Molecular Pharmacology Fast Forward. Published on February 2, 2005 as doi:10.1124/mol.104.009498

"MOL (Manuscript # 9498)"

U937 cell necrosis mediated by peroxynitrite is not caused by depletion of ATP and is prevented by arachidonate via an ATP-dependent mechanism

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Molecular Pharmacology Fast Forward. Published on February 2, 2005 as DOI: 10.1124/mol.104.009498
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"MOL (Manuscript # 9498)"

Running title: Role of ATP in U937 cell necrosis induced by peroxynitrite

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Number of text pages: 11
Number of Tables: 1
Number of Figures: 3
Number of References: 27
Number of words in the Abstract: 217
Number of words in the Introduction: 702
Number of words in the Result: 1029
Number of words in the Discussion: 1332

Abbreviations used: $AACOCF_3$, arachidonyl trifluoromethyl ketone; $cPLA_2$, cytosolic phospholipase A_2; DHR, dihydrorhodamine 123, ETYA, 5,8,11,14-eicosatetraynoic acid, GSH, reduced glutathione; MPT, mitochondrial permeability transition.

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ABSTRACT

Exposure of U937 cells to an otherwise non-toxic concentration of peroxynitrite promotes a rapid necrotic response in the presence of pharmacological inhibitors of phospholipase A_2. A 12 fold higher concentration of the oxidant, in the absence of additional treatments, caused remarkably greater DNA single-strand breakage, delayed formation of H_2O_2 and depletion of reduced glutathione but an identical level of toxicity. Cell death was in both circumstances prevented by nanomolar levels of arachidonic acid, or by cyclosporin A, via mechanisms unrelated to elimination of the above effects and causally-linked to prevention of mitochondrial permeability transition. Treatment with the high dose of peroxynitrite for 30 min caused an about 40% decline in ATP, both in the absence and presence of arachidonate, whereas only a small, arachidonic acid-sensitive, reduction of the ATP pool was detected in cells treated with the low dose of peroxynitrite and the phospholipase A_2 inhibitor. ATP-predepleted cells, however, were hypersensitive to peroxynitrite and, under these conditions, toxicity was not prevented by arachidonate. The above findings were reproduced in another promonocytic cell line, THP-1 cells. It is concluded that the rapid necrotic response triggered by peroxynitrite in monocytes is mediated by a regulated process -and not by ATP depletion- associated with reduced arachidonate availability. Supplementation of exogenous arachidonic acid always rescued cells via an ATP-dependent survival pathway.

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Cell death, a process of paramount importance in a variety of physiological and pathological processes, is mediated by different mechanisms in which apoptosis and necrosis represent the two extremes of a continuum (Leist and Nicotera, 1997). Since apoptosis requires energy-dependent events, the choice among these different modes of cell death may be determined on the basis of the ATP availability (Nicotera and Melino, 2004). In the presence of ATP, a toxic treatment leads to apoptosis whereas, in its absence, cell death would take place via a passive mechanism, i.e. necrosis. In support of this notion are the following observations: i) toxicity paradigms causing necrosis are associated with depletion of the ATP pool (Lelli et al., 1998), which is however well preserved using lower concentrations of the toxic agent, under conditions in which apoptosis, but not necrosis, is concomitantly induced; ii) depletion of ATP, early in the apoptotic process, switches the predominant form of cell death from apoptosis to necrosis (Leist et al., 1997); activation of poly(ADP-ribose)polymerase leads to ATP depletion and necrosis whereas inhibition of its activity prevents both ATP depletion and necrosis and eventually switches cells into apoptosis (Ha and Snyder, 1999). Thus, the above results define an important role of ATP in the regulation of cell death and imply that necrosis takes place in damaged cells unable to perform the highly energy demanding processes involved in apoptosis.

However, necrosis may also represent the primary mode of cell death induced by an otherwise apoptotic treatment, when parameters other than ATP are affected, e.g. when caspases are inhibited (Hirsch et al., 1997; Nicotera and Melino, 2004). Furthermore, it is becoming increasingly clear that necrosis may also represent a primary mode of cell death in a variety of physiopathological conditions (Proskuyakov et al., 2003), and several early events described in the apoptotic process are also critical in the regulation of necrosis (Leist and Nicotera, 1997; Proskuyakov et al., 2003). A good example is given by mitochondrial permeability transition (MPT), which may trigger both apoptosis and necrosis (Kroemer et al., 1998) via mechanisms that are similarly regulated by proteins of the Bcl-2 family (Single et al., 2001; Proskuyakov et al., 2003). Although severe depletion of ATP was described in toxicity paradigms resulting in primary necrosis (Barros et al., 2001; Proskuyakov et al., 2003), whether or not ATP depletion does always occur and is in fact the most prominent cause of this lethal response, remains to be established. This is an important question to be asked since a general requirement for ATP depletion would demonstrate that necrosis is always a passive response, regardless of whether it is primarily induced or it takes place as a consequence of the inability of the cells to perform the apoptotic process.

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The present study was designed to investigate whether depletion of ATP is needed in primary necrosis resulting from a toxicity paradigm that has been extensively characterized in our laboratory. We reported that exposure of U937 cells to peroxynitrite promotes a MPT-dependent necrosis within minutes, followed by immediate cell lysis (Sestili et al., 2001). An initial event triggered by peroxynitrite, i.e. inhibition of complex III of the mitochondrial respiratory chain, was responsible for the time-dependent formation of H_2O_2 that was essential for the occurrence of cell death (Tommasini et al., 2002a). We also showed that otherwise non-toxic concentrations of peroxynitrite nevertheless commit cells to MPT-dependent necrosis, that is however prevented by a cytoprotective signaling driven by arachidonic acid released by the cytosolic phospholipase A_2 isoform (cPLA_2; Tommasini et al., 2002b; 2004a). We do not know whether arachidonate itself, or some downstream product of the cyclooxygenase or lipoxygenase pathways, is responsible for the survival signaling.

Thus, this toxicity paradigm, while representing a model of "severe primary necrosis" in which extensive ATP depletion may occur, since both peroxyntrite and H_2O_2 potently inhibit the respiratory chain and glycolysis (Souza and Radi, 1998; Hyslop et al., 1998), nevertheless presents some features of a highly regulated event.

The present study demonstrates a requirement for ATP in the above cytoprotective signaling and indicates that the ATP pool is well preserved when the necrotic response, that takes place via a highly regulated mechanism independent of the damage accumulated, is elicited by suppression of the protective signaling.

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Materials and Methods

Chemicals. 5,8,11,14-Eicosatetraynoic acid (ETYA), arachidonic acid, 2-deoxy-D-glucose, catalase, N-acetyl-L-cysteine as well as most of reagent grade chemicals, were obtained from Sigma-Aldrich (Milan, Italy). Arachidonyl trifluoromethyl ketone ($AACOCF_3$), cyclosporin A and dihydrorhodamine 123 (DHR) were purchased from Calbiochem (San Diego, CA, USA), Sandoz A.G. (Bern, Switzerland) and Molecular Probes Europe (Leiden, The Netherlands), respectively.

Cell culture and treatment conditions. U937 or THP-1 cells were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (50 units/ml) and streptomycin (50 µg/ml) (Sera-Lab Ltd., Crawley Down, England), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air- 5% CO_2. Peroxynitrite was synthesised by the reaction of nitrite with acidified H_2O_2, as described in (Radi et al., 1991), with minor modifications (Tommasini et al., 2002a). Treatments were performed in 2 ml of pre-warmed saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose) containing 5 x 10^5 cells.

Viability 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 viable cells were counted with a hemocytometer.

Alkaline halo assay. DNA single-strand breakage was determined using the alkaline halo assay, as described previously (Sestili and Cantoni, 1999). After treatments, the cells were re-suspended at 2.0 x 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-labeled DNA was visualized using a Bio Rad DVC 250 confocal laser microscope (Bio Rad, Richmond, CA, USA) and the resulting images were taken and processed with a Hamamatsu chilled CCD 5985 camera (Hamamatsu Italy S.p.a., Milan, Italy) 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/).

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The level of DNA single-strand breakage was quantified 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.

DHR oxidation and imaging. Peroxynitrite-pretreated cells (3 min) were post-incubated in fresh saline A containing 10 µM DHR (7 min). After treatments, the cells were washed three times, 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., Frederick, MD). Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/ treatment condition/experiment.

ATP determination. Cold 2.5% perchloric acid were added to the cells (10^6 cells/ml). After a 20 min incubation in an ice bath, the solution was removed. Cell extracts were neutralized with K_2CO_3 and the precipitate was removed by centrifugation. The nucleotide-containing supernatants were filtered through 0.22 µm pore microfilters and analyzed for ATP content by reversed-phase HPLC (Stocchi et al., 1985) using a Supelcosil LC-18 column (Supelco, Bellafonte, PA, USA).

Biochemical assays. The assay described by Beutler (1984) was used to measure non-protein sulphydryl levels. Since reduced glutathione (GSH) represents more than 90% of the non-protein-SH, the latter will be referred to as GSH. In brief, cells (4×10^6) were washed three times with saline A and centrifuged; the pellet was then re-suspended with 150 µl of metaphosphoric acid solution [1.67% (v/v) metaphosphoric acid /0.2% EDTA/30% (w/v) NaCl], kept on ice for 5 min and centrifuged at 10,000 g for 5 min . The non-protein thiol content was measured spectrophotometrically in the supernatant, at 412 nm by using 5,5'-dithiobis(2-nitrobenzoic acid) (ε_{412} = 13600 M^{-1} . cm^{-1}). Protein content was assayed as indicated in (Bradford, 1976), with bovine serum albumin standard. Phosphofructokinase activity was measured as described by Beutler (1984).

Statistical analysis. Results 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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Results

The results illustrated in Table 1 confirm our previous findings (Sestili et al., 2001) indicating that the loss of viable cells is detectable 15 min after addition of 1.2 mM peroxynitrite, progressively increases for an additional 45 min and is not paralleled by the appearance of non-viable cells (not shown), an indication that cell lysis had indeed taken place. Also consistent with our previous findings (Tommasini et al., 2002b; 2004a) is the observation that an otherwise non-toxic concentration of peroxynitrite (100 μM) causes, upon supplementation of a phospholipase A_2 inhibitor (50 μM $AACOCF_3$), a lethal response identical to that obtained with 1.2 mM peroxynitrite. Once again, toxicity was associated with immediate cell lysis. The effects of $AACOCF_3$ were mimicked by other phospholipase A_2 inhibitors, including ETYA (50 μM, not shown).

In the next set of experiments, the cells were treated as detailed above for 10 min, a time at which toxicity is not as yet apparent, and analyzed for various biochemical parameters. The results illustrated in Fig. 1 indicate that treatment with the high dose of peroxynitrite produced a remarkably greater DNA single-strand breakage (A), delayed formation of H_2O_2 (B) and loss of GSH (C) than treatment of phospholipase A_2 inhibitor-supplemented cells with the otherwise non-toxic concentration of peroxynitrite or with the low concentration of peroxynitrite alone. The latter two conditions failed to elicit a detectable DHR fluorescence response and produced identical effects on DNA strand scission. Depletion of GSH, however, was more pronounced in cells supplemented with the phospholipase A_2 inhibitor. ETYA was used as a phospholipase A_2 inhibitor, since $AACOCF_3$ is intrinsically fluorescent and interferes with the assays measuring DNA cleavage and oxidation of DHR. Taken together, the above results indicate that the same level of cell death mediated by two different toxicity paradigms is associated with an early accumulation of remarkably different levels of damage.

A very low concentration of exogenous arachidonic acid (100 nM) prevented toxicity induced in both conditions (Table 1). N-acetyl-L-cysteine (5 mM) or catalase (10 U/ml), however, abolished the lethal response mediated by the intrinsically toxic concentration of peroxynitrite but were ineffective in cells exposed to the otherwise non-toxic concentration of peroxynitrite and $AACOCF_3$. These results confirm our previous findings (Tommasini et al. 2004b) indicating that in both conditions, toxicity arises as a consequence of insufficient arachidonate release due to either pharmacological inhibition of phospholipase A_2 (treatment with the low concentration of peroxynitrite/$AACOCF_3$) or H_2O_2-dependent inhibition of arachidonic acid release (treatment with the high concentration of peroxynitrite).

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The cytoprotective effects of arachidonic acid were mimicked by the MPT inhibitor cyclosporin A (0.5 μM) (Table 1). Neither of the two agents, however, affected the DNA single-strand breakage (A) and delayed formation of H_2O_2 (B) induced by the three different treatments. In contrast arachidonic acid, or cyclosporin A, significantly reduced the extent of GSH depletion (C) observed in phospholipase A_2-inhibited cells exposed to 100 μM peroxynitrite but had hardly any effect in the two remaining conditions. Exposure to 1.2 mM peroxynitrite led to total depletion of cellular GSH. These results further establish the notion that toxicity is not a direct consequence of the overall cellular damage.

We next investigated the effect of the above treatments on the cellular ATP pool. Exposure to 100 μM peroxynitrite did not affect ATP levels (Fig. 2A). Under the same conditions, however, addition of $AACOCF_3$ (Fig. 2B) caused a small reduction in cellular ATP after 15 min of incubation, a time at which many cells are committed to death and only a few dead cells are present since, as previously mentioned, necrotic cells lyse immediately. Identical levels of ATP were found after 30 min. Similar results were obtained by replacing $AACOCF_3$ with ETYA (not shown). When toxicity was prevented by arachidonic acid, or cyclosporin A, the decline in ATP levels was also prevented. Exposure to 1.2 mM peroxynitrite caused a greater decline in ATP content insensitive to either arachidonic acid or cyclosporin A (Fig. 2C). This response was time dependent and, after 30 min, a 40% reduction in the ATP content was detected. These results indicate that, under the above treatment conditions, the ATP pool is very well preserved.

U937 cells, as most tumor cells, meet their high energy demand for cell proliferation by glycolysis (Brand and Hermfisse, 1997). Indeed a 60 min exposure to the complex I inhibitor rotenone (0.5 μM) only reduced ATP content by 12%, suggesting a minor contribution of oxidative phosphorylation in the overall ATP synthesis. This is in contrast with the about 90% decrease mediated by 5 mM 2-deoxy-D-glucose, a glucose analogue which competitively inhibits cellular uptake and utilization of glucose. Thus, the decline in ATP content mediated by 1.2 mM peroxynitrite is only in part due to inhibition of electron transport. Inhibition of glycolysis is therefore more likely to mediate the decline in ATP levels, an inference supported by the reduction in phosphofructokinase activity (30%, 0.046 U/mg of protein in untreated cells) observed 10 min after addition of peroxynitrite. Finally, accelerated ATP hydrolysis is expected to take place as a consequence of the extensive damage induced by peroxynitrite.

We next investigated the toxicity of peroxynitrite in ATP-predepleted cells and asked the question of whether arachidonic acid could promote survival also under these treatment

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conditions. For this purpose, the cells were incubated for 60 min in a glucose-free saline supplemented with 2-deoxy-D-glucose that, as previously mentioned, caused a 90% reduction in the ATP pool. We found that ATP depletion markedly sensitizes cells to peroxynitrite since a lethal response was observed after exposure to as low as 10-100 μM peroxynitrite (Fig. 2D). Interestingly, exogenous arachidonic acid did not prevent toxicity in ATP-predepleted cells, indicating that the ability of the lipid messenger to promote survival is restricted to conditions associated with ATP availability.

The final set of experiments was performed with the aim of reproducing the most relevant findings in THP-1 cells, an additional promonocytic cell line. As illustrated in Fig. 3, the arachidonate-dependent cytoprotective signaling is not restricted to U937 cells (A), the ATP pool is well preserved also in THP-1 cells (B) and pre-depletion of ATP in the latter cell type, as previously observed in U937 cells, abolishes the cytoprotective effects of arachidonic acid.

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Discussion

The present study employed two different toxicity paradigms (i.e. a high dose of peroxynitrite or an otherwise non-toxic concentration of peroxynitrite associated with a phospholipase A_2 inhibitor) to show that the rapid necrotic response that in both conditions takes place as a consequence of the inhibition of a survival signaling is not dependent on ATP depletion.

We first provided evidence indicating that MPT-dependent necrosis is not a direct consequence of the overall cellular damage. Indeed the two toxicity paradigms were associated with the accumulation of different levels of DNA single-strand breakage and H_2O_2 as well as with different depletion of GSH (Fig. 1). Furthermore, addition of exogenous arachidonic acid, or cyclosporin A, was on the one hand invariably followed by prevention of toxicity whereas, on the other hand, survival was not always paralleled by a reduction of the induced damage. By comparing the results obtained using the low dose of peroxynitrite alone, or associated with the phospholipase A_2 inhibitor, the same conclusion can be reached. The non-toxic and the toxic treatments produced identical levels of DNA strand scission and failed to promote detectable delayed formation of H_2O_2. The decline in GSH was however greater in the second condition. The observation that arachidonic acid, or cyclosporin A, abolished the enhancing effects mediated by the phospholipase A_2 inhibitor on GSH depletion, with no effect on GSH decline mediated by the non-toxic dose of peroxynitrite, strongly suggests a role for events downstream to MPT in causing a further decrease in the GSH pool. The high concentration of peroxynitrite led to total depletion of cellular GSH, insensitive to arachidonic acid or cyclosporin A. This would indicate that oxidation of cellular thiols is directly mediated by peroxynitrite.

Consistent with the notion that toxicity is not a direct consequence of the overall cellular damage were also the results reported in a recent study from our laboratory (Tommasini et al., 2004b). We showed that formation of H_2O_2, critical for cell death induction by high concentrations of peroxynitrite (Tommasini et al., 2002a), was in fact a dispensable event under conditions in which $cPLA_2$ was pharmacologically inhibited or genetically depleted. The effect of H_2O_2 was indeed upstream to arachidonate and restricted to inhibition of arachidonic acid release. Supplementation of exogenous arachidonate was therefore able to promote full survival even under conditions associated with extensive H_2O_2-dependent damage (e.g. in the presence of *bona fide* complex III inhibitors, which remarkably enhance formation of H_2O_2, or upon supplementation of high levels of exogenous H_2O_2). These observations therefore readily explain our findings that catalase or N-acetyl-L-cysteine

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prevented toxicity mediated by 1.2 mM peroxynitrite but not that mediated by 100 μM peroxynitrite in phospholipase A_2 inhibitor-supplemented cells (Table 1).

The fact that these two conditions produced identical levels of toxicity indicates that the specific lesion(s) committing cells to MPT is already induced by the low dose of peroxynitrite. An effect of peroxynitrite on the adenine nucleotide translocator was recently identified in Kroemer's laboratory (Vieira et al., 2001) and this event, in conjunction with alterations in the redox state (Costantini et al., 1996), may well be involved in the commitment to MPT, critically regulated by the arachidonate-dependent cytoprotective signaling. Indeed, using the calcein assay we produced direct evidence of MPT induction, prevented by cyclosporin A as well as by arachidonate (Tommasini et al., 2004a).

The above findings indicate that necrosis takes place as a consequence of a regulated process, an inference supported by the observation that the ATP pool was well preserved during drug exposure (Fig. 2A-C). Indeed, a 15-30 min treatment with 100 μM peroxynitrite/$AACOCF_3$ alone caused a very small reduction of cellular ATP. Importantly, at 15 min a significant proportion of the cells was committed to death whereas, at 30 min, the cell population was mainly represented by cells surviving treatment with peroxynitirte. The observation that the small decline in ATP was prevented by arachidonic acid would suggest that this response does not precede, but rather follows, MPT. Consistently, MPT inhibition by cyclosporin A also prevented the ATP decline. These results clearly demonstrate that cellular ATP is virtually identical in cells committed to death, in survivors or in untreated cells. Exposure to 1.2 mM peroxynitrite caused a greater decrease in ATP content and this response was insensitive to either arachidonic acid or cyclosporin A. This indicates that the severe nitrosative stress imposed by this treatment causes ATP depletion by impairing the machinery of ATP synthesis and/or via accelerated ATP hydrolysis. As a consequence, inhibition of MTP prevents toxicity but not the effects of peroxynitrite on cellular ATP. Hence, cells committed to death and surviving cells are equally affected in their machinery to produce/hydrolyse ATP.

We confirmed the notion that U937 cells, as most tumor cells, mainly produce their ATP by glycolysis. Indeed, 2-deoxy-D-glucose virtually depleted the ATP pool that was however only marginally affected by the respiratory chain inhibitor rotenone. Thus, the significant decline in ATP content mediated by the high dose of peroxynitrite is more likely to depend on inhibition of glycolysis than of electron transport. This conclusion is supported by the observed reduction in the activity of phosphofructokinase, a rate-limiting enzyme of glycolysis (Stryer, 1995). In addition, H_2O_2 may also contribute to inhibition of glycolysis

(Hyslop et al., 1988). Finally, as mentioned above, accelerated ATP hydrolysis is a very likely cause of ATP depletion since the cells are heavily injured by peroxynitrite. Although the ATP synthetic machinery appears to be partially compromised, this event is not expected to lead to toxicity since identical ATP levels were found in cells rescued with arachidonic acid or cyclosporin A. Furthermore, we previously showed that prevention of MPT does not simply delay toxicity, but rather promotes full survival and allows cells to proliferate as untreated cells (Sestili et al., 2001). Hence, inhibition of ATP synthesis must be reversible.

These results are consistent with the notion that ATP depletion is not the cause of necrotic cell death. The execution of the arachidonate-dependent signaling, however, requires ATP. Important indications in this direction were provided by the observations that otherwise non-toxic levels of peroxynitrite kill cells previously depleted in ATP and that arachidonic acid fails to mediate cytoprotection under these conditions (Fig. 2D).

The arachidonate-dependent cytoprotective signaling is not restricted to U937 cells, but is rather a general feature of cells belonging to the monocyte/macrophage lineage (Tommasini et al., 2004c). We confirmed the results obtained in U937 cells using an additional promonocytic cell line, i.e. THP-1 cells (Fig. 3). Hence, these cells may cope with peroxyntrite using both endogenous and exogenous arachidonic acid to trigger an energy-dependent survival signaling pathway.

In conclusion, the results presented in this study further our knowledge on the mechanism whereby monocytic cells cope with peroxynitrite and provide important information on the role of ATP in primary necrosis in a selected toxicity paradigm. Previous studies had described necrosis as a sort of an obligatory and passive response in which, regardless of the necrotic insult, the ATP levels rapidly drop to 0, as a consequence of defective ATP synthesis associated with elevated ATP consumption (for a review see ref., Proskuyakov et al., 2003). Cells unable to sustain the activity of ion pumps and other energy demanding functions would therefore rapidly loose their membrane integrity and lyse. This is obviously not the case of U937 (or THP-1) cells challenged with peroxynitrite, which preserve their ATP levels and eventually die by necrosis via a highly regulated mechanism.

Thus, this lethal response may not just represent a cell failure to perform apoptosis. Monocytes sustain the inflammatory response by releasing an array of toxic molecules, including peroxynitrite, and survive in an environment in which other cells die by using stimuli that are detrimental for other cell types. The final physiological response of monocytes would then be a necrotic death, as an extreme strategy to further support the inflammatory response. Thus, necrosis not only takes place via a highly regulated mechanism

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but might also represent a well-defined physiological event with important pathological implications (e.g. conditions in which inflammation is of pathological significance).

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Footnotes

This work was supported by a grant from Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Progetti di Ricerca di Interesse Nazionale (OC).

Molecular Pharmacology Fast Forward. Published on February 2, 2005 as DOI: 10.1124/mol.104.009498
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Legends for figures

Fig. 1. DNA single-strand breakage, delayed formation of H_2O_2 and depletion of GSH induced by peroxynitrite. Cells were exposed for 3 min to either 100 μM or 1.2 mM peroxynitrite, centrifuged and then post-incubated for a further 7 min in saline A in the absence or presence (only for cells exposed to 100 μM peroxynitrite) of ETYA (50 μM). In some experiments, arachidonic acid (0.1 μM was also added during the 7 min post-treatment incubation. In other experiments cyclosporin A (0.5 μM) was given to the cultures 5 min prior to peroxynitrite and freshly re-added during the 7 min post-treatment incubation. Cells were then analyzed for DNA single-strand breakage (A), DHR oxidation (B) or GSH content (C). In experiments measuring DHR oxidation, the fluorescent probe was given to the cultures 3 min after peroxynitrite. This prevents direct oxidation of DHR and allows measurement of H_2O_2 that is continuously being formed upon dismutation of superoxides generated via peroxynitrite-dependent inhibition of complex III (Tommasini et al., 2002a). Results represent the mean ± S.E.M. from at least four separate experiments. * $P < 0.05$ and ** $P < 0.001$ as compared to untreated cells or to cells exposed to arachidonic acid, or cyclosporin A, alone; (*) $P < 0.05$ as compared to cells exposed to peroxynitrite, (unpaired *t*-test).

Fig. 2. Toxicity induced by peroxynitrite is not associated with depletion of ATP. A-C, Cells were treated as detailed in the legend to Fig. 1 and then analyzed for their ATP content 12 or 27 min after addition of peroxynitrite. $AACOCF_3$ (50 μM) was used in the place of ETYA. Exposure to the PLA_2 inhibitor, arachidonate or cyclosporin A alone did not affect the ATP content. Results represent the mean ± S.E.M. from four separate experiments. *$P < 0.05$ and **$P < 0.01$ as compared to untreated cells or to cells exposed to arachidonic acid, or cyclosporin A, alone; (*) $P < 0.05$ as compared to cells exposed to peroxynitrite, (unpaired *t*-test). D, Cells were exposed for 60 min in a glucose-free saline to 0 or 5 mM 2-deoxy-D-glucose, treated for a further 60 min with 10-100 μM peroxynitrite, in the absence or presence of arachidonic acid, and finally analyzed for viability . Results represent the mean ± S.E.M. from four separate experiments. * $P < 0.01$ or ** $P < 0.001$ as compared to cells exposed to peroxynitrite (unpaired *t*-test).

Fig. 3. The role of ATP in the lethal response evoked by peroxynitrite in THP-1 cells.
A, THP-1 cells were treated, as indicated in the figure, using the same protocol reported in Table 1, and analyzed for viability. B, The cells were treated, as indicated in the figure, using

the same protocol illustrated in the legend to Fig. 2A-C and analyzed for their ATP content. C, The cells were treated as indicated in the figure, using the same protocol illustrated in the legend Fig. 2D and analyzed for viability. Arachidonate was always used (A and C) at 0.3 μM. Results represent the mean ± S.E.M. from four separate experiments. *P < 0. 01 or ** P < 0.001 as compared to untreated cells (A and B) or to cells exposed to peroxynitrite (C) (unpaired *t*-test).

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TABLE 1

Kinetics of cell death induced by peroxynitrite in the absence or presence of $AACOCF_3$ and cytoprotection by arachidonic acid , cyclosporin A, N-acetyl-L-cysteine or catalase.

Cells were exposed for 3 min to either 100 μM or 1.2 mM peroxynitrite, centrifuged and then post-incubated for a further 57 min in saline A in the absence or presence (only for cells exposed to 100 μM peroxynitrite) of $AACOCF_3$ (50 μM). In some experiments, arachidonic acid (0.1 μM) or catalase (10 U/ml) was also added during the 57 min post-treatment incubation. In other experiments cyclosporin A (0.5 μM) was given to the cultures 5 min prior to peroxynitrite and freshly re-added during the 57 min post-treatment incubation. The last experimental condition involved a 60 min exposure to N-acetyl-L-cysteine (5 mM) prior to treatment with peroxynitrite, as detailed above. After treatments, cells were analyzed for viability using the trypan blue exclusion assay. Results represent the mean ± S.E.M. from at least four separate experiments. * $P < 0.01$ and ** $P < 0.001$ as compared to cells that did not receive peroxynitrite.

Treatment	Trypan blue negative cells (% of control)				
(Time min)	5	10	15	30	60
Arachidonate	97 ± 3.6	96 ± 3.9	98 ± 4.1	95 ± 4.8	97 ± 3.2
Cyclosporin A	95 ± 4.6	98 ± 3.3	96 ± 2.7	99 ± 2.7	95 ± 3.5
$AACOCF_3$	95 ± 3.8	94 ± 2.9	94 ± 4.1	96 ± 4.4	94 ± 3.8
N-acetyl-L-cysteine	99 ± 4.0	97 ± 2.8	95 ± 2.7	94 ± 3.3	93 ± 2.5
Catalase	96 ± 2.9	95 ± 3.4	93 ± 3.8	98 ± 4.1	96 ± 4.7
Peroxynitrite (100 μM)	94 ± 5.2	93 ± 6.3	95 ± 4.7	91 ± 3.8	91 ± 4.5
+ Arachidonate	96 ± 4.5	97 ± 4.2	91 ± 5.7	92 ± 2.7	93 ± 5.1
+ Cyclosporin A	97 ± 4.3	96 ± 3.6	93 ± 2.8	92 ± 5.8	93 ± 3.3
+ N-acetyl-L-cysteine	95 ± 5.0	94 ± 3.8	94 ± 4.5	90 ± 3.9	92 ± 4.9
+ Catalase	93 ± 5.8	95 ± 3.6	91 ± 3.3	94 ± 4.9	96 ± 4.1
+ $AACOCF_3$	93 ± 5.5	89 ± 3.4	80 ± 4.5*	67 ± 5.2**	51 ± 2.9**
+ $AACOCF_3$ + Arachidonate	93 ± 6.1	91 ± 2.4	91 ± 5.3	92 ± 4.6	91 ± 3.8
+ $AACOCF_3$ + Cyclosporin A	93 ± 4.7	92 ± 3.5	90 ± 3.6	91 ± 2.4	92 ± 3.9
+ $AACOCF_3$ + N-acetyl-L-cysteine	90 ± 3.6	87 ± 4.8	81 ± 5.3*	62 ± 3.7**	54 ± 4.4**
+ $AACOCF_3$ + Catalase	94 ± 2.9	83 ± 4.8	77 ± 3.1*	69 ± 4.0**	52 ± 5.1**

Molecular Pharmacology Fast Forward. Published on February 2, 2005 as DOI: 10.1124/mol.104.009498
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Peroxynitrite (1.2 mM)	94 ± 5.8	88 ± 3.6	81 ± 4.2*	64 ± 5.0**	55 ± 2.1**
+ Arachidonate	91 ± 4.0	91 ± 3.3	92 ± 4.5	89 ± 5.3	93 ± 6.7
+ Cyclosporin A	89 ± 4.9	91 ± 3.0	90 ± 5.8	93 ± 6.1	90 ± 4.6
+ N-acetyl-L-cysteine	93 ± 3.6	92 ± 2.5	94 ± 4.4	95 ± 5.8	91 ± 3.8
+ Catalase	94 ± 5.3	89 ± 4.7	92 ± 4.1	91 ± 5.3	93 ± 6.1

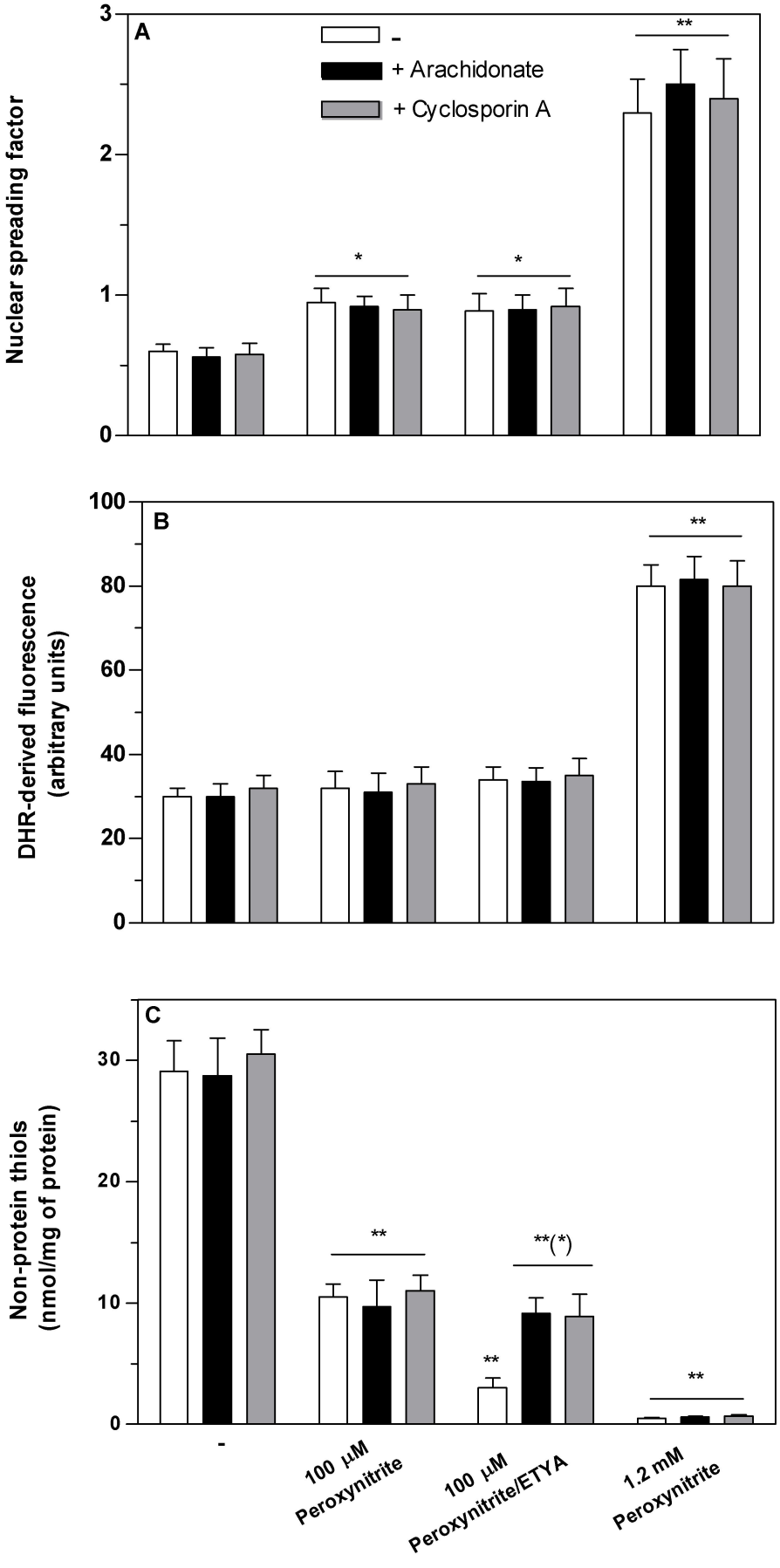


Figure 1

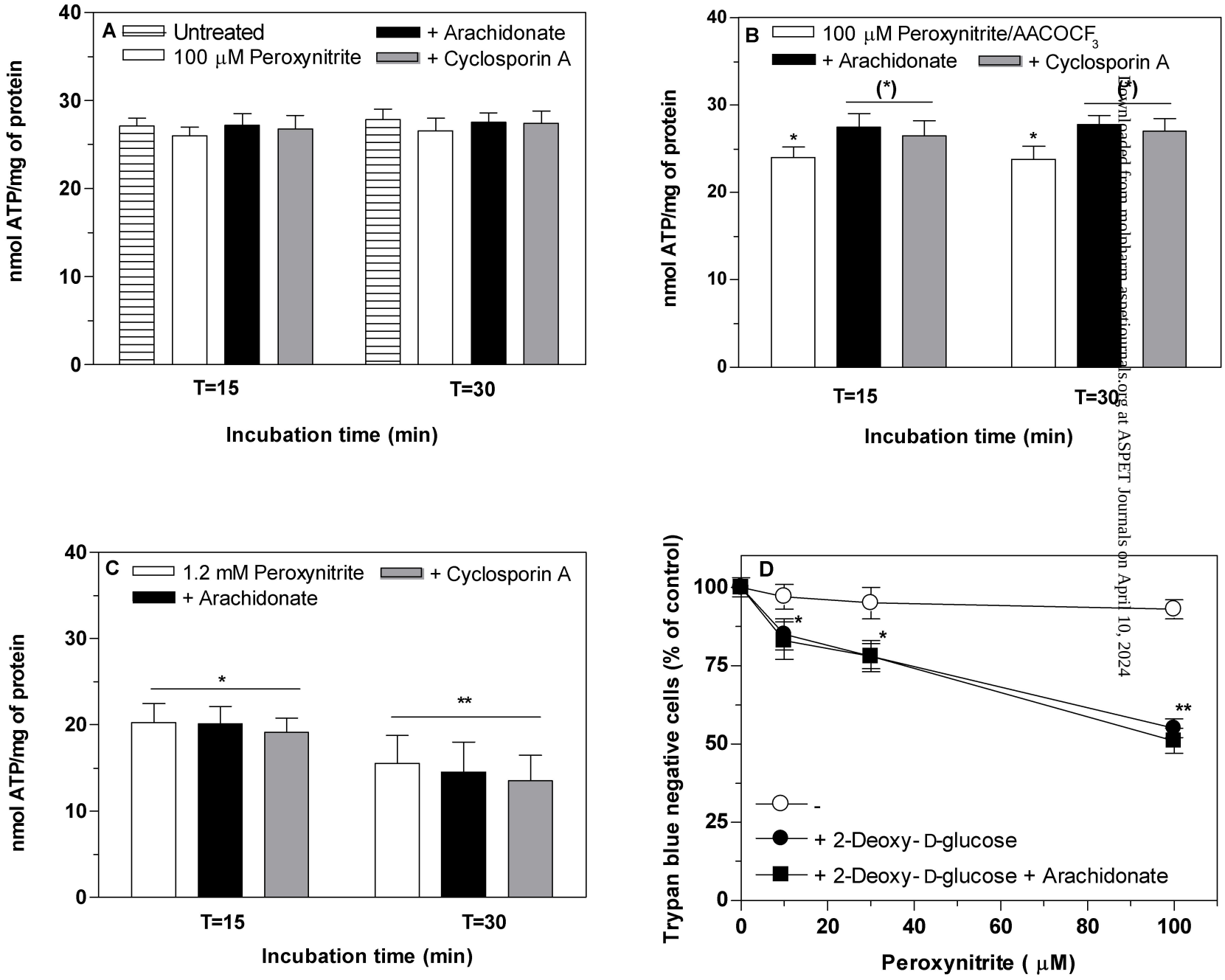
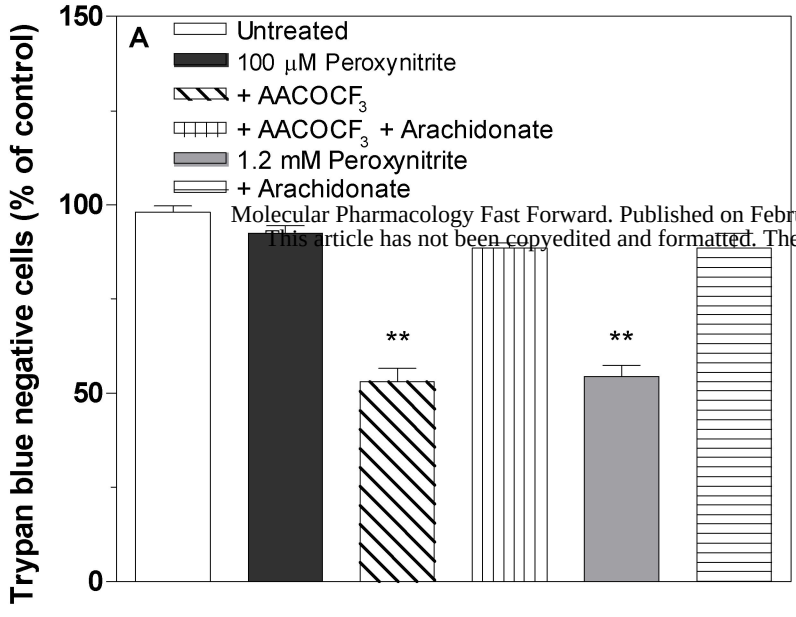
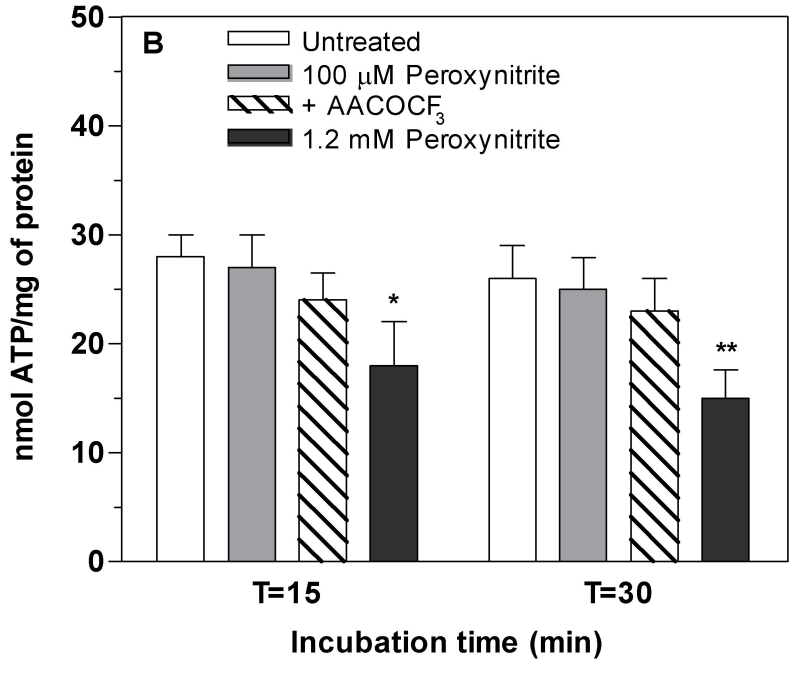


Figure 2





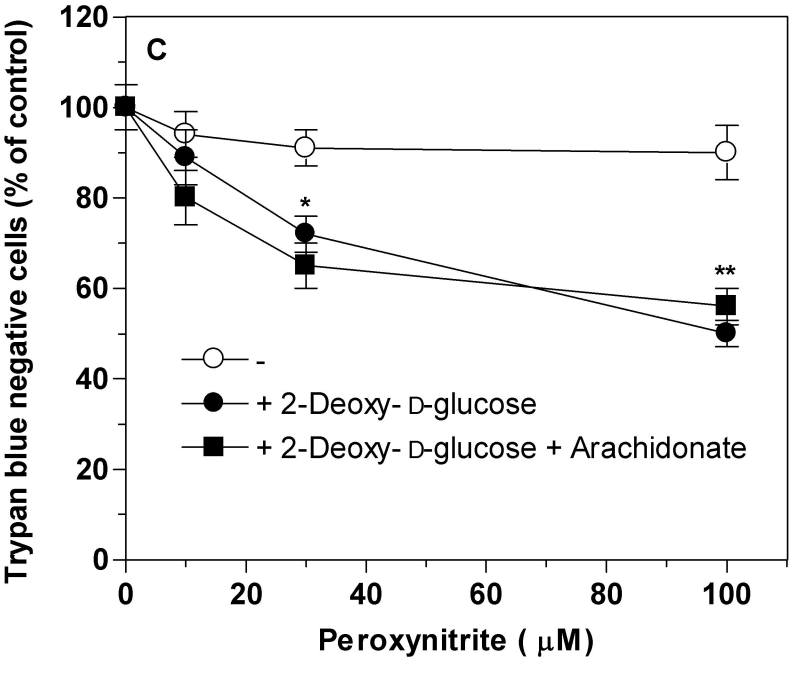


Figure 3