Chymase is Activated in the Pulmonary Inflammation and Fibrosis Induced by Paraquat in Hamsters

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The involvement of chymase has been implicated in fibrotic response to tissue injuries. Besides its direct action, chymase indirectly promotes fibrotic response by generating angiotensin (Ang) II from Ang I. In the present study, we examined whether chymase and angiotensin converting enzyme (ACE), that also generates Ang II, were activated in the pulmonary inflammation and fibrosis induced by paraquat (PQ) in hamsters. In an acute PQ intoxication group, PQ was administered subcutaneously and the lungs were excised four days after administration. In a chronic PQ intoxication group, PQ was administered at the same dose once a week for six weeks. The lungs were excised five weeks after the last administration. On dissection, alveolar hemorrhage and capillary stasis were found in the acute PQ intoxication group while interstitial pulmonary fibrosis was found in the chronic PQ intoxication group. The pulmonary tissue chymase activity was elevated in both intoxication groups when compared with a respective age-matched, vehicle (saline)-administered group. Pulmonary tissue ACE activity, on the other hand, decreased in both intoxication groups. These data suggested that activated chymase may be involved in the establishment of PQ-induced pulmonary fibrosis in hamsters. ——— paraquat; pulmonary fibrosis; ACE; chymase

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Paraquat (1,1-dimethyl-4,4’-bipyridylum chloride, PQ), a para-substituted quaternary bipyridyl cation, is a herbicide introduced commercially in 1962. Because of its solubility in water and its efficacy in killing weeds, it is still in use worldwide (Winchester 1995). However, PQ poses a serious hazard to humans, especially when the concentrate is accidentally or intentionally ingested (Tinoco et al. 1993; Stephens and Moormeister 1997; Hwang et al. 2002). The primal toxicity of PQ is pulmonary fibrosis which develops after the acute phase (Toner et al. 1970). It has been reported that PQ concentrates in the lungs, forming pulmonary regional edema which may progress to interstitial fibrosis. The mechanisms of the production of fibrosis, however, have not yet been elucidated. There is an accumulation of supporting evidence that chymase, a chymotrypsin-like serine protease, may contribute to the production of fibrosis by directly activating procollagenase (Saarinen et al. 1994) and initiating collagen fibril formation (Kofford et al. 1997). One of the major effects of chymase is its ability to convert angiotensin (Ang) I to Ang II, which is believed to play a crucial role in the pathogenesis of fibrosis within organs such as cardiac muscles (Tachikawa et al. 2003), arteries (Shiota et al. 1993), and lungs (Marshall et al. 2000). This evidence strongly suggests that chymase is the leading candidate involved in the production of fibrosis induced by PQ. In the present study, the effects of PQ on pulmonary chymase activity were examined in acute and chronic PQ intoxication in hamsters. The effects of PQ on angiotensin converting enzyme (ACE) activity that converts Ang I to Ang II was also examined.

**Materials and Methods**

### Chemicals

PQ dichloride was obtained from Zeneca (Osaka) and diluted with saline to a final concentration of 9 mg/mL that was used for administrations of 18 mg/kg of PQ. Ang II, aprotinin, o-phenanthroline, chymostatin, captopril, and hippuric acid hydrate were obtained from Sigma (St. Louis, MO, USA). Ang I was obtained from Bachem (King of Prussia, PA, USA). Hippuryl-His-Leu was obtained from Peptide Institute (Osaka).

### Animals

Four-week-old male Syrian hamsters (SLC, Hamamatsu) were used. Six hamsters each were housed in cages at 23±2°C, 60±10% humidity, and lit daily from 7:00 a.m. to 7:00 p.m. in a controlled room. They received laboratory chow and water *ad libitum*. The care and handling of the animals were in accordance with the “Azabu University Animal Experiment Guidelines; April 2000”.

### Acute PQ intoxication

Four days after subcutaneous PQ administration of a single dose of 18 mg/kg, lungs of six hamsters were isolated as described below and subjected to histopathological examination and measurements of chymase and ACE activities. Saline, as a vehicle of PQ, was administered subcutaneously to six other hamsters at the same volume of PQ, and they too were subjected to histopathological examination and measurements of chymase and ACE activities four days after administration.

### Chronic PQ intoxication

Although a single PQ administration induced pulmonary hemorrhage (Kokubo et al. 1984), repeated administrations were required to produce the chronic lung injury that leads to fibrosis in hamsters (Butler 1975). Thus, PQ at a subcutaneous dose of 18 mg/kg was scheduled to be administered once a week for six weeks to seventeen hamsters. Saline at the same volume of PQ was administered once a week for six weeks to five other hamsters. The lungs of both chronic PQ-intoxicated and saline-administered hamsters were subjected to histopathological examination and measurements of chymase and ACE activities five weeks after the above series of administrations.
Lung isolation and histopathological examinations

Before dissection, the animals were deeply anesthetized with pentobarbital (50 mg/kg, ip). After exsanguinations, the lungs were quickly removed and the right lungs were isolated and frozen in liquid nitrogen. The specimens were kept at −80°C until measurements of chymase and ACE activities. The left lungs were inflated via the trachea with 20% natural-buffered formalin at a water pressure of 18 cm and immersed in the same fixing solution. Using the standard method, the anterior or posterior lobes of lungs were paraffin-embedded and cut into thin sections (6 μm) which were stained with Hematoxylin-Eosin and Masson’s trichrome stain.

Measurements of chymase and ACE activity

Chymase and ACE activities were measured using a modified method of previous reports (Horiuchi et al. 1982; Balcells et al. 1997; Jin et al. 2001). For the measurement of chymase activity, the lung tissues were minced and homogenized in 10 volumes of chilled 20 mmol/mL sodium phosphate buffer (pH 7.3). After centrifugation at 20 000 g for 20 minutes, the supernatant was discarded and the remained tissue pellet was suspended in 10 volumes of the same buffer. After centrifugation at 20 000 g for 20 minutes, the pellets were resuspended in chilled 10 mM sodium phosphate buffer (pH 8.0) containing 2 M KCl and 0.1% (v/v) Nonidet P-40. The suspensions were allowed to stand at 4°C overnight and centrifuged at 20 000 g for 20 minutes. The resultant supernatants were used for chymase assay. Twenty-five microliters of aliquots of the enzyme preparations were preincubated in potassium phosphate buffer containing 1 mM o-phenanthroline, 20 μM aprotinin, and 2 mM EDTA with or without 100 μM chymostatin at room temperature for 30 min. After an addition of 600 mM Ang I, samples were incubated for 60 min at 37°C. To terminate the reaction, 99.9% ethanol was added to the sample. Generated Ang II was quantified using a reverse-phase HPLC column (Asahipak ODP-50 6D, Showa-Denko). Chymase activity was defined as the formation of chymostatin-inhibitable Ang II. One unit of chymase activity was defined as the amount of enzyme needed to produce 1 μmol per minute Ang II from Ang I.

For the measurement of ACE activity, the pulmonary tissues were minced and homogenized in 50 volumes of chilled 20 mM Tris-HCl buffer (pH 8.3) containing 30 mM KCl, 5 mM Mg(CH₃COO)₂, 250 mM scrose, and 0.5% (v/v) Nonidet P-40. The homogenates were centrifuged at 20 000 g for 20 minutes at 4°C, and the resultant supernatants were used for ACE assay. Fifty microliters of aliquots of the enzyme preparations were preincubated in 100 mM potassium phosphate buffer (pH 8.3) containing 600 mM NaCl with or without 100 μM captopril, an ACE inhibitor, at room temperature for 30 minutes. After an addition of 5 mM Hip-His-Leu, samples were incubated for 30 minutes at 37°C. To terminate the reaction, 3% metaphosphoric acid was added to the sample. Generated hippuric acid was quantified using a reverse-phase HPLC column (Asahipak F-411, Showa-Denko, Japan). ACE activity was defined as the formation of captopril-inhibitable hipuric acid. One unit of ACE activity was defined as the amount of enzyme needed to produce 1 μmol per min hippuric acid from Hip-His-Leu. The protein concentration of the samples was determined according to Lowry et al. (1951).

Statistical analyses

All data were shown as mean±s.e.m. The population variances of chymase and ACE activities of PQ- and saline-administration groups were analyzed using the F-test. For cases in which the population variances of the two groups were assumed to be the same, the difference between the PQ- and saline-administered groups was analyzed using the Student’s t-test. Otherwise the difference was analyzed using Welch’s t-test.
RESULTS

Clinical signs and histopathological examinations

In the acute PQ intoxication group, the hamsters showed no clinical signs until four days after a subcutaneous PQ administration. On dissection, however, multiple and variously-sized congestive or hemorrhagic foci were found in all hamsters examined (Fig. 1, left panel). Microscopic examinations revealed the presence of alveolar hemorrhage and capillary stasis (Fig. 1, right panel). In the hamsters assigned to the chronic PQ intoxication group, 13 out of 17 died after the second PQ administration. They had been showing tachypnea and cyanosis prior to death, and diffused pulmonary hemorrhage was observed in the pathological autopsies of all dead animals. Four hamsters were successfully administered a series of PQ. Upon dissection five weeks later, irregular pulmonary surfaces were noted (Fig. 2, left panel). Masson’s trichrome stain revealed an increased content of collagenous fiber in the pulmonary interstitial areas (Fig. 2, right panel), suggesting interstitial fibrosis. Three hamsters that showed these abnormalities were assigned to

Fig. 1. A hamster lung four days after a single paraquat administration (18 mg/kg, sc). Multiple and variously sized congestive or hemorrhagic foci were found (left panel). Lung section (right panel). Note the congestion and hemorrhage in the alveoli (arrow). Hematoxylin-Eosin stain. (Bar equals 30 μm).

Fig. 2. A hamster lung five weeks after repeated administrations of paraquat (once a week for six weeks administrations at a dose of 18 mg/kg, sc). An irregular surface of the lung was noted (Left panel). Collagenous fiber in pulmonary interstitial areas is demonstrated (arrows, right panel). Masson’s trichrome stain. (Bar equals 30 μm).
the PQ intoxication-induced pulmonary fibrosis group.

The effect of PQ on chymase and ACE activities

The pulmonary chymase in the acute PQ intoxication group was significantly activated when compared with that in the age-matched control group (Fig. 3, upper panel). In the chronic PQ intoxication-induced pulmonary fibrosis group, chymase was also significantly activated when compared with that of age-matched controls (Fig. 3, lower panel). Pulmonary ACE activities were significantly lower in both the acute PQ intoxication group (Fig. 4, upper panel) and the chronic PQ intoxication-induced pulmonary fibrosis group (Fig. 4, lower panel) compared with the respective age-matched controls.

**DISCUSSION**

Acute hemorrhage was induced by a single dose of PQ in hamsters as was evoked in dogs (Nagata et al. 1992) and humans (Yasaka et al. 1986; Harsanyi et al. 1987). PQ produces oxidative stress by redox cycling and elevating the intracellular superoxide (O$_2^-$) level (Bus et al. 1974). Nitric oxide (NO) was remarkably increased in the lung after PQ intoxication. The signs of pulmonary injury, including increased airway and perfusion pressures and pulmonary edema were attenuated by N-nitro-L-arginine, a nitric oxide synthase inhibitor (Berisha et al. 1994).
Thus, elevating the intracellular superoxide and excessive NO may be possible mechanisms of pulmonary hemorrhage. In chronic PQ intoxication study, PQ was administered repeatedly because no hamsters showed chronic interstitial changes five weeks after a single PQ administration (data not shown). This is consistent with another study by Bulter (Butler 1975). Thirteen out of 17 died after the second PQ administration. All animals exhibited tachypnea and cyanosis prior to death. With the evidences obtained in the pathological autopsies, we defined that these animals died of severe pulmonary dysfunction caused by diffuse hemorrhages. Three hamsters survived after six weekly administrations of PQ resulted in interstitial fibrosis, a typical chronic pulmonary injury caused by PQ in human (Toner et al. 1970).

Chymase was activated in the lung of patients with interstitial fibrosis (Mitani et al. 1999) and was also activated in bleomycin-induced pulmonary fibrosis in hamsters (Sakaguchi et al. 2002). Chymase contributes to the production of fibrosis by directly activating procollagenase (Saarinen et al. 1994) and initiating collagen fibril formation (Kofford et al. 1997). These lines of evidence suggest that chymase may directly involve in the fibrotic responses. In addition, Ang II, which is generated by chymase from Ang I, stimulates lung fibroblast proliferation through the activation of transforming growth factor β (Marshall et al. 2000), suggesting that chymase indirectly contributes to the establishment of pulmonary fibrosis. The present study showed for the first time that chymase activity was elevated after acute PQ intoxication and that it remained elevated even after the establishment of pulmonary fibrosis. Thus, it is conceivable that the activated chymase may, at least in a part, contribute to the establishment of PQ-induced pulmonary fibrosis. Further studies are, however, necessary to demonstrate the direct and indirect contributions of chymase to the fibrotic responses induced by PQ.

The present study revealed that ACE activity was lowered by PQ intoxication, as was reported in previous studies (Hollinger et al. 1980; Masaoka et al. 1989). It has been suggested that PQ might damage pulmonary endothelial cells, and ACE might leak out to the circulation (Masaoka et al. 1989).

Although PQ poisoning of humans is still reported in many countries (Bruyndonckx et al. 2002; Hwang et al. 2002; Hsu et al. 2003; Lugo-Vallin et al. 2003), there are no effective antidotes and no measures to enhance the elimination of PQ from the body (Jaeger et al. 1995). As described above, chymase may contribute both directly and indirectly to fibrotic changes. Especially in humans, chymase-like enzyme-dependent Ang II forming activity is about five times of the ACE dependent Ang II forming activity (Akasu et al. 1998), suggesting that chymase plays a more crucial role than ACE in the production of Ang II. From this point of view, a medication that suppresses elevated chymase may prove to be effective in preventing the formation of pulmonary fibrosis induced by PQ in humans.

References


