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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

Daisuke Hama,∗ Kensuke Koyama,∗ Mai Mukai,† Naomi Shimokawa,∗ Masanori Miyata,∗ Yuki Nakamura,∗ Yuko Ohnuma,∗ Hideoki Ogawa,§ Shuji Matsuoka,§ Adrienne W. Paton,¶ James C. Paton,¶ Masanori Kitamura,2† and Atsuhito Nakao2∥

Subtilase cytotoxin (SubAB) is the prototype of a newly identified family of AB5 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. SubAB, 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 SubAB, 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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protein response (UPR) signaling pathways (PERK, IRE1, and ATF6 signaling pathways) (3, 4). UPR is an adaptive mechanism which protects the cells against ER dysfunction in several ways including the induction of ER chaperone molecules such as BiP (5, 6). However, prolonged UPR activation eventually triggers apoptosis, which may be responsible for the cytotoxicity of SubAB.

Endotoxin (LPS from Gram-negative bacteria, or LPS) has been implicated as a major cause of septic syndrome that remains the most common cause of death in intensive care units in the USA, with a current estimate of at least 750,000 cases per year and 215,000 deaths annually (7, 8). Exposure to LPS results in the activation of the NF-κB pathway and the release of a number of proinflammatory cytokines and chemokines, including MCP-1 and TNF-α from monocytes/macrophages, which can cause fever, shock, organ failure, and death (8). A number of different approaches have been investigated to try to treat and/or prevent the septic syndrome associated with infections caused by Gram-negative bacteria, including blockade of one or more of the cytokines induced by LPS.

Although UPR can activate NF-κB in the early phase (9, 10), we have recently shown that UPR can suppress cellular responses to inflammatory stimuli in the later phase in association with suppression of the NF-κB pathway using cultured glomerular podocytes and mesangial cells (11, 12). These findings prompted us to hypothesize that a selective UPR inducer, SubAB, if used at subcytotoxic doses and administered at an appropriate time, could prevent LPS-induced inflammatory responses through UPR-dependent inhibition of the NF-κB pathway. To test this hypothesis, this study first determined the dose of SubAB that can cleave BiP protein and consequently induce the UPR without affecting cellular

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Abbreviations used in this paper: SubAB, subtilase cytotoxin; ER, endoplasmic reticulum; UPR, unfolded protein response; WST, 2-(4-isopropylphenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-benzotriazolium monosodium salt; CHOP, CEBP homologous protein.

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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

AB5 SubAB was purified as previously described (1), except that the toxin was expressed in a lpxM/H11002 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). SubA A272B, 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 SubA A272B (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, SubA A272B, 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 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 SubA A272B 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 × 10⁵ 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-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium monosodium salt (WST) using Tetra Color ONE kit (Seikagaku Corporation) according to the manufacturer’s instructions.

Flow cytometric analysis

The cells (1 × 10⁶) were incubated with 5 μl FITC-conjugated annexin V and 5 μl propidium iodine (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-IκBo 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 ATF4, 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 × 10⁴/well in 24-well plates. We then transfected the cells with 200 ng of NF-κB reporter plasmid (NF-κB Trans-Lucent 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 μg/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 SubAA272B 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 SubAA272B. Two days after the treatment, the mice were injected i.p. with 5 mg/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...
the Logrank test. A value of
groups. The statistical analysis of mouse survival data was performed using
the nonparametric Mann-Whitney
Values represent the mean
SD. Statistical analysis was performed using
for 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 SubA$_{272}B$, 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

FIGURE 4. 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 IκBα 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. * $p < 0.05$.

Similarly results were obtained from three independent experiments.

SubAB, SubA$_{272}B$ (1 μg/mouse), or PBS was i.p. administered on day 2.

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 arthritis 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.

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 SubA$_{272}B$ 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. 3A, 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 endotoxemic 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 SubAΔ272B, 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 a 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, SubAΔ272B, 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.
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 FcyRI, 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 SubA272B 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 1xβz kinase (IKK) complex and activated NF-κB by promoting the degradation of 1xβz. IRE1α also binds to TRAF2 and activate 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 activation of IKK, even without TNF-α (22, 23).

In contrast, we have recently shown that preconditioning of the UPR with tunicamycin, thapsigargin, or SubAB blocked TNF-α-induced activation of NF-κB and consequent induction of MCP-1 in cultured rat mesangial cells and murine podocytes (11, 12). Most recently, we showed that A20, one of the major negative regulators for NF-κB, was induced by tunicamycin, thapsigargin, or SubAB in rat mesangial cells and the knockdown of A20 by RNA interference significantly reversed the blunted response of NF-κB by preconditioning with these reagents, thus suggesting that the UPR-induced A20 expression is involved in the inhibition of NF-κB by the preconditioning of the UPR (24). We found that the induction of A20 was observed from 2 to 9 h after the stimulation with tunicamycin, thapsigargin, or SubAB in the rat mesangial cells (24). These findings suggest a molecular mechanism underlying the blunted response of NF-κB in ER stress–primed cells, while also providing further experimental support for the current in vitro and in vivo results. Indeed, in the current study, the inhibitory effects of SubAB on LPS-induced MCP-1 and TNF-α production in RAW264.7 cells and on LPS-induced lethality and arthritis in mice were not observed when the mice were treated with SubAB after LPS challenge (data not shown).

A lesser effect of SubAB on LPS-induced MCP-1 and TNF-α production was observed in primary mouse macrophages (peritoneal or bone marrow–derived) in comparison to that seen in RAW264.7 cells (Fig. 3). In the case of mouse peritoneal macrophages, this might be due to prior in vivo activation of the macrophages by the thiglycollate medium, which is required for the collection of these cells from the mouse peritoneal cavity. Alternatively, it is possible that cell type (e.g., macrophages from different origins) or cellular conditions (e.g., primary cells or cell lines; resting or activated cells) can affect the sensitivity to SubAB, thereby resulting in different responses. The latter interesting issue is presently under investigation.

The i.p. injection of 1 μg SubAB was previously reported to be fatal for mice (1), which differs from the current results. The SubAB used in the current study had been stored frozen for ~2–3 mo, and may have partially lost its activity. Indeed, we observed that the stock samples of SubAB showed less cytotoxic activity than freshly prepared SubAB samples based on an in vitro WST assay (our unpublished observation). Clearly, the dose and potency of SubAB preparations will be an important issue for future studies.

ER stress has been implicated in many diseases, such as neurodegenerative disorders, diabetes, ischemia, cancer, and immune disorders and modulation of ER stress (or the UPR) including the preconditioning with ER stress may provide a therapeutic modality in such diseases (25, 26). For instance, in a mouse acute kidney injury model, preconditioning with ER stress reduced the injury, possibly by enhancing the adaptive UPR, shutting down the translation and expressing ER chaperones to aid in protein folding, which improves the efficiency of energy consumption (26, 27). Our current results also support the notion. To investigate how and when the preconditioning with ER stress should be applied in various ER stress–associated diseases thus remains an important challenge for future studies.

Most recently, Inagi et al. (28) reported that preconditioning with ER stress ameliorated glomerulonephritis in rats, which is consistent with the current study. They used tunicamycin or thapsigargin to induce the ER stress and consequent UPR in their experimental system. However, in contrast to SubAB, these agents can affect multiple cellular functions and it is not clear whether these agents selectively affect only the UPR. In addition, the current study extends their study by addressing a possible mechanism how SubAB ameliorates inflammatory responses; that is, to show the UPR–associated inhibition of NF-κB activation in macrophages.

In summary, this study suggests that preconditioning of the UPR 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
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