Role of matrix metalloprotease-2 in oxidant activation of Ca^{2+}ATPase by hydrogen peroxide in pulmonary vascular smooth muscle plasma membrane

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Exposure of bovine pulmonary artery smooth muscle plasma membrane suspension with the oxidant H_2O_2 (1 mM) stimulated Ca^{2+}ATPase activity. We sought to determine the role of matrix metalloprotease-2 (MMP-2) in stimulating Ca^{2+}ATPase activity by H_2O_2 in the smooth muscle plasma membrane. The smooth muscle membrane possesses a Ca^{2+}-dependent protease activity in the gelatin containing zymogram having an apparent molecular mass of 72 kDa. The 72 kDa protease activity was found to be inhibited by EGTA, 1 : 10-phenanthroline, a_2-macroglobulin and tissue inhibitor of metalloprotease-2 (TIMP-2) indicating that the Ca^{2+}-dependent 72 kDa protease is the MMP-2. Western immunoblot studies of the membrane suspension with polyclonal antibodies of MMP-2 and TIMP-2 revealed that MMP-2 and TIMP-2, respectively, are the ambient matrix metalloprotease and the corresponding tissue inhibitor of metalloprotease in the membrane.

In addition to increasing the Ca^{2+}ATPase activity, H_2O_2 also enhanced the activity of the smooth muscle plasma membrane associated protease activity as evidenced by its ability to degrade ^{14}C-gelatin. The protease activity and the Ca^{2+}ATPase activity were prevented by the antioxidant, vitamin E, indicating that the effect produced by H_2O_2 was due to reactive oxidant species(es). Both basal and H_2O_2 stimulated MMP-2 activity and Ca^{2+}ATPase activity were inhibited by the general inhibitors of matrix metalloproteases: EGTA, 1 : 10-phenanthroline, a_2-macroglobulin and also by TIMP-2 (the specific inhibitor of MMP-2) indicating that H_2O_2 increased MMP-2 activity and that subsequently stimulated Ca^{2+}ATPase activity in the plasma membrane. This was further confirmed by the following observations: (i) adding low doses of MMP-2 or H_2O_2 to the smooth muscle membrane suspension caused submaximal increase in Ca^{2+}ATPase activity, and pretreatment with TIMP-2 prevents the increase in Ca^{2+}ATPase activity; (ii) combined treatment of the membrane with low doses of MMP-2 and H_2O_2 augments further the Ca^{2+}ATPase activity caused by the respective low doses of either H_2O_2 or MMP-2; and (iii) pretreatment with TIMP-2 prevents the increase in Ca^{2+}ATPase activity in the membrane caused by the combined treatment of MMP-2 and H_2O_2.

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1. Introduction

H_2O_2 can be generated either enzymatically or nonenzymatically by two electron reduction of molecular oxygen (Halliwell and Gutteridge 1990). Upon challenging cells and tissues with H_2O_2, the experimental condition of 'oxidative stress' may be produced (Cross *et al* 1987). H_2O_2 may be generated *in situ* under a variety of pathophysiological conditions such as chemotactic responses of various foreign matters, inhalation of volatile toxic

Keywords. Antiprotease; Ca^{2+}ATPase; hydrogen peroxide; matrix metalloprotease-2; oxidant, protease, smooth muscle plasma membrane; tissue inhibitor of metalloprotease-2

Abbreviations used: BBI, Bowman Birk inhibitor; HBPS, Hank's buffered physiological saline; H_2O_2, hydrogen peroxide; MMP-2, matrix metallo-protease-2; PMSF, phenyl methyl sulfonylfluoride; TBS, tris buffered saline; TIMP-2, tissue inhibitor of metalloprotease-2.

gases and reoxygenation of ischemic tissues (Fantone and Ward 1982).

Infusion of oxidants in isolated rabbit lungs have been shown to produce pulmonary hypertension (Burghuber *et al* 1985). The condition of pulmonary hypertension arises due to a marked increase in blood pressure within the pulmonary artery. One etiological factor in the pathogenesis of this process is pulmonary fibrosis itself. Under sustained pulmonary hypertension, excessive collagen degradation occurs within the wall of the vessel. Collagenases with their unique ability to degrade native fibrillar collagen have been implicated in several pathological states, for example, vascular diseases associated with collagen degradation (Galis *et al* 1995).

The ability of aortic smooth muscle to activate matrix degrading metalloproteases under stimulated conditions, suggest that they participate in regulating physiological and pathological turnover of extracellular matrix molecules in the human arterial wall. Such degradation may be one of the key requirements for smooth muscle to migrate from the media to the intima upon stimulation (Yanagi *et al* 1992). The activities of matrix metalloproteases (MMPs) are thought to be counter-balanced by their common inhibitors, tissue inhibitor of matrix metalloproteases (TIMPs) (Bond and Butler 1987). It has been demonstrated that MMPs play an important role in the degradation of extracellular matrix molecules associated with tissue destruction in pulmonary vascular diseases (Yanagi *et al* 1992).

Ca^{2+} plays the role of a second messanger in many biochemical and physiological events (Berridge 1993; Chakraborti and Chakraborti 1995; Chakraborti *et al* 1992). An increase in Ca^{2} level *in situ* caused by a variety of agonists is due to an influx of extracellular Ca^{2+} and/or release of Ca^{2+} from its sub-organelle stores (Chakraborti *et al* 1989, 1992). Oxidants have been shown to cause Ca^{2+} overload in cells and tissues (Bennet and Williams 1993; Ghosh *et al* 1989). In some systems, plasma membrane associated Ca^{2+}ATPase contributes toward counteracting different agonists induced increase in intracellular free Ca^{2+} level (Evers *et al* 1988; Qu *et al* 1992), but its role in pulmonary vascular smooth muscle membrane is not known.

Ca^{2+}ATPases were activated by a variety of proteases in some systems (Carafoli 1994). In some systems, Ca^{2+}-ATPases were modulated by a variety of signal transducers such as cAMP, cGMP and protein kinase C (Neyses *et al* 1985; Farrukh *et al* 1987; Qu *et al* 1992). Furthermore, the activities of these signal transducers were found to be regulated by different types of protease (Chakraborti *et al* 1996; Ghosh *et al* 1996).

Previous researchers have provided evidence that the occurrence of Ca^{2+} overload caused by oxidants in isolated lung plays a critical role in pulmonary hypertension (Farrukh *et al* 1985). Pulmonary hypertension often causes substantial morbidity and mortality (Murray *et al* 1986). Because of inadequate understanding of the regulation of Ca^{2+} dynamics under oxidant-triggered conditions in pulmonary smooth muscle, we sought to determine whether pulmonary vascular smooth muscle plasma membrane associated Ca^{2+}ATPase could play a role in the Ca^{2+} overload under H_2O_2-triggered conditions. Our results suggest that (i) H_2O_2 causes stimulation of Ca^{2+}-ATPase activity in the smooth muscle membrane; and (ii) the activation of Ca^{2+}ATPase in pulmonary vascular smooth muscle membrane occurs through the involvement of MMP-2.

2. Materials and methods

2.1 *Materials*

Hydrogen peroxide, vitamin E (dl-*a*-tocopherol acetate), rotenone, NADPH, cytochrome *c*, Tris-ATP, p-nitrophenyl phosphate, p-nitrophenol, adenosine monophosphate, HEPES, $CaCl_2$, phenylmethylsulphonylfluoride, antipain, leupeptin, pepstatin, chymostatin, EGTA, 1 : 10-phenanthroline, N-ethylmaleimide, a_2-macroglobulin, gelatin, 4-chloro-1-napthol were obtained from Sigma Chemical Co., St. Louis, MO, USA. ^{14}C-gelatin (1 mCi/mg) was obtained from New England Nuclear, Wilmington, DE, USA. TIMP-2, MMP-2 and their polyclonal antibodies were obtained from Chemicon International Inc, CA, USA. Nitrocellulose paper was obtained from Bio-Rad, USA. All other chemicals used were of analytical grade.

2.2 *Isolation of smooth muscle membrane*

Bovine pulmonary artery collected from slaughter house was washed several times with Hank's buffered physiological saline (HBPS) (pH 7·4) and kept at 4°C. The washed pulmonary artery was used for further processing within 4 h after collection. The intimal and external portions were removed and the tunica media, i.e. the smooth muscle tissue was collected and characterized histologically (figure 1), and the smooth muscle plasma membrane fraction was isolated by following the procedure previously described by Chakraborti and Michael (1993a,b). Protein concentrations were determined by following the procedure described by Lowry *et al* (1951).

2.3 *Assay of marker enzymes*

Rotenone-insensitive NADPH-cytochrome *c* reductase activity was assayed in bovine pulmonary artery smooth muscle plasma membrane fraction by following the pro-

cedure previously described (Master *et al* 1967). Cytochrome *c* oxidase activity was determined by measuring oxidation of reduced cytochrome *c* by following the method previously described by Wharton and Tazagoloff (1967). Acid phosphatase activity was determined at pH 5·5 using p-nitrophenylphosphate as the substrate by following the procedure previously described by Hodges and Leonard (1974). 5′-Nucleotidase activity was assayed by following the procedure described by Chen *et al* (1956).

2.4 *Zymogram of protease activity*

Polyacrylamide gels (12%; 0·75 mm thick) were cast containing 0·1% gelatin. Gelatin solution was made up as a 2% stock solution in distilled water and dissolved by heating. Bovine pulmonary artery smooth muscle plasma membrane suspension (~ 10 μg of protein) was applied to the gel in standard SDS loading buffer containing 0·1% SDS but lacking 2-mercaptoethanol; it was not boiled before loading. The gels were run at 200 V for 1 h at 4°C in a Bio-Rad Mini protean II apparatus and then soaked in 200 ml of 2·5% triton X-100 in distilled water in a shaker for 1h with one change after 30 min at 20°C. Next, the gels were soaked in reaction buffer (100 mM Tris/HCl, 10 mM $CaCl_2$, pH 8·0) for 12 h at 37°C and then stained with Coomassie brilliant blue, and this was followed by washing with distilled water for one min. The clear zone against the dark Coomassie background indicates protease activity. The marker lane was separated from the gel and then destained with destaining solution containing methanol : acetic acid : water (4 : 1 : 5 by volume) (Billings *et al* 1991).

Figure 1. Transverse section through the tunica media of bovine pulmonary artery showing the characteristic smooth muscle cells (magnification × 200). (Eosin-hematoxilin preparation).

EGTA (10 mM), 1 : 10 phenanthroline (10 mM), a_2-macroglobulin (100 μg/ml), TIMP-2 (100 μg/ml), PMSF (1 mM), pepstatin (100 μg/ml), N-ethylmaleimide (5 mM), leupeptin (100 μg/ml), antipain (100 μg/ml), chymostatin (100 μg/ml) and BBI (100 μg/ml) were added to the triton X-100 soaked gels for 1 h, then incubated in reaction buffer containing these inhibitors for 12 h at 37°C. This was then stained with Coomassie brilliant blue followed by washing with distilled water for one min. The clear zone against the dark Coomassie background indicated protease activity.

2.5 *Assay of protease activity by ^{14}C-gelatin degradation*

Ca^{2+}-dependent matrix metalloprotease activity was determined as follows: The radiolabelled gelatin substrate was prepared by diluting 20 μl (1·2 μCi) of ^{14}C-labelled gelatin with 480 μl of 1 mg/ml cold gelatin. The substrate mixture was then heated at 55°C for 25 min and allowed to cool slowly to room temperature. The final assay mixture contained 40 μl of substrate, 10 mM $CaCl_2$ and the smooth muscle plasma membrane suspension (~ 50 μg protein). Samples were incubated for 1 h at 37°C and then the reaction was stopped by the addition of 20 μl of 0·25 M EGTA. Undigested gelatin was precipitated by the addition of 60 μl of 10% TCA. After chilling on ice for 10 min, samples were centrifuged at 10,000 *g* for 10 min and the supernatant was counted (Brown *et al* 1993).

Bovine pulmonary artery smooth muscle plasma membrane suspension (~ 50 μg protein) was treated with H_2O_2 (1 mM) for 1 h at 37°C, and the matrix metalloprotease activity was determined.

Vitamin E (1 mM), EGTA (10 mM), 1 : 10-phenanthroline (10 mM), a_2-macroglobulin (100 μg/ml), TIMP-2 (100 μg/ml), PMSF (1 mM), leupeptin (100 μg/ml), pepstatin (100 μg/ml), antipain (100 μg/ml), N-ethylmaleimide (5 mM), chymostatin (100 μg/ml) and BBI (100 μg/ml) were added to the membrane suspension (~ 50 μg protein) for 15 min and this was followed by treatment with H_2O_2 (1 mM) for 1 h; the protease activity was then measured.

2.6 *Assay of Ca^{2+}ATPase activity*

Ca^{2+}ATPase activity was determined colorimetrically by measuring Ca^{2+}-dependent release of P_i as previously described (Chakraborti *et al* 1996). Bovine pulmonary artery smooth muscle plasma membrane suspension (~ 50 μg protein) was treated with H_2O_2 (1 mM) for 1 h at 37°C, and Ca^{2+}ATPase activity was determined.

Vitamin E (1 mM), EGTA (10 mM), 1 : 10-phenanthroline (10 mM), a_2-macroglobulin (100 μg/ml), TIMP-2 (100 μg/ml), PMSF (1 mM), leupeptin (100 μg/ml), pep-

statin (100 μg/ml), antipain (100 μg/ml), N-ethylmaleimide (5 mM), chymostatin (100 μg/ml) and BBI (100 μg/ml) were added to the membrane suspension (~ 50 μg protein) for 15 min and this was followed by treatment with H_2O_2 (1 mM) for 1 h; Ca^{2+}ATPase activity was then determined.

2.7 *Identification of MMP-2 and TIMP-2*

MMP-2 and TIMP-2 were identified in bovine pulmonary artery smooth muscle plasma membrane fraction by Western immunoblot method by using their polyclonal antibodies. Western immunoblot was performed according to Towbin *et al* (1979) with some modifications. Briefly, the bovine pulmonary artery smooth muscle membrane suspension (~ 50 μg protein) were electrophoresed in 12% SDS–PAGE. The resolved proteins were then transferred electrophoretically (1 h, 100 V) to nitrocellulose paper (0·2 μm pore size). Nonspecific protein binding sites on the nitrocellulose were blocked by incubating the paper with 3% BSA in Tris buffered saline [TBS: 10 mM Tris-HCl (pH 7·4), 0·9% NaCl] for 40 min with constant shaking at 40°C. The nitrocellulose paper was then incubated with polyclonal rabbit anti bovine (MMP-2 or TIMP-2) IgG for 2 h at room temperature with constant shaking. Then the nitrocellulose paper was washed three times (20 min each) with TBS. The nitrocellulose paper was then incubated for 2 h with horseradish peroxidase conjugated goat anti (rabbit IgG) antibody. This was then washed three times with TBS (20 min each), and then the blots were developed with 0·2 mM 4-chloro-1-naphthol.

2.8 *Statistical analysis*

Data were analysed by unpaired *t*-test and analysis of variance followed by the test of least significant difference (David 1978) for comparisons within and between the groups. Statistical significance was assumed when the $P < 0{\cdot}05$.

3. Results

We characterized the pulmonary vascular smooth muscle plasma membrane fraction at different steps in the preparation process by measuring the activities of cytochrome *c* oxidase [a mitochondrial marker (De Reuck and Cameron 1963)], acid phosphatase [a lysosomal marker (Ketis *et al* 1986)], NADPH-cytochrome *c* reductase [a microsomal marker (Clark *et al* 1969)] and 5′-nucleotidase [a plasma membrane marker (Ketis *et al* 1986)]. Compared with the 600–15000 *g* pellet and the microsomal fractions, the plasma membrane fraction showed respectively 14-fold and 17-fold increase in the specific activity of 5′-nucleotidase (table 1). The plasma membrane fraction showed a 33-fold decrease in the specific activity of cytochrome *c* oxidase compared with 600–15000 g pellet, a 23-fold decrease in the specific activity of acid phosphatase compared with the 600–15000 g pellet and a 26-fold decrease in the specific activity of NADPH-cytochrome *c* reductase compared with microsomal fraction (table 1).

Immunoblot studies with polyclonal MMP-2 antibody and TIMP-2 antibody revealed that MMP-2 and TIMP-2, respectively, are the ambient matrix metalloprotease (figure 2) and the corresponding tissue inhibitor of metalloprotease (figure 3) in the smooth muscle membrane. The immunoblot studies also revealed that MMP-2 has an apparent molecular mass of 72 kDa (figure 2) while that of TIMP-2 has an apparent molecular mass of 21 kDa (figure 3).

Using ^{14}C-gelatin as the substrate for the protease, we found that treatment of the smooth muscle membrane with H_2O_2 stimulated gelatin degradation (tables 2 and 3). Treatment of the membrane suspension with H_2O_2 also stimulated Ca^{2+}ATPase activity (tables 2 and 3). Pretreatment of the smooth muscle membrane with the

Table 1. Specific activities of cytochrome *c* oxidase acid phosphatase, rotenone-insensitive NADPH-cytochrome *c* reductase and 5′-nucleotidase at different steps in the preparation of bovine pulmonary artery smooth muscle plasma membrane.

Fraction	Cytochrome *c* oxidase	Acid phosphatase	Rotenone-insensitive NADPH-cytochrome *c* reductase	5′-nucleotidase
600–15,000 g pellet	3·01 ± 0·15	3·51 ± 0·22	0·22 ± 0·01	0·14 ± 0·01
15,000–100,000 g pellet	0·29 ± 0·02 (10)	0·39 ± 0·03 (11)	2·87 ± 0·16 (1305)	1·82 ± 0·12 (1300)
Microsomes	0·11 ± 0·01 (4)	0·19 ± 0·01 (5)	3·58 ± 0·21 (1627)	0·12 ± 0·01 (86)
Plasma membrane	0·09 ± 0·01 (3)	0·15 ± 0·01 (4)	0·14 ± 0·01 (64)	2·02 ± 0·11 (1443)

Results are mean ± SE ($n = 4$). The values in the parentheses indicate the activity as percentage of that of the 600–15,000 g pellet (values of 600–15,000 g pellet are set at 100%). Cytochrome *c* oxidase activity is expressed as μmol of cytochrome *c* utilized/30 min per mg protein. Acid phosphatase activity is expressed as μmol of *p*-nitrophenol/30 min per mg of protein. NADPH cytochrome *c* reductase (rotenone insensitive) activity is expressed as reduction of cytochrome *c* at 550 nm/30 min per mg of protein. 5′-nucleotidase activity is expressed as μmol of P_i/30 min per mg protein.

antioxidant, vitamin E markedly prevents the H_2O_2 triggered stimulation of Ca^{2+}ATPase activity and the protease activity (as determined by ^{14}C-gelatin degradation) (table 2). The smooth muscle membrane possesses a protease activity in gelatin containing zymogram with an apparent molecular mass of 72 kDa (figure 5). The protease activity as observed in gelatin zymogram and as determined by ^{14}C-gelatin degradation studies were found to be inhibited by the general inhibitors of matrix metalloproteases, EGTA, 1 : 10 phenanthroline, α_2-macroglobulin and also by TIMP-2 (the specific inhibitor of MMP-2), the specific inhibitor for MMP-2 (figure 4 and table 3). In contrast, the protease activity was found to be insensitive to the inhibitors of serine, thiol and cysteine groups of proteases such as PMSF, BBI, chymostatin, N-ethylmaleimide, antipain, leupeptin and pepstatin (figure 5 and table 2).

To test further the hypothesis that H_2O_2-mediated activation of Ca^{2+}ATPase occurs through the involvement of MMP-2, we treated the smooth muscle membrane suspension with low doses of H_2O_2 and/or MMP-2. Treatment of the smooth muscle membrane with a low dose of H_2O_2 or MMP-2 causes submaximal increase in Ca^{2+}ATPase activity and these responses were found to be prevented upon pretreatment with TIMP-2 (table 4). Combined treatment of the membrane suspension with low doses of H_2O_2 and MMP-2 augments Ca^{2+}ATPase activity compared with the effect produced by either MMP-2 or H_2O_2 at their respective low doses (table 4). Interestingly pretreatment of the membrane suspension with TIMP-2 prevents the increase in Ca^{2+}ATPase activity in the smooth muscle membrane elicited by the low doses of MMP-2 plus H_2O_2 (table 4).

4. Discussion

Our results indicate that treatment of bovine pulmonary artery smooth muscle membrane with H_2O_2 (1 mM) causes stimulation of Ca^{2+}ATPase activity (table 2 and 3). This is in contrast with the previous reports that oxidants depress Ca^{2+} pump activity in sarco-endoplasmic reticulum and heart sarcolemma (Chakraborti *et al* 1996). The differences between these studies and our present observations using pulmonary smooth muscle membrane may possibly be explained by considering the differences in biochemical characteristics and functional responsiveness of pulmonary vessels and other vascular and non-vascular systems. Because of their different functions, pulmonary vessels and systemic vessels respond differently to stimuli such as hypoxia, α-adrenergic activators and prostaglandin F2α (Murray *et al* 1986; Robinson *et al* 1973). Pulmonary vessels also differ from other types of blood vessels in their receptor types and surface enzymes (Murray *et al* 1986). Therefore, to understand the role that the pulmonary smooth muscle membrane plays in Ca^{2+}ATPase activity under oxidant stimulation caused by H_2O_2, we have carried out experiments on

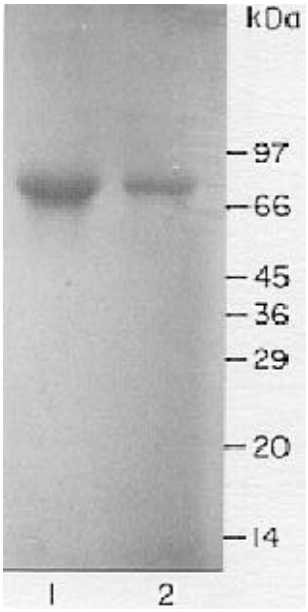

Figure 2. Identification of matrix metalloprotease-2 (MMP-2) in bovine pulmonary vascular smooth muscle plasma membrane with polyclonal rabbit anti (bovine MMP-2) IgG by Western immunoblot. Lane 1, purified bovine MMP-2 (obtained from Chemicon International, CA, USA); lane 2, bovine pulmonary artery smooth muscle plasma membrane suspension.

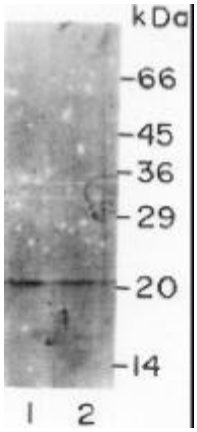

Figure 3. Identification of tissue inhibitor of metalloprotease-2 (TIMP-2) in bovine pulmonary vascular smooth muscle plasma membrane with polyclonal rabbit anti (bovine TIMP-2) IgG by Western immunoblot. Lane 1, purified bovine TIMP-2 (obtained from Chemicon International, CA, USA); lane 2, bovine pulmonary artery smooth muscle plasma membrane suspension.

Table 2. Effect of vitamin E and different protease inhibitors on H_2O_2 triggered Ca^{2+}-dependent matrix metalloprotease activity and Ca^{2+}ATPase activity in bovine pulmonary artery smooth muscle plasma membrane.

Treatment	Ca^{2+}-dependent matrix metalloprotease activity	Change vs basal value (%)	Ca^{2+}ATPase activity	Change vs basal value (%)
Basal	136 ± 8		1·98 ± 0·09	
H_2O_2 (1 mM)	960 ± 22[a]	+ 606	5·84 ± 0·26[a]	+ 195
Vitamin E (1 mM)	114 ± 11	– 16	1·76 ± 0·08	– 11
Vitamin E (1 mM) + H_2O_2 (1 mM)	158 ± 11	+ 16	2·12 ± 0·09	+ 7
PMSF (1 mM)	132 ± 9	– 3	1·94 ± 0·08	– 2
PMSF (1 mM) + H_2O_2 (1 mM)	956 ± 22[a,b]	+ 603	5·78 ± 0·24[a,b]	+ 192
Leupeptin (100 μg/ml)	128 ± 8	– 6	1·86 ± 0·10	– 6
Leupeptin (100 μg/ml) + H_2O_2 (1 mM)	948 ± 24[a,b]	+ 597	5·72 ± 0·26[a,b]	+ 189
Pepstatin (100 μg/ml)	142 ± 11	+ 4	2·01 ± 0·09	+ 2
Pepstatin (100 μg/ml) + H_2O_2 (1 mM)	972 ± 23[a,b]	+ 615	5·98 ± 0·24[a,b]	+ 202
Antipain (100 μg/ml)	148 ± 10	+ 9	2·06 ± 0·11	+ 4
Antipain (100 μg/ml) + H_2O_2 (1 mM)	978 ± 28[a,b]	+ 619	6·02 ± 0·22[a,b]	+ 204
N-ethylmaleimide (5 mM)	138 ± 9	+ 1	1·99 ± 0·08	+ 1
N-ethylmaleimide (5 mM) + H_2O_2 (1 mM)	967 ± 26[a,b]	+ 611	5·89 ± 0·27[a,b]	+ 197
Chymostatin (100 μg/ml)	144 ± 9	+ 6	2·02 ± 0·11	+ 2
Chymostatin (100 μg/ml) + H_2O_2 (1 mM)	974 ± 26[a,b]	+ 616	5·99 ± 0·21[a,b]	+ 203
BBI (100 μg/ml)	140 ± 8	+ 3	2·01 ± 0·10	+ 2
BBI (100 μg/ml) + H_2O_2 (1 mM)	966 ± 22[a,b]	+ 610	5·87 ± 0·23[a,b]	+ 196

Results are mean ± SE (n = 4). Ca^{2+} dependent matrix metalloprotease activity is expressed as cpm (^{14}C–gelatin degradation)/30 min per mg of protein). Ca^{2+}ATPase activity is expressed as μmol of P_i/30 min per mg protein.
[a]$P < 0·001$ compared with basal value.
[b]$P < 0·001$ compared with respective control.

Table 3. Effect of EGTA and different metalloprotease inhibitors on Ca^{2+}-dependent metalloprotease activity and Ca^{2+}ATPase activity in bovine pulmonary vascular smooth muscle membrane under H_2O_2-triggered conditions.

Treatment	MMP-2 activity	Change vs basal value (%)	Ca^{2+}ATPase activity	Change vs basal value (%)
Basal	136 ± 12		1·98 ± 0·11	
H_2O_2 (1 mM)	960 ± 22[a]	+ 606	5·84 ± 0·26[a]	+ 195
EGTA (10 mM)	ND	–	ND	–
EGTA (10 mM) + H_2O_2 (1 mM)	ND	–	ND	–
1 : 10 phenanthroline (10 mM)	16 ± 2[a]	– 88	0·02 ± 0·002[a]	– 99
1 : 10 phenanthroline (10 mM) + H_2O_2 (1 mM)	19 ± 2[a]	– 86	0·03 ± 0·003[a]	– 98
a_2-macroglobulin (100 μg/ml)	15 ± 2[a]	– 89	0·02 ± 0·001[a]	– 99
a_2-macroglobulin (100 μg/ml) + H_2O_2 (1 mM)	18 ± 2[a]	– 87	0·03 ± 0·002[a]	– 98
TIMP-2 (100 μg/ml)	8 ± 2[a]	– 94	0·01 ± 0·001[a]	– 99
TIMP-2 (100 μg/ml) + H_2O_2 (1 mM)	10 ± 2[a]	– 93	0·01 ± 0·002[a]	– 99

Results are mean ± SE (n = 4). The protease activity is expressed as ^{14}C-gelatin degradation (cpm/30 min per mg protein). Ca^{2+}ATPase activity is expressed as μmol of P_i/30 min per mg protein. ND, not detected.
[a]$P < 0·001$ compared with basal value.

pulmonary vascular smooth muscle membrane rather than extrapolate findings from other type of systems.

In the present study we tested the hypothesis that the Ca^{2+}-dependent matrix metalloprotease plays a pivotal role in regulating Ca^{2+}ATPase activity by determining its possible involvement in the enhanced Ca^{2+}ATPase activity caused by the oxidant H_2O_2. Previous researchers (Carafoli 1994) have demonstrated activation of erythrocyte Ca^{2+}ATPase by selective proteolytic cleavage of the calmodulin-binding domain. Earlier reports have also provided evidence that proteolysis of endogenous protease inhibitor(s) cause an imbalance between protease(s) and antiprotease(s) with the resultant shift of the equilibrium towards protease(s) (Mellgren *et al* 1986; Chakraborti *et al* 1996).

Several lines of evidences suggest that MMP-2 plays an important role in the oxidant H_2O_2 triggered Ca^{2+}ATPase activity in pulmonary vascular smooth muscle plasma membrane. (i) The smooth muscle plasma membrane associated matrix metalloprotease showed a proteolytic

activity in gelatin containing zymogram with an apparent molecular mass of 72 kDa (figure 4). This 72 kDa proteolytic activity is inhibited by the general inhibitors of matrix metalloproteases: namely, EGTA, 1 : 10-phenanthroline; a_2-macroglobulin; and also by the specific inhibitor of MMP-2, TIMP-2 (figure 4). By contrast, the 72 kDa proteolytic activity, as revealed in the zymogram (figure 5) and also the ^{14}C-gelatin degradation (table 2), was found to be insensitive to PMSF, BBI, chymostatin, N-ethylmaleimide, leupeptin, antipain and pepstatin. Western immunoblot study of the smooth muscle plasma membrane with polyclonal antibody of MMP-2 revealed a characteristic band at the apparent molecular mass of 72 kDa (figure 2), indicating that MMP-2 is an ambient metalloprotease in the membrane. (ii) H_2O_2 stimulates MMP-2 activity in bovine pulmonary artery smooth muscle membrane as evidenced by ^{14}C-gelatin degradation studies (tables 2 and 3). H_2O_2 not only augments MMP-2 activity but also stimulates Ca^{2+}ATPase activity in the smooth muscle membrane (tables 2 and 3). H_2O_2 appears to produce these effects via oxidant species, because pretreatment of the smooth muscle membrane with vitamin E prevents the increase in the matrix metalloprotease activity and Ca^{2+}ATPase activity (table 2). (iii) Matrix metalloprotease inhibitors such as EGTA, 1 : 10-phenanthroline, a_2-macroglobulin and TIMP-2 (Fujimoto *et al* 1995) inhibit basal gelatinolytic activity and Ca^{2+}ATPase activity in the smooth muscle plasma membrane (table 3). Western immunoblot study of the smooth muscle membrane suspension with polyclonal antibody of TIMP-2 revealed a characteristic band at the apparent molecular mass of 21 kDa (figure 3), which is typically similar to the antiprotease obtained from other sources (Fujimoto *et al* 1995; Gomez *et al* 1997), indicating that TIMP-2 is the ambient MMP-2 inhibitor in the membrane. (iv) The oxidant-stimulated smooth muscle plasma membrane associated MMP-2 and Ca^{2+}ATPase activities were also inhibited by TIMP-2 (table 3). (v) Adding MMP-2 to the smooth muscle plasma membrane suspension markedly increases Ca^{2+}ATPase activity (table 4), indicating that MMP-2 can activate Ca^{2+}ATPase activity in the membrane. (vi) Adding low doses of MMP-2 or H_2O_2 to the smooth muscle membrane suspension sub-maximally increases Ca^{2+} ATPase activity and these responses were found to be prevented upon pretreatment with TIMP-2 (table 4). (vii) Combined treatment of the smooth muscle membrane suspension with low doses of MMP-2 and H_2O_2 further augments Ca^{2+}ATPase activity caused by either MMP-2 or H_2O_2

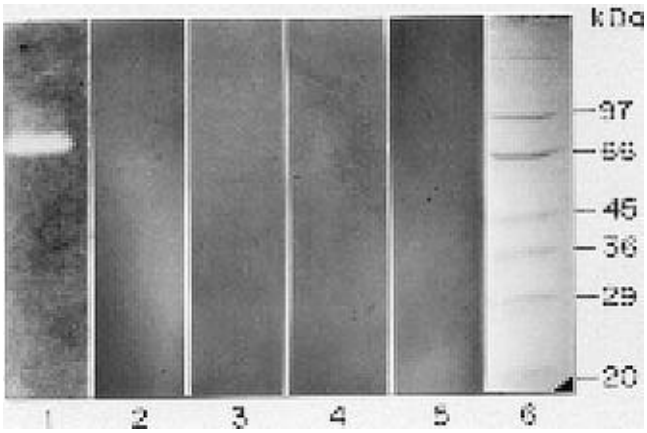

Figure 4. Effect of EGTA, 1 : 10 phenanthroline, a_2-macroglobulin and TIMP-2 on bovine pulmonary artery smooth muscle plasma membrane associated 72 kDa protease activity in gelatin zymogram. Lane 1, control; lane 2, EGTA; lane 3, 1 : 10-phenanthroline; lane 4, a_2-macroglobulin; lane 5, TIMP-2; lane 6, mol. wt. markers.

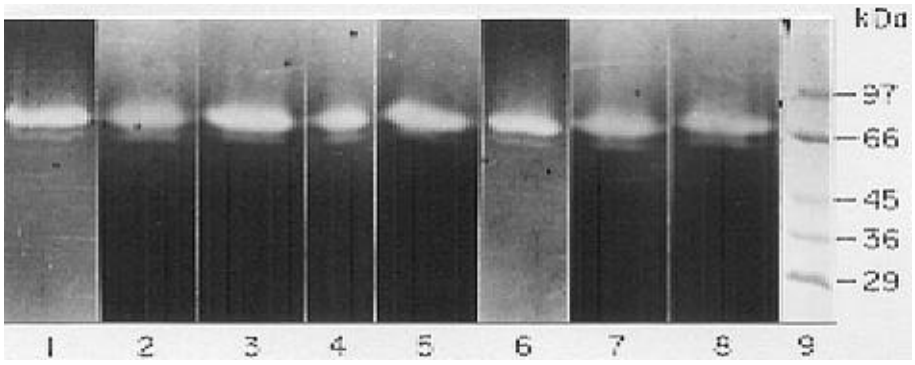

Figure 5. Effect of different protease inhibitors on bovine pulmonary artery smooth muscle plasma membrane associated 72 kDa protease activity in gelatin zymogram. Lane 1, control; lane 2, PMSF; lane 3, Bowman-Birk inhibitor; lane 4, chymostatin; lane 5, N-ethylmaleimide; lane 6, leupeptin; lane 7, antipain; lane 8, pepstatin; lane 9, mol. wt. markers.

Table 4. Effect of TIMP-2 on MMP-2 and/or H_2O_2-mediated activation of Ca^{2+}ATPase in bovine pulmonary vascular smooth muscle plasma membrane.

Treatment	Ca^{2+}ATPase activity	Change vs basal value (%)
Basal	1·98 ± 0·11	
MMP-2 (1 μg/ml)	7·02 ± 0·32[a]	+ 255
TIMP-2 (100 μg/ml)	0·01 ± 0·001[a]	– 99
TIMP-2 (100 μg/ml) + MMP-2 (1 μg/ml)	0·01 ± 0·002[a]	– 99
H_2O_2 (1 mM)	5·84 ± 0·26[a]	+ 195
TIMP-2 (100 μg/ml) + H_2O_2 (1 mM)	0·01 ± 0.002[a]	– 99
H_2O_2 (0.5 mM)	4.02 ± 0.18[a]	+ 103
TIMP-2 (100 μg/ml) + H_2O_2 (0.5 mM)	0.01 ± 0.001[a]	– 99
MMP-2 (0.5 μg/ml)	4.86 ± 0.21[a]	+ 145
TIMP-2 (100 μg/ml) + MMP-2 (0.5 μg/ml)	0.01 ± 0.001[a]	– 99
H_2O_2 (0.5 mM) + MMP-2 (0.5 μg/ml)	7.98 ± 0.35[a,b,c]	+ 303
TIMP-2 (100 μg/ml) + {H_2O_2 (0.5 mM) + MMP-2 (0.5 μg/ml)}	0.02 ± 0.001[a,d]	– 99

Results are mean ± SE (n = 4). Ca^{2+}ATPase activity is expressed as μmol of P_i/30 min per mg protein.
[a]$P < 0.001$ compared with basal value; [b]$P < 0.05$ compared with MMP-2 (1 μg/ml); [c]$P < 0.001$ compared with H_2O_2 (1 mM); [d]$P < 0.001$ compared with H_2O_2 (0.5 mM) + MMP-2 (0.5 μg/ml) treatment.

(table 4). Pretreatment of the membrane suspension with TIMP-2 prevent the effect produced by the low doses of MMP-2 plus H_2O_2 (table 4). These evidences strongly support the concept that MMP-2 plays a pivotal role in stimulating Ca^{2+}ATPase activity in the smooth muscle membrane under the oxidant H_2O_2-triggered conditions.

Proteolytic processes play important roles in experimentally induced and physiologically occurring changes in cells and tissues (Bond and Butler 1987). Oxidants have been demonstrated to produce pulmonary hypertension in isolated lung (Burghuber *et al* 1985). In isolated lung, protease inhibitors have been shown to prevent pulmonary hypertension caused by oxidants and Ca^{2+} mobilizing agents, for example, A23187 (Seeger *et al* 1983; White 1988). Oxidants, for example, hypochlorous acid have been shown to inactivate tissue inhibitors of metalloproteases (Shabani *et al* 1998). How the oxidant H_2O_2 stimulates MMP-2 activity is not clearly known. One of the mechanisms by which H_2O_2 stimulates MMP-2 activity could be that H_2O_2 inactivates the corresponding ambient antiproteases, for example, TIMP-2, thereby causing an alteration in the protease-antiprotease balance in favour of the protease. The mechanisms by which MMP-2 stimulates Ca^{2+}ATPase activity in the smooth muscle membrane under H_2O_2-triggered condition is currently unknown. Conceivably, it may act directly on Ca^{2+}ATPase (Moreau *et al* 1998) or indirectly via factors that influence Ca^{2+}ATPase activity: namely, calmodulin (Kosk-Kosicka and Inesi 1985), protein kinase C (Qu *et al* 1992), cyclic AMP and cyclic GMP and their phosphodiesterases (Neyses *et al* 1985; Farrukh *et al* 1987), and the proteolipid phospholamban (Koh *et al* 1987).

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