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Pro-Carboxypeptidase R is an Acute Phase Protein in the Mouse, Whereas Carboxypeptidase N Is Not1,2

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Carboxypeptidase R (EC 3.4.17.20; CPR) and carboxypeptidase N (EC 3.4.17.3; CPN) cleave carboxyl-terminal arginine and lysine residues from biologically active peptides such as kinins and anaphylatoxins, resulting in regulation of their biological activity. Human proCPR, also known as thrombin-activatable fibrinolysis inhibitor, plasma pro-carboxypeptidase B, and pro-carboxypeptidase U, is a plasma zymogen activated during coagulation. CPN, however, previously termed kininase I and anaphylatoxin inactivator, is present in a stable active form in plasma. We report here the isolation of mouse proCPR and CPN cDNA clones that can induce their respective enzymatic activities in culture supernatants of transiently transfected cells. Potato carboxypeptidase inhibitor can inhibit carboxypeptidase activity in culture medium of mouse proCPR-transfected cells. The expression of proCPR mRNA in murine liver is greatly enhanced following LPS injection, whereas CPN mRNA expression remains unaffected. Furthermore, the CPR activity in plasma increased 2-fold at 24 h after LPS treatment. Therefore, proCPR can be considered a type of acute phase protein, whereas CPN is not. An increase in CPR activity may facilitate rapid inactivation of inflammatory mediators generated at the site of Gram-negative bacterial infection and may consequently prevent septic shock. In view of the ability of proCPR to also inhibit fibrinolysis, an excess of proCPR induced by LPS may contribute to hypofibrinolysis in patients suffering from disseminated intravascular coagulation caused by sepsis. The Journal of Immunology, 2000, 165: 1053–1058.

1 It has been well established that human carboxypeptidase N (CPN),4 which consists of two small active subunits and two large glycosylated subunits, is an important inactivator of several potent peptides, including anaphylatoxins, kinins, and fibrinopeptides (1–4). CPN is present in the active form in plasma (5, 6). Mathews et al. (7) has reported familial partial carboxypeptidase N deficiency resulting in angioedema/chronic urticaria, hay fever/asthma, or both. Over the last decade, a new basic carboxypeptidase that is generated from its zymogen during coagulation was identified independently by a number of groups (8–12). We designated this enzyme carboxypeptidase R (CPR) and its zymogen, proCPR. The activated form appeared to remove arginine residues (R) more rapidly than lysine residues (K) at the carboxyl terminus of substrates compared with CPN (5, 11, 13). It has also been termed plasma carboxypeptidase B (11), carboxypeptidase U (CPU) (8), and thrombin-activatable fibrinolysis inhibitor (TAFI) (12). It is known that human proCPR can be hydrolyzed by trypsin-like enzymes such as thrombin (11), thrombin/thrombomodulin complex (14), and plasmin (11), thereby acquiring enzymatic activity by which it cleaves carboxyl-terminal arginine and lysine residues from various peptides. This activity can be completely inhibited by potato carboxypeptidase inhibitor (PCI) at concentrations at which CPN is not affected (15). Because the activity of CPR is similar to that of CPN, this enzyme has been implicated in the inactivation of several natural potent peptides, including anaphylatoxins and bradykinin, as well as in the removal of arginine and lysine residues from partially plasmin-degraded fibrin (8, 15, 16). To elucidate the in vivo roles of proCPR and CPN, the establishment of an animal model is essential. For this purpose we identified cDNAs of mouse proCPR and CPN and studied the effects of LPS on the expression of these enzymes during the inflammatory process.

Materials and Methods

Probes for screening a cDNA library and Northern blot analysis

We obtained two partial mouse sequences similar to that of human plasma pro-carboxypeptidase B (proCPR) using Entrez (http://www.ncbi.nlm.nih.gov/Entrez/) from the National Center of Biotechnology Information. The GenBank accession numbers are AA244760 and AA254734. In the same manner we obtained data on three partial mouse sequences from clones similar to the human small subunit of CPN. The numbers for these sequences are AA238838, AA285540, and AA396985. The sets of primers generated based on the above were: mCPNR6′, 5′-CTGGCTCTTCCAA GAACCTCC-3′; mCPNR292′, 5′-CCAGCCTGGTCTATCGAGAAGG-3′; mCPNR533′, 5′-GAATTCCTGGCCAGAGATGGG-3′; mCPNR574′, 5′-TTGTTCTCTGAGCAGACGAGG-3′; mCPNR108′, 5′-CAAGGTGGCCA ACAAATCC-3′; mCPNR510′, 5′-GAAGTTAGGGTGTGAGACTGCGG-3′; mCPNR609′, 5′-GATGGTGTTCATGCAGTCCTC-3′; and mCPNR1118′, 5′-GTTCACCTGAGTACTGGCCAGG-3′. RT-PCR was performed with these sets of primers using mouse liver total RNA. PCR products were subcloned with a TA cloning kit (Invitrogen, San Diego, CA). The presence of these inserts was confirmed by sequencing. Clones were digested with several restriction enzymes followed by electrophoresis on a 1.0% agarose gel. The inserts were purified using a Prep-A-Gen kit (Bio-Rad, Hercules, CA) and the probes were labeled CPR1, CPR2, CPN1, and CPN2, respectively.
Molecular cloning and base sequence analysis

Dr. Mayumi Nonaka of our laboratory constructed the cDNA library, which was constructed using the ZAP II vector with poly(A)\(^+\) RNA isolated from adult BALB/c mouse liver. Recombinants (1 × 10\(^5\)) were plated at 50,000 plaques/137-mm plate. Screening was conducted under stringent conditions by the plaque hybridization method using each of the (α-\(^32\)P)dCTP-labeled CPR (CPR1 and CPR2) described above. Hybridization was performed at 65°C for 16 h in a buffer containing 50 mM Tris (pH 7.4), 10× Denhardt’s solution, 1 M NaCl, 10 mM EDTA, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA (Wako, Osaka, Japan). Filters were washed twice with 2× SSC (0.3 M NaCl and 30 mM sodium citrate, pH 7.0) containing 0.1% SDS at 65°C for 30 min and once with 0.2× SSC containing 0.1% SDS at room temperature for 60 min. The dried filters were then exposed for 16 h at ~80°C to x-ray films (X-OMAT AR, Eastman Kodak, Rochester, NY). The plBluescript phagemids were excised from positive clones using R408 helper phage (Stratagene, La Jolla, CA).

RT-PCR

Total RNA extracts were prepared from normal BALB/c mouse brain, lung, liver, stomach, intestine, spleen, and kidney using Trizol reagent (Life Technologies, Gaithersburg, MD). Each cDNA was synthesized from 1 μg of total RNA treated with DNase I using Superscript II reverse transcriptase (Life Technologies) and random hexamer; 2.5% of this reaction product was used as a template for PCR. To confirm the integrity of each total RNA obtained, GAPDH mRNA was amplified from the same cDNA product was used as a template for PCR. To confirm the integrity of each total RNA obtained, GAPDH mRNA was amplified from the same cDNA preparation. The following primers were used: mCPN, mCPN1\(^*\) (5′-AT GCCAGACTGCTCCCTGAG-3′) and mCPN510; mouse proCPR, mCPR86′ and mCPR740′; and mouse GAPDH (5′-CATCACATTCTTCAAGA-3′ and 5′-TGTGTACATGGATGACCTTGGC-3′). DNA was amplified by PCR with AmpliTaq Gold DNA polymerase (Perkin–Elmer Applied Biosystems, Foster City, CA) for 30 cycles of 95°C for 30 s, 55°C and 0.1 mg/ml denatured salmon sperm DNA (Wako, Osaka, Japan). Filters were then exposed for 16 h at ~80°C to x-ray films (X-OMAT AR, Eastman Kodak, Rochester, NY). The plBluescript phagemids were excised from positive clones using R408 helper phage (Stratagene, La Jolla, CA).

Preparation of fresh serum and plasma samples to measure CP activity

BALB/c mice, 9–12 wk of age, were injected i.p. with 0.5 or 4 mg/kg of LPS (E. coli O111:B4) in sterile saline. They were anesthetized with pentobarbital before cardiac puncture at 0, 1, 12, 24, 36, and 48 h after LPS injection. Each blood sample was immediately separated into a 1.5-ml plastic tube containing 10 μl of heparin and a silicone-coated glass tube kept on ice. The sample in the plastic tube was immediately mixed and then centrifuged at 8,000 rpm for 10 min at 4°C. This was used as the source of mouse plasma. The glass tube sample was incubated for 12 h at 4°C and then centrifuged at 3,000 rpm for 15 min at 4°C. This was used as the source of mouse serum. Results were obtained from three mice at each time point.

Measurement of CP activity

CP activity was determined using hippuryl-L-arginine (Sigma) as a synthetic substrate. The amount of hippuric acid generated by the enzyme was determined by a method of a liquid chromatography method described previously (18). For concentrated culture medium, 20 μl of each sample and 40 μl of 30 mM hippuryl-L-arginine in 50 mM HEPES (pH 8.2) as the substrate solution were mixed with or without 20 μl of 1 mg/ml trypsin solution (Sigma) and then incubated at 37°C for 45 min. With normal fresh serum and plasma, the sample volume was 10 μl. After incubation, 20 μl of 2.5 M HCl was added to stop the reaction enzyme. After incubation, 300 μl of ethanol, the top 200 μl was removed and evaporated, and the residue was dissolved in 200 μl of double-distilled water. The OD\(_{235}\) of the reaction product (hippuric acid) was compared with that of dilutions of a standard solution of hippuric acid. When the potato carboxypeptidase inhibitor (PCI, Calbiochem, La Jolla, CA) was used, each sample was incubated with 1 μl of PCI solution (adjusted to the appropriate concentration with 50 mM Tris buffer, pH 7.5) for >5 min before measurement of CP activity.

Results

Cloning of mouse proCPR and mouse CPN cDNAs

Based on several partial mouse sequences similar to those of human plasma procarboxypeptidase B (proCPR) and the human CPN active subunit acquired from the mouse EST database, two sets of primers for each enzyme were synthesized. Using these primers, we performed PCR amplification on cDNA that had been reverse transcribed from mouse liver total RNA. Probes were then made of the PCR products. These probes were labeled with [α-\(^32\)P]dCTP and used to screen ~1,000,000 plaques from the mouse liver cDNA library. The screening identified two clones containing a full-length cDNA of either the mouse homologue of human proCPR or the mouse homologue of the active subunit of mouse proCPR.
human CPN. We tentatively designated the isolated cDNAs as mouse proCPR and mouse CPN, respectively. These were completely sequenced using both universal primers and specific internal primers of our own design. The DNA sequences of these clones are shown in Fig. 1. Sequence analysis of the mouse proCPR clone revealed an insert of 1432 bp with an open reading frame of 1266 bp coding for a protein of 422 aa (Fig. 1A). The 3′-untranslated sequence includes the canonical polyadenylation signal, AATAAA, 33 bp upstream from the poly(A) tail. The NH2-terminal of 21 aa probably represents a portion of the signal peptide as determined by the weight-matrix method (19). The deduced protein sequence for mouse proCPR has 84% identity to human proCPR. Based on analysis of human proCPR, mouse proCPR appears to consist of a 92-aa activation peptide and a 309-aa mature enzyme. Four N-linked glycosylation sites (Asn-X-Ser/Thr) are probably present in the activation peptide as in human proCPR. From the amino acid sequence, two glycosylation sites were predicted for mouse CPN.

**FIGURE 1.** Nucleotide sequences of mouse proCPR (A) and mouse CPN (B) cDNAs and their deduced amino acid sequences. A. Nucleotides are numbered from the Met initiation codon. The first arrowhead indicates the signal peptidase cleavage site, and others indicate trypsin cleavage sites deduced from human plasma proCPB (proCPR) sequence analysis (11). The polyadenylation site is underlined. Residues implicated in zinc (circles) and substrate (squares) binding previously determined for human proCPR are indicated. Predicted N-glycosylation sites are shown by asterisks. B. The arrowhead indicates the signal peptidase cleavage site. The other symbols are the same in A.
Transient expression and characterization of mouse proCPR and mouse CPN

We confirmed that the products of our isolated genes have the same properties as human proCPR and CPN. Since we detected little CP activity in the culture medium of COS-7 cells alone (data not shown), we used these cells as host cells for cDNA transfection. COS-7 cells were transiently transfected with the following expression vectors: pDR2EF1 alone as a control, m-proCPR/pDR2EF1, or m-CPN/pDR2EF1. Because mouse proCPR and CPN cDNAs both harbor a portion of the signal peptide, it was predicted that the recombinant COS-7 cells would secrete the gene products. Therefore, serum-free medium was used to measure the CP activity of recombinant proteins secreted from the transfected cells, since its use ensured that there would be little interference from FBS CPs. Enzyme activity was determined from the amount of hippuric acid generated by the cleavage of hippuryl-L-arginine as substrate. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate/min at 37°C. Data are the mean ± SD of triplicate samples.

Expression of mouse proCPR and CPN mRNAs in various normal mouse tissues

By means of RT-PCR, we detected mRNA for mouse proCPR and CPN in all tissues studied except mouse brain CPN or mouse spleen proCPR (Fig. 3A). A comparison of mouse proCPR and CPN expression showed that they shared almost the same pattern, in that liver and stomach showed stronger expression than did other tissues. Northern blot analysis of mouse CPN mRNA showed only a 1.5-kb species that was expressed abundantly in the liver and was undetected in other tissues. This 1.5-kb Northern blot species corresponded to the length of our isolated gene.

Expression of proCPR and CPN mRNAs in liver of LPS-inoculated mice

We examined how LPS influences mouse proCPR and CPN gene expression in murine liver, which is the major site of synthesis. As shown in Fig. 4, i.p. administration of LPS (0.5 mg/kg) caused an up-regulation of mouse proCPR gene expression, which reached a maximal level at 12 h after LPS treatment. The level at 24 h was still higher than that in the control. On the other hand, expression of both 1.6- and 1.9-kb mouse CPN mRNAs remained unchanged.
following LPS injection (0.5 mg/kg). A high dose (4 mg/kg) and a low dose (0.5 mg/kg) of LPS elicited the same response (data not shown). Furthermore, to examine whether the levels of expression of mouse proCPR and CPN mRNAs in various other tissues are affected by LPS treatment, we compared levels at 12 h after LPS or saline injection by Northern blot analysis. Northern blotting did not detect mouse proCPR mRNA in brain, lung, stomach, intestine, or kidney despite LPS treatment. However, LPS stimulation reduced transcription of mouse CPN mRNA in the stomach to half that in the untreated control, although it did not change the level in the kidney or lung (data not shown).

Kinetics of CP activity in fresh mouse serum and plasma after LPS stimulation

Human CPR was initially identified following the observation that the CP activity of fresh serum is higher than that of plasma (8), and we have now observed the same phenomenon in the mouse. The CP activity of fresh mouse serum was approximately twice that of plasma. This augmented activity decreased in a time-dependent manner at 37°C and returned to the plasma level within 1 h. Furthermore, PCI could lower this enhanced activity in a dose-dependent fashion, and activity was completely suppressed to the plasma level using 50 μg/ml of this inhibitor (data not shown). These results agree with the finding that PCI inhibits human CPR but not human CPN. Therefore, we determined that the CP activity in fresh serum and plasma of LPS-treated mice is representative of the CP activity of CPR plus CPN and of CPN alone, respectively. At 24 h following LPS administration, the CP activity of fresh serum was significantly increased to 5 times the plasma level (Fig. 5). The CP activity of serum did not rise above the plasma level in the presence of 100 μg/ml of PCI (preliminary results). The CP activity of fresh serum was reduced at 6 h following a transient increase at 1 h, but it increased again at 24 h to a level about 2.5 times higher than that in sera of untreated mice. The CPR activity, therefore, was 4 times greater than that in untreated mouse serum, as calculated by subtracting the plasma CP activity, corresponding to CPN activity, from the total activity in serum. ProCPR consumption during inflammation induced by LPS may be responsible for the decrease in serum CP activity observed in the early stage after injection. Similarly, plasma CP activity corresponding to CPN had decreased slightly at 6 and 12 h after LPS injection. This difference in activity between serum and plasma was the same whether mice were injected with 0.5 or 4 mg/kg of LPS.

To show that the enhanced CPR activity was not due to an increased efficiency in the conversion of proCPR to CPR during coagulation, the CP activity in plasma was also determined in the presence of trypsin. As expected, CP activity in the presence of trypsin was much higher in plasma from mice administered LPS 24 h earlier than in plasma from untreated mice. Furthermore, the enhanced CP activity in trypsin-treated plasma returned to the level of activity in untreated plasma in the presence of PCI, indicating that the increase in CP activity was due to CPR.

Discussion

In the present study we isolated mouse homologues of human proCPR cDNA and human CPN small active subunit cDNA. The deduced amino acid sequences revealed >80% homology between mice and humans. Substrate and zinc binding sites were conserved. Culture media of COS-7 cells transfected with these homologues had enzyme activities similar to those of the human enzymes, as mentioned below. The trypsin-treated culture medium of the proCPR transfectant had high CP activity, and that of the CPN transfectant also had significant carboxypeptidase activity without trypsin treatment. Furthermore, PCI inhibited the CP activity of mouse CPR as is seen with human CPR; therefore, we considered the isolated genes to be those of mouse proCPR and CPN, respectively. Northern blot analysis revealed that mouse CPN mRNA consists of two species of 1.6 and 1.9 kb. The mouse CPN cDNA that we isolated was regarded as the 1.9-kb species because it was more abundantly expressed than was the 1.6-kb species. It has been demonstrated that human proCPR and human CPN are synthesized in the liver (11, 20, 21). This organ appears to be the major site of synthesis in the mouse as well. Although mouse CPN mRNA expression was not affected by LPS, mouse proCPR mRNA expression steadily increased for at least 12 h after LPS treatment. It is conceivable that the CP activity inhibited by PCI of trypsin-treated plasma reflects the level of proCPR in plasma. Its value increased 2-fold at 24 h after LPS injection, suggesting that proCPR expression was up-regulated at the protein level in addition to the mRNA level. Furthermore the CPR activity (fresh serum CP activity minus the plasma CP activity) at 24 h after LPS stimulation increased 4-fold, whereas the plasma CP activity was essentially unaffected. This increase in CPR activity demonstrates that LPS induces activation of proCPR as well as enhanced expression, and CPN is not affected. These results indicate that
mouse proCPR is an acute phase protein, whereas mouse CPN is not. CPN may be required for homeostasis to remove terminal lysine and arginine from a variety of peptides. This was supported by the finding that CPN is related to the pathophysiology of chronic diseases such as chronic urticaria or asthma (7). An increase in CPR activity may facilitate rapid inactivation of inflammatory mediators such as anaphylatoxins and kinins generated at sites of Gram-negative bacterial infections and may consequently prevent septic shock. Based on these findings, it is conceivable that administration of proCPR could prevent death from septic shock.

However, several recent reports suggest that excess activation of proCPR may exacerbate a state of disseminated intravascular coagulation (DIC) resulting from a Gram-negative bacterial infection (14, 15, 22–27). Following activation by thrombin, proCPR, otherwise known as TAFI, exerts an antifibrinolytic effect by removing carboxyl-terminal lysines on fibrin, preventing plasminogen binding and activation (15). On the other hand, it has been established that CPN does not remove carboxyl-terminal lysine residues from the clot surface despite the preference for lysine residues rather than arginine residues (15, 26). Furthermore, according to the present study, LPS stimulation did not essentially alter CPN expression or consumption of CPN compared with proCPR following LPS injection (Fig. 5), suggesting that CPN may not be actively involved in the pathophysiology of DIC. Activated protein C specifically inactivates factors VIIIa and Va, two essential co-factors in the intrinsic coagulation pathway, thereby attenuating further thrombin formation and consequent activation of TAFI (proCPR) (22). Expression of pro C mRNA in the mouse, mainly in liver, dropped to a minimal level at 8 h after LPS treatment (24). Analysis of serial plasma samples from patients with DIC revealed that the activity of protein C and its Ag decreases progressively during the initial stages of DIC and remains at a low level for 24–48 h (25). Our studies with a murine model demonstrated that proCPR increases and reaches a maximum level 24 h after LPS treatment. Therefore, endotoxin induces down-regulation of protein C and up-regulation of proCPR. Reduced protein C activity favors the intrinsic pathway of coagulation, with a relative increase in thrombin formation and a consequent activation of proCPR, which is up-regulated following LPS stimulation. Endotoxin thereby causes the hypercoagulability and hypofibrinolysis seen in DIC. Thrombomodulin (TM) is expressed on the surface of endothelial cells, and soluble functional proteolytic fragments of TM are also present in circulating plasma. It has been reported that the thrombin/TM complex activates TAFI (proCPR) 1250 times more rapidly than does free thrombin (14) and that plasma TM, including soluble TM fragments, inhibits fibrinolysis in a dose-dependent manner via activation of plasma proCPR (= proCPR) (26). Plasma levels of soluble TM fragments are elevated in patients with DIC (27). These findings suggest that high concentrations of these fragments in patients with DIC facilitate the activation of proCPR.

Our results suggest that CPR may actually play two important roles in vivo: first, as an inactivator of inflammatory mediators to prevent excessive inflammation, and secondly, as an inhibitor of fibrinolysis. Therefore, although a higher level of CPR activity may reduce susceptibility to shock, an increase in its activity could also facilitate DIC by preventing fibrinolysis. Further research on CPR both in vitro and in vivo should provide insight into its important dual function as a regulatory enzyme.

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