Role of MMP-2 in oxidant-mediated regulation of Ca\textsuperscript{2+} uptake in microsomes of bovine pulmonary artery smooth muscle

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Received 3 September 2004; revised 28 December 2004

Treatment of bovine pulmonary artery smooth muscle microsomes with tert-butylhydroperoxide (t-buOOH) (300 µM) markedly stimulated matrix metalloproteinase-2 (MMP-2) activity and enhanced Ca\textsuperscript{2+}-ATPase activity and ATP-dependent Ca\textsuperscript{2+} uptake. Pre-treatment with vit. E (1 mM) and tissue inhibitor of metalloproteinase-2 (TIMP-2) (50 µg/ml) prevented t-buOOH-induced stimulation of MMP-2 activity, Ca\textsuperscript{2+}-ATPase activity and ATP-dependent Ca\textsuperscript{2+} uptake. In contrast, Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake was inhibited by t-buOOH and the inhibition was reversed by vit. E (1 mM) and TIMP-2 (50 µg/ml). However, t-buOOH-triggered changes in MMP-2 activity, and ATP- and Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake were not reversed upon pre-treatment of the microsomes with a low concentration of 5 µg/ml of TIMP-2, which on the contrary reversed MMP-2 (1 µg/ml)-mediated alteration on these parameters. The inhibition of Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake by MMP-2 under t-buOOH treatment overpowered the stimulation of ATP-dependent Ca\textsuperscript{2+} uptake in the microsomes. Combined treatment of the microsomes with low doses of MMP-2 (0.5 µg/ml) and t-buOOH (100 µM) augmented Ca\textsuperscript{2+}-ATPase activity and ATP-dependent Ca\textsuperscript{2+} uptake, but inhibited Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake, compared to that elicited by either MMP-2 (0.5 µg/ml) or t-buOOH (100 µM). Pre-treatment with TIMP-2 (50 µg/ml) reversed the effects of MMP-2 (0.5 µg/ml) and/or t-buOOH (100 µM). Although pre-treatment with 5 µg/ml of TIMP-2 reversed the effects produced by MMP-2 (0.5 µg/ml), but it did not inhibit the responses elicited by t-buOOH (300 µM) or t-buOOH (100 µM) plus MMP-2 (0.5 µg/ml) in the microsomes. Treatment with TIMP-2 (5 µg/ml) inhibited MMP-2 (1 µg/ml) activity (assessed by [\textsuperscript{14}C]-gelatin degradation), whereas treatment of t-buOOH (300 µM) with TIMP-2 (5 µg/ml) abolished the inhibitory effect of TIMP-2 (5 µg/ml) on MMP-2 (1 µg/ml) activity (assessed by [\textsuperscript{14}C]-gelatin degradation). Overall, these results suggested that t-buOOH inactivated TIMP-2, the ambient inhibitor of MMP-2, leading to activation of the ambient protease, MMP-2 which subsequently stimulated Ca\textsuperscript{2+}-ATPase activity and ATP-dependent Ca\textsuperscript{2+} uptake, but inhibited Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake, resulting in a marked decrease in Ca\textsuperscript{2+} uptake in the microsomes.

Keywords: Pulmonary artery smooth muscle, microsomes, oxidant, tert-butylhydroperoxide, antioxidant, vitamin E, matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-2, Ca\textsuperscript{2+}-ATPase, ATP-dependent Ca\textsuperscript{2+} uptake, Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake.

IPC Code: C12N 9/00

Ca\textsuperscript{2+} plays a role as a second messenger in many biochemical and physiological events\textsuperscript{1,4}. An increase in its levels \textit{in situ}, caused by a variety of agonists is due to an influx of extracellular Ca\textsuperscript{2+} and/or release of Ca\textsuperscript{2+} from its suborganelle stores\textsuperscript{1,3,5}. Oxidants have been shown to stimulate Ca\textsuperscript{2+} release from suborganelles of smooth muscle and endothelium of pulmonary vessel\textsuperscript{6,7}. Microsomes sequester a considerable amount of mobilizable Ca\textsuperscript{2+} and play a pivotal role in regulating Ca\textsuperscript{2+} dynamics under stimulated conditions\textsuperscript{8,9}. As ATP-dependent Ca\textsuperscript{2+} uptake (via Ca\textsuperscript{2+}-ATPase) and Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake are involved in Ca\textsuperscript{2+} sequestration in microsomes of pulmonary smooth muscle, any defect in their activities could lead to an increase in Ca\textsuperscript{2+} mobilization \textit{in situ}\textsuperscript{8,10} and that may consequently produce pulmonary hypertension\textsuperscript{7,11}.

Ca\textsuperscript{2+}-ATPases and ATP- and Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake have been shown to be modulated by a variety of proteases in different systems\textsuperscript{9,10,12,13}. Activation of proteases under stimulated conditions has been found to inactivate ambient protease inhibitors, resulting in an alteration of the protease-antiprotease balance in favour of proteases\textsuperscript{14,15}. In isolated lung, infusion of

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Abbreviations: t-buOOH, tert-butylhydroperoxide; MMP-2, matrix metalloproteinase-2; TIMP-2, tissue inhibitor of metalloproteinase-2; HBPS, Hank’s buffered physiological saline; TBS, tris buffered saline; MOPS-Tris buffer, 3-(N-morpholino)propanesulfonic acid-Tris buffer; BSA, bovine serum albumin.
oxidants has been reported to produce pulmonary hypertension and edema\textsuperscript{11,18}. Protease inhibitors could prevent pulmonary hypertension and edema triggered by oxidants\textsuperscript{7,10,17}. In the lung, matrix metalloproteinase-2 (MMP-2) is found to be involved in the production of active vasopressors, such as angiotensin (from angiotensinogen) and endothelin (from proendothelin)\textsuperscript{18,19}, raising the possibility that oxidant-induced pulmonary hypertension could be mediated by MMP-2. As a change in \(\text{Ca}^{2+}\) levels in situ causes an alteration of pulmonary vascular tone\textsuperscript{11,16} and since oxidants enhance the tone by altering \(\text{Ca}^{2+}\) dynamics in suborganelles, for example, microsomes of pulmonary smooth muscle\textsuperscript{2,20-22}, in the present study, we have attempted to determine the roles of MMP-2 on \(\text{Ca}^{2+}\) dynamics in microsomes of bovine pulmonary vascular smooth muscle under an oxidant tert-butylhydroperoxide (\(\text{t}-\text{buOOH}\))-triggered conditions.

**Materials and Methods**

**Materials**

Molecular wt. markers, tert-butylhydroperoxide (\(\text{t}-\text{buOOH}\)), \(\text{LaCl}_3\), phosphocreatine, creatine phosphokinase, \(\text{CaCl}_2\), vitamin E, Tris-ATP, rotenone, \(\text{NADPH}\), \(\text{AMP}\), \(p\text{-nitrophenylphosphate}\), \(p\text{-nitrophenol}\), cytochrome \(c\) and gelatin were obtained from Sigma Chemical Co., St. Louis, MO, USA. [\(^{45}\text{Ca}\)] (sp. activity 0.8-1.0 Ci/mmole) and [\(^{14}\text{C}\)]-gelatin (sp. activity 1 mCi/mg) were the products of ICN, California, USA. MMP-2, TIMP-2, and their polyclonal antibodies were procured from Chemicon International, Temecula, California, USA. All other chemicals used were of analytical grade.

**Methods**

**Isolation of pulmonary artery smooth muscle tissue**

Bovine pulmonary artery collected from local slaughterhouse was washed several times with Hank’s buffered physiological saline (HBPS), \(pH\) 7.4 and the washed artery was used for further processing within 2 hr of collection. The intimal and external portions were removed and the tunica media i.e., the smooth muscle tissue was used\textsuperscript{23}.

**Preparation of microsomes**

Microsomes from the smooth muscle tissue were prepared as described\textsuperscript{10}. Briefly, the smooth muscle tissue was homogenized with a cyclomixer in ice-cold medium containing 0.25 \(M\) sucrose and 10 \(mM\) Tris/HCl buffer, \(pH\) 7.4 and the homogenate was centrifuged at 600 \(g\) for 15 min at 4°C. The resulting supernatant was centrifuged at 15,000 \(g\) for 20 min to sediment mitochondria and lysosomes\textsuperscript{24} and then centrifuged at 100,000 \(g\) for 1 hr. The pellet was suspended in 10\% (w/v) sucrose containing 10 \(mM\) Tris/HCl buffer, \(pH\) 7.4 and was layered on a discontinuous gradient consisting of 40\% (w/v) and 20\% (w/v) sucrose, both containing 10 \(mM\) Tris/HCl buffer, \(pH\) 7.4. The gradient was centrifuged at 105,000 \(g\) for 2 hr. The fraction collected at the 20-40\% sucrose interface was used as the plasma membrane\textsuperscript{25} and the pellet was used as the microsomal fraction. The microsomal pellet was suspended in the homogenizing buffer consisting of 0.25 \(M\) sucrose and 10 \(mM\) Tris/HCl buffer \(pH\) 7.4. All operations for the isolation of the microsomes were performed at 4°C, and the experiments were carried out with freshly prepared microsomes.

**Assay of marker enzymes**

Rotenone-insensitive cytochrome \(c\) reductase activity\textsuperscript{26} and cytochrome \(c\) oxidase\textsuperscript{27} were assayed as previously described. Acid phosphatase activity was determined at \(pH\) 5.5 using \(p\text{-nitrophenyl phosphate} as the substrate\textsuperscript{28}. Release of \(\text{Pi}\) from 5’AMP, an index of 5’ nucleotidase activity was determined by the method reported earlier\textsuperscript{29}.

**Electron microscopic study**

Electron microscopic study of the microsomes was performed using the procedure previously described\textsuperscript{30}, with some modifications\textsuperscript{31}. Briefly, both control and \(\text{t}-\text{buOOH}\) (300 \(\mu M\)) treated (30 min at 37°C) microsomal suspensions were prefixed with 3\% glutaraldehyde in 0.1 \(M\) Na-cacodylate buffer, \(pH\) 7.2, post-fixed with 1\% buffered osmium tetroxide, and then centrifuged at 1,10,000 \(g\) for 1 hr. Pellets were then dehydrated through graded series of ethanol and embedded in Agar 100 resin. Ultrathin sections were cut in a Leica ultracut UCT ultramicrotome, stained with a saturated solution of uranyl acetate and 0.2\% lead citrate, \(pH\) 12.0. Grids were examined in a FEI Tecnai 12 Biotwin transmission electron microscope fitted with a SIS (SIS GmbH, Germany) CCD camera.

**Identification of MMP-2 and TIMP-2 in microsomes**

MMP-2 and TIMP-2 were identified in the microsomal suspension by western immunoblot, as described\textsuperscript{32} with some modifications using their respective polyclonal antibodies. Briefly, the microsomal suspension (~5 \(\mu g\) protein) was electrophoresed in SDS-PAGE and the resolved
proteins were then transferred electrophoretically (1 hr, 100 V) to nitrocellulose paper (0.2 μm pore size). Non-specific 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. Nitrocellulose paper was then incubated with polyclonal rabbit anti bovine (MMP-2 or TIMP-2) IgG for 2 hr at room temperature with constant shaking, followed by washings with TBS (3 times; 20 min each). Thereafter, nitrocellulose paper was incubated for 2 hr with horse raddish peroxidase conjugated goat (rabbit IgG) antibody and then washed 3 times with TBS (20 min each). The enzymatic activity was revealed with 4-chloro-1-naphthol.

Zymogram of protease activity

Gelatin zymogram was performed as previously described. Briefly, the microsomal suspension (~50 µg of protein) in standard SDS loading buffer lacking 2-mercaptoethanol and not boiled before loading was applied to the gel containing 0.1% gelatin. The gels were run at 200 V for 1 hr in a Bio-Rad Mini protein II apparatus and then soaked in 200 ml of 2% Triton X-100 in distilled water in a shaker for 1 hr at 20°C. Thereafter, the gels were soaked in reaction buffer (100 mM Tris/HCl, pH 8.0) with or without TIMP-2 (5 µg/ml) for 12 hr at 37°C and then stained with Coomassie brilliant blue, followed by washing with distilled water for 1 min. The clear zone against the dark Coomassie background indicated MMP-2 activity. The marker lane was separated from the gel and destained with destaining solution containing methanol:acetic acid:water (4:1:5).

To determine the effect of t-buOOH on MMP-2 activity, the microsomal suspension was treated with t-buOOH (300 µM) for 30 min at 37°C, and the protease activity in gelatin zymogram was determined. To determine whether TIMP-2 can inhibit t-buOOH induced MMP-2 activity, the microsomal suspension was treated with t-buOOH (300 µM) for 30 min at 37°C and then electrophoresis was carried out. After appropriate washing with Triton X-100, the gel slice was incubated with TIMP-2 (50 µg/ml) for 12 hr at 37°C in reaction buffer, and the zymogram was performed as mentioned.

Assay of protease activity by [14C]-gelatin degradation

Protease activities in the microsomes were determined by following the procedure previously described. Briefly, the radiolabelled gelatin substrate was prepared by diluting 20 µl (1.2 µCi) of [14C]-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 reaction contained 40 µl of substrate, 10 mM CaCl2 and the microsomal suspension (~50 µg protein). Samples were incubated for 1 hr at 37°C and the reaction was stopped by adding 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 radioactivity in the supernatant was determined.

Microsomal suspension (~50 µg/ml) was treated with t-buOOH (300 µM) for 30 min at 37°C and [14C]-gelatin degradation was determined. Vit. E (1 mM) or TIMP-2 (5 µg/ml and 50 µg/ml) was added to the microsomal suspension (~50 µg protein) for 30 min, followed by the treatment with or without t-buOOH (300 µM) for 30 min and the [14C]-gelatin degradation was assessed. To determine the effect of TIMP-2 (5 µg/ml) on MMP-2 activity, the microsomes were pre-treated with or without TIMP-2 (5 µg/ml) for 30 min, followed by the addition of MMP-2 (0.5 µg/ml and 1 µg/ml) for 30 min, and [14C]-gelatin degradation was determined.

To determine the combined effect of t-buOOH and MMP-2, the microsomal suspension was treated with low doses of t-buOOH (100 µM) and MMP-2 (0.5 µg/ml) for 30 min, and then MMP-2 activity was determined. TIMP-2 (5 µg/ml and 50 µg/ml) were added to the microsomal suspension for 30 min, followed by addition of t-buOOH (100 µM) plus MMP-2 (0.5 µg/ml) for 30 min, and then MMP-2 activity was determined. To elucidate whether t-buOOH inactivates TIMP-2, t-buOOH (300 µM) was added to the assay buffer containing TIMP-2 (5 µg/ml and 50 µg/ml) for 30 min, followed by addition of MMP-2 (1 µg/ml) for 30 min and the gelatin degradation was determined.

Assay of Ca2+-ATPase activity

Ca2+-ATPase activity was determined colorimetrically by measuring Ca2+-dependent release of Pi as previously described. Vit. E (1 mM) and TIMP-2 (5 µg/ml and 50 µg/ml) were added to the microsomal suspension (~50 µg protein) for 30 min, followed by treatment with or without t-buOOH (300 µM) for 30 min and the activity was determined. To determine
the effect of MMP-2 and TIMP-2 on Ca\textsuperscript{2+}-ATPase activity, the microsomes were pre-incubated with or without TIMP-2 (5 µg/ml), followed by addition of MMP-2 (0.5 µg/ml and 1 µg/ml) for 30 min and then activity was measured. To determine the combined effect of t-buOOH and MMP-2 on Ca\textsuperscript{2+}-ATPase in the presence and absence of TIMP-2, the microsomal suspension were pre-treated with or without TIMP-2 (5 µg/ml and 50 µg/ml) for 30 min, followed by the addition of low doses of t-buOOH (100 µM) plus MMP-2 (0.5 µg/ml) for 30 min and the activity was measured.

**Determination of ATP-dependent Ca\textsuperscript{2+} uptake**

ATP-dependent Ca\textsuperscript{2+} uptake was measured as described\textsuperscript{38}. Briefly, the microsomal suspension (~50 µg protein) was pre-incubated at 37°C for 30 min with or without vit. E (1 mM) and/or TIMP-2 (5 µg/ml and 50 µg/ml) in 0.5 ml of a medium (containing 140 mM KCl, 10 mM MOPS-Tris buffer, pH 7.4, 2 mM MgCl\textsubscript{2}) and then t-buOOH (300 µM) was added. After 30 min, 10 µCi of [\textsuperscript{45}Ca\textsuperscript{2+}] was added, and Ca\textsuperscript{2+} uptake was initiated by adding 1 mM Tris-ATP, 5 mM phosphocreatine, and 20 U/ml creatine phosphokinase. After 30 min of incubation at 37°C, 250 µl aliquots were filtered through Millipore filters (pore size:0.45 µm) and radioactivity in the filters was determined.

To determine the effect of MMP-2 and TIMP-2 on ATP-dependent Ca\textsuperscript{2+} uptake, the microsomal suspension (~50 µg protein) was pre-incubated with or without TIMP-2 (5 µg/ml) for 30 min, followed by the addition of MMP-2 (0.5 µg/ml and 1 µg/ml) for 30 min, and then Ca\textsuperscript{2+} uptake was determined. To determine the combined effect of t-buOOH and MMP-2 in the presence and absence of TIMP-2, the microsomal suspension was pre-incubated with or without TIMP-2 (5 µg/ml and 50 µg/ml) for 30 min, followed by addition of low doses of t-buOOH (100 µM) plus MMP-2 (0.5 µg/ml) for 30 min, and then ATP-dependent Ca\textsuperscript{2+} uptake was determined.

**Determination of Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake**

Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake measurements were carried out as described\textsuperscript{29}. Briefly, the microsomal suspension (~50 µg protein) was pre-incubated at 37°C for 30 min with or without vit. E (1 mM) and/or TIMP-2 (5 µg/ml and 50 µg/ml) in a medium containing 140 mM NaCl and 20 mM MOPS-Tris buffer, pH 7.4 then t-buOOH (300 µM) was added. After 30 min, 0.5 ml aliquots of Na\textsuperscript{+} loaded microsomes were added to a series of tubes containing 30 ml of a medium containing 140 mM KCl, 20 mM MOPS-Tris buffer, pH 7.4 plus 10 µCi of [\textsuperscript{45}Ca\textsuperscript{2+}] for 30 min. Ca\textsuperscript{2+} uptake was then terminated by adding 5 ml of ice-cold stopping solution containing 140 mM KCl, 1 mM LaCl\textsubscript{3}, 20 mM MOPS-Tris buffer, pH 7.4. Aliquots (250 µl) were filtered through Millipore filters (pore size:0.45 µm) and radioactivity in the filters was determined.

To determine the effect of MMP-2 and TIMP-2 on Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake, the microsomal suspension was pre-incubated with or without TIMP-2 (0.5 µg/ml and 1 µg/ml) for 30 min, and [\textsuperscript{45}Ca\textsuperscript{2+}] uptake was determined. To determine the combined effect of t-buOOH and MMP-2 in the presence and absence of TIMP-2, the microsomal suspension was pre-incubated with or without TIMP-2 (5 µg/ml and 50 µg/ml) for 30 min, followed by addition of low doses of t-buOOH (100 µM) plus MMP-2 (0.5 µg/ml) for 30 min, and then Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake was measured.

Proteins were estimated by following the method of Lowry et al\textsuperscript{37}.

**Statistical analysis**

Data were analyzed by unpaired t test and analysis of variance, followed by the test of least significant differences\textsuperscript{36} for comparison within and between the groups. P<0.05 was considered as significant.

**Results**

We characterized the microsomal fraction of bovine pulmonary artery smooth muscles by measuring at different steps the activities of cytochrome c oxidase (a mitochondrial marker\textsuperscript{39}), acid phosphatase (a lysosomal marker\textsuperscript{40}), rotenone insensitive NADPH-cytochrome c reductase (a microsomal marker\textsuperscript{41}) and 5'-nucleotidase (a plasma membrane marker\textsuperscript{40}). Microsomal fraction showed, respectively 37-fold decrease in specific activity of cytochrome c oxidase and 26-fold decrease in the specific activity of acid phosphatase activity compared with 600-15,000 g pellet. It also showed 45-fold decrease in specific activity of 5'-nucleotidase, compared with plasma membrane fraction. Furthermore, the microsomal fraction showed, respectively 14-fold and 22-fold increase in the specific activity of rotenone insensitive NADPH-
cytochrome c reductase, compared with the 600-15,000 g pellet and the plasma membrane fraction (Table 1).

Immunoblot study of the microsomal suspension with polyclonal MMP-2 and TIMP-2 antibodies revealed that they are the ambient proteinase and the antiproteinase respectively (Figs 1 & 2). Gelatin zymogram study showed that t-buOOH stimulated 72 kDa MMP-2 activity and that was inhibited by TIMP-2 (Fig. 3).

Treatment of microsomes with t-buOOH (300 µM) stimulated MMP-2 activity, as evidenced from the [14C]-gelatin degradation assay. In addition it also stimulated Ca2+-ATPase activity and ATP-dependent Ca2+ uptake, but inhibited Na+-dependent Ca2+ uptake (Table 2). However, Ca2+ uptake, (ATP- and Na+-dependent) during treatment of the microsomes with t-buOOH (300 µM) was inhibited, compared to the basal condition. Pre-treatment with Vit. E (1 mM) and TIMP-2 (50 µg/ml) reversed the MMP-2 activity, Ca2+-ATPase activity, ATP- and Na+-dependent Ca2+ uptakes caused by t-buOOH (300 µM), but pretreatment with TIMP-2 (5 µg/ml) did not reverse any of the effects produced by t-buOOH (Table 2).

Treatment of the microsomes with MMP-2 (1 µg/ml) stimulated Ca2+-ATPase activity and ATP-

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cytochrome c oxidase</th>
<th>Acid phosphatase</th>
<th>NADPH-cytochrome c reductase</th>
<th>5'-Nucleotidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>600-15,000 g pellet</td>
<td>3.34 ± 0.21</td>
<td>3.40 ± 0.23</td>
<td>0.16 ± 0.02</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>15,000-100,000 g pellet</td>
<td>0.24 ± 0.04 (7)</td>
<td>0.33 ± 0.05 (10)</td>
<td>1.68 ± 0.12 (1050)</td>
<td>2.25 ± 0.16 (1250)</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.09 ± 0.01 (3)</td>
<td>0.13 ± 0.02 (4)</td>
<td>2.20 ± 0.16 (1375)</td>
<td>0.07 ± 0.01 (39)</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>0.08 ± 0.01 (2)</td>
<td>0.14 ± 0.02 (4)</td>
<td>0.10 ± 0.01 (63)</td>
<td>3.12 ± 0.20 (1733)</td>
</tr>
</tbody>
</table>

Table 2—Effect of vit. E, TIMP-2 (5 µg/ml and 50 µg/ml) on MMP-2 activity, Ca2+-ATPase activity, ATP- and Na+-dependent [45Ca2+] uptake caused by t-buOOH in bovine pulmonary artery smooth muscle microsomes

<table>
<thead>
<tr>
<th>Additions</th>
<th>MMP-2 activity</th>
<th>Ca2+-ATPase activity</th>
<th>ATP-dependent [45Ca2+] uptake</th>
<th>Na+-dependent [45Ca2+] uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (none)</td>
<td>164±9</td>
<td>2.28±0.15</td>
<td>67.34±2.34</td>
<td>134.71±3.51</td>
</tr>
<tr>
<td>t-buOOH (300 µM)</td>
<td>126±72a (+672)</td>
<td>6.26±0.41a (+175)</td>
<td>98.26±2.91a (+46)</td>
<td>43.25±1.42a (-68)</td>
</tr>
<tr>
<td>Vit. E (1 mM)</td>
<td>151±8 (-8)</td>
<td>2.13±0.13 (-7)</td>
<td>66.93±2.29 (-1)</td>
<td>135.31±3.56 (0)</td>
</tr>
<tr>
<td>Vit. E (1 mM) + t-buOOH (300 µM)</td>
<td>175±9 (+7)</td>
<td>2.37±0.16a (+44)</td>
<td>67.54±2.37b (0)</td>
<td>134.12±3.48b (0)</td>
</tr>
<tr>
<td>TIMP-2 (5 µg/ml)</td>
<td>17±3a (-90)</td>
<td>1.59±0.12b (-30)</td>
<td>59.26±1.89b (-12)</td>
<td>147.95±3.71b (+10)</td>
</tr>
<tr>
<td>TIMP-2 (5 µg/ml) + t-buOOH (300 µM)</td>
<td>1198±66a (-630)</td>
<td>6.01±0.39b (+164)</td>
<td>96.88±2.84a (+44)</td>
<td>46.33±1.52a (-66)</td>
</tr>
<tr>
<td>TIMP-2 (50 µg/ml)</td>
<td>16±3a (-90)</td>
<td>1.57±0.11b (-31)</td>
<td>58.94±1.83a (-12)</td>
<td>148.21±3.38b (+10)</td>
</tr>
<tr>
<td>TIMP-2 (50 µg/ml) + t-buOOH (300 µM)</td>
<td>21±3a (-87)</td>
<td>1.61±0.12b (-29)</td>
<td>59.81±1.94b (-11)</td>
<td>147.09±3.66b (+9)</td>
</tr>
</tbody>
</table>

*p<0.001 compared to control (none); b*p<0.05 compared to control (none); c*p<0.001 compared to t-buOOH (300 µM); d*p<0.001 compared to respective control.

Total [45Ca2+] uptake (ATP- and Na+-dependent) decreased compared to that observed under basal conditions (141.51 and 202.05 n mol [45Ca2+]30 min/mg protein, respectively; percent change from basal value: -30).
dependent Ca^{2+} uptake, while Na^{+}-dependent Ca^{2+} uptake was inhibited, but the total Ca^{2+} uptake (ATP- and Na^{+}-dependent) during treatment of the microsomes with MMP-2 (1 µg/ml) decreased in comparison to the basal condition. However, pretreatment with TIMP-2 (5 µg/ml) reversed these responses produced by MMP-2 (1 µg/ml) (Table 3).

Electron micrograph revealed that treatment with t-buOOH (300 µM) did not cause any damage to the microsomes, compared to the untreated condition (Fig. 4).

Combined treatment of low doses of MMP-2 (0.5 µg/ml) and t-buOOH (100 µM) augmented Ca^{2+}-ATPase activity and ATP-dependent Ca^{2+} uptake, but inhibited Na^{+}-dependent Ca^{2+} uptake, compared to the response produced by either of them. However, Ca^{2+} uptake (ATP- and Na^{+}-dependent) activity was significantly decreased, compared to the basal condition and to the effects produced by either of these agents at their respective concentrations. Pre-treatment with TIMP-2 (5 µg/ml) reversed the responses produced by the low dose of MMP-2, but not by t-buOOH or the combined treatments on the parameters. In contrast, pre-treatment with TIMP-2 (50 µg/ml) reversed the changes in the parameters caused by combined treatment of the low doses of MMP-2 and t-buOOH (Table 3).

TIMP-2 (5 µg/ml) inhibited MMP-2 (1 µg/ml) activity, but the inhibitory activity of TIMP-2 (5 µg/ml) was abolished in the presence of t-buOOH.
Table 3—Effect of TIMP-2 on MMP-2 and/or t-buOOH (100 µM) mediated Ca\(^{2+}\)-ATPase activity and ATP- and Na\(^{+}\)-dependent \([^{45}\text{Ca}^{2+}]\) uptakes in bovine pulmonary artery smooth muscle microsomes

[Results are mean ± SE (n = 4); Ca\(^{2+}\)-ATPase activity is expressed as µ mol Pi/30 min/mg protein and ATP- and Na\(^{+}\)-dependent \([^{45}\text{Ca}^{2+}]\) uptake as n mol \([^{45}\text{Ca}^{2+}]\)/30 min/mg protein. Values in the parentheses indicate % change vs control (none)]

<table>
<thead>
<tr>
<th>Additions</th>
<th>Ca(^{2+})-ATPase activity</th>
<th>ATP-dependent ([^{45}\text{Ca}^{2+}]) uptake</th>
<th>Na(^{+})-dependent ([^{45}\text{Ca}^{2+}]) uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (none)</td>
<td>2.28 ± 0.15</td>
<td>67.34 ± 2.34</td>
<td>134.71 ± 3.51</td>
</tr>
<tr>
<td>MMP-2 (1 µg/ml)</td>
<td>7.27 ± 0.57(^{a}) (+219)</td>
<td>109.76 ± 3.21(^{a}) (+63)</td>
<td>35.34 ± 1.03(^{a}) (-74)</td>
</tr>
<tr>
<td>TIMP-2 (5 µg/ml)</td>
<td>1.59 ± 0.12(^{b}) (-30)</td>
<td>59.26 ± 1.89(^{b}) (-12)</td>
<td>147.95 ± 3.71(^{b}) (+10)</td>
</tr>
<tr>
<td>TIMP-2 (5 µg/ml) + MMP-2 (1 µg/ml)</td>
<td>1.62 ± 0.12(^{bc}) (-29)</td>
<td>59.87 ± 1.94(^{bc}) (-11)</td>
<td>147.14 ± 3.66(^{bc}) (+9)</td>
</tr>
<tr>
<td>MMP-2 (0.5 µg/ml)</td>
<td>4.34 ± 0.34(^{b}) (-90)</td>
<td>87.47 ± 2.85(^{b}) (+30)</td>
<td>69.62 ± 1.53(^{b}) (-48)</td>
</tr>
<tr>
<td>TIMP-2 (5 µg/ml) + MMP-2 (0.5 µg/ml)</td>
<td>1.60 ± 0.11(^{b}) (-30)</td>
<td>59.45 ± 1.91(^{b}) (-12)</td>
<td>147.52 ± 3.70(^{bd}) (+10)</td>
</tr>
<tr>
<td>t-buOOH (100 µM)</td>
<td>3.94 ± 0.31(^{b}) (+73)</td>
<td>83.48 ± 2.79(^{b}) (+24)</td>
<td>63.25 ± 1.61(^{b}) (-53)</td>
</tr>
<tr>
<td>t-buOOH (100 µM) + MMP-2 (0.5 µg/ml)</td>
<td>8.07 ± 0.72(^{bd}) (+254)</td>
<td>118.64 ± 3.38(^{bd}) (+76)</td>
<td>21.82 ± 1.97(^{bd}) (-84)</td>
</tr>
<tr>
<td>TIMP-2 (5 µg/ml) + [t-buOOH (100 µM) + MMP-2 (0.5 µg/ml)]</td>
<td>7.11 ± 0.51(^{ad}) (+212)</td>
<td>108.75 ± 3.08(^{ad}) (+61)</td>
<td>37.82 ± 1.08(^{ad}) (-72)</td>
</tr>
<tr>
<td>MMP-2 (0.5 µg/ml)]</td>
<td>1.57 ± 0.11(^{b}) (-31)</td>
<td>58.94 ± 1.83(^{b}) (-12)</td>
<td>148.21 ± 3.88(^{b}) (+10)</td>
</tr>
<tr>
<td>TIMP-2 (50 µg/ml) + [MMP-2 (0.5 µg/ml) + t-buOOH (100 µM)]</td>
<td>1.65 ± 0.12(^{d}) (-28)</td>
<td>60.26 ± 2.11(^{d}) (-11)</td>
<td>146.77 ± 3.64(^{d}) (+9)</td>
</tr>
</tbody>
</table>

\(^{a}\)p<0.001 compared to control (none); \(^{b}\)p<0.05 compared to control (none); \(^{c}\)p<0.001 compared to MMP-2 (1 µg/ml); \(^{d}\)p<0.001 compared to MMP-2 (0.5 µg/ml); \(^{e}\)p<0.05 compared to MMP-2 (0.5 µg/ml); \(^{f}\)p<0.05 compared to t-buOOH (100 µM); \(^{g}\)p<0.001 compared to t-buOOH (100 µM); \(^{h}\)p<0.001 compared to TIMP-2 (5 µg/ml). Total Ca\(^{2+}\) uptake (ATP- and Na\(^{+}\)-dependent) during treatment of the microsomes with the MMP-2 (1 µg/ml), MMP-2 (0.5 µg/ml) and {t-buOOH (100 µM) + MMP-2 (0.5 µg/ml)} (145.1, 157.09 and 140.46 nmol \([^{45}\text{Ca}^{2+}]\)/30 min/mg protein, respectively) was found to be decreased compared to that observed under basal conditions (202.05 n mol \([^{45}\text{Ca}^{2+}]\)/30 min/mg protein); percent change from basal value: -28, -22 and -30, respectively.

Table 4—Effect of pre-treatment of TIMP-2/TIMP-2 plus t-buOOH on \([^{14}\text{C}]\)-gelatin degradation by MMP-2

[MMP-2 activity was measured by \([^{14}\text{C}]\)-gelatin degradation and expressed as (cpm/30 min/mg protein). Results are mean ± S.E. (n = 4). Values in parentheses indicate % change vs control]

<table>
<thead>
<tr>
<th>Additions</th>
<th>([^{14}\text{C}])-gelatin degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control [MMP-2 (1 µg/ml)]</td>
<td>1410 ± 68</td>
</tr>
<tr>
<td>MMP-2 (5 µg/ml)</td>
<td>12 ± 2(^{d}) (-99)</td>
</tr>
<tr>
<td>MMP-2 (5 µg/ml) + MMP-2 (1 µg/ml)</td>
<td>14 ± 3(^{b}) (-99)</td>
</tr>
<tr>
<td>MMP-2 (50 µg/ml)</td>
<td>11 ± 3(^{b}) (-99)</td>
</tr>
<tr>
<td>MMP-2 (50 µg/ml) + MMP-2 (1 µg/ml)</td>
<td>10 ± 3(^{b}) (-99)</td>
</tr>
<tr>
<td>[t-buOOH (300 µM) + TIMP-2 (50 µg/ml)] + MMP-2 (1 µg/ml)</td>
<td>26 ± 5(^{a}) (-98)</td>
</tr>
<tr>
<td>[t-buOOH (300 µM) + TIMP-2 (50 µg/ml)] + [MMP-2 (1 µg/ml)]</td>
<td>1302 ± 59 (-8)</td>
</tr>
</tbody>
</table>

\(^{a}\)p<0.001 compared to the response produced by MMP-2 (1 µg/ml)

Discussion

The aim of the present study was to determine the role of MMP-2 in the oxidant t-buOOH-mediated alteration of Ca\(^{2+}\)-ATPase activity and Ca\(^{2+}\) uptake (ATP- and Na\(^{+}\)-dependent) in bovine pulmonary artery smooth muscle microsomes. Our results show that treatment of the microsomes with t-buOOH stimulates MMP-2 activity, Ca\(^{2+}\)-ATPase activity and ATP-dependent Ca\(^{2+}\) uptake, whereas Na\(^{+}\)-dependent Ca\(^{2+}\) uptake is inhibited. We have used 300 µM of t-buOOH, since at this concentration the oxidant causes pulmonary hypertension and edema in isolated rabbit lung to an optimum extent\(^{11}\). Moreover, the concentration of t-buOOH (300 µM) employed in the present study was based on the oxidant’s effects on different biochemical parameters in a variety of systems\(^{7,20,21,42}\). t-buOOH appears to produce these effects via oxidant species because pre-treatment of the microsomes with vit. E reversed these responses (Table 2). The increase in Ca\(^{2+}\)-ATPase activity by t-buOOH does not occur merely due to membrane-associated changes since Ca\(^{2+}\)-ATPase activity and the ATP-dependent Ca\(^{2+}\) uptake both under basal and...
$t$-buOOH stimulation appears to be positively correlated (Table 2), suggesting that the Ca$^{2+}$ pump activity is indeed associated with ATP-dependent Ca$^{2+}$ uptake under $t$-buOOH-stimulated conditions.

Activation of Ca$^{2+}$-ATPase by selective proteolytic cleavage of the calmodulin binding domain has been demonstrated$^{43}$. The proteolysis of Ca$^{2+}$-ATPase is also reported to cause activation of ATP hydrolytic activity in human erythrocyte membrane$^{44}$. In addition, Na$^+$-dependent Ca$^{2+}$ uptake could be modulated proteolytically$^{12,45}$. Evidences to prove that a protease plays an important role in regulating Ca$^{2+}$ uptake in the microsomes under $t$-buOOH are: (i) treatment with $t$-buOOH stimulates MMP-2 activity, an ambient MMP having molecular mass of 72 kDa (Figs. 1 & 3), as well as Ca$^{2+}$-ATPase activity and ATP-dependent Ca$^{2+}$ uptake, but inhibits Na$^+$-dependent Ca$^{2+}$ uptake (Table 2); (ii) $t$-buOOH-induced changes on these parameters were found to be inhibited by TIMP-2 (Table 2), an ambient inhibitor of MMP-2 in the microsomes (Fig. 2); (iii) treatment of the microsomes with MMP-2 stimulates Ca$^{2+}$-ATPase activity, ATP-dependent Ca$^{2+}$ uptake, but inhibits Na$^+$-dependent Ca$^{2+}$ uptake (Table 3); (iv) pre-treatment with TIMP-2 reverses these effects produced by MMP-2 (Table 3); (v) combined treatment of low doses of $t$-buOOH and MMP-2 stimulates Ca$^{2+}$-ATPase activity, ATP-dependent Ca$^{2+}$ uptake, and inhibits Na$^+$-dependent Ca$^{2+}$ uptake compared to the effects produced by them individually (Table 3); (vi) pre-treatment with TIMP-2 reverses these effects produced by combined treatment of the low doses of $t$-buOOH and MMP-2 (Table 3); and (vii) inhibition of Na$^+$-dependent Ca$^{2+}$ uptake overpowers the stimulation of ATP-dependent Ca$^{2+}$ uptake under $t$-buOOH and/or MMP-2 stimulated conditions (Tables 2 & 3). These results strongly suggest that MMP-2 plays a pivotal role in stimulating Ca$^{2+}$-ATPase activity and ATP-dependent Ca$^{2+}$ uptake, and in inhibiting Na$^+$-dependent Ca$^{2+}$ uptake in the microsomes under $t$-buOOH-triggered conditions.

The present study also demonstrates that pre-treatment with 50 µg/ml TIMP-2 reversed the effects of the low doses of $t$-buOOH and/or MMP-2 on the studied parameters (Table 3). By contrast, pre-treatment with 5 µg/ml TIMP-2 do not reverse the effects produced by the combined treatment of low doses of $t$-buOOH and/or MMP-2, while it inhibits the effects elicited by the low dose of MMP-2 (Table 3). Interestingly, both low and high doses of $t$-buOOH-mediated effects on MMP-2 activity, Ca$^{2+}$-ATPase activity, ATP and Na$^+$-dependent Ca$^{2+}$ uptake were reversed by pretreatment with 50 µg/ml of TIMP-2, but not by 5 µg/ml of TIMP-2 (Tables 2 & 3). Further, our study suggests that treatment of MMP-2 (1 µg/ml) with 5 µg/ml TIMP-2 inhibits the MMP-2 activity, but prior treatment of MMP-2 (1 µg/ml) with $t$-buOOH (300 µM) does not elicit any inhibition by TIMP-2 (5 µg/ml) on MMP-2 activity (Table 4). This apparently suggests that $t$-buOOH causes oxidative inactivation of TIMP-2 by some mechanism(s) leading to an increase in MMP-2 activity and that subsequently stimulates Ca$^{2+}$-ATPase activity and ATP-dependent Ca$^{2+}$ uptake, but inhibits Na$^+$-dependent Ca$^{2+}$ uptake in the microsomes.

MMPs play a pivotal role in the physiologically occurring changes in cells and tissues$^{46-48}$. Oxidants have been demonstrated to produce pulmonary hypertension in isolated lung, which can be prevented by protease inhibitors as well as by the intracellular mobilizing agent, A23187$^{11,49-51}$. Pulmonary vascular tissue produces MMPs, which also contribute to different cardiovascular diseases$^{47,48,52}$. In mammalian systems, hypertensive cells contain low amounts of TIMPs$^{53}$, which can be degraded in the myocardium by a variety of stimulants, such as oxidants including hypochlorous acid$^{52,54}$. Proteolytic degradation (inactivation) of ambient proteinase inhibitors has been observed in the myocardium from patients with essential hypertension and has been used as a marker to establish the severity of the disease and/or effectiveness of therapeutic treatment$^{53,55}$. Overall, it appears conceivable that $t$-buOOH inactivates TIMP-2, the ambient antiproteinase activity, thereby causing an alteration in the MMP-2/TIMP-2 balance in favour of MMP-2. However, the mechanism(s) by which MMP-2 regulates Ca$^{2+}$-ATPase activity, ATP- and Na$^+$-dependent Ca$^{2+}$ uptake in the microsomes under $t$-buOOH–triggered condition needs further investigation.

**Acknowledgement**

Financial assistance from the Life Science Research Board (Defence Research and Development Organization, Ministry of Defence, Govt. of India) is gratefully acknowledged. Thanks are due to Dr. Amar Nath Ghosh (Scientist, Electron Microscopy Division, NICED, ICMR, Kolkata), Dr. D Sarkar (Scientist, Indian Institute of Chemical Biology, Kolkata) and...
Dr. Sudip Das (Department of Biochemistry and Biophysics, University of Kalyani, Kalyani 741235, West Bengal) for their help in this work.

References