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SPECIAL REPORT

Peroxynitrite-mediated release of arachidonic acid from PC12 cells

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A short term exposure of PC12 cells to a concentration of *tert*-butylhydroperoxide (tB-OOH) causing peroxynitrite-dependent DNA damage and cytotoxiticity promoted a release of arachidonic acid (AA) that was sensitive to phospholipase A_2 (PLA_2) inhibitors and insensitive to phospholipase C or diacylglycerol lipase inhibitors. The extent of AA release was also mitigated by nitric oxide synthase (NOS) inhibitors and peroxynitrite scavengers. Low levels (10 μM) of authentic peroxynitrite restored the release of AA mediated by tB-OOH in NOS-inhibited cells whereas concentrations of peroxynitrite of 20 μM, or higher, effectively stimulated a PLA_2 inhibitor-sensitive release of AA also in the absence of additional treatments. These results are consistent with the possibility that endogenous as well as exogenous peroxynitrite promotes activation of PLA_2.
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Abbreviations: AA, arachidonic acid, ETYA, 5,8,11,14-eicosatetraynoic acid; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; PLA_2, phospholipase A_2; RHC-80267, 1,6-*bis*(cyclohexyloximonocarbonylamino)hexane; tB-OOH, *tert*-butylhydroperoxide

Introduction Peroxynitrite, the coupling product of superoxides and nitric oxide, is a potent oxidant that induces an array of deleterious events including peroxidation of membrane lipids (Rubbo *et al*., 1994), depletion of glutathione (Salgo *et al*., 1995), DNA single strand breakage (Szabó & Ohshima, 1997), mitochondrial dysfunction (Brown, 1999) and cell death (Cookson *et al*., 1998; Pieper *et al*., 1999). All these events are thought to be directly mediated by peroxynitrite.

In the present study, we report experimental evidence consistent with the possibility that peroxynitrite may also act as a signalling molecule. In particular, endogenous as well as exogenous peroxynitrite promotes a release of arachidonic acid (AA) most likely attributable to stimulation of phospholipase A_2 (PLA_2).

Methods PC12 rat pheochromocytoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 5% foetal bovine serum, penicillin (50 units ml^{-1}) and streptomycin (50 μg ml^{-1}), at 37°C in T-75 tissue culture flasks gassed with an atmosphere of 95% air-5% CO_2. Peroxynitrite was synthesized by the reaction of nitrite with acidified H_2O_2 as described by Radi *et al*. (1991). Peroxynitrite was added as a bolous to samples and, to avoid changes in pH due to the high alkalinity of peroxynitrite stock solution, an appropriate amount of 1 N HCl was added. PC12 cells were subcultured in 6 well plates at 2×10^5 cells per well with [^{3}H]-AA (0.5 μCi ml^{-1}) and grown for 18 h. Before treatments, the cells were washed twice with saline A supplemented with 1 mg ml^{-1} fatty acid-free bovine serum albumin and exposed to tB-OOH or peroxynitrite, in the absence or presence of drugs, in a final volume of 1 ml of saline A. The solution was then separated and centrifuged at 12,000 r.p.m. for 1.5 min; 500 μl of the resulting supernatant were removed and radioactivity was determined in a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland). Results are shown as c.p.m. $\pm$ s.e.mean values from 3–5 independent experiments.

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Results and discussion It was previously reported that tB-OOH stimulated PLA_2 activity in cultured mammalian cells (Chakraborti *et al*., 1993; Kondakova *et al*., 1995). Consistently with these findings, we report (Table 1) that a 10 min exposure of PC12 cells to 200 μM tB-OOH induced a release of AA that was prevented by two general PLA_2 inhibitors, mepacrine (100 μM) or 5,8,11,14-eicosatetraynoic acid (ETYA, 50 μM). Because a combined action of phospholipase C and diacylglycerol lipase has also been implicated in AA release it was of interest to determine the effect of the PLC inhibitor neomycin (1 mM) and that of 1,6-*bis*(cyclohexyloximonocarbonylamino)hexane (RHC-80267, 100 μM), an inhibitor of diacylglycerol lipase. The results illustrated in Table 1 indicate that none of these inhibitors affected the tB-OOH-induced release of AA. Thus, as it was previously observed in endothelial (Chakraborti *et al*., 1993) and P815 tumor (Kondakova *et al*., 1995) cells, AA release stimulated by tB-OOH appears to be specifically mediated by activation of PLA_2 also in PC12 cells. Since the DNA-damaging (Sestili *et al*., 2000) and lethal (Palomba *et al*. unpublished) responses caused by 200 μM tB-OOH in PC12 cells are in part mediated by peroxynitrite, experiments were designed to determine if endogenous peroxynitrite was involved in the tB-OOH-induced release of AA. Cells were pre-treated with either the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1 mM) or the peroxynitrite scavengers L-methionine (20 mM) and Trolox (1 mM) and the tB-OOH-stimulated release of AA measured. Each of these treatments significantly reduced – but did not abolish – the release of AA. Taken together, these results suggest that tB-OOH stimulates PLA_2 activity in PC12 cells *via* peroxynitrite-dependent and -independent mechanisms. If this conclusion is correct, then addition of exogenous peroxynitrite should restore release of AA in L-NAME-supplemented cells challenged with tB-OOH. The results illustrated in Figure 1 are consistent with this possibility. Indeed, while suppression of NOS activity caused a significant reduction in the amount of AA released by tB-OOH, as low as 10 μM peroxynitrite promoted a release of AA comparable to that observed after treatment with tB-OOH in the absence of L-NAME. An even higher increase was mediated by 20 or

Table 1 tB-OOH and peroxynitrite promote a release of AA sensitive to PLA_2 inhibitors

	[^{3}H]-Arachidonic acid release (% of control)		
Treatment	–	+ *tB-OOH* (200 μM)	+ *Peroxynitrite* (50 μM)
Control	100±3.5	179±6.9	187±9.3
100 μM Mepacrine	98±7.8	102±8.9**	105±12.8**
50 μM ETYA	97±5.7	118±10.2**	102±9.4**
100 μM RHC-80267	101±4.5	173±11.3	189±3.8
1 mM Neomycin	102±6.2	174±3.5	175±9.5
1 mM L-NAME	103±4.5	138±3.9*	193±12.7
1 mM Trolox	101±6.7	132±6.7*	101±0.6**
20 mM L-Methionine	99±79	127±5.7*	98±5.0**

[^{3}H]-AA-labelled cells were incubated for 5 min in the absence or presence of the indicated concentrations of drugs and then treated for a further 10 min with either 200 μM tB-OOH or 50 μM peroxynitrite. After treatments, the [^{3}H]-AA release was quantified as described in the Methods section. The results are expresed as per cent of control (875±68 c.p.m.) and are the mean±s.e.mean of 3-5 seperate experiments, each performed in duplicate. *$P<0.01$, **$P<0.001$ *vs* tB-OOH or peroxynitrite-treated cells (unpaired *t*-test).

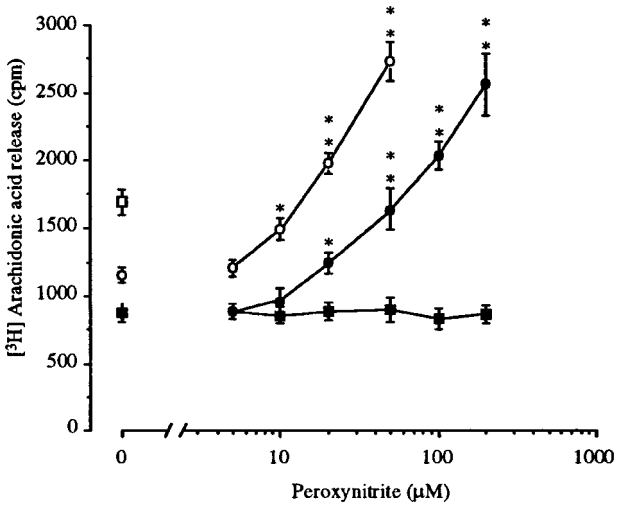

Figure 1 Release of AA mediated by increasing concentrations of peroxynitrite in the absence or presence of L-NAME/tB-OOH. [^{3}H]-AA-labelled cells were exposed for 5 min to 1 mM L-NAME and then treated for a further 10 min with 200 μM tB-OOH in the absence or presence of increasing concentrations of peroxynitrite (open circles). The open square indicates the [^{3}H]-AA release measured in cells treated with tB-OOH alone. Also shown are the results obtained in cells exposed for 10 min to authentic (closed circles) or decomposed (closed squares) peroxynitrite in the absence of additional treatments. After treatments, the [^{3}H]-AA release was quantified as described in the Methods section. Each point is the mean±s.e.mean of 3–5 separate experiments, each performed in duplicate. *$P<0.05$, **$P<0.01$ vs untreated or tB-OOH/L-NAME-treated cells (ANOVA followed by Dunnet's test).

50 μM peroxynitrite. These responses were sensitive to inhibition by 100 μM mepacrine (not shown). It is important to note that, although 10 μM peroxynitrite did not increase the release of AA in the absence of tB-OOH, peroxynitrite concentrations of $\geqslant$20 μM effectively stimulated this response (Figure 1). These effects were not observed using decomposed peroxynitrite. These results illustrated in Table 1 provide the additional information that (a) authentic peroxynitrite did not enhance AA release in cells pretreated with PLA_2 inhibitors or peroxynitrite scavengers and that (b) the release of AA mediated by peroxynitrite is insensitive to neomycin, RHC-80267 and L-NAME. Taken together, these results strongly suggest that exogenous peroxynitrite stimulates PLA_2 activity in PC12 cells. These results also emphasize the specificity of L-NAME in preventing the peroxynitrite-dependent activation of PLA_2 in cells treated with tB-OOH (see above). In summary, the data presented in this study demonstrate that exogenous as well as endogenous peroxynitrite promoted release of AA in PC12 cells and strongly suggest that this response is mediated by activation of PLA_2. This implies that peroxynitrite may act as a signalling messenger rather than, or in addition to, as a direct effector molecule. Whether peroxynitrite directly or indirectly activated PLA_2 is a matter of future investigations. The experimental approach utilized in the present study does not provide sufficient information to speculate on the identity of the PLA_2 responsive to peroxynitrite. Studies are in progress to assess the possible involvement of secretory or cellular Ca^{2+}-dependent/independent PLA_2. It will also be of importance to investigate whether the peroxynitrite-dependent release of AA is casually linked to peroxynitrite-dependent induction of DNA cleavage (Sestili *et al*., 2000) and toxicity (Palomba *et al*. unpublished) that were previously observed in PC12 cells treated with tB-OOH. As a final note, peroxynitrite was shown to progressively stimulate release of AA over a concentration-range which is compatible with the amounts of peroxynitrite produced in various pathologies; thus, the relevance of the peroxynitrite-dependent release of AA in an array of pathological conditions (including inflammation) needs to be investigated.

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References

BROWN, G.C. (1999). Nitric oxide and mitochondrial respiration. *Biochim. Biophys. Acta,* **1411,** 351–369.

CHAKRABORTI, S., MICHAEL, J.R., GURTNER, G.H, GHOSH, S.S., DUTTA, G. & MERKER, A. (1993). Role of a membrane-associated serine esterase in the oxidant activation of phospholipase A_2 by t-butyl hydroperoxide. *Biochem. J.,* **292,** 585–589.

COOKSON, M.R., INCE, P.G. & SHAW, P.J. (1998). Peroxynitrite and hydrogen peroxide induced cell death in the NSC34 neuroblastoma x spinal cord cell line: role of poly (ADP-ribose) polymerase. *J. Neurochem.,* **70,** 501–508.

KONDAKOVA, I.V., PEIRETTI, F., NALBONE, G. & LAFONT, H. (1995). Phospholipase A stimulation in tumor cells by subtoxic concentration of tert-butyl hydroperoxide. *Biochim. Biophys. Acta,* **1258,** 297 – 302.

PIEPER, A.A., VERMA, A., ZHANG, J. & SNYDER, S.H. (1999). Poly(ADP-ribose)polymerase, nitric oxide and cell death. *Trends Pharmacol. Sci.,* **20,** 171 – 181.

RADI, R., BECKAM, J.S., BUSH, K.M. & FREEMAN, B.A. (1991). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.,* **266,** 4244 – 4250.

RUBBO, H., RADI, R., TRUJILLO, M., KALYANARAMAN, B., BARNES, S., KIRK, M. & FREEMAN, B.A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.,* **269,** 26066 – 26075.

SALGO, M.G., BERMUDEZ, E., SQUADRITO, G.L. & PRYOR, W.A. (1995). Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes. *Arch. Biochem. Biophys.,* **322,** 500 – 505.

SESTILI, P., CLEMENTI, E., GUIDARELLI, A., SCIORATI, C. & CANTONI, O. (2000). Endogenous and exogenous nitric oxide enhance the DNA stran scission induced by tert-butylhydroperoxide in PC12 cells via peroxynitrite-dependent and independent mechanisms, respectively. *Eur. J. Neurosci.,* **12,** 145 – 154.

SZABÓ, C. & OHSHIMA, H. (1997). DNA damage induced by peroxynitrite: subsequent biological effects. *Nitric Oxide,* **1,** 373 – 385.

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