Oxidant-mediated proteolytic activation of Ca^{2+}-ATPase in microsomes of pulmonary smooth muscle

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Abstract Treatment of bovine pulmonary artery smooth muscle tissue microsomes with H_2O_2 (1 mM) markedly stimulated protease activity tested with a synthetic substrate *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), and also enhanced Ca^{2+}-ATPase activity. ATP-dependent Ca^{2+} uptake was found to be stimulated upon treatment of the microsomes with H_2O_2. Pretreatment of the microsomes with vitamin E and aprotinin prevented the H_2O_2-induced stimulation of Ca^{2+}-ATPase activity and also ATP-dependent Ca^{2+} uptake. In contrast, H_2O_2-induced inhibition of Na^+-dependent Ca^{2+} uptake was reversed by vitamin E and aprotinin.

Key words: Ca^{2+}-ATPase; Protease; Aprotinin; Pulmonary smooth muscle tissue; Microsome; Oxidant; Hydrogen peroxide

1. Introduction

Ca^{2+} plays a role as a second messanger in many biochemical and physiological events [1–3]. Microsomes sequester a large portion of mobilizable Ca^{2+}, which plays an important role in releasing Ca^{2+} under stimulatory conditions [4–6]. Ca^{2+}-ATPases have been reported to be present in microsomes of many vascular and nonvascular systems, and provide a main line of defence against an increase in Ca^{2+} level in situ under stimulatory conditions [7–9]. Ca^{2+}-ATPases have been shown to be activated by a variety of proteases in different systems [10–12]. We have previously shown that rabbit pulmonary vascular smooth muscle cells possess protease activity, which plays an important role in stimulating phospholipase A_2 activity [13,14]. The occurrence of Ca^{2+} overload by oxidants in situ has been shown to play a critical role in a variety of pulmonary diseases such as pulmonary hypertension and edema, microembolism and adult respiratory distress syndrome [15,16]. Since Ca^{2+}-ATPases play a role as a protective device, at least partly, in many systems against an increase in Ca^{2+} level in situ under stimulatory conditions [7–9], it appears interesting to investigate the effect of the oxidant H_2O_2 on Ca^{2+}-ATPase activity and Ca^{2+} uptake profiles in microsomes of bovine pulmonary artery smooth muscle tissue and the role of a protease in these phenomenon. The results are presented in this paper.

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Abbreviations: HBPS, Hank's buffered physiological saline; BAPNA, *N*-benzoyl-DL-arginine *p*-nitroanilide

2. Materials and methods

2.1. Materials

Vitamin E (dl-α-tocopherol acetate), NADPH, cytochrome *c*, Tris-ATP, *N*-benzoyl-DL-arginine *p*-nitroanilide, *p*-nitrophenyl phosphate, *p*-nitrophenol, adenosine monophosphate, rotenone and lanthanum chloride were obtained from Sigma Chemical Co., USA. $^{45}Ca^{2+}$ (spec. act. 0.6–0.8 Ci/mmol) was obtained from ICN, USA. All other chemicals used were of analytical grade.

2.2. Methods

Bovine pulmonary artery collected from a slaughterhouse was washed several times with HBPS. The washed pulmonary artery was then kept in HBPS and used for further processing within 2 h after collection. The intimal and external portions of the pulmonary artery were taken out and the tunica media, i.e. the smooth muscle tissue was collected, characterized histologically (Fig. 1) and used for the present studies.

The smooth muscle tissue was homogenized with a cyclomixer in ice-cold medium containing 0.25 M sucrose-10 mM Tris buffer (pH 7.4). The homogenate was centrifuged at $600\times g$ for 15 min at 4°C. The supernatant was centrifuged at $15\,000\times g$ for 20 min to sediment mitochondria and lysosomes [17]. The supernatant was centrifuged at $100\,000\times g$ for 1 h. The pellet was suspended in 10% (w/v) sucrose containing 10 mM Tris buffer (pH 7.4) and was layered on a discontinuous gradient consisting of 40 and 20% (w/v) sucrose both containing 10 mM Tris buffer (pH 7.4). The gradient was centrifuged at $105\,000\times g$ for 2 h. The fraction collected at the 20 and 40% sucrose interface was used as the plasma membrane [18] and the pellet was used as the microsomes. The microsomal pellet was suspended in the homogenizing buffer. All operations were carried out at 4°C. Microsomes were stored under liquid N_2 and thawed prior to the experiments.

Rotenone-insensitive NADPH-Cyt. *c* reductase activity was measured by following a previously described procedure [19].

Cytochrome *c* oxidase assay was performed according to the procedure described in [20].

Acid phosphatase activity was determined at pH 5.5 using *p*-nitrophenyl phosphate as the substrate [21].

Release of P_i from 5′-AMP, an index of 5′-nucleotidase activity, was determined by following the method of Chen et al. [22].

Ca^{2+}-ATPase activity was determined colorimetrically by measuring Ca^{2+}-dependent release of P_i by following the procedure previously described [23]. To determine the effect of H_2O_2, the microsomes were treated with H_2O_2 (1 mM) for 15 min then Ca^{2+}-ATPase activity was determined. Vitamin E (1 mM) or aprotinin (1 mg/ml) was added for 15 min followed by treatment with H_2O_2 (1 mM) for 15 min then Ca^{2+}-ATPase activity was measured.

Protease activity was assessed by determining hydrolysis of the synthetic substrate *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) by following the method previously described [24]. To determine the effect of H_2O_2, the microsomes were treated with 1 mM H_2O_2 for 15 min then protease activity was determined. Vitamin E (1 mM) or aprotinin (1 mg/ml) were added for 15 min followed by treatment with H_2O_2 (1 mM) for 15 min then protease activity was measured.

ATP-dependent Ca^{2+} uptake was measured by following the procedure described in [25]. Briefly, bovine pulmonary artery smooth muscle tissue microsomes (100 μg protein) was preincubated at 37°C for 15 min with or without vitamin E (1 mM) and/or aprotinin (1 mg/ml) in 0.5 ml of a medium containing 140 mM KCl, 10 mM MOPS-Tris buffer (pH 7.4), 2 mM $MgCl_2$ with or without H_2O_2 (1 mM). After 15 min, 10 μCi of $^{45}CaCl_2$ was added, and Ca^{2+} uptake was

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initiated by adding 1 mM Tris ATP, 5 mM phosphocreatine, and 20 U/ml creatine phosphokinase. After 15 min of incubation at 37°C, 250 μl aliquots were filtered through Millipore filters (pore size = 0.45 μm). The radioactivity in the filters was determined by following a previously described procedure [25].

Na^+-dependent Ca^{2+} uptake measurements were carried out by following the procedure previously described [26]. Briefly, bovine pulmonary artery smooth muscle tissue microsomes (100 μg/tube) were preincubated at 37°C for 15 min with or without vitamin E (1 mM) and aprotinin (1 mg/ml) in a medium containing 140 mM NaCl and 200 mM MOPS-Tris buffer (pH 7.4) with or without H_2O_2 (1 mM). After 15 min, 0.5 ml aliquots of the Na^+-loaded microsomes (with or without the stated agents) were added to a series of tubes containing 15 ml of a medium containing 140 mM MOPS-Tris buffer (pH 7.4) plus 10 μCi $^{45}Ca^{2+}$ for 15 min. Ca^{2+} uptake was terminated by adding 5 ml of ice-cold stopping solution containing 140 mM KCl, 1 mM $LaCl_3$, 20 mM MOPS-Tris buffer (pH 7.4). 250 μl aliquots were filtered through Millipore filters (pore size = 0.45 μm). The radioactivity in the filters were determined as described [25].

Proteins were estimated by following the method of Lowry et al. [27].

2.3. Statistical analysis

Data were analyzed by unpaired '*t*'-test, analysis of variance and the Duncan multiple range test for group comparisons [28].

3. Results

We characterized the microsomal fraction isolated from bovine pulmonary artery smooth muscle tissue by measuring at different steps in the preparation process the activity of cytochrome *c* oxidase, a mitochondrial marker [29], of acid phosphatase, a lysosomal marker [30], of NADPH-Cyt. *c* reductase, a microsomal marker [31] and of 5′-nucleotidase, a plasma membrane marker [30]. Compared with the 600×*g* to 15000×*g* pellet and plasma membrane fraction, the microsomal fraction shows a 14- and 25-fold increase, respectively, in the specific activity of rotenone-insensitive NADPH-Cyt. *c* reductase (Table 1). The microsomal fraction shows a 26-fold decrease in the specific activity of Cyt. *c* oxidase compared with the 600×*g* to 15000×*g* pellet, a 16-fold decrease in that of acid phosphatase compared with the 600×*g* to 15000×*g* pellet and a 28-fold decrease in that of 5′-nucleo-

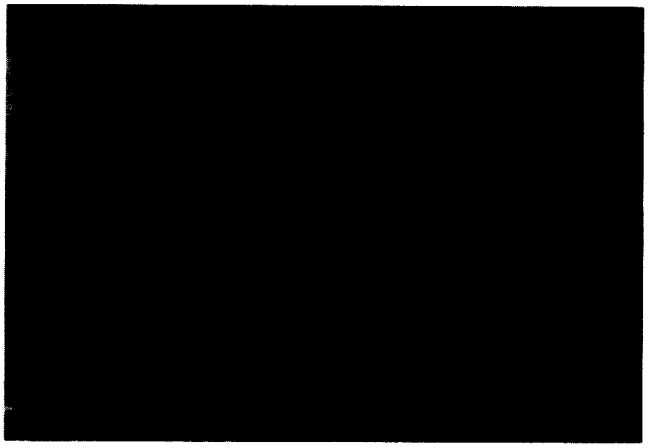

Fig. 1. Transverse section through tunica media of bovine pulmonary artery smooth muscle tissue (magnification ×200) (eosin-hematoxylin preparation).

Table 1
Specific activities of cytochrome *c* oxidase, acid phosphatase, rotenone-insensitive NADPH-Cyt. *c* reductase and 5′-nucleotidase at different steps in the preparation process of microsomes

Fraction	Cyt. *c* oxidase	Acid phosphatase	Rotenone-insensitive NADPH-Cyt. *c* reductase	5′-Nucleotidase
600×*g* to 15000×*g* pellet	1.58 ± 0.08	1.82 ± 0.10	0.14 ± 0.008	0.08 ± 0.008
15000×*g* to 100000×*g* pellet	0.16 ± 0.01 (10)	0.22 ± 0.02 (12)	1.56 ± 0.10 (1114)	1.01 ± 0.07 (1262)
Microsomes	0.06 ± 0.007 (4)	0.11 ± 0.008 (6)	2.02 ± 0.14 (1442)	0.05 ± 0.005 (62)
Plasma membrane	0.07 ± 0.008 (4)	0.18 ± 0.01 (10)	0.08 ± 0.005 (57)	1.42 ± 0.10 (1775)

Cyt. *c* oxidase activity is expressed as μmol Cyt. *c* utilized/mg protein per 15 min. Acid phosphatase is expressed as μmol *p*-nitrophenol/mg protein per 15 min. NADPH-Cyt. *c* reductase (rotenone-insensitive) activity is expressed as reduction of Cyt. *c* at 550 nm/mg protein per 15 min. 5′-Nucleotidase activity is expressed as μmol P_i/mg protein per 15 min. Values are presented as mean ± S.E.M. ($n = 4$). Values in parentheses indicate the activity as a percentage of that of the 600×*g* to 15000×*g* pellet (values of the 600×*g* to 15000×*g* pellet are set at 100%).

Table 2
Effect of the oxidant H_2O_2 and vitamin E and aprotinin on protease activity and Ca^{2+}-ATPase activity in microsomes of bovine pulmonary artery smooth muscle tissue

Treatment	Protease activity (change in absorbance at 410 nm/mg protein per 15 min)	Change (% vs. basal condition)	Ca^{2+}-ATPase activity (μmol P_i/mg protein per 15 min)	Change (% vs. basal condition)
Basal condition	0.17 ± 0.01		1.48 ± 0.11	
H_2O_2 (1 mM)	1.03 ± 0.05[a]	+506	4.22 ± 0.23[a]	+185
Vitamin E (1 mM)	0.15 ± 0.01	−12	1.39 ± 0.09	−6
Vitamin E (1 mM)+H_2O_2 (1 mM)	0.21 ± 0.02[b]	+23	1.61 ± 0.14[b]	+9
Aprotinin (1 mg/ml)	0.10 ± 0.01[c]	−41	1.12 ± 0.06[d]	−24
Aprotinin (1 mg/ml)+H_2O_2 (1 mM)	0.27 ± 0.02[be]	+59	1.76 ± 0.12[bf]	+19

Results are mean ± S.E.M. (n=4).
[a] $P < 0.001$ compared with basal condition.
[b] $P < 0.001$ compared with H_2O_2 treatment.
[c] $P < 0.01$ compared with basal condition.
[d] $P < 0.05$ compared with basal condition.
[e] $P < 0.001$ compared with respective control.
[f] $P < 0.001$ compared with respective control.

Table 3
Effect of H_2O_2 and vitamin E and aprotinin on ATP-dependent Ca^{2+} uptake and Na^+-dependent Ca^{2+} uptake in microsomes of bovine pulmonary artery smooth muscle tissue

Treatment	ATP-dependent $^{45}Ca^{2+}$ uptake (nmol $^{45}Ca^{2+}$/mg protein per 15 min)	Change (% vs. basal value)	Na^+-dependent $^{45}Ca^{2+}$ uptake (nmol $^{45}Ca^{2+}$/mg protein per 15 min)	Change (% vs. basal value)
Basal condition	36.17 ± 1.24		74.26 ± 2.18	
H_2O_2 (1 mM)	55.24 ± 2.01[a]	+53	31.63 ± 1.16[a]	−57
Vitamin E (1 mM)	34.28 ± 1.22	−5	75.42 ± 2.64	+1
Vitamin E (1 mM)+H_2O_2 (1 mM)	38.14 ± 1.26[b]	+5	70.18 ± 2.48[b]	−5
Aprotinin (1 mg/ml)	34.68 ± 1.28[a]	−4	75.64 ± 2.52[d]	+2
Aprotinin (1 mg/ml)+H_2O_2 (1 mM)	37.53 ± 1.14[bc]	+4	62.94 ± 2.46[bc]	−15

Results are mean ± S.E.M. ($n = 4$).
[a] $P < 0.001$ compared with basal condition.
[b] $P < 0.001$ compared with H_2O_2 treatment.
[c] $P < 0.001$ compared with respective control.
[d] $P < 0.05$ compared with basal condition.

tidase compared with the plasma membrane fraction (Table 1). Collectively, these results suggest that our studied fraction is enriched with microsomes.

H_2O_2 stimulates protease activity and also Ca^{2+}-ATPase activity in microsomes of bovine pulmonary artery smooth muscle tissue (Table 2). Pretreatment of the microsomes with vitamin E prevents the increase in protease activity and Ca^{2+}-ATPase activity caused by H_2O_2 (Table 2). Pretreatment of the microsomes with aprotinin decreases the protease activity and Ca^{2+}-ATPase activity under basal conditions (Table 2). Aprotinin also prevents the increase in protease activity in microsomes caused by H_2O_2 (Table 2). A change in the protease activity correlates directly with a change in the activity of Ca^{2+}-ATPase (Fig. 2).

Treatment of the microsomes with H_2O_2 stimulated ATP-dependent Ca^{2+} uptake while Na^+-dependent Ca^{2+} uptake was inhibited by H_2O_2 (Table 3). Pretreatment with vitamin E or aprotinin prevented the stimulation of ATP-dependent Ca^{2+} uptake, while the inhibition of Na^+-dependent Ca^{2+} uptake caused by H_2O_2 was reversed by vitamin E and aprotinin in the microsomes (Table 3). Total Ca^{2+} uptake (ATP-dependent and Na^+-dependent) under H_2O_2 treatment in the microsomes decreases compared with basal conditions (basal: 110.43 vs. H_2O_2 (1 mM) treatment: 86.87 nmol $^{45}Ca^{2+}$/mg protein per 15 min; % change vs. basal value: −21).

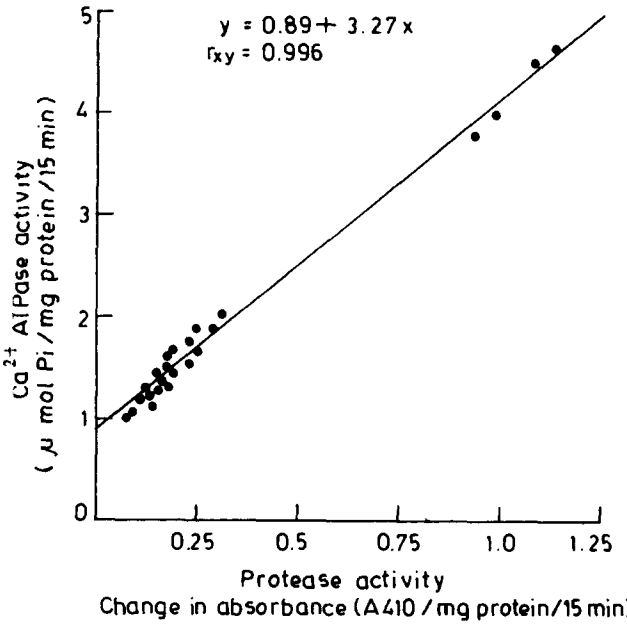

Fig. 2. The regression line of Ca^{2+}-ATPase activity (y) against protease activity (x) based on the observed data (Table 2).

4. Discussion

The aim of the present study was to determine the role that the oxidant H_2O_2 plays in the Ca^{2+}-ATPase activity and Ca^{2+} uptake pattern in microsomes of bovine pulmonary artery smooth muscle tissue and the involvement of a protease in this phenomenon. Our results show that treatment of the microsomes with H_2O_2 stimulates Ca^{2+}-ATPase activity (Table 2) and also enhances ATP-dependent Ca^{2+} uptake (Table 3). H_2O_2 appears to produce these effects via oxidant species(es) because pretreatment of the microsomes with vitamin E reversed the responses produced by H_2O_2 (Tables 2 and 3). These observations are apparently in contrast to the previous reports that oxidants depress Ca^{2+} pump activity in systems such as sarcoendoplasmic reticulum of lobster abdominal muscle [32] and heart sarcolemma [25]. The differences between these studies and our present observation of the effect of H_2O_2 on Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake may apparently be explained upon considering the differences of the functional aspects and responsiveness and biochemical characteristics of pulmonary vessels compared to that of systemic vessels and other nonvascular systems. Because of the differences in the biochemical characteristics and metabolic needs, pulmonary vessels respond differently to stimuli such as hypoxia, α-adrenergic activators and prostaglandin $F_{2\alpha}$ with respect to other types of tissues [33,34]. Pulmonary vessels also differ in their receptor types and surface enzymes compared to other types of tissues [35]. Therefore, to understand the role that the oxidant H_2O_2 plays in Ca^{2+}-ATPase activity and Ca^{2+} uptake in microsomes of pulmonary vascular smooth muscle tissue, we studied the effect of H_2O_2 on microsomes of this tissue rather than to extrapolate findings from other tissues.

Several lines of evidence suggest that a protease plays an important role in stimulating Ca^{2+}-ATPase activity under treatment with H_2O_2 in microsomes of bovine pulmonary artery smooth muscle tissue. First, the smooth muscle tissue

microsomes contain protease activity tested with a synthetic substrate *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) (Table 2). Second, H_2O_2 not only augments Ca^{2+}-ATPase activity but also increases the protease activity in the microsomes (Table 2). Third, the protease inhibitor aprotinin decreases basal protease activity and Ca^{2+}-ATPase activity (Table 2). Fourth, aprotinin also prevents the protease activity and Ca^{2+}-ATPase activity caused by H_2O_2 (Table 2). Fifth, a change in protease activity correlates directly with a change in Ca^{2+}-ATPase activity (Fig. 2). These five pieces of evidence strongly support the concept that a protease plays a crucial role in stimulating Ca^{2+}-ATPase activity in the microsomes by H_2O_2. The identity of the protease is unknown at present but it has the characteristic that it is sensitive to aprotinin (Table 2), an ambient protease inhibitor of bovine lung [36].

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