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Involvement of a serine esterase in oxidant-mediated activation of phospholipase A_2 in pulmonary endothelium

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Exposure of bovine pulmonary arterial endothelial cells to 1 mM H_2O_2 stimulated associated TAME-esterase and PLA_2 activities. Pretreatment with the serine esterase inhibitors: PMSF (1 mM), DFP (1 mM), and α_1-PI (1 mg/ml) inhibited H_2O_2-induced stimulation of TAME-esterase and PLA_2 activities. The TAME-esterase and PLA_2 activities under H_2O_2 exposure were determined to be linearly correlated. Affinity labelling of the endothelial cell membrane with [^{3}H]DFP demonstrated that the serine esterase resides in a protein having molecular weight of 29000 daltons (29 kDa) which is similar to that of elastase. Treatment of the endothelial cell homogenate with trypsin (1 μg/ml) also stimulated PLA_2 activity.

Serine esterase; Phospholipase A_2; Hydrogen peroxide; Oxidant; Antiproteases; Trypsin; Endothelial cells

1. INTRODUCTION

Oxidant-mediated activation of phospholipase A_2 (PLA_2) in bovine pulmonary arterial endothelial cells has been well documented [1]. PLA_2 plays a pivotal role in the production of various types of eicosanoids under stimulating conditions which contribute to lung injury [2,3]. Despite intense interest in elucidating the role of eicosanoids in the development of pathological consequences in the lung, neither the ability of pulmonary arterial endothelial cells to activate proteases nor the process involved in the activation of PLA_2 under agonist-exposed conditions have been adequately appreciated. Earlier studies revealed that in pancreas PLA_2 is present in an inactive form and becomes activated by the addition of a small amount of trypsin [4]. To gain an insight into the biochemical mechanisms associated with the oxidant-caused activation of PLA_2 and the role played by the vascular endothelium, the effect of the oxidant H_2O_2 on serine esterase activity and the role of the stimulated serine esterase, if any, on the activation of PLA_2 in pulmonary vascular endothelial cells have been investigated in this communication.

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; TAME, *p*-tosyl-L-arginine methylester; H_2O_2, hydrogen peroxide; α_1-PI, alpha$_1$ proteinase inhibitor; AA, arachidonic acid; PBS, phosphate buffered saline; PLA_2, phospholipase A_2

2. MATERIALS AND METHODS

2.1. *Cell culture*

Bovine pulmonary arterial endothelial cells (ATCC 209) obtained from the American Type Culture Collection (Rockville, MD) were studied between passages 19 and 25. Cells were maintained in DMEM supplemented with 20% fetal calf serum (GIBCO) and non-essential amino acids (GIBCO). Cells were subcultured after treatment with 0.25% trypsin (SIGMA). All experiments were performed on confluent monolayers and in serum-free media supplemented with 1 mg/ml essentially fatty-acid-free bovine serum albumin.

2.2. *Preparation of membranes*

The cells were grown in T-150 flasks. The monolayers were washed twice with ice-cold 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and incubated at 37°C for 30 min with a hypotonic solution of 5 mM HEPES buffer (pH 8.0). The cells were then scraped from the flasks and homogenized using a Dounce homogenizer with a tight fitting pestle. The homogenate was then centrifuged at 100000 × *g* for 1 h at 4°C. The pellet was resuspended by briefly vortexing in 20 mM Tris-HCl buffer (pH 8.0) containing 250 mM sucrose and 1 mM $MgCl_2$, aliquotted and stored at −70°C. Protein concentration was measured by following the method of Lowry et al. [6].

2.3. *Determination of TAME esterase activity*

Trypsin-like activity was assessed by determining the rate of hydrolysis of *p*-tosyl-L-arginine methylester (TAME, SIGMA) based on the method of Hummel [7]. Briefly, 300 μl of the substrate (37.9 mg/ml in H_2O) was added to 2.4 ml of 0.05 M Tris-buffer containing 10 mM $CaCl_2$ (pH 8.5). The reaction was initiated by the addition of appropriate amount of membrane protein to a final volume of 3.0 ml. Hydrolysis was measured by an increase in OD at 247 nm. A blank without enzyme activity was included to monitor for spontaneous hydrolysis of the substrate. For the determination of the effect of H_2O_2 on TAME-esterase activity, the cells were exposed to H_2O_2 (1 mM) for 20 min. The membrane fraction was isolated and TAME-esterase activity was determined. The pulmonary arterial endothelial cells were pretreated with PMSF (1 mM), DFP (1 mM) and α_1-PI (1 mg/ml) for 20 min before the addition of H_2O_2. Membrane fractions under the inhibitors and/or H_2O_2-exposed conditions were isolated and TAME-esterase activity was measured.

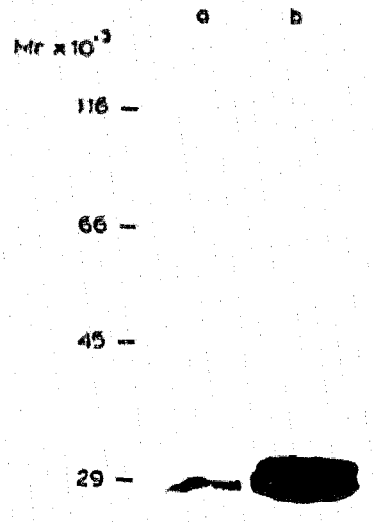

Fig. 1. Affinity labelling of endothelial cell membrane and pure elastase with [^{3}H]DFP; (lane a) membrane isolated from endothelial cells (unstimulated), (lane b) pure elastase.

2.4. *Measurement of [^{14}C]AA release and PLA$_2$ activity*

Cells grown in 6-well plates (Coster) were washed twice with phosphate buffered saline (PBS, Sigma) and incubated for 20 h with [^{14}C]arachidonic acid (specific activity 54.6 mCi/mmol; 2 μCi/ well). After incubation, the supernatant was removed and the cells were washed twice with PBS. To measure H_2O_2-induced AA release, cells were exposed to 1 mM H_2O_2 for 20 min. The medium was then removed and AA release was measured by following the method described by Chakraborti et al. [1]. To determine H_2O_2-induced PLA$_2$ activity, the endothelial cells grown in 6-well plates were incubated with H_2O_2 (1 mM) for 20 min. After incubation, endothelial cell monolayers were washed twice with PBS (pH 7.2). The cells were then removed from the PBS with a rubber policeman and centrifuged in a microfuge at 12000 × g for 2 min at room temperature. The pellet was suspended in PBS and disrupted by sonication with a cell sonicator. Twenty microliters of the broken-cell preparation (1–3 mg protein, ~ 10^5 cells) was added to 30 μl of the reaction mixture, which contained (final concentration): Tris-buffer (100 μM), NaCl (100 mM), deoxycholate (1 mM), and the phospholipid L-3-phosphatidyl choline-L-1-steroyl-2-[1-^{14}C]arachidonyl (specific activity 58.3 mCi/mmol; 10 μM). Phospholipase A$_2$ activity was assayed at pH 9.0 by following the method described by Chakraborti et al. [1].

To determine the effect of serine esterase inhibitors on AA release and PLA$_2$ activity, cells were pretreated with PMSF (1 mM), DFP (1 mM) and α_1-PI (1 mg/ml) for 20 min before the addition of H_2O_2 (1 mM).

2.5. *Cell viability*

The dose of the agents used in this present study did not affect the cell viability as assessed by Trypan blue exclusion studies.

2.6. *In vitro assay of phospholipase A$_2$ activity in endothelial cell homogenate*

Bovine pulmonary arterial endothelial cells grown in 6-well plates were washed twice with PBS (pH 7.2). The cells were then removed from the PBS with a rubber policeman and protein concentration was maintained at 3 mg/ml. The resulting cell suspension was then treated with trypsin (1 μg/ml) for 20 min at 37°C. PLA$_2$ activity was measured by following the method of Chakraborti et al. [1].

2.7. *Affinity labelling of endothelial cell membrane and pure elastase with [^{3}H]DFP*

100 μg of membrane preparation isolated from control cells were placed in a microfuge tube. In another microfuge tube, 100 μg of pure elastase (Sigma) were placed. The contents of the tubes were then lyophylized and reconstituted with 100 μl of 0.1 M Tris-HCl, pH 7.5, and then 10 μCi of [1,3-^{3}H]DFP (5.2 Ci/mmol; NEN) were added. The reconstituted samples were then incubated for 60 min at 37°C. After incubation, 10 μl of 100 mM cold DFP (Sigma) was added to the tubes to terminate the labelling reaction. The samples were then dialyzed against 0.001% SDS for 24 h and lyophylized. The lyophylized samples were dissolved in sample buffer containing 5% mercaptoethanol and analyzed by electrophoresis on a 10% polyacrylamide gel in the presence of 0.1% SDS by the procedure described by Laemmli [5]. Gels were impregnated with Enhancer (NEN) for 30 min, washed 4 times with water, dried and exposed for 10 days at −70°C using XAR 5 film (Eastman-Kodak). Proteins used as molecular weight standards were: *Escherichia coli* beta-galactosidase, 116000 Da; bovine serum albumin, 66000 Da; ovalbumin, 45000 Da; and carbonic anhydrase, 29000 Da (Sigma).

3. RESULTS AND DISCUSSION

Bovine pulmonary arterial endothelial cells exhibit a serine esterase profile as evidenced by affinity labelling of [^{3}H]DFP with membranes isolated from normal cells (Fig. 1). The enzyme has been found to be preferentially localized in the membrane fraction and *p*-tosyl-L-arginine methylester (TAME) is the synthetic substrate

Table I

Effect of H_2O_2 and/or serine esterase inhibitors on TAME-esterase and phospholipase A$_2$ activities in bovine pulmonary arterial endothelial cells

Conditions	TAME-esterase activity (OD_{247}/min/mg)	PLA$_2$ activity (pmol AA/min/mg)
None	0.47 ± 0.11	3.96 ± 0.47
H_2O_2 (1 mM)	3.54 ± 0.59[a]	10.48 ± 0.85[a]
PMSF (1 mM)	0.09 ± 0.008[b]	2.85 ± 0.42
PMSF (1 mM) + H_2O_2 (1 mM)	0.82 ± 0.089[c,d]	4.81 ± 0.59[d]
DFP (1 mM)	0.08 ± 0.007[b]	2.91 ± 0.31
DFP (1 mM) + H_2O_2 (1 mM)	0.91 ± 0.14[c,d]	4.87 ± 0.64[d]
α_1-PI (1 mg/ml)	0.19 ± 0.032[c]	3.08 ± 0.42
α_1-PI (1 mg/ml) + H_2O_2 (1 mM)	1.18 ± 0.21[c,d]	4.97 ± 0.72[d]

Results are mean ± S.E.M. (n = 4); statistical analyses were by Student's t-test; [a] $P < 0.001$ compared with control; [b] $P < 0.01$ compared with control; [c] $P < 0.05$ compared with control; [d] $P < 0.001$ compared with H_2O_2 treatment condition.

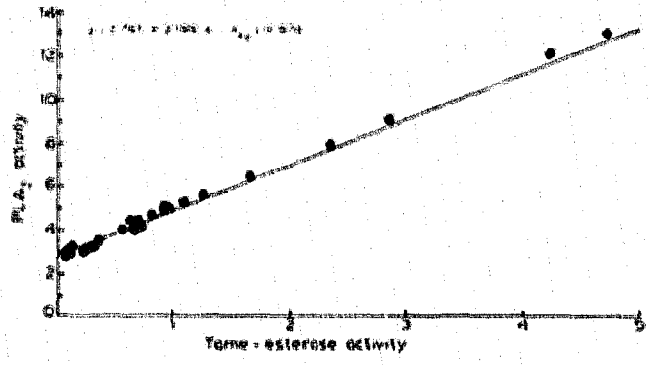

Fig. 2. The regression line of phospholipase A_2 activity (y) against TAME-esterase activity (x) based on the observed data (Table I).

for the enzyme (data not shown). The autoradiogram of [^{3}H]DFP binding with endothelial cell membrane shows that the TAME-esterase activity resides in a protein of active molecular mass (M_r) 29 000 Da (Fig. 1). TAME-esterase activity in the cell membrane isolated from H_2O_2-exposed cells has been found to be stimulated compared to the control condition (Table I). Pretreatment of the cells with serine esterase inhibitors: PMSF and DFP inhibit the TAME-esterase activity for both control and H_2O_2-stimulated conditions (Table I). Thus, from the above results it appears that the endothelial cells possess a serine esterase in membrane-bound form and H_2O_2 stimulates the esterase activity. From Table I it also appears that H_2O_2 stimulates phospholipase A_2 activity in bovine pulmonary arterial endothelial cells and that pretreatment of the cells with serine esterase inhibitors (PMSF and DFP) prevents the PLA_2 activity caused by H_2O_2. The stimulatory profile of PLA_2 activity has been found to correspond well with the TAME-esterase activity as evidenced by linear regression and correlation analysis (Fig. 2).

Because of the previous report of the involvement of trypsin in the activation of pancreatic phospholipase A_2 [4] the effect of trypsin on PLA_2 activity in bovine pulmonary arterial endothelial cell homogenate has been measured. Treatment of the cell homogenate with trypsin has been found to activate PLA_2 activity (Table II). Overall, it appears from the present study that H_2O_2 activates a trypsin-like serine esterase activity which subsequently enhances PLA_2 activity in bovine pulmonary vascular endothelial cells. The underlying mechanisms of the oxidant-mediated activation of TAME-esterase and the subsequent stimulation of PLA_2 activity is currently under investigation.

To determine whether the physiologically occurring antiprotease has any effect on the H_2O_2-induced stimulatory profile of TAME-esterase and PLA_2 activities in

Table II

Effect of trypsin on PLA_2 activity in bovine pulmonary arterial endothelial cell homogenate

Conditions	PLA_2 activity (pmol AA/min/mg)	% change
None	3.52 ± 0.38	
Trypsin (1 μg/ml)	12.46 ± 1.12*	+254

Results are mean ± S.E.M. ($n = 4$); statistical analyses were by Student's t-test; *$P < 0.001$ compared with control.

bovine pulmonary arterial endothelial cells, the cells were pretreated with α_1-PI (1 mg/ml) before the addition of H_2O_2. The results show that α_1-PI inhibits the increase in TAME-esterase and PLA_2 activities caused by H_2O_2 (Table I).

Oxidants, including H_2O_2, have been shown to increase pulmonary vasoconstriction and pulmonary edema [3,8]. The vasoconstriction and pulmonary edema caused by a variety of agonists have been found to be prevented by antiproteinases [9]. There are numerous antiproteinases present in the body and one of these, α_1-PI is believed to be particularly important in controlling the proteolytic events in tissues, especially against proteolytic attack of the lungs [10]. The present study of the effect of α_1-PI in preventing H_2O_2-mediated stimulatory profile of TAME-esterase and PLA_2 activities in pulmonary vascular endothelial cells raises the possibility that antiproteases may prove useful in the treatment of arachidonic acid mediated lung diseases. It could be of physiological significance to determine the effect of the oxidant H_2O_2 on the activation of TAME-esterase, the subsequent stimulation of PLA_2 activity, and the role of antiproteases in this scenario involving other pulmonary cells.

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