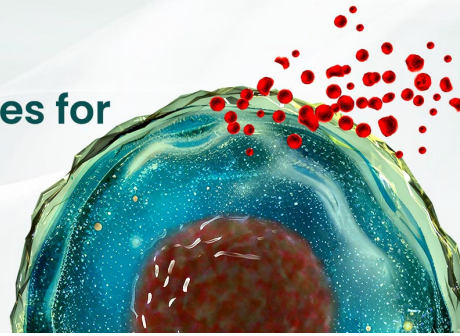




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Absence of Procarboxypeptidase R Induces Complement-Mediated Lethal Inflammation in Lipopolysaccharide-Primed Mice¹

Suzuka Asai,* Tomoo Sato,*[§] Toyohiro Tada,[†] Tomomi Miyamoto,[‡] Noriaki Kimbara,* Noboru Motoyama,[¶] Hidechika Okada,*[§] and Noriko Okada^{2*}

Carboxypeptidase R (CPR) is a heat-labile enzyme found in serum in addition to stable carboxypeptidase N. CPR cleaves the C-terminal basic amino acids, arginine and lysine, from inflammatory peptides such as complement C3a and C5a, bradykinin, and enkephalin. This enzyme is generated from procarboxypeptidase R (proCPR), also known as thrombin-activatable fibrinolysis inhibitor, following cleavage by proteolytic enzymes such as thrombin, plasmin, and trypsin. We generated proCPR-deficient mice by knocking out exons 4 and 5 of the proCPR gene, which are regarded as essential for CPR function. At LPS challenge, there was virtually no difference in lethality among proCPR^{+/+}, proCPR^{+/-}, and proCPR^{-/-} mice. However, challenge with cobra venom factor, which can activate and deplete almost all complement *in vivo*, induced a lethal effect on proCPR^{-/-} mice following LPS sensitization which up-regulates C5a receptor expression. In contrast, proCPR^{+/+} and proCPR^{+/-} mice were able to tolerate the cobra venom factor challenge with the limited dose (30 U). Although carboxypeptidase N plays a role in inactivation of inflammatory peptides *in vivo*, CPR may also be important in the regulation of hyperinflammation. *The Journal of Immunology*, 2004, 173: 4669–4674.

During inflammation, the levels of complement activation products C3a and C5a increase in plasma (1–5). C3a and C5a not only have potent anaphylactic effects, but they can also cause cell chemotaxis, adhesion, and aggregation via receptors on leukocytes or platelets, which release other inflammatory mediators. Basic carboxypeptidases (CPs)³ are present in plasma and cleave C-terminal arginine and lysine residues from various peptides including inflammatory mediators (6–9). Carboxypeptidase N (CPN) is present in an active form, whereas carboxypeptidase R (CPR), also known as plasma carboxypeptidase B (CPB), carboxypeptidase U (CPU), or activated thrombin-activatable fibrinolysis inhibitor, exists in a precursor form (procarboxypeptidase R (proCPR)). CPR is generated from its zymogene (proCPR) by thrombin (10), thrombin/thrombomodulin complex (11), or plasmin (10) during coagulation or in response to inflammation (12, 13). Recently, it was demonstrated that CPR inactivated C5a octapeptide more rapidly than did CPN (14), and another study showed that elastase, an enzyme secreted from neutrophils, could convert proCPR to CPR (15). Elastase released from neutrophils accumulates at the site of infection, and might

play a role in suppression of inflammation by generating CPR, which inactivates inflammatory peptides such as C5a and bradykinin. *In vivo* studies showed that in response to inflammation induced by injection of LPS in mice and rats, the mRNA level of proCPR but not that of CPN increased significantly (12, 13). Furthermore, the administration of a lethal dose of LPS to rats exhausted CPR activity in serum (12). These studies suggested that CPR may have an important role in preventing hyperinflammation induced by C5a or other inflammatory peptides. To obtain more *in vivo* evidence of this CPR function, we generated proCPR-deficient mice to evaluate the role of CPR in regulation of the inflammatory response.

Materials and Methods

Construction of the proCPR gene-targeting vector

The mouse proCPR gene was isolated by screening a mouse 129/SV λFIX II genomic library with mouse proCPR cDNA as a probe. A 23.2-kb *NotI* DNA fragment containing exons 3A–7 was subcloned into pBluescript II SK (–) vector (Stratagene, La Jolla, CA). A 4.3-kb *EcoRV-SalI* proCPR DNA fragment containing exon 6 from the above plasmid was subcloned into a pLoxNeoB-P1/R vector, which is composed of a phosphoglycerate kinase (PKG) promoter, a neomycin (neo) resistant gene, and a PGK poly(A) sequence. The resulting plasmid was used as a positive control for PCR detection of the wild-type allele. A 3.0-kb *BamHI* DNA region of the above 4.3-kb *EcoRV-SalI* fragment was removed from the plasmid. A 5.0-kb *EcoRI* proCPR DNA fragment containing exon 3B was inserted at the 3' end of the neo cassette. As a result, the neo cassette was flanked by 1.3 and 5.0 kb of homologous sequences. The 8.0-kb *BamHI-XhoI* fragment containing the neo cassette and the homologous sequences was subcloned into the *BamHI-XhoI*-restricted pPGKtk *Apal/R* vector composed of a PGK promoter, an HSV thymidine kinase gene, and a PGK poly(A) sequence. The construction of the targeting vector is shown in Fig. 1.

Generation of proCPR-deficient mice

The *BamHI*-linearized targeting vector was introduced by electroporation into E14 embryonic stem (ES) cells derived from the murine 129/Ola strain with Gene Pulser (two pulses of 300 V and 125 μF; Bio-Rad, Hercules, CA). Selection was started 24 h later with G418 (0.3 mg/ml) and ganciclovir (2 μM), and cells were subsequently cultured for 7 days. Correctly

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³ Abbreviations used in this paper: CP, carboxypeptidase; proCPR, procarboxypeptidase R; neo, neomycin; ES, embryonic stem; CVF, cobra venom factor; T-TM, thrombin and thrombomodulin complex; PGK, phosphoglycerate kinase.

targeted ES clones were identified by PCR with a pair of primers specific to the proCPR gene-flanking sequence of the targeting construct (5'-CCA AAGTAAAACCTCCTCACCAAG-3') and the pLoxNeoB-P1/R vector (5'-GCTATACGAAGTTATTAGGTCCTCG-3'), respectively, and were confirmed by Southern blot analysis. Germline chimera were generated by Japan SLC (Hamamatsu, Japan). Chimeric mice were produced by micro-injecting ES cells from four different targeted clones into C57BL/6 blastocysts. Male chimeras were mated to BALB/c females and germline transmission of the mutant proCPR gene in all progeny was confirmed by PCR and Southern blot analysis of tail DNA. Germline transmission was obtained from one of four clones injected. Brother-sister mating was conducted to generate homozygous proCPR-deficient mutants. The background of their progeny was 129/Ola, BALB/c, and C57BL/6. The experimental protocol was approved by the Animal Studies Committee of Nagoya City University Graduate School of Medical Sciences.

Analysis of genotypes

Genomic DNA was isolated from mouse tail and used for genotypic PCR analysis. PCR products from the wild-type allele derived from primers exon 5⁺ (GCTTCTGTTTGTGGTTCATAGGCTAC) and SA⁻ (GAAT GTTCTCCAGCAGGTGAAATCA), and from the mutant allele derived from primers neo/4.3 (368⁺) (GCTATACGAAGTTATTAGGTCCTCG) and neo/4.3 (1775⁻) (CCAAAGTAAAACCTCCTCACCAAG), were both 1.4 kb. The DNA fragment was amplified with *rTaq* DNA polymerase (Toyobo, Osaka, Japan) for 32 cycles of 94°C for 1 min, 60°C for 90 s, and 72°C for 90 s, followed by 72°C for 10 min. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

RT-PCR

Total RNA was extracted from mouse liver using TRIzol reagent (Invitrogen Life Technologies, Gaithersburg, MD). cDNA was synthesized from total RNA with Superscript II reverse transcriptase (Invitrogen Life Technologies, San Diego, CA) and used as a template for PCR. The following primers were used; exons 2–3, ex2S (CTGCTCTTCCAAGAACCCTCA) and ex3A (ACACTGTGACATCAGACGC); exons 1–6, ex1S (GCT TCGCCTTTCAGAGTGG) and ex6A (ACGTGTAGTCATAGCCATC CACG); exons 4–5, ex4S (CTGAACAGCATCTGACA) and ex5A (GCAGGTGAAATCCATTCTCTGGC); exons 6–10, ex6S (GTGGATG GCTATGACTACAGT) and ex10A (TGATATCTCAGGCAGC). DNA was amplified with *rTaq* DNA polymerase for 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. PCR products were analyzed by electrophoresis on 2% agarose gels.

Preparation of fresh sera for measurement of CP activity

ProCPR^{+/+}, proCPR^{+/-}, and proCPR^{-/-} mice, 8–12 wk of age, were anesthetized with pentobarbital and blood was collected by venipuncture of the inferior vena cava. The blood sample was immediately put into a 1.5-ml Eppendorf tube on ice and incubated overnight at 4°C before centrifugation at 3000 rpm for 15 min at 4°C to remove clots. The supernatant was used as the source of serum for determination of CP activity.

Determination of CP activity

CP activity was determined by means of a colorimetric assay using hippuryl-L-arginine (Peptide Institute, Osaka, Japan) as a synthetic substrate (16, 17). In fresh sera, we found CP activity of the CPR generated during coagulation in addition to that of CPN, which is constantly present in the active form in plasma. Total CP activity in sera was determined before or after preincubation at 37°C for 1 h, conditions which inactivate CPR. All sera were diluted 1/10 in 50 mM Tris-HCl buffer (pH 8.0) before using. In fresh sera, an appreciable amount of unactivated proCPR remained. To convert all proCPR to CPR, thrombin and thrombomodulin complexes (T-TM) were added for determination of total proCPR as follows. For determination of both proCPR and CPN activities, sera were diluted 1/10 in 50 mM Tris-HCl (pH 8.0) containing 2 NIH U/ml thrombin (Nihon Pharmaceutical, Tokyo, Japan), 1.4 μg/ml thrombomodulin (a generous gift from A. Kasei, Tokyo, Japan), and 4 mM CaCl₂ (each quantity represented the final concentration), and incubated for 20 min at room temperature. Ten microliters of these samples were mixed with 5 μl of 50 mM hippuryl-L-arginine in 50 mM HEPES (pH 8.2) as the substrate solution before incubation for 1 h at room temperature. After the incubation, 100 μM of 0.25 M phosphate buffer (pH 8.3) and 75 μM 3% cyanuric chloride in 1,4 dioxane were added to the mixtures which were then stirred for 20 s on a vortex mixer and centrifuged at 5,000 rpm for 10 min at 4°C. A 100-μl aliquot of the supernatant of each tube was transferred to a 96-well microtiter plate for measurement of absorbance at 405 nm. Each value was

compared with a hippuric acid standard curve and the CP activity of each serum sample was then determined as the amount of hippuric acid produced within 1 h.

Histopathology

ProCPR^{+/+} and proCPR^{-/-} mice, 8–12 wk of age, were killed under anesthesia with pentobarbital. Samples of tissues were immediately fixed in 10% phosphate-buffered formalin, then dehydrated in ethanol, cleared in xylene, and embedded in paraffin. The treated tissues were as follows: brain, heart, lungs, liver, kidneys, intestines, stomach, esophagus, spleen, salivary glands, seminal vesicles, testes, thymus, urinary bladder, trachea, adrenal glands, pancreas, and skeletal muscle. Sections were cut at 3-μm thickness and subjected to H&E staining.

Inflammatory model using LPS injection

For the acute inflammatory model, mice were injected i.p. with 15 mg/kg body weight of LPS (*Escherichia coli* O111:B4, Sigma-Aldrich, St. Louis, MO) dissolved in sterile saline. For the chronic inflammatory model, mice were injected i.p. with 3 mg/kg LPS in sterile saline every 24 h for 1 wk (seven times).

Complement activation model using cobra venom factor (CVF)

CVF was purified from lyophilized cobra venom (*Naja naja*; Sigma-Aldrich) as described elsewhere (16). Each mouse was injected i.p. with 100 U of CVF or injected i.v. into the tail vein with 200 U of CVF.

Complement activation model using C5aR up-regulation

For up-regulation of C5aR, 5 mg/kg LPS in sterile saline were injected i.v. into the tail (18). After 6 h, CVF was injected i.p. at a dose of 30 U/mouse. As a control group, some mice were not injected with CVF.

Guinea pig skin test for anaphylatoxin activity

The mouse serum was incubated with 1 mg/ml zymosan A (Sigma-Aldrich) at 37°C for 1 h and centrifuged at 1000 rpm for 5 min to remove this agent. As a control, the mouse serum was incubated without zymosan A using the same conditions. Guinea pigs were injected i.v. with 4 mg of Evans blue (Nacalai Tesque, Kyoto, Japan) 30 min before intradermal injection of 50 μl of mouse serum. After 1 h, guinea pigs were killed by stunning and cervical dislocation before removal of the skin. The blue spots were evaluated from the inner surface of the skin.

Results

Analysis of genotypes

Genomic DNA from mouse tails was used for genotyping by PCR. One of the primers used to detect the wild-type allele matched sequences within exon 5 of proCPR and the other matched that within the intron preceding exon 6 of proCPR. Therefore, the bands synthesized with these primers were detected only with genomic DNA of proCPR^{+/+} and proCPR^{+/-} mice. In contrast, both of the primers for detecting the mutant allele matched the sequences included in the neo vector sequences, and the bands were synthesized with only the genomic DNA of proCPR^{-/-} and proCPR^{+/-} mice (Fig. 1b).

RT-PCR with liver mRNA

To confirm the deficiency of proCPR mRNA, RT-PCR was performed with four sets of primers to detect four positions on the mRNA isolated from the liver. All of the PCR products were full length for the mRNA from the proCPR^{+/+} mouse (Fig. 2). In contrast, with the mRNA of the proCPR^{-/-} mouse, the sizes of the PCR product of exons 1–6 were shorter by ~200 bp than those of proCPR^{+/+}, although those of exons 2–3 and 6–10 were the same as those of the proCPR^{+/+} mouse. The 200 nucleotide bp length was consistent with the length of exons 4 and 5 coding for proCPR. A band for exons 4–5 was not detected with mRNA isolated from the proCPR^{-/-} mouse. With mRNA from proCPR^{+/-} mice, the bands detected on the agarose gel consisted of all the sizes found with both proCPR^{+/+} and proCPR^{-/-} mice. This confirmed generation of the proCPR^{+/-} and proCPR^{-/-} mice. ProCPR synthesized in the proCPR^{-/-} mice is defective in the region encoded by

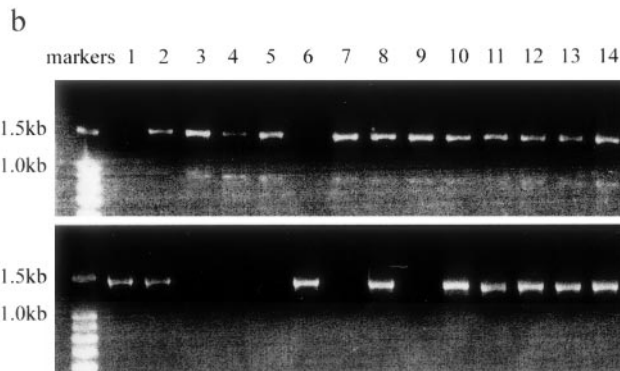
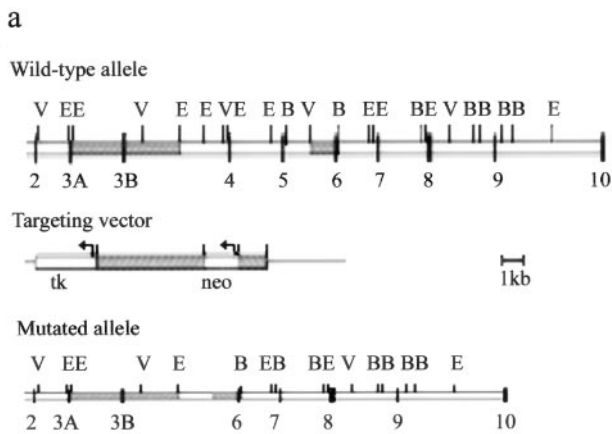


FIGURE 1. *a*, Targeting strategy to disrupt the murine proCPR gene. Restriction maps depict the wild-type allele (*top*), targeting vector (*middle*), and the mutated allele (*bottom*). Exons 4 and 5 encoding some of the zinc and substrate binding sites of proCPR were replaced with a neomycin resistance gene (*neo*). The shaded region represents the 5' and 3' flanks used for homologous recombination. Exons are represented by black boxes. B, E, and V indicate *Bam*HI, *Eco*RI, and *Eco*RV sites. *b*, The genomic DNA isolated from mouse tails were used for genotypic PCR analysis. The PCR products from the wild-type allele are shown in the *upper panel* and those from the mutant allele are shown below. Both bands are ~1400 bp. proCPR^{+/+}, proCPR^{+/-}, and proCPR^{-/-} mice were nos. 3, 4, 5, 7, and 9, nos. 2, 8, and 10–14, and nos. 1 and 6, respectively.

exons 4 and 5, which includes the genes for two of three zinc-binding sites and one of nine substrate-binding sites in humans (10).

Ratios of the three genotypes among generational offspring

We generated 129/B6/BALB/c (129/Ola, C57BL/6, and BALB/c) proCPR-deficient mice. By the mating of male 129/B6 (129/Ola and C57BL/6) chimeras and female wild-type BALB/c, we obtained one proCPR^{+/-} F₁ mouse. This proCPR^{+/-} mouse was mated with other wild-type littermates, and proCPR^{+/+} and proCPR^{+/-} F₂ offspring with the strain backgrounds of 129/B6/BALB/c were obtained. ProCPR^{+/+}, proCPR^{+/-}, and proCPR^{-/-} F₃ mice were generated by the mating of proCPR^{+/-} F₂ mice. However, as shown in Table I, proCPR^{-/-} F₃ mice represented only 14 of 124, and proCPR^{-/-} F₄ mice represented 9 of 138. The ratios of these offspring did not conform to Mendel's laws.

CP activity of sera from proCPR-deficient mice

The CP activity of sera from a proCPR^{-/-} mouse and a proCPR^{+/-} mouse was determined and compared with that of a proCPR^{+/+} mouse. Because only a portion of proCPR is activated

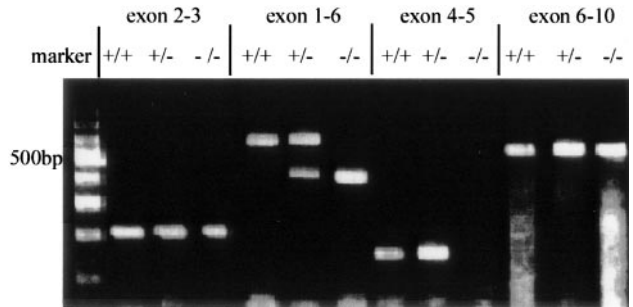


FIGURE 2. RT-PCR analysis of liver mRNA. All of the PCR products were obtained in full length with the mRNA from proCPR^{+/+} and proCPR^{+/-}. In contrast, there was no product using mRNA from proCPR^{-/-} lacking exons 4–5. The product obtained with mRNA from proCPR^{-/-} and one of two obtained with that of proCPR^{+/-} corresponding to exons 1–6 were shorter by ~200 bp than that of proCPR^{+/+} (full length). The 200-bp nucleotide length was consistent with the length of exons 4 and 5 coding for proCPR.

to CPR during coagulation, the addition of T-TM and Ca²⁺ can be used to activate the remaining proCPR (17, 19). In this experiment, the serum was incubated with T-TM and Ca²⁺ for 20 min at room temperature and then used to determine the total proCPR and CPN activity. The half-lives of CPR in sera of humans and other animals tested were within 6.3–16 min at 37°C (6, 17, 19). Mouse CPR was also labile at 37°C. Therefore, serum incubated for 1 h at 37°C was used to determine CPN activity, because almost all CPR would have been inactivated during the incubation. The CPR activities of proCPR^{+/+}, proCPR^{+/-}, and proCPR^{-/-} mice were 12.48 ± 1.73 nmol/h (n = 7), 5.03 ± 0.30 nmol/h (n = 8), and 0.60 ± 0.17 nmol/h (n = 12), respectively, and CPN activities were 4.79 ± 0.35 nmol/h (n = 7), 5.01 ± 0.16 nmol/h (n = 8), and 5.00 ± 0.13 nmol/h (n = 12), respectively (Fig. 3).

Histopathology

Possible morphological abnormality was observed in the kidney. The epithelial cells lining the inner surface of most of the renal Bowman's capsules of the proCPR^{-/-} mouse kidney, many of which were flat in the proCPR^{+/+} mice, showed a cuboidal shape continuing to the proximal urinary tubular cells (Fig. 4). This change was seen only in some renal glomeruli of proCPR^{+/+} mice (n = 5), however, proCPR^{-/-} mice (n = 4) showed a significant increase in the number of changes; the mean (mean ± SD) percentages of glomeruli with this change were 73 ± 7.5% in proCPR^{-/-} mice, and 24 ± 3.5% in proCPR^{+/+} mice. Statistical analysis using the Mann-Whitney U test showed significant difference between the two groups (p = 0.0143).

Inflammatory models induced by LPS injection

In preliminary experiments, a portion of proCPR^{+/+} mice survived the i.p. challenge with 15 mg/kg LPS although none survived challenge with 30 mg/kg LPS. Therefore, we used 15 mg/kg LPS to demonstrate increased susceptibility to LPS in proCPR^{-/-} mice. However, some of the proCPR^{-/-} mice survived the LPS

Table I. Genotypes of F₃ and F₄ offspring of proCPR^{+/-} mating

Genotype		+/+	+/-	-/-
F ₃	Number of mice	43	67	14
	Percentage of total (%)	35	54	11
F ₄	Number of mice	44	85	9
	Percentage of total (%)	32	62	6

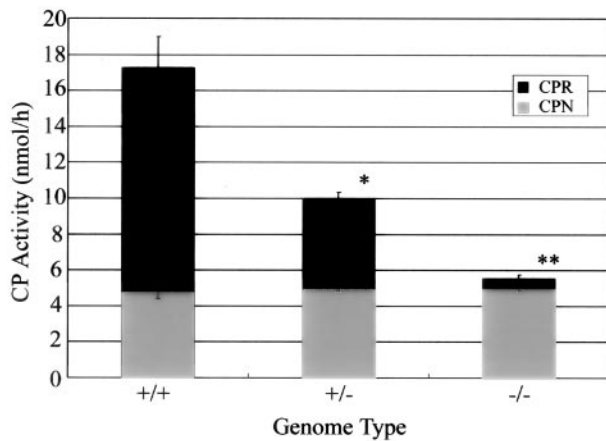


FIGURE 3. CPR activities in all mice groups. Sera were collected from proCPR^{+/+}, proCPR^{+/-}, and proCPR^{-/-} mice and their CP activities were determined. CPR activities per 1 μ l of serum were 12.48 \pm 1.73 nmol/h, 5.03 \pm 0.30 nmol/h, and 0.60 \pm 0.17 nmol/h, respectively (*, $p < 0.001$, **, $p < 0.0001$; Student's t test), and the CPN activities per 1 μ l of sera were 4.79 \pm 0.35 nmol/h, 5.01 \pm 0.16 nmol/h, and 5.00 \pm 0.13 nmol/h, respectively. Each point represents the mean \pm SE ($n = 7-12$).

challenge as did other types of mice. (Table II). For a chronic inflammation model, a lower concentration of LPS (3 mg/kg) was injected i.p. every 24 h for 1 wk (seven times). After the first injection, some mice in all groups died, and additional deaths occurred from the second to fifth injections (Fig. 5). However, the remaining mice recovered and lived for at least 1 wk after the final LPS injection. From this data, no difference was noted between the proCPR^{-/-} group and any other group of mice.

Complement activation model obtained with CVF following LPS sensitization

As CVF is a strong activator of the alternative complement pathway (20), its injection induces an enhanced production of anaphylatoxins C3a and C5a. Because CVF injection did not result in serious symptoms under normal conditions, we hypothesized that CPR could play an important role in inactivation of these anaphylatoxins in the acute phase. However, i.v. injection of CVF even at a high dose (200 U) did not induce lethal shock in proCPR^{-/-}

mice (Table III). It was reported that an i.v. injection of LPS induced the up-regulation of C5aR in mice and rats (18, 21). Furthermore, LPS sensitization of rats induced lethal shock with i.v. injection of a mAb to Crry, a membrane inhibitor of C (22). Therefore, we injected CVF i.p. 6 h after i.v. injection of LPS (5 mg/kg). In preliminary experiments, sensitization of mice with 5 mg/kg LPS rendered the mice, including proCPR^{+/+} mice, susceptible to lethal challenge with 200 U of CVF and proCPR^{+/-} mice susceptible to challenge with 50 U of CVF. Therefore, we used 30 U of CVF to demonstrate the difference in the susceptibility between the proCPR^{-/-} mice and other mice following LPS sensitization (Table III). The survival rate of proCPR^{+/+} and proCPR^{+/-} mice 24 h after the CVF injection was 100% and only one proCPR^{+/-} mouse died 3 days later. In contrast, 6 of 10 proCPR^{-/-} mice died within 24 h of CVF injection.

Inflammatory activity of proCPR^{-/-} mice sera in guinea pig skin

Local inflammation in the skin of guinea pig induced by inoculation of mouse serum was visualized with exudation of Evans blue injected i.v. (Fig. 6). Exudation only occurred where proCPR^{-/-} serum was inoculated, and the lesion was apparently stronger at sites inoculated with proCPR^{-/-} mouse serum treated with zymosan A. However, proCPR^{+/+} serum induced no exudation even after treatment with zymosan A. Repeated experiments gave the same result.

Discussion

The proenzyme of CPR (proCPR) is synthesized in the liver (10, 12, 13) as is the case with that of CPN (23, 24). Human CPR has nine substrate-binding sites and three zinc-binding sites (10), and of these, one substrate-binding and two zinc-binding sites are encoded by the exon 5 region. Therefore, we generated proCPR-deficient mice by knocking out the portion of the gene containing exons 4 and 5. Results of carrying out RT-PCR with total RNA prepared from the liver suggested that proCPR synthesized in the liver of proCPR^{-/-} mice would be an incomplete peptide. CPR activity in sera of proCPR^{+/-} mice was \sim 30% that of proCPR^{+/+} mice, whereas proCPR^{-/-} mice had little CPR activity at a level which was 3% that of proCPR^{+/+} mice. It remains to be determined whether sera of mice harboring the mutant proCPR gene

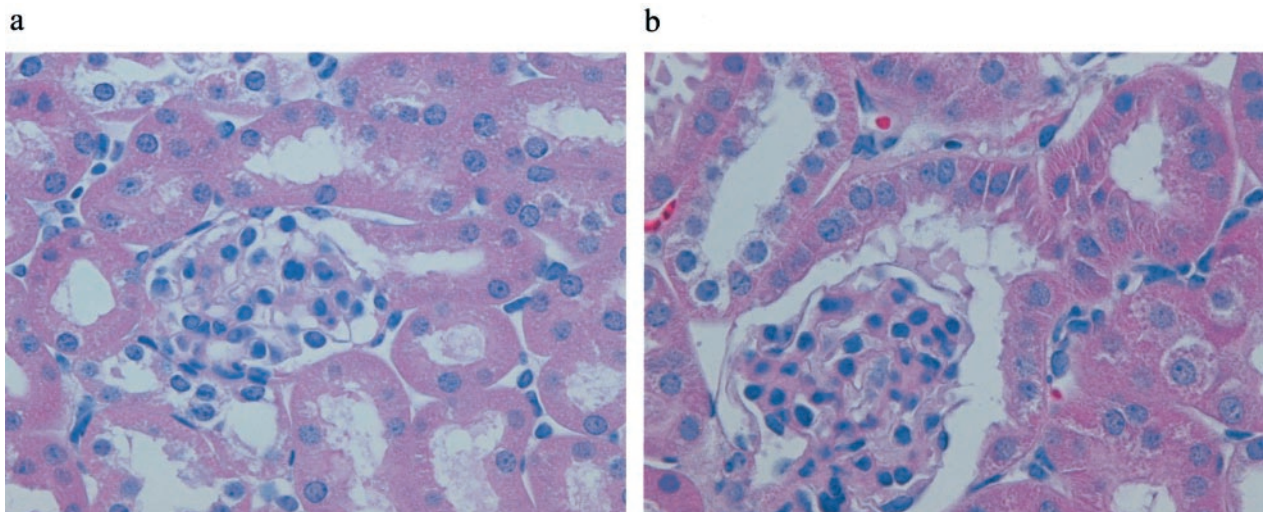


FIGURE 4. Histological features of Bowman's capsule of kidneys of mice. Many cells of Bowman's parietal epithelium of the proCPR^{+/+} mouse are flat in shape (a). In contrast, most epithelial cells lining the inner surface of Bowman's capsule of the proCPR^{-/-} mouse are cuboidal extending to the proximal urinary tubular cells (b).

Table II. Effect of LPS on survival rate

Genotype	Survival	Total	Rate (%)
+/+	6	9	67
+/-	7	10	70
-/-	7	9	78

deficient in exons 4 and 5 could retain a slight amount of CPR activity. Although it was reported that CPN is stable at 37°C for at least 25 min (6, 19), murine CPN activity might have been impaired during the 1-h incubation at 37°C performed to inactivate CPR completely. If this were the case, CPN activity in fresh serum of proCPR^{-/-} mice would be slightly higher than that in serum incubated for 1 h at 37°C.

Thrombin-activatable fibrinolysis inhibitor (proCPR)-deficient mice generated from 129/Sv and C57BL/6 (25) were reported to have delivered the Mendelian ratio with the heterozygote (+/-) intercross. However, although the proCPR-deficient mice produced here developed normally, the genomic ratios of F₃ and F₄ offspring of proCPR^{+/-} intercrosses were not in accordance with Mendel's laws. Disaccordances for F₃ and F₄ offspring were $p < 0.001$ and $p < 0.00001$ using the χ^2 goodness-of-fit test. The proCPR-deficient mice used in this study have a background of 120/Ola, C57BL/6, and BALB/c. Therefore, the BALB/c background might be related to the suppressed number of proCPR^{-/-} offspring. ProCPR might have a role in protecting the fetus in early ontogeny from some stress affecting mice with a BALB/c background.

Some cuboidal transformation of epithelial cells lining Bowman's capsules is normally seen in kidneys of mice (26) and humans (27), but in the present study, the incidence of this was significantly higher as seen from the increased number of affected glomeruli in the proCPR^{-/-} mice compared with the control mice. It could be hypothesized that these cuboidal epithelial cells might aid in some active transport process such as reabsorption of protein (28), but the pathological significance of this change is not well understood. These cuboidal cells were similar to those of proximal convoluted tubules, and might therefore be a metaplasia of Bowman's parietal epithelium (29). In contrast, this epithelial morphology had some resemblance to fetal glomerular structure, and it is possible that a disturbance in histological differentiation was induced in the proCPR^{-/-} mice. Because the mice of 129 background could have certain abnormalities, further analysis is remaining to determine whether the morphological abnormality in the kidney could be a direct consequence of the gene targeting, and whether the morphological abnormality might induce functional disturbance.

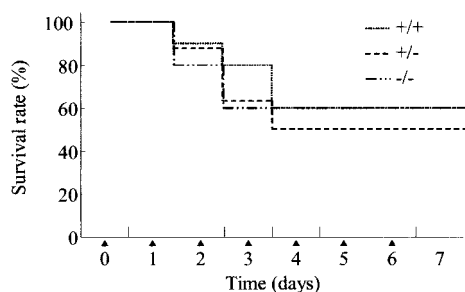


FIGURE 5. Effect of low concentration of LPS. Survival rates of mice were recorded after every injection of LPS (3 mg/kg). LPS dissolved in saline was injected i.p. every 24 h for 1 wk (▲).

Table III. Effect of CVF injection on survival rate

	Genotype	Survival	Total	Rate (%)
LPS (5 mg/kg) i.v.	+/+	5	5	100
	+/-			
	-/-	6	6	100
CVF (100 U) i.p.	+/+	3	3	100
	+/-	2	2	100
	-/-	5	5	100
CVF (200 U) i.v.	+/+	12	12	100
	+/-	10	10	100
	-/-	10	10	100
LPS (5 mg/kg) i.v. ^a + CVF (30 U) i.p.	+/+	7 ^b	7	100
	+/-	7 ^{b,c}	7	100
	-/-	4 ^{b,d}	10	40

^a The interval between LPS injection and CVF injection was 6 h.

^b Difference between proCPR^{-/-} group and other groups is significant ($p < 0.05$; Cochran method).

^c One mouse died 3 days later.

^d Six mice died within 24 h.

ProCPR has been established as an acute phase protein (12, 13) because of the up-regulation of mouse proCPR mRNA expression in the liver and proCPR protein in plasma after LPS injection. Therefore, proCPR-deficient mice might be more sensitive to LPS-induced inflammation. When we injected a sublethal dose of LPS into proCPR^{+/-} and proCPR^{-/-} mice, the survival rate was not different from that of proCPR^{+/+} mice. This indicated that LPS could induce cascades of inflammation independent of C5a and other inflammatory peptides which could be inactivated by CPR.

In a rat model, i.v. administration of mAb against Crry, a membrane inhibitor of C, induced lethal shock following LPS sensitization (22), although the mAb alone did not induce this serious outcome (30). This phenomenon might involve the increased expression of C5aR by LPS (18, 21, 31–33). Therefore, we sensitized mice with 5 mg/kg LPS 6 h before i.v. administration of CVF (20), which activates the alternative complement pathway so completely as to exhaust the total amount of complement in vivo. In preliminary experiments, mice sensitized with the LPS preparation hardly survived administration of 200 U of CVF (to proCPR^{+/+} mice) and 100 U of CVF (to proCPR^{+/-} mice), although 50 U of CVF

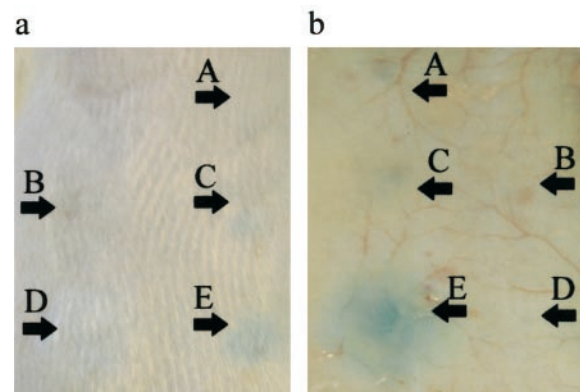


FIGURE 6. *a*, Guinea pig skin observed from the outer surface 1 h after the intradermal injection of 50 µl of mice sera. *b*, Reverse side of the skin. As a control, saline was injected at point A. ProCPR^{+/+} and proCPR^{-/-} sera were injected at points B and C, respectively. ProCPR^{+/+} and proCPR^{-/-} sera which were incubated with zymosan A were injected at points D and E, respectively. Significant Evans blue exudation from vascular sites (blue spots) was observed at E, and faintly at C from the over-surface. No spot was observed at the proCPR^{+/+} serum site even after treatment with zymosan A. Reverse side of the skin showed a strong blue spot at E and was scarcely detected elsewhere even at C.

killed all proCPR^{-/-} mice sensitized with LPS. In mice pretreated with LPS, C5aR had been up-regulated and the extensive generation of C5a could overcome restriction by CPR in proCPR^{+/+} mice. Therefore, we used 30 U of CVF to compare the sensitivity in proCPR^{+/+}, proCPR^{+/-}, and proCPR^{-/-} mice presensitized with LPS. We found that the injection of 30 U of CVF 6 h after i.v. injection of LPS induced lethality only in proCPR^{-/-} mice but not in proCPR^{+/+} or proCPR^{+/-} mice. This result suggests that CPR generated from proCPR in vivo plays a role in regulation of excessive inflammation due to inflammatory peptides such as C5a anaphylatoxin generated by C activation, which could be inactivated by CPR.

The presence of inflammatory peptides such as C5a in the serum of proCPR^{-/-} mice was demonstrated in the guinea pig skin by means of Evans blue exudation (34), and the exudation was strongly enhanced by preincubation of the serum with zymosan A that has a capacity to activate C. In contrast, exudation was not observed with the serum of proCPR^{+/+} and proCPR^{+/-} mice, even after treatment with zymosan A. This evidence indicates that CPR efficiently inactivates inflammatory peptides such as C5a in serum, and the absence of the enzyme delayed the inactivation although the deficient mouse serum contained CPN. This phenomenon could be explained by the finding that C5a octapeptide was efficiently inactivated by CPR but not by CPN (14).

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