC receptor antagonist (Yang et al., 1996; Mahajan and London, 1997). As illustrated in Fig. 1D, RU 486 prevented, in a concentration-dependent fashion, the effects of Dex on peroxynitrite (100 µM) toxicity. RU 486 was not toxic in the absence of additional treatments (Fig. 1D) and did not affect

the toxicity mediated by an intrinsically toxic concentration of peroxynitrite (not shown).

Taken together, the above results indicate that Dex, upon its interaction with the GC receptor of U937 cells, promotes delayed events resulting in toxicity after exposure to otherwise nontoxic levels of peroxynitrite.

Dex Fails to Enhance Formation of Reactive Oxygen Species Elicited by Peroxynitrite. Previously, we showed that delayed formation of H₂O₂ is associated with, and causally linked to, U937 cell death mediated by peroxynitrite (Tommasini et al., 2002a). We therefore investigated whether the increased susceptibility of Dex pretreated cells to peroxynitrite toxicity was associated with enhanced accumulation of reactive oxygen intermediates. Formation of H₂O₂ was assessed using the fluorescent probe DHR (10 µM), given to the cultures 5 min after exposure to peroxynitrite, a con-

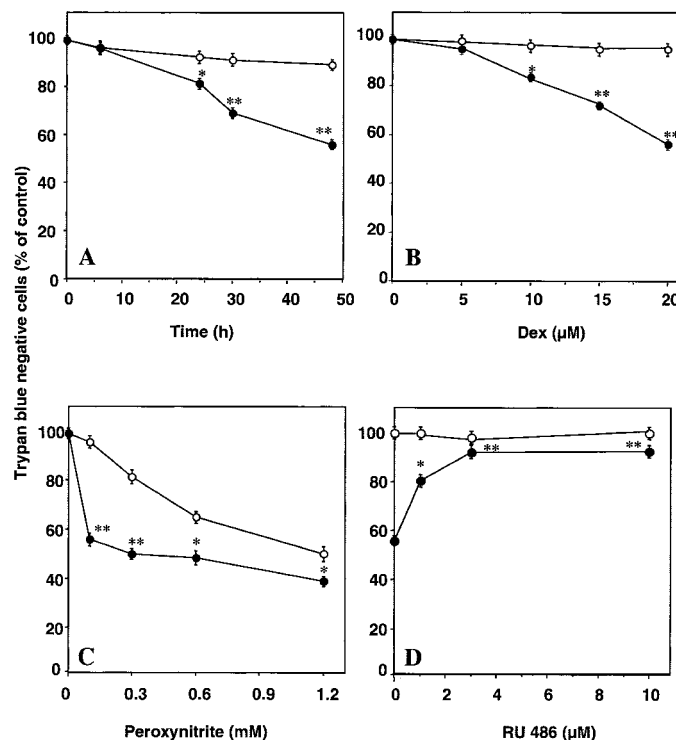


Fig. 1. Dex pretreatment enhances the toxicity mediated by peroxynitrite. A, cells were exposed for increasing time intervals to 0 (○) or 20 µM Dex (●), treated for 5 min with 100 µM peroxynitrite and then postincubated for a further 55 min in fresh saline A. Cells were then analyzed for cytotoxicity using the trypan blue exclusion assay. Results represent the means ± S.E.M. from at least five separate experiments. *, *p* < 0.001; **, *p* < 0.0001 compared with unstimulated cells exposed to peroxynitrite (unpaired *t* test). B, U937 cells were exposed for 48 h to increasing concentrations of Dex and then exposed for 60 min to saline A (○) or treated with 100 µM peroxynitrite as described above (●). Cytotoxicity was then determined. Results represent the means ± S.E.M. from at least three separate experiments. *, *p* < 0.001; **, *p* < 0.0001 compared with Dex-pretreated cells that did not receive subsequent peroxynitrite exposure (unpaired *t* test). C, cells were exposed for 48 h to 0 (○) or 20 µM Dex (●) and treated with increasing concentrations of peroxynitrite as described in A. Cytotoxicity was then determined. Results represent the means ± S.E.M. from at least four separate experiments. *, *p* < 0.001; **, *p* < 0.0001 compared with unstimulated cells exposed to peroxynitrite (unpaired *t* test). D, cells were exposed for 48 h to increasing concentrations of RU 486 and 20 µM Dex (●) and then treated with 100 µM peroxynitrite as described in A. Cytotoxicity was then determined. Also shown is the effect of increasing concentration of RU 486 alone (○). Results represent the means ± S.E.M. from at least four separate experiments. *, *p* < 0.001; **, *p* < 0.0001 compared with cells pre-exposed to Dex without RU 486 and treated with peroxynitrite (unpaired *t* test).

dition that does not allow peroxynitrite to directly oxidize DHR. As reported in Table 1, there was no difference in the extent of DHR oxidation mediated by increasing concentrations of peroxynitrite in cells preincubated for 48 h with 0 or 20 μ M Dex. Taken together, these results are consistent with the notion that the mechanism whereby Dex pretreatment enhances toxicity in cells exposed to peroxynitrite does not involve accumulation of reactive oxygen species.

Dex Impairs the AA-Dependent Cytoprotective Signaling Induced by Peroxynitrite. We showed previously that pharmacological inhibition, or genetic depletion, of cPLA₂ promotes U937 cell death after exposure to an otherwise nontoxic concentration of peroxynitrite (Tommasini et al., 2002b). The results illustrated in Fig. 2A indicate that the effects mediated by Dex are not dependent on inhibition of cPLA₂ expression. Indeed, immunocytochemical and Western blot analyses using a monoclonal antibody to human cPLA₂ showed that the level of the protein was similar in cells grown for 48 h in the absence or presence of 20 μ M Dex.

Exposure to the synthetic GC, however, caused a significant reduction in cPLA₂ activation mediated by peroxynitrite. Indeed, as shown in Fig. 2B, peroxynitrite caused a time-dependent release of AA, and this response was remarkably lower in Dex-pretreated cells. RU 486 prevented the effects of Dex. It is important to note that the AA release was monitored over 15 min after application of peroxynitrite because, under all of the above conditions, toxicity was not apparent (data not shown).

The possibility therefore exists that the mechanism whereby Dex promotes toxicity in cells exposed to otherwise nontoxic levels of peroxynitrite involves inhibition of the cPLA₂-dependent cytoprotective signaling identified in our laboratory (Tommasini et al., 2002b, 2004). To determine whether this was indeed the case, we used two different approaches involving the use of pharmacological inhibitors of PLA₂ and cPLA₂ antisense oligonucleotides.

Inhibitor studies involved the use of AACOCF₃, or MAFP, compounds extremely more potent in inhibiting cPLA₂ than other calcium-dependent PLA₂ isoforms, such as secretory PLA₂ (Street et al., 1993, Deutsch et al., 1997), but that can also inhibit calcium-independent PLA₂ (Ackermann et al., 1995; Lio et al., 1996). As shown previously, however, cPLA₂ is the specific PLA₂ isoform involved in AA release stimulated by peroxynitrite in U937 cells (Tommasini et al., 2002b, 2004). As expected (Tommasini et al., 2002b), a remarkable lethal response was observed after a 5-min exposure 100 μ M peroxynitrite, followed by an additional 55 min of incubation in the presence of either 50 μ M AACOCF₃ or 30 μ M MAFP

(Fig. 2C). An identical toxicity was also observed in cells pretreated with 20 μ M Dex exposed to the same concentration of peroxynitrite, and this response was insensitive to AACOCF₃ or MAFP. As shown in Table 2, the same level of toxicity was also observed in cells pre-exposed to increasing Dex concentrations and treated with peroxynitrite in the presence of a PLA₂ inhibitor. These results are therefore consistent with the possibility that Dex and AACOCF₃ (or MAFP) act on the same target; e.g., they both inhibit PLA₂ and thus prevent the AA-dependent cytoprotective signaling. Experimental support for this notion was provided by the observation that nanomolar levels of AA prevent toxicity induced by peroxynitrite in both Dex-pretreated cells and in PLA₂ inhibitor-supplemented cells (Fig. 2C).

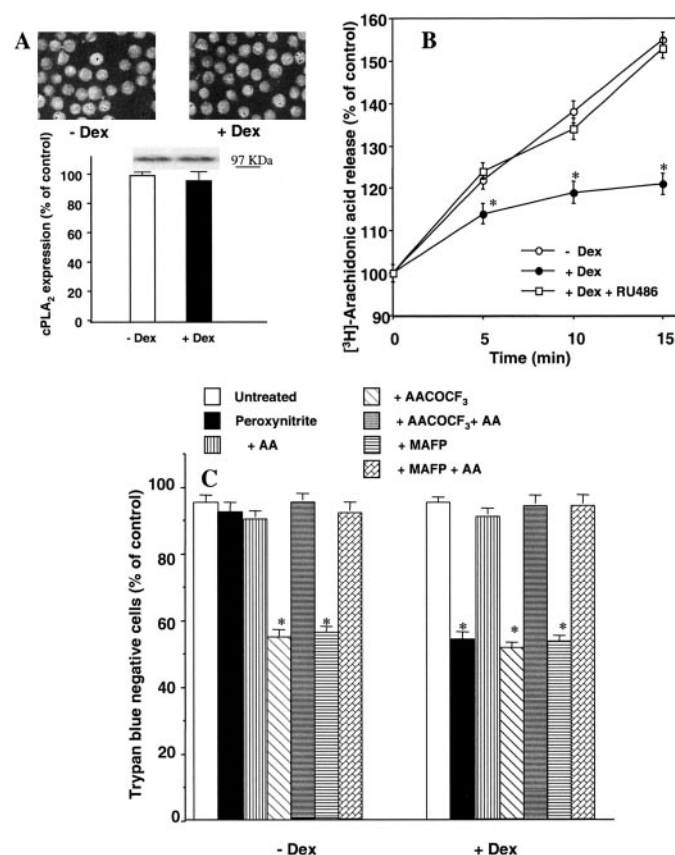


Fig. 2. Dex does not affect cPLA₂ protein expression but impairs the AA-dependent cytoprotective signaling induced by peroxynitrite. A, cells were exposed for 48 h to 0 or 20 μ M Dex and then processed for immunocytochemical or Western blot analyses using a monoclonal antibody against human cPLA₂, as detailed under *Materials and Methods*. The results shown are representative of three separate experiments. The amount of cPLA₂ protein detected in the Western blot assay was quantified by densitometric analysis. B, cells were exposed for 48 h to 0 or 20 μ M Dex, with or without 3 μ M RU 486, and labeled with [³H]AA in the last 18 h of incubation. Cells were subsequently treated for increasing time intervals with 100 μ M peroxynitrite. After treatments, [³H]AA release was quantified as described under *Materials and Methods*. Results represent the means \pm S.E.M. from three separate experiments, each performed in duplicate. *, $p < 0.001$ compared with untreated cells or cells pretreated with Dex and RU 486 (unpaired t test) C, cells pre-exposed for 48 h to 0 or 20 μ M Dex were treated for 5 min with 100 μ M peroxynitrite and then postincubated for a further 55 min in the absence or presence of 0.1 μ M AA, 50 μ M AACOCF₃, or the two agents combined. In some experiments, AACOCF₃ was replaced with MAFP (30 μ M). Cells were then analyzed for cytotoxicity. Results represent the means \pm S.E.M. from three to five separate experiments. *, $p < 0.001$ compared with untreated cells or cells pre-exposed to Dex.

TABLE 1

Dex fails to enhance formation of reactive oxygen species in U937 cells exposed to peroxynitrite

Cells were exposed for 48 h to 0 or 20 μ M Dex, treated with peroxynitrite for 5 min, and postincubated for a further 10 min in fresh saline A containing 10 μ M DHR. The DHR-derived fluorescence was determined as detailed under *Materials and Methods*. Results are presented as mean arbitrary units \pm S.E.M. from three separate experiments.

Treatment	DHR-Derived Fluorescence	
	-Dex	+Dex
Untreated	35.3 \pm 2.1	38.9 \pm 2.4
100 μ M peroxynitrite	43.8 \pm 3.1	47.3 \pm 2.9
300 μ M peroxynitrite	52.6 \pm 2.9	50.4 \pm 2.4
600 μ M peroxynitrite	60.6 \pm 2.5	64.3 \pm 2.9

Additional support for the above inference is given by experiments using cells in which the expression of cPLA₂ was suppressed using specific antisense oligonucleotides. The cells were transfected with phosphorothioate-modified antisense oligonucleotides (10 μ M), designed to bind specifically to the cPLA₂ mRNA initiation codon (Griffoni et al., 2001) and complementary nonsense oligonucleotides were used as a negative control. Immunocytochemical and Western blot analyses using a monoclonal antibody to human cPLA₂ showed that the level of the protein was indeed significantly lower in cells treated with antisense oligonucleotides, compared with cells treated with nonsense oligonucleotides (Fig. 3A). Similar results were obtained under conditions in which exposure to the oligonucleotides was performed in the presence of Dex (Fig. 3B). The expression of the protein was identical in nontransfected cells or nonsense oligonucleotide-transfected cells (not shown). As expected (Tommasini et al., 2002b), 100 μ M peroxynitrite, although not toxic for nonsense oligonucleotide-transfected cells, caused an AACOCF₃-insensitive lethal response in cPLA₂-down-regulated cells (Fig. 3C). The cPLA₂ inhibitor, however, promoted toxicity in cPLA₂ nonsense oligonucleotide-transfected cells exposed to peroxynitrite. As observed in nontransfected cells (Fig. 2C), 100 μ M peroxynitrite caused an AACOCF₃-insensitive lethal response in cPLA₂ nonsense oligonucleotide-transfected cells pretreated with Dex. Growth in the presence of the GC, however, did not further enhance toxicity in cells transfected with cPLA₂ antisense oligonucleotides. These effects were observed in both the absence and presence of AACOCF₃.

These results, therefore, confirm the information obtained in inhibitor studies and strongly suggest that Dex enhances peroxynitrite toxicity via inhibition of cPLA₂. Coherent with this inference is the observation that exogenous AA afforded cytoprotection under all of the above conditions. An additional indication from the results reported in this section is that Dex does not impair cPLA₂ expression but rather mitigates the extent of cPLA₂ activation mediated by peroxynitrite.

A Role for LC1 in Mediating the Effects of Dex. LC1 is constitutively expressed in U937 cells (Wu et al., 2000), and this information is also provided by immunocytochemical and Western blot analyses performed in our cells using a monoclonal antibody against human LC1 (Fig. 4A). It is interesting that, consistent with previous studies (Wu et al., 2000),

LC1 expression was significantly (about 60%) increased in cells grown for 48 h in the presence of Dex (20 μ M; Fig. 4A). We therefore investigated the possibility that enhanced expression of LC1 is the mechanism whereby Dex inhibits cPLA₂ activity and enhances toxicity induced by peroxynitrite.

For this purpose, antisense oligonucleotides recognizing the region coding the unique N-terminal portion of LC1 were employed (Croxtall and Flower, 1994); nonsense oligonucleotides were used as a negative control. Immunocytochemical and Western analyses revealed that the level of LC1 protein was slightly decreased by transfection with LC1 antisense oligonucleotide and that the LC1 up-regulation mediated by Dex in nontransfected cells (Fig. 4A) is also found in LC1 nonsense oligonucleotide-transfected cells (Fig. 4B). Dex, however, failed to significantly enhance LC1 expression in LC1 antisense oligonucleotide-transfected cells (Fig. 4B).

These studies set the bases for addressing the question of whether Dex-mediated LC1 up-regulation is responsible for the effects caused by Dex in cells exposed to peroxynitrite. The results illustrated in Fig. 4C indicate similar levels of AA release in LC1 antisense and nonsense oligonucleotide-transfected cells exposed to peroxynitrite (100 μ M). Interestingly, however, the Dex-mediated suppression of peroxynitrite-induced AA release previously observed in nontransfected cells was once again identified in LC1 nonsense oligonucleotide-transfected cells but not in LC1 antisense oligonucleotide-transfected cells. The lack of LC1 up-regulation was indeed associated with the inability of dex to impair the peroxynitrite-dependent AA release. Further studies revealed that 100 μ M peroxynitrite was not toxic for LC1 antisense or nonsense oligonucleotide-transfected cells, except under conditions in which the cells were also exposed to AACOCF₃. Dex pretreatment, however, on the one hand promoted an AACOCF₃-insensitive toxicity in LC1 nonsense oligonucleotide-transfected cells and, on the other hand, caused toxicity in LC1 antisense oligonucleotide-transfected cells only in the presence of AACOCF₃ (Fig. 4D). Similar experiments using increasing concentrations of peroxynitrite showed that pre-exposure to Dex increased peroxynitrite toxicity in LC1 nonsense oligonucleotide-transfected cells but had no effect in LC1 antisense oligonucleotide-transfected cells (Fig. 4E).

These results therefore indicate that enhanced expression of LC1 plays a pivotal role in the mechanism whereby Dex promotes cPLA₂ inhibition and enhances the lethal response evoked by peroxynitrite. Coherent with this inference is the observation that exogenous AA afforded cytoprotection under all of the above conditions (e.g., LC1 antisense or nonsense oligonucleotide-transfected cells with or without pre-exposure to Dex).

Discussion

The results reported in this study indicate that Dex enhances the sensitivity of cultured U937 cells to peroxynitrite-induced toxicity. The effects of Dex were time- (Fig. 1A) and concentration- (Fig. 1B) dependent and blocked by RU 486 (Fig. 1D), a GC receptor antagonist (Yang et al., 1996; Mahajan and London, 1997). Additional evidence that RU 486 blocks the effects of Dex is discussed below. It is important to note that the concentrations of Dex and RU 486 employed in the above experiments are considerably higher than those

TABLE 2

Effect of a phospholipase A₂ inhibitor on the lethal response mediated by otherwise nontoxic concentrations of peroxynitrite in cells pre-exposed to increasing concentrations of dex

Cells were exposed for 48 h to increasing concentrations of Dex, treated for 5 min with 100 μ M peroxynitrite, and then postincubated for a further 55 min in the absence or presence of 50 μ M AACOCF₃. Cells were then analyzed for cytotoxicity. Results represent the means \pm S.E.M. from three separate experiments

Trypan Blue Negative Cells		
Pretreatment	Treatment	
	Peroxyntirite	Peroxyntirite + AACOCF ₃
% of control		
5 μ M Dex	94.1 \pm 2.3	50.3 \pm 2.1
10 μ M Dex	91.6 \pm 2.5	57.3 \pm 2.2
15 μ M Dex	81.5 \pm 3.6	54.3 \pm 3.9
15 μ M Dex	77.3 \pm 4.2	53.3 \pm 2.9
20 μ M Dex	55.9 \pm 3.3	51.2 \pm 3.4

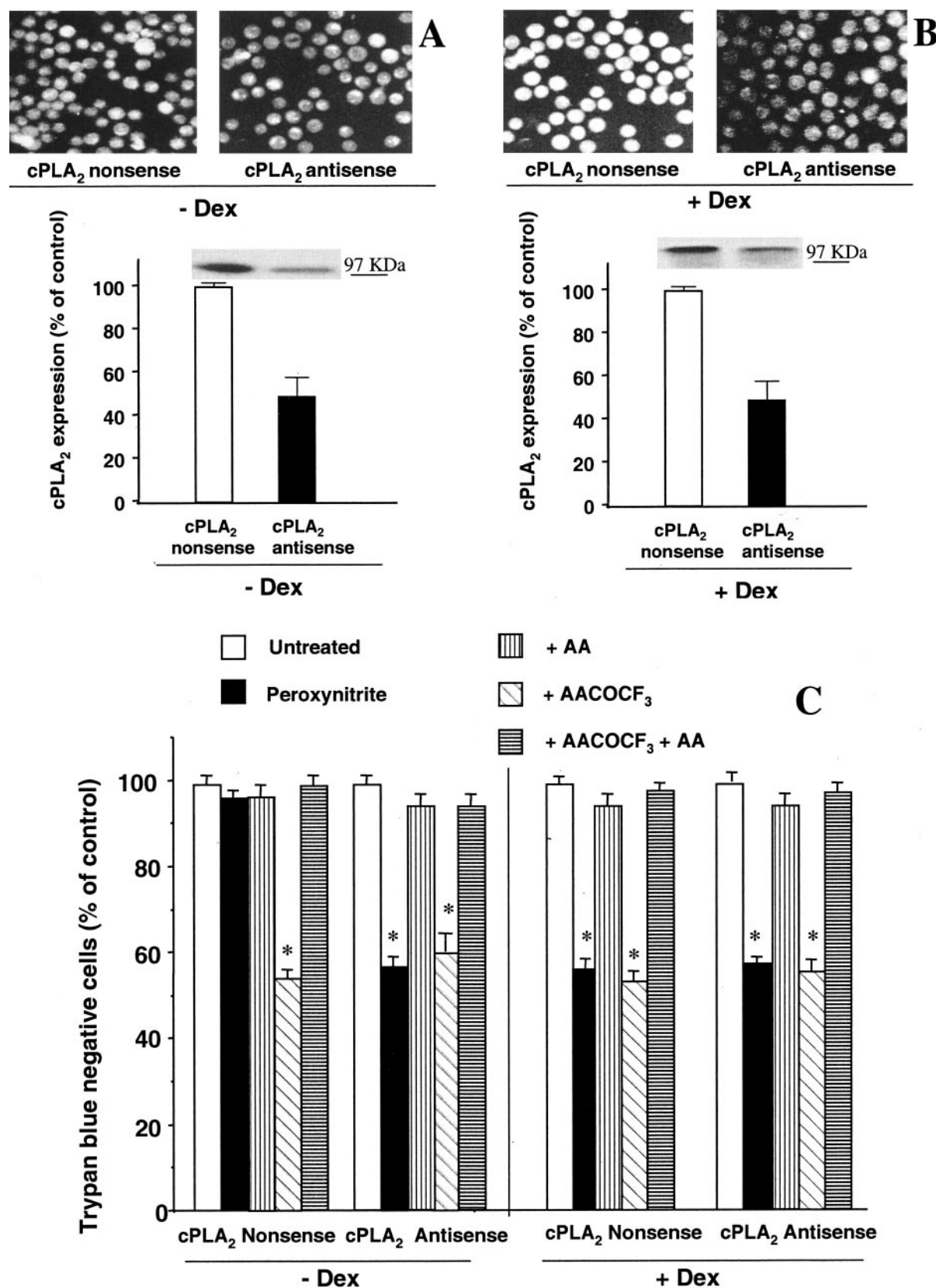


Fig. 3. Dex fails to enhance toxicity mediated by peroxynitrite in cPLA₂ antisense oligonucleotide-transfected cells. A–B, cells were exposed for 6 h in FBS-free medium to 10 μ M phosphorothioate-modified cPLA₂ nonsense or antisense oligonucleotides, allowed to incubate for 48 h in 5% FBS-containing medium in the absence (A) or presence (B) of 20 μ M Dex, and finally processed for immunocytochemical and Western blot analysis using a monoclonal antibody against human cPLA₂. The results shown are representative of three separate experiments. cPLA₂ protein in the blots was quantified by densitometric analysis. C, cPLA₂ nonsense or antisense oligonucleotide-transfected cells were exposed for 48 h to 0 or 20 μ M Dex and subsequently treated as detailed in the legend to Fig. 2C. Cells were then analyzed for cytotoxicity. Results represent the means \pm S.E.M. from three-five separate experiments. *, $p < 0.001$ compared with cPLA₂ nonsense or antisense oligonucleotide-transfected cells pretreated with 0 or 20 μ M Dex.

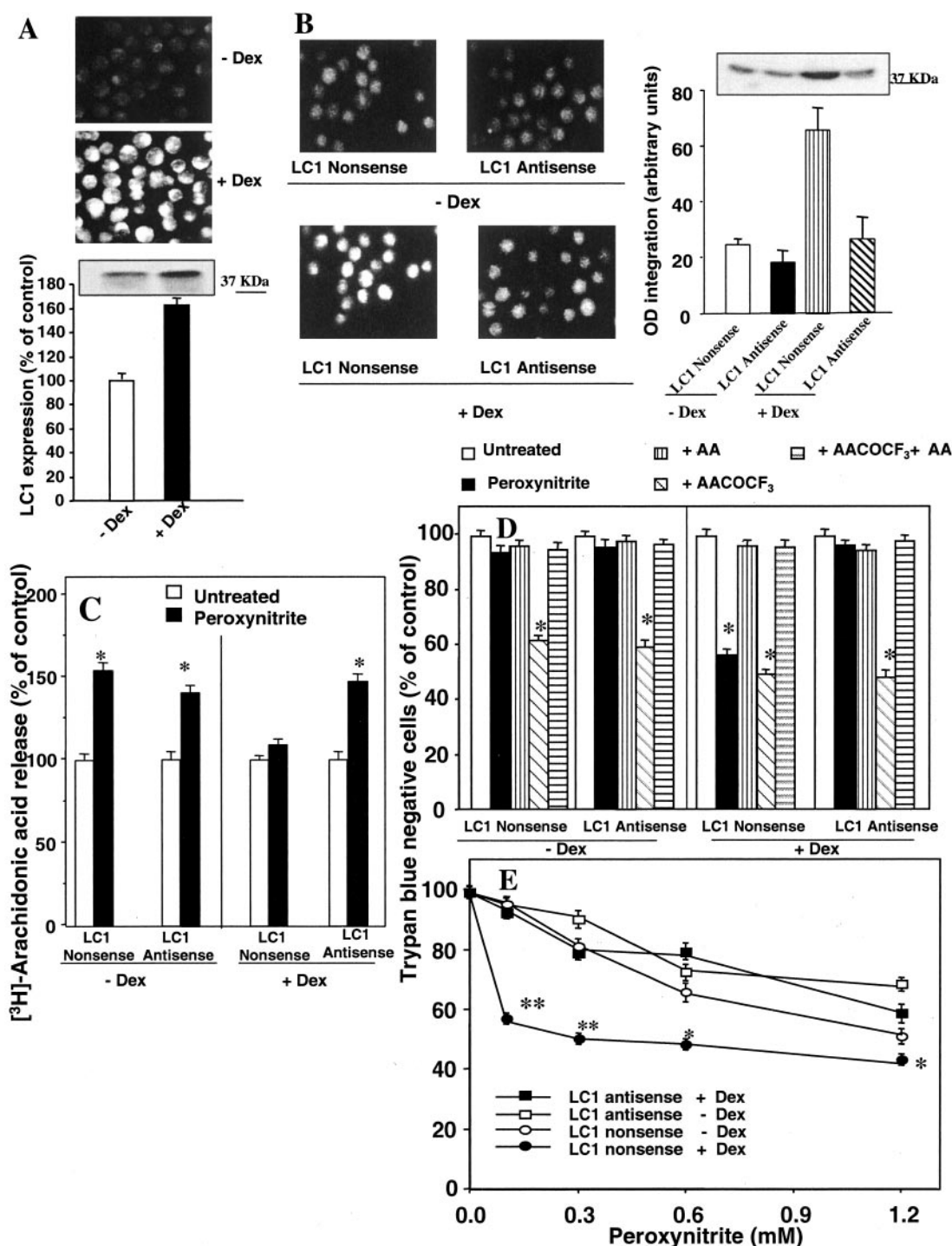


Fig. 4. Transfection with LC1 antisense oligonucleotide abolishes the effects of Dex on peroxynitrite-induced toxicity. **A**, U937 cells were exposed for 48 h to 0 or 20 μ M Dex and then processed for immunocytochemical and Western blot analysis using a monoclonal antibody against human LC1. The results shown are representative of three separate experiments. LC1 expression in the blots was quantified by densitometric analysis. **B**, Cells were treated for 6 h in FBS-free medium with 20 μ M phosphorothioate-modified LC1 nonsense or antisense oligonucleotides, allowed to incubate for 48 h in 5% FBS containing medium in the absence or presence of Dex, and then processed for immunocytochemical and Western blot analysis using a monoclonal antibody against human LC1. The results shown are representative of three separate experiments. The relative amount of LC1 protein was quantified by densitometric analysis expressed as optical density integration. **C**, LC1 nonsense and antisense oligonucleotide-transfected cells were exposed for 48 h to 0 or 20 μ M peroxynitrite. After treatments, [³H]AA release was quantified as described under *Materials and Methods*. Results represent the means \pm S.E.M. from four separate experiments. *, $p < 0.001$ compared with untreated LC1 nonsense or antisense oligonucleotide-transfected cells pretreated with 0 or 20 μ M Dex. **D**, LC1 nonsense or antisense oligonucleotide-transfected cells were exposed for 48 h to 0 or 20 μ M Dex and subsequently treated as detailed in the legend to Fig. 2C. Cells were then analyzed for cytotoxicity. Results represent the means \pm S.E.M. from three to five separate experiments. *, $p < 0.001$ compared with untreated LC1 nonsense or antisense oligonucleotide-transfected cells pretreated with 0 or 20 μ M Dex. **E**, LC1 nonsense or antisense oligonucleotide-transfected cells were exposed for 48 h to 0 or 20 μ M Dex and subsequently treated with increasing concentrations of peroxynitrite as described in the legend to Fig. 1A. Cells were then analyzed for cytotoxicity. Results represent the means \pm S.E.M. from five separate experiments. *, $p < 0.001$; **, $p < 0.0001$ compared with nonsense oligonucleotide-transfected cells exposed to peroxynitrite.

previously used with different cell lines (Yao et al., 1999), including U937 cells (Bienkowski et al., 1989). However, other studies using U937 cells also employed micromolar concentrations of Dex (Wu et al., 2000). The attenuated hormone responsiveness is a likely consequence of reduced receptor protein availability, which may either represent a feature of the specific U937 cell clone used in this study or be generated by homologous down-regulation (e.g., presence of the cognate ligand in the serum). Indeed, this event was described previously in various cell types, including U937 cells (Fukawa et al., 1994). It therefore seems that the action of the synthetic GC is at the transcriptional level.

There are remarkable similarities among the enhancing effects on peroxynitrite-induced U937 cell death mediated by Dex and by *bona fide* PLA₂ inhibitors. First, as observed previously with mepacrine, 5,8,11,14-eicosatetraynoic acid, and AACOCF₃ (Tommasini et al., 2002b), Dex produced dramatic effects in cells challenged with nontoxic or marginally toxic concentrations of peroxynitrite but displayed minor effects after exposure to intrinsically toxic levels of the oxidant (Fig. 1C). Second, in analogy with the results obtained using PLA₂ inhibitors (Tommasini et al., 2004), Dex did not enhance delayed formation of H₂O₂ (Table 1), an event playing a pivotal role in the ensuing lethal response (Tommasini et al., 2002a). Finally, Dex pretreatment does indeed impair the cPLA₂ activity stimulated by peroxynitrite.

We determined that the latter effect is not mediated by inhibition of cPLA₂ expression using both immunocytochemical and Western blot assays (Fig. 2A). Earlier studies (Gewert and Sundler, 1995) showed that GCs reduce cPLA₂ protein expression and activity whereas other studies (Yao et al., 1999) demonstrated inhibition of activity in the absence of effects on cPLA₂ protein expression. Similarly, we found that Dex reduces the release of AA induced by peroxynitrite via a RU 486-sensitive mechanism (Fig. 2B) in the absence of obvious effects on cPLA₂ protein expression (Fig. 2A). Thus, it seems that Dex promotes a receptor-mediated inhibition of PLA₂ activity, which may well explain the molecular bases of the observed effects on peroxynitrite toxicity.

The first clue in this direction was provided by studies showing that AACOCF₃, MAFP, and Dex promote similar levels of toxicity in cells treated with an otherwise sublethal concentration of peroxynitrite. AACOCF₃, however, did not further enhance toxicity in cells pretreated with 20 μ M Dex (Fig. 2C), and the same lethal response was obtained in cells pre-exposed to suboptimal concentrations of Dex (Table 2). These results strongly suggest the involvement of identical targets in the action of a PLA₂ inhibitor or Dex. That inhibition of PLA₂ may be critically involved in both conditions is strongly suggested by the observation that nanomolar levels of AA prevent toxicity in PLA₂ inhibitor- as well as Dex-supplemented cells (Fig. 2C).

The outcome of experiments using cPLA₂ antisense oligonucleotide-transfected cells confirms the notion that inhibition of PLA₂ activity plays a pivotal role in the mechanism whereby Dex enhances peroxynitrite toxicity. Indeed, although showing a robust decrease in cPLA₂ immunoreactivity (Fig. 3A), these cells—regardless of whether they were pretreated with Dex—displayed a parallel enhanced sensitivity to peroxynitrite toxicity, unaffected by AACOCF₃, but prevented by AA (Fig. 3C). cPLA₂ nonsense oligonucleotide-transfected cells displayed a cPLA₂ immunoreactivity iden-

tical to that detected in nontransfected cells (not shown) and also produced identical results in toxicity studies.

The experimental results discussed below are consistent with the notion that the effects of Dex leading to inhibition of cPLA₂ and increased sensitivity to peroxynitrite toxicity are the consequence of enhanced LC1 protein expression. LC1 expression, as observed by others (Wu et al., 2000), can be easily monitored using both immunocytochemical and Western blot assays in untreated U937 cells (Fig. 4A). Dex, as previously shown in monocytes and macrophages (Ambrose et al., 1992; Mancuso et al., 1995), greatly enhanced LC1 synthesis (Fig. 4A); this response was prevented by transfection with LC1 antisense oligonucleotides but not by LC1 nonsense oligonucleotides (Fig. 4B). Consistent with a role of LC1 up-regulation in the Dex-mediated enhancement of peroxynitrite-induced U937 cell death was the observation that LC1 down-regulation completely abolished the effects of Dex on both AA release (Fig. 4C) and toxicity (Fig. 4, D and E). Indeed, unlike LC1 nonsense oligonucleotide-transfected cells, LC1 antisense oligonucleotide-transfected cells grown in the presence of Dex were not killed by peroxynitrite. Toxicity was observed upon PLA₂ inhibitor supplementation, and this response was sensitive to exogenous AA. Thus, it seems that enhanced LC1 expression is a critical event in the Dex-induced lethal response mediated by otherwise nontoxic concentrations of peroxynitrite. In the absence of Dex, pre-exposure toxicity was observed, in both LC1 antisense and nonsense oligonucleotide-transfected cells, only in the presence of the PLA₂ inhibitor, and AA supplementation abolished it. These results can be explained by the observation that LC1 antisense oligonucleotides produced minor effects on constitutive levels of LC1 (Fig. 4B). Similar findings were obtained by Taylor et al. (1997), suggesting that the LC1 protein has a relatively long half-life. Indeed, the ratio of protein to mRNA is quite high in U937 cells (Wu et al., 2000).

We noted previously that the AA-dependent cytoprotective signaling described in U937 cells (Tommasini et al., 2002b), a promonocytic cell line, is of potential importance for survival of peroxynitrite-producing cells, such as monocytes or macrophages. It is therefore possible that an additional mechanism whereby GCs mediate their anti-inflammatory/immunosuppressive effects involves enhanced sensitivity of these cells to peroxynitrite. Interestingly, monocytes in culture rapidly undergo apoptosis unless stimulated by various pro-inflammatory cytokines (Mangan and Wahl, 1991); anti-inflammatory cytokines (Estaquier and Ameisen, 1997), as well as prolonged exposure to GCs, promote monocyte apoptosis (Schmidt et al., 1999). It seems therefore that specific products of the inflammatory response trigger survival mechanisms protecting monocytes against various toxic stimuli. Consequently, GCs either directly impair monocyte survival or, as suggested by this study, enhance the sensitivity of monocytes to the toxicity mediated peroxynitrite.

The above information emphasizes the specificity of the response of cells belonging to the monocyte lineage to the toxicity mediated by reactive species. A wealth of experimental evidence indeed suggests that cPLA₂ activation plays a pivotal role in eliciting tissue damage evoked by toxic species generated in the inflammatory process. These include H₂O₂ (Sapirstein et al., 1996), organic hydroperoxides (Martin et al., 2001), tumor necrosis factor- α (Wu et al., 1998), and pharmacological inhibition of PLA₂-elicited cytoprotection.

Consequently, GCs are expected to promote cytoprotection, an event that was observed in mouse fibroblasts exposed to tumor necrosis factor- α (Pagliacci et al., 1993) or in a rat intestinal epithelial cell line exposed to an oxidative stress (Urayama et al., 1998).

In conclusion, the results presented in this study indicate that Dex promotes U937 cell death after exposure to otherwise nontoxic concentrations of peroxynitrite. It seems that Dex acts by selectively inhibiting the AA-dependent cytoprotective signaling pathway via up-regulation of LC1, a cPLA₂ inhibitory protein. This study may thus contribute to the definition of the molecular mechanisms underlying the therapeutic action of GCs.

References

- Ackermann EJ, Conde-Freixas K, and Dennis EA (1995) Inhibition of macrophage Ca²⁺-independent phospholipase A₂ by bromoenol lactone and trifluoromethyl ketones. *J Biol Chem* **270**:445–450.
- Ambrose MP, Bahns CL, and Hunninghake GW (1992) Lipocortin I production by human alveolar macrophages. *Am J Respir Cell Mol Biol* **6**:17–21.
- Barnes PJ and Adcock I (1993) Anti-inflammatory actions of steroids: molecular mechanism. *Trends Pharmacol Sci* **14**:436–441.
- Bienkowski MJ, Petro AM, and Robinson CJ (1989) Inhibition of thromboxane A synthesis in U937 cells by glucocorticoids. *J Biol Chem* **264**:6536–6544.
- Boumpas DT, Chrousos GP, Wilder RL, Cupps TR, and Balow JE (1993) Glucocorticoid therapy for immune-mediated diseases: basic and clinical correlates. *Ann Intern Med* **119**:1198–1208.
- Croxtall JD and Flower RJ (1994) Antisense oligonucleotides to human lipocortin-1 inhibit glucocorticoid-induced inhibition of A549 cell growth and eicosanoid release. *Biochem Pharmacol* **48**:1729–1734.
- Deutsch DG, Omeir R, Arreaza G, Salehani D, Prestwich GD, Huang Z, and Howlett A (1997) Methyl arachidonyl fluorophosphonate: a potent irreversible inhibitor of anandamide amidase. *Biochem Pharmacol* **53**:255–260.
- Estaquier J and Ameisen JC (1997) A role for T-helper type-1 and type-2 cytokines in the regulation of human monocyte apoptosis. *Blood* **90**:1618–1625.
- Flower RJ (1988) Eleventh Gaddum memorial lecture. Lipocortin and the mechanism of action of the glucocorticoids. *Br J Pharmacol* **94**:987–1015.
- Flower RJ and Rothwell NJ (1994) Lipocortin-1: cellular mechanisms and clinical relevance. *Trends Pharmacol Sci* **15**:71–76.
- Fukawa E, Tanaka H, Makino Y, Hirano F, Akama H, Kawai S, and Makino I (1994) Homologous down-regulation of glucocorticoid receptor down-modulates cellular hormone responsiveness in human histiocytic lymphoma U937 cells. *Endocr J* **41**:623–630.
- Gewert K and Sundler R (1995) Dexamethasone down-regulates the 85 kDa phospholipase A₂ in mouse macrophages and suppresses its activation. *Biochem J* **307**:499–504.
- Griffoni C, Laktionov PP, Rykova EY, Spisni E, Riccio M, Santi S, Bryksin A, Volodko N, Kraft R, Vlassov V and Tomasi V (2001) The Rossmann fold of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a nuclear docking site for antisense oligonucleotides containing a TAAAT motif. *Biochim Biophys Acta* **1530**:32–46.
- Heales SJ, Bolanos JP, Stewart VC, Brookes PS, Land JM, and Clark JB (1999) Nitric oxide, mitochondria and neurological disease. *Biochim Biophys Acta* **1410**:215–228.
- Hong SL and Levine L (1976) Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory corticosteroids. *Proc Natl Acad Sci USA* **73**:1730–1734.
- Hughes MN (1999) Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. *Biochim Biophys Acta* **1411**:263–272.
- Kim WS, Rhee HJ, KO J, Kim YJ, Kim HG, Yang MJ, Choi EC and Na DS (2001) Inhibition of cytosolic phospholipase A₂ by annexin I. *J Biol Chem* **276**:15712–15719.
- Lio YC, Reynolds LJ, Balsinde J, and Dennis EA (1996) Irreversible inhibition of Ca²⁺-independent phospholipase A₂ by methyl arachidonyl fluorophosphonate. *Biochim Biophys Acta* **1302**:55–60.
- Mahajan DK and London SN (1997) Mifepristone (RU 486): a review. *Fertil Steril* **68**:967–976.
- Mancuso F, Flower RJ, and Perretti M (1995) Leukocyte transmigration, but not rolling or adhesion, is selectively inhibited by dexamethasone in the hamster post-capillary venule. Involvement of endogenous lipocortin 1. *J Immunol* **155**:377–386.
- Mangan DF and Wahl SM (1991) Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. *J Immunol* **147**:3408–3412.
- Martin C, Martinez R, Navarro R, Ruiz-Sanz JI, Lacort M, and Ruiz-Larrea MB (2001) tert-Butyl hydroperoxide-induced lipid signaling in hepatocytes: involvement of glutathione and free radicals. *Biochem Pharmacol* **62**:705–712.
- Moncada S, Palmer RM, and Higgs EA (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* **43**:109–142.
- Pagliacci MC, Migliorati G, Smacchia M, Grignani F, Riccardi C, and Nicoletti I (1993) Cellular stress and glucocorticoid hormones protect L929 mouse fibroblasts from tumor necrosis factor alpha cytotoxicity. *J Endocrinol Invest* **16**:591–599.
- Radi R, Beckman JS, Bush KM, and Freeman BA (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* **266**:4244–4250.
- Salgo MG, Bermudez E, Squadrito GL, and Pryor WA (1995) Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes. *Arch Biochem Biophys* **322**:500–505.
- Sapirstein A, Spech RA, Witzgall R, and Bonventre JV (1996) Cytosolic phospholipase A₂ (cPLA₂), but not secretory PLA₂, potentiates hydrogen peroxide cytotoxicity in kidney epithelial cells. *J Biol Chem* **271**:21505–21513.
- Schmidt M, Pauels HG, Luger N, Luger A, Domschke W, and Kucharzik T (1999) Glucocorticoids induce apoptosis in human monocytes: potential role of IL-1 beta. *J Immunol* **163**:3484–3490.
- Sestili P, Tommasini I, and Cantoni O (2001) Peroxynitrite promotes mitochondrial permeability transition-dependent rapid U937 cell necrosis: survivors proliferate with kinetics superimposable on those of untreated cells. *Free Radic Res* **34**:513–527.
- Street IP, Lin HK, Laliberte F, Ghomashchi F, Wang Z, Perrier H, Tremblay NM, Huang Z, Weech PK, and Gelb MH (1993) Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A₂. *Biochem* **32**:5935–5940.
- Szabó C (1996) The pathophysiological role of peroxynitrite in shock, inflammation and ischemia-reperfusion injury. *Shock* **6**:79–88.
- Taylor AD, Christian HC, Morris JF, Flower RJ, and Buckingham JC (1997) An antisense oligodeoxynucleotide to lipocortin 1 reverses the inhibitory actions of dexamethasone on the release of adrenocorticotropin from rat pituitary tissue in vitro. *Endocrinol* **138**:2909–2918.
- Tommasini I, Sestili P, and Cantoni O (2002a) Delayed formation of hydrogen peroxide mediates the lethal response evoked by peroxynitrite in U937 cells. *Mol Pharmacol* **61**:870–878.
- Tommasini I, Sestili P, Guidarelli A, and Cantoni O (2002b) Peroxynitrite stimulates the activity of cytosolic phospholipase A₂ in U937 cells: the extent of arachidonic acid formation regulates the balance between cell survival or death. *Cell Death Differ* **9**:1368–1376.
- Tommasini I, Sestili P, Guidarelli A, and Cantoni O (2004) Hydrogen peroxide generated at the level of mitochondria in response to peroxynitrite promotes U937 cell death via inhibition of the cytoprotective signalling mediated by cytosolic phospholipase A₂. *Cell Death Differ*, in press.
- Urayama S, Musch MW, Retsky J, Madonna MB, Straus D, and Chang EB (1998) Dexamethasone protection of rat intestinal epithelial cells against oxidant injury is mediated by induction of heat shock protein 72. *J Clin Invest* **102**:1860–1865.
- Webster DA (1975) The formation of hydrogen peroxide during the oxidation of reduced nicotinamide adenine dinucleotide by cytochrome c from *Vitreoscilla*. *J Biol Chem* **250**:4955–4958.
- Wu YL, Jiang XR, Lillington DM, Newland AC, and Kelsey SM (2000) Up-regulation of lipocortin 1 inhibits tumour necrosis factor-induced apoptosis in human leukaemic cells: a possible mechanism of resistance to immune surveillance. *Br J Haematol* **111**:807–816.
- Wu YL, Jiang XR, Newland AC, and Kelsey SM (1998) Failure to activate cytosolic phospholipase A₂ causes TNF resistance in human leukaemic cells. *J Immunol* **160**:5929–5935.
- Yang J, Serres C, Baulieu EE, and Jouannet P (1996) RU 486 inhibits penetration of human spermatozoa into zona-free hamster oocytes. *Int J Androl* **19**:61–66.
- Yao XL, Cowan MJ, Gladwin MT, Lawrence MM, Angus CW, and Shelhamer JH (1999) Dexamethasone alters arachidonate release from human epithelial cells by induction of p11 protein synthesis and inhibition of phospholipase A₂ activity. *J Biol Chem* **274**:17202–17208.

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