Pivotal role of superoxides generated in the mitochondrial respiratory chain in peroxynitrite-dependent activation of phospholipase A₂

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Exposure of PC12 cells to reagent peroxynitrite promotes the release of arachidonic acid (AA) mediated by activation of phospholipase A₂ [Guidarelli, Palomba and Cantoni (2000) *Br. J. Pharmacol.* **129**, 1539–1542]. We now present experimental evidence consistent with the notion that this response is not directly triggered by peroxynitrite but, rather, by reactive oxygen species generated at the level of complex III of the mitochondrial respiratory chain. In particular, superoxide (and not hydrogen peroxide) has a pivotal role in peroxynitrite-dependent activation of phospholipase A₂. This observation was confirmed by results showing that superoxide, or peroxynitrite, promotes release of AA in isolated mitochondria. Consistently, the release of AA elicited by either peroxynitrite or A23187 in intact cells was shown to be calcium-dependent and differentially affected by

phospholipase A₂ inhibitors with different levels of specificity. In particular, the effects of peroxynitrite, unlike those of A23187, were both sensitive to low concentrations of two general phospholipase A₂ inhibitors and insensitive to arachidonyltrifluoromethyl ketone, which shows some selectivity towards cytosolic phospholipase A₂. In addition, peroxynitrite and A23187 synergistically enhanced the release of AA. Collectively, the above results demonstrate that peroxynitrite causes inhibition of complex III, followed by enforced formation of superoxides that stimulate the activity of a calcium-dependent PLA₂ isoform, probably localized in the mitochondria.

Key words: arachidonic acid, complex III, reactive nitrogen species, respiratory chain.

INTRODUCTION

Peroxynitrite is a strong reactive nitrogen species produced *in vivo* by the diffusion-limited reaction of superoxide with nitric oxide [1], which causes an array of deleterious effects, including lipid peroxidation [2], DNA damage [3,4], alteration of calcium ion homeostasis [5], inhibition of enzymes of the mitochondrial electron-transport chain [4,6–8] and nitration of protein and non-protein thiol residues [9].

While each, or the combination of two or more, of these effects has the potential to result in cytotoxicity, it is unclear whether the occurrence of the latter event is restricted to conditions in which the cells build up peroxynitrite concentrations sufficient to overwhelm its scavenging and repair capacities. In principle, low net amounts of peroxynitrite in confined micro-environments may generate local concentrations, resulting in formation of specific lesions and/or activation of signalling pathways causally linked to the lethal response.

We reported recently that both endogenous and exogenous peroxynitrite has the ability to activate phospholipase A₂ (PLA₂), an enzyme promoting downstream formation of potentially toxic species [10]. In conflict with the commonly held hypothesis that peroxynitrite directly causes oxidation of dihydrorhodamine 123 (DHR), the DHR fluorescence response triggered by endogenous as well as authentic peroxynitrite was found to be mediated by peroxynitrite-dependent activation of PLA₂ [11]. Once again, these results suggest the occurrence of delayed formation of reactive oxygen species that may be of importance in the ensuing lethal response.

The present study was performed with the aim of gathering more information on the mechanism whereby peroxynitrite

stimulates PLA₂. It was found that peroxynitrite does not directly enhance the activity of this enzyme, which appears in fact to be activated by events triggered by superoxides generated at the level of complex III of the mitochondrial respiratory chain. Additional experimental evidence suggests that the PLA₂ isoform activated by superoxides, although calcium-dependent, is different from the cytosolic isoform of PLA₂ (cPLA₂) and is most likely to be represented by a low-molecular-mass PLA₂ localized within the mitochondria.

MATERIALS AND METHODS

Materials

A23187, xanthine, xanthine oxidase (XO), eicosa-5,8,11,14-tetraenoic acid (ETYA), rotenone, thenoyltrifluoroacetone (TTFA), myxothiazol, 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), antimycin A, as well as most of reagent grade chemicals, were obtained from Sigma-Aldrich (Milan, Italy). [³H]Arachidonic acid (AA) was purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). 2,7-Diamino-10-ethyl-9,10-dihydrophenanthridine (DHE) and DHR were obtained from Molecular Probes Europe (Leiden, The Netherlands). Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP), arachidonyltrifluoromethyl ketone (AACOCF₃), and bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM) were purchased from Calbiochem (San Diego, CA, U.S.A.).

Abbreviations used: AA, arachidonic acid; AACOCF₃, arachidonyltrifluoromethyl ketone; BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); cPLA₂, cytosolic phospholipase A₂; DHE, 2,7-diamino-10-ethyl-9,10-dihydrophenanthridine; DHR, dihydrorhodamine 123; ETYA, eicosa-5,8,11,14-tetraenoic acid; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; PLA₂, phospholipase A₂; TTFA, thenoyltrifluoroacetone; XO, xanthine oxidase.

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Cell culture and treatment conditions

PC12 rat pheochromocytoma cells were cultured in Dulbecco's modified Eagle's medium (HyClone Laboratories, Logan, UT, U.S.A.) supplemented with 10% (v/v) horse serum (HyClone), 5% (v/v) fetal bovine serum (HyClone), penicillin (50 units/ml) and streptomycin (50 µg/ml; Sera-Lab Ltd, Crawley Down, U.K.) at 37 °C in T-75 tissue-culture flasks (Corning, Corning, NY, U.S.A.) that was gassed with an atmosphere of air/CO₂ (19:1).

Respiration-deficient PC12 cells were isolated by culturing the cells in Dulbecco's modified Eagle's medium containing 400 ng/ml ethidium bromide, 110 µg/ml pyruvate and 5 µg/ml uridine for 50–60 days.

Stock solutions of xanthine, XO, MnTBAP, MnTMPyP, KCN and mepacrine were freshly prepared in distilled water. A23187, ETYA, rotenone, TTFA, myxothiazol, HQNO, antimycin A, AACOCF₃ and BAPTA/AM were dissolved in 95% (v/v) ethanol. At the treatment stage, the final concentration of ethanol was less than 0.05%.

Peroxynitrite was added as a bolus to samples and, to avoid changes in pH due to the high alkalinity of peroxynitrite stock solution, an appropriate amount of 1 M HCl was also added.

For experiments, cells were inoculated in six-well plates at 2×10^5 cells per well and grown for 18–24 h. At the treatment stage, the total cell number was between 4.0×10^5 and 4.5×10^5 cells per well. Treatments were performed in 2 ml of saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l NaHCO₃ and 0.9 g/l glucose) at 37 °C.

Synthesis of peroxynitrite

Peroxynitrite was synthesized by the reaction of nitrite with acidified H₂O₂ as described previously [9]. MnO₂ (1 mg/ml) was added to the mixture for 30 min at 4 °C to eliminate the excess of H₂O₂. MnO₂ was then removed by centrifugation and filtration (0.45 µm pore filters). The peroxynitrite solution formed, by freeze fractionation (–80 °C), a yellow top layer, which typically contained 150–200 mM peroxynitrite, as determined spectrophotometrically at 302 nm in 1.5 M NaOH (ϵ_{302} 1670 M^{–1} · cm^{–1}). The peroxynitrite solution was stored for 2 months (–80 °C) with negligible changes in its concentration.

Measurement of extracellular release of [³H]AA

PC12 cells were sub-cultured in six-well plates at 2×10^5 cells per well with [³H]AA (0.5 µCi/ml) and grown for 18 h. Before treatment, the cells were washed twice with saline A supplemented with 1 mg/ml fatty-acid-free BSA and exposed to peroxynitrite or A23187 in the absence or presence of drugs. The solution was then separated and centrifuged at 5000 g for 1.5 min; 500 µl of the resulting supernatant was removed and radioactivity was determined in a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland).

DHE or DHR oxidation and confocal imaging

Cells were exposed for 3 min in saline A to 200 µM peroxynitrite, and then post-incubated for 7 min in fresh saline A containing 10 µM DHE or 30 µM DHR in the absence or presence of additional drugs. After accurate washings, cellular fluorescence was imaged using a confocal laser microscope (Bio Rad DVC 250; Bio Rad, Richmond, CA, U.S.A.) equipped with a Hamamatsu 5985 (Hamamatsu Italy, Milan, Italy)-chilled CCD camera. Cells were illuminated with the 488 nm line of the argon

laser, and the fluorescence emitted was monitored at $\lambda > 515$ 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 DHE or 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 U.S. National Institutes of Health, and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). Mean fluorescence values were determined by averaging the fluorescence of at least 50 cells/treatment condition per experiment.

Measurement of oxygen consumption

The cells were washed once in saline A and then suspended in the same medium at a density of 1×10^7 cells/ml. Oxygen consumption was measured using a Y.S.I. oxygraph equipped with a Clark electrode (model 5300; Yellow Springs Instruments Co, Yellow Springs, OH, U.S.A.). The cell suspension (3 ml) was transferred to the polarographic cell and the rate of oxygen utilization was monitored under constant stirring for 3 min (basal respiration). The rate of oxygen utilization was calculated as described previously [12].

Preparation of mitochondria

The cells were sub-cultured in T-175 tissue culture flasks at 1×10^7 cells per flask with [³H]AA (0.5 µCi/ml) and grown for 48 h. Cells (3×10^7) were removed from the flask by trypsin treatment and washed twice with saline A supplemented with 1 mg/ml fatty-acid-free BSA. The pellet was suspended in 5 ml of an ice-cold solution containing 5 mM K⁺-Hepes, pH 7.4, 210 mM mannitol, 1 mM EGTA, 70 mM sucrose and 55 µg/ml digitonin, and homogenized by 10 strokes in an ice-cold glass homogenizer. Non-lysed cells and nuclei were pelleted by centrifugation at 750 g for 10 min at 4 °C and the supernatant was centrifuged again at 10000 g for 15 min at 4 °C. The resulting mitochondrial pellet was resuspended in 1 ml of 5 mM K⁺-Hepes, pH 7.4, 210 mM mannitol and 70 mM sucrose at 37 °C, and treated for 10 min with peroxynitrite or xanthine/XO. After the treatments, the [³H]AA release was quantified as described above.

Statistical analysis

Statistical analysis of the data for multiple comparisons was performed by ANOVA followed by a Dunnett's test. For single comparison, the significance of differences between means was determined by Student's *t* test.

RESULTS

Figure 1 illustrates the extent of AA release mediated by a 10 min exposure to increasing concentrations of either authentic peroxynitrite or the calcium ionophore A23187, a widely utilized PLA₂ activator. From these dose–response curves, we established the maximal non-effective concentrations (10 µM peroxynitrite; 0.5 µM A23187) and the concentrations promoting an approx. 160% increased AA release (50 µM peroxynitrite; 2.5 µM A23187) that were used in the experiments reported below. As reported previously [10], the effects mediated by peroxynitrite were prevented by the peroxynitrite scavengers Trolox (1 mM) and L-methionine (20 mM), and were not observed in cells

Table 1 Effect of respiratory-chain inhibitors on the AA release mediated by peroxynitrite or A23187

[³H]AA-labelled cells were exposed for 5 min to the indicated concentrations of respiratory-chain inhibitors and then treated for a further 10 min with peroxynitrite (10 or 50 μ M) or A23187 (0.5 or 2.5 μ M). After treatments, the [³H]AA release was quantified as described in the Materials and methods section. The results are expressed as percentages of control (815 $\pm$ 48 c.p.m.) and are the means $\pm$ S.E.M. of three to five separate experiments, each performed in duplicate. **P* < 0.001 compared with peroxynitrite-treated cells (unpaired *t* test).

Treatment	Control	Concentration (μ M) ...	[³ H]AA release (% of control)			
			Peroxynitrite		A23187	
			10	50	0.5	2.5
Untreated	100 $\pm$ 5.4		112 $\pm$ 3.8	159 $\pm$ 10.3	115 $\pm$ 4.6	169 $\pm$ 9.8
Rotenone (0.5 μ M)	101 $\pm$ 4.1		109 $\pm$ 9.1	118 $\pm$ 6.9*	118 $\pm$ 5.7	171 $\pm$ 10
TTFA (250 μ M)	103 $\pm$ 6.6		105 $\pm$ 4.9	163 $\pm$ 9.4	103 $\pm$ 7.1	160 $\pm$ 8.8
Myxothiazol (5 μ M)	98 $\pm$ 5.3		114 $\pm$ 8.4	115 $\pm$ 7.1*	113 $\pm$ 6.8	162 $\pm$ 9.7
Antimycin A (1 μ M)	99 $\pm$ 7.0		165 $\pm$ 7.2*	162 $\pm$ 8.9	114 $\pm$ 4.9	168 $\pm$ 7.8
HQNO (10 μ M)	102 $\pm$ 6.2		161 $\pm$ 5.5*	165 $\pm$ 7.5	109 $\pm$ 9.2	165 $\pm$ 6.2
KCN (1 mM)	96 $\pm$ 4.7		108 $\pm$ 3.7	168 $\pm$ 9.6	104 $\pm$ 4.3	167 $\pm$ 5.4

Figure 1 Release of AA mediated by increasing concentrations of peroxynitrite or A23187 in respiration-proficient or -deficient cells

[³H]AA-labelled respiration-proficient (open symbols) or deficient (closed symbols) cells were exposed in saline A for 10 min to increasing concentrations of peroxynitrite (circles) or A23187 (squares). After treatments, the [³H]AA release was quantified as described in the Materials and methods section. The results are the means $\pm$ S.E.M. for three to five separate experiments, each performed in duplicate. **P* < 0.001 compared with control cells (ANOVA followed by Dunnett's test).

exposed to the vehicle or to decomposed peroxynitrite (results not shown). The results shown in Table 1 indicate that exposure to selected concentrations of peroxynitrite promotes a release of AA differentially affected by inhibitors of the mitochondrial respiratory chain. We found that two different complex III inhibitors, antimycin A (1 μ M) and HQNO (10 μ M), markedly increased the release of AA in cells treated with a concentration of peroxynitrite (10 μ M) that was otherwise unable to significantly elevate this response over control values. Interestingly, these enhancing effects were prevented by the complex I inhibitor

rotenone (0.5 μ M) and by myxothiazol (5 μ M), a complex III inhibitor which binds to a site different from that of antimycin A or HQNO [13] and prevents the electron flow from the reduced coenzyme Q to cytochrome *c*₁ [14] (Table 2). In contrast with these results, rotenone, myxothiazol and the inhibitors of complex II (TTFA, 250 μ M) or complex IV (KCN, 1 mM) did not produce significant effects on AA release in cells exposed to 10 μ M peroxynitrite. Each of the above respiratory-chain inhibitors did not significantly modify the release of AA in untreated cells (Table 1) or in cells exposed to the maximal non-effective dose of A23187 (0.5 μ M; Table 1), and caused inhibition of oxygen consumption greater than 95 %, with the exception of TTFA (48.8 %; Table 3). Inhibition of oxygen consumption was also observed in cells exposed to peroxynitrite (Table 3). It is important to note that these studies employed cell densities that were 6 times greater than those utilized for the assessment of AA release. Since the effects of peroxynitrite are an inverse function of cell density [15] it may be expected that significantly lower concentrations of peroxynitrite will effectively reduce the extent of oxygen utilization at a 6-times-lower cell density.

Experiments were next performed using conditions identical with those described above, except that peroxynitrite was utilized at a concentration (50 μ M) promoting a significant increase in the release of AA (Table 1). It was found that this response was sensitive to rotenone or myxothiazol, and insensitive to antimycin A, HQNO, TTFA or KCN. Importantly, each of the respiratory-chain inhibitors utilized in the above experiments failed to affect the release of AA in cells treated with 2.5 μ M A23187, a treatment promoting a release of AA comparable with that mediated by 50 μ M peroxynitrite. Control experiments were also performed to rule out the possibility that the inhibitory effects mediated by rotenone or myxothiazol were due to scavenging of peroxynitrite. In these experiments, the cells were first exposed for 3 min to 50 μ M peroxynitrite and then post-incubated for 7 min in fresh saline A containing each of the respiratory-chain inhibitors. As shown in Table 4, the release of AA mediated by peroxynitrite under these conditions was similar to that reported in Table 1, and rotenone, as well as myxothiazol, abolished this response.

With the aim of gathering further information on the role of the mitochondrial respiratory chain in peroxynitrite-dependent activation of PLA₂, experiments were performed using PC12 cells made respiration-deficient by continuous growth in a culture medium supplemented with ethidium bromide. The results shown in Figure 1 indicate that the same concentrations of peroxynitrite resulting in significant AA release in the parental cell line had

Table 2 Effect of rotenone or myxothiazol on the AA release mediated by peroxynitrite associated with antimycin A or HQNO

[³H]AA-labelled cells were treated and analysed as detailed in the legend to Table 1. The results are expressed as percentages of the control (913 ± 84 c.p.m.) and are the means ± S.E.M. of three to five separate experiments, each performed in duplicate. **P* < 0.001 compared with peroxynitrite associated with antimycin A or HQNO (unpaired *t* test).

Treatment	[³ H]AA release (% of control)
Peroxyntirite (10 μM) + antimycin A (1 μM)	168 ± 4.2
+ Rotenone (0.5 μM)	106 ± 6.6*
+ Myxothiazol (5 μM)	107 ± 5.8*
Peroxyntirite (10 μM) + HQNO (10 μM)	159 ± 8.3
+ Rotenone (0.5 μM)	101 ± 2.4*
+ Myxothiazol (5 μM)	110 ± 7.7*

Table 3 Effect of peroxynitrite or respiratory-chain inhibitors on PC12 cell oxygen consumption

The cells were harvested by trypsin treatment, resuspended in saline A and analysed for oxygen consumption for 3 min at the beginning of the experiment and following addition of the indicated concentrations of peroxynitrite or each of the respiratory chain-inhibitors. The results are expressed as percentage inhibition of basal respiration (32 nmol/O₂ per min per 10⁷ cells) and are the means for two separate experiments with identical results.

Treatment	Oxygen consumption (% inhibition)
Peroxyntirite (50 μM)	12.3
Peroxyntirite (100 μM)	34.9
Peroxyntirite (200 μM)	52.6
Rotenone (0.5 μM)	97.3
TTFA (250 μM)	48.8
Myxothiazol (5 μM)	96.3
Antimycin A (1 μM)	95.5
HQNO (10 μM)	94.7
KCN (1 mM)	98.2

hardly any effect in respiration-deficient cells. Addition of antimycin A or HQNO did not cause appreciable changes (results not shown). In contrast with these results showing a lack of response to peroxynitrite, respiration-deficient cells were found to be as sensitive as respiration-proficient cells to the AA release stimulated by increasing concentrations of A23187 (Figure 1).

Taken together, the above results are consistent with the notion that peroxynitrite stimulates the release of AA via a mechanism that involves inhibition of complex III, an event known to result in the formation of reactive species [16,17].

We therefore investigated the effect of superoxide dismutase-mimetic agents on the release of AA induced by peroxynitrite. In these experiments, the cells were exposed for 3 min to 50 μM peroxynitrite and then post-incubated for 7 min, in the absence or presence of 30 μM MnTBAP [18] or MnTMPyP [19]. It was found that each of these treatments abolishes the ensuing release of AA (Table 4). Control experiments revealed that, under similar conditions, MnTBAP does not affect the release of AA stimulated by 2.5 μM A23187 (results not shown).

The next set of experiments was performed with the aim of assessing whether delayed formation of superoxides does indeed take place after peroxynitrite exposure. For this purpose we utilized DHE, a fluorogen previously shown to be rapidly taken up by cells in culture and specific for superoxides [20]. In order to increase the sensitivity of the assay, peroxynitrite was used at 200 μM. We observed that a 3 min treatment with peroxynitrite, followed by a 7 min post-incubation with 10 μM DHE, causes

Table 4 Effect of rotenone, myxothiazol or superoxide dismutase-mimetic agents on the AA release and DHE-/DHR-fluorescence responses mediated by peroxynitrite

[³H]AA-labelled cells were exposed for 3 min to 50 μM peroxynitrite and then post-incubated for 7 min in fresh saline A containing the indicated concentrations of drugs. After treatments, the [³H]AA release was quantified as described in the Materials and methods section. The results are expressed as percentages of control (875 ± 88 c.p.m.) and are the means ± S.E.M. for three to five separate experiments, each performed in duplicate. **P* < 0.001 compared with peroxynitrite-treated cells (unpaired *t* test). In other experiments, the cells were exposed for 3 min to 200 μM peroxynitrite and then post-incubated for 7 min in fresh saline A containing 10 μM DHE or 30 μM DHR in the absence or presence of the indicated concentrations of drugs. After treatments, the DHE- or DHR-derived fluorescence was monitored by confocal microscopy and quantified as detailed in the Materials and methods section. The results are expressed as percentages of control (DHE-fluorescence = 14.96 ± 1.95 arbitrary units; DHR-fluorescence = 13.16 ± 2.89 arbitrary units) and are the means ± S.E.M. for three to five separate experiments, each performed in duplicate. **P* < 0.001 compared with peroxynitrite-treated cells (unpaired *t* test).

Treatment	[³ H]AA release (% of control)	DHE-fluorescence (% of control)	DHR-fluorescence (% of control)
Peroxyntirite	168 ± 6.4	287 ± 15.4	392 ± 7.4
+ Rotenone (0.5 μM)	109 ± 7.8*	103 ± 6.8*	115 ± 9.2*
+ Myxothiazol (5 μM)	111 ± 8.1*	114 ± 11.5*	112 ± 8.8*
+ MnTBAP (30 μM)	105 ± 2.8*	110 ± 9.6*	383 ± 12.8
+ MnTMPyP (30 μM)	107 ± 5.2*	121 ± 10.2*	378 ± 13.7

an intense fluorescence response due to oxidation of DHE to form ethidium, which then intercalates in the DNA and generates red fluorescence. Image analysis performed in large numbers of randomly selected cells revealed that this fluorescence response was increased 2.87 times over control values and abolished upon addition of rotenone, myxothiazol, MnTBAP or MnTMPyP during recovery (Table 4). These results are indicative of delayed formation of superoxides taking place at the level of complex III.

Experiments were then performed using identical exposure protocols, except that DHE was replaced with DHR, a fluorogen that is rapidly oxidized to rhodamine 123 following exposure to hydrogen peroxide and other reactive species, but not by superoxides [21,22]. It was found that, under conditions in which peroxynitrite cannot directly oxidize the probe, a remarkable DHR fluorescence response is readily detected and its occurrence is prevented by rotenone or myxothiazol, but unaffected by MnTBAP or MnTMPyP (Table 4).

Thus the above results indicate that peroxynitrite, unlike A23187, stimulates the release of AA via a mechanism that involves inhibition of complex III and formation of superoxides. As a consequence, these agents most likely activate two different PLA₂ pools. This notion finds experimental support in the results shown in Table 5, showing that peroxynitrite and A23187 enhance synergistically the release of AA. Table 5 also shows that the AA release stimulated by peroxynitrite, A23187 or by their combination is sensitive to intracellular calcium chelation afforded by exposure to BAPTA/AM (10 μM). Analysis of AA release mediated by 50 μM peroxynitrite or 2.5 μM A23187 revealed that these responses displayed remarkable differences in their sensitivities to PLA₂ inhibitors characterized with different levels of specificity (Figure 2). In particular, high concentrations of mepacrine (Figure 2A) and ETYA (Figure 2B), two general PLA₂ inhibitors, were able to suppress the AA release stimulated by either peroxynitrite or A23187. At low concentrations (≤ 10 μM), however, mepacrine and ETYA effectively reduced the extent of AA release mediated by peroxynitrite and only slightly reduced the response evoked by A23187. Figure 2(C)

Table 5 Effect of BAPTA/AM on AA release mediated by peroxynitrite, A23187 or by their combination

[³H]AA-labelled cells were exposed for 5 min to 0 or 10 μ M BAPTA/AM and then treated for a further 10 min with 50 μ M peroxynitrite or 2.5 μ M A23187. After treatments, the [³H]AA release was quantified as described in the Materials and methods section. The results are the means $\pm$ S.E.M. for three to five separate experiments, each performed in duplicate. * $P < 0.001$ compared with peroxynitrite or A23187-treated cells (unpaired t test).

Treatment	[³ H]AA release (c.p.m.)
Untreated	881 $\pm$ 46
BAPTA/AM (10 μ M)	897 $\pm$ 33
Peroxyntirite (50 μ M)	1609 $\pm$ 84
+ 10 μ M BAPTA/AM	903 $\pm$ 51
A23187 (2.5 μ M)	1719 $\pm$ 89
+ 10 μ M BAPTA/AM	896 $\pm$ 29
Peroxyntirite (50 μ M) + A23187 (2.5 μ M)	3394 $\pm$ 116*
+ 10 μ M BAPTA/AM	918 $\pm$ 38

indicates that AA release stimulated by 50 μ M peroxynitrite was only marginally reduced by 3–30 μ M AACOCF₃, an inhibitor thought to be selective for cPLA₂ [23]. In contrast with these results, AACOCF₃ was extremely effective in reducing AA release stimulated by 2.5 μ M A23187.

Finally, we investigated whether peroxynitrite or superoxide promotes AA release in isolated mitochondria. The results shown in Figure 3 indicate that this response was indeed effectively induced by very low concentrations of peroxynitrite or superoxides generated by the cocktail xanthine/XO.

DISCUSSION

The present study represents an extension to our previous investigations, indicating that endogenous as well as exogenous peroxynitrite promotes a release of AA causally linked to activation of PLA₂ [10,11]. In particular, we investigated the mechanism whereby exogenous peroxynitrite enhances the release of AA in PC12 cells, and found that peroxynitrite-dependent inhibition of complex III generates a flux of superoxides which plays a pivotal role in this response.

We report the results of experiments showing that complex III inhibitors antimycin A and HQNO, while causing an about 95 % inhibition in oxygen consumption (Table 3), markedly increased the release of AA in cells treated with a concentration of peroxynitrite (10 μ M) that was otherwise unable to significantly elevate this response over control values (Table 1). These enhancing effects appeared to be causally linked to the ability of antimycin A or HQNO to bind to their specific site in complex III, since the ensuing release of AA evoked in cells exposed to 10 μ M peroxynitrite was suppressed by both rotenone and myxothiazol (Table 2). These agents are expected to prevent the entry of electrons in the Q cycle, since rotenone is a complex I inhibitor whereas myxothiazol is a complex III inhibitor that blocks the electron flow from the reduced coenzyme Q to cytochrome c_1 [14]. In addition, rotenone, myxothiazol and KCN were also used at levels causing a > 95 % inhibition of oxygen consumption (Table 3), but did not promote release of AA in cells supplemented with 10 μ M peroxynitrite (Table 1). Finally, the specificity of the effects mediated by antimycin A or HQNO is also emphasized by the observation that each of these inhibitors failed to enhance AA release induced by a maximal non-effective dose of A23187 (0.5 μ M; see Table 1).

Taken together, the results thus far discussed indicate that inhibition of oxygen consumption does not directly result in

Figure 2 Effect of PLA₂ inhibitors on the AA release mediated by peroxynitrite or A23187

[³H]AA-labelled cells were incubated for 5 min in the absence or presence of increasing concentrations of mepacrine (A), ETYA (B) or AACOCF₃ (C), and then treated for a further 10 min with 50 μ M peroxynitrite (open circles) or 2.5 μ M A23187 (closed circles). After treatments, the [³H]AA release was quantified as described in the Materials and methods section. The results are expressed as percentage inhibition and are the means $\pm$ S.E.M. for three to five separate experiments, each performed in duplicate.

enhanced release of AA in cells treated with an inactive concentration of peroxynitrite. Rather, it would appear that the enhancing effects of antimycin A or HQNO are due to their binding to the specific site in complex III.

Since previous studies from this [4] and other [7,8] laboratories demonstrated that peroxynitrite promotes inhibition of complex III, we postulated that this event was causally linked to stimulation of AA release. The outcome of experiments using cells

Figure 3 Release of AA mediated by increasing concentrations of peroxynitrite or xanthine/XO in isolated mitochondria

[³H]AA-labelled mitochondria were exposed in 1 ml of 5 mM K⁺-Hepes (pH 7.4), 210 mM mannitol and 70 mM sucrose at 37 °C for 10 min to increasing concentrations of peroxynitrite (open circles) or XO [closed circles, in the presence of 100 μM xanthine (X)]. After treatments, the [³H]AA release was quantified as described in the Materials and methods section. The results are expressed as percentages of the control and are the means ± S.E.M. for three to five separate experiments, each performed in duplicate. **P* < 0.001; ***P* < 0.0001 compared with control cells (ANOVA followed by Dunnett's test).

treated with 50 μM peroxynitrite, which promotes a significant AA release, is consistent with this notion (Table 1). Indeed this response was sensitive to rotenone or myxothiazol, and insensitive to antimycin A, HQNO, TTFA and KCN. The fact that rotenone, or myxothiazol, were equally effective when given after peroxynitrite exposure (Table 4) rules out the possibility that these compounds may act as peroxynitrite scavengers. Furthermore, the specificity of the effects of rotenone and myxothiazol is also emphasized by the observation that these agents did not affect the release of AA in cells treated with 2.5 μM A23187 (Table 1). Similar results were obtained using the remaining respiratory-chain inhibitors.

The final clue establishing a pivotal role of the mitochondrial respiratory chain in peroxynitrite-dependent activation of PLA₂ is given by the experiments using respiration-deficient PC12 cells. It was found that these cells, although responding to A23187 as effectively as respiration-proficient cells, failed to release AA in response to peroxynitrite (Figure 1). Addition of antimycin A or HQNO did not cause appreciable changes (results not shown), a finding consistent with the notion that these cells were indeed respiration-deficient.

Taken together, the above results are consistent with the notion that peroxynitrite-dependent inhibition of complex III is a critical event in the ensuing stimulation of AA release. The fact that antimycin A and HQNO inhibit complex III at the same level [24], along with the observation that the action of each of the two complex III inhibitors is on a saturable mechanism, strongly suggests that peroxynitrite-dependent inhibition of

complex III plays a pivotal role in the above response. Under the conditions that were used in this study, increasing concentrations of peroxynitrite may lead to a progressive impairment in complex III activity, and this would explain both the strong enhancement of the AA release mediated by antimycin A or HQNO in cells treated with a low concentration of peroxynitrite (10 μM), and the lack of effect of antimycin A or HQNO after exposure to 50 μM peroxynitrite. Although oxygen consumption experiments were performed using cell densities greater than those employed in AA release studies, we nevertheless could show that low concentrations of peroxynitrite effectively reduce oxygen consumption (Table 3). Our previous studies showed that this effect is largely mediated by inhibition of complex III [4].

Thus it would appear that the mechanism whereby peroxynitrite causes AA release in intact cells involves inhibition of complex III at a level close to the antimycin, or HQNO, binding site. This region represents 'the weak site' of the respiratory chain, and prevention of the electron flow from cytochrome *b*₅₆₂ to oxidized coenzyme Q leads to formation of superoxides and hydrogen peroxide [17]. Each, or both, of these species may subsequently lead to activation of PLA₂.

In order to investigate whether superoxides mediate the peroxynitrite-dependent stimulation of AA release, we utilized MnTBAP, an agent that rapidly converts superoxides into hydrogen peroxide [18]. MnTBAP, given after peroxynitrite exposure, was found to prevent the ensuing release of AA (Table 4). Three different lines of evidence emphasize the specificity of the effects mediated by MnTBAP. First, identical results were obtained when MnTBAP was replaced with another superoxide dismutase-mimetic agent, MnTMPyP [19]. Secondly, neither MnTBAP nor MnTMPyP could act as peroxynitrite scavengers, since they were given to the cultures 3 min after addition of peroxynitrite, a time at which the oxidant had disappeared from the culture medium. Finally, MnTBAP did not promote significant effects on the release of AA stimulated by 2.5 μM A23187 (results not shown).

These experiments demonstrate that enforced dismutation of superoxides prevents the release of AA in cells exposed to peroxynitrite. The use of a superoxide-specific fluorogen, DHE [20], allowed us to demonstrate that, under the same conditions, peroxynitrite causes a delayed formation of superoxides via a mechanism sensitive to rotenone, myxothiazol, MnTBAP or MnTMPyP (Table 4). Thus formation of superoxides does indeed occur after exposure to peroxynitrite and this process is mediated by events taking place at the level of complex III.

Interestingly, when DHE was replaced with DHR, a fluorogen sensitive to hydrogen peroxide but not to superoxides [21,22], the ensuing fluorescence response was prevented by rotenone or myxothiazol, but was unaffected by MnTBAP or MnTMPyP (Table 4). Thus these results indicate that enforced conversion of superoxides into hydrogen peroxide does not affect the extent of oxidation of a hydrogen peroxide-sensitive probe. The latter response is, however, blunted by specific electron-transport inhibitors, also preventing the DHE fluorescence response. The fact that MnTBAP or MnTMPyP abolished AA release (Table 4) is therefore consistent with the notion that superoxide, and not hydrogen peroxide, is the species that mediates the peroxynitrite-dependent stimulation of AA release.

The above results collectively indicate that peroxynitrite and A23187 stimulate two different PLA₂ pools, since the first, unlike the second, is stimulated by superoxides. Both PLA₂ isoforms, however, were calcium-dependent, since the AA release stimulated by peroxynitrite and A23187 either alone or in combination was suppressed by BAPTA/AM, an agent which is readily taken up by the cells and efficiently chelates intracellular calcium ions

(Table 5). The above results are also consistent with the possibility that the superoxide-sensitive PLA₂ stimulated by peroxynitrite is located within the mitochondria. Indeed, superoxides are rapidly converted into hydrogen peroxide ($2 \times 10^9 \text{ mol}^{-1} \cdot \text{s}^{-1}$) by Mn-superoxide dismutase, and their exit from the mitochondria is unlikely [25]. The involvement of two different PLA₂ pools is supported further by the observations that peroxynitrite and A23187 synergistically enhance the release of AA (Table 5), and that the response mediated by each of these stimuli was differentially affected by PLA₂ inhibitors characterized with different levels of specificity (Figure 2). We found that the AA release stimulated by peroxynitrite was sensitive to low concentrations ($\leq 10 \mu\text{M}$) of two general PLA₂ inhibitors, mepacrine and ETYA, but insensitive to AACOCF₃, an inhibitor thought to be specific for cPLA₂ [23]. In contrast with these results, the release of AA elicited by A23187, although inhibited only by high concentrations of mepacrine or ETYA, was extremely sensitive to inhibition afforded by AACOCF₃.

Altogether, the above results are consistent with the hypothesis that the specific PLA₂ isoform stimulated by peroxynitrite, although being calcium-dependent, is different from cPLA₂ and is possibly localized within the mitochondria. Such an enzyme is reported to belong to group IIA PLA₂s [26]. Our results indicating that either peroxynitrite or superoxides generated by the cocktail xanthine/XO effectively stimulate AA release in isolated mitochondria is consistent with this notion.

Previous studies by Madesh and Balasubramanian [27] showed that superoxides activate a liver mitochondrial PLA₂. Although the mechanism involved in this response remains unclear, it may be predicted that important events in this direction are represented by the mitochondrial accumulation of calcium ions, by changes in the physicochemical state of the membrane-lipid phase [28], and by enhanced susceptibility of phospholipids to PLA₂ resulting from peroxidation of membrane lipids [29,30]. All these events are known to be triggered by peroxynitrite [31].

In conclusion, the results presented in the present study provide compelling evidence indicating that peroxynitrite does not stimulate directly the release of AA in PC12 cells; rather, this response appears to be mediated by superoxides generated at the level of complex III of the respiratory chain in a reaction in which ubiquinone serves as an electron donor. As a corollary, superoxides appear to be, on the one hand, necessary ingredients to allow formation of endogenous peroxynitrite, and, on the other hand, secondary products triggering downstream events with a potential cytotoxic impact. It may therefore be predicted that conditions leading to formation of nitric oxide and superoxides, and thus peroxynitrite, support a self-perpetuating cycle in which the latter leads to progressive inhibition of complex III and promotes formation of additional superoxides, thereby increasing the rate of peroxynitrite generation and resulting in activation of mitochondrial PLA₂. Peroxynitrite induces peroxidation of membrane lipids and this event is known to enhance the susceptibility of phospholipids to PLA₂ [29]. Furthermore, mitochondria are considered to be important targets of mitochondrial PLA₂ [26], and, indeed, mitochondrial PLA₂ contributes to cell injury mediated by chemical hypoxia in isolated rat hepatocytes [32,33]. Thus it may well be that the sequence of events described above has a pivotal role in the mechanism whereby peroxynitrite causes mitochondrial-permeability-transition-dependent toxicity. Consistently, previous studies from our laboratory demonstrated that exposure of PC12 cells to a short-chain lipid hydroperoxide analogue induces peroxynitrite-dependent toxicity mediated by mitochondrial permeability transition [34]. Similar results were obtained using exogenous peroxynitrite (L. Palomba and O. Cantoni, unpublished work).

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