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A Subcytotoxic Dose of Subtilase Cytotoxin Prevents Lipopolysaccharide-Induced Inflammatory Responses, Depending on its Capacity to Induce the Unfolded Protein Response

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Subtilase cytotoxin (SubAB) is the prototype of a newly identified family of Aβ cytotoxins produced by Shiga toxigenic Escherichia coli. SubAB specifically cleaves the essential endoplasmic reticulum (ER) chaperone BiP (GRP78), resulting in the activation of ER stress-induced unfolded protein response (UPR). We have recently shown that the UPR following ER stress can suppress cellular responses to inflammatory stimuli during the later phase, in association with inhibition of NF-κB activation. These findings prompted us to hypothesize that SubAB, as a selective UPR inducer, might have beneficial effects on inflammation-associated pathology via a UPR-dependent inhibition of NF-κB activation. The pretreatment of a mouse macrophage cell line, RAW264.7, with a subcytotoxic dose of SubAB-triggered UPR and inhibited LPS-induced MCP-1 and TNF-α production associated with inhibition of NF-κB activation. SubA272B, a SubAB active site mutant that cannot induce UPR, did not show such effects. In addition, pretreatment with a sublethal dose of SubAB, but not SubA272B, protected the mice from LPS-induced endotoxic lethality associated with reduced serum MCP-1 and TNF-α levels and also prevented the development of experimental arthritis induced by LPS in mice. Collectively, although SubAB has been identified originally as a toxin associated with the pathogenesis of hemolytic uremic syndrome, the unique ability of SubAB to selectively induce the UPR may have the potential to prevent LPS-associated inflammatory pathology under subcytotoxic conditions. The Journal of Immunology, 2009, 183: 1368–1374.

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viability in macrophages. Next, the effects of such a subcytotoxic dose of SubAB on LPS-induced MCP-1 and TNF-α production in macrophages were examined. Finally, the effects of a sublethal dose of SubAB on LPS-mediated endotoxic lethality and experimental arthritis were determined in vivo.

Materials and Methods

Reagents

SubAB was purified as previously described (1), except that the toxin was expressed in a lpxM mutant of Escherichia coli BL21(DE3). Because the E. coli BL21(DE3) lpxM- host strain produces a penta- rather than hexa-acylated (nonmyristoylated) LPS, it has very low LPS activity as described by Cognet et al., (13). SubAA272B, a nonfunctional variant of SubAB that has a point mutation in the active site serine residue of SubA subunit and has lost its protease (and also UPR-inducing) activity, was developed by site-directed mutagenesis of SubA as previously described (1). Both the native and mutant SubAB proteins are purified as B-subunit C-terminal His6 fusions by Ni-NTA chromatography (there is no His6 on the A subunit). LPS was purchased from Sigma-Aldrich. Mouse recombinant IFN-γ and M-CSF were purchased from R&D Systems.

FIGURE 1. Effects of SubAB on cellular viability in RAW264.7 cells. A, RAW264.7 cells were treated with 1, 10, 100, and 1000 ng/ml SubAB (left) or SubAA272B (middle) for 24 h. RAW264.7 cells were pretreated with 100 ng/ml SubAB for 8 h and then LPS (10 ng/ml) was added into the culture for 3, 6, an 12 h (right). Following these treatments, cell viability was measured by the WST assay. The data were represented as OD450. Values represent the mean ± SD of triplicate samples per group (n = 3), *p < 0.05. Similar results were obtained from three independent experiments. B, RAW264.7 cells treated with 100 ng/ml SubAB, SubAA272B, or LPS (150 μg/ml) plus IFN-γ (10 μg/ml) for 24 h were stained with FITC-conjugated anti-annexinV and PI. Percentage of annexin V positive cells were then determined by a FACS analysis. Similar results were obtained from three independent experiments. C, RAW264.7 cells were treated with 100 ng/ml SubAB or LPS (150 μg/ml) plus IFN-γ (10 μg/ml) for 24 h. Cell lysates were then subjected to a Western blot analysis with anti-cleaved caspase 3 Ab or anti-β-actin Ab. A quantitative analysis of the Western blot analysis using densitometry (normalized to actin) is also shown. Similar results were obtained from three independent experiments.

FIGURE 2. A, RAW264.7 cells were treated with 100 ng/ml SubAB or SubAA272B for the indicated times. Cell lysates were then subjected to a Western blot analysis with anti-BiP Ab which can both detect BiP (78 kDa) and cleaved Bip (28 kDa) and anti-β-actin Ab. A quantitative analysis of the Western blot analysis using densitometry (normalized to actin) is also shown. Similar results were obtained from three independent experiments. B, RAW264.7 cells were stimulated with 100 ng/ml SubAB for 3 h. RNA was then extracted from the cells and ATF4, BiP, or CHOP mRNA expression was quantified by real-time PCR. Values represent the mean ± SD of triplicate samples per group. *p < 0.05.
**Mice**

Female 4- to 6-wk-old BALB/c mice were purchased from SLC and were bred under specific pathogen-free conditions. All animal experiments were approved by the Institutional Review Board of University of Yamanashi.

**Cell culture**

The mouse macrophage cell line RAW264.7 (American Type Culture Collection) was maintained in MEM (Invitrogen) containing 10% FCS and antibiotics. Mouse peritoneal macrophages were harvested by peritoneal lavage 4 days after i.p. administration of 3 ml PBS containing 3% thioglycollate medium (BD Pharmingen) and were maintained in DMEM (Invitrogen) containing 10% FCS and antibiotics. To generate bone marrow-derived macrophages, the femoral bone marrow cells of mice were cultured in RPMI 1640 containing 10% FCS and antibiotics in the presence of 5 ng/ml M-CSF. After 3 days, the nonadherent cells were washed out and fresh medium was added. The medium was changed every 2 days and the cells were harvested with 1 × trypsin-EDTA.

**Cell viability assay**

The cells (5 × 10^5 cells/well) were cultured in DMEM containing 0.1% FCS in a flat-bottom 96-well microtiter plate. Cell viability was determined by measuring the metabolic activity using 2-(4-iodophenyl)-3-(4-nitophenyl)-5-(2, 4-disulfophenyl)[2H]tetrazolium monosodium salt (WST) using Tetra Color ONE kit (Seikagaku Corporation) according to the manufacturer’s instructions.

**Flow cytometric analysis**

The cells (1 × 10^6) were incubated with 5 μl FITC-conjugated annexin V and 5 μl propidium iodide (BD Pharmingen) in 500 μl PBS for 15 min. After washing with PBS, the cells were analyzed on FACSCalibur (BD Biosciences) and the data were analyzed using the CellQuest Pro software program (BD Biosciences).

**Western blotting**

Western blot analysis was performed by using anti-KDEL (BiP/GRP78) Ab (Stressgen), anti-cleaved caspase 3 Ab (Cell Signaling Technology), anti-IκBα Ab, and anti-β-actin Ab (Santa Cruz Biotechnology) as previously described (14). These primary Abs were used at a 1/1000 dilution.

**Quantitative real-time PCR**

Quantitative real-time PCR with specific primers and probes (for mouse AIF4, BiP, and C/EBP homologous protein (CHOP) purchased from Applied Biosystems) was performed using the AB7500 real-time PCR system (Applied Biosystems) as previously described (15).

**ELISA**

The amounts of MCP-1 and TNF-α in the culture supernatants or the mouse serum were determined using the mouse MCP-1 or TNF-α ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Transcriptional reporter assay**

RAW264.7 cells were seeded at 3 × 10^4/well in 24-well plates. We then transfected the cells with 200 ng of NF-κB reporter plasmid (NF-κB TransLucent Reporter Vector, Panomics), which detected the activation of NF-κB, and 5 ng pRL-CMV (Promega) using FuGENE6 transfection reagent (Roche Diagnostic Systems). After 12 h, the cells were stimulated with 100 ng/ml SubAB. Eight hours later, 10 ng/ml LPS was then added to the culture. Six hours after the stimulation, the firefly and Renilla luciferase activities were measured as previously described (12).

**Northern blot analysis**

Total RNA was extracted from the spleens of mice i.p. injected with PBS, 1 μg/mouse SubAB, or SubA272B 9 h after the injection. Northern blot analysis was then performed as described before (12). cDNAs for BiP and CHOP were used for preparation of radio-labeled probes. Expression of GAPDH was used as a loading control.

**Induction of endotoxemia**

The mice were pretreated i.p. with either PBS, 1 μg/mouse SubAB, or SubA272B. Two days after the treatment, the mice were injected i.p. with 5 ng/kg LPS (Sigma-Aldrich) and the survival of mice was observed over 7–10 days as previously described (16). For analysis of cytokine production, blood from living mice was collected at 2 h after the LPS injection and then the serum specimens were collected.

**Induction of arthritis**

To establish anti-collagen type II Ab-induced arthritis (17), the mice were injected i.p. with 2 mg per mouse of anti-collagen type II mAb mixture (Chondrex; day 1) and 3 days later (day 4) with 50 μg per mouse of LPS.
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A subcytotoxic dose of SubAB inhibits LPS-induced NF-κB activation in RAW264.7 cells. A. RAW264.7 cells pretreated with 100 ng/ml SubAB or PBS for 8 h were stimulated with 10 ng/ml LPS for the indicated times. The cell lysates were then subjected to a Western blot analysis with specific Abs for IkBα and β-actin. A quantitative analysis of the Western blot analysis using densitometry (normalized to actin) is also shown. Similar results were obtained from three independent experiments. B. RAW264.7 cells were transiently transfected with pNF-κB-luc, pretreated with 100 ng/ml SubAB or PBS for 8 h, and then exposed to 10 ng/ml LPS for 6 h. The cell lastaes were then subjected to luciferase assay to evaluate activity of NF-κB. Values represent the mean ± SD of triplicate samples per group. *, p < 0.05. Similar results were obtained from three independent experiments.

Clinical evaluation of arthritis

Starting on day 1 after mAb injection, the mice were blindly inspected for disease progression. The clinical severity of the disease was scored using a scoring system based on the number of inflamed joints in forepaws, hindpaws, and ankles, inflammation being defined by swelling: 0, normal; 1, slight swelling; 2, mild swelling; 3, moderate swelling; and 4, severe swelling. All paws and ankles were graded, thus resulting in a maximal clinical score of 24 per mouse and then were expressed as the mean ar-thritic index on a given day.

Statistical analysis

Values represent the mean ± SD. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. The statistical analysis of mouse survival data was performed using the Logrank test. A value of p < 0.05 was considered to be significant.

Results

Determination of a subcytotoxic dose of SubAB in the RAW264.7 macrophage cell line

The effects of highly purified LPS-free SubAB on the viability of a mouse macrophage cell line RAW264.7 were examined using a metabolic activity-based WST-1 assay. SubAB at the concentrations of 1, 10, and 100 ng/ml did not affect the cell viability in this assay at 24 h although SubAB at 1000 ng/ml decreased the cell viability (Fig. 1A, left). In contrast, treatment with SubAα272B, a nontoxic variant of SubAB that has a point mutation in the active site serine residue of the A subunit (SubA) and has lost its protease (and also UPR-inducing) activity, did not affect the cell viability at concentrations of 1, 10, 100, and 1000 ng/ml (Fig. 1A, middle). In addition, stimulation of RAW264.7 cells with LPS (10 ng/ml) following 100 ng/ml SubAB pretreatment also did not affect the cellular viability and, rather, appeared to slightly enhance it (Fig. 1A, right, see below). Therefore, SubAB up to the concentration of 100 ng/ml appeared to be subcytotoxic to RAW264.7 cells.

To further confirm these findings, the effects of 100 ng/ml SubAB on cellular apoptosis/necrosis were next examined using an Annexin V-FITC assay. Stimulation with LPS plus IFN-γ (positive control) significantly induced apoptosis/necrosis as judged by

the Annexin V positive fraction in RAW264.7 cells at 8 h after the stimulation, whereas SubAB or SubAα272B at 100 ng/ml did not affect apoptosis/necrosis at either 3 or 8 h after the stimulation (Fig. 1B). Although there appeared to be more death in the SubAB- or SubAα272B-treated cells in the PI-positive fraction, they were not statistically significant. Consistent with these findings, activation of caspase 3 (cleaved caspase 3) was detected in RAW264.7 cells treated with LPS plus IFN-γ, but it was not detected in RAW264.7 cells treated with 100 ng/ml SubAB (Fig. 1C).

Although 100 ng/ml SubAB did not affect cellular viability in RAW264.7 cells at 24 h, SubAB at this dose efficiently cleaved BiP protein (Fig. 2A). The BiP cleavage occurred ~8 h after SubAB treatment. In contrast to SubAB, 100 ng/ml SubAα272B did not induce the BiP cleavages (Fig. 2A). In addition, 100 ng/ml SubAB increased the mRNA expression of ATF4, BiP, and CHOP, representative markers for the UPR (6) (Fig. 2B), confirming that this dose of SubAB induced the cleavage of BiP and subsequent UPR in RAW264.7 cells.

A subcytotoxic dose of SubAB inhibits LPS-induced MCP-1 and TNF-α production in RAW264.7 cells

Because 100 ng/ml SubAB had the capacity to induce the UPR without affecting cellular viability in RAW264.7 cells, the effects of this dose of SubAB on LPS-induced MCP-1 and TNF-α production in RAW264.7 cells were examined. Pretreatment with SubAB inhibited LPS-induced MCP-1 and TNF-α production in RAW264.7 cells (Fig. 3A). In contrast to SubAB, pretreatment with SubAα272B did not inhibit either the LPS-induced MCP-1 and TNF-α production (Fig. 3B). As shown earlier (Fig. 1A, right), stimulation with LPS following SubAB pretreatment did not affect cell viability of RAW264.7 cells. In addition, pretreatment with 100 ng/ml SubAB also significantly inhibited LPS-induced MCP-1 and TNF-α production in primary mouse peritoneal macrophages (Fig. 3C). SubAB at this dose did not affect the cell viability as judged by WST assay (Fig. 3D). Furthermore, pretreatment with 100 ng/ml SubAB also significantly inhibited LPS-induced MCP-1 production in mouse bone marrow-derived macrophages (Fig. 3E). Although it appeared that SubAB also tended to inhibit the basal levels of MCP-1 production in some experiments (Fig. 3, A, left,
and C, left), the findings were not consistently observed in any other experiments.

Because LPS-induced MCP-1 and TNF-α production involves the NF-κB pathway (8, 18), the effects of SubAB on LPS-induced IkBα activation (degradation) were then examined in RAW264.7 cells (Fig. 4A). PBS or 100 ng/ml SubAB alone did not affect the protein levels of IkBα, whereas LPS induced IkBα degradation, beginning at 15 min after the stimulation, and it then completely recovered at 60 min. Pretreatment with SubAB partially inhibited the LPS-induced IkBα degradation at 15 min. More distinctly, pretreatment of RAW264.7 cells with SubAB significantly inhibited LPS-induced increase in NF-κB reporter activity (Fig. 4B).

These results indicated that pretreatment with a subcytotoxic dose of SubAB significantly inhibited LPS-induced MCP-1 and TNF-α production in RAW264.7 cells, depending on its protease activity and in association with the inhibition of NF-κB activation.

**Pretreatment of mice with a sublethal dose of SubAB protects against LPS-induced mortality and LPS-induced experimental arthritis in mice**

Because pretreatment with SubAB down-regulated LPS-induced MCP-1 and TNF-α production in macrophages, the protective effects of SubAB on LPS-induced endotoxic lethality were examined in rodents (16).

Mice that received SubAB (1 μg per mouse) survived at least 14 days, whereas most of the mice that received SubAB (10–100 μg per mouse) died before 14 days after SubAB treatment (data not shown). However, the mice that received SubAB (1 μg per mouse), but not SubAA272B, showed strong increases in BiP and CHOP mRNA expression in the spleen 9 h after the challenge (Fig. 5A), suggesting that this dose of SubAB indeed induced the UPR in vivo. Therefore, this sublethal dose of SubAB was used for the following studies.

Eighty percent of the mice pretreated with control PBS died within 7 days after LPS challenge (Fig. 5B). In contrast, 50% of the mice pretreated with SubAB survived this dose of LPS injection. Consistent with the involvement of cytokines in endotoxemia (19), the serum concentrations of MCP-1 and TNF-α were greater in the PBS-pretreated mice than in those of SubAB-pretreated mice (Fig. 5C). In contrast to SubAB, 80% of the mice pretreated with the nonfunctional variant of SubAB, SubAA272B, died within 7 days after LPS challenge (Fig. 5B). These results indicated that pretreatment of mice with a sublethal dose of SubAB protected the mice from LPS-induced lethality.

**FIGURE 5.** Pretreatment with a sublethal dose of SubAB protects mice against LPS-induced lethality and experimental arthritis. A–C, Mice were i.p. injected with PBS, 1 μg/mouse SubAB, or SubAA272B. Forty-eight hours after the injection, the mice were i.p. challenged with a lethal dose of LPS (5 mg/kg). A, Northern blot analysis showing BiP and CHOP expression in the spleen obtained from mice 9 h after i.p. injection with PBS, 1 μg/mouse SubAB, or SubAA272B (n = 2 in each group). B, Survival rates of the mice following LPS injection (n = 8–10 in each group). C, Serum MCP-1 and TNF-α concentrations 2 h after LPS injection. Values represent the mean ± SD of three mice per group. *, p < 0.05. D and E, The mice were i.p. injected with 2 mg per mouse of anti-collagen type II mAb mixture (day 1) and 3 days later (day 4) with 50 μg per mouse of LPS. SubAB, SubAA272B (1 μg per mouse), or PBS was i.p. administered on day 2. D, Representative photographs showing the fore paws of the mice treated with PBS, SubAB, or SubAA272B on day 8. E, Clinical scoring measured during the course of study. The values represent the mean ± SD (n = 6–8 per group); *, p < 0.05.
To further confirm the effects of SubAB on LPS-mediated pathology, the effects of sublethal dose of SubAB were tested using another model. For this purpose, collagen type II Ab-induced arthritis model was used. This model was developed by the injection of mAbs against type II collagen followed by the subsequent injection of LPS (17). It was reported that LPS signaling via TLR4 is essential for the development of this arthritis model (20). In addition, the importance of FcγRI, III, and IV bearing cells, including monocytes/macrophages in the development of this model, have previously been demonstrated (15).

The mice pretreated with control PBS before LPS challenge developed arthritis, beginning on day 5 after the administration of the mAbs, with a peak at around day 8 and thereafter persisting at least until day 11 (Fig. 5, D and E). In contrast, the mice pretreated with a sublethal dose of SubAB (1 μg per mouse) developed limited clinical manifestations of arthritis based on the clinical scoring (Fig. 5, D and E). The mice pretreated with the same dose of SubAA272B developed the full clinical manifestations of arthritis comparable to the mice pretreated with PBS (Fig. 5, D and E). Therefore, pretreatment of the mice with the sublethal dose of SubAB before LPS challenge ameliorated the development of LPS-induced experimental arthritis.

Discussion

Several reports have previously shown that ER stress causes the activation of NF-κB in the early phase by several mechanisms (9, 10). In response to ER stress, IRE1α binds to the 1xBα kinase (IKK) complex and activated NF-κB by promoting the degradation of 1xBα. IRE1α also binds to TRAF2 and activates apoptosis signal-regulating kinase 1, thus leading to the activation of IKK (21). Other investigators have also suggested that, under ER stress, TNFR1 accumulates in the ER and thus forms a complex with TRAF2 and TNFR-interacting protein. This molecular event may therefore lead to the induction of TNF signaling, especially the induction of TNF in macrophages by a selective UPR inducer, SubAB, inhibited LPS-induced inflammatory responses both in vitro and in vivo. Therefore, although SubAB has been identified originally as a toxin associated with hemolytic uremic syndrome pathogenesis (2, 29), a subcytotoxic/lethal dose of SubAB or possible chemical reagents that can selectively induce the UPR might have the potential to prevent LPS-mediated inflammatory pathology such as septic shock. The current study also suggests that the NF-κB pathway is an important target for preconditioning with ER stress (30).

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Disclosures

The authors have no financial conflict of interest.

References

1374 SubAB INHIBITS LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION


