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Staphylococcal enterotoxin B causes differential expression of Rnd3 and RhoA in renal proximal tubule epithelial cells while inducing actin stress fiber assembly and apoptosis☆

Boris Ionin [a], Rasha Hammamieh [a], Jeffrey W. Shupp [a], Rina Das [a], Carol H. Pontzer [b], Marti Jett [a,*]

[a] Department of Molecular Pathology, Division of Pathology, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910, USA
[b] NIH, NCCAM, 6707 Democracy Boulevard, Suite 40, Bethesda, MD 20892, USA

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ABSTRACT

Staphylococcal enterotoxin B (SEB) is a toxic shock-inducing agent produced by *Staphylococcus aureus*. The hallmark of SEB-induced lethal shock is acute vasodilation leading to severe hypotension. Animal studies reveal that ≈70% of intravenously administered toxin localizes to renal proximal tubule epithelial cells (RPTEC). This evidence, together with the well-documented role of the kidney in regulation of vascular tone, suggests that molecular events induced in RPTEC by SEB may contribute to the blood pressure dysregulation seen in enterotoxic shock. In an attempt to elucidate these molecular mechanisms, differential display was performed on SEB-treated and untreated RPTEC, and 32 differentially expressed transcripts (DETs) were identified. One of the down-regulated DETs matched the sequence for Rnd3, which normally inhibits Rho protein function. Consistent with Rnd3 down-regulation, message for RhoA was shown to increase upon SEB exposure, and actin stress fiber formation was dramatically increased. Further, SEB-exposed cells showed both increased enzymatic activity of caspase-3 and an increase in the percentage of apoptotic cells. Taken together, these results support the hypothesis that RPTEC undergo apoptosis upon exposure to SEB. Furthermore, these data implicate the involvement of the Rho family proteins in the molecular signaling pathway induced by SEB in RPTEC.

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1. Introduction

Staphylococcal enterotoxins (SEs) are of considerable clinical significance as causative agents in food poisoning and lethal shock [1–4]. Staphylococcal enterotoxin B (SEB) is one of the most clinically important members of the SE family and one of the best studied. SEB exhibits potent enterotoxic, exotoxic, and superantigenic properties, and has been reported as a causative agent in both food poisoning and toxic shock [5], as well as certain autoimmune processes [6].

Of particular interest is the ability of SEB to interact directly with certain target tissues and organs, especially renal epithelium [7,8], a characteristic which may contribute to the shock-inducing properties of this toxin. Its resistance to heat, stability in aerosols (which ensures ease of delivery), and very low lethal (1.7 μg/person) and incapacitating (30 ng/person) inhalation doses, as well as lack of effective therapeutic intervention (other than supportive care), render SEB a potential biological threat agent [9] and, as such, is the subject of intense clinical and basic research interest.

While the causative relationship between SEs and toxic shock syndrome has been well studied, the molecular events underlying the progression of the disease remain largely unknown. The massive release of inflammatory cytokines, resulting from the superantigenic activity of SEs, has been strongly implicated in the development of systemic toxicity [4,10]. However, clinical trials of therapies targeting inflammatory cytokines involved in the mediation of toxic shock have yielded disappointing results [11].

The apparent limitations of anticytokine therapies necessitate an investigation of other potential targets for therapeutic intervention, thus warranting a study of alternative mechanisms involved in the development of SE-induced toxic shock. Evidence from various animal models implicates the kidney, particularly renal proximal tubule epithelial cells (RPTEC), as the major target of SEB uptake [7,8,12–14]. Indeed, 75% of SEB administered to rhesus monkeys localizes to RPTEC [8]. Binding inhibition studies with ^{125}I-SEB revealed that RPTEC contain a glycosphingolipid receptor, later identified as digalactosylceramide, that binds SEB with high specificity and affinity [7,15]. This receptor was shown to be absent in the rodent kidney, confirming earlier observations that revealed

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* Corresponding author. Tel.: +1 301 319 9997; fax: +1 301 319 9699.
E-mail address: marti.jett@us.army.mil (M. Jett).

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differences in SEB localization in rodents and primates [8], and providing further support for the hypothesis that lack of RPTEC–SEB interaction in rodents may contribute to their resistance to SEB intoxication.

It has also been shown that SEB-treated RPTEC undergo morphological changes and even express certain apoptotic markers, suggesting that the toxin may activate an apoptotic signaling pathway in the cells of the proximal tubule epithelium [16]. These data suggest that SEB induces apoptosis in RPTEC. In view of the RPTEC involvement in blood pressure regulation, especially via secretion of endothelins 1 and 2 [17], it may be hypothesized that the effects exerted by SEB on these cells may contribute to the SEB-induced dysregulation of vascular tone. In an attempt to test this hypothesis, RPTEC were exposed to SEB and analyzed for differential gene expression and subsequent morphological changes.

2. Experimental procedures

2.1. Cells

Primary cultures of normal RPTEC from a 34-year-old African American male donor were obtained from Clonetics Corp. (Walkersville, MD). The cultures were maintained in 75-cm^2 or 162-cm^2 tissue culture flasks at 37 °C in 5% CO_2 atmosphere in renal epithelial growth medium (REGM, Clonetics). The cultures were maintained for up to seven passages and used for experiments after the second passage.

2.2. Toxin

SEB from *S. aureus* strain 10-275 was purified by the method of Schantz and co-workers [18] and stored in vacuum ampules. The stock solution was prepared in sterile, pyrogen-free deionized water at a concentration of 5 mg/ml and stored frozen at −80 °C. When used, the stock solution was diluted with cell culture medium to the desired concentration.

2.3. Toxin treatment and total RNA isolation

RPTEC ($3–10 \times 10^6$) were treated with 50 μg/ml of SEB for 2, 6, 12, 24, 48, and 72 h. The reaction was terminated by removing the culture medium and washing the cells with HEPES-buffered saline solution (HBSS, Clonetics). The cells were then removed from the flask by trypsinization for 1 min at room temperature, trypsin inactivated by the addition of trypsin-neutralizing solution (Clonetics), transferred to 50-ml centrifuge tubes, and centrifuged at 500 × g for 10 min at room temperature. The supernatants were decanted and the pellets resuspended in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) per 1×10^6 cells. Total RNA was extracted with Trizol/chloroform mixture (0.2 ml of chloroform/1 ml of Trizol), the aqueous phase transferred into a new 1-ml centrifuge tube (Eppendorf Scientific, Westbury, NY), and the RNA precipitated with 0.5–1 ml of 100% isopropanol, in accordance with the standard protocol. The RNA pellets were resuspended in 50 μl of diethylpyrocarbonate (DEPC, Sigma Chemical Co., St. Louis, MO)-treated deionized water. After 2- to 5-μl aliquots were removed to measure absorbance at 260 nm, the RNA samples were stored frozen at −80 °C.

2.4. Differential display-PCR

DD-PCR was performed using the Hieroglyph DD kit (Beckman Coulter, Fullerton, CA), in accordance with the manufacturer's specifications. All reactions were prepared in duplicate. The PCR products were resolved by PAGE and analyzed by autoradiography. The bands showing 5-fold or greater differences in intensity between the control and the toxin-treated samples were excised from the gel and reamplified by PCR using the corresponding anchored and arbitrary primers.

2.5. DNA sequencing

Differentially expressed cDNA samples were then purified by series of PAGE, excisions, and PCR reamplifications, and sequenced using the ThermoSequenase sequencing kit with ^{33}P-labeled ddNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting sequences were compared to the existing cDNA sequences in the GENBANK database using basic BLAST software (all non-redundant nucleotide sequence databases). The query sequences were filtered for low compositional complexity regions (that eliminate false high scores based on compositional bias, such as polyadenosine tails), and no sequence gaps were allowed. Statistical significance (i.e. the BLAST *E* value score) was calculated based on the number of local alignments. Sequence matches with $E < 60$ were rejected as not statistically significant.

2.6. RT-PCR

The results, showing differentially expressed transcripts (DETs) matching known GENBANK sequences, were confirmed by RT-PCR using the SuperScript II kit (Invitrogen, Carlsbad, CA) and either the PCR Master kit (Roche Pharmaceuticals, Nutley, NJ), or the Z-Taq PCR kit (TaKaRa Biomedicals, Osaka, Japan). The reactions were performed in accordance with the manufacturers' protocols using 0.5 μg of the SuperScript II oligo-dT primer/1–5 μg of the total RNA isolated from cells incubated with or without 50 μg/ml of SEB for 2, 6, 12, 24, 48, and 72 h, allowing for the time-course analysis of SEB-induced gene expression in RPTEC. The custom-designed PCR primers were obtained from Invitrogen (Carlsbad, CA). The Rnd3 primer sequences used were as follows: forward: 5′-CCTCTCTTACCCTGATTC-3′; reverse: 5′-TCTTCGCTTTGTCCTTTC-3′. The PCR products were resolved on 1% agarose gels containing 5–10 μl of ethidium bromide, visualized under ultraviolet light, and photographed. To ensure a linear correlation between the relative amount of the total RNA used in the reaction and that of the resultant amplified cDNA, the lowest possible number of PCR cycles that still produced detectable levels of the product was utilized for each gene of interest. For each RNA sample, parallel reactions using primers for housekeeping genes were performed as controls for the normalization of results.

2.7. Analysis of actin stress fiber formation

RPTEC were grown to 90% confluence in Lab-Tek 8-well chamber slides. The cells were incubated for 4 h or overnight, with or without 50 μg/ml of SEB. The reaction was terminated by removing the culture medium and washing the cells twice with phosphate-buffered saline (PBS) supplemented with 5% bovine-serum albumin (BSA). The cells were fixed with 3% formaldehyde in REGM for 3 h at 37 °C, washed twice with PBS/5% BSA, and permeabilized with 0.1% Triton X-100 in PBS/5% BSA for 5 min. Following two PBS/5% BSA washes, the cells were stained in the dark for 40 min at room temperature with 0.1 μg/ml Texas Red isothiocyanate (TRITC)-labeled phalloidin (Sigma). The results were analyzed using a fluorescent microscope equipped with a digital camera (Olympus Optical Company, Melville, NY).

2.8. TUNEL assay

RPTEC were grown in Lab-Tek 8-well chamber slides as described previously and incubated with or without 50 μg/ml of SEB for 48 h. Following the termination of the reaction, fixation,

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