Procarboxypeptidase R Deficiency Causes Increased Lethality in Concanavalin A-Induced Hepatitis in Female Mice

Suzuka ASAI, a Noriaki KIMBARA, a Toyohiro TADA, b Masaki IMAI, c,William CAMPBELL, c
Hidechika OKADA, d and Noriko OKADA a

*a Department of Immunology, Nagoya City University Graduate School of Medical Sciences; b Department of Pathology, Nagoya City University School of Nursing; c 1 Kawasumi, Mizhuo-cho, Mizhuo-ku, Nagoya 467–8601, Japan; c Chojyu Medical Institute, Fukushima Hospital; 19–14 Azayamanaka, Noyori, Toyohashi, Aichi 441–8194, Japan; and d Institute for Protein Science Co.; 2–18 Nakayama-cho, Mizhuo-ku, Nagoya 467–0803, Japan.

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Carboxypeptidase R (CPR), also known as thrombin-activatable fibrinolysis inhibitor (TAFI), is an enzyme generated by proteolytic cleavage of its zymogen (proCPR). CPR removes the C-terminal arginine from inflammatory peptides such as C3a and C5a, bradykinin, enkephalin, and the thrombin-cleaved N-terminal fragment osteopontin (cleaved N-OPN). In the mouse model of concanavalin A (Con A)-induced immune-mediated fulminating hepatitis, cleaved N-OPN is one of the important peptides that induce the production of chemokines or cytokines. In the current study using proCPR deficient mice, we showed that injection of Con A into the mouse tail vein can induce a significantly higher lethality in proCPR-deficient female but not in male mice. Furthermore, a lack of CPR activity increased serum macrophage inflammatory protein-2 (MIP-2) and high-mobility group box 1 (HMGB1) levels after Con A injection. These in vivo findings suggest that CPR helps to protect against Con A-induced hepatitis.

Key words Rodent; complement; cytokine; inflammation; knockout mouse

Basic carboxypeptidases (CPs) are present in plasma and cleave C-terminal arginine and lysine residues from various peptides including inflammatory mediators. 1-4 Carboxypeptidase N (CPN) is present in an active form in plasma, whereas carboxypeptidase R (CPR) exists in a precursor form (procarboxypeptidase R (proCPR)). CPR is known as plasma carboxypeptidase B (CPB), carboxypeptidase U (CPU), or activated thrombin-activatable fibrinolysis inhibitor (TAFIa), and is generated from its zymogen (proCPR) by thrombin,5 thrombin/thrombomodulin complex,6 or plasmin7 during coagulation or in response to inflammation.7,8 Plasma CPN and CPR can inactivate anaphylatoxins C3a and C5a, but CPR is more effective at inactivating C5a than is CPN both in vitro9 and in vivo.10 In addition to these reports, we obtained in vivo evidence that CPR plays an important role in preventing hyperinflammation caused by LPS and C5a, using proCPR-deficient mice.11 A recent study suggested that in addition to C5a, osteopontin (OPN), one of the extracellular matrix proteins, is a target for CPR since CPR removed the C-terminal arginine of the thrombin-cleaved N-terminal fragment of OPN (cleaved N-OPN) in vitro.12 In the Concanavalin A (Con A)-induced fulminant hepatitis mouse model, plasma pro-inflammatory cytokine levels and high-mobility group box 1 (HMGB1) expression in liver tissue were increased.13 In addition to these cytokines, OPN is secreted from natural killer T (NKT) cells, and then cleaved by thrombin after Con A injection. OPN is well known as a secretory extracellular matrix protein, however, on the other hand, the cleaved N-OPN exposes the cryptic epitope SVVYGCLR at its C-terminus thereby enhancing its adhesive interaction with NKT, spreading, and migration of a variety of cells, leading to production of macrophage inflammatory protein-2 (MIP-2).14

In two Con A-induced murine hepatitis studies, female mice showed more severe liver injury and a higher concentration of Th1 type cytokines than did male mice.15,16 Furthermore, both of OPN and cleaved N-OPN concentrations were significantly higher in female rats than in male rats after injection of lipopolysaccharide (LPS) in alcoholic liver disease model.17 These reports suggest that there is a close relationship between the secretory protein OPN and hepatic injury, especially in terms of the severity of hepatitis in the females.

Since the liver is the major site of proCPR synthesis and since both proCPR and OPN are activated by thrombin, CPR is presumed to play an important role in Con A-induced hepatitis. In this study, we examined the role of CPR in liver damage using proCPR-deficient mice. We found that a lack of proCPR in female permitted the significantly higher levels of lethality and CPR deficiency enhances the serum level of some inflammatory cytokines in Con A-induced hepatitis.

MATERIALS AND METHODS

Animals As described previously, proCPR−/− mice (129/Ola, BALB/c, and C57BL/6 background) were generated by knocking out the portion of the gene containing exons 4 and 5 to eliminate all CPR activity. In this study, we used 5- to 8-week-old proCPR−/− mice. Similar background proCPR+/+ mice were used as controls. It has been confirmed that there was no gender difference in CPN and CPR activities in these proCPR+/+ and proCPR−/− mice. The experimental protocol was approved by the Animal Studies Committee of Nagoya City University Graduate School of Medical Sciences.

Measurement of Aspartate Amino Transferase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP), Chemokine, and Cytokines of Mice Sera Con A (Sigma-Aldrich, St. Louis, MO, U.S.A.) (400 μg/mouse) in 50 μl saline was injected into female mice through the tail vein and the blood samples were collected 4 h later. Sera were used to determine AST, ALT and ALP. The same sam-
ples were also used to measure MIP-2 (IBL, Gunma, Japan), HMGBl (SHINO-TEST, Kanagawa, Japan) and interferon-gamma (IFN-γ) (BD Biosciences) by enzyme-linked immunosorbent assays (ELISAs) according to the instructions of the manufacturer.

Measurement of CP Activity  Con A (250 μg/mouse) in 50 μl saline was injected into female proCPR+/+ mice through the tail vein and the blood samples were collected 1 h, 2 h and 4 h later. CP activity was determined by means of a colorimetric assay using hippuryl-L-arginine (Peptide Institute, Osaka, Japan) as a synthetic substrate.1,18)

Briefly, for determination of both CPR and CPN activities, plasma was diluted 1/10 in 50 mM Tris–HCl buffer (pH 8.0) containing 1.3 NIH U/ml thrombin (Nihon Pharmaceutical, Tokyo, Japan), 8 nM thrombomodulin (a generous gift from Asahi Kasei, Tokyo, Japan), and 10 mM CaCl2 (each quantity represents the final concentration), and incubated for 20 min at room temperature. For determination of CPN activity, plasma was diluted 1/5 in 50 mM Tris–HCl buffer and incubated for 1 h at 37 °C to inactivate CPR. Ten microliters of each sample were mixed with 5 μl of 30 mM hippuryl-L-arginine in 50 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) (pH 8.2) as the substrate solution prior to incubation for 45 min at 37 °C. After incubation, 100 μl of 250 mM phosphate buffer (pH 8.3) and 75 μl of 3% cyanuric chloride in 1.4 dioxane were added and the mixture was centrifuged at 5000 rpm for 10 min at 4 °C. A 100 μl aliquot of the supernatant of each sample was transferred to a 96-well microtiter plate for measurement of absorbance at 405 nm.

RESULTS

CPR Can Limit Liver Damage Induced with Con A Injection  Under inflammatory conditions, CPR cleaves the C-terminal arginine of inflammatory peptides including anaphylatoxins and then inhibits the cytokine response. To estimate the role of CPR during Con A-induced hepatitis, we injected Con A intravenously (i.v.) (500 μg/mouse) into proCPR+/+ and proCPR−/− mice. The survival rate was significantly lower in proCPR−/− female mice than in proCPR+/+ female mice. However, there was no significant reduction in the survival rate of proCPR−/− male mice (Fig. 1).

To confirm the extent of liver damage, liver enzymes such as AST, ALT, and ALP were measured. Some mice had died within 4 h after 500 μg of Con A injection, then the sera used to determine these enzymes were collected from female mice 4 h after 400 μg of Con A injection. As shown in Table 1, there is a tendency toward higher levels of these liver enzymes in proCPR−/− mice.

CPR and CPN Activities after Con A Injection  As plasma CPs are synthesized in the liver7,8) and are secreted into plasma, the activities of these enzymes may change during Con A-induced hepatitis. Although a portion of proCPR is activated during inflammation/coagulation, the addition of thrombin, thrombomodulin and Ca2+ can activate all the remaining proCPR. In this experiment, plasma incubated with thrombin, thrombomodulin and Ca2+ was used to determine the total CP activity (CPR and CPN activity). The half-life of CPR is within 6.3—16 min at 37 °C.1,19,20) Therefore, plasma incubated for 1 h at 37 °C had exhausted CPR and CP activity remaining was CPN activity. CPR activity was determined as the difference between total CP activity and the
CPN activity. The decreases of CPR activities at 1 h and 4 h caused the significant reduction of the total CP activities after Con A injection. However, the total CP activity even at 4 h was preserved the same level as CPN activity at 2 h after Con A injection (Fig. 2).

**Serum Chemokine and Cytokine Concentrations after Con A Injection** The severity of liver injury might result in the change of serum chemokine and cytokine concentrations. To investigate the influence of lack of CPR activity on these chemokine and cytokine levels, MIP-2, HMGB1 and IFN-γ were measured. Between proCPR+/+ and proCPR−/−, MIP-2 and HMGB1 concentrations tended to higher in proCPR−/− compared with proCPR+/+ mice (Table 2).

**DISCUSSION**

CPR removes the C-terminal arginine of anaphylatoxins, C3a and C5a, bradykinin, enkephalin, and cleaved N-OPN. In this report, the lethality of proCPR−/− was significantly increased compared with proCPR+/+ female mice. The blood AST, ALT, and ALP levels 4 h after Con A injection tended to be higher in proCPR−/− than proCPR+/+ mice, indicating that hepatocytes of proCPR−/− mice are sensitive to Con A attack. Liver damage over such a short time period reduced the CPR activity of proCPR+/+ mice within 1 h of Con A injection, however, total CP activity remained unchanged between 1 h and 2 h. In addition, the total CP activity at 4 h still retained the same level of CPN activity at 2 h. This suggests that, in proCPR+/+ mice, any residual CPR and CPN might restrict the function of anaphylatoxins and cleaved N-OPN. The suppression of anaphylatoxins and cleaved N-OPN could limit the overflow of cytokines and chemokines in immune-mediated hepatic injury.

Additional histological examination revealed massive liver damage in Con A-injected mice, where, 6 h after 200 μg/mouse Con A injection, neutrophil infiltration was observed in hepatic sinusoids of both of proCPR+/+ and proCPR−/− mice (data not shown). We could not detect significant differences between proCPR+/+ and proCPR−/− animals 6 h after Con A injection. Since cleaved N-OPN can prevent neutrophil apoptosis, higher levels of cleaved N-OPN in proCPR−/− mice might allow neutrophils to remain in the liver longer than in proCPR+/+ mice. Neutrophils in hepatic sinusoids should reinforce liver parenchymal cell damage, suggesting that further histological investigation over in a longer time period is needed.

Another group using proCPR deficient mice demonstrated that in septic models using *Escherichia coli* (*E. coli*) intraperitoneally (i.p.) injection, proCPR deficiency resulted in fewer and smaller liver necrosis foci.21) These investigators suggested that higher C3a and C5a activity in proCPR deficient mice could offer protection from liver necrosis, since in C3a or C5a deficient mice, liver regeneration was impaired.22—25) Although CPR can inactivate C3a, C5a and cleaved N-OPN, Con A injected hepatitis is supposed to be mediated by NKT cells and OPN. Furthermore, Con A induced footpad edema is independent of the C5/C5a pathway.26) Therefore in our case, the difference in survival rate between proCPR−/− and proCPR+/+ female mice may not be directly related to C5a activity.

The survival rate of proCPR−/− female mice following 500 μg/mouse Con A injection was significantly lower than that of proCPR+/+ female mice. However, proCPR deficiency in male mice was not accompanied by such a significant difference. Interestingly, some reports have demonstrated that Con A-induced liver injury was more severe in female mice than in male mice, and that cytokine levels were significantly higher in female mice.15,16) In another report, it was found that in alcoholic liver disease (ALD) model rats, both OPN and cleaved N-OPN levels were significantly higher in female rats than in male rats after injection of LPS. Indeed in the absence of ethanol, a single LPS injection also induced an increase in cleaved N-OPN only in females but not in males.17) Therefore, the reduction in survival rate seen in female proCPR−/− mice only supports these findings.

The Con A-induced liver injury causes massive increases of plasma pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), INF-γ, interleukin-2 (IL-2), IL-4, IL-6, IL-10, and IL-12.13) In addition to these proteins, they showed the up-regulation of the HMGB1 expression in the liver tissue. Recent studies indicate that the OPN is necessary for the translocation of HMGB1 from the nucleus to the cytoplasm,27) and HMGB1 activates NKT cells to produce the IFN-γ.28) Although our results did not show the difference of the IFN-γ concentration between proCPR+/+ and proCPR−/− mice, the lack of CPR activity tended to increase the serum MIP-2 and HMGB1 levels, then these findings aroused more interest in the role of CPR on OPN or cleaved N-OPN. Unfortunately, in spite of many evidences for CPR capability to remove the C-terminal arginine of C3a, C5a, and cleaved N-OPN in vitro, there is no assay to detect the exact quantities of C3a, C5a, or cleaved N-OPN remaining in plasma directly.

Further investigation is necessary to clarify the relationship between cleaved N-OPN, CPR activity and other inflammatory cytokines such as TNF-α and IL-6 in vivo, since we have demonstrated a critical role for CPR in the Con A-induced murine hepatitis model. It has been demonstrated that plasma CPR levels in patients with a variety of chronic liver diseases including ALD, HCV or HBV infections were significantly higher in male patients than in female patients.29) Our findings indicate that in patients with chronic hepatitis, CPR therapy might be a powerful strategy for preventing the development of a severe form of the disease.

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**REFERENCES**


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**Table 2. Serum Chemokine and Cytokines Levels in Con A-Injected Mice**

<table>
<thead>
<tr>
<th></th>
<th>MIP-2 (pg/ml)</th>
<th>HMGB1 (ng/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>+/+</td>
<td>313±107</td>
<td>92±23</td>
<td>321±73</td>
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<td>−/−</td>
<td>396±57</td>
<td>188±30</td>
<td>292±52</td>
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Con A (400 µg/mouse) was injected into 5—8-week-old female mice through tail vein and the serum MIP-2, HMGB1, and IFN-γ levels were measured 4 h later. The data are expressed as mean±S.D.


