

Neutral Endopeptidase Modulation of Septic Shock

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Summary

Neutral endopeptidase (NEP; EC. 3.4.24.11) is a type 2 cell surface metalloprotease known by a variety of eponyms, including enkephalinase, common acute lymphoblastic leukemia antigen, and CD10. Identified substrates are largely neural or humoral oligopeptide agonists, and the enzyme functions to terminate signaling by degrading the ligand, analogously to acetylcholine/acetylcholinesterase. Targeted disruption of the NEP locus in mice results in enhanced lethality to endotoxin shock with a pronounced gene dosage effect. The site(s) of action appears downstream from release of tumor necrosis factor and interleukin-1 since NEP-deficient animals demonstrate increased sensitivity to these mediators as well. This unexpected finding indicates an important protective role for NEP in septic shock.

Peptide signaling molecules are controlled at the level of synthesis and release. Another level of control can exist through their degradation, analogous to the elimination of acetylcholine by acetylcholinesterase. Neutral endopeptidase (NEP) appears particularly well suited as a model system for studies of proteolytic control of biologically active peptides (1). NEP is a member of a newly recognized family of metalloproteinases that includes the endothelin-converting enzyme and the Kjell blood group antigen (2). The NEP enzyme is colocalized with peptidergic neurons in the central nervous system, along mucosal epithelial surfaces, and in the brush border membrane of the kidney. Pre-B cell expression of NEP was first noted as the common acute lymphoblastic leukemia antigen, a marker for a childhood leukemia (3, 4). Inflammatory cells such as the PMN are also positive for NEP expression (5).

Highly specific, rationally designed NEP inhibitors such as thiorphan (6) have been used as pharmacologic tools, and they potentiate the activity of particular peptide agonists, in some cases, by several orders of magnitude. Such studies have identified two broad classes of substrates subject to NEP control in vivo, neuropeptides including enkephalins, tachykinins, and bombesin-like peptides (7-9), and humoral mediators including bradykinin, atriopeptin, and endothelins (10-12).

In the present report, we characterize mice that bear a targeted disruption of the NEP gene in two models of shock. The models were chosen because of the wide variety of potential NEP substrates that may be present in septic shock.

Materials and Methods

Gene Targeting of the NEP Locus. A murine Sv 129 genomic library was screened with the human NEP cDNA, resulting in

multiple overlapping clones. A 6.5-kb genomic fragment containing exons 10-14 was digested with BglII, and the 0.6-kb fragment was replaced with a neomycin resistance cassette driven by the glucokinase promoter.

The targeting fragment was subcloned into the pPNT vector (13) for positive/negative selection with geneticin and gancyclovir. Electroporation into J1 ES cells ($\sim 10^7$ cells, 16 μ g targeting vector, 400 V at 25 μ F) was followed by plating on 75 cm² of neomycin-resistant primary embryonic fibroblasts derived from Sv 129 mice homozygous for β_2 -microglobulin deficiency and irradiated with 2,400 rad. 12 h after electroporation, confluent ES cells were passed 1:4 after brief trypsin treatment and were plated on irradiated feeders cells. Selection was initiated with 0.5 mg/ml geneticin and 2 μ M gancyclovir. On day 8, 200 resistant clones were picked with a micropipette and were expanded. Approximately 3% of clones were correctly targeted and were introduced into C57Bl/6 blastocysts. Chimeric males were bred with C57Bl/6 females to yield germ line transmission of the targeted allele. Heterozygous progeny were inbred yielding littermates used in these experiments.

Biochemical and Biological Assays. NEP enzymatic activity was assayed fluorometrically using glutaryl-Ala-Ala-Ala-Phe- β -naphthylamide in a two-stage assay using aminopeptidase M (5), as described in the legend of Fig. 2. Renal membranes were prepared by homogenization of kidneys in 10 mM Tris-HCl, pH 7.5, containing 0.2 M sucrose, followed by centrifugation. Membranes obtained from a 20,000 g pellet were resuspended in 100 mM morpholinethanesulfonate buffer, pH 6.5, containing 300 mM NaCl.

Endotoxin lipopolysaccharide (*Salmonella enteritidis*, L6011; Sigma Chemical Co., St. Louis, MO) plus 8 mg D-galactosamine/20 g body wt were injected i.p. at time 0 into male littermates at 8-10 wk of age, ~ 2.5 h into the animals' light cycle (14). TNF- α (CS064071; R&D Systems, Minneapolis, MN) and IL-1 β (BN024071; R&D Systems) were injected via the tail vein in similar groups. Lethality was assessed after 24 h. In the endotoxin experiments, wild-type mice (19 total, minimum of 3 per dose), NEP heterozy-

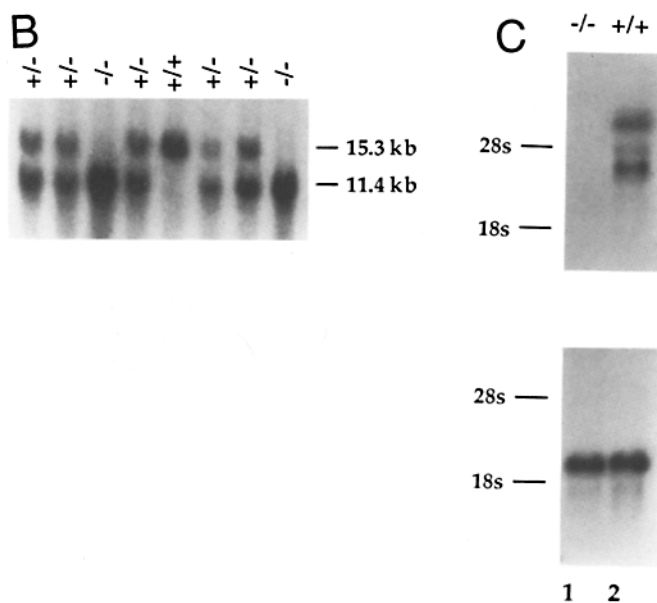
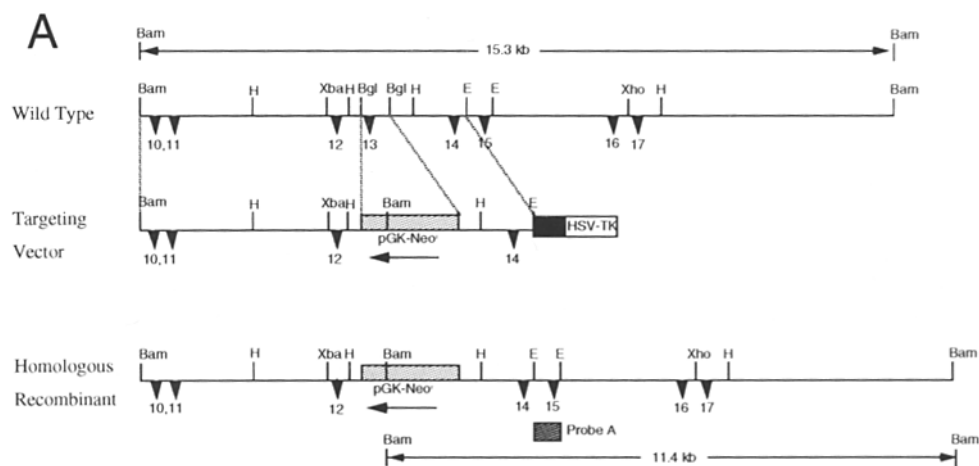


Figure 1. Gene targeting of the neutral endopeptidase locus. (A) Restriction map of the murine NEP locus encompassing exons 10–17, targeting vector, and predicted map following homologous recombination. Homologous recombination introduces a new BamHI site into the gene, which can be detected with the flanking probe A (*Bam*, BamHI; *Bgl*, BglII; *E*, EcoRI; *H*, HindIII; *Xba*, XbaI; *Xho*, XhoI). (B) Southern analysis of a representative litter by tail snip DNA digestion with BamHI. The wild-type allele migrates at 15.3 kb, while the replacement of exon 13 yields a 11.4-kb fragment with flanking probe A. (C) Northern analysis of renal poly(A⁺) RNA (~5 μg) from NEP (-/-) and (+/+) littermates. (Upper panel) The “knockout” mouse does not display a detectable NEP message using a cDNA probe (~500-bp EcoRI fragment) spanning the active site region, exons 14–20, while a series of mRNAs that correspond to multiple different 5' and 3' untranslated regions are seen in the wild type (18-h exposure, -70°C) (28). (Lower panel) Rehybridization of the membrane with cDNA corresponding to β-actin indicates similar amounts of RNA in each lane (2-h exposure, -70°C).

gotes (+/-) (34 total), and NEP homozygous deficient (-/-) (33 total) animals were housed under barrier conditions. All animal experiments and animal care procedures were performed under the guidelines of Children's Hospital (Boston, MA). Other experimental details are given in the figure legends.

Statistical Analysis. Multiple logistic regression was used to assess the significance of dose and gene copies for risk factors of death. The probability of death was modeled as a function of LPS and gene doses, with LPS dose as a continuous variable and gene dose as a categorical covariate with three levels (i.e., none, one, or two

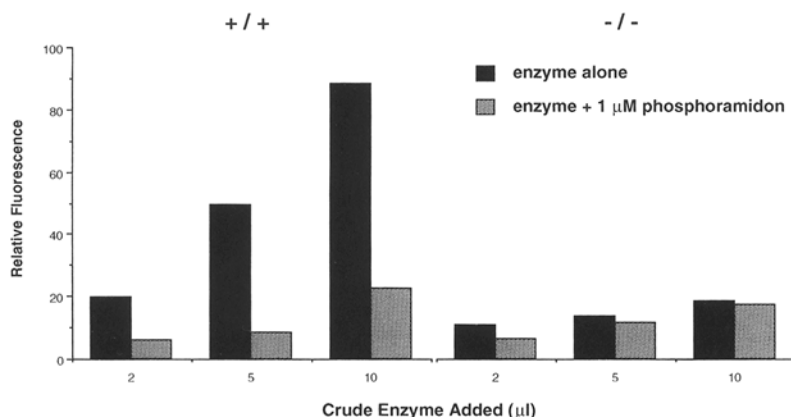


Figure 2. Enzymatic analysis of neutral endopeptidase littermates. Renal membranes were suspended in 100 mM MES buffer, pH 6.5, containing 300 mM NaCl with or without 1 μM phosphoramidon. Glutaryl-Ala-Ala-Phe-MNA (100 μM) was incubated with enzyme for 30 min at 37°C, and the reaction was terminated with boiling. The mixture was next treated with 6 mU aminopeptidase M at 37°C for 30 min. Aliquots of the reaction were assayed for relative fluorescence (extinction = 340 nm, emission = 425 nm).

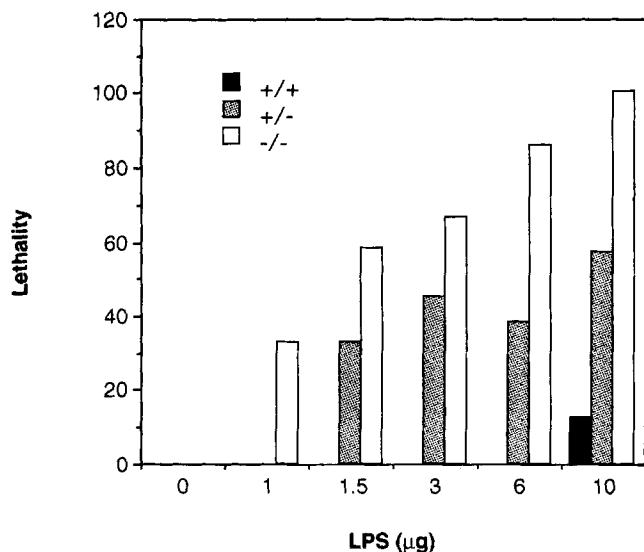


Figure 3. Lethal toxicity of LPS in neutral endopeptidase transgenics. Transgenic littermates (male, 8–10 wk old) were sensitized with 8 mg of D-galactosamine and the indicated i.p. dose of LPS (*S. enteritidis*, Sigma L6011) calculated per 20 g body wt. Mortality was recorded for 24 h after dosing.

alleles). A main effects model was fitted and the likelihood ratio test was used to evaluate each risk factor. Odds ratios were calculated to compare risks between the three genotype groups. LD₅₀ was estimated by probit analysis.

Results and Discussion

To extend our insight into the *in vivo* functions of this protease, the murine NEP gene was disrupted by replacing exon 13 and part of intron 13 with a gene encoding neomycin resistance (*neo^r*) in a 6.5-kb targeting vector (Fig. 1 A). Electroporation into J1 embryonic stem (ES) cells was followed by positive/negative selection with geneticin and gancyclovir. The correct targeting event was detected in 6 out of 200 ES cell clones that were screened. After blastocyst injection, mutant alleles were identified in the resulting chimeric mice and through the germline F1 animals. Heterozygous NEP (*-/+*) animals were bred to generate homozygous NEP-deficient (*-/-*) animals. The mutated allele was found in the expected Mendelian ratios, indicating no enhanced prenatal mortality (Fig. 1 B).

The transgenic NEP (*-/-*) mice were grossly developmentally normal, although subtle differences in lymphoid development were observed (15). To confirm the abrogation of NEP activity in the targeted animals, Northern analyses and enzymatic assays were performed (Figs. 1 C and 2) using renal tissues as a rich source of enzyme message and protein. Using a probe containing the known active site of the enzyme, we detected no NEP mRNA in the targeted animals. In addition, virtually all phosphoramidon-sensitive estero-lytic activity was absent in crude renal membranes from the NEP (*-/-*) strain (Fig. 2). The low level of phosphoramidon-insensitive enzymatic activity observed is likely a reflection of additional (non-NEP) metalloprotease activity (16).

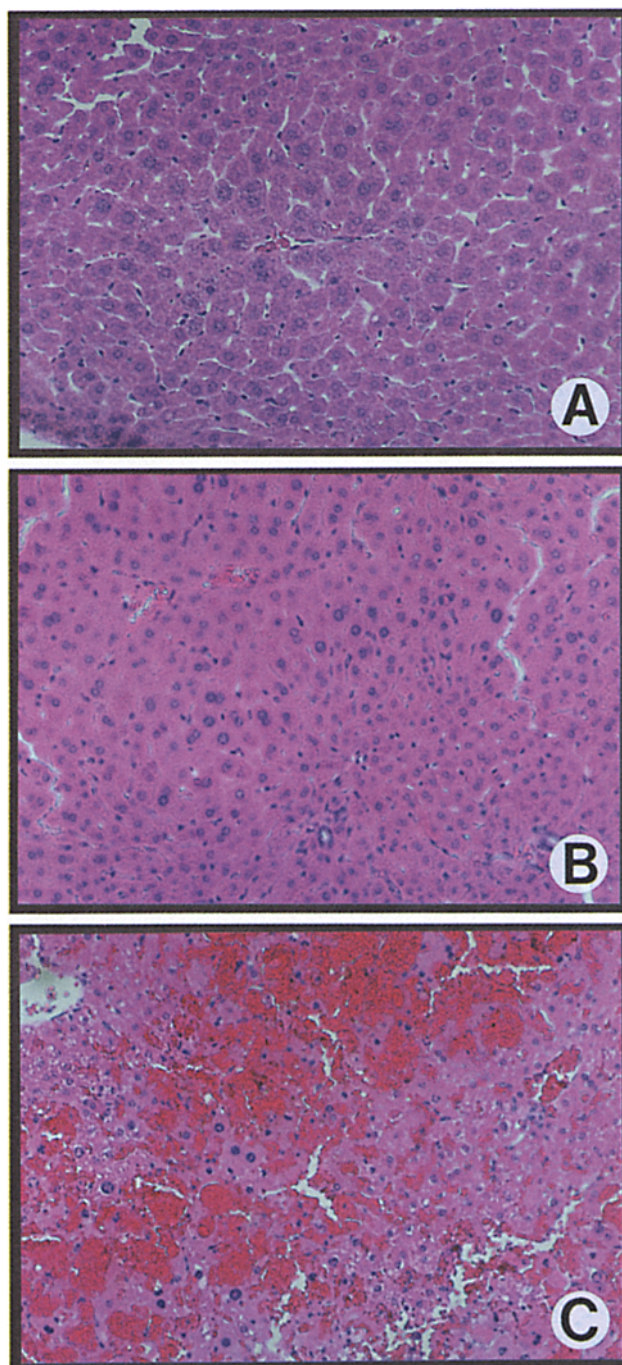


Figure 4. Hepatic pathology after LPS shock. Representative hematoxylin and eosin sections of hepatic tissues in LPS shock. (A) Wild type, no LPS/galactosamine. (B and C) Livers from wild-type and NEP-deficient animals, respectively, 5 h after 10 mg LPS + 8 mg D-galactosamine/20 g body wt. Note the hemorrhagic necrosis in the NEP-deficient (*-/-*) animal (originally $\times 40$).

Because of the role of NEP in terminating the actions of a wide variety of proinflammatory peptides, we challenged mice with endotoxin LPS and D-galactosamine. LPS is a complex inflammatory stimulus that activates parallel proinflammatory humoral pathways including the coagulation, kininogen,

and complement cascades, and that stimulates macrophages to release cytokines, including TNF and IL-1 (17, 18). NEP-deficient (-/-) animals demonstrated >10-fold increased sensitivity to endotoxin relative to wild-type littermates (Fig. 3). There was a highly significant effect of dose on mortality (likelihood ratio test = 13.49 on 1 d.f., $P < 0.001$), as well as the number of gene copies on mortality (likelihood ratio test = 32.21 on 2 d.f., $P < 0.001$). Probit analysis revealed an LD₅₀ of 1.2 µg for NEP (-/-) animals, 6.7 µg for NEP (-/+) heterozygotes, and >16.7 µg for wild-type NEP (+/+) mice. Homozygous NEP deficient mice were >100 times more likely to die than wild-type mice (odds ratio = 127.82, $P < 0.001$); heterozygotes were almost 25 times more likely to die than wild type (odds ratio = 24.36, $P < 0.01$); gene-deleted animals were five times as likely to die as heterozygotes (odds ratio = 5.25, $P < 0.01$). At the time of autopsy, the knockout animals had gross discoloration (blue black) of the liver, as did the (-/+) heterozygotes. Histopathologic analysis revealed diffuse hemorrhagic necrosis in these livers, but not in the livers of their wild-type littermates (Fig. 4). Additionally, both wild-type and NEP (-/-) mice that were given LPS or D-galactosamine alone did not show evidence of gross pathology.

No differences were observed in the coagulation profile in endotoxin-treated wild-type or knockout animals (thrombocytopenia, elevated coagulation times, elevated fibrin split products; data not shown). To determine whether the observed sensitivity to LPS-induced shock occurred in parallel or downstream from cytokine release, mice were injected with the combination of TNF-α and IL-1β (1 and 0.5 µg, respectively) (Table 1) (19). A marked difference in susceptibility to this combination was also observed for NEP (-/-) mice. While all of the NEP-deficient animals that were injected succumbed to this cytokine mixture, only one of six wild-type mice developed irreversible shock. Thus, the enhanced lethality to galactosamine-sensitized endotoxin shock is carried over in a model using the more defined stimulus TNF-α + IL-1β. Given the complexity of the endotoxin response, these results confirm a critical role for NEP in modulating circulatory shock states. Clearly, since neither TNF-α nor IL-1β is a substrate for the protease, the data indicate that these cytokines must stimulate release and potentially upregulation of additional NEP-sensitive peptides that

Table 1. Lethal Toxicity of TNF-α + IL-1β in NEP Transgenics

Genotype	TNF-α ± IL-1β	Lethality (deaths/total)
	<i>mg</i>	
Wild type (+ / +)	1.0 ± 0.5	1/5
NEP (- / -)	1.0 ± 0.5	6/6

Male mice (8–10 wk old) were treated with recombinant mouse TNF-α and IL-1β. The mixture of both reagents in 0.15 ml saline was administered via the tail vein. The mice died within 5–12 h after dosing. The two-tailed probability based on Fisher's exact test indicates a significantly higher proportion of death in the gene-deleted group ($P = 0.015$).

enhance lethality. The potentiation of lethal shock is likely caused by a complex interplay of NEP substrate peptides that are released in shock states, including endothelin, bradykinin, tachykinins, atriopeptin, and enkephalins (20–23). These peptides are capable of acting synergistically to cause ischemia, hemoconcentration, and vascular permeability changes that would contribute to the perfusion defects in shock states.

The significant gene dosage effect noted in heterozygous deficient animals upon LPS challenge indicates that NEP enzyme levels in vivo operate near saturation in shock states. Heterozygous phenotypes consistent with gene dosage effects have also been seen with endothelin-1 and G-CSF gene disruption (24, 25).

NEP (-/-) animals at LD₁₀₀ for LPS were protected from lethal shock by i.p. injection of recombinant human NEP (Lu, B., unpublished observation). The metalloprotease that processes TNF to its mature form is related but not identical to NEP, based on the inhibitor profile (26–28). Inhibition of this enzyme afforded protection against lethal endotoxin shock, indicating that zinc metalloproteases regulate both pro- and antiinflammatory pathways.

Mice genetically deficient in NEP will also be useful for understanding the role of this enzyme in pain pathways (29), neurogenic inflammation (30, 31), and lymphoid development (32).

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