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Targeted Disruption of the Gene Encoding the Murine Small Subunit of Carboxypeptidase N (CPN1) Causes Susceptibility to C5a Anaphylatoxin-Mediated Shock[1]

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Abstract

Carboxypeptidase N (CPN) is a plasma zinc metalloprotease, which consists of two enzymatically active small subunits (CPN1) and two large subunits (CPN2) that protect the protein from degradation. Historically, CPN has been implicated as a major regulator of inflammation by its enzymatic cleavage of functionally important arginine and lysine amino acids from potent phlogistic molecules, such as the complement anaphylatoxins C3a and C5a. Because of no known complete CPN deficiencies, the biological impact of CPN in vivo has been difficult to evaluate. Here, we report the generation of a mouse with complete CPN deficiency by targeted disruption of the CPN1 gene. $CPN1^{-/-}$ mice were hypersensitive to lethal anaphylactic shock due to acute complement activation by cobra venom factor. This hypersensitivity was completely resolved in $CPN1^{-/-}/C5aR^{-/-}$ but not in $CPN1^{-/-}/C3aR^{-/-}$ mice. Moreover, $CPN1^{-/-}$ mice given C5a i.v., but not C3a, experienced 100% mortality. This C5a-induced mortality was reduced to 20% when $CPN1^{-/-}$ mice were treated with an antihistamine before C5a challenge. These studies describe for the first time a complete deficiency of CPN and demonstrate 1) that CPN plays a requisite role in regulating the lethal effects of anaphylatoxin-mediated shock, 2) that these lethal effects are mediated predominantly by C5a-induced histamine release, and 3) that C3a does not contribute significantly to shock following acute complement activation.

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Disclosures

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Carboxypeptidase N (CPN)[3] is a zinc metalloprotease that cleaves lysine and arginine amino acid residues from the C terminus of biologically active peptides and proteins (reviewed in Refs. 1–3). CPN is a tetramer composed of two large subunits, designated CPN2 (83 M_r each), and two small subunits, designated CPN1 (52 M_r each). The small subunits contain the enzymatic active sites, and the large subunits protect the protein from degradation or filtration from the bloodstream (4). CPN is primarily expressed in the liver and is secreted into the bloodstream at a concentration of 30 μg/ml.

CPN has been demonstrated in vitro to cleave several biologically active molecules, which significantly decreases their physiological activity. In 1962, CPN was shown to inactivate bradykinin by cleaving its C-terminal arginine (5). Bradykinin is a 9-aa peptide that is produced in response to tissue injury, and this peptide can induce bronchoconstriction and hypotension and increase vascular permeability. CPN has also been shown to cleave and regulate other kinins, including kallidin (Lys-bradykinin) and Met-Lys-bradykinin (5). Bokisch and Muller-Eberhard (6) identified CPN as an inactivator of the complement anaphylatoxins C3a and C5a. Removal of the C-terminal arginine from these peptides reduces their biological activity considerably in that C3a-desArg can no longer bind to the C3a receptor, and C5a-desArg has significantly reduced affinity for the C5a receptor (reviewed in Ref. 7). C5a and C3a are 74- and 77-aa peptides, respectively, that mediate release of histamine from mast cells, contraction of smooth muscle, dilation of blood vessels, and chemotaxis of various myeloid cells. Most recently, CPN has also been shown to be a regulator of the chemokine stromal-derived factor-1α (SDF-1α) (8). SDF-1α that lacks the C-terminal lysine has a reduced capacity to stimulate B cell proliferation and chemotaxis compared with full-length SDF-1α (9).

There are no reported cases of complete deficiency of CPN in humans, and there are only two documented cases of partial CPN deficiency. The first case was a 65-year-old Caucasian man with 21% normal CPN activity and protein due to reduced synthesis of CPN (10, 11). This patient presented with chronic recurrent severe angioedema. Studies showed that inactivation of C3a and lysyl-bradykinin by his serum was significantly prolonged (10). During attacks of angioedema, the level of plasma histamine, but not serotonin or kinin activity, increased (10). This patient's CPN1 gene was identified as having a frameshift mutation in exon 1, which should result in a truncated protein, and a missense mutation in exon 3 in which a conserved glycine at residue 178 was replaced with an aspartic acid (12). The second reported case of CPN deficiency has not been as extensively studied as the first and was only documented in early 2008 (13). This female patient also developed angioedema, but unlike the first case who developed severe angioedema independent of diet or environmental factors, the second CPN deficient patient only presented with angioedema 2 years after starting on an angiotensin-converting enzyme inhibitor. Despite having reported very low CPN functional levels, her symptoms improved after stopping the angiotensin-converting enzyme inhibitor and being treated with H1 antihistamines. Because of the severe angioedema associated with partial CPN deficiency, because there are no

[3]Abbreviations used in this paper: CPN, carboxypeptidase N; SDF-1α, stromal-derived factor-1α; WT, wild type; ES cell, embryonic stem cell; FA, furylacryloyl; C3aR, C3a receptor; C5aR, C5a receptor; CVF, cobra venom factor; CPU, carboxypeptidase U; CPR, carboxypeptidase R; TAFI, thrombin activatable fibrinolysis inhibitor.

documented cases of total CPN deficiency, and because of the early expression of CPN1 in embryonic development (14), it has been speculated that CPN must fulfill a required biological function and that a complete absence of CPN may prove fatal. Therefore, to evaluate the biological importance of CPN in normal development as well as in states of inflammation and to delineate the contributions of CPN vs those of other inflammatory regulators, we have generated mice deficient in CPN by genetic targeting of the CPN1 gene.

Materials and Methods

Animals

Mice were maintained in a barrier animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all animal protocols were approved by the University of Texas Health Science Center at Houston Institutional Review Board. The mice used in these studies were crossed at least 10 generations onto the C57BL/6 background. C57BL/6 mice were used as wild-type (WT) controls, and the mice in these experiments were age- and sex-matched.

Generation of CPN1-deficient mice

A targeting vector was designed to replace exon 3 of the murine *cpn1* gene (15) with a neomycin-resistance gene from pKO SelectNeo V800 (Lexicon Genetics) (see Fig. 1*A*). The linearized targeting vector was electroporated into EZ-1 embryonic stem (ES) cells derived from 129S1/SvImJ mice (The Jackson Laboratory), and neomycin-resistant clones were selected. Two out of 360 targeted ES cell clones were identified by Southern blot analysis as described below. Both ES cell clones were microinjected into C57BL/6 blastocysts, and the resulting male chimeras were mated with C57BL/6 females to generate heterozygous F_1 offspring. All progeny were screened for germline transmission via Southern blot analysis as described below. Mice from only one clone were further backcrossed onto the C57BL/6 background so that all mice described in these studies are from the same original ES cell clone.

Southern blot and PCR analysis for genotyping

Genomic DNA was isolated from mouse tail snips and was used for genotyping by either Southern blot analysis or PCR analysis. A 387-bp PCR fragment encompassing all of exon 4 and part of intron 4 was used as a hybridization probe for Southern blotting. Restriction enzyme cutting of genomic DNA with *Eco*RI and subsequent hybridization with the above probe yielded a 5.5-kb fragment for the WT allele and a 3.7-kb fragment for the targeted allele. Three primers located in the following regions were used for PCR analysis of the *cpn1* gene: 1) the 5′ region of intron 3 (5′-CAACTCCAGCAGCAAGTCCTCAAT GACC-3′), which is missing in the targeted allele; 2) the *neo* cassette (5′-GCGCATGCTCCAGACTGCCTTG-3′); and 3) a more 3′ region of intron 3 (5′-GGTCGCCTAAGAGCACCAAAACACACA-3′), which is present in both the WT and targeted alleles. Primer pairs 1 and 3 yield a 736-bp PCR product for the WT allele, and primer pairs 2 and 3 yield a 488-bp PCR product for the targeted allele.

RT-PCR

Total RNA was isolated from mouse liver using RNA-Bee (Tel-Test). The Access RT-PCR System (Promega) was used to amplify both cDNA and DNA in the same tube using a two-enzyme system. The following primers were used for RT-PCR: 1) exon 2 (5′-GGAAGTCAAGTATGTGG GAAACATGCACGG-3′), 2) exon 3 (5′-ACCAACCAGATACCCAGA CATGTTTGGGCC-3′), 3) exon 4 (5′-GTCATCAGGTGTCGGGGAAT TGGAGGT-3′), and 4) exon 9 (5′-TACACCTGAGATGAGCTT CGCTTGAGTTGG-3′).

Determination of CPN activity in plasma

Plasma CPN activity was measured using a protocol modified from Camp-bell et al. (16). Briefly, 75 μl of citrated plasma from $CPN1^{+/+}$ and $CPN1^{-/-}$ mice was incubated with 30 μl of 0.5 mg/ml stock of human C3a (Complement Technology) or human recombinant C5a (Calbiochem) for 0 or 5 min at 37°C. Reactions were stopped by the addition of 150 μl of 5 N HCl, and the samples were then centrifuged at 12,000 relative centrifugal force for 5 min at room temperature to remove precipitates. Following centrifugation, 200 μl of each supernatant was transferred to fresh tubes, and 120 μl of 5 N NaOH and 100 μl of 1 M Tris (pH 7.5) were added to each tube. The samples were centrifuged again as above. The supernatants (400 μl) were filtered through 0.2-μm microfuge filters (Millipore) by centrifugation at 12,000 relative centrifugal force for 4 min. The samples were analyzed by Agilent 1100 reverse-phase HPLC (Agilent Technologies) on a Vydac C_{18} column (10 μm, 4.6 × 250 mm) (The Nest Group) using the following conditions: solvent A was water containing 0.1% trifluoroacetic acid, and solvent B was an acetonitrile/water mixture (90/10, v/v) containing 0.086% trifluoroacetic acid. The gradient was 15% B to 50% B in 30 min, then 50% B to 85% B for 5 min at a flow rate of 0.5 ml/min. Peptides of interest were isolated by HPLC, concentrated with a centrifugal vacuum concentrator, and identified using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems). Pooled plasma obtained from three mice per group was used for these experiments.

Plasma CPN activity was also measured using furylacryloyl (FA)-Ala-Lys and FA-Ala-Arg as substrates and a protocol modified from Skidgel (17). Briefly, 125 μl of 0.1 M HEPES (pH 7.75) (Invitrogen) containing 0.5 M NaCl was added to the appropriate number of wells of a 96-well flat bottom microtiter plate (Nunc). Then, 25 μl of either 5 mM FA-Ala-Lys (Sigma-Aldrich) or 5 mM FA-Ala-Arg (Bachem) and 95 μl of deionized water were added to each well. The microtiter plate was incubated at 37°C for 10 min. Lastly, 5 μl of plasma obtained from either $CPN1^{+/+}$, $CPN1^{-/-}$, or $CPN1^{+/-}$ mice was added to the appropriate wells. The microtiter plate was placed in a SpectraMax M2 plate reader (Molecular Devices) at 37°C, and the change in absorbance at 336 nm was recorded every minute for 30 min. The number of micromoles of substrate cleaved per minute was determined by allowing the reaction to go to completion. Plasma obtained from three mice per group was used for these assays.

Generation of CPN1/C3a receptor (C3aR) and CPN1/C5a receptor (C5aR) double-deficient mice

$CPN1^{-/-}/C3aR^{-/-}$ double-deficient mice were generated by crossing $CPN1^{-/-}$ mice with $C3aR^{-/-}$ mice (18), both of which had been crossed at least 10 generations onto the C57BL/6 background. Likewise, $CPN1^{-/-}/C5aR^{-/-}$ double-deficient mice were generated by crossing $CPN1^{-/-}$ mice with $C5aR^{-/-}$ mice (19), both of which had been crossed at least 10 generations onto the C57BL/6 background. Genotypes of the double-deficient mice have been confirmed on multiple occasions by PCR.

Survival studies following injection of cobra venom factor (CVF)

$CPN1^{+/+}$, $CPN1^{-/-}$, $CPN1^{-/-}/C5aR^{-/-}$, and $CPN1^{-/-}/C3aR^{-/-}$ male mice, 11–15 wk old, were injected i.p. with 91 U of CVF in a volume of 1 ml (Quidel). The mice were monitored continuously for mortality for 3 h. No deaths occurred beyond this time point. The surviving mice were examined the next day and had completely recovered. Several experiments were performed using the same lot of CVF, and the data were combined to generate the survival curves shown in Fig. 4.

Survival studies following injection of C3a and C5a

$CPN1^{+/+}$ and $CPN1^{-/-}$ female mice, 13–14 wk old, were injected i.v. via the tail vein with 100 μg of purified human C3a (Complement Technology) in a volume of 200 μl. This lot of C3a has been used previously in our laboratory to stimulate Muc5ac expression from airway epithelial cells (20) and is known to be active. $CPN1^{+/+}$ and $CPN1^{-/-}$ female mice, 13–14 wk old, were injected i.v. via the tail vein with 7.5 μg of human C5a (Complement Technology) in a volume of 100 μl. In some studies, $CPN1^{-/-}$ mice were pretreated with antihistamine via an i.p. injection of diphenhydramine (10 mg/kg in PBS) (Sigma-Aldrich) or with PBS as a vehicle control. These mice were then injected with 7.5 μg of C5a i.v. 1 h later. The mice in these studies were monitored continuously for mortality for 2 h. No deaths occurred beyond this time point. The surviving mice were examined the next day and showed complete recovery.

Statistical analyses

For Table I, comparisons between $CPN1^{-/-}$ and $CPN1^{+/+}$ mice were assessed with GraphPad Prism software using the unpaired two-tailed Student's *t* test, with p values <0.05 considered significant. Data are expressed as means ± SEM. Survival curves were generated using GraphPad Prism software, and the log rank test was used to assess statistical significance among groups of mice, with p values <0.05 considered significant.

Results

Generation of $CPN1^{-/-}$ mice

The murine CPN1 gene was disrupted by replacing exon 3 (15), which participates in substrate binding, with a neomycin resistance cassette as described in *Materials and Methods* (Fig. 1*A*). Disruption of the gene was confirmed by Southern blot analysis using a probe, which is outside of the targeting vector, that encompasses all of exon 4 and part of

intron 4. This probe, when hybridized with genomic tail DNA that had been digested with *Eco*RI, bound to a 5.5-kb fragment of DNA from CPN1$^{+/+}$ mice and to a 3.7-kb fragment of DNA from CPN1$^{-/-}$ mice (Fig. 1*B*). PCR analysis was also used to confirm disruption of the CPN1 gene as described in *Materials and Methods*. DNA from CPN1$^{+/+}$ mice produced a 736-bp PCR product, and DNA from CPN1$^{-/-}$ mice produced a 488-bp PCR product (Fig. 1*C*). Both PCR products were present when DNA from CPN1$^{+/-}$ mice was used (Fig. 1*C*). Total liver RNA was analyzed by RT-PCR using three sets of primers corresponding to exons 2 and 3, exons 2 and 4, and exons 2 and 9 of CPN1 cDNA. All of the RT-PCR products were full-length for RNA from CPN1$^{+/+}$ mice (Fig. 2). However, the RT-PCR product was absent when primers corresponding to exons 2 and 3 were used with RNA from CPN1$^{-/-}$ mice (Fig. 2). Additionally, the RT-PCR products were shorter for exons 2 and 4 and exons 2 and 9 by ~156 bp, which corresponds to the size of exon 3. RNA from CPN1$^{+/-}$ mice produced both the full-length and the shorter length PCR products for exons 2 and 4 and exons 2 and 9 (Fig. 2). These results indicate that RNA from CPN1$^{-/-}$ mice contains some CPN1 RNA, but this RNA is devoid of coding sequence for exon 3.

CPN1$^{-/-}$ mice exhibit normal development, fertility, and survival

Intercrossing of CPN1$^{+/-}$ mice yielded the expected Mendelian ratio of CPN1 genotypes, with equal numbers of males and females produced, indicating that absence of CPN1 does not lead to embryonic lethality. CPN1$^{-/-}$ mice develop normally, displaying no gross abnormalities. Additionally, CPN1$^{-/-}$ mice are similar in weight to CPN1$^{+/+}$ mice of the same age and gender. Matings of CPN1$^{-/-}$ males with CPN1$^{-/-}$ females produce viable offspring of normal litter size, indicating that CPN1$^{-/-}$ mice are fertile. Survival of CPN1$^{+/+}$ and CPN1$^{-/-}$ mice followed for a minimum of 12 mo was identical.

Plasma from CPN1$^{-/-}$ mice lacks carboxypeptidase activity

Purified CPN has been shown to inactivate C3a and C5a in vitro by removing the terminal arginine from these peptides. To determine whether CPN1$^{-/-}$ mice are deficient in inactivating C3a and C5a, plasma from CPN1$^{+/+}$ and CPN1$^{-/-}$ mice was analyzed for the ability to cleave C3a and C5a to their desarginine forms as described in *Materials and Methods*. As shown in Fig. 3, plasma from CPN1$^{+/+}$ mice removed the terminal arginine from C3a (Fig. 3*A*) and C5a (Fig. 3*C*) within 5 min, which resulted in the HPLC peak shifting to the right, but plasma from CPN1$^{-/-}$ mice was unable to cleave the terminal arginine, so there was no shift in the peak for C3a (Fig. 3*B*) or C5a (Fig. 3*D*). The HPLC fractions were collected, and the identities of C3a, C3a-desArg, C5a, and C5a-desArg were verified by mass spectrometry analysis. These data confirm that plasma from CPN1$^{-/-}$ mice lacks CPN activity.

Plasma CPN activity was also measured using the substrate analogs FA-Ala-Lys and FA-Ala-Arg. Plasma from CPN1$^{-/-}$ mice had essentially no carboxypeptidase activity when compared with plasma from CPN1$^{+/+}$ mice (Table I), and plasma from CPN1$^{+/-}$ mice had carboxypeptidase activity intermediate to that of CPN1$^{+/+}$ and CPN1$^{-/-}$ plasma (Table I). Additionally, CPN1$^{+/+}$ plasma cleaved the FA-Ala-Lys substrate much more efficiently than the FA-Ala-Arg substrate (Table I). These data support previous findings (21–23) that purified CPN cleaves terminal lysine more effectively than terminal arginine.

CPN1$^{-/-}$ mice are more susceptible to CVF

CVF is a complement-activating protein present in cobra venom (reviewed in Ref. 24). CVF is a functional analog of C3b, which forms a stable fluid phase C3/C5 convertase leading to the generation of C3a and C5a. Because of the potent inflammatory nature of C3a and C5a, dysregulation of these molecules may have lethal consequences in the CPN1$^{-/-}$ mice. CPN1$^{+/+}$ and CPN1$^{-/-}$ mice were given 91 U of CVF i.p. and were continuously followed for mortality for 3 h. As shown in Fig. 4*A*, CPN1$^{+/+}$ mice experienced 12.5% mortality (2 of 16 died), whereas CPN1$^{-/-}$ mice experienced 53.3% mortality (8 of 15 died) ($p = 0.02$), indicating that the CPN1$^{-/-}$ mice were much more susceptible to CVF compared with the CPN1$^{+/+}$ mice. To determine whether the lethal effects of CVF were due to C3a, C5a, or both, the CPN1$^{-/-}$ mice were crossed with either C3aR$^{-/-}$ mice (18) or C5aR$^{-/-}$ mice (19). The CPN1$^{-/-}$/C3aR$^{-/-}$ mice given 91 U of CVF had 33.3% mortality (6 of 18 died) (Fig. 4*B*); however, this was not statistically different from the mortality seen in the CPN1$^{-/-}$ mice ($p = 0.34$). In stark contrast, the CPN1$^{-/-}$/C5aR$^{-/-}$ mice showed no mortality (0 of 10 died) (Fig. 4*B*) (*p*value compared with CPN1$^{-/-}$ mice = 0.007). These data indicate that the mortality observed in the CPN1$^{-/-}$ mice following CVF administration was primarily due to the effects of C5a, with little to no effect from C3a.

Administration of C5a but not C3a is fatal in CPN1$^{-/-}$ mice

To further examine the importance of CPN in providing protection from anaphylatoxin-mediated shock, purified C3a and C5a were given i.v. to CPN1$^{+/+}$ and CPN1$^{-/-}$ mice to test if acute production of either C3a or C5a in the circulation would be lethal in the CPN1$^{-/-}$ mice. In agreement with the CVF results in the CPN1$^{-/-}$/C3aR$^{-/-}$ mice, CPN1$^{-/-}$ mice given 100 μg of human C3a i.v. showed no mortality (data not shown). C3 is present in serum at a concentration of 0.75–1.53 mg/ml (25, 26), and C3a comprises ~5% of C3 based on size (27), which means 37.5–76.5 μg/ml C3a would be generated from 0.75–1.53 mg/ml C3. The quantity of C3a (100 μg) that we used is equivalent to the amount that would be generated by total activation of circulating C3, assuming that 50 μg/ml C3a is generated from 1 mg/ml C3 in an adult mouse that has a blood volume of 2 ml (for mouse blood volume, see Ref. 28). The CVF data indicated that the CPN1$^{-/-}$ mice are extremely sensitive to the lethal effects of C5a. We therefore injected CPN1$^{+/+}$ and CPN1$^{-/-}$ mice with 7.5 μg of human C5a i.v. and monitored the mice for mortality. The CPN1$^{+/+}$ mice exhibited no mortality (0 of 6 died), while all of the CPN1$^{-/-}$ mice died (5 of 5 died; $p = 0.0008$) (Fig. 5*A*). C5 is present in serum at a concentration of 70–340 μg/ml (26, 29), and C5a comprises ~5% of C5 based on size (27), which means 3.5–17 μg/ml C5a would be generated from 70–340 μg/ml C5. The quantity of C5a (7.5 μg) that we used is equivalent to the amount that would be generated by total activation of circulating C5, assuming that 3.75 μg/ml C5a is generated from 75 μg/ml C5 in an adult mouse that has a blood volume of 2 ml. C5a mediates numerous effects upon binding to its receptor, C5aR, including release of histamine from mast cells and basophils. Histamine causes vasodilation, increased vascular permeability, and smooth muscle contraction leading to broncho-constriction (reviewed in Ref. 30). To determine whether the mortality observed in the CPN1$^{-/-}$ mice was due to the effects of histamine, the CPN1$^{-/-}$ mice were pretreated with the antihistamine diphenhydramine at 10 mg/kg i.p. 1 h before injection of C5a i.v. CPN1$^{-/-}$ mice pretreated with the vehicle (PBS)

exhibited 100% mortality (6 of 6 died) following injection of C5a (Fig. 5*B*), whereas CPN1$^{-/-}$ mice pretreated with antihistamine experienced 20% mortality (1 of 5 died; $p = 0.004$) (Fig. 5*B*). These data indicate that the lethal effects of C5a observed in the CPN1$^{-/-}$ mice were caused by C5a-induced histamine release from mast cells and/or basophils.

Discussion

This study has described the generation of mice deficient in CPN for the first time. CPN1$^{-/-}$ mice were generated by homologous recombination using a targeting vector that resulted in ablation of exon 3 of the CPN1 gene (see Fig. 1), which participates in substrate binding. Despite the fact that a complete deficiency of CPN has not been reported in humans and that CPN1 is expressed early in embryonic development (14), CPN1$^{-/-}$ mice are viable, fertile, and develop normally.

Carboxypeptidase activity was measured from the plasma of untreated CPN1$^{+/+}$ and CPN1$^{-/-}$ mice. CPN1$^{+/+}$ plasma completely removed the terminal arginine from both C3a and C5a within 5 min (see Fig. 3). In contrast, plasma from CPN1$^{-/-}$ mice was unable to cleave the terminal arginine from C3a and C5a (see Fig. 3), demonstrating that plasma from CPN1$^{-/-}$ mice lacks carboxypeptidase activity. We also tested mouse plasma for carboxypeptidase activity using the substrate analogs FA-Ala-Lys and FA-Ala-Arg. The results demonstrated little to no carboxypeptidase activity in the CPN1$^{-/-}$ plasma compared with the CPN1$^{+/+}$ plasma (Table I). Additionally, we observed a gene dosage effect for CPN1 in that plasma from CPN1$^{+/-}$ mice had carboxypeptidase activity intermediate to that of CPN1$^{-/-}$ and CPN1$^{+/+}$ plasma (Table I). Moreover, these data with the substrate analogs support previous findings (21–23) that purified CPN cleaves terminal lysine more effectively than terminal arginine.

The CPN1$^{-/-}$ mice display no overt phenotype in a clean, unchallenged environment. However, these mice were significantly more susceptible to the lethal effects of acute complement activation caused by injection of CVF as compared with CPN1$^{+/+}$ mice (see Fig. 4*A*). As stated previously, CVF treatment causes rapid generation of C3a and C5a, which are both potent proinflammatory molecules. Generating the CPN1$^{-/-}$/C3aR$^{-/-}$ and the CPN1$^{-/-}$/C5aR$^{-/-}$ double knockout mice allowed us to delineate the importance of C3a and C5a in the CVF-induced mortality observed in the CPN1$^{-/-}$ mice (see Fig. 4*B*). The CPN1$^{-/-}$/C3aR$^{-/-}$ double knockout mice were partially protected from death compared with CPN1$^{-/-}$ mice (33.3% mortality vs 53.3% mortality, respectively), but this difference was not statistically significant. In contrast to the CPN1$^{-/-}$/C3aR$^{-/-}$ double knockout mice that were partially protected in the CVF model, the CPN1$^{-/-}$/C5aR$^{-/-}$ double knockout mice were completely protected from CVF-induced mortality (i.e., no deaths occurred), demonstrating the importance of C5a and its receptor in the lethality caused by CVF.

Besides CPN, there is only one other carboxypeptidase in the blood stream. This carboxypeptidase is known either as CPU, due to its instability in serum, CPR, for its preference for terminal arginine, or thrombin-activatable fibrinolysis inhibitor (TAFI) for its role in removing terminal lysine residues from the surface of fibrin clots, preventing binding and activation of plasminogen, and thereby inhibiting whole blood clot lysis. Unlike CPN,

which is secreted in an active and stable form, CPU/CPR/TAFI is secreted as a proenzyme that is activated during coagulation by plasmin, thrombin, or thrombin-thrombomodulin complexes (31) or by trypsin (32, 33) and possibly other proteases (34). Several groups have generated CPU/CPR/TAFI-deficient mice (35–38), but only one group has used these mice to examine the role of CPU/CPR/TAFI in a lethal complement activation model (35). The CPU/CPR/TAFI-deficient mice and their WT controls were injected i.p. with 100 U of CVF and were followed for mortality. However, none of the mice died, indicating that CPN was sufficient to protect these mice from the lethal effects of acute complement activation in the absence of CPU/CPR/TAFI. In contrast, our findings using a very similar CVF model clearly showed 53.3% mortality in the $CPN^{-/-}$ mice, indicating that the presence of CPU/CPR/TAFI was not sufficient to protect these animals from the lethal effects of acute complement activation in the absence of CPN. There was also no difference in mortality between WT and CPU/CPR/TAFI-deficient mice when injected with LPS, suggesting that CPN provided sufficient protection in this model as well. Interestingly, if the mice were first primed with a sublethal dose of LPS for 6 h and then given CVF, 60% of the CPU/CPR/TAFI-deficient mice but none of the WT mice died. Although there is no definitive explanation for why CPN sufficiently compensates for the loss of CPU/CPR/TAFI when LPS or CVF are administered alone, but not when CVF is given 6 h after LPS treatment, it suggests that in a highly inflamed environment both CPN and CPU/CPR/TAFI would be necessary to provide the host complete protection from sudden and massive complement activation.

This is the first published report that uses C3aR and C5aR knockout mice to simultaneously compare the effects of C3a and C5a in a CVF model of complement activation. Guinea pigs given a carboxypeptidase inhibitor before administration of CVF experienced 100% mortality, whereas guinea pigs given CVF alone had none (39). The authors attributed the lethal effects of the carboxypeptidase inhibitor plus CVF to both C3a and C5a. C5-deficient mice were protected from acute lung injury following injection of CVF (40), and injection of an Ab to C5a before administration of CVF protected rats against the acute lung injury caused by CVF (41). However, neither of those papers examined the effects of C3a. Proctor et al. (42) used antagonists to C3aR and C5aR in rats to simultaneously examine the importance of these receptors in the pathology that follows CVF administration. The C5aR antagonist protected the rats from CVF-induced hypotension, neutropenia, serum TNF elevation, and acute lung injury. However, the results with the C3aR antagonist were not as straightforward. The C3aR antagonist did not rescue the rats from CVF-induced hypotension, and administration of the C3aR antagonist itself caused a decline in circulating neutrophils. Also, the C3aR antagonist was only partially protective in the acute lung injury caused by CVF. The results with the C3aR antagonist are complicated to interpret because Proctor et al. (43) demonstrated in a previous publication, using a rat model of intestinal ischemia-reperfusion injury, that the C3aR antagonist can induce anti-inflammatory effects that are not due to antagonism of the C3aR. Additionally, this same C3aR antagonist has been shown to have agonist activity (44). Regardless of the ambiguity of the C3aR antagonist results, the C5aR antagonist data obtained by Proctor et al. (42) are unequivocal and agree with our findings in the $CPN1^{-/-}/C5aR^{-/-}$ double knockout mice demonstrating that C5a and its receptor are necessary for the pathology and ultimate death induced by

CVF. Our data with the CPN1$^{-/-}$/C3aR$^{-/-}$ double knockout mice suggest that C3a and its receptor play little, if any, role in CVF-induced mortality.

We challenged CPN1$^{-/-}$ mice and CPN1$^{+/+}$ mice with purified C3a i.v. and monitored the mice for mortality to validate our findings with the CVF. Injection of CPN1$^{-/-}$ mice with 100 μg of human C3a did not induce mortality in these mice. These data corroborate our findings in the CVF model, indicating no major role for C3a in the mortality observed in the CPN1$^{-/-}$ mice. These results, however, contradict the findings by Huey et al. (39). In their study, guinea pigs that were first given an inhibitor to CPN followed by 1.25 or 2.5 mg of human C3a experienced 50% mortality. At the lower dose of C3a, one of two animals died, and at the higher dose of C3a, two of four animals died. As reported by Huey et al. (39), 2.5 mg of C3a would represent maximum activation of C3 in the guinea pig and correlates with the dose we used in our mice on a weight basis. A possible explanation for this difference in findings is that guinea pigs are more susceptible to anaphylaxis compared with mice (45) and that mice and guinea pigs respond differently to anaphylaxis (46). Henderson and Smith reported in 1970 (46) that anaphylaxis in guinea pigs leads to acute respiratory distress due to bronchoconstriction, but anaphylaxis in mice leads to a generalized systemic shock.

As expected, CPN1$^{-/-}$ mice are extremely sensitive to the potent effects of C5a, and the mortality induced by C5a in these mice is histamine-dependent (see Fig. 5). Our group recently reported that C5aR$^{-/-}$ mice are protected from shock induced by i.v. injection of either *Escherichia coli* or LPS (19). Both of these models of shock induced death in WT mice within 1 h of injection, similar to our findings with injection of purified C5a in the CPN1$^{-/-}$ mice. Additionally, prior administration of antihistamine protected the WT mice from *E. coli*-induced death, demonstrating the importance of both C5a and histamine in this model of acute bacteremic shock (19).

In summary, this study describes the generation of mice deficient in CPN, facilitating the examination of complete CPN deficiency for the first time. Because no complete CPN deficiency has been discovered in humans and because the clinical manifestations of partial CPN deficiency were severe, there has been considerable speculation that a complete absence of CPN would be lethal. However, the CPN1$^{-/-}$ mice are viable, fertile, develop normally, and appear vigorous and healthy, indicating that CPN is not required for normal development and that in a healthy individual complete CPN deficiency could possibly be tolerated. Our findings, however, also indicate that acute complement activation would likely prove fatal to an individual devoid of CPN and that the presence of CPU/CPR/TAFI, the only other known bloodstream carboxypeptidase, would not provide sufficient protection from anaphylatoxin-mediated shock during acute complement activation.

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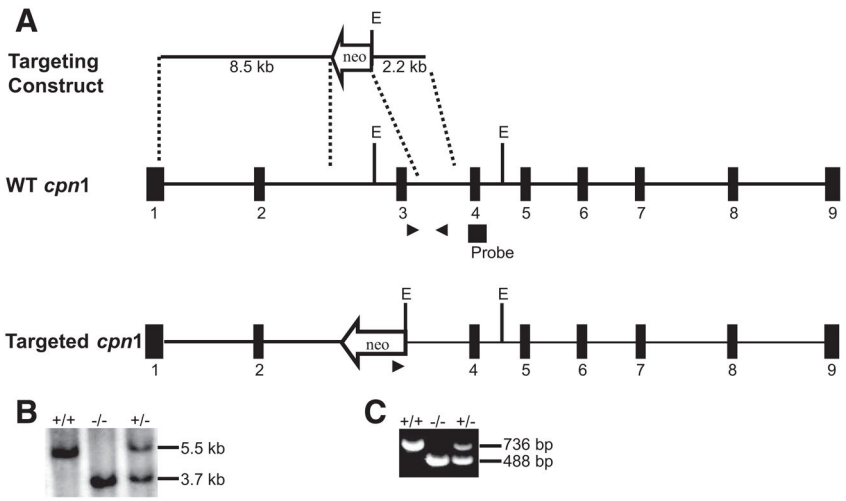

FIGURE 1.

Targeting strategy, Southern blot analysis, and PCR analysis. *A*, Schematic of the CPN1 genomic locus, the targeting vector, and the targeted null locus. Exons are indicated by black boxes. E represents *Eco*RI. The position of the 3′ external probe is shown. The arrowheads indicate the position of the primers used for PCR genotyping. *B*, Southern blot analysis of genomic mouse tail DNA digested with *Eco*RI. Bands of 5.5 and 3.7 kb correspond to the wild-type and the targeted allele, respectively. Results from $CPN1^{+/+}$, $CPN1^{-/-}$, and $CPN1^{+/-}$ mice are shown. *C*, PCR genotyping using the primers indicated in *A*. For the WT allele, a 736-bp fragment is generated, and for the targeted allele, a 488-bp fragment is generated. Results from $CPN1^{+/+}$, $CPN1^{-/-}$, and $CPN1^{+/-}$ mice are shown.

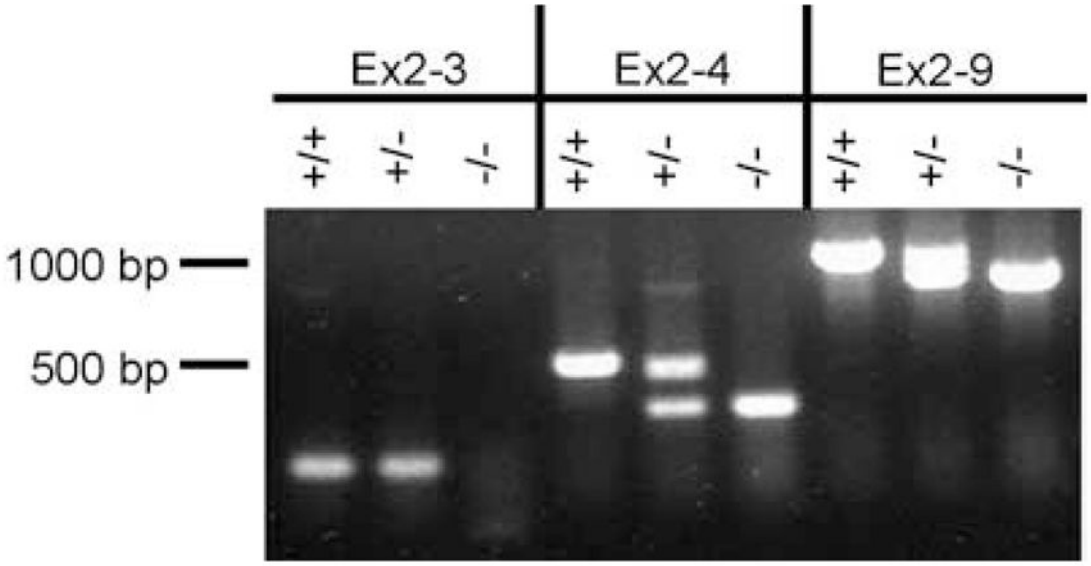

FIGURE 2.

RT-PCR analysis of total liver RNA. Total RNA was prepared from the liver of $CPN1^{+/+}$, $CPN1^{+/-}$, and $CPN1^{-/-}$ mice. Primers specific for exons 2 and 3 (*left panel*), exons 2 and 4 (*center panel*), and exons 2 and 9 (*right panel*) were used. Exon 3 is missing in RNA from $CPN1^{-/-}$ mice, resulting in no PCR product when primers for exons 2 and 3 were used and a smaller PCR product when primers for exons 2 and 4 and exons 2 and 9 were used. RNA from $CPN1^{+/-}$ mice produced both the WT PCR products and the mutated PCR products.

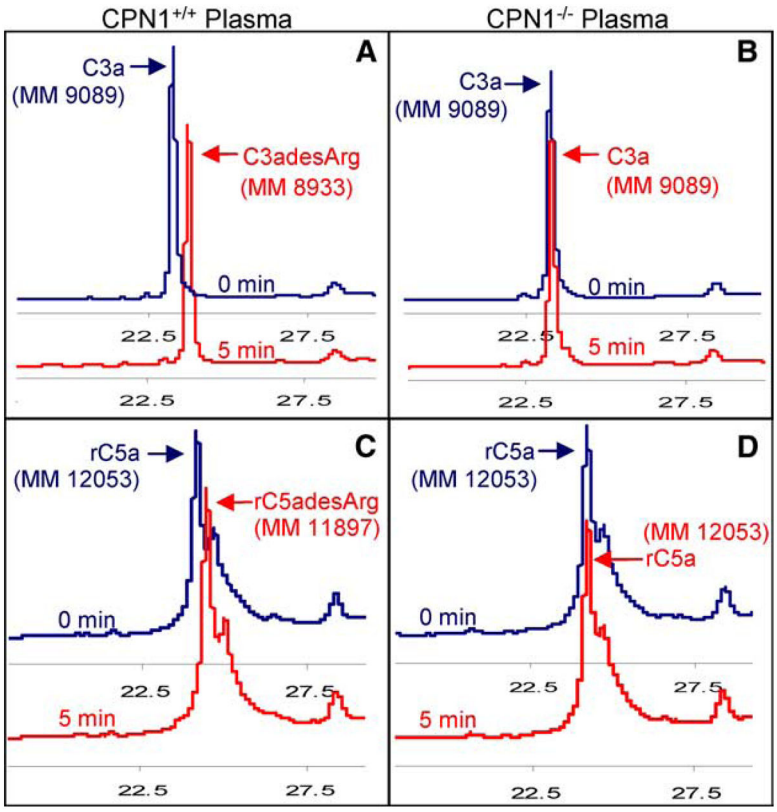

FIGURE 3.

Cleavage of C3a and C5a to their desArg forms by plasma from CPN1$^{+/+}$ mice but not from CPN1$^{-/-}$ mice. Plasma from CPN1$^{+/+}$(*A* and *C*) and CPN1$^{-/-}$(*B* and *D*) mice was incubated with C3a (*A* and *B*) or C5a (*C* and *D*) for 0 (shown in blue) or 5 min (shown in red), and the samples were analyzed by HPLC. Removal of the terminal arginine from these peptides resulted in the peak for C3a and C5a shifting to the right. The identities of the peptides were confirmed by mass spectrometry. Pooled plasma from three mice per group was used for these experiments. The data are shown as overlays of the 0 min (blue) and 5 min (red) time points.

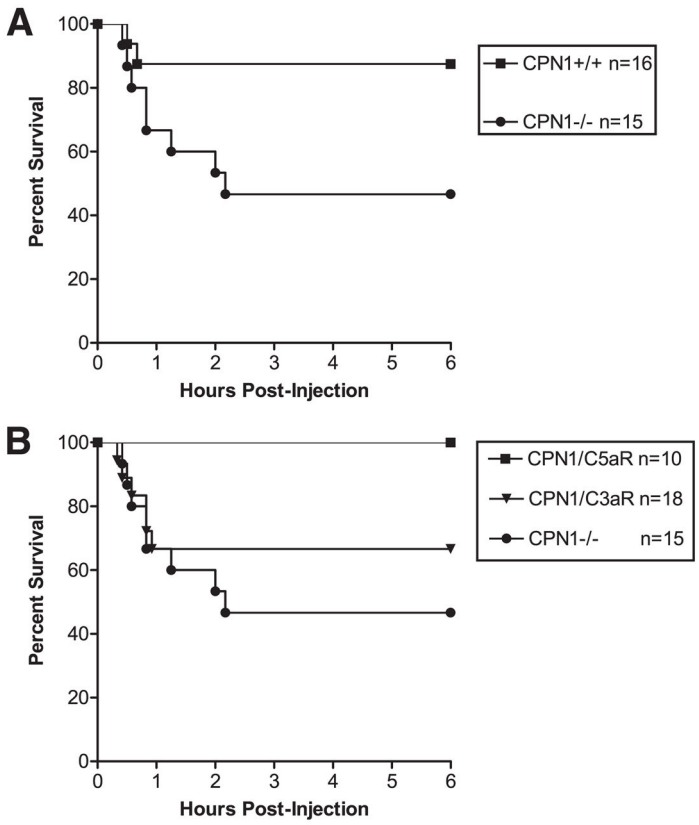

FIGURE 4.

$CPN1^{-/-}$ mice are highly susceptible to mortality following CVF-induced complement activation. *A*, $CPN1^{+/+}$ and $CPN1^{-/-}$ mice were injected i.p. with 91 U of CVF and were monitored for mortality for 3 h. $CPN1^{-/-}$ mice had much higher mortality compared with $CPN1^{+/+}$ mice (*, $p = 0.02$). *B*, CPN1/C3aR double knockout mice and CPN1/C5aR double knockout mice were injected i.p. with 91 U of CVF and were monitored for mortality for 3 h. The CPN1/C3aR double knockout mice were slightly more protected than the $CPN1^{-/-}$ mice, but this difference in mortality was not significant ($p = 0.34$). However, the CPN1/C5aR double knockout mice were completely protected from CVF-induced mortality (**, *p* value compared with $CPN1^{-/-}$ mice = 0.007). The $CPN1^{-/-}$ curve shown in *B* is the same as that shown in *A* and is shown again for comparison to the curves obtained for the double knockout mice.

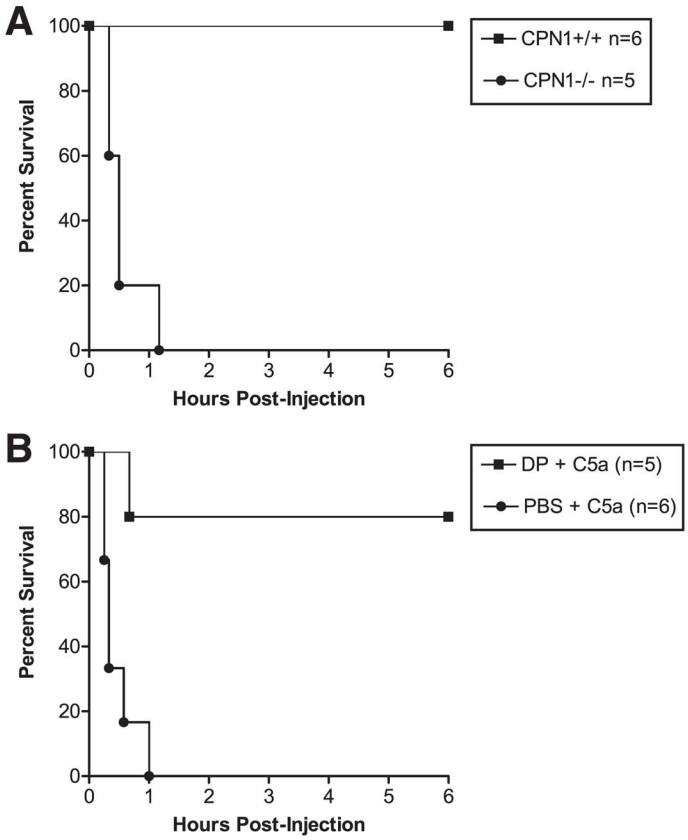

FIGURE 5.

CPN1$^{-/-}$ mice are highly susceptible to the lethal effects of C5a. *A*, CPN1$^{+/+}$ and CPN1$^{-/-}$ mice were injected i.v. with 7.5 μg of human C5a and were monitored for mortality for 2 h. CPN1$^{-/-}$ mice experienced 100% mortality, whereas the CPN1$^{+/+}$ mice exhibited no mortality (***, $p = 0.0008$). *B*, CPN1$^{-/-}$ mice were pretreated via i.p. injection with PBS (vehicle control) or the antihistamine diphenhydramine (DP) 1 h before i.v. injection of 7.5 μg of human C5a. CPN1$^{-/-}$ mice pretreated with PBS exhibited 100% mortality, whereas CPN1$^{-/-}$ mice pretreated with antihistamine exhibited 20% mortality (**, $p = 0.004$).

Table I

Plasma carboxypeptidase activities[a]

Plasma	FA-Ala-Lys μmol Cleaved/min (mean ± SEM)	FA-Ala-Arg μmol Cleaved/min (mean ± SEM)
$CPN1^{+/+}$	9.81 ± 0.55	2.49 ± 0.15
$CPN1^{+/-}$	6.02 ± 0.91+	ND
$CPN1^{-/-}$	0.62 ± 0.58***	0.91 ± 0.38*

[a]CPN activity in plasma was measured using FA-Ala-Lys and FA-Ala-Arg as substrates. The change in absorbance at 336 nm was recorded every minute for 30 min, and the number of micromoles of substrate cleaved per minute was determined by allowing the reaction to go to completion.

Significant differences between $CPN1^{+/+}$ and $CPN1^{-/-}$ mice are indicated as *, $p = 0.019$ and ***, $p = 0.0003$. +, The difference between $CPN1^{+/+}$ and $CPN1^{+/-}$ is significant, with $p = 0.024$, as well as the difference between $CPN1^{+/-}$ and $CPN1^{-/-}$, with $p = 0.008$.