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Role of mast cell chymase in angiotensin-induced vascular contraction of hamster cheek pouch microvessels

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Abstract

We investigated the contribution of chymase-dependent conversion of angiotensin I to angiotensin II in hamster cheek pouch. To investigate the converting activities in intact tissues, angiotensin I or II was applied to microvessels of the intact cheek pouch, and the vascular contractile response was recorded. Angiotensin I or angiotensin II (20 nM) induced a rapid contraction of arterioles, irrespective of their diameter. In the presence of 1 mM captopril, there was no contraction in response to angiotensin I in arterioles < 25 μm in diameter, whereas contraction was still observed in larger arterioles. Chymostatin (100 μM) treatment also reduced the response to angiotensin I in arterioles > 40 μm in diameter. Treatment with 1 mM captopril and 100 μM chymostatin resulted in the loss of response to angiotensin I, but not to angiotensin II, in all arterioles. Treatment of microvessels with 100 μg/ml compound 48/80 enhanced angiotensin I-induced vascular contraction response, suggesting the significance of mast cells as a source of cheek pouch chymase.

Keywords: Chymase; Mast cell; Angiotensin; Vascular contraction; Microvessel; (Hamster, pouch)

1. Introduction

Angiotensin II plays important roles in the regulation of various aspects of the cardiovascular system, such as regulation of blood pressure and renal blood flow (Ichikawa and Harris, 1991), vascular remodelling (Schelling et al., 1991) and leukocyte adhesion to the endothelium (Gräfe et al., 1997). Angiotensin II is generated enzymatically from angiotensin I, and angiotensin-converting enzyme (ACE) is a unique enzyme responsible for the generation of angiotensin II. However, recent studies have revealed alternative angiotensin II generating pathways (Cornish et al., 1979; Cornish and Gilmore, 1981; Bührle et al., 1987; Dzau, 1989) and locally generated angiotensin II by these angiotensin-converting enzyme-independent pathways also plays various physiological and pathophysiological roles.

Chymase (EC 3.4.21.39) is a serine protease, originally found in mast cell granules. In humans, monkeys, dogs and hamsters, chymase can generate angiotensin II, whereas chymase of other species such as rats, mice or rabbits cleaves angiotensin I into inactive fragments (Le Trong et al., 1987; Urata et al., 1990; Okunishi et al., 1993; Chandrasekharan et al., 1996; Balcells et al., 1997). Chymase (or chymase-like activity) is present in vascular tissues (Shiota et al., 1997c; Takai et al., 1997) and cardiac muscles (Shiota et al., 1997a,b) of several species including humans and hamsters, suggesting it plays crucial roles as another key enzyme in the angiotensin system and in the pathogenesis of cardiovascular diseases (for review, see Husain, 1993; Urata et al., 1996). The exact role of chymase in vivo, however, remains unknown.

Hamster cheek pouch has also been shown to contain relatively large amounts of chymase, which can convert angiotensin I to angiotensin II in vitro similar to human chymase (Takai et al., 1996). However, its localization within the pouch is not clear and whether chymase can form angiotensin II to cause contraction of microvessels in the intact pouch remains to be determined. The aims of the present work were to determine the source of chymase in the cheek pouch and demonstrate the contribution of chymase to the generation of angiotensin II from angiotensin I in the intact pouch. Our results showed the presence of an angiotensin-converting enzyme-independent, chymase-dependent angiotensin II-forming pathway in the intact cheek

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pouch and that exogenously administered angiotensin I, which was converted to angiotensin II through this alternative pathway, caused vascular contraction, especially in arterioles. The distribution of mast cells along large arterioles suggests that they are the possible source of chymase in the cheek pouch.

2. Materials and methods

2.1. Determination of angiotensin-converting enzyme and chymase-like activities

Tissue extracts were prepared according to the procedure of Okunishi et al. (1987) with minor modifications. Unlike angiotensin-converting enzyme, chymase binds to heparin-like molecules at low salt concentrations and is extracted by treatment with high salt buffer. Therefore, in this procedure angiotensin-converting enzyme and chymase were extracted using low salt buffer and high salt buffer, respectively. Briefly, hamsters were sacrificed by exsanguination under anesthesia and various tissues including the cheek pouches were excised. They were cut into small pieces with scissors and homogenized in 10 volumes (w/v) of 20 mM Na–phosphate buffer (pH 7.4) using a homogenizer (Polytron). The homogenate was centrifuged at $20{,}000 \times g$ for 30 min. The supernatant (ACE fraction) was stored at −20°C prior to the determination of angiotensin-converting enzyme activity. The pellet was washed with the same low salt buffer and centrifuged under the same condition. The supernatant was discarded and the pellet was resuspended and homogenized in 5 volumes of 10 mM Na–phosphate buffer containing 2 M KCl and 0.1% Nonidet P-40. The homogenate was stored on a shaker overnight at 4°C and then centrifuged at $20{,}000 \times g$ for 30 min. The supernatant was used as a high salt extract and stored at −20°C prior to the determination of chymase-like activity. Angiotensin-converting enzyme activity was determined as previously described (DePierre and Roth, 1975), using the angiotensin-converting enzyme-specific synthetic substrate Hippuryl-His-Leu (HHL) (Okunishi et al., 1984). Briefly, 50 μl of the ACE fraction was mixed with 200 μl of 5 mM HHL and incubated for 30 min at 37°C in 100 mM potassium phosphate buffer (pH 8.3) containing 0.5 M NaCl (Miyazaki et al., 1984) in the presence or absence of 10 mM captopril. The reaction was terminated by the addition of 0.15 ml of 15% trichloroacetic acid and the supernatant, obtained after centrifugation for the removal of denatured proteins, was mixed with 10% *o*-phthalaldehyde under alkaline conditions. After incubation for 10 min at room temperature, 0.2 ml of 6 N HCl was added, followed by measurement of fluorescence (excitation at 340 nm and emission at 455 nm).

Chymase-like activity was determined as described previously (Okunishi et al., 1987). Twenty microliters of high salt extract was incubated with 770 μM angiotensin I in borax–borate buffer (pH 8.5) containing 5 mM EDTA, 8 mM dipyridyl and 0.77 mM diisopropylfluorophosphate for 1 h at 37°C. Then, 150 μl of 15% trichloroacetic acid was added to each sample, and the concentration of released His-Leu was determined as mentioned above. Blank values were obtained from measurements made in the presence of 500 μM chymostatin.

2.2. Examination of microcirculation of hamster cheek pouch

Male Syrian hamsters (supplied by Japan SLC, Shizuoka Japan), weighing 80–120 g, were anesthetized with sodium pentobarbital (60 mg/kg i.p.). The cheek pouch was prepared for direct visual examination using the method described previously (Bouskela and Grampp, 1992; Bouskela et al., 1997). Briefly, the hamster was placed on the stage of a dissecting microscope and the cheek pouch was carefully excised by an incision made at the cheek skin, then spread inside-out. An incision was made in the upper layer of the pouch to produce a single-layer preparation, and the fibrous connective tissue was carefully removed using ophthalmic scissors and micro-tweezers to obtain a clear field. The pouch was fixed in place with needles into a round bath and was immediately perfused with a warm Tyrode solution (37°C) containing 136 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.3 mM NaH_2PO_4, 11.9 mM $NaHCO_3$ and 5.6 mM glucose. The net volume of the bath was 3 ml. The hamster was transferred onto a warm pad at 37°C and the cheek pouch preparation was allowed to equilibrate with Tyrode solution for 30 min to dilate the microvessels, which had somewhat contracted by mechanical stimulation, to their resting state. After a period of 30 min, the hamster was placed on a microscope stage and microvessels were photographed at resting state. In our preliminary experiments, we checked the responsiveness of arterioles and venules to angiotensins and found that venules were less sensitive than arterioles, a finding similar to that of Myers et al. (1988). We also determined the optimal dose of angiotensins that induces vascular contraction. These studies showed that 20 nM angiotensin I or angiotensin II induced the maximum contraction. Therefore, in order to examine angiotensin II-generating activities of microvessels from angiotensin I, the contractile effect of 20 nM angiotensin I was studied using arterioles of different diameters. Sixty μl of 1 μM angiotensin I or 1 μM angiotensin II solution was applied to the bath, which yielded a final concentration of 20 nM, and the maximum contraction of microvessels, which was obtained 2–3 min after the application in the majority of arterioles, was photographed on 35 mm film, using a ×20 objective lens, once every several minutes. Finally, norepinephrine (final concentration 1 μM) was applied to the bath to obtain the maximum contraction 10 min after the application of angiotensin II. To examine the effect of

inhibition of angiotensin-converting enzyme and/or chymase on the contraction induced by angiotensins, the cheek pouch was first treated for 15 min with 1 mM captopril (angiotensin-converting enzyme inhibitor) and/or 10 μM chymostatin (an inhibitor of chymase) prior to the application of angiotensins.

The internal diameter of each microvessel in the resting state was defined as the maximum vessel diameter (0%), and that observed after treatment with norepinephrine was considered as the minimum diameter (100% contraction). The degree of contraction after each treatment was expressed as percent (%) contraction.

2.3. Effects of pretreatment with compound 48/80 on angiotensin I-induced vascular contraction

To confirm the involvement of mast cells as a source of chymase, the cheek pouch was treated with compound 48/80, a potent mast cell activator, and the response of microvessels to angiotensin I was examined. Because histamine, released from activated mast cells, also induces vasoconstriction and increased microvascular permeability (Grega and Adamski, 1991; Shepherd and Duling, 1996), treatment with compound 48/80 was carried out in the presence of pyrilamine, a histamine H1 receptor antagonist. Briefly, exposed microvessels of the cheek pouch were treated with 100 μg/ml compound 48/80 in the presence of 1 mM pyrilamine for 5 min at 37°C. Then, the pouch was washed three times with Tyrode solution and incubated for 30 min at 37°C to eliminate any possible effects of histamine. Angiotensin I solutions at various concentrations were applied to arterioles in the presence of 1 mM captopril, and the contraction of arterioles was recorded on 35 mm film every 2 min after application of angiotensin I. In control experiments, the response of vessels treated with 1 mM pyrilamine alone was also examined.

2.4. Histochemistry

Detection of mast cells within the tissue of cheek pouch was carried out by staining with 0.5% toluidine blue acidified with 0.1 M citrate (pH 4.8). A drop of 0.5% toluidine solution was applied to the exposed area of the pouch, which allowed blue staining of mast cells without tissue fixation. Stained mast cells around randomly selected microvessels were recorded on 35 mm negative film

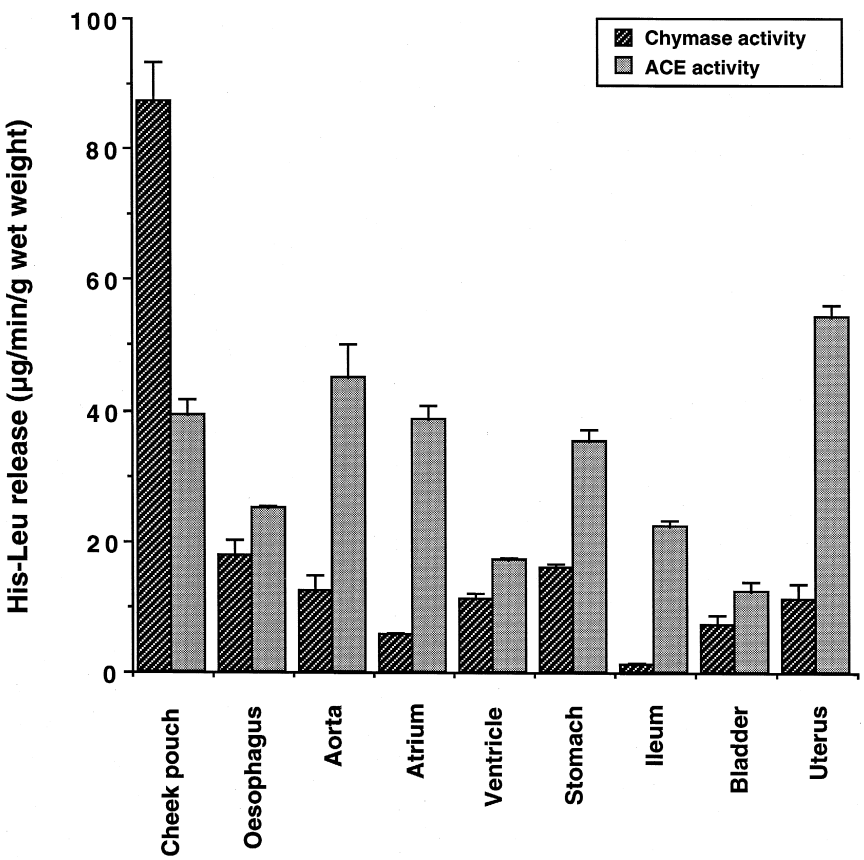

Fig. 1. Chymase-like and angiotensin-converting enzyme activity in homogenates from various hamster tissues. angiotensin-converting enzyme activity in the low salt extract and chymase-like activity in the high salt extract, prepared according to the protocol described in Section 2, were determined by the release of His-Leu dipeptide from the angiotensin-converting enzyme-specific substrate Hippuryl-His-Leu and angiotensin I, respectively. Data are mean ± S.E.M. of three measurements.

and the number of mast cells along 200 μm of microvessels was counted.

2.5. Chemicals

Angiotensin I, angiotensin II, compound 48/80, pyrilamine, bovine serum albumin (Fraction V), captopril and chymostatin were purchased from Sigma (St. Louis, MO). Borate, EDTA, dipyridyl, diisopropylfluorophosphate and toluidine blue were purchased from Wako (Tokyo, Japan). Hippuryl-His-Leu was purchased from Peptide Research Institute (Tokyo). N-terminal peptide of hamster chymase was synthesized by the solid phase method utilizing an Fmoc strategy with an automated ABU instrument (Model 431A).

2.6. Statistical analysis

All data were presented as mean ± S.E.M. Comparisons between paired groups were performed by Student's *t*-test. Comparisons between % contraction values in four categories were performed using Scheffe's *F*-test and one-way ANOVA (analysis of variance). A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Angiotensin converting activities in cheek pouch homogenate

As previously reported (Cornish et al., 1979; Takai et al., 1996), hamster cheek pouch shows angiotensin-converting enzyme-independent angiotensin II-generating activity and most of this is thought to be catalyzed by chymase. To evaluate the contribution of chymase-like activity to the conversion of angiotensin I to angiotensin II, we determined angiotensin-converting enzyme and chymase-like activity in cheek pouch homogenate. For comparison, the same activities were also determined in homogenates of other tissue, which were prepared using a protocol similar to that for cheek pouch. As shown in Fig. 1, chymostatin-sensitive, chymase-like activity per gram of wet tissue was highest in the cheek pouch homogenate. A lower chymase-like activity was also observed in homogenates of other tissues including esophagus, aorta, ventricle and stomach. On the other hand, captopril-sensitive angiotensin-converting enzyme activity was observed in various tissue homogenates but was particularly high in homogenates of the lung and kidney (data not shown). Calculation of the ratio of chymase-like activity to angiotensin-converting enzyme activity indicated that the ratio was > 1.0 only in the cheek pouch among 18 tissues tested.

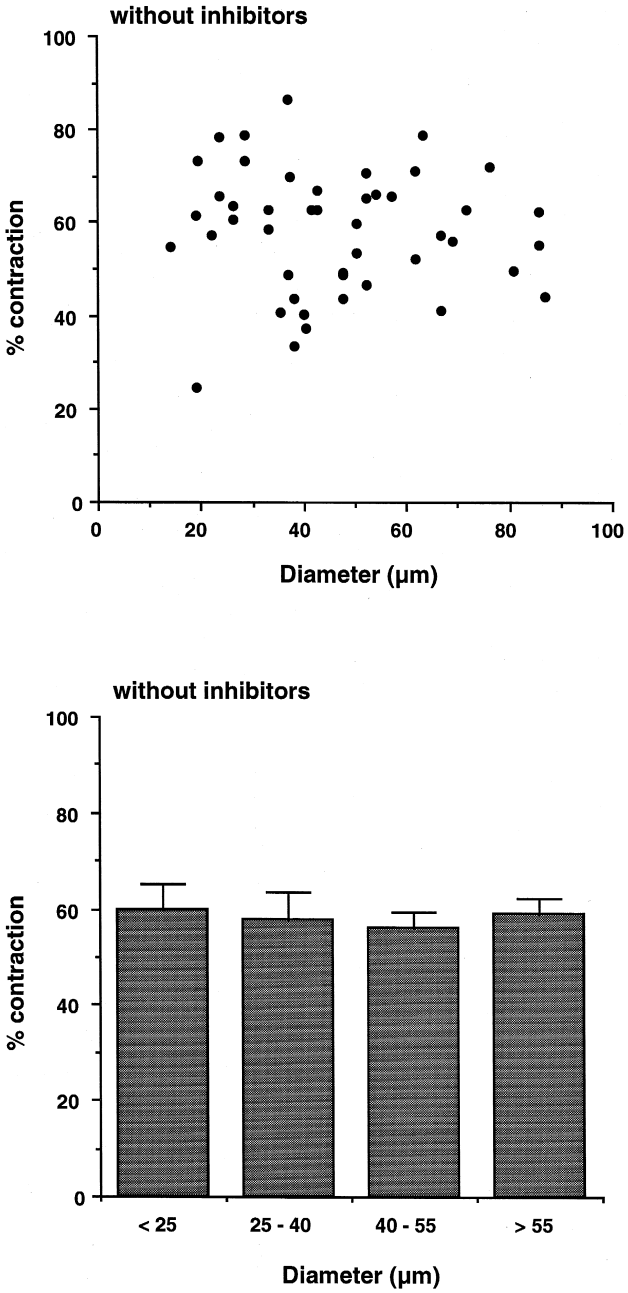

Fig. 2. Vascular contraction of hamster cheek pouch microvessels induced by angiotensin I. Vascular contraction was recorded after application of 20 nM angiotensin I to exposed microvessels of the cheek pouch. The degree of contraction was represented as a percentage of the initial diameter of each vessel. (A) Relationship between vessel diameter and response to angiotensin I. Data of eight hamsters (correlation coefficient $(r) = 0.055$, $P > 0.1$). (B) Microvessels were divided into four groups according to their diameter and the response to angiotensin I was compared in these groups. Data are mean ± S.E.M. There was no statistical difference between groups by one-way ANOVA.

3.2. Responses of microvessels to exogenous angiotensin I and angiotensin II

Microvessels of the cheek pouch respond to exogenously applied vasoactive substances including angiotensin

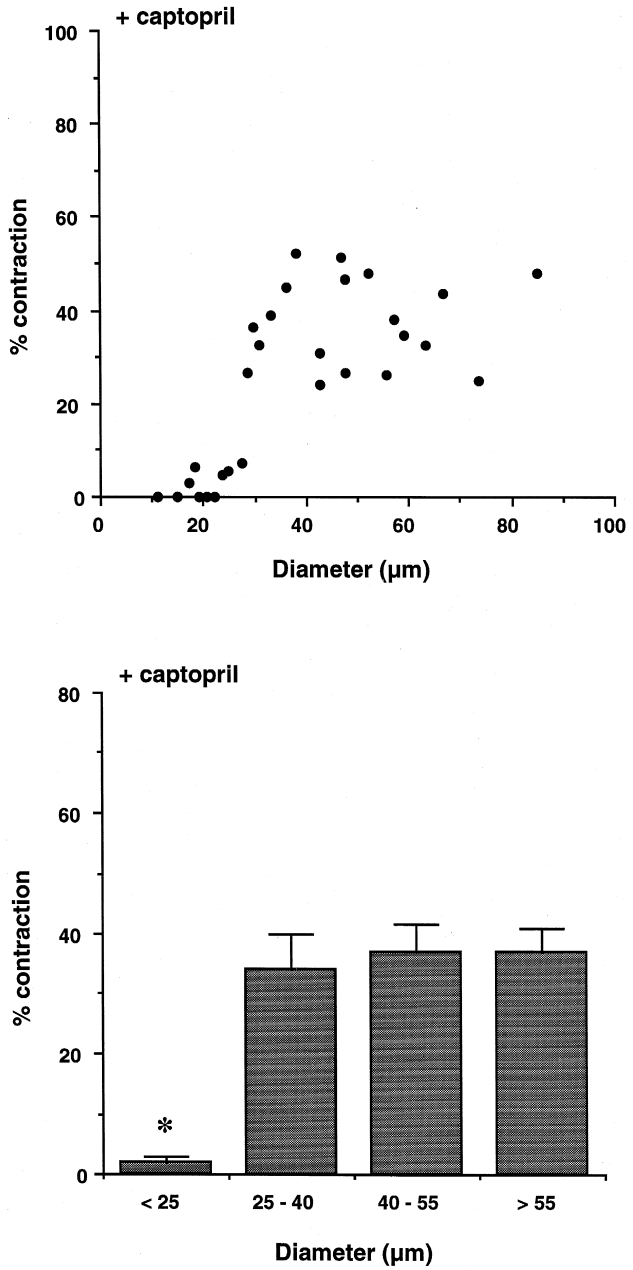

Fig. 3. Effect of captopril on vascular contraction of microvessels induced by angiotensin I. Cheek pouch microvessels were incubated with 1 mM captopril for 15 min, and then vascular contraction was induced by the application of 20 μM angiotensin I. (A) Data from six hamsters are plotted ($r = 0.704$, $p < 0.005$). (B) Mean ± S.E.M. $^{*}P < 0.01$ compared with other groups by one-way ANOVA. See also the legend of Fig. 2.

II (Tang et al., 1995; Myers et al., 1988). In order to examine the responsiveness of arterioles to exogenously applied angiotensin I, we treated arterioles of different diameters with angiotesin I. Application of 20 nM angiotensin I induced vascular contraction with a latency of 60–90 s, which was slightly longer than that observed after application of the same concentration of angiotensin II. As shown in Fig. 2, application of angiotensin I induced contraction of microvessels of various diameters. Although the contractility of each vessel was considerably different, there was no apparent relationship between the vessel diameter and response to angiotensin I. When microvessels were divided into four groups according to their diameters, there were no differences in contractility among these groups and the mean value of % contraction was approximately 60% (Fig. 2b). These results suggested that all arterioles exhibited angiotensin II-forming activity from

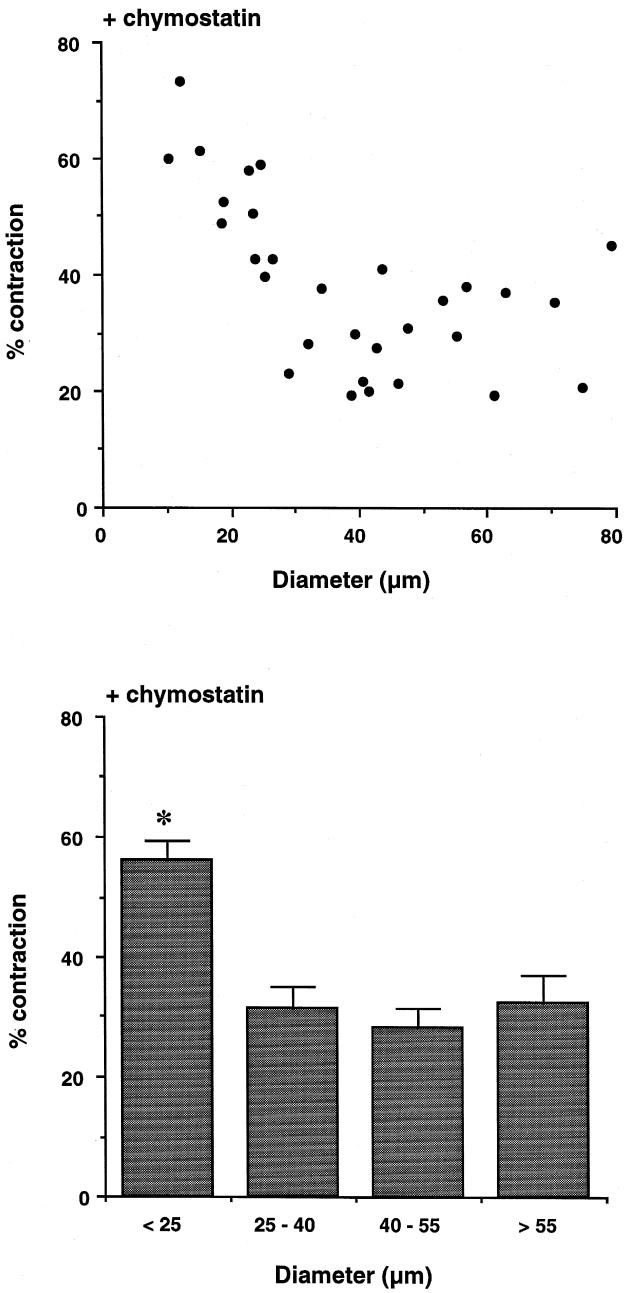

Fig. 4. Effects of chymostatin on contraction of arterioles induced by angiotensin I. Cheek pouch arterioles were incubated with 100 μM chymostatin for 15 min. Vascular contraction induced by angiotensin I (20 nM) was observed. (A) Data from six hamsters ($r = -0.589$, $P < 0.005$). (B) Mean ± S.E.M. $^{*}P < 0.01$ as compared with other groups by one-way ANOVA. See also the legend of Fig. 2.

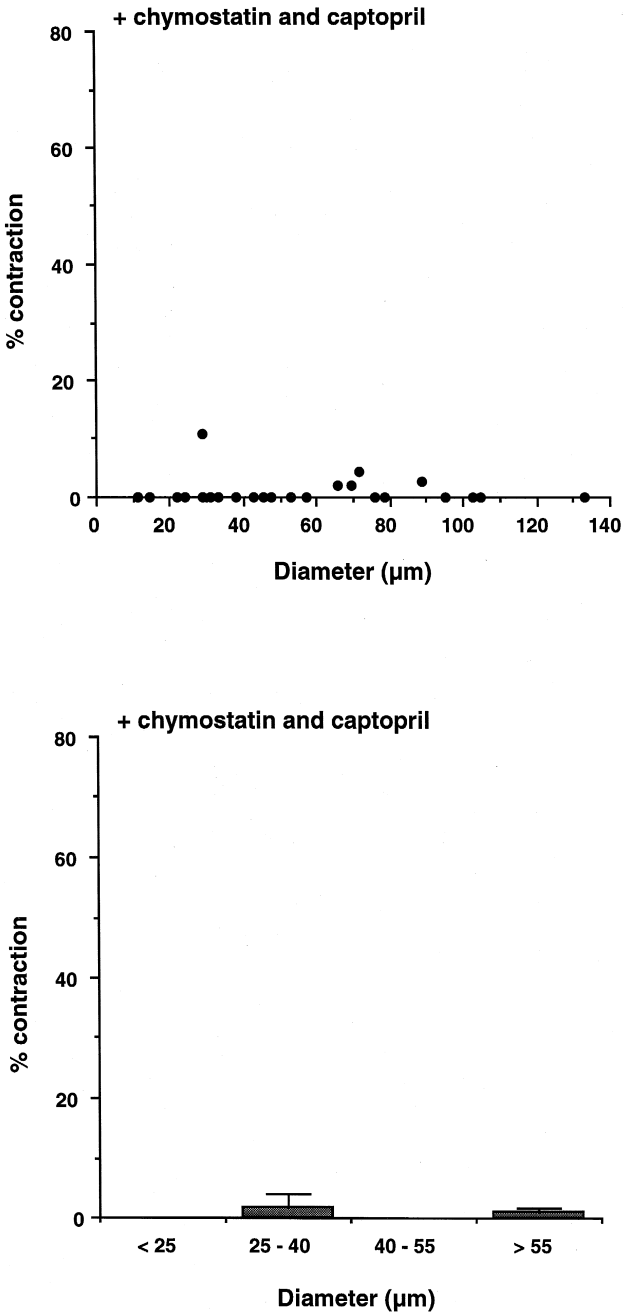

Fig. 5. Effect of captopril and chymostatin on contraction of microvessels induced by angiotensin I. Cheek pouch microvessels were incubated with a solution containing 1 mM captopril and 100 μM chymostatin for 15 min, and vascular contraction was induced by local application of 20 μM angiotensin I. (A) Data of four hamsters ($r = 0.045$, $p > 0.1$). (B) Mean ± S.E.M. There was no statistical difference between four groups. See also the legend of Fig. 2.

angiotensin I, irrespective of their diameter. The results also suggested that each microvessel contained receptors for angiotensin II and could respond to angiotensin II.

In the presence of 1 mM captopril, an angiotensin-converting enzyme inhibitor, application of angiotensin I induced only slight or no contraction of microvessels < 25 μm in diameter (Fig. 3a), whereas vascular contraction was observed in vessels > 30 μm in diameter. However, there were no significant differences in contractility of microvessels with diameter > 25 μm (Fig. 3b). In addition, the latency between application of angiotensin I and development of contraction did not differ in the presence or absence of captopril. These results suggested that in vessels < 25 μm in diameter, angiotensin-converting enzyme-dependent angiotensin I formation might be dominant and indicated the possible lack of other alternative pathways.

As shown in Fig. 4, treatment of arterioles with 100 μM chymostatin decreased the contractile response evoked by angiotensin I in arterioles > 25 μm in diameter. Contractility in these vessels decreased to 50% of that observed in the absence of the inhibitor. On the other hand, angiotensin I-induced responses were not affected in arterioles of a smaller diameter and % contraction values of about 60% were also noted even in the presence of chymostatin. These results suggested that chymostatin-sensitive, chymase-dependent angiotensin II-formation pathway did not exist in the vascular wall of these smaller arterioles ($\phi < 25$ μm). They also suggested that 50% of angiotensin II-forming activity from angiotensin I was chymase-dependent in arterioles with a larger diameter ($\phi > 25$ μm).

In the presence of 1 mM captopril and 10 μM chymostatin, almost no contraction of microvessels was noted upon application of angiotensin I irrespective of the vessel diameter (Fig. 5a). Because the same dose of angiotensin II induced contraction in the presence of these inhibitors, the inhibitory effect was not due to cytotoxic effects of these inhibitors and dimethylsulfoxide (final concentration 0.5%) used to solubilize chymostatin. These results suggest that there is no other angiotensin II-forming pathway in addition to the captopril-sensitive, angiotensin-converting enzyme-dependent pathway and the chymostatin-sensitive, chymase-dependent pathway. These results are summarized in Table 1. In microvessels < 25 μm in diameter, the formation of angiotensin II from angiotensin I, which resulted in vascular contraction, was mostly dependent on angiotensin-converting enzyme. In vessels > 40 μm in

Table 1
Contribution of chymase and angiotensin-converting enzyme (ACE) in angiotensin I-induced contraction
Cheek pouch arterioles were treated with 20 nM angiotensin I and % contraction was calculated. Chymase: % contraction in the presence of 1 mM captopril, ACE: % contraction in the presence of 100 μM chymostatin.

Diameter (μm)	% contraction		Chymase/ACE ratio	
	Chymase	ACE	% Chymase	% ACE
< 25	2.5	58	4.0	96
25–40	30	24	56	44
40–55	33	20	62	38
> 55	35	22	61	39

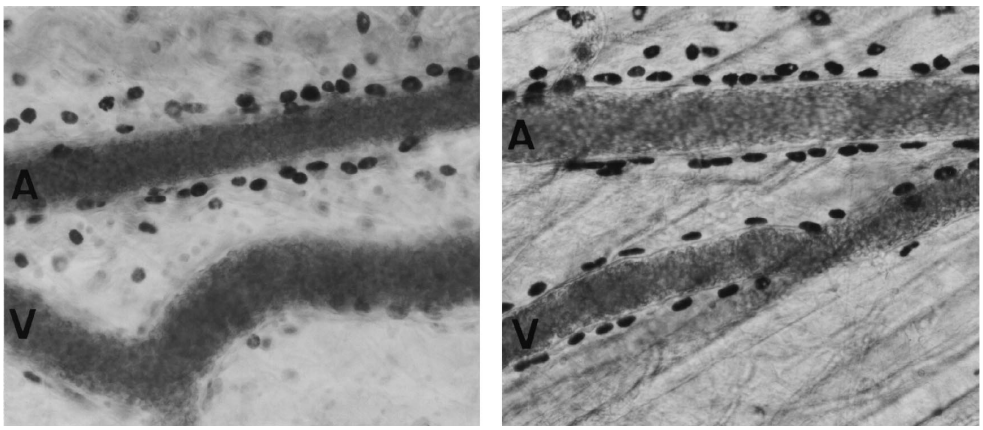

Fig. 6. Presence of mast cells along arterioles in the cheek pouch. The intact cheek pouch preparation was stained with toluidine blue solution acidified with 0.1 M citrate. Note the presence of numerous toluidine blue-positive cells in the connective tissue around the arterioles. A: arteriole, V: vein. Bar = 100 μm.

diameter, about 60% of angiotensin II-forming activity was due to chymase-like enzyme.

3.3. Localization of mast cells around microvessels of cheek pouch

Our aforementioned results demonstrated the presence of chymase activity near arterioles of the cheek pouch. Mast cells are a possible source of chymase in the cheek pouch because chymase is the major protease in mast cell granules and can be released into the extracellular space upon degranulation. Therefore, we investigated the presence and distribution of these cells in the pouch. Numerous toluidine blue-positive cells were present in the cheek pouch (Fig. 6). These cells are likely mast cells based on their morphological features. Most mast cells were located close to the vascular wall of microvessels. As shown in Fig. 7, the number of mast cells was proportional to the diameter of arterioles. In particular, the maximal number of mast cells was observed around arterioles with a diameter larger than 30 μm, in which considerable chymase-dependent angiotensin-converting activity was also observed. In contrast, a limited number of mast cells was located in the vicinity of venules.

3.4. Response of compound 48/80-treated vessels to angiotensin I

Our immunohistochemical studies using anti-chymase antibody demonstrated that mast cells in the cheek pouch

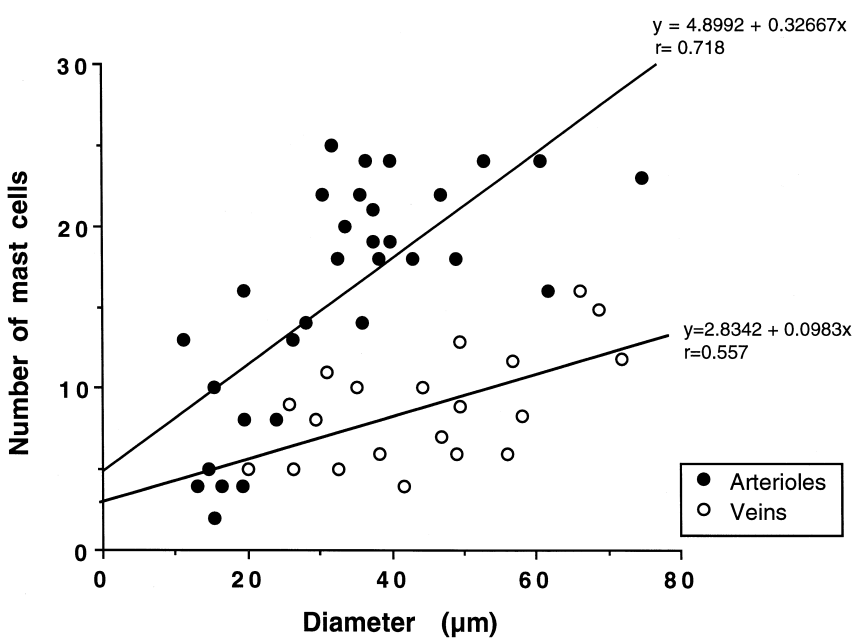

Fig. 7. Number of mast cells around microvessels in the cheek pouch. The exposed cheek pouch was stained with toluidine blue acidified with citrate. Near-straight portions of microvessels were randomly selected, and the number of mast cells along the 200 μm straight vessels was counted. Closed circles; arterioles, open circles; veins. Data of three animals are plotted. Regression lines were drawn by the least-squares method.

contained chymase in their granules (data not shown). Therefore, it is likely that angiotensin II-forming chymase in arterioles of cheek pouch is derived from mast cells. In the next series of experiments, we investigated the effects of treatment with compound 48/80, which activates mast cells and stimulates the release of chymase, on the response of microvessels to exogenously administered angiotensin I. Treatment of the cheek pouch with 100 μg/ml compound 48/80 for 5 min caused degranulation of mast cells, which was confirmed by staining with toluidine blue (data not shown). Because histamine, also released from activated mast cells, induces vasocontraction and increased microvascular permeability (Grega and Adamski, 1991; Shepherd and Duling, 1996), treatment with compound 48/80 was carried out in the presence of 1 mM pyrilamine, a histamine H1 receptor antagonist. As shown in Fig. 8, in the presence of 1 mM captopril, application of angiotensin I induced contraction of the arterioles in a concentration-dependent manner. The apparent contraction was observed at doses >10 ng/ml. Treatment of arterioles with compound 48/80 increased their responsiveness to angiotensin I and the vascular contraction was observed even at a dose of 1 ng/ml. IC_{50} values for control and compound 48/80-treated microvessels were 8.2 ng/ml and 2.6 ng/ml, respectively. Treatment with pyrilamine alone did not have any effect on angiotensin I-induced contraction (data not shown). These results suggested that chymase, released from activated mast cells, might reside near the microvessels as an active enzyme and can convert angiotensin I to angiotensin II.

4. Discussion

The major finding of the present study was the presence of functional chymase in the vicinity of arterioles in the intact cheek pouch. This enzyme generates angiotensin II from angiotensin I, resulting in contraction of the arterioles. Although chymase had been detected and purified in the hamster cheek pouch homogenates as an angiotensin II-forming enzyme (Takai et al., 1996), its distribution and function have not been elucidated. Our present results showed that angiotensin-converting enzyme was dominant in vessels <25 μm diameter (4th and 5th-order arterioles) but more than 50% of angiotensin II-forming activity was chymase-dependent in larger vessels (2nd and 3rd-order arterioles). These results are in agreement with those of a previous study by Tang et al. (1995), showing that impairment of the endothelium, a major site for angiotensin-converting enzyme, by light-dye treatment greatly reduces angiotensin conversion in 4th-order arterioles. Although the physiological role of chymase-dependent angiotensin II-generating pathway is still unknown, the present results suggest that this pathway may regulate blood flow through the cheek pouch through contraction of arterioles.

Local angiotensin II formation, in addition to the systemic renin–angiotensin system, has been reported by many investigators and its importance in the regulation of heart and vascular activities has been suggested (Dzau, 1984; Unger et al., 1986). In systemic angiotensin II formation, circulating angiotensin I is thought to be cleaved to angiotensin II, mainly by angiotensin-converting en-

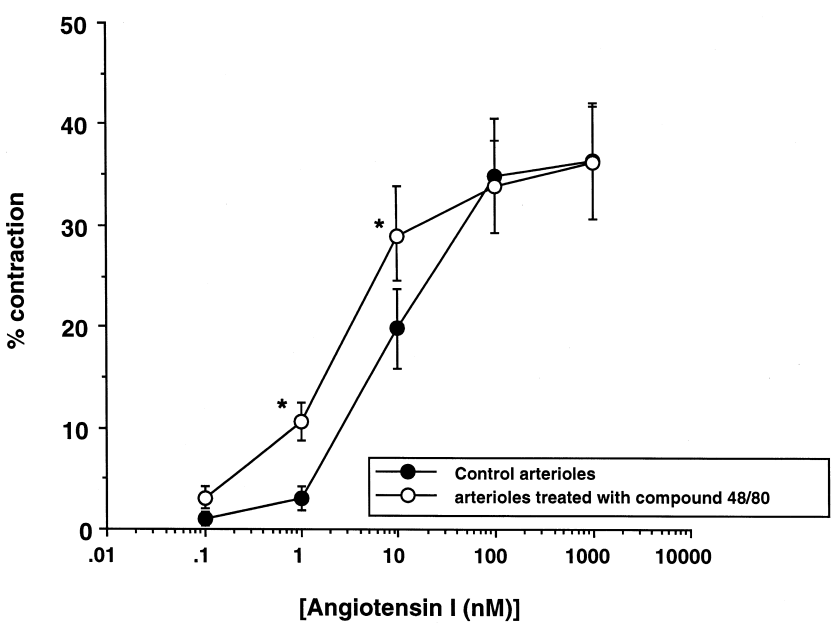

Fig. 8. Effects of treatment with compound 48/80 on the concentration-response curve of angiotensin I. The exposed cheek pouch was pretreated with 100 μg/ml compound 48/80 for 5 min in the presence of 1 mM pyrilamine. After washout with PBS, contraction of arterioles (25–50 μm in diameter) elicited by angiotensin I at various concentrations in the presence of 1 mM captopril was determined. Closed circles; vehicle-treated arterioles, open circles; compound 48/80-treated arterioles. Each value represents the mean ± S.E.M. $n=10$, $^{*}P<0.05$ vs. vehicle control.

zyme in plasma or at the endothelial cell surface. In contrast, angiotensin I produced within the vessel wall is converted to angiotensin II by enzymatic activity in the interstitial fluid or extracellular matrix where chymase activity had been demonstrated (Kokkonen et al., 1997). To our knowledge, there are no reports that have previously investigated the local generation of angiotensinogen or angiotensin I in hamster cheek pouch and it is not clear at this stage whether angiotensin I is locally formed by vascular wall cells or it is derived from plasma by unknown uptake mechanism.

One of our interests in this field is the origin of chymase in hamster cheek pouch. Chymase is expressed in one of two subtypes of mast cells, designated MC_{TC} or connective tissue type, and a relatively large amount of chymase is stored in their granules (Irani et al., 1986 ;Irani and Schwartz, 1989). As shown in Fig. 6, staining of the cheek pouch with toluidine blue under acidic conditions showed that many mast cells reside in the connective tissue of the cheek pouch, especially in the vicinity of arterioles. These mast cells contained chymase because they were positively stained when treated with anti-chymase antibody (data not shown). Therefore, it is likely that chymase in the cheek pouch is derived from mast cells. Chymase is stored in the granules of mast cells and released into the extracellular space upon activation of these cells. It has been shown that chymase has a heparin-binding domain and can bind to heparin or heparin-like molecules (Sayama et al., 1987; Caughey et al., 1988). Therefore, chymase may be retained in tissues near mast cells by binding to sulfated proteoglycans on the cell surface or extracellular matrix after degranulation of mast cells. As shown in Fig. 7, mast cell density is apparently higher around large arterioles (>30 μm in diameter) compared with veins or small arterioles (<30 μm in diameter). This distribution pattern of mast cells in the cheek pouch is almost identical to that of angiotensin-converting enzyme-independent angiotensin-converting activity, as shown in Fig. 7. Furthermore, treatment of the pouch with compound 48/80 potentiated angiotensin I-induced vascular contraction, supporting the notion that functional chymase is released from activated mast cells and is retained in the vascular wall tissue. Staining of the cheek pouch preparation used in the present study with toluidine blue showed only few degranulated mast cells, suggesting that mast cells were not activated during the operation. Furthermore, pretreatment of hamsters with cromoglicic acid for 10 days, which is known to inhibit mast cell degranulation, did not affect chymase-dependent angiotensin I-induced vascular contraction (data not shown). Therefore, it is unlikely that chymase activity observed in the cheek pouch is due to activation of mast cells during the experiment.

Although the present results suggest that the source of chymase is mast cells, it is also probable that cells constituting the microvessel wall may also express and release chymase. Using in situ hybridization techniques, Urata et al. (1993) demonstrated that endothelial cells or interstitial cells of the human heart also express chymase. Therefore, we cannot exclude the possible production of chymase by cells in the adventitia or connective tissue adjacent to the vessels. Further studies using in situ hybridization techniques are necessary to establish the contribution of such cells to the local chymase activity.

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References

Balcells, E., Meng, Q.C., Johnson, W.H. Jr., Oparil, S., Dell'Italia, L.J., 1997. Angiotensin II formation from ACE and chymase in human and animal heart: methods and species considerations. Heart Circ. Physiol. 42, H1769–H1774.

Bouskela, E., Grampp, W., 1992. Spontaneous vasomotion in hamster cheek pouch arterioles in varying experimental conditions. Am. J. Physiol. 262, H478–H485, Heart Circ. Physiol. 31.

Bouskela, E., Cyrino, F.Z.G.A., Lerond, L., 1997. Effects of oral administration of different doses of purified micronized flavonoid fraction on microvascular reactivity after ischaemia/reperfusion in the hamster cheek pouch. Br. J. Pharmacol. 122, 1611–1616.

Bührle, C.P., Rosivall, L., Taugner, R., 1987. Intrarenal generation of angiotensin II evaluated by an electrophysiological technique. Am. J. Physiol. 252, F635–F644.

Caughey, G.H., Viro, N.F., Lazarus, S.C., Nadel, J.A., 1988. Purification and characterization of dog mastocytoma chymase: identification of an octapeptide conserved in chymotryptic leukocyte proteinases. Biochim. Biophys. Acta 952, 142–149.

Chandrasekharan, U.M., Sanker, S., Glynias, M.J., Karnik, S.S., Husain, A., 1996. Angiotensin II-forming activity in a reconstructed ancestral chymase. Science 271, 502–505.

Cornish, K.G., Gilmore, J.P., 1981. Angiotensin I conversion by the microcirculation of the nonhuman primate. Blood Vessels 18, 128–133.

Cornish, K.G., Joyner, W.L., Gilmore, J.P., 1979. Direct evidence for the presence of a different converting enzyme in the hamster cheek pouch. Circ. Res. 44, 540–544.

DePierre, D., Roth, M., 1975. Fluorimetric determination of dipeptidyl carboxypeptidase. Enzyme 19, 65–70.

Dzau, V.J., 1984. Vascular renin–angiotensin: a possible autocrine or paracrine system in control of vascular function. J. Cardiovasc. Pharmacol. 6, S377–S382, Suppl. 2.

Dzau, V.J., 1989. Multiple pathways of angiotensin production in the blood vessel wall: evidence, possibilities and hypotheses. J. Hypertens. 7, 933–936.

Gräfe, M., Auch-Schwelk, W., Zakrzewicz, A., Regitz-Zagrosek, V., Bartsch, P., Graf, K., Loebe, M., Gaehtgens, P., Fleck, E., 1997. Angiotensin II-induced leukocyte adhesion on human coronary endothelial cells is mediated by E-selectin. Circ. Res. 81, 804–811.

Grega, G.J., Adamski, S.W., 1991. Effects of local mast cell degranulation on vascular permeability to macromolecules. Microcirc., Endothelium, Lymphatics 7, 267–291.

Husain, A., 1993. The chymase–angiotensin system in humans. J. Hypertens. 11, 1155–1159.

Ichikawa, I., Harris, R.C., 1991. Angiotensin actions in the kidney: renewed insight into the old hormone. Kidney Int. 40, 583–596.

Irani, A.-M.A., Schwartz, L.B., 1989. Mast cell heterogeneity. Clin. Exp. Allergy 19, 143–155.

Irani, A.-M.A., Schechter, N.M., Craig, S.S., DeBlois, G., Schwartz, L.B., 1986. Two types of mast cells that have distinct neutral protease compositions. Proc. Natl. Acad. Sci. U.S.A. 83, 4464–4468.

Kokkonen, J.O., Saarinen, J., Kovanen, P.T., 1997. Regulation of local angiotensin II formation in the human heart in the presence of interstitial fluid. Inhibition of chymase by protease inhibitors of interstitial fluid and of angiotensin-converting enzyme by Ang-(1-9) formed by heart carboxypeptidase A-like activity. Circulation 95, 1455–1463.

Le Trong, H., Neurath, H., Woodbury, R.G., 1987. Substrate specificity of the chymotrypsin-like protease in secretory granules isolated from rat mast cells. Proc. Natl. Acad. Sci. U.S.A. 84, 364–367.

Miyazaki, M., Okunishi, H., Nishimura, K., Toda, N., 1984. Vascular angiotensin-converting enzyme activity in man and other species. Clin. Sci. 66, 39–45.

Myers, T.O., Joyner, W.L., Gilmore, J.P., 1988. Angiotensin reactivity in the cheek pouch of the renovascular hypertensive hamster. Hypertension 12, 373–379.

Okunishi, H., Miyazaki, M., Toda, N., 1984. Evidence for a putatively new angiotensin II-generating enzyme in the vascular wall. J. Hypertens. 2, 277–284.

Okunishi, H., Miyazaki, M., Okumura, T., Toda, N., 1987. Different distribution of two types of angiotensin II-generating enzymes in the aortic wall. Biochem. Biophys. Res. Commun. 149, 1186–1192.

Okunishi, H., Oka, Y., Shiota, N., Kawamoto, T., Song, K., Miyazaki, M., 1993. Marked species-difference in the vascular angiotensin II-forming pathways: humans versus rodents. Jpn. J. Pharmacol. 62, 207–210.

Sayama, S., Iozzo, R.V., Lazarus, G.S., Schechter, N.M., 1987. Human skin chymotrypsin-like protease chymase: subcellular localization to mast cell granules and interaction with heparin and other glycosaminoglycans. J. Biol. Chem. 263, 6808–6815.

Schelling, P., Fischer, H., Ganten, D., 1991. Angiotensin and cell growth: a link to cardiovascular hypertrophy?. J. Hypertens. 9, 3–15.

Shepherd, R.K., Duling, B.R., 1996. Inosine-induced vasoconstriction is mediated by histamine and thromboxane derived from mast cells. Am. J. Physiol. 270, H560–H566.

Shiota, N., Fukamizu, A., Takai, S., Okunishi, H., Murakami, K., Miyazaki, M., 1997a. Activation of angiotensin II-forming chymase in the cardiomyopathic hamster heart. J. Hypertens. 15, 431–440.

Shiota, N., Jin, D., Takai, S., Kawamura, T., Koyama, M., Nakamura, N., Miyazaki, M., 1997b. Chymase is activated in the hamster heart following ventricular fibrosis during the chronic stage of hypertension. FEBS Lett. 406, 301–304.

Shiota, N., Saegusa, Y., Nishimura, K., Miyazaki, M., 1997c. Angiotensin II-generating system in dog and monkey ocular tissues. Clin. Exp. Pharmacol. Physiol. 24, 243–248.

Takai, S., Shiota, N., Yamamoto, D., Okunishi, H., Miyazaki, M., 1996. Purification and characterization of angiotensin II-generating chymase from hamster cheek pouch. Life Sci. 58, 591–597.

Takai, S., Shiota, N., Kobayashi, S., Matsumura, E., Miyazaki, M., 1997. Induction of chymase that forms angiotensin II in the monkey atherosclerotic aorta. FEBS Lett. 412, 86–90.

Tang, T., Connelly, B.A., Joyner, W.L., 1995. Heterogeneity of endothelial cell function for angiotensin conversion in serial-arranged arterioles. J. Vasc. Res. 32, 129–137.

Unger, T., Ganten, D., Lang, R.E., 1986. Tissue converting enzyme and cardiovascular action of converting enzyme inhibitors. J. Cardiovasc. Pharmacol. 8, S7–S81, Suppl. 10.

Urata, H., Kinoshita, A., Misono, M.S., Bumpus, F.M., Husain, A., 1990. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. J. Biol. Chem. 265, 22348–22357.

Urata, H., Boehm, K.D., Philip, A., Kinoshita, A., Gabrovsek, J., Bumpus, F.M., Husain, A., 1993. Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. J. Clin. Invest. 91, 1269–1281.

Urata, H., Nishimura, H., Ganten, D., 1996. Chymase-dependent angiotensin II forming system in humans. Am. J. Hypertens. 9, 277–284.