H_2O_2 renders cells accessible to lysis by exogenous phospholipase A_2: a novel mechanism for cell damage in inflammatory processes

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Abstract Phospholipase A_2 (PLA_2) and H_2O_2, secreted from activated inflammatory cells, play a central role in the tissue damage occurring in inflammatory processes. However, while exogenous PLA_2 alone does not cause cell lysis, it readily does so when acting with H_2O_2. We have found that H_2O_2 degrades cell surface proteoglycans, thus rendering the membrane PL accessible to hydrolysis by exogenous PLA_2. This novel mechanism introduces a role for cell surface proteoglycans in protection of cells from damage by pro-inflammatory agents, and may assign a central role for the combined action of H_2O_2 and PLA_2 in inflammatory and bacteriocidal processes.

Key words: Phospholipase A_2; H_2O_2; Cell surface proteoglycan; Inflammation

1. Introduction

Phospholipase A_2 (PLA_2), which hydrolyses cell membrane phospholipids (PL) to produce free fatty acid and lysophospholipids, plays a major role in inflammatory processes [1,2]. The intracellular cytosolic PLA_2 ($cPLA_2$) preferentially hydrolyses arachidonic acid (ArAc) containing PL, thus producing the precursor of the eicosanoids, which are mediators of cell activation in inflammatory processes [3]. The secreted PLA_2 ($sPLA_2$) is found at high levels in body fluids in inflammatory diseases, such as septic shock, arthritis, acute lung injury, psoriasis and bowel disease, and has been implicated in the pathophysiology of these diseases [4]. The $SPLA_2$, secreted from activated inflammatory cells into the extracellular fluid [5] hydrolyses cell membrane PL of target cells, and thus takes a major part in the cell lysis and the subsequent tissue damage occurring in these diseases.

However, previous studies raised the possibility that exogenous PLA_2 alone does not cause cell lysis [6]. In animal models the administration of exogenous PLA_2 does not induce inflammation unless a pro-inflammatory background has already been established [4]. In accordance with this, it has been shown that the induction of lysis in cultured cells requires the combined action of inflammatory agents, such as reactive oxygen species (H_2O_2, peroxy radicals), proteases and phospholipases [7]. Each of these agents alone may damage cells only at pharmacological levels, while at physiological levels they are not toxic, and the synergistic action of more than one agent is required to induce cell damage. In accordance with this we have found that while various types of exogenous PLA_2 alone do not cause cell damage, they can do so when the cells are treated with subtoxic concentrations of H_2O_2 (see Fig. 1). This is in contrast to results obtained with a variety of phospholipid membranes (liposomes), which are readily hydrolysed by different types of PLA_2 from various sources [8]. This may indicate that target cells have a protective mechanism against PLA_2, which is eliminated by reactive oxygen species when the inflammatory process is activated.

The present study was undertaken to elucidate the combined action of H_2O_2 and extracellular PLA_2 in the hydrolysis of cell membrane PL and the subsequent cell damage. For this purpose we have studied the PL hydrolysis and cell damage induced by treatment of cultured cells with glucose oxidase (GO), an H_2O_2 generator, and exogenous PLA_2.

2. Materials and methods

All materials were purchased from Sigma (St. Louis, MO), unless specified otherwise.

2.1. Cell culture

BGM (green monkey kidney epithelial cells) were grown in 5% CO_2 in DMEM supplemented with 10% FCS and antibiotics (Biological Industries, Israel).

2.2. Induction of cell damage and release of arachidonic acid

Three kinds of commercially available PLA_2s were used: (1) type II, *Crotalus atrox* PLA_2, which is of the same type as the human synovial fluid PLA_2 [4,9]; (2) type I, *Naja mocambique* PLA_2; and (3) bovine pancreas PLA_2 (type I). Cells were incubated in 1 ml/well of serum-free DMEM supplemented with 3 mg/ml BSA. Cells were treated with GO (0.4 units/well) or directly with H_2O_2 (1 mM) for 1 h. The cells were then washed (to avoid possible interference of H_2O_2 with the action of PLA_2 itself) and incubated in fresh medium to which the PLA_2 (2 units/well) was added.

2.3. Release of cellular arachidonic acid

BGM cells were labelled with [^{3}H]ArAc (0.5 μCi/24 well plate) (Amersham) during inoculation and grown to confluence. Following treatment with GO, and/or with exogenous PLA_2, the culture medium was collected and subjected to lipid extraction in chloroform:methanol (1:1). The lipids were chromatographed on silica thin layer plates (Whatman), developed in chloroform:methanol:water (65:35:5), and the band corresponding to the radioactive ArAc was scraped and counted.

2.4. Hydrolysis of liposomal PL by PLA_2

Phosphatidylcholine (PC) liposomes were interacted with exogenous PLA_2 as follows [10]: 30 nmol 1-stearoyl,2-ArAc-PC vesicles containing 15 nmol lyso-PC and 10 nCi of 1-stearoyl, [2-^{14}C]ArAc PC, in Tris-HCl buffer (100 nM, pH 8.0) containing 10 mM $CaCl_2$ and 0.1% BSA, were incubated with increasing concentrations of either *Naja mocambique* or *Crotalus atrox* PLA_2. Following this treatment the released fatty acid was extracted and separated from PL and its radioactivity was counted.

2.5. Degradation of cell surface glycosaminoglycans (GAG)

Cultured BGM cells were labelled overnight with $Na_2^{35}SO_4$. The

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cells were then washed thoroughly and subjected to treatment with GO. The culture medium was then collected and chromatographed on a Sepharose 6B column (Pharmacia), using PBS as the eluant.

2.6. PL oxidation

Following exposure of the cells or liposomes to H_2O_2, PL were extracted into chloroform:methanol (1:1) and oxidized PL were determined by the formation of conjugated dienes [11] and by the formation of malondialdehydes (MDA) [12].

2.7. Determination of cell damage

2.7.1. Absorption of Neutral red. After treatment, cells were incubated with a solution of Neutral red in PBS (0.02 mg/ml) for 1 h. The cells were then washed extensively, and the absorbed color extracted from the cells with ethanol:water:acetic acid (1 : 1 : 0.1) and the absorbance read at 540 nm [13].

2.7.2. Release of ^{51}Cr. Cells were labelled with 10 μCi/ml ^{51}Cr at the time of seeding, and the release of radioactive chromium into the culture medium of treated cells was determined [14,15].

3. Results and discussion

Fig. 1 depicts the release of ArAc by cells treated with PLA_2 after prior treatment with H_2O_2 or with GO, as well as without prior treatment. When the cells were treated with exogenous PLA_2 alone, little PL hydrolysis was observed. This level of PL hydrolysis is practically insignificant as it does not induce any cell damage, as measured by both viability staining and the release of ^{51}Cr (not shown). However, marked PL hydrolysis occurred when the cells were treated with either GO or H_2O_2 prior to the treatment with PLA_2.

Fig. 1 shows that the same effect is produced by H_2O_2 or GO, demonstrating that it is due to the presence of H_2O_2. This was further confirmed by the addition of catalase (which decomposes H_2O_2) where no PL hydrolysis was obtained. Treatment with GO produces a steady concentration of H_2O_2 throughout the course of the experiment, while H_2O_2 itself, added in one dose at the beginning of the experiment, is gradually depleted. Accordingly, in some experiments we found that direct treatment with GO produces less variable results. Therefore in the following experiments only GO was used.

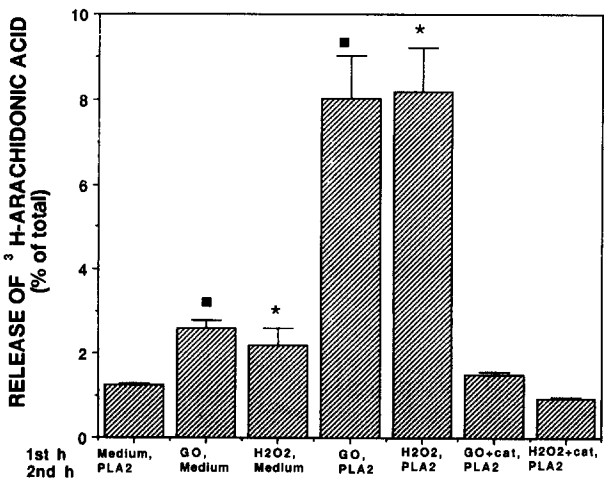

Fig. 1. Release of ArAc by the sequential treatment of cells with H_2O_2 and PLA_2. BGM cells, prelabelled with [^{3}H]ArAc were washed and treated with H_2O_2 or GO for 1 h. The cells were then washed again and treated with *Crotalus atrox* PLA_2 for 1 h. The release of [^{3}H]ArAc was determined as described in section 2. * and ■, significant at $P < 0.005$; ▲, significant at $P < 0.01$.

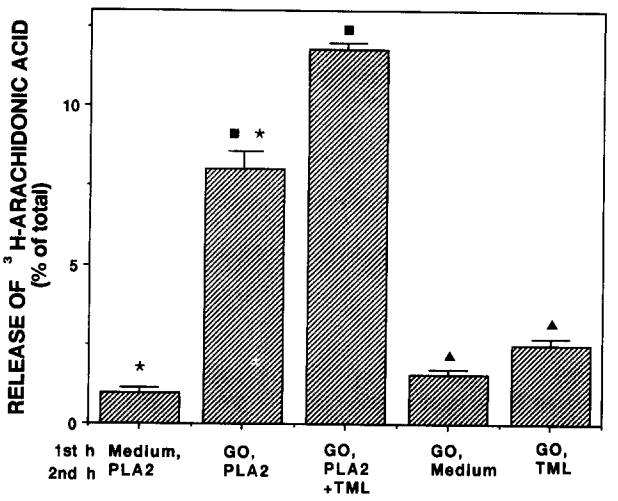

Fig. 2. Release of ArAc in cells treated with thimerosal. The same procedure was followed as in the experiment of Fig. 1, except that in the experiments with thimerosal (TML), 40 μM thimerosal was added along with the enzyme.

Fig. 1 depicts results obtained with type II PLA_2, and similar results were obtained with both the *Naja* and the pancreatic PLA_2 (5–6-fold and 4–5-fold increase, respectively, when the cells were treated with both GO and PLA_2 compared with treatment with PLA_2 or GO alone).

As we have previously shown, the release of ArAc paralleled the cell damage as measured by the release of ^{51}Cr from labelled cells [14,15]. This was reconfirmed in the present study by determining the percent of viable cells using the Neutral red method. Untreated cells were taken as 100%, and cells treated with PLA_2 had the same absorption at 540 nm, indicating no loss of viability. Cells treated with PLA_2 following exposure to GO or H_2O_2 were only 20% viable, while the viability following treatment with GO or H_2O_2 alone was more than 95%, or 85%, when measured by ^{51}Cr or Neutral red, respectively. The viablity test, particularly with ^{51}Cr, shows that the treatment with H_2O_2 is not sufficient to make the cells permeable to the exogenous PLA_2. It thus seems that H_2O_2 conditions the cell surface to the action of PLA_2.

In search of the mechanism of this phenomenon, we considered previous studies which investigated the effect of H_2O_2 on the level of endogenously produced ArAc. These studies reported that H_2O_2 enhances the level of fatty acids released from the sn2 position of cell membrane PL, and suggested two mechanisms which might be relevant to exogenous PLA_2: (a) H_2O_2 oxidizes membrane PL, which makes them a better substrate for PLA_2 [16,17]; (b) H_2O_2 inhibits fatty acid reacylation by depleting the cells of the ATP necessary for the reacylation reactions, and perhaps by a direct effect on arachidonyl-CoA synthetase [18,19].

To examine the possibility that the enhanced PL hydrolysis observed in the present study is due to lipid oxidation, the cells were treated with GO or H_2O_2 directly, and PL oxidation was determined by the two methods described in section 2.6. It was found, by both methods, that in the culture medium used in this study (DMEM, not containing Cu^+), these treatments did not cause any lipid oxidation. To confirm this, liposomes composed of 1-stearoyl,2-arachidonyl PC were treated with H_2O_2 in DMEM medium, and again, no lipid

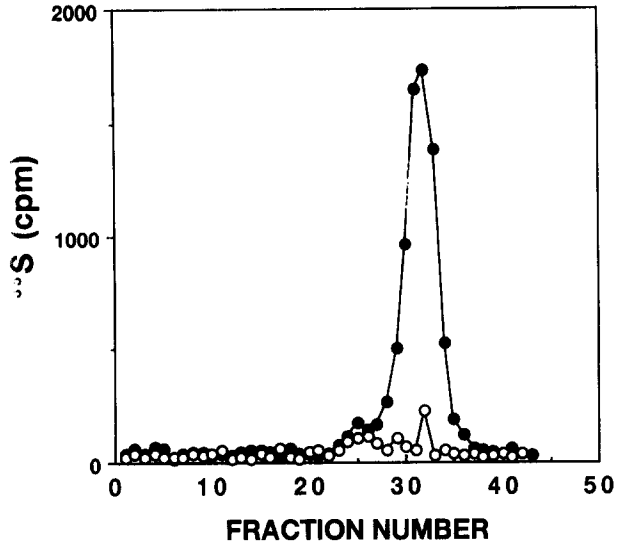

Fig. 3. Degradation and release of cell surface GAG by H_2O_2. Cultured BGM cells were labelled overnight with $^{35}SO_4$, 1.2 mCi per 24 well plate, then washed and treated with GO for 1 h, as in the experiment of Fig. 1. The GAG released into the culture medium were chromatographed and determined as described in section 2. ●, Cultured medium of H_2O_2-treated cells; ○, culture medium of untreated cells.

oxidation was detected by either of the two methods. In positive control experiments, CuCl (100 μM) was added to the cell culture medium and this induced a significant lipid oxidation (10 nmol MDA/mg cell protein). It should be noted that the studies reporting that oxidized PL are better substrates for PLA_2 were practically all done with lipid membranes [16,17] while no clear evidence for PL oxidation in intact cells by H_2O_2 has been provided. Moreover, as noted above, PLA_2 of different types and sources easily hydrolyses all kinds of liposomal PL without prior oxidation. Hence, lipid oxidation does not account for the conditioning of cell membrane PL to hydrolysis by exogenous PLA_2.

The possible inhibition of fatty acid reacylation by H_2O_2 does not explain its effect on the release of fatty acid by exogenous PLA_2 observed here for the following reasons. In the present study the hydrolysis of cell membrane PL was determined in the presence of albumin (3 mg/ml), known to bind free fatty acids and prevent their reacylation [20]. In addition, to specifically examine the role of reacylation in the elevation of free fatty acid levels, the effect of PLA_2 and GO on ArAc release was determined in the presence of thimerosal, an inhibitor of fatty acid reacylation [20]. In this experiment thimerosal was added after the treatment with H_2O_2, along with the treatment with PLA_2. As shown in Fig. 2, thimerosal further elevated the level of free ArAc considerably beyond the level obtained by the combination of H_2O_2 and PLA_2 alone, thus demonstrating that in the presence of H_2O_2 alone, reacylation is not blocked. Thus, a possible inhibition of reacylation by H_2O_2 does not account for the enhancement of ArAc release by the application of the PLA_2 following treatment with GO. Fig. 1 also shows that the treatment with GO induced the release of a small but significant amount of ArAc. This might be due to specific activation of $CPLA_2$ via PKC and MAP kinase as previously reported [21].

We then considered previous reports that H_2O_2, probably through its reactive oxygen derivatives, may oxidize and break down proteoglycans [22,23]. We hypothesized that these macromolecules, which cover the cell surface, normally protect the cell surface lipids from attack by PLA_2, and that H_2O_2 degrades the proteoglycans, thus allowing the enzyme access to the membrane PL. To examine this hypothesis, the cell GAG were labelled overnight with $^{35}SO_4^{2-}$, then washed and treated with H_2O_2 as in the experiment of Fig. 1 and the release of labelled GAG into the extracellular medium was determined. It was found that H_2O_2 induces the release of a considerable amount (about 30%) of labelled GAG from the cell surface, while no labelled GAG were released from untreated cells. To confirm the degradation and to characterize the molecular weight of the released GAG, the extracellular medium from GO-treated and from untreated cells was subjected to column chromatography. As shown in Fig. 2, H_2O_2 broke the GAG into fragments of 7000–10000 MW, while no labelled GAG, in any MW range, were detected in the medium of untreated cells. H_2O_2 has been also shown to inhibit proteoglycan synthesis [24]. However, the possible inhibition of GAG synthesis does not seem to contribute to the effect observed here for the following reasons. As described in section 2, in our experiment, during the GAG labelling period all the cells underwent the same treatment, i.e. overnight labelling in the absence of H_2O_2. The cells were then washed thoroughly and subjected to H_2O_2 for 1 h, which is a relatively short period of time compared to proteoglycan turnover, the half-life of which is usually from hours to days [25,26]. This is supported by the present finding (Fig. 3) that in the control cells (no H_2O_2) no release of GAG to the extracellular medium was obtained, showing that the GAG turnover in our system is too slow to exhibit inhibition of synthesis during this short treatment with H_2O_2. It is thus plausible to conclude that H_2O_2 strips GAG from the cell surface thus exposing it to the action of exogenous PLA_2.

This conclusion is also supported by an ongoing study, showing that hyaluronic acid (HA) protects liposomal PL from hydrolysis by snake venom PLA_2, but this protection is abolished if the HA was degraded by H_2O_2 prior to introduction of the enzyme (unpublished results). In addition, in view of the reports that heparin may bind and inhibit PLA_2 [27,28] it is possible that by stripping the cells of GAG, H_2O_2 eliminates this inhibition.

In conclusion, the present study demonstrates that (1) cell surface proteoglycans have a protective role against exogenous PLA_2; (2) in the process of cell damage by the combined action of H_2O_2 and exogenous PLA_2, a major role of H_2O_2 is in the oxidation and degradation of the cell surface proteoglycan, which renders the cell membrane PL accessible to hydrolysis by PLA_2. This mechanism may also apply to the process by which polymorphonuclear (PMN) cells kill bacteria. It may be that the PLA_2 secreted by PMN is actually designed as an anti-bacterial weapon to act in concert with reactive oxygen species to kill invading bacteria. This novel mechanism, in addition to possible inhibition of fatty acid reacylation and PL oxidation by H_2O_2, may assign a central role to the combined action of H_2O_2 and PLA_2 in inflammatory and bactericidal processes.

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References

[1] Wong, P.K.Y. and Dennis, E.A. (1990) Adv. Exp. Med. Biol. 275.
[2] Flower, R.J. and Blackwell, G.J. (1979) Nature 278, 456–459.
[3] Clark, J.D., Milona, N. and Knopf, J.L. (1990) Proc. Natl. Acad. Sci. USA. 87, 7708–7712.
[4] Vadas, P., Browning, J., Edelson, J. and Pruzanski, W. (1993) J. Lipid Mediators 8, 1–30.
[5] Scott, D.L., White, S.P., Browning, J.L., Rosa, J.J., Gelb, M.H. and Sigler, P.B. (1991) Science 254, 1007–1010.
[6] Oka, S. and Arita, H. (1991) J. Biol. Chem. 226, 9956–9960.
[7] Ginsburg, I., Gibbs, D.F., Schuger, L., Johnson, K.J., Ryan, U.S., Ward, P.A. and Varani, J. (1989) Free Radical Biol. Med. 7, 369–376.
[8] Pfeilschifter, J., Schalkwijk, C., Briner, V.A. and van den Bosch, H. (1993) J. Clin. Invest. 92, 2516–2523.
[9] Glaser, K.B., Mobilio, D., Chang, J.Y. and Senko, N. (1993) Trends Pharmacol. Sci. 14, 92–98.
[10] Shakir, K.M. (1981) Anal. Biochem. 14, 64–70.
[11] Corongio, P. and Banni, S. (1994) Methods Enzymol. 233, 303–310.
[12] Naito, C., Kawamura, M. and Yamamoto, Y. (1993) Ann. NY Acad. Sci. 676, 27–45.
[13] Fautz, R., Husein, B. and Hechenberger, C. (1991) Mutation Res. 253, 173–179.
[14] Ginsburg, I., Mitra, M., Gibbs, D.F., Varani, J. and Kohen, R. (1993) Inflammation 17, 295–319.
[15] Yedgar, S., Dagan, A., Dan, P., Eitan, A., Herschkoviz, R. and Ginsburg, I. (1994) in: Lipid Mediators in Health and Disease (U. Zor, ed.), pp. 39–43, Freund, London.
[16] Salgo, M.G., Corongiu, F.P. and Sevanian, A. (1993) Arch. Biochem. Biophys. 304, 123–132.
[17] van Kuijk, F.J.G.M., Sevanian, A., Handelman, G.J. and Dratz, A.E. (1987) Trends Biochem. Sci. 12, 31–34.
[18] Sporn, P.H.S., Marshall, T.M. and Peters-Golden, M. (1992) Am. J. Respir. Cell. Mol. Biol. 7, 307–316.
[19] Duane, P.G., Rice, K.L., Charboneau, D.E., King, M.B., Gilboe, D.P. and Niewoeher, D.E. (1991) Am. J. Respir. Cell. Mol. Biol. 4, 408–416.
[20] Kroner, E.E., Peskar, B.A., Fischer, H. and Ferber, E. (1981) J. Biol. Chem. 256, 3690–3697.
[21] Rao, G.N., Runge, M.S. and Alexander, R.W. (1995) Biochim. Biophys. Act. 1265, 67–72.
[22] Roberts, C.R., Roughley, P.J. and Mort, J.S. (1989) Biochem. J. 259, 805–811.
[23] Klebanoff, S.J., Kinsella, M.G. and Wight, J.N. (1993) Am. J. Pathol. 143, 907–917.
[24] Matsubara, T., Kimura, T., Kuroda, T. and Hirota, K. (1992) Br. J. Rheumatol. 31 Suppl 1, 27–32.
[25] Vijayagopal, P. (1993) Biochem. J. 294, 603–611.
[26] Kowanko, I.C., Bates, E.J. and Ferrante, A. (1989) Immunol. Cell Biol. 67, 321–329.
[27] Murakami, M., Kudo, I. and Inoue, K. (1993) J. Biol. Chem. 268, 839–844.
[28] Barbour, S.E. and Dennis, E.A. (1993) J. Biol. Chem. 268, 21875–21882.