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Shiga toxin activates p38 MAP kinase through cellular Ca^{2+} increase in Vero cells

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Abstract We examined whether the mitogen-activated protein kinase (MAPK) pathway is involved in Shiga toxin (Stx)-induced Vero cell injury. Consonant with cell injury, Stx caused a transient extracellular signal-regulated kinase1/2 (ERK1/2) and a sustained p38 MAPK phosphorylation. p38 MAPK inhibitors (SB 203580 and PD 169316), but not an ERK1/2 kinase inhibitor (PD 98059), partially inhibited the Stx-induced cell death. BAPTA-AM, a Ca^{2+} chelator, reduced both cell injury and p38 MAPK phosphorylation. Antioxidants reduced Stx1-induced p38 MAPK phosphorylation. These data indicate that Stx activates p38 MAPK through an increase in intracellular Ca^{2+} and reactive oxygen species, and this signaling is involved in Stx-induced cell death.

Key words: p38 Mitogen-activated protein kinase; Extracellular signal-regulated kinase1/2; Shiga toxin; Intracellular Ca^{2+}; Cell death; Reactive oxygen species

1. Introduction

Shiga toxins (Stxs) are a family of bacterial protein toxins produced by *Shigella dysenteriae* type 1 and enterohemorrhagic *Escherichia coli* (Stx-producing *E. coli*). The Stx family is classified into two groups on the basis of immunological property, namely the Stx1 family and the Stx2 family. Stx1 has about 60% homology to Stx2 at the amino acid level. Stxs are composed of one enzymatically active A subunit responsible for RNA *N*-glycosidase activity, thereby inhibiting protein synthesis, and five B subunits which carry the binding property of holotoxin to receptor molecule such as globotriaosyl ceramide (Gb3) and globotetraosyl ceramide (Gb4). Therefore, Stx was originally thought to cause cell death by inhibiting protein synthesis [1]. However, recent observations indicate that the situation is more complex. Addition of B subunit, which had been thought to have no effect on protein synthesis, to Burkitt's lymphoma cells induced cell death [2]. Yoshida et al. also observed that cycloheximide, an inhibitor of protein synthesis, did not cause apoptosis in human endothelial cells that underwent apoptosis due to Stx [3]. These reports suggest that inhibition of protein synthesis is not the sole cause of Stx-induced cell death, and that Stx exerts cell injury through a more complex mechanism.

The mitogen-activated protein kinase (MAPK) family has an important role in signal transduction and its pathway is activated by a variety of stimuli, such as growth factors and cellular stresses [4–7]. Among the MAPK family, three members in particular, the extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs, also called SAPKs), and p38 MAPK, have been well characterized. Activation of the ERK signaling pathway functions to protect cells from a variety of cellular stresses. By contrast, the JNK and p38 MAPK signaling pathways have been suggested to be pathways of cell death [8,9]. Recently, Katagiri et al. have demonstrated that Stx activates the Src family kinase, Yes, which is thought to be an upstream player of MAPK activation [10]. However, to date, there is no direct evidence linking MAPK family members with the signaling pathway of Stx-induced cell injury.

Ca^{2+}-mobilizing compounds, including A23187 and thapsigargin, have been shown to induce cell death in a certain type of cells [11,12]. It has also been observed that Stx increased the intracellular Ca^{2+} concentration and that inhibition of this increase reduced Stx cytotoxicity [2,13], suggesting that an increase in cellular Ca^{2+} may be involved in Stx-induced cell death. However, it is not yet clear where this pathway is positioned in Stx-induced intracellular signal transduction.

In the present study, we show that Stx1 and Stx2 induce phosphorylation of both ERK1/2 and p38 MAPK, and that Stx-induced Vero cell death is partially associated with the p38 MAPK pathway but not with the ERK pathway. We also provide evidence that cellular Ca^{2+} has an important role as an upstream molecule of p38 MAPK.

2. Materials and methods

2.1. Purification of Stxs

Stx1 and Stx2 were prepared and purified as described previously [14,15]. A non-toxic mutant Stx1, E167Q, in which glutamic acid at position 167 from the N-terminus of the A subunit of Stx1 is replaced by glutamine was prepared and purified as described previously [16].

2.2. Cell culture

Vero cells were grown and maintained in Eagle minimum essential medium (MEM; Gibco BRL) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 8 μg/ml tyrosine and 10% fetal bovine serum (FBS). All experiments were performed after serum starvation. Cells were grown to 80% confluence in MEM containing 10% FBS and transferred to MEM containing 0.5% FBS for 48–72 h.

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2.3. Measurement of cell viability

Cell viability was determined by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay [17]. Quiescent Vero cells were incubated with Stx in the presence or absence of test agent (s) for 24 or 48 h and a mixture of WST-1 and 1-methoxy-5-methylphenazinium methosulfate was then added to each well (final concentrations: WST-1, 0.5 mM; 1-methoxy-5-methylphenazinium methosulfate, 0.02 mM). After incubation for 1 h, the absorbance was measured at 405 nM with a reference wavelength of 655 nm.

2.4. Evaluation of phosphorylation of ERK1/2 and p38 MAPK

In preliminary experiments, MAPK activity was assessed by in vitro kinase assay. As the activity had been comparable to that obtained by evaluating phosphorylation of MAPK, we assessed activation of MAPK by Western blotting with anti-phospho-MAPK antibody. Briefly, quiescent Vero cells were stimulated with Stx in the absence or presence of test agent (s), and were rinsed quickly in 100-mm dishes with ice-cold Tris-buffered saline (TBS; 25 mM Tris, pH 7.4, 150 mM NaCl) containing 1 mM Na_3VO_4 and then lysed in lysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml antipain, 100 μg/ml benzamidine, 10 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 1% glycerol) for 20 min at 4°C. The cells were then scraped off the dish, centrifuged at 12 000 × *g* for 10 min, and the protein concentration in the supernatants was determined by DC protein assay (Nippon, Bio-Rad). The remaining supernatants were combined with 4×SDS–PAGE sample buffer (0.5 M Tris, pH 6.8, 50% glycerol, 8% SDS, 0.4 M dithiothreitol, 0.05% bromophenol blue) and heated at 95°C for 3 min. The samples were then frozen at −80°C until use.

After separation by SDS–PAGE, the proteins were transferred to nitrocellulose membranes and analyzed by immunoblotting. The membranes were incubated in blocking solution and then with primary antibodies for 1.5 h at 37°C. Excess primary antibody was removed by washing the membranes in TBS containing 0.05% Tween 20. The blots were incubated with appropriate secondary antibodies for 1 h at 37°C, and the membranes were then washed and proteins associated with antibodies were detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). For repeated immunoblotting, the membranes were stripped in 50 mM Tris, pH 6.5, 100 mM 2-mercaptoethanol and 2% SDS for 30 min at 50°C.

2.5. Chemicals and reagents

Rabbit polyclonal anti-phospho-ERK1/2 antibody specific for dual-phosphorylated ^{202}Thr and ^{204}Tyr of ERK1/2, rabbit polyclonal anti-phospho-p38 MAPK antibody specific for dual-phosphorylated ^{180}Thr and ^{182}Tyr of p38 MAPK, and rabbit polyclonal anti-p38 MAPK antibody which detects phosphorylation-state independent p38 MAPK, were purchased from New England Biolabs Inc. Horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit secondary antibodies were obtained from Amersham Pharmacia Biotech. PD 98059, SB 203580, PD 169316, thapsigargin, A23187 and 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM) were obtained from Calbiochem, and were dissolved in 100% DMSO and diluted with culture medium to the final concentration (1% DMSO). All other chemicals and reagents were purchased from Sigma Chemical Co. or Wako Pure Chemicals.

3. Results

3.1. Stx-induced cell injury

The cytotoxic effects of Stx1, Stx2 and E167Q in Vero cells were determined by the WST-1 assay. Quiescent Vero cells were incubated with various concentrations of Stx1, Stx2 or E167Q for 24 or 48 h. Both Stx1- and Stx2-reduced cell viability in a dose- and time-dependent manner. The 50% cytotoxic concentrations for 48 h of treatment with Stx1 or Stx2 were 11 pg/ml and 1.3 pg/ml, respectively. In contrast, 1 μg/ml E167Q reduced cell viability by only 30%, indicating that E167Q had a much weaker effect, about $1/10^5$ as reported by us previously [16], on Vero cell viability.

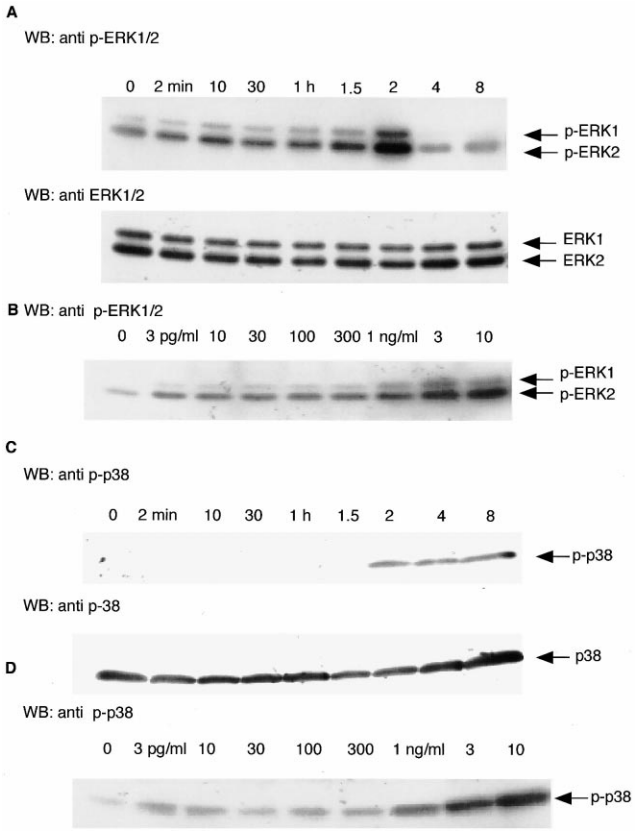

Fig. 1. Effect of Stx1 on phosphorylation of ERK1/2 and p38 MAPK. A and C: Typical examples of Stx1-induced ERK1/2 (A) and p38 MAPK phosphorylation (C) in a time-dependent manner by Western blot analysis (WB). Quiescent Vero cells were incubated with 3 ng/ml Stx1 for the time indicated. An equal amount of protein was subjected to SDS–PAGE in a 11% acrylamide gel and immunoblotted with anti-phospho-ERK1/2 antibody or -p38 MAPK antibody (upper panel). The same membrane was then stripped and reprobed with anti-ERK1/2 or -p38 MAPK antibody (lower panel). B and D: Typical example of Stx1-induced dose-dependent ERK1/2 (B) and p38 MAPK phosphorylation (D). Quiescent Vero cells were incubated with Stx1 at the indicated concentration for 2 h. Similar results were obtained in three separate experiments.

3.2. Stx1 induces phosphorylation of MAPK

When quiescent Vero cells were stimulated with 3 ng/ml Stx1, marked phosphorylations of ERK1/2 and p38 MAPK were observed (Fig. 1). Both phosphorylations peaked at 2 h of Stx1 stimulation, and ERK1/2 phosphorylation then declined to the basal level and p38 MAPK phosphorylation was sustained thereafter. In contrast, Stx1 had little effect on the MAPK protein level (Fig. 1A,C). Fig. 1B,D represents the dose-dependent effect of Stx1 on both MAPK phosphorylations. At a concentration of 3 pg/ml or more, Stx1 induced phosphorylations of ERK1/2 and p38 MAPK.

3.3. Phosphorylation of MAPK by Stx2 but not by E167Q

When quiescent Vero cells were stimulated with various concentrations of Stx2 for 2 h, phosphorylations of ERK1/2 and p38 MAPK were also observed (Fig. 2A,B). At an Stx2 concentration of 30 pg/ml or more, the ERK1/2 phosphorylation level was comparable to that observed with 300 pg/ml

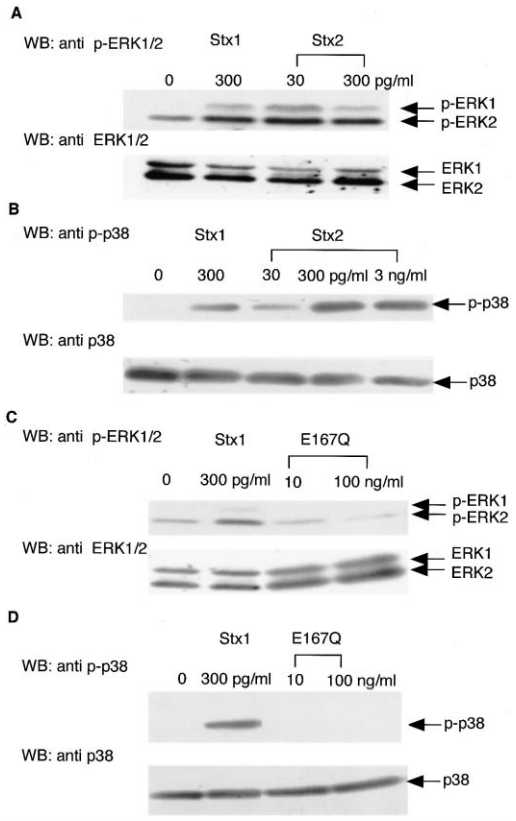

Fig. 2. Effects of Stx2 and E167Q on phosphorylations of ERK1/2 or p38 MAPK. A and B: Typical example of Stx2-induced ERK1/2 (A) or p38 MAPK phosphorylation (B) by Western blot analysis. Quiescent Vero cells were incubated with Stx1 or Stx2 at the indicated concentration for 2 h. C and D: Typical example of the effect of E167Q on ERK1/2 (C) or p38 MAPK phosphorylation (D). Quiescent Vero cells were incubated with Stx1 or E167Q at the indicated concentration for 2 h. Similar results were obtained in three separate experiments.

Stx1. The p38 MAPK phosphorylation level in response to 300 pg/ml Stx2 was also larger than that with the same dose of Stx1. This indicates that Stx2 phosphorylates ERK1/2 and p38 MAPK more potently than Stx1, and this efficacy was proportional with that observed in the cell viability determination. The protein levels of both MAPKs were not significantly affected by treatment with Stx2. When 300 pg/ml Stx2 was applied to the cells for 10 or 30 min, phosphorylation levels of ERK1/2 and p38 MAPK were minimal (data not shown). Therefore, the time courses of phosphorylations of ERK1/2 and p38 MAPK in response to Stx2 were similar to those of Stx1-induced phosphorylations.

When quiescent Vero cells were stimulated with 10 or 100 ng/ml E167Q known to be a non-toxic Stx1 mutant for 2 h, phosphorylations of ERK1/2 and p38 MAPK were not observed (Fig. 2C,D). This result was consistent with the data from the cell viability experiment.

3.4. *Effects of p38 MAPK inhibitors on Stx1-induced cell responses*

The effects of SB 203580 and PD 169316, both inhibitors of p38 MAPK [18,19], on Stx1-induced cell injury were examined by the WST-1 assay. To this end, we used a concentration of 20 pg/ml, because this dose of Stx1 fully activated p38 MAPK and induced cell death. Quiescent Vero cells were pretreated with each agent for 1 h, and 20 pg/ml Stx1 was then added to the cells in the presence of each drug. As shown in Fig. 3, 30 μM SB 203580 and 3 μM PD 169316 partially but significantly reduced Stx1-induced cell injury. 3 μM SB 203580 had no inhibitory effect on Stx1-induced cell injury. On the contrary, it significantly enhanced cell injury. Although the exact reason for this enhancement is not clear, the following might be one possible explanation. When the cells were treated with 30 μM SB 202474 whose structure is related to SB 203580 [18], but which lacks the inhibitory activity of p38 MAPK, instead of 3 μM SB 203580, we observed a response similar to that with 3 μM SB 203580 (vehicle; 26.5 ± 2.5%, 30 μM SB 202474; 17.6 ± 4.9%, in duplicate and repeated three times, $P < 0.05$). Therefore, it is conceivable that this enhancement of Stx1-induced cell injury was due to the inert molecule of this analogue.

To determine the inhibitory effect of 30 μM SB 203580 and 3 μM PD 169316 on p38 MAPK, quiescent Vero cells were incubated with each drug for 1 h, and were then stimulated with 20 pg/ml Stx1 for 2 h in the presence of drug. As shown in Fig. 3C, each drug reduced phosphorylation of p38 MAPK.

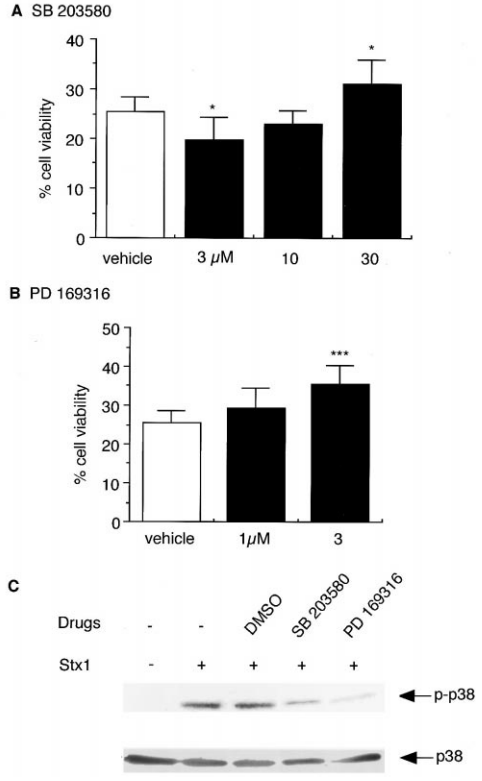

Fig. 3. Effects of p38 MAPK inhibitors on Stx1-induced cell injury and phosphorylation of p38 MAPK. A and B: Quiescent Vero cells were incubated with or without SB 203580 (A) or PD 169316 (B) at the indicated concentration for 1 h. Cells were then treated with or without 20 pg/ml Stx1 for 24 h in the presence of drug. Data are expressed as percent of the mean value obtained from cells untreated (with both Stx and test agents). Values are presented as mean ± S.D. from four independent experiments performed in duplicate. * and *** represent the significance level of $P < 0.05$ and $P < 0.001$, respectively, tested for the difference between vehicle and test agents (Dunnett's method). C: Typical example of the effect of SB 203580 (30 μM) or PD 169316 (3 μM) on Stx1-induced p38 MAPK phosphorylation by Western blot analysis. Similar results were obtained in two separate experiments.

The effect of PD 98059, a specific inhibitor of ERK1/2 kinase [20], on Stx1-induced cell injury was also examined by the WST-1 assay. Quiescent Vero cells were incubated with different doses of PD 98059 and Stx1 for 24 and 48 h. We cold not detect any significant effect of PD98059 on Stx1-induced cell injury (data not shown).

3.5. Effect of Ca^{2+} chelator on Stx1-induced cell responses

Next, we examined the effect of the intracellular Ca^{2+} chelator, BAPTA-AM, on Stx1-induced phosphorylation of p38 MAPK. Quiescent Vero cells were incubated with each drug for 1 h, and were then stimulated with 20 pg/ml Stx1 for 2 h in the presence of drug. As shown in Fig. 4A, treatment of the cells with BAPTA-AM reduced phosphorylation of p38 MAPK to the basal level.

The effect of BAPTA-AM on Stx1-induced cell injury was also examined by the WST-1 assay. Quiescent Vero cells were pretreated with BAPTA-AM for 1 h and 20 pg/ml Stx1 was then added to the cells in the presence of drug. As shown in Fig. 4B, BAPTA-AM markedly reduced Stx1-induced cell injury.

To examine the phosphorylation of p38 MAPK by intracellular Ca^{2+} elevation, quiescent Vero cells were stimulated with 1 μM A23187 or 30 nM thapsigargin, both of which are Ca^{2+}-mobilizing compounds, for 2 h. As shown in Fig. 4C, each drug induced phosphorylation of p38 MAPK, while the protein level of p38 MAPK was not significantly affected by each treatment.

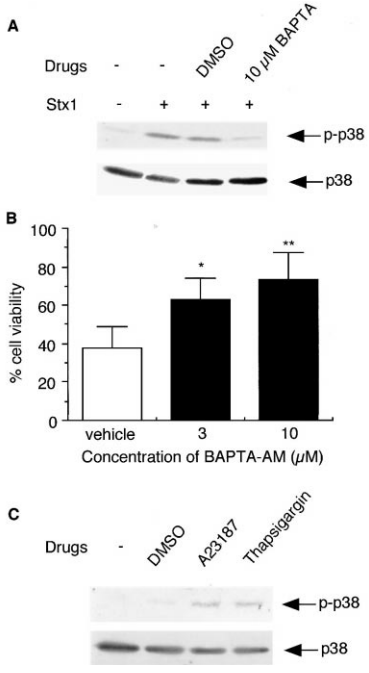

Fig. 4. Effect of intracellular Ca^{2+} chelator on Stx1-induced cell injury and phosphorylation of p38 MAPK A: Typical example of the effect of BAPTA-AM (BAPTA) on Stx1-induced p38 MAPK phosphorylation by Western blot analysis. Similar results were obtained in three separate experiments. B: Effect of BAPTA-AM on Stx1-induced cell injury. Quiescent Vero cells were incubated with or without BAPTA-AM at the indicated concentration for 1 h and were then stimulated with or without 20 pg/ml Stx1 in the presence of drug. Data are expressed as percent of the mean value obtained from cells untreated (with both Stx and test drug). Values are presented as mean ± S.D. from two independent experiments performed in duplicate. * and ** represent the significance level of $P < 0.05$ and $P < 0.01$, respectively, tested for the difference between vehicle and test agents (Dunnett's method). C: Typical example of A23187- or thapsigargin-induced p38 MAPK phosphorylation by Western blot analysis. Similar results were obtained in two separate experiments.

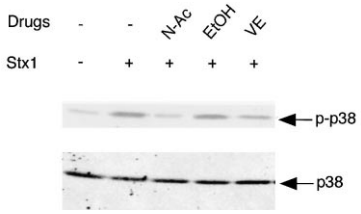

Fig. 5. Effects of antioxidants on Stx1-induced p38 MAPK phosphorylation. Typical example of the effect of *N*-Ac or vitamin E on Stx1-induced p38 MAPK phosphorylation by Western blot analysis. Quiescent Vero cells were incubated with 10 mM *N*-Ac, 100 μM vitamin E, or 1% ethanol (EtOH, vehicle for vitamin E) for 1 h, and were then stimulated with 20 pg/ml Stx1 for 2 h in the presence of drug. Similar results were obtained in two separate experiments.

3.6. Effects of antioxidants on Stx1-induced p38 MAPK phosphorylation

The effects of *N*-acetyl cysteine (*N*-Ac) and vitamin E, both of which are antioxidants, on Stx1-induced p38 MAPK phosphorylation were examined. Quiescent Vero cells were incubated with 10 mM *N*-Ac or 100 μM vitamin E for 1 h, and were then stimulated with 20 pg/ml Stx1 for 2 h in the presence of drug. As shown in Fig. 5, each drug reduced Stx1-induced p38 MAPK phosphorylation, while the protein level of p38 MAPK was not significantly affected by each treatment.

4. Discussion

In the present study, the intracellular signaling pathway activated by Stx1 and Stx2 in Vero cells was examined. Stx1, Stx2, but not E167Q were shown to cause cell injury. Concurrent with cell injury, Stx1 and Stx2 clearly induced sustained phosphorylation of p38 MAPK. The p38 MAPK inhibitors, SB203580 and PD 169316 partially prevented the Stx1-induced cell injury. Stx1 and Stx2 also caused phosphorylation of ERK1/2, but the phosphorylation peaked at 2 h of Stx1 stimulation and then declined to the basal level. PD 98059, an ERK1/2 kinase inhibitor, did not prevent or potentiate the Stx1-induced cell injury. These results suggest that the activation of p38 MAPK plays a role in Stx-induced cell death.

Stx is composed of one enzymatically active A subunit responsible for Stx-induced inhibition of protein synthesis and five B subunits which carry the binding property of the holotoxin [1]. A mutant Stx1 with a single mutation at position 167 from the N-terminus of the A subunit of Stx1, glutamic acid being replaced by glutamine (E167Q) has been shown to have little activity in both cytotoxicity to Vero cells and inhibitory activity of protein synthesis [16]. The present study showed that E167Q caused neither cell injury nor phosphorylation of p38 MAPK, suggesting that an intact A subunit is necessary for the phosphorylation of p38 MAPK.

It is well known that reactive oxygen species (ROS) activate p38 MAPK [21–23]. Recently, King et al. have described that oxygen free radical production by polymorphonuclear cells is

observed 30 min after addition of Stx1 [24]. Matsunaga et al. have also observed that treatment of human aortic endothelial cells with Stx1 for 1 h results in increased levels of hydroxyl radical and that this response is associated with the reduction of catalase mRNA and protein but with a lesser alteration in the levels of Cu, Zn-superoxide dismutase and NADPH oxidase mRNA [25]. In the present study, we showed that Stx1-induced activation of p38 MAPK peaked within 2 h and that *N*-Ac and vitamin E inhibited Stx1-induced phosphorylation of p38 MAPK. These data suggest that Stx1, and possibly the A subunit of Stx1 in particular, specifically inhibits transcription of catalase and produces ROS, leading to activation of p38 MAPK.

Stx1-induced phosphorylation of p38 MAPK and cell injury were inhibited by treatment with intracellular Ca^{2+} chelator. In addition, Ca^{2+}-mobilizing compounds including A23187 and thapsigargin induced phosphorylation of P38 MAPK in Vero cells. Although we did not perform direct measurement of intracellular Ca^{2+} concentration, these results suggest that Stx1 elevated the intracellular Ca^{2+} concentration and that this increase triggered the phosphorylation of p38 MAPK and cell injury. This may also be supported by previous reports that Stx mobilized intracellular Ca^{2+} and the inhibition of its Ca^{2+} mobilization by extracellular EGTA or verapamil reduced Stx-induced cytotoxicity [2,13], and, more recently, that Ca^{2+}-mobilizing compounds including A23187 and thapsigargin have been shown to activate p38 MAPK along with induction of cell death [11].

Recently, Herson et al. clearly indicated that hydrogen peroxide increases cellular [NAD] and opens NAD-activated Ca^{2+}-permeable cation channels, causing unregulated Ca^{2+} entry and consequent cell death [26]. Taken together with this report and our data, the Stx1-induced cell injury cascade in Vero cells appears to operate as follows: Stx1 activates ROS production, activating Ca^{2+} entry pathway, leading to intracellular Ca^{2+} elevation and p38 MAPK activation.

In contrast to p38 MAPK inhibitors, Ca^{2+} chelator was a more potent inhibitor of Stx1-induced cell injury. Intracellular Ca^{2+} elevation is also known to activate other signaling pathways responsible for cell injury, such as the calpain, calcineurin and JNK pathways [27–30]. Therefore, in addition to inhibition of the p38 MAPK pathway, inhibitors of intracellular Ca^{2+} elevation may suppress other signaling pathways in response to Stx. Furthermore, the residual component of Stx-induced cell injury, which was insensitive to p38 MAPK inhibitors, may be due to inhibition of protein synthesis. These hypotheses need further evaluations.

To our knowledge, this is the first report that MAPK pathways can be activated by Stx1 and Stx2, and that activation of the p38 MAPK pathway, which is triggered by intracellular Ca^{2+} elevation, plays a role in Stx-induced cell death.

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