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European Journal of Pharmacology 358 (1998) 229–233

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Chymase processes big-endothelin-2 to endothelin-2-(1-31) that induces contractile responses in the isolated monkey trachea

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Received 26 May 1998; revised 17 August 1998; accepted 18 August 1998

Abstract

Purified monkey chymase cleaved the Tyr^{31}–Gly^{32} bond of big-endothelin-1 and big-endothelin-2 to yield endothelin-1-(1–31) and endothelin-2-(1–31), respectively. In the isolated monkey trachea, endothelin-1-(1–31) and endothelin-2-(1–31), as well as big-endothelin-1 and big-endothelin-2, induced contractile responses. Chymostatin, which inhibits chymase, suppressed the contractile response induced by big-endothelin-2 to 16.6% but not the responses induced by big-endothelin-1, endothelin-1-(1–31) and endothelin-2-(1–31). These results suggest that the contractile response of big-endothelin-2 is predominantly dependent on the conversion of big-endothelin-2 to endothelin-2-(1–31) by chymase.

Keywords: Chymase; Big-endothelin-1; Big-endothelin-2; Constriction; (Monkey)

1. Introduction

Chymase is a serine protease with chymotrypsin-like specificity and is stored in mast cell granules. Chymase has been isolated and its enzymatic characteristics have been studied in human (Urata et al., 1990), monkey (Takai et al., 1997), hamster (Takai et al., 1996) and rat (Le Trong et al., 1987). It is well known that all of these chymases hydrolyze the carboxy-terminal side of aromatic amino acids. However, using the substrate angiotensin I, human, monkey and hamster chymases cleave the Phe^{8}–His^{9} bond of angiotensin I to yield angiotensin II, whereas rat chymase cleaves the Tyr^{4}–Ile^{5} bond to form inactive fragments. Such species differences in the substrate specificity of chymases should be kept mind. For example, in humans, monkey, hamster and rat, isolated vessels show contractile responses after injection of angiotensin I. However, the angiotensin-I-dependent contractile responses of isolated arteries in human, monkey and hamster, which have chymase-dependent angiotensin II-forming pathways, are partially suppressed by an angiotensin converting enzyme inhibitor and the remaining responses are blocked by a chymase inhibitor, whereas the response in rat is completely suppressed by an angiotensin converting enzyme inhibitor only (Okunishi et al., 1993).

It has been thought that endothelin is generated from big-endothelin through cleavage of the Trp^{22}–Val^{23} bond, which is hydrolyzed by endothelin converting enzyme-1 and endothelin converting enzyme-2 (Emoto and Yanagisawa, 1995). However, recently, Nakano et al. (1997) demonstrated that human chymase cleaved the Tyr^{31}–Gly^{32} bond of big-endothelin-1, big-endothelin-2 and big-endothelin-3 to produce endothelin-1-(1–31), endothelin-2-(1–31) and endothelin-3-(1–31), respectively. In contrast, rat chymase hydrolyzed various bonds of big endothelins to yield inactive fragments (Nakano et al., 1997). Hanson et al. (1997) also reported that big-endothelin-1 was converted to endothelin-1-(1–31) in the membrane fraction from human lung and this conversion was inhibited by a chymase inhibitor. However, it is unclear whether or not the conversion has a physiological role in humans. Recently, we isolated and characterized monkey chymase, which has a substrate specificity very similar to human chymase (Takai et al., 1997).

In the present study, we examined if purified monkey chymase, like human chymase, cleaved the Tyr^{31}–Gly^{32} bond of big-endothelin-1 and big-endothelin-2 to yield endothelin-1-(1–31) and endothelin-2-(1–31), respectively,

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0014-2999/98/$ - see front matter
PII: S0014-2999(98)00622-0

and then studied whether or not chymase affects the contractile responses of big-endothelin-1, big-endothelin-2, endothelin-1-(1–31) and endothelin-2-(1–31) in the isolated monkey trachea.

2. Materials and methods

The experimental protocol of this study was approved by the local ethics committee and conformed to the guidelines for the care and use of laboratory animals (Animal Research Laboratory, Osaka Medical College).

2.1. Enzymatic studies of monkey chymase

Purified monkey chymase was obtained according to Takai et al. (1997). Twelve monkeys (*Macaca fascicularis*) were sacrificed by bleeding under pentobarbital anesthesia, and their cheek pouch vascular tissues were excised. Monkey chymase was purified from the extract of cheek pouch vascular tissues using heparin affinity and gel filtration columns. To determine the fragments hydrolyzed from big-endothelin-1 and big-endothelin-2 by monkey chymase, 12 ng of the purified enzyme were incubated with 1 nmol big-endothelin-1 or big-endothelin-2 in 20 mM Tris–HCl buffer, pH 8.0, containing 100 μM concentrations of these synthetic peptides, 0.5 M KCl and 0.1% (v/v) Triton X-100 for 0–180 min at 37°C. Reactions were terminated by adding ice-cold methanol and the supernatant ($20\,000 \times g$ for 10 min) was applied to an octadecyl silica reversed-phase column (4.6 mm × 250 mm I.D., Tohso, Tokyo, Japan), which had been equilibrated with 35% methanol in 0.1% trifluoroacetic acid. Separation was conducted with the same solution at a rate of 0.5 ml/min by high performance liquid chromatography (HPLC).

2.2. Functional studies of airway tissue

The monkey tracheas were cut into strips, 10 mm in length and 2.0 mm in width. The strip was placed on a myograph under a resting tension of 1.0 g. The bathing medium was Tyrode's solution consisting of 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$ and 5.7 mM glucose, pH 7.4. The medium was continuously aerated with O_2/CO_2 (95:5), which was maintained at 37°C. The strip was equilibrated for 2 h before the experiments. The contractile response to 50 mM KCl was obtained first, and then the bathing medium was washed out. The medium was washed out three times for 20 min each time with fresh Tyrode's solution, and equilibrated for 40 min. Big-endothelin-1, big-endothelin-2, endothelin-1-(1–31) or endothelin-2-(1–31) (at final concentrations of 1, 3, 10, 30 or 100 nM) was added as bolus doses to the bathing medium. To study whether chymase affects the contractile responses induced by 10 nM big-endothelin-1, big-endothelin-2, endothelin-1-(1–31) or endothelin-2-(1–31), 100 μM chymostatin, which completely inhibited purified monkey chymase (Takai et al., 1997), was added and preincubation was conducted for 20 min and the contractile responses of these peptides (each at a final concentration of 10 nM) were recorded.

2.3. Statistical analysis

All results are represented as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed by between-within analysis of variance (ANOVA). Significant differences between the means of two groups were evalu-

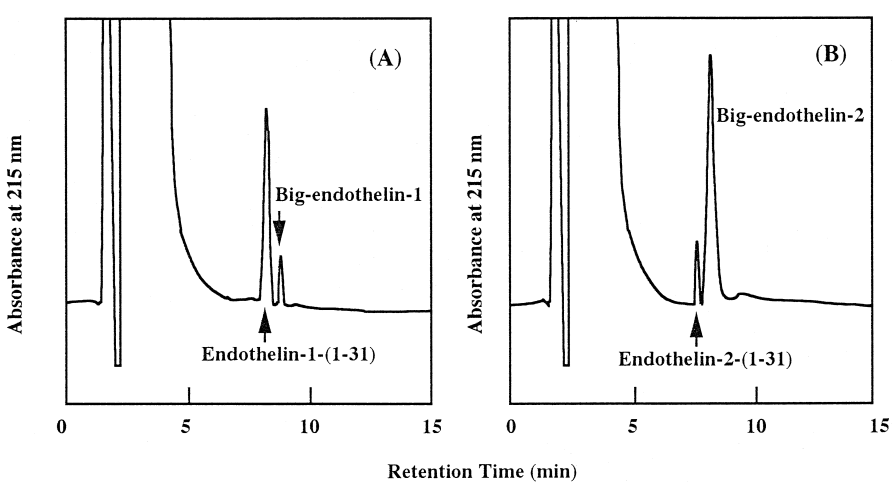

Fig. 1. HPLC analysis of reaction solutions after incubation for 30 min of 0.1 nmol big-endothelin-1 (A) or big-endothelin-2 (B) with 12 ng of purified monkey chymase.

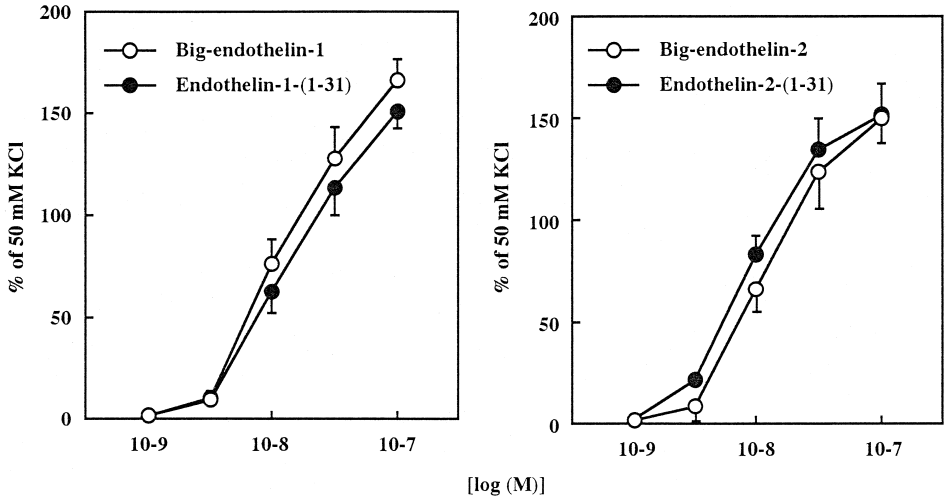

Fig. 2. Concentration–response curves for the contractile responses induced by big-endothelin-1, endothelin-1-(1–31), big-endothelin-2 and endothelin-2-(1–31) in the isolated monkey trachea. Values expressed as percentage of the contractile responses obtained with 50 mM KCl. Values are means ± S.E.M. ($n = 5$).

ated by Student's *t*-test after a one-way analysis of variance. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Analysis of products of big-endothelins by chymase

Fig. 1 shows the cleavage of big-endothelin-1 and big-endothelin-2 after incubation with purified monkey chymase for 30 min. The big-endothelin-1 substrate was eluted at 8.7 min and the product of big-endothelin-1 by purified monkey chymase constituted a major peak eluting at 7.9 min (Fig. 1A). The big-endothelin-2 substrate was eluted at 8.3 min. The big-endothelin-2 products constituted a major peak eluting at 7.7 min (Fig. 1B).

3.2. Contractile responses of big-endothelins and endothelins-(1–31)

The contractile responses induced by big-endothelin-1, big-endothelin-2, endothelin-1-(1–31) and endothelin-2-(1–31) are shown in Fig. 2. The contractile responses

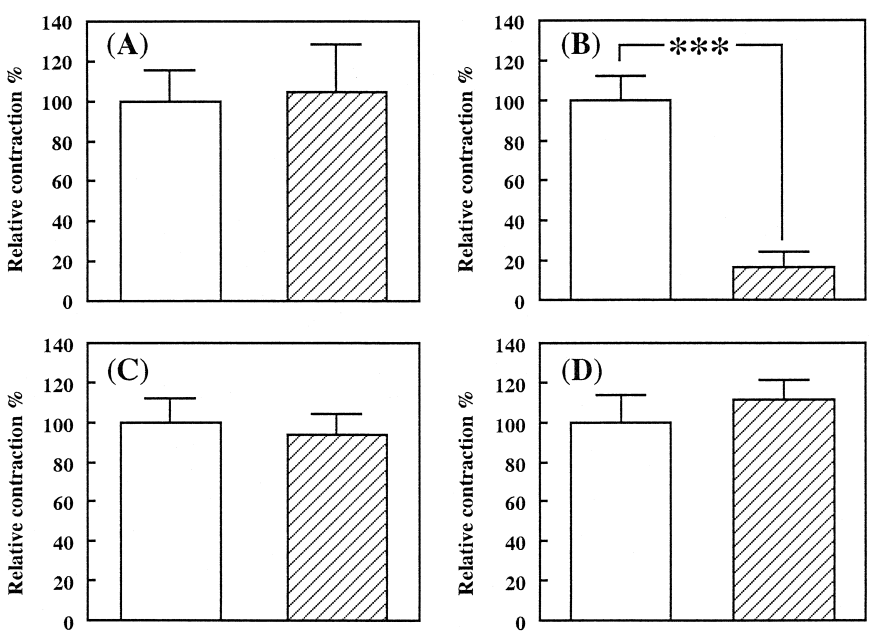

Fig. 3. Effects of 100 μM chymostatin on the contractile responses induced by 10 nM big-endothelin-1 (A), big-endothelin-2 (B), endothelin-1-(1–31) (C) and endothelin-2-(1–31) (D) in the isolated monkey trachea (open bars: control response, $n = 5$; hatched bars: responses in the presence of chymostatin, $n = 5$). Values are expressed as percentage of control responses. Values are means ± S.E.M. $***P < 0.001$ vs. the control response.

induced by 10, 30 and 100 nM big-endothelin-1 were slightly stronger than those induced by the same doses of endothelin-1-(1–31), whereas those by 10 and 30 nM endothelin-2-(1–31) were slightly stronger than those of big-endothelin-2. However, these differences were not significant. The contractile responses induced by endothelin-1-(1–31) and by endothelin-2-(1–31) were almost identical.

3.3. Effects of chymostatin in contractile responses of big-endothelins and endothelins-(1–31)

The effects of chymostatin on the contractile responses to big-endothelin-1, big-endothelin-2, endothelin-1-(1–31) and endothelin-2-(1–31) are shown in Fig. 3. The responses of big-endothelin-1, big-endothelin-2, endothelin-1-(1–31) and endothelin-2-(1–31) were 0.32 ± 0.03, 0.23 ± 0.02, 0.28 ± 0.04 and 0.24 ± 0.03 g, respectively, and each of these responses was regarded as 100%. Chymostatin significantly suppressed the contractile responses induced by big-endothelin-2 to 16.6%, but not by big-endothelin-1, endothelin-1-(1–31) and endothelin-2-(1–31).

4. Discussion

Our recent report (Takai et al., 1997) demonstrated that primate chymases have a higher substrate specificity than non-primate chymases. For example, rat chymase (Le Trong et al., 1987) hydrolyzes the Phe^{7}–Phe^{8} bond of substance P and the Tyr^{22}–Leu^{23} bond of vasoactive intestinal peptide, and both rat chymase (Le Trong et al., 1987) and hamster chymase (Takai et al., 1996) cleave the Tyr^{4}–Ile^{5} bond of angiotensin II, while human chymase (Urata et al., 1990) and monkey chymase (Takai et al., 1997) hydrolyze none of these bonds. The present study showed that purified monkey chymase, like human chymase, cleaved only the Tyr^{31}–Gly^{32} bond of big-endothelin-1 and big-endothelin-2 to form endothelin-1-(1–31) and endothelin-2-(1–31), respectively. On the other hand, rat chymase cleaved various bonds of big-endothelins to yield inactive fragments (Nakano et al., 1997), and hamster chymase also hydrolyzed various bonds of big-endothelins (unpublished observation). Therefore, using big-endothelins as a substrate for chymase, the fragments generated from the substrates by primate chymase may be different from those by non-primate chymases, and such species differences for substrate specificity may be involved in different functional roles of big-endothelins.

Mast cells, which contain and release chymase, are widely distributed in the submucosa of monkey and human trachea, and have also been widely detected in bronchial tissues, airway subepithelium and smooth muscle (Matin et al., 1992). The present study showed that big-endothelin-1 and big-endothelin-2 induced contractile responses in the isolated monkey trachea dose-dependently, and the products generated by purified monkey chymase, endothelin-1-(1–31) and endothelin-2-(1–31), also induced these responses. The contractile responses induced by big-endothelin-2, but not those induced by big-endothelin-1, were suppressed to 16.6% by adding 100 μM chymostatin, which completely inhibited purified monkey chymase (Takai et al., 1997). The contractile responses induced by endothelin-1-(1–31) and endothelin-2-(1–31) were not influenced by chymostatin. These results suggested that big-endothelin-2 was converted to endothelin-2-(1–31) by chymase in the monkey trachea and the product, endothelin-2-(1–31), induced contractile response. In the present study, the contractile responses induced by endothelin-2-(1–31) in the isolated monkey trachea slightly more strongly than those by big-endothelin-2, but this difference was not significant. Nakano et al. demonstrated that, in rat trachea, the contractile response induced by endothelin-2-(1–31) was significantly stronger than that induced by big-endothelin-2. These findings suggested that endothelin-2-(1–31) generated by human and monkey chymases may have a stronger contractile action than endothelin-2 or may have its own receptor. On the other hand, in the present study, a chymostatin-sensitive contractile response induced by big-endothelin-1, which shows chymase-dependent constriction, was not observed. Endothelin converting enzyme converted big-endothelin-1 to endothelin-1 to a far greater extent than big-endothelin-2 to endothelin-2 (Sawamura et al., 1993) and may rapidly convert big-endothelin-1 to endothelin-1 in the isolated monkey trachea. However, the monkeys used in this study were normal, whereas the chymase-dependent constriction induced by big-endothelin-2 can be detected in hypersecretory airways where the number of mast cells are increased. Further studies on the effect of chymase inhibitors on the airway hypersecretion model are needed.

In the present study, we demonstrated for the first time that monkey chymase, like human chymase, cleaved the Tyr^{31}–Gly^{32} bond of big-endothelin-1 and big-endothelin-2 to produce endothelin-1-(1–31) and endothelin-2-(1–31), respectively, and the contractile response induced by big-endothelin-2 in the isolated monkey trachea was dependent on the conversion by chymase.

Acknowledgements

The authors are grateful to Dr. H. Kido (University of Tokushima) for his valuable comments on the study.

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