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Site-Specific Carboxypeptidase B1 Tyrosine Nitration and Pathophysiological Implications following Its Physical Association with Nitric Oxide Synthase-3 in Experimental Sepsis

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LPS-induced sepsis results in oxidative modification and inactivation of carboxypeptidase B1 (CPB1). In this study, immunoprecipitated CPB1 was probed for tyrosine nitration using monoclonal nitrotyrosine-specific Abs in a murine model of LPS-induced sepsis. Tyrosine nitration of CPB1 was significantly reduced in the presence of NO synthase (NOS) inhibitors and the xanthine oxidase (XO) inhibitor allopurinol and in NOS-3 knockout (KO) mice. CPB1 tyrosine nitration and loss of activity by the concerted action of NOS-3 and XO were also confirmed in vitro using both the NO donor 3-morpholinosydnonimine and peroxynitrite. Liquid chromatography/tandem mass spectrometry data indicated five sites of tyrosine nitration in vitro including Tyr248, the tyrosine at the catalytic site. The site- and protein-specific nitration of CPB1 and the possible high nitration yield to inactivate it were elucidated by confocal microscopy. The studies indicated that CPB1 colocalized with NOS-3 in the cytosol of sinus-lining cells in the red pulp of the spleen. Further analysis of CPB1-immunoprecipitated samples indicated immunoreactivity to a monoclonal NOS-3 Ab, suggesting protein complex formation with CPB1. XO and NOS inhibitors and NOS-3 KO mice injected with LPS had decreased levels of C5a in spleens of septic mice, indicating peroxynitrite as a possible cause for CPB1 functional alteration. Thus, CPB1 colocalization, coupling, and proximity to NOS-3 in the sinus-lining cells of spleen red pulp could explain the site-specific tyrosine nitration and inactivation of CPB1. These results open up new avenues for the investigation of several enzymes involved in inflammation and their site-specific oxidative modifications by protein-protein interactions as well as their role in sepsis. The Journal of Immunology, 2009, 183: 4055–4066.

The possible formation of peroxynitrite and the resultant posttranslational nitration of protein tyrosine residues are associated with the pathogenesis of a series of diseases, including acute and chronic inflammatory processes, sepsis, ischemia-reperfusion, and neurodegenerative diseases (1, 2). Nitration of tyrosine residues by radical mechanisms is always tyrosyl- and NO- or nitrogen dioxide-dependent (3). Despite the number of diseases and pathological conditions in which tyrosine nitration has been observed, our knowledge of specific enzyme targets is limited, especially where a distinctive association is found between tyrosine nitration and functional alterations.

Tyrosine nitration does not depend on the abundance or number of tyrosine residues present on a particular protein (2). The commonalities among various nitrated proteins include glutamate or aspartate residues in the vicinity of Tyr residues and/or the presence of turn-inducing amino acids such as proline or glycine (4–6). Tyrosine nitration yields in proteins, organs, and disease conditions have typically been low; the poor yield has raised questions about nitration as a posttranslational modification in the molecular basis of disease (2).

Carboxypeptidase B (CPB)3 in tissue, designated CPB1, was initially described as a pancreatic metallocarboxypeptidase and is a marker for acute pancreatitis. This stable protease has high homology with plasma CPB and has substrates in common with it. This was assessed in recent studies where supplementation of the matrix with additional thrombin-activatable fibrinolysis inhibitor (TAFI) or CPB produced a reduction in capillary tube formation (7). Plasma CPB, CPU (carboxypeptidase U), or active TAFI (designated TAFIa) has a half-life of 8 min and plays a role in inflammation (8–10). Earlier work from this laboratory has identified CPB1 in the septic spleen and found it to form a radical in the presence of xanthine oxidase (XO) and NO synthase (NOS)-3. This study further investigates the nature of the radical and its posttranslational modification.

In this work, we address the site-specific nature of protein tyrosyl radical formation and nitration and the higher nitration yield

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3 Abbreviations used in this paper: CPB, carboxypeptidase B; DMPO, 5-dimethyl-1-pyrroline N-oxide; FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) chloride; KO, knockout; l-NIO, N(2)-(1-imino-3-butenyl)-L-ornithine; MGA, DL-2-mercaptopropionyl-3-glutamylthiopropionic acid; NOS, NO synthase; SIN-1, 5-morpholinosydnonimine; TAFI, thrombin-activatable fibrinolysis inhibitor; TRIM, 1-(2-trifluoromethylphenyl)imidazole; 1400W, N3-(aminomethyl)benzylacetamide · 2HCl; XO, xanthine oxidase.

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generated in CPB1, a zinc-containing tissue metallopeptin, following LPS-induced systemic inflammation. Our previous work has shown that LPS-induced systemic inflammation leads to the formation of CPB1 radicals, which are mediated by XO and endothelial NOS (NOS-3) with a concomitant loss of enzyme activity (11). Immuno-spin trapping of the CPB1 radical was a significant step in the demonstration of the involvement of NOS-3 and XO-derived oxidizing species in vivo. However, molecular and post-translational footprints of reactive oxygen species and reactive nitrogen species-based oxidative stress needed to be identified. We used relatively specific NOS inhibitors to identify the relative contributions of different NOS isoforms and peroxynitrite decomposition catalysts to identify the role of peroxynitrite in CPB1.

Furthermore, to understand the molecular basis of CPB1, we have examined the role of 3-morpholinosydnonimine (SIN-1) and guanidinoethylthiopropionic acid (MGTA) was used to study the involvement of the catalytic process in the nitration of CPB1.

Materials and Methods

Materials

LPS (Escherichia coli strain 55:B5), porcine CPB, SIN-1 hydrochloride, and allopurinol were obtained from Sigma-Aldrich. The trap spin 5,5-di-(methyl-1-pyryl)-N-oxide (DMPO) was obtained from Alexis Biochemicals. Trypsin (from bovine pancreas, a modified sequencing grade) and chymotrypsin (from bovine pancreas, a modified sequencing grade) were obtained from Roche Molecular Biochemicals. All other chemicals were of analytical grade and were purchased from Sigma-Aldrich or Roche Molecular Biochemicals. All other chemicals were of analytical grade and were purchased from Sigma-Aldrich or Roche Molecular Biochemicals. All aqueous solutions were prepared using water passed through a Picopure2 UV Plus system (Hydro Services and Supplies) equipped with a 0.2-μm pore size filter. Absorption spectra were recorded on a Cary 100 UV-visible spectrometer (Varian). HPLC was conducted on an Agilent ChemStation 1100 series liquid chromatography system (Agilent Technologies) equipped with a control module, binary pump, manual injector, and diode array UV-visible detector. HPLC fractions were collected using a fraction collector, Bio-Rad model 2110.

Mice

Adult male, pathogen-free, 8- to 10-wk-old C57BL6/J mice (The Jackson Laboratory) weighing 23–27 g on arrival were used in these experiments. The animals were housed for 1 wk, one to a cage, before any experimental use. Experiments using mice that contained the disrupted NOS-2 (NOS-2 −/−; B6.129P2-Nos2tm1Lau/J), gp91phox (gp91phox−/−; B6.129S6-Cybb−/−); B6.129S6-Cybb−/−, NOS-3 (NOS-3 +/+, NOS-3 −/−, NOS-3 −/+), B6.129P2-Nos2tm1Din/J, B6.129P2-Nos2tm1Lau/J, B6.129P2-Nos2tm1Din/J (3 volumes of 4% streptomycin 12 g of protein A/G-agarose followed by incubation for 1 h at room temperature. The homogenate was then incubated overnight with 30 μl of polyclonal anti-mouse CPB1 Ab (0.1 μg/ml), and the Ag-antibody mixture was further incubated with the protein A-G-agarose slurry overnight. Immune complexes were eluted with elution buffer according to the manufacturer’s instructions. Anti-CBP1 immunoprecipitates were subjected to SDS/PAGE on 4–12% Bis-Tris gels (Invitrogen) and electroblotted onto nitrocellulose membranes. Abs for the corresponding Western blots used in these experiments were mouse monoclonal anti-CPB1 (1:1000 dilution; Abcam). In some experiments, lysates were subjected to immunoblotting without immunoprecipitation. The Abs used in these experiments were anti-mouse polyclonal CPB1 (1:1000 dilution; R&D Systems), mouse monoclonal anti-NOS-3 (1:1000; Cell Signaling), and purified rat anti-mouse C5a (1:2000, BD PharMingen). The immunocomplexes were probed (1 h at room temperature) with either goat anti-rabbit (1:1000; Upstate Biotechnologies), donkey anti-goat (1:3000; R&D Systems), or goat anti-mouse (1:5000; Pierce) HRP-conjugated secondary Abs. Immunoreactive proteins were detected using ECL (Immobilon Western chemiluminescence HRP substrate; Millipore). The images were subjected to densitometry analysis using LabImage 2006 Professional software for the measurement of specific bands.

Carboxypeptidase activity assay

Carboxypeptidase activity of immunoprecipitated CPB1 was measured using the Actichrome TAFI activity kit (American Diagnostica) according to the procedure described by the manufacturer with some modifications. Briefly, the reaction was initiated by adding 50 μl of the TAFI substrate to 25 μl of anti-CBP1 immunoprecipitates. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 250 μl of 100 mM glutamic acid and released chromogenic product was measured at 405 nm. The A405 of blanks, which consisted of complete mixtures incubated for 0 min, was subtracted from each value. CPB concentration in immunoprecipitates was estimated by ELISA using anti-CBP1 Ab (1/1000; R&D Systems). A calibration curve was constructed with known concentrations of recombinant mouse CPB1 (R&D Systems).

The activity of purified porcine CPB was estimated by monitoring the formation of the concomitant loss of enzyme activity (11). Immuno-spin trapping of the CPB1 radical was a significant step in the demonstration of the involvement of NOS-3 and XO-derived oxidizing species in vivo. However, molecular and post-translational footprints of reactive oxygen species and reactive nitrogen species-based oxidative stress needed to be identified. We used relatively specific NOS inhibitors to identify the relative contributions of different NOS isoforms and peroxynitrite decomposition catalysts to identify the role of peroxynitrite in CPB1.

We have examined the role of 3-morpholinosydnonimine (SIN-1) and guanidinoethylthiopropionic acid (MGTA) was used to study the involvement of the catalytic process in the nitration of CPB1. Furthermore, to understand the molecular basis of CPB1, we examined the role of 3-morpholinosydnonimine (SIN-1) and guanidinoethylthiopropionic acid (MGTA) was used to study the involvement of the catalytic process in the nitration of CPB1.

The above-mentioned events might result in higher nitration yields sufficient to inactivate CPB1 in sepsis.

LPS-induced systemic inflammation model

Systemic inflammation was induced in mice following LPS administration as described previously (11, 13). Briefly, mice received a bolus infusion of LPS (6 and 12 mg/kg) at 0 h. A sham group was also included in which normal mice received saline in place of LPS. LPS was dissolved in pyrogen-free saline and administered through the i.p. route. At 6 h, mice from the sham group and the LPS groups were sacrificed. The spleens were collected and snap-frozen in liquid nitrogen. Tissues were homogenized in phosphate buffer containing 100 μM diethylenetriaminepentaacetic acid and centrifuged at 3000 rpm at 4°C for 20 min. The samples were stored at −80°C until further use.

Administration of allopurinol, NOS inhibitors, CPB inhibitor, and the peroxynitrite scavenger FeTPPS

Allopurinol, a specific inhibitor of XO, the nonselective NOS-3 inhibitors N’-[1-imino-3-butenyl]-l-ornithine (L-NIO) and vinyl-l-l-arginine (Cayman Chemical), the putatively selective inhibitor of neuronal NOS-1, 1,2-(tri-fluoromethyl)phenyl)imidazole (TRIM, Calbiochem), and the NOS-2 inhibitor N-[2-(aminomethyl)benzylidene]-2HCl (1400W; Sigma-Aldrich) were administered in a single bolus dose of 20 mg/kg through the i.p. route 30 min before LPS treatment. In different experiments, the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) chloride (FeTPPS; Calbiochem) and the CPB1 inhibitor MGTA (Sigma-Aldrich), an inhibitor of CPB, were administered at bolus doses of 30 and 100 mg/kg and 20 mg/kg, respectively, through the i.p. route 15 min before LPS administration.

Immunoprecipitation and immunoblotting

CPB1 was immunoprecipitated with polyclonal anti-CPB1 Ab (R&D Systems) using the Seize X mammalian immunoprecipitation kit (Pierce BioMedical) with some modifications. Solubilized spleen cell homogenates were adjusted to a protein concentration of 150 μg per sample were preclarified by adding 200 μl of protein A/G-agarose followed by incubation for 1 h at room temperature. The homogenate was then incubated overnight with 30 μl of polyclonal anti-mouse CPB1 Ab (0.1 μg/ml), and the Ag-antibody mixture was further incubated with the protein A/G-agarose slurry overnight. Immune complexes were eluted with elution buffer according to the manufacturer’s instructions. Anti-CBP1 immunoprecipitates were subjected to SDS/PAGE on 4–12% Bis-Tris gels (Invitrogen) and electroblotted onto nitrocellulose membranes. Abs for the corresponding Western blots used in these experiments were mouse monoclonal anti-CPB1 (1:1000 dilution; Abcam). In some experiments, lysates were subjected to immunoblotting without immunoprecipitation. The Abs used in these experiments were anti-mouse polyclonal CPB1 (1:1000 dilution; R&D Systems), mouse monoclonal anti-NOS-3 (1:1000; Cell Signaling), and purified rat anti-mouse C5a (1:2000, BD PharMingen). The immunocomplexed membranes were probed (1 h at room temperature) with either goat anti-rabbit (1:1000; Upstate Biotechnologies), donkey anti-goat (1:3000; R&D Systems), or goat anti-mouse (1:5000; Pierce) HRP-conjugated secondary Abs. Immuno-reactive proteins were detected using ECL (Immobilon Western chemiluminescence HRP substrate; Millipore). The images were subjected to densitometry analysis using LabImage 2006 Professional software for the measurement of specific bands.
FIGURE 1. Immunoblotting of immunoprecipitated CPB1 from spleen tissue homogenates with anti-nitrotyrosine Ab. A, Spleen tissue proteins were isolated from control and LPS-treated mice and then immunoprecipitated with anti-CPB1 Ab and immunoblotted with an anti-nitrotyrosine Ab. A 47-kDa band corresponding to CPB1 is shown by a black arrow. Equal loading was confirmed using a direct ELISA of CPB1 present in the immunoprecipitate. The CPB1 band density was compared with nitrotyrosine band density for normalization. B, Densitometric analysis of normalized band densities against anti-CPB1 immunoblots. *, p < 0.05 when compared with sham. Similar results were obtained in three independent experiments. C, In a control experiment the specificity of CPB1 tyrosine nitration was confirmed by preabsorbing the anti-CPB1 antiserum with recombinant mouse CPB1 (i, lane 2; ii, lane 4), BSA (i, lane 1) and normal rabbit serum (ii, lane 3).

Confocal microscopy

For confocal studies, C57BL/6j wild-type mice were injected with LPS at 12 mg/kg and sacrificed 6 h after LPS administration. Spleens were collected and washed in PBS and the tissues were fixed in 10% neutral buffered formalin. After fixation, spleens were removed and placed in 30% sucrose for 24 h. Tissues were then sliced on a microtome into 20-μm sections, placed in PBS, and then permeabilized with 0.1% Surfact-Amps X-100 detergent solution (Pierce Biomedical) for 1 h. After blocking with 0.1% BSA in PBS, NOS-3 was stained using monoclonal anti-NOS-3 Ab (BD Transduction Laboratories) as the primary Ab and Alexa Fluor 488 anti-goat secondary Ab. Secondary controls were used to determine background fluorescence by applying the secondary Ab only (data not shown). Slices were mounted on microscope cover glasses (22 × 22 mm; 1-mm thickness) (Erie Scientific) and sections were analyzed under a confocal laser microscope (Zeiss).

C5a chemotaxis assay

Cell migration assay was conducted using the CytoSelect 96-well cell migration assay kit (Cell Biolabs) following the manufacturer’s protocol. HL-60 cells were stimulated for 72 h with 1000 U/ml IFN-γ to have C5a receptor expression and were used for the assay. Mouse rC5a (R&D Chemicals) was used as a positive control and incubated in the feeder layer. A group where no rC5a was added served as the negative control.

Experiments with pancreatic CPB, mouse rCPB1 peroxynitrite, and SIN-1 in vitro

To illustrate the possible posttranslational modification of CPB in vitro, porcine CPB was incubated with different concentrations of SIN-1 and peroxynitrite in the presence or absence of 25 mM sodium bicarbonate. Peroxynitrite was prepared in the laboratory according to the method described by Bonini et al. (16, 17). CPB (100 μM) was dissolved in phosphate buffer (pH 7.4) and incubated with 300, 1,250, or 2,500 μM peroxynitrite or peroxynitrite donor SIN-1. In experiments with mouse rCPB, 1 μM mouse rCPB1 was dissolved in phosphate buffer (pH 7.4) and incubated with 3, 12.5, or 25 μM peroxynitrite. Samples consisting of only enzyme, only peroxynitrite, or only SIN-1 formed the negative controls. The reaction mixture was incubated at 37°C for 1 h. The resultant reaction mixture was then separated by SDS-PAGE and tested for nitrosyl oxygen reactivity by Western analysis.

Sample preparation and proteolytic digestion

Typically, incubations contained 100 μM CPB in 50 mM potassium phosphate buffer (pH 7.4) and were conducted at 37°C in the presence or absence of 25 mM sodium bicarbonate, as indicated in the figure legends. The reactions were initiated by the addition of different concentrations of peroxynitrite (3–25 equivalents) and allowed to proceed for 30 min, after which the solutions were passed over a Sephadex G-25 gel filtration column (GE Healthcare Bio-Sciences) previously equilibrated and eluted with 100 mM Tris-HCl (pH 8.5). The samples were denatured with 6 M guanidine hydrochloride in 100 mM Tris-HCl (pH 8.5) at 60°C for 30 min, reduced with 5 mM DTT at 37°C for 30 min, and alkylated with 25 mM iodoacetamide at 37°C for 30 min. The resulting solutions were then loaded onto a previously equilibrated PD-10 gel filtration column and eluted with 100 mM Tris-HCl (pH 8.5). After passage through the PD-10 column, carboxyamidated samples were digested using a 20:1 substrate-to-protease ratio for 16 h at 37°C (trypsin) or for 16 h at 25°C (chymotrypsin). Reactions were stopped by injecting the final mixture directly onto a reverse-phase HPLC column.

Reverse-phase HPLC analysis of proteolytic hydrolysates

Digested samples (200 μl) were injected directly onto a Vydac 218TP54, 4.6 mm × 250 mm, C18 reverse-phase HPLC column. Peptides were eluted at a flow rate of 0.8 ml/min with a linear gradient from 100% solvent A (0.1% trifluoroacetic acid in water) to 50% solvent B (0.085% trifluoroacetic acid in acetonitrile) over 80 min. A rapid gradient to 90% solvent B and then back to 100% solvent A was used to regenerate the column. The eluent was monitored at 214, 280, and 365 nm with an Agilent HP1100 diode array detector. HPLC fractions were collected with a fraction collector, and the fractions containing nitrated peptides (as judged by the appearance of chromatographic peaks at 365 nm) were pooled, lyophilized, and stored at −70°C for subsequent analysis.
FIGURE 2. CPB1 tyrosine nitration is a result of peroxynitrite formation by dual action of NOS-3 and XO. A, Spleen proteins from sham-, LPS- and LPS plus FeTPPS (peroxynitrite decomposition catalyst)-treated mice were immunoprecipitated with anti-CPB1 Ab, separated by SDS-PAGE, and electroblotted on a nitrocellulose membrane. Western blot analysis was performed using an Ab specific to 3-nitrotyrosine (inset). The bar chart shows the band intensity of the immunoreactive proteins. B, Spleen proteins from sham-, LPS-, LPS plus L-NIO-, and LPS plus vinyl-L-NIO-treated mice (relatively specific NOS-3 inhibitors) and LPS plus allopurinol-treated mice (XO-specific inhibitor) were immunoprecipitated with anti-CPB1 Ab, separated by SDS-PAGE, and electroblotted on a nitrocellulose membrane. Western blot analysis was performed using an Ab specific to 3-nitrotyrosine. C, Graphical representation of the band intensities of immunoreactive proteins. Equal loading was confirmed using a direct ELISA of CPB1 present in the immunoprecipitate. The CPB1 band density was compared with nitrotyrosine band density for normalization. \( \star \), \( p < 0.05 \) when compared with sham; \( \# \), \( p < 0.05 \) when compared with LPS (12 mg/kg)-treated mice. D, Spleen proteins from sham-, LPS-, LPS plus L-NIO-, LPS plus 1400W (NOS-2 inhibitor)-, and LPS plus TRIM (NOS-1 inhibitor)-treated mice were immunoprecipitated with anti-CPB1 Ab, separated by SDS-PAGE, and electroblotted on a nitrocellulose membrane. Western blot analysis was performed using an Ab specific to 3-nitrotyrosine. E, Graphical representation of the band intensities of immunoreactive proteins is also provided. Equal loading was confirmed using a direct ELISA of CPB1 present in the immunoprecipitate. The CPB1 band density was compared with nitrotyrosine band density for normalization. \( \star \), \( p < 0.05 \) when compared with sham; \( \# \), \( p < 0.05 \) when compared with LPS (12 mg/kg)-treated mice. F, Spleen proteins from sham- and LPS-treated wild-type (WT) and NOS-3 KO LPS mice were immunoprecipitated with anti-CPB1 Ab, separated by SDS-PAGE, and electroblotted on a nitrocellulose membrane. Western blot analysis was performed using an Ab specific to 3-nitrotyrosine (3-NT). G, Graphical representation of the band intensities of immunoreactive proteins. Equal loading was confirmed using a direct ELISA of CPB1 present in the
Electrospray mass spectrometry

A Micromass quadrupole/time-of-flight micro hybrid tandem mass spectrometer (Waters Micromass) was used for the acquisition of the electrospray ionization mass spectra and tandem mass spectra. All experiments were performed in the positive ion mode. Lyophilized HPLC fractions of nitratetted peptides were resuspended in a minimal volume (~50 μl) of 50:50 water:acetoniitrile containing 0.1% formic acid and infused into the mass spectrometer at ~300 nA/min using a pressure injection vessel (18). The needle voltage was ~3.200 volts and the collision energy was 10 eV for the mass spectrometry analyses. Collision-induced dissociation experiments used argon with collision energy between 30 and 50 electron volts. Data analysis was accomplished with a MassLynx data system and MassEnt deconvolution software supplied by the manufacturer.

Statistical analyses

All in vivo experiments were repeated three times with three mice per group (n = 3). All in vitro experiments were repeated three times and the statistical analysis was conducted using the Microcal Origin software package. Quantitative data from Western blotting as depicted from the relative intensity of the bands were analyzed by performing a one-tailed Student’s t test. Values of p < 0.05 were considered statistically significant.

Results

LPS-induced septic shock produces CPB1 tyrosine nitration in vivo

CPB1 forms a DMPO-trappable protein radical in response to LPS treatment as detected by immuno-spin trapping (11). To detect whether the CPB1 radical correlated with the nitration of tyrosine residues, which is dependent on tyrosyl radical formation (19), spleen homogenates from sham and LPS (6 and 12 mg/kg) treated mice were probed for 3-nitrotyrosine immunoreactivity (data not shown). The data shows anti-nitrotyrosine Ab immunoblotting of spleen tissue homogenates from control and LPS-treated mice. A number of proteins showed detectable immunoreactivity toward the Ab at 6 h, but not in 3 or 24 h, in both the control and the LPS-treated mice. The most prominently nitrated protein had a molecular mass of ~47 kDa. MALDI-TOF analysis of this 47-kDa band performed in an earlier study (11) resulted in the detection of >10 proteins, one of which was CPB1.

To determine whether the CPB1 protein was nitrosated, mouse spleen tissue lysates were immunoprecipitated with the anti-CPB1 Ab. The immunoprecipitates were then immunoblotted with the anti-nitrotyrosine Ab. Western blot analysis of the immunoprecipitates (Fig. 1A) indicated the presence of only one nitrotrated protein band of ~47 kDa. The results also indicated that CPB1 nitration was significantly higher in the LPS-treated group than in the sham-treated group at 6 h (Fig. 1B). This result suggests that the 47-kDa nitrotrated protein detected in the splenocyte lysate is indeed CPB1. As an additional control of specificity for the nitration of CPB1, anti-CPB1 antiserum was preabsorbed with mouse rCPB1. The preabsorbed Ab was used for immunoprecipitation of CPB1. Anti-CPB1 Ab was also preabsorbed with BSA and normal rabbit serum for negative controls. Results indicated that immunoprecipitation of CPB1 using a CPB1 Ab preabsorbed with mouse rCPB1 had little or no reactivity to anti-nitrotyrosine Ab (Fig. 1C, lanes 2 and 4), whereas immunoprecipitation of CPB1 using an Ab preabsorbed with either BSA or normal rat serum showed distinct bands on the Western blot (Fig. 1C, lanes 1 and 3).

Tyrosine nitration of CPB1 is apparently due to formation of peroxynitrite and is mediated by XO and NOS-3

It is known that protein tyrosine nitration can be mediated by peroxynitrite (1). Superoxide and NO in a diffusion-controlled reaction yield peroxynitrite that, in biological systems, promptly reacts with carbon dioxide, leading to the formation of carbonate (CO₃²⁻) and nitrogen dioxide (NO₂⁻) radicals (16, 20, 21, 22). Although there are no direct methods for detecting peroxynitrite in vivo, there are reports of certain classes of iron porphyrins that act as peroxynitrite decomposition catalysts (23).

To determine whether peroxynitrite plays a role in LPS-induced tyrosine nitration of CPB1, we treated mice with FeTPPS, a porphyrin peroxynitrite scavenger, before LPS treatment. Proteins from spleen homogenates were immunoprecipitated with an anti-CPB1 Ab and analyzed by Western blotting using an anti-nitrotyrosine Ab. As depicted in Fig. 2A, administration of FeTPPS significantly inhibited the nitration of the protein, supporting the involvement of peroxynitrite.

Peroxynitrite is a powerful oxidant that is formed by the diffusion-limited reaction between nitrogen monoxide (NO') and superoxide (O2•⁻). To identify the possible O2•⁻ and NO' generators in LPS-treated mice, we used allopurinol, a specific XO inhibitor, 1400 W, a specific inhibitor for NOS-2, TRIM, a putatively specific inhibitor for NOS-1, and vinyl-L-NIO and l-NIO, two nonselective inhibitors of all NOS isoforms. As seen in Fig. 2B, administration of the XO inhibitor allopurinol and two nonselective inhibitors of NOS, the isoforms l-NIO and vinyl-L-NIO, significantly inhibited the nitration of CPB1 (Fig. 2, B and C), whereas the administration of 1400W, a specific inhibitor for NOS-2, and TRIM, a relatively specific inhibitor for NOS-1, did not inhibit nitration (Fig. 2, D and E). Because the inhibitors showing marked isoforn selectivity toward NOS-1 and NOS-2 did not inhibit the nitration of CPB1, whereas, in strong contrast, the nonselective inhibitors were highly effective, it is thus reasonable to suspect that NOS-3 plays a central role in the nitration process.

In the septic spleen, NOS-2 has been shown to be a major source of NO, whereas at early sepsis (4–6 h after LPS administration) NOS-3 and XO were major sources of NO' and O2•⁻, respectively (11, 24). The respective roles of the three enzymes in the CPB1 nitration process were delineated using gene KO mice. Western blot analysis of anti-CPB1 immunoprecipitates indicated that the nitration of tyrosine residues was not blocked in either NOS-2 KO mice (Fig. 2, F and G, lane 4 from the left) or NADPH oxidase KO mice (data not shown). In contrast, no staining was observed for either sham or LPS-treated NOS-3 KO mice (Fig. 2, F and G, lanes 2 and 3 from the left). These data indicate that NOS-3 is the primary NOS isoform responsible for CPB1 nitration in vivo.

To investigate the involvement of CPB1 in the catalysis of its self-nitrification in vivo, we used MGT4, an inhibitor of CPB1, which binds to the catalytic site of the enzyme. Tyrosine nitration of immunoprecipitated CPB1 was completely blocked by the administration of this inhibitor in LPS-treated mice as shown in Fig. 2, H and I.
FIGURE 3. Peroxynitrite-mediated tyrosine nitration inactivates CPB1 in the septic spleen and in vitro. A, CPB1 activity in spleen tissue homogenates from LPS, LPS plus allopurinol, LPS plus l-NIO, 1400W, LPS plus vinyl-l-NIO, and NOS-3 KO mice was measured using an Actichrome TAFI activity kit with slight modifications, and activity indices were calculated using sham-treated values (activity index = treated activity/sham activity). B, Tyrosine nitration mediated by peroxynitrite as a function of loss of activity of CPB. Porcine CPB (100 μM) was incubated with different concentrations of peroxynitrite. Samples were separated by SDS-PAGE, and the proteins were electroblotted on a nitrocellulose membrane. The blotted proteins were probed for 3-nitrotyrosine immunoreactivity using a 3-nitrotyrosine Ab (Anti 3-NT). C, Graphical representation of the band intensities of immunoreactive proteins. Equal loading was confirmed using Coomassie blue-stained bands on the SDS-polyacrylamide gel. Furthermore, the CPB1 band density of the Western blot with anti-CPB Ab was compared with the nitrotyrosine band density for normalization. *, p < 0.05 when compared with the CPB only group; $, p < 0.05 when compared with the CPB plus ONOO$\textsuperscript{−}$(0.6 mM) group; #, p < 0.001 when compared with the CPB plus ONOO$\textsuperscript{−}$(1.25 mM) group. D, Percentage activity of CPB as a function of 0, 3, 6, 12.5, and 25 molar equivalents (eqs) of peroxynitrite (Per). E, Mouse recombinant CPB1 (1 μM) was incubated with different concentrations of peroxynitrite. Samples were separated by SDS-PAGE and the proteins were electroblotted on a nitrocellulose membrane. The blotted proteins were probed for 3-nitrotyrosine immunoreactivity using a 3-nitrotyrosine Ab (Anti 3-NT). F, Graphical representation of the band intensities of immunoreactive proteins. Equal loading was confirmed using Ponceau-stained bands on the nitrocellulose membrane. Furthermore, the CPB1 band density of the Western blot with the anti-CPB1 Ab was compared with the nitrotyrosine band density for normalization. *, p < 0.05 when compared with the recombinant mouse CPB1 only group; #, p < 0.001 when compared with the CPB plus ONOO$\textsuperscript{−}$(25 μM) group.
LPS-induced peroxynitrite formation and nitration lead to inactivation of CPB1 in vivo and in vitro

To study the biological consequences of CPB1 tyrosine nitration, we measured the enzyme activity of CPB1. The activity index of immunoprecipitated CPB1 in LPS-treated mice and LPS plus 1400W-treated mice was significantly lower than that in the sham-treated mice \((p < 0.05)\) (Fig. 3A). Administration of allopurinol, l-NIO, or vinyl-l-NIO to LPS-treated mice significantly increased activity compared with the LPS-treated group alone \((p < 0.05)\). LPS-treated mice lacking the NOS-3 gene also had activity comparable to that of the corresponding sham-treated NOS-3 KO mice (Fig. 3A). The results obtained in vivo were further confirmed with parallel measurements of peroxynitrite-mediated nitration of CPB in vitro and loss of activity. When porcine CPB and/or mouse rCPB1 was treated with 3, 6, 12.5, or 25 molar equivalents of peroxynitrite, there was a concentration-dependent increase in tyrosine nitration (Fig. 3B, C, E, and F). The increased tyrosine nitration was associated with parallel decreases in the activity of CPB, showing >50% loss of activity with 12.5 molar equivalents of peroxynitrite (Fig. 3D).

LPS-induced tyrosine nitration of CPB1 and inactivation are linked to colocalization and protein complex formation with NOS-3

The loss of CPB activity in the presence of authentic peroxynitrite in vitro (50% activity loss with 12.5 equivalents or 1.25 mM peroxynitrite) raises the question of the possible existence of such concentrations of peroxynitrite in inflammatory physiology. Typically, protein tyrosine nitration is a relatively widespread in vivo modification, with its overall yield (expressed as moles of 3-nitrotyrosine/mole of tyrosine) being typically low. The low yield poses serious doubts about whether these modifications are relevant to the molecular basis of disease in pathophysiological states (2). To establish the connection between the inactivation of CPB1 in vivo and the concentration of peroxynitrite required to inactivate CPB in vitro, we sought to determine the localization of CPB1 in the spleen. We propose that a close proximity to the availability of NO and \(\text{O}_2^-\) sources can create sufficiently high nitration yields in a site-specific manner so as to inactivate the enzyme. It has been shown that NO production in rat lung microvascular endothelial cells is stimulated more efficiently by arginine released from carboxypeptidase substrates than by free arginine after LPS stimulation, thus indicating a mechanism by which the arginine supply for NO production in inflammatory conditions may be maintained (12).

These observations prompted us to investigate whether subcellular colocalization of two enzymes in the splenic vascular bed resulted in a steady flow of NO and the formation of higher concentrations of peroxynitrite. This could lead to high nitration yields that are not obtained even in inflammatory microenvironments where the enzymes are widely separated. Confocal microscopy results indicated that levels of both CPB1 and NOS-3 were high in the septic spleen and that CPB1 colocalized with NOS-3 in the sinus-lining cells of the red pulp. NOS-3 was mostly localized in the membrane in sham-treated spleens (Fig. 4A), and LPS treatment resulted in the translocation of NOS-3 to the cytosol and colocalization with CPB1 (Fig. 4AII) (25). Cells lining the venous sinusoids also showed significant colocalization of CPB1 and NOS-3 (Fig. 4AIII). We did not observe the same colocalization pattern in the white pulp of the spleen (data not shown).

To further address whether the colocalization resulted from the binding of CPB1 to NOS-3 following its activation and translocation into the cytosol in LPS- treated mice, immunoprecipitates with CPB1 were probed with monoclonal anti-NOS-3 Ab. In both the sham-treated and LPS-treated groups there was an immunoreactive band at 140 kDa corresponding to NOS-3. The increased tyrosine nitration of CPB1 oxidative inactivation leads to accumulation of C5a in the LPS-treated spleen

It has recently been shown that thrombin-activatable CPB is catalytically more efficient than plasma carboxypeptidase N (CPN), the major plasma anaphylatoxin inhibitor, in inhibiting bradykinin, activated complements C3a and C5a, and thrombin-cleaved osteopontin in vitro (26). CPB inactivates these active inflammatory mediators by specific cleavage of the carboxyl-terminal arginines (26). Similarly, pro-CPB-deficient mice displayed enhanced pulmonary inflammation with C5a-induced alveolitis (10). Based on the above evidence, we chose to probe the functional consequences of LPS treatment in the spleen. When we separated spleen tissue...
CPB1 tyrosine nitration results in C5a accumulation in the mouse spleen following LPS administration. A, Spleen proteins from sham-, LPS-, LPS plus NOS-2 inhibitor 1400W-, LPS plus l-NIO-, LPS plus vinyl-l-NIO (relatively specific NOS-3 inhibitors)-, and LPS plus allopurinol (XO inhibitor)-treated mice were separated by SDS-PAGE and electroblotted on a nitrocellulose membrane. Western blot analysis was performed using an Ab specific to C5a. B, Bar graph represents the normalized relative band intensities of the Western blot analysis of the proteins immunoreactive to C5a Ab. C, Spleen proteins from sham (wild type), LPS (wild type), sham (NOS-3 KO) and LPS (NOS-3 KO) were separated by SDS-PAGE and electroblotted on a nitrocellulose membrane. Western blot analysis was performed using an Ab specific to C5a. D, Bar graph represents the normalized relative band intensities of the Western blot analysis of the proteins immunoreactive to C5a Ab. * p < 0.05 when compared with the sham-treated wild-type (WT) mice; #, p < 0.05 when compared with the sham-treated KO mice. E, Chemotaxis of HL-60 cells in response to rC5a. Also, rC5a was incubated with both mouse rCPB1 and nitro-CPB1 to assess the effect of nitration of CPB1 on C5a-induced chemotaxis. *, p < 0.05 when compared with rC5a alone; #, p < 0.05 when compared with rC5a plus mouse rCPB1. F, Chemotaxis of HL-60 cells in response to immunoprecipitated C5a from LPS- (wild type)-, XO inhibitor-, NOS-3 inhibitor-, NOS-2 inhibitor-, and LPS-treated KO mice spleen tissue homogenates was measured in terms of relative fluorescent intensity by a CytoSelect 96-well cell migration assay following the manufacturer’s protocol (Cell Biolabs). *, p < 0.05 when compared with sham-treated mice alone; #, p < 0.05 when compared with LPS-treated wild-type (WT) mice.
homogenate proteins by SDS-PAGE and immunoblotted them against the C5a Ab, we found that LPS-treated and LPS plus 1400W-treated mouse spleen tissue homogenates showed significant levels of C5a compared with no detectable levels in sham-treated spleens (Fig. 5, A and B). Accumulation of C5a under these conditions would lead to severe inflammatory reactions as those seen in sepsis and septic shock.

The XO inhibitor allopurinol and the NOS-3 inhibitors l-NIO and vinyl-l-NIO, which significantly decreased tyrosine nitration of CPB1 in spleens of LPS-treated mice (Fig. 2B), also decreased the levels of C5a in the LPS-treated spleen (Fig. 5, A and B). Furthermore, levels of C5a were significantly increased in NOS-3 KO mice compared with the corresponding KO sham treatment, but were significantly lower than those in LPS-treated wild-type mice (Fig. 5, C and D).

Because polyclonal anti-serum cannot distinguish between C5a (74 aa) and C5a des Arg (73 aa), neutrophil chemotaxis assay was performed to evaluate concentrations of C5a relative to its less active (5%) des Arg form (27, 28). In the first set of experiments, HL-60 cell chemotactic response to purified C5a was examined. Results indicated that at $2 \times 10^{-9}$ M, mouse rC5a could induce chemotaxis of HL-60 cells, whereas preincubation of C5a with mouse rCPB1 reduced chemotactic response by up to 40% (Fig. 5E). Little chemotactic activity was detectable in the absence of C5a. Furthermore, when nitro-mouse rCPB1 was incubated with mouse rC5a, chemotaxis was comparable to the control/unmodified C5a. In a second set of experiments, the chemotactic response of HL-60 cells to immunoprecipitated C5a from spleen tissue homogenates was evaluated. These experiments were performed using equal amounts of immunoprecipitated C5a as assessed by ELISA. The results indicated that the chemotactic response was significantly higher for samples from LPS- and LPS plus 1400W-treated mice than that observed for sham, allopurinol, l-NIO, Vinyl-l-NIO, and LPS KO mouse samples (Fig. 5F).

**In vitro characterization of peroxynitrite-induced chemical modifications of CPB1**

To study the molecular basis of tyrosine oxidation and nitration of CPB by peroxynitrite in vitro, porcine CPB (100 μM) was incubated with peroxynitrite (300 μM) in the presence or absence of 25 mM sodium bicarbonate. Carbonate radicals (CO₃⁻) can be formed biologically by the decomposition of the peroxynitrite-carbon dioxide adduct (ONOOCO₂⁻) or by enzymatic activities, i.e., peroxidase activity of copper-zinc-dependent superoxide dismutase (CuZnSOD) and XO turnover in the presence of bicarbonate (29, 30, 31). Peroxynitrite-dependent tyrosine nitration is likely to occur through the initial reaction of peroxynitrite with carbon dioxide or metal centers, leading to secondary nitrating species (19, 32).

We found that a reaction mixture containing sodium bicarbonate as a bicarbonate source showed several immunoreactive bands when compared with samples incubated with only peroxynitrite. CPB (32 and 35 kDa) and mouse CPB1 (43 and 48 kDa) migrate as doublets on an SDS-polyacrylamide gel. Apart from the monomer (35 kDa) and a slightly less intense band at 30–32 kDa, another distinct band of ~70 kDa was immunoreactive to an Ab specific to 3-nitrotyrosine (Fig. 6).

Following this observation, we used SDS-PAGE to analyze a reaction mixture containing both peroxynitrite and SIN-1 as a potential mimetic of O₂⁻/NO generation. The analysis revealed the formation of dimers, characterized by the 70-kDa Coomassie blue-stained band, compared with none in a sample containing only CPB (Fig. 7A). The dimers were inhibited in a dose-dependent manner when incubated with 10 and 100 mM DMPO (Fig. 7A). It has been reported that the formation of dimers is the result of dityrosine crosslinks that form by the reaction of two tyrosyl radicals. Dityrosine crosslinks are considered to be stable markers for the oxidative modification of proteins (33). To identify the nature of the dimer, we measured the fluorescence of the intact protein containing the reaction mixture at 410 nm. The spectra showed a pattern that is distinct for dityrosine formation in samples incubated with SIN-1, but was absent in samples containing CPB alone (Fig. 7B). When the reaction mixture was digested with trypsin and run through a reverse-phase HPLC column, a spectrum indicative of dityrosine crosslink formation was found in one of the peptides (data not shown).

**Nitration of the catalytic site tyrosines (Tyr²⁴⁸ and Tyr¹⁹⁸) as an index of tyrosine oxidation and inactivation of CPB**

To examine the possible sites of tyrosine nitration of CPB, the enzyme was incubated with 12.5 molar equivalents of peroxynitrite, digested with either chymotrypsin or trypsin, and subjected to reverse-phase HPLC. The fractions corresponding to the absorbance at 365 nm were collected and subjected to tandem mass spectrometry analysis. At least 11 peptides with five sites of nitration on tyrosine residues were identified. These include Tyr⁹², Tyr⁴¹⁰, Tyr³⁷⁷, Tyr²⁴⁸, and Tyr¹⁹⁸. Of these, Tyr²⁴⁸ and Tyr¹⁹⁸ are located in the catalytic site of CPB (Table I). The observed nitration of Tyr²⁴⁸ was consistent with previous findings showing that Tyr²⁴⁸ is more reactive and undergoes nitration with an 8-fold molar excess of tetranitromethane (34).

**Discussion**

We have previously shown that CPB1 produces a DMPO-trappable protein radical in vivo in LPS-induced systemic inflammation and that this radical production is mediated by the dual role of NOS-3 and XO (11). In the present work, we report the site-specific nitration of splenic CPB1, possibly mediated by peroxynitrite, in vivo and in vitro in LPS-induced acute inflammation in mice, a model that resembles systemic inflammation response syndrome or SIRS.

We chose to use FeTPPS, a relatively specific peroxynitrite decomposition catalyst in vivo (35), to investigate the involvement of
peroxynitrite in CPB1 nitration. We limited our studies with FeT-PPS in vivo to relating the tyrosine nitration process of CPB1 to peroxynitrite in the presence or absence of DMPO. The reaction mixture was then separated by SDS-PAGE and stained with Coomassie Blue. CPB (0.1 mM) was incubated with SIN-1 (0.3 mM) for 1 h. The reaction mixture was then checked for an increase in fluorescence at 410 nm, characteristic of di-tyrosine dimer formation. The Coomassie blue-stained gel photograph and the di-tyrosine dimer detection by fluorescence are representative of a total of three experiments.

Tyrosine nitration at catalytic sites has been shown to result in loss of activity in many enzyme targets (2). To determine whether by using both NOS-3 and NOS-2 KO mice. NOS-2 KO mice showed no significant difference in immunoreactivity to 3-nitrotyrosine Ab, suggesting the important role played by NOS-3 in peroxynitrite formation and nitration of CPB1.

Tyrosine nitration at catalytic sites has been shown to result in loss of activity in many enzyme targets (2). To determine whether

Table I. Summary of identified peptides originating from proteolytic digestion of CPB and nitrated tyrosine residues

<table>
<thead>
<tr>
<th>Protease</th>
<th>Elution Time</th>
<th>Position of YNO₂</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>45.6</td>
<td>210</td>
<td>(Y) SYDKLPENNAMLNLA(K)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>48.3</td>
<td>248</td>
<td>(K) YTYGHAATTIYPAAGGSDLWAYDQGK(Y)</td>
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<tr>
<td>Trypsin</td>
<td>52.6</td>
<td>92</td>
<td>(R) EAVLTGYESHMTDI(N)</td>
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<tr>
<td>Trypsin</td>
<td>58.79</td>
<td>277</td>
<td>(R) YGFLPSGQATCETMLAIK(Y)</td>
</tr>
<tr>
<td>Trypsin</td>
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<td>277</td>
<td>(R) YGFLPSGQATCETMLAIK</td>
</tr>
<tr>
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<td>(F) ELRDKGK(G)</td>
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<tr>
<td>Chymotrypsin</td>
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<td>92</td>
<td>(Y) GYSHMTEF(L)</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>41.77</td>
<td>248</td>
<td>(T) IYPAAAGGSDL(A)</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>41.83</td>
<td>92</td>
<td>(Y) GYSHMTEF(L)</td>
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<tr>
<td>Chymotrypsin</td>
<td>42.06</td>
<td>248</td>
<td>(T) IYPAAAGGSDL(A)</td>
</tr>
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</table>

Summary of identified peptides originating from proteolytic digestion of porcine CPB. Tryptic and chymotryptic digestions of untreated CPB and CPB reacted with peroxynitrite were subjected to reverse-phase HPLC. Absorbance at 365 nm was used to monitor the elution of the nitrated peptide fragment. The fractions showing intense signals at 365 nm were collected and further characterized by tandem mass spectrometry. The nitrated tyrosine residues (YNO₂) are underlined in the “Sequence” column.
Altered patho-physiology in septic spleen following NOS-3 and CPB1 coupling

FIGURE 8. Tyrosine nitration of CPB1 in the sinus lining cells of the spleen. Proximal association and binding of CPB1 and NOS-3 possibly resulted in higher nitration yield in the local milieu, leading to loss of CPB1 activity. This would possibly lead to the accumulation of inflammatory mediators like C5a in the spleen, thus amplifying the systemic inflammatory response and altering immune pathology.

The significant inactivation of CPB1 in vivo was probed further for any functional alteration of the enzyme. The hydrolysis of peptide bonds at the C terminus of peptides and proteins conducted by carboxypeptidases may be a step in the degradation of some substrate molecules or result in the maturation of others. The physiological effect of these enzymes, as for every type of protease, is thus varied and site- and organism-dependent (40). The presence of significantly increased levels of CPB1 has been identified in the spleens of septic mice (11). Because enzymes of the carboxypeptidase family, especially CPB2 or TAFI, have been known to cleave basic arginine and lysine residues from peptides like C5a and bradykinin and regulate inflammation (10), the functional significance of enzyme inactivation was probed in septic mice. The results of increased accumulation of C5a in LPS-administered mice (Fig. 5) and their regulation by NOS-3 and XO inhibitors may indicate a broader role for tyrosine nitration of CPB1.

In conclusion, we report the posttranslational tyrosine nitration of CPB1 with significant loss of its activity and concomitant accumulation of C5a in the spleen. The pathological consequences observed might be a result of high nitration rates due to the proximal association of CPB1 and NOS-3 in the sinus lining cells of the red pulp.
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