Cooperation between Mast Cell Carboxypeptidase A and the Chymase Mouse Mast Cell Protease 4 in the Formation and Degradation of Angiotensin II*

Anders Lundequist, Elena Tchougounova, Magnus Åbrink, and Gunnar Pejler†

From the Department of Molecular Biosciences, The Biomedical Centre, Swedish University of Agricultural Sciences, Box 575, 75 123 Uppsala, Sweden

Received for publication, May 19, 2004
Published, JBC Papers in Press, June 1, 2004, DOI 10.1074/jbc.M405576200

The octapeptide angiotensin II (Ang II) exerts a wide range of effects on the cardiovascular system but has also been implicated in the regulation of cell proliferation, fibrosis, and apoptosis. Ang II is formed by cleavage of Ang I by angiotensin-converting enzyme, but there is also evidence for non-angiotensin-converting enzyme-dependent conversion of Ang I to Ang II. Here we address the role of mast cell proteases in Ang II production by using two different mouse strains lacking mast cell heparin or mouse mast cell protease 4 (mMCP-4), the chymase that may be the functional homologue to human chymase. Ang I was added to ex vivo cultures of peritoneal cells, and the generation of Ang II and other metabolites was analyzed. Activation of mast cells resulted in marked increases in both the formation and subsequent degradation of Ang II, and both of these processes were strongly reduced in heparin-deficient peritoneal cells. In the mMCP-4−/− cultures no reduction in the rate of Ang II generation was seen, but the formation of Ang-(5–10) was completely abrogated. Addition of a carboxypeptidase A (CPA) inhibitor to wild type cells caused complete inhibition of the formation of Ang-(1–9) and Ang-(1–7) but did not inhibit Ang II formation. However, when the CPA inhibitor was added to the mMCP-4−/− cultures, essentially complete inhibition of Ang II formation was obtained. Taken together, the results of this study indicate that mast cell chymases and CPA have key roles in both the generation and degradation of Ang II.

EXPERIMENTAL PROCEDURES

Materials—Ang I (Sigma), Ang II (Sigma), Ang(5–8) (Bachem, Bubendorf, Switzerland), Ang-(1–7), Ang-(1–9), Ang-(5–10) (XCP/Xia Care HB, Gothenburg, Sweden), α-antichymotrypsin (Calbiochem, Nottingham, UK), calcium ionophore A23187 (Sigma), Captopril

* This work was supported by grants from the AgriFunGen program at the Swedish University of Agricultural Sciences, the Swedish Research Council, King Gustaf V’s 80th Anniversary Fund, and the Magnus Bergvall Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 46-18-4714090; Fax: 46-18-550762; E-mail: Gunnar.Pejler@bmc.uu.se.

‡ To whom correspondence should be addressed. Tel.: 46-18-550762; E-mail: Gunnar.Pejler@bmc.uu.se.

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The abbreviations used are: Ang, angiotensin; ACE, angiotensin-converting enzyme; MC, mast cell; CPA, carboxypeptidase A; mMCP, mouse mast cell protease; NDST, N-deacetylasyl/N-sulfotransferase; ELISA, enzyme-linked immunosorbent assay; wt, wild type; HPLC, high pressure liquid chromatography; CPI, carboxypeptidase A inhibitor.
Mast Cell Proteases in Angiotensin Metabolism

FIG. 1. Morphology of chymase- and heparin-deficient peritoneal cells. Cytospin slides of peritoneal cells from wild type (A), NDST-2−/− (B), mMCP-4−/− (C), and calcium ionophore (A23187)-treated wt cells (D) were stained with May-Grunwald/Giemsa. Mast cells are indicated by arrows.

Ang(1–9), Ang-(1 standard Ang I, Ang II, Ang-(1–7), Ang-(1–10), and Ang(6–8). treatment, each peak area was compared with an unrelated reference quantity. The quantities of peaks were assessed by integrating peak areas. For normalization, a linear gradient extending from 10% acetonitrile, 0.1% trifluoroacetic acid. The column was eluted at 1 ml/min with a Source-5RPC ST 4.6/150 reversed phase column (Amersham Biosciences) were from the commercial sources indicated. A selective trypsin inhibitor, 52335, was a gift from Morphochem AG (Munich, Germany).

Mice—NDST-2−/− (C57BL6 background; back-crossed 10 generations) and mMCP-4−/− mice (mixed genetic background, C57BL/6–129SVJ) were generated by gene targeting (7, 12). For experiments on the NDST-2−/− strain, C57BL/6 mice were used as wild type (wt) controls. For the experiments on the mMCP-4−/− strain, mMCP-4+/− or mMCP-4−/− littermates were used as the wt control. No differences in Ang I processing were observed between mMCP-4−/− and mMCP-4−/− cells.

Cells—Peritoneal cells were collected by lavages with phosphate-buffered saline. Cells were centrifuged (300 × g, 4 °C, 10 min), resuspended, and cultured in serum-free medium (Ham’s/F12 supplemented with 4 mM l-glutamine and 100 IU/ml penicillin-streptomycin). The cells were distributed in 24-well plates (Nunc; ∼2 × 10⁵ cells in 0.5 ml of medium/well) and incubated at 37 °C in a humidified atmosphere of 5% CO₂. To study the processing of Ang I, exogenous Ang I (100 ng for ELISA; 20 μg for HPLC; in 10–20 μl of phosphate-buffered saline) was added, followed by either calcium ionophore (1 μl of a 2 mM solution of A23187), anti-IgE (4 μl of a 0.5 μg/μl solution) or an equal volume of phosphate-buffered saline (control). Medium samples (50 or 100 μl) were taken after 10 min, 30 min, 1 h, 3 h, and 6 h and were stored at −20 °C until analysis. In the inhibition studies, cells were first treated with calcium ionophore, followed by the addition of inhibitor after 10 min and the addition of Ang I 15 min later.

HPLC—Samples of conditioned medium (50 μl) were applied to a Source-5RPC ST 4.6/150 column equilibrated with 10% acetonitrile, 0.1% trifluoroacetic acid. The column was eluted at 1 ml/min with a linear gradient extending from 10% acetonitrile, 0.1% trifluoroacetic acid to 80% acetonitrile. The eluate was monitored at 214 nm, and the quantities of peaks were assessed by integrating peak areas. For normalization, each peak area was compared with an unrelated reference quantity.

RESULTS

Peritoneal cell cultures from wt mice typically contained ∼2–3% MCs, with the remainder being lymphocytes and macrophages in approximately equal proportions (Fig. 1A). In the NDST-2−/− cells, the densely stained MCs were replaced by cells containing empty appearing vacuoles, in agreement with the MC morphology reported previously in the NDST-2 knock-out (Fig. 1B) (7). In the mMCP-4−/− cell populations, MCs had normal morphology (Fig. 1C). Cells other than MCs had indistinguishable morphology in all three genotypes (Fig. 1A–C). Addition of a calcium ionophore resulted in MC degranulation but no observable changes in the morphology of the other peritoneal cell types (Fig. 1D).

Angiotensin conversion in the peritoneal cell ex vivo system was first studied by ELISA. Ang I was added, and the level of Ang II in conditioned medium was measured at different time points. Addition of Ang I to wt cells resulted in the generation of immunoreactive Ang II, and it is clear from Fig. 2 that the level of Ang II was maximal at −1 h, after which leveling off...
was observed. After activation of MCs, either with calcium ionophore (Fig. 2) or anti-IgE antibody (not shown), Ang II generation was much more rapid, with peak Ang II levels reached at −10−30 min. Furthermore, after maximal Ang II levels had been reached, the Ang II peak decreased rapidly and was essentially undetectable after 3 h (Fig. 2). When similar experiments were performed on peritoneal cells recovered from NDST-2−/− animals, the levels of Ang II that were generated, either in the absence or presence of MC activation agent, were markedly lower than in wt cells. However, the kinetics for Ang II formation and subsequent decrease were similar as in wt cell cultures (Fig. 2).

To identify Ang I processing products other than Ang II, we used HPLC analysis. Addition of Ang I to wt cells resulted in formation of a peak corresponding to Ang II, in agreement with the ELISA analysis (Fig. 3), and the Ang II peak was markedly increased after activation of MCs (Fig. 3B). Addition of Ang I to activated wt cells also resulted in the formation of Ang-(1–9), Ang-(5–10), and Ang-(1–7), respectively (Fig. 3B). The levels of all of these peptides were markedly lower in non-activated peritoneal cell cultures (Fig. 3A) and were considerably lower in conditioned medium from NDST-2−/− cells than in medium from wt cells (Fig. 3C). To follow the kinetics of Ang I processing, the levels of each of the Ang I processing products were assessed at various time points (Fig. 4). From Fig. 4A it is apparent that the level of Ang I decreased continuously after addition to peritoneal cells. The rate for this decrease was considerably higher in wt than in NDST-2−/− cells and was markedly increased by calcium ionophore (Fig. 4A). In agreement with the ELISA data, both the Ang II formation and its subsequent decrease were markedly increased upon MC activation, and those processes were defective in NDST-2−/− cell cultures (Fig. 4B). The generation and subsequent decrease in Ang-(1–9) showed similar kinetics as was observed for Ang II, indicating that Ang II generation and Ang-(1–9) generation are closely associated events (Fig. 4C). The kinetics of Ang-(5–10) formation and subsequent decrease showed similarities to the kinetics for Ang II. However, Ang-(5–10) did not accumulate to the same extent in medium from non-activated cells as was seen for Ang II and Ang-(1–9). In contrast to the generation of Ang II, Ang-(1–9), and Ang-(5–10), the generation of Ang-(1–7) was relatively slow, being first detectable ~60 min after addition of Ang I. Furthermore, the generation of Ang-(1–7) was not defective in the NDST-2−/− cultures. Instead, the level of this peptide was higher in NDST-2−/− cells than in wt cell cultures (Fig. 4E).

The results above clearly indicate that MCs are involved in Ang I processing in the peritoneal cell ex vivo system and that their stored proteases participate in the process. Next, we investigated which of the MC proteases are responsible for each of the different Ang I processing steps. For this purpose we used a mouse strain with a targeted inactivation of mMCP-4, the murine chymase that may be the closest functional homologue to the human chymase (12). To address the relative contribution of other MC proteases, various protease inhibitors were employed.
The rate of Ang I decrease, after its addition to activated or non-activated peritoneal cell cultures, was similar in wt and mMCP-4 cultures (Fig. 5A), indicating that overall Ang I hydrolysis is not critically dependent on mMCP-4. Addition of a CPA inhibitor (CPI) resulted in a markedly slower rate of Ang I decrease in activated wt cell cultures, and the addition of CPI to activated mMCP-4-null cultures gave an even more pronounced reduction in Ang I hydrolysis (Fig. 5A). As shown in Fig. 5B, Ang II was readily formed in mMCP-4 cell cultures, indicating that mMCP-4 is not essential for Ang II generation. The kinetics for Ang II formation and its subsequent decrease were highly similar in wt and mMCP-4 cell cultures except that higher levels of Ang II were accumulated in non-activated cell cultures from mMCP-4 mice than in wt counterparts. When CPI was added to wt cell cultures, no reduction in Ang II generation was observed. In fact, addition of CPI resulted in higher levels of accumulated Ang II than in wt counterparts, possibly explained by the fact that mMCP-4 participates in further processing of this peptide after its generation.

Substantially higher levels of Ang-(1–9) were accumulated in medium from mMCP-4 cells than in wt cell cultures, indicating that mMCP-4 may catalyze further processing of this peptide. Otherwise, the kinetics for the generation and subsequent decrease of Ang-(1–9) were not affected to any major extent in the mMCP-4 cell cultures. However, the addition of CPA inhibitor resulted in essentially complete inhibition of the formation of Ang-(1–9), indicating that CPA is solely responsible for the generation of this processing product (Fig. 5C). The generation of Ang-(5–10) was virtually undetectable in mMCP-4 cell cultures, indicating that the formation of this peptide is critically dependent on mMCP-4. Addition of CPA inhibitor to wt cells resulted in a large increase in the accumulation of Ang-(5–10), probably explained by an involvement of CPA in downstream processing of generated Ang-(5–10). Similar to Ang-(1–9), the generation of Ang-(1–7) was completely inhibited by the CPA inhibitor, indicating that CPA is essential for its generation (Fig. 5E). Interestingly, Ang-(1–7) was accumulated at markedly higher levels in conditioned medium from mMCP-4 mice than in wt counterparts, a finding possibly explained by the fact that mMCP-4 participates in further processing of this peptide after its generation.

Addition of the chymase inhibitors chymostatin or α1-antichymotrypsin to wt cells resulted in effects on the Ang processing patterns that were similar to the effects seen in the mMCP-
4-null cell cultures (not shown). Captopril, an ACE inhibitor, did not affect any of the studied processing events, including Ang II formation, indicating that ACE is not operative in this biological system (not shown). Furthermore, we did not see any effect of a selective inhibitor of MC tryptase on any of the parameters studied (not shown).

**DISCUSSION**

The present study strongly supports a major contribution of MCs in the extravascular generation of Ang II. Moreover, MCs appear to play a major role not only in the generation of Ang II but also in its subsequent degradation. The subsequent degradation of Ang II may indeed be an important process that serves to limit the effects of the generated Ang II in time. Among the various MC proteases it is clear that the chymase mMCP-4 plays a major role in Ang II formation. However, we also provide evidence that MC CPA, an enzyme to which few biological functions have been ascribed previously, can convert Ang I to Ang II, a finding that is also supported by a previous study performed in a purified system (13). Importantly, neither of these enzymes is itself essential for Ang II formation, but when both of them are inactivated, negligible amounts of Ang II are generated. We can thereby propose a model in which these two MC proteases act in concert during the formation of Ang II and in which the inhibition of either chymase or CPA may be compensated by the other protease. Indeed, it has been proposed previously that MC chymase and CPA, which are both bound to heparin proteoglycan (14), cooperate in the degradation of low density lipoprotein (15).

Based on the data obtained in this work we can propose a scheme for extravascular Ang I processing (Fig. 6). CPA hydrolyzes Ang I stepwise from the C terminus, generating Ang-(1–9) followed by Ang-(1–7). Ang-(1–9) and Ang II are both rapidly formed, even when mMCP-4 is inactive, indicating that CPA readily hydrolyzes the His9–Leu10 and Phe8–His9 bonds. In contrast, the subsequent formation of Ang-(1–7) is markedly delayed, indicating that the Pro7–Phe8 bond is not an optimal, although feasible, CPA substrate. Clearly, an alternative pathway for Ang II formation is the direct cleavage of

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**Fig. 5.** Kinetics for the processing of angiotensin I in wild type and mMCP-4−/− peritoneal cells. Peritoneal cells from wild type and mMCP-4−/− mice were cultured at 4 × 10⁶ cells/ml. Ang I (20 μg) was added to resting or calcium ionophore-treated (degranulated) cells. Cells were cultured in the absence or presence (CPI) of a carboxypeptidase A inhibitor. Media samples (100 μl) were collected at different times and were analyzed by HPLC. The levels of Ang I (A), Ang II (B), Ang-(1–9) (C), Ang-(5–10) (D), and Ang-(1–7) (E) were estimated based on peak areas (A₂₁₄ × ml), normalized against a reference peak (see Fig. 3). Control, Ang I incubated in cell-free culture medium. The results shown are from a single experiment, representative of three independent experiments. mAU, milliabsorbance units.
the Phe<sup>8</sup>–His<sup>10</sup> bond in Ang I by mMCP-4. Evidence for this pathway was obtained by the finding that Ang II was generated even when CPA was inhibited, but not when both CPA and mMCP-4 are inactivated. Interestingly, the CPA- and mMCP-4-catalyzed pathways appear to generate Ang II by similar kinetics because Ang II is formed at an equal rate when either CPA or mMCP-4 is inhibited. Our data thus show that mMCP-4 indeed cleaves the Phe<sup>8</sup>–His<sup>10</sup> bond of Ang I, leading to the generation of Ang II. It is also apparent that the inactivating Tyr<sup>4</sup>–Ile<sup>5</sup> cleavage occurs in the system, leading to the formation of Ang-(5–10). In contrast to the formation of Ang II, the generation of Ang(5–10) is exclusively dependent on mMCP-4. These findings are thus in agreement with a recent report in which it was shown by using purified components that mMCP-4 can hydrolyze both the Tyr<sup>4</sup>–Ile<sup>5</sup> and Phe<sup>8</sup>–His<sup>9</sup> bonds (10), and thus these results rule out the previous concept that β-chymases such as mMCP-4 exclusively hydrolyze the Tyr<sup>4</sup>–Ile<sup>5</sup> bond.

An important issue that was addressed by the present study is the relative contribution of mMCP-4 and other chymotrypsin-like murine proteases in the generation of Ang II. Because MCs express a number of different chymases and, possibly, cathepsin G, it is clear that any of these proteases may have the potential to contribute to net Ang II production. However, our data indicate that mMCP-4 is by far the dominating Ang I converter in the peritoneal cell ex vivo system. On the other hand, we cannot rule out that other MC chymases, such as mMCP-1 (11), may be quantitatively important for Ang II formation in other tissue locations.

It is important to note that Ang I processing, although entirely dependent on MC proteases, is observed not only when MCs have been activated but also in cultures containing non-activated MCs. This indicates that mMCP-4 and/or CPA activity is present on the surface of non-activated cells and implicates MCs in the continuous regulation of Ang II levels in extravascular tissues even in the absence of MC-degranulating agents. In fact, we have shown previously that chymase activity is present on the surface of non-activated MCs but that surface-associated chymase activity is markedly increased upon MC activation (16), findings that are well in agreement with the present observations.

Our data thus indicate a key role for MC CPA in the extravascular formation of Ang-(1–9) and Ang-(1–7). Importantly, these peptides are not inert degradation products. On the contrary, they are both highly biologically active with a variety of effects where their antagonistic effects on Ang II formation and function, respectively, are most well known (17, 18). MC CPA-catalyzed formation of Ang-(1–9) has been documented previously in a purified system (19), but to our knowledge, this is the first report demonstrating that MC CPA produces Ang-(1–7). The generation of these peptides along with Ang II raises important questions regarding the net Ang II effect. Because Ang-(1–9) and Ang-(1–7) both have been reported to antagonize Ang II, the net Ang II response may be determined by the balance between Ang II and its antagonizing peptides, Ang-(1–9) and Ang-(1–7). Clearly, the present study supports an important role for MCs in regulating the balance between these biologically active peptides.

In summary, this study provides support for a key role of MCs in the extravascular generation of Ang II and various other Ang I processing products, as well as a major role for MCs in the degradation of the respective peptides. Hence, our data are in agreement with the concept that MC chymase primarily acts in the extravascular formation of Ang II, whereas ACE mainly generates Ang II in the vasculature. Based on the present study we may propose that inhibition of MC chymase could be an important way to limit the actions of Ang II, in addition to the employment of ACE inhibitors. Furthermore, we may propose that additional inhibition of MC CPA could cause an even more effective prevention of Ang II effects.

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J. Biol. Chem. 2004, 279:32339-32344.
doi: 10.1074/jbc.M405576200 originally published online June 1, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405576200

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