Protein Kinase Signaling by Shiga Toxin Subunits

Abstract

Background: Escherichia coli produces Shiga toxin (Stx), a pentamer composed of one A subunit and four B subunits. The B subunit of Stx (StxB) mediated the attachment of the holotoxin to the cell surface while the A subunit (StxA) has N-glycosidase activity, resulting in protein synthesis and cell death inhibition. Stx-induced cytotoxicity and apoptosis have been observed in various cell lines, although the signaling effectors are not precisely defined. Activated by protein kinases (PK), the signaling pathway in human tumors plays an oncogenic role. Tumor proliferation, survival, and metastasis are promoted by kinase receptors. In this regard, PK regulatory effects on the cellular constituents of the tumor microenvironment can affect immunosuppressive purposes. **Methods:** In this study, kinase inhibitors were used to evaluate the influence of Stx and its subunits on HeLa and Vero cells. Selective inhibitors of protein kinase C (PKC), CaM kinase (calmodulin kinase), protein kinase A (PKA), and protein kinase G (PKG) were used to compare the signaling activity of each subunit. **Results:** The ribotoxic activity in the target cells will lead to rapid protein synthesis inhibition and cell death in the mammalian host. The expression of Bcl2 family members was also assessed. Protein kinase signaling by Stx and its A and B subunits was induced by PKA, PKG, and PKC in HeLa cells. CaM kinase induction was significant in Vero cells. StxB significantly induced the pro-apoptotic Bax signaling factor in HeLa cells. **Conclusion:** The assessment of different signaling pathways utilized by Stx and its subunits could help in a better understanding of various cell death responses. The use of inhibitors can block cell damage and disease progression and create therapeutic compounds for targeted cancer therapy. Inhibition of these pathways is the primary clinical goal.

Keywords: *Kinase inhibitors, Shiga toxin, signaling pathways, subunits*

**Mana Oloomi,
Neda Moazzezy,
Saeid Bouzari**

Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran

Introduction

Shiga toxins (Stxs) are produced by *Shigella* or *Escherichia coli*.[1] Potent bacterial exotoxin, such as Vero (Cyto) toxins or Stxs, is the principal virulence factor of *enterohemorrhagic E. coli* (EHEC). *E. coli* O157:H7, O104:H4 that may cause large outbreaks of life-threatening diseases in humans. A subset of Stx-producing *E. coli* and EHEC strains is most frequently associated with severe human disease.[2] Food-borne pathogens are EHEC strains that can cause clinical conditions like hemolytic-uremic syndrome (HUS) known as diarrhea, hemorrhagic colitis, and systemic complications.[3] EHEC operates by adhesion to epithelial host cells in the first step of the pathogenic cascade in the gastrointestinal tract.

Stx 1 is one of the variants of Stx which has shown cytotoxicity. Inhibition of protein synthesis can induce Stx and result in apoptosis and necrosis.[4-6] The mechanism of cytotoxicity involves the induction of apoptosis in several types of cells in addition to blocking protein synthesis.[4] Cell death with protein synthesis is a highly regulated form of apoptosis. Protein synthesis inhibitors could protect cells from apoptosis.[5,6] The role of protein synthesis inhibition in apoptosis has yet to be established.[7] However, Stxs may induce apoptosis independent from protein synthesis inhibition.[7] Thus, Stx-induced apoptosis and cytotoxicity are two distinct processes.[2]

The genes encode a mature subunit peptide chain with two polypeptide chains giving rise to the Stx subunits, preceded by an N-terminal signaling sequence of StxA and StxB subunits with five B subunits of Stx 1 to form a pentameric ring. The A subunit of Stx is necessary for apoptosis. Time- and dose-dependent cytotoxicity induced by the recombinant Stx 1 (rStx 1) has

Address for correspondence:
Prof. Mana Oloomi,
Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran.
E-mail: manaoloomi@yahoo.com

been described in the sensitive HeLa and Vero cell lines. Cytotoxicity induced by rStx and its A and B subunits has been also demonstrated, and the effects of rStx appeared at lower doses compared to A and B subunits.[8] However, the protein synthesis inhibition results in cytotoxicity and cell death by subunit A. It seems that StxB has no effect on cytotoxicity and is responsible for the binding to the cell surface receptor.[9] StxA or StxB separately are used due to their different biological functions. These subunits are responsible for the attachment of the holotoxin to the cell surface and cell cytotoxicity. They have been also employed in drug delivery and targeted cancer therapy.[10] The Stx-induced protein kinase A (PKA) signaling pathway has been already shown in Burkitt's lymphoma cell. In addition, PKA activity is associated with an intracellular increase of Ca^{+2} influxes in the early stages of apoptosis.[11] Protein kinase C (PKC) activation following Stx treatment has been also reported in monocytic THP-1 cells.[12] High levels of Stx induce endothelial apoptosis and cell detachment.[12] It was first demonstrated that cell lines transfection with Stx results in mRNA corresponding to the biologic activity of StxA and StxB in the supernatant. The intestinal epithelial (HCT-8) cell line which is involved in EHEC infection was considered as an *in vitro* model similar to the *in vivo* physiopathologic conditions. A study of the pathogenesis of HUS in host-pathogen interactions for different *in vitro* models showed markedly different cellular responses to bacterial infections.[13]

In this study, for the first time, proliferation assays using PKA, protein kinase G (PKG), PKC, and CaM kinase inhibitor treatment were examined by epithelial, HeLa, and Vero cells. In parallel, the Bcl2 family apoptosis induction by rStx and its recombinant subunits (rStxA and rStxB) was evaluated in HeLa cells.

Materials and Methods

Shiga toxin and its subunits

The preparation of the Stx 1, StxA, and StxB subunits was described previously.[14] Three separate constructs were prepared to produce holotoxin and subunits A and B. Briefly, rStx and its rStxA and rStxB were expressed in *E. coli*, harboring an *stx*-carrying plasmid under the control of an arabinose-inducible promoter.[8,14] Cell lysates were obtained from a transformed *E. coli* strain using polymyxin B (0.1 mg/ml) treatment. Periplasmic extracts were already provided using polymyxin B. The extracts were determined to be free of detectable lipopolysaccharide and used for further assessment. In this study, two different dilutions of Stx and its subunits were considered (10 µg/ml and 20 µg/ml) as the range of toxicity to detect the inhibitory effect.

Cell culture

The HeLa and Vero cell lines were used. The cytotoxic potencies of Stx 1 and its subunits were adjusted to 10^5 and 10^7 cells. 50% cytotoxic dose (CD50)/g of

protein was used. Cytotoxicity was assayed in the HeLa and Vero cells as described previously.[8] Fetal bovine serum (10%), heat-inactivated (56°C, 45 min), and 2% penicillin-streptomycin (GIBCO BRL) were used to culture cells (10^7 cells) in RPMI 1640 medium (GIBCO BRL) in a 5% CO_2 incubator at 37°C. Cells were incubated for up to 24 h, after overnight serum starvation, with the Stx, StxA, or StxB subunits (10 µg/ml). They were filtered with 0.2 µm syringe filters (Gelman Sciences).

Kinase inhibitor assay

Mixed kinase inhibitor Tocriset™ contains a selective inhibitor of PKC, GF 109203x, and KN-62 (a selective inhibitor of CaM kinase II), KT5720 (a potent and selective inhibitor of PKA), and KT 5823 (a highly selective inhibitor of PKG). The assays were performed according to the manufacturer's protocol. 10^7 cells were incubated with Stx and its subunits for 24 h. The cells were then washed with PBS, and the cytotoxicity assay was applied as previously described.[8] Then, they were seeded (10^4 cells per well) in a 96-well plate followed by overnight incubation at 5% CO_2 and temperature of 37°C. The serially diluted toxin samples were added to the cells and inhibitors (2.5 µg/ml and 5 µg/ml), then the plates were incubated for an additional 48 h. The absorbance was spectrophotometrically measured at 630 nm. Specific toxin activity was calculated as the CD50. For Stx and its subunits, the toxin dilution was determined as CD50 (i.e., 50% of the cell death in the monolayer compared to the controls).

Total RNA extraction and one-step reverse transcription-polymerase chain reaction

According to the manufacturer's instructions, a filter-based RNA isolation system (Takara kit) was used for the total RNA extraction from the cells. The extracted RNA was then treated with RNase-free DNase at 37°C for 30 min. The contaminating genomic DNA was removed followed by heat inactivation at 95°C for 20 min. The purity and concentration of RNA were measured by spectrophotometry (Nanodrop Technologies Inc.). Total extracted RNA was amplified by one-step AccuPower® reverse transcription-polymerase chain reaction (RT-PCR) Premix (BioNEER) Kit. A one-step RT-PCR was performed according to the manufacturer's protocol. The extracted RNA and the reverse primer were mixed and incubated at 70°C for 5 min. The incubated mixture and the forward primer were then transferred to a PreMix tube. The cDNA synthesis was performed at 42°C for 60 min and at 94°C for 5 min. PCR cycles (30 cycles) were conducted as follows: 94°C for 60 s, 54°C for 30 s, and 72°C for 60 s with a final 10 min extension at 72°C. The gene-specific PCR primers were used for the reactions whose sequences are listed in Table 1.

Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control of RNA extraction. The

Table 1: Primers used in this study for real-time polymerase chain reaction

	Primer F	Primer R	Reference
Bcl-2	CCTGTGGATGACTGAGTACC	GAGACAGCCAGGAGAATCA	[15]
Bcl-xl	ACAGCCCCGCGGTGAATGG	TGCCCCGCCGAAGGAGAAA	[15]
Bak	TCCAGATGCCGGGGAATGCACTGACG	TGGTGGGAATGGGCTACTAGG	[16]
Bax	AAGCTGAGCGAGTGTCTCAAGCGC	TCCCGCCACAAAGATGGTCACG	[16]
GAPDH	GGTCGGAGTCAACGGATTTG	ATGAGCCCCAGCCTTCTCCAT	[15]

GAPDH – Glyceraldehyde phosphate dehydrogenase

extracted RNA (1–2 μg) was used with the same amount of GAPDH RT-PCR product to obtain the same sensitivity for Bcl-2, Bcl-xl, Bak, and Bax. It is important to choose the proper number of cycles so that the amplified product is easily detectable on an agarose gel and can be quantified. The aliquots of PCR products were visualized after electrophoresis on 1.5% agarose gel, stained with ethidium bromide in tris-borate-ethylenediaminetetraacetic acid buffer under ultraviolet, and the intensities of the bands were quantified by Lab Gel Doc Image software as determined in the previous study.[17]

Statistical analysis

The results were expressed as the mean ± standard error of mean were presented for three independent experiments. Mean values were compared by the Student's *t*-test. *P* values lower than 0.05 were considered significant for the inhibitor assays.

Results

Kinase inhibitor effect on Shiga toxin and its subunits

The following inhibitors were used in this experiment: GF109203x (10 mM), KN-62 (1 mM), KT5720 (0.1 mM), and KT5823 (0.1 mM). Enzyme inhibitors include PKC (IC50 = 3nM), PKA (IC50 = 7nM), IC50 = 6nM for p60[v-src] tyrosine- PK, and IC50 = 20nM for CaM kinase II. The inhibitors were used in two different concentrations (2.5 μg/ml and 5 μg/ml) to treat Vero and HeLa cells. Different concentrations of rStx and its subunits (10 μg/ml and 20 μg/ml) were used to cytotoxically decrease cell proliferation. The inhibitors could block the cytotoxic effect of rStx and its subunits. The effect of KT5720 on the cell proliferation of Vero and HeLa cells is depicted in Figure 1. rStx and its subunit concentrations (10 μg/ml and 20 μg/ml) showed significant results in both cell types with different inhibitor concentrations. Compared to HeLa cells proliferation by rStxB (10 μg/ml), inhibitor KT5720 (2.5 μg/ml) showed a significant (#) difference relative to the inhibitor-free situation [Figure 1f].

The cell proliferation assay using KT5823 is shown in Figure 2 for the Vero and HeLa cells. Treatment with KT5823 along with rStx (20 μg/ml) in the HeLa cells caused significant effects One asterisk (*) [Figure 2b]. In addition, treatment of HeLa cells with rStx and inhibitor KT5823 was significant hash tag sign (#) compared to the
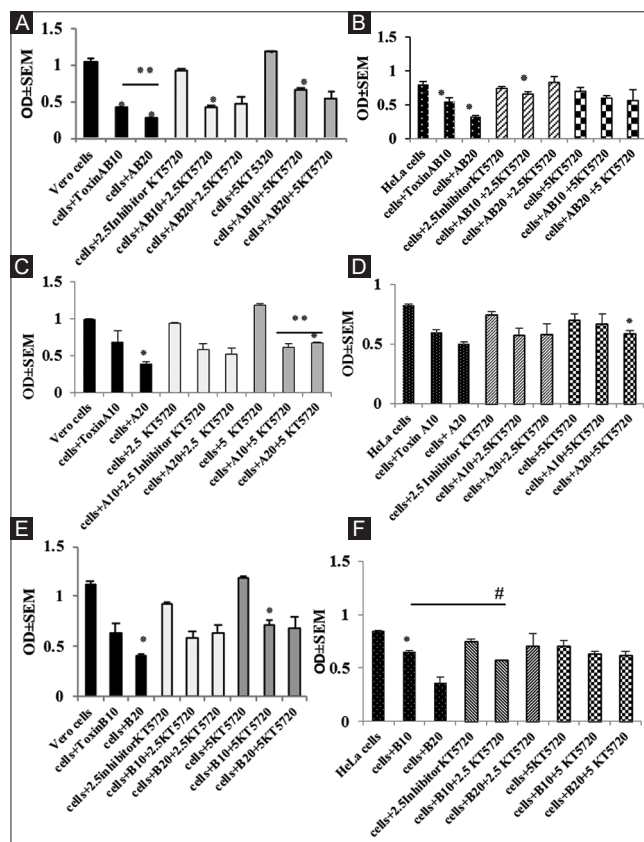

Figure 1: Stx and its subunits treatment with Inhibitor KT5720 (PKA). In A) Vero cells and in B) HeLa cells treatment with rStx, rStx (10μg/ml), (20μg/ml), In C) Vero cells and in D) HeLa cells treatment with rStxA, In E) Vero cells and in F) HeLa cells treatment with rStxB shows. Mean of ± standard error for duplicate experiments presents. Compared significance by Student's paired t test (p < 0.05) was calculated. Different concentration of rStx and its subunits treatment or different inhibitor concentration treatment compared with controls and its significant show by (*). Significant comparison of rStx, its subunits, and/or different inhibitor concentration treatment compared with each other show by (**). Treatment with rStx, its subunits, and inhibitor compared with situation without inhibitors and its significance show by (#).

proliferation of HeLa cells without inhibitor [Figure 2b]. For each subunit, two inhibitor concentrations (2.5 μg/ml and 5 μg/ml) were tested. Inhibitory effects on cell proliferation by rStx were also explored using each subunit (in different concentrations: 0, 10, and 20 μg/ml). The rStx and its subunits could significantly decrease cell proliferation. Conversely, cell proliferation could be enhanced by the cell treatment with inhibitors, but the significance of the increase was variable for StxA and StxB. In Figure 3, Vero cell treatment with rStx (20 μg/ml) is compared which
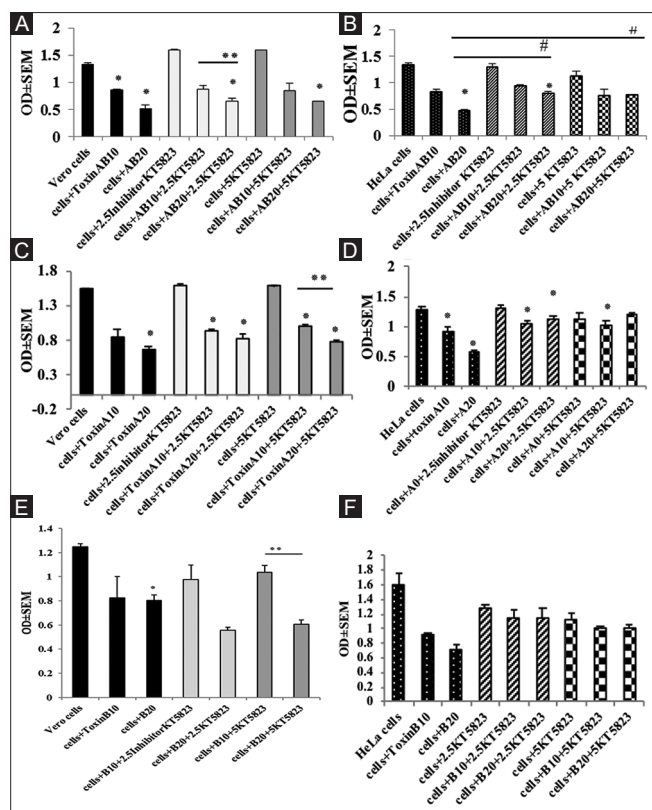
Figure 2: Stx and its subunits treatment with Inhibitor KT5823 (PKG). In A) Vero cells and in B) HeLa cells treatment with rStx, rStx (10μg/ml), (20μg/ml), In C) Vero cells and in D) HeLa cells treatment with rStxA, In E) Vero cells and in F) HeLa cells treatment with rStxB show. Mean of ± standard error for duplicate experiments shows. Compared significance by Student's paired t test (p < 0.05) was calculated. Different concentration of rStx and its subunits treatment or different inhibitor concentration treatment compared with controls and its significant show by (*). Significant comparison of rStx, its subunits, and/or different inhibitor concentration treatment with each other show by (**). Treatment with rStx, its subunits, and inhibitor compared with situation without inhibitors and its significance show by (#).

Figure 3: Stx and its subunits treatment with Inhibitor KN-62, (CaM kinase II). In A) Vero cells and in B) HeLa cells treatment with rStx, rStx (10μg/ml), (20μg/ml), In C) Vero cells and in D) HeLa cells treatment with rStxA, In E) Vero cells and in F) HeLa cells treatment with rStxB show. Mean of ± standard error for duplicate experiments shows. Compared significance by Student's paired t test (p < 0.05) was also calculated. Different concentration of rStx and its subunits treatment or different inhibitor concentration treatment compared with controls and its significant show by (*). Significant comparison of rStx, its subunits, and/or different inhibitor concentration treatment with each other shows by (**). Treatment with rStx, its subunits, and inhibitor compared with situation without inhibitors and its significance show by (#).

was significant. Furthermore, the Vero cell treatment with rStx (20 μg/ml) and inhibitor KN-62 (5 μg/ml) led to significantly different results compared to Vero cells treated with rStx (20 μg/ml) (#) [Figure 3a]. Based on Figure 4, HeLa cell treatment with rStx (10 μg/ml) and inhibitor GF (2.5 μg/ml) resulted in significantly different outcomes compared to single rStx (10 μg/ml) treatment [Figure 4a]. HeLa cell treatment with rStx and inhibitor GF (2.5 μg/ml) was compared with HeLa cell treatment with rStx (20 μg/ml) and inhibitor GF (2.5 μg/ml) which showed significant difference Two asterisks (**). HeLa cell exposure to rStxA (10 μg/ml) and inhibitor GF (2.5 μg/ml) led to significantly different results compared to their treatment with rStxA (10 μg/ml) (#) [Figure 4b]. HeLa cell treatment with rStxA (10 μg/ml) and inhibitor GF (2.5 μg/ml) were compared with HeLa cells treatment with rStxA (20 μg/ml) and inhibitor GF (2.5 μg/ml) which revealed significant difference (**). HeLa cells treated with different concentration of rStxB (10 and 20 μg/ml) was compared with controls which showed a significant difference (*). Different contents of rStxB (10 and 20 μg/ml) led to significantly
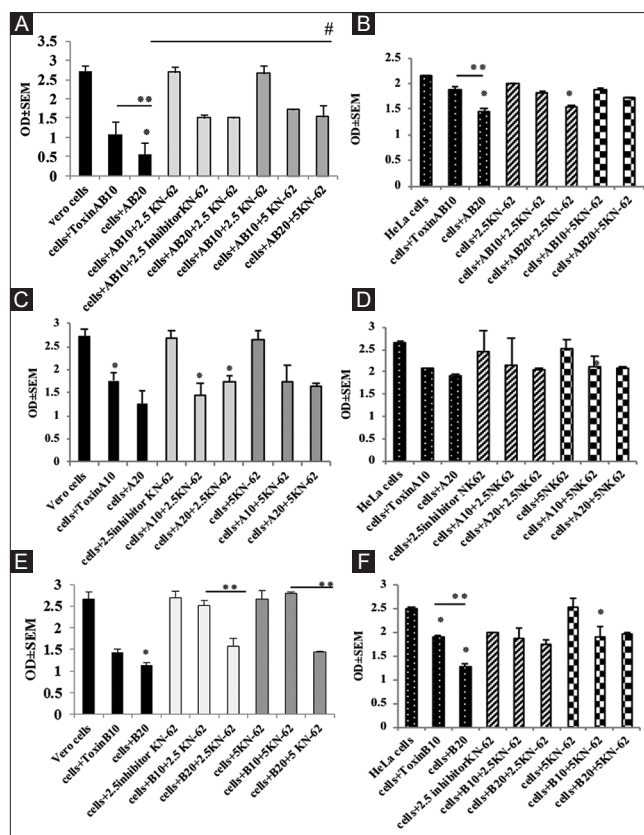
different results in HeLa cell (**). HeLa cell treatment with rStxB (10 μg/ml) and inhibitor GF (2.5 μg/ml) was compared to HeLa cells treatment with rStxB (20 μg/ml) and inhibitor GF (2.5 μg/ml) which suggested significant difference (**). HeLa cells treatment with rStxB (20 μg/ml) and inhibitor GF (2.5 μg/ml) was compared with HeLa cells treatment with rStxB (20 μg/ml) which showed significant difference (#). HeLa cells treatment with rStxB (10 μg/ml) and inhibitor GF (2.5 μg/ml) was compared with HeLa cells treatment with rStxB (10 μg/ml) which suggested a significant difference (#) [Figure 4c]. Concerning HeLa cells, Stx and its subunits significantly increased cell toxicity upon the use of GF inhibitor [Figure 4]. PKC signaling by GF inhibitor was not addressed in Vero cells as it has already been explored.[12]

Apoptotic factor expression by Shiga toxin and its subunits

The expression levels of Bcl-2 family members, anti-apoptotic Bcl-2 and Bcl-xl, pro-apoptotic Bax, and
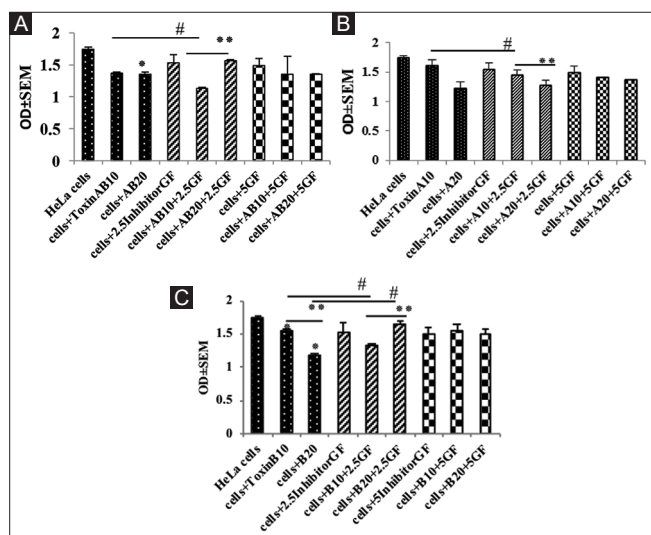
Figure 4: Stx and its subunits treatment with Inhibitor GF (PKC). In A) Vero cells treatment with rStx, rStx (10µg/ml), and (20µg/ml), In B) Vero cells treatment with rStxA, In C) Vero cells treatment with rStxB show. Means for duplicate experiments as mean ± standard error presents. Compared significance by Student's paired t test (p < 0.05) was calculated. Different concentration of rStx and its subunits treatment compared with controls and its significant show by (*). Significant comparison of rStx, its subunits, and/or different inhibitor concentration treatment compared with each other shows by (**). Treatment with rStx, its subunits, and inhibitor compared with situation without inhibitors and its significance show by (#).

Bak were assessed in the HeLa cells using RT-PCR. HeLa cell apoptosis with rStx treatment was already addressed while Vero cells were not studied in this experiment.[8] The expression of Bcl-2 and Bcl-xl by rStx treatment and its subunits is presented in Figure 5. Bcl-2 expression was affected by rStx and its subunits, while the expression of Bcl-xl showed no difference with the controls. The expression of Bax and Bak was also different. Bak expression was negative, and Bax expression by rStx and its subunit varied notably. Bcl-2 and BAX expression was increased by both rStxA and rStxB. However, the difference between rStxA and control was not statistically significant. rStx increased the expression of Bcl-2 ($P < 0.01$), while the expression of Bax was decreased by rStx compared to controls significantly ($P < 0.05$).

Discussion

Stx is one of the ribosomes inactivating proteins.[18] Toxins can efficiently inhibit protein synthesis by inactivating the ribosomes and induce signaling that could lead to apoptosis.[19] An apoptotic phenotype can convert to a necrotic phenotype by eliminating caspase-dependent apoptosis. Therefore, necrosis and apoptosis represent morphologic expressions of a shared biochemical network.[20] These molecular mechanisms of the two primary modes of cell death were extensively studied.[21] Bcl-2 family members regulate the intrinsic pathway of apoptosis and can be determined by pro- and anti-apoptotic factors.[22]

Bcl-xl and Bcl-2 are necessary for anti-apoptotic functions. The mitochondrial release of proteins can negatively
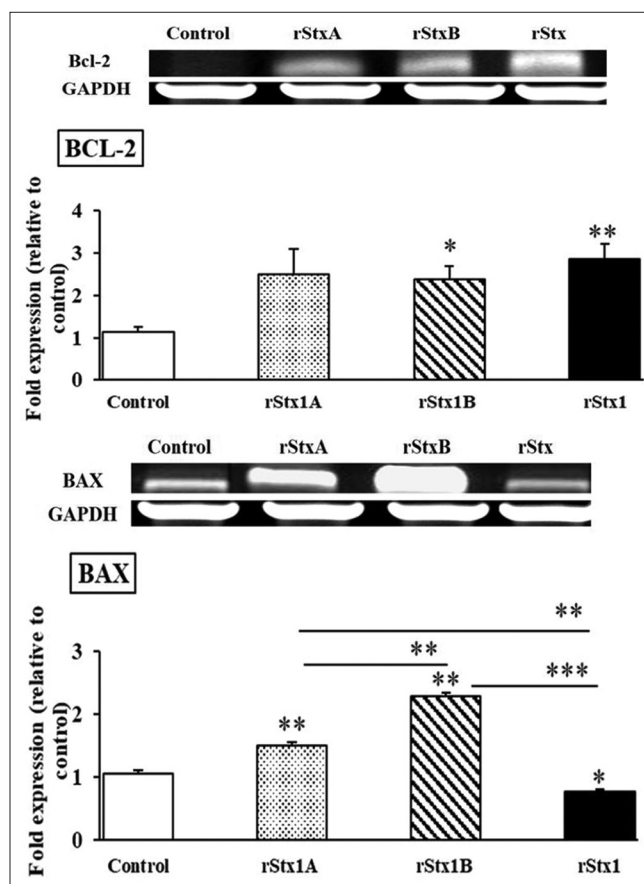

Figure 5: The results of RT-PCR by the challenge of rStx and its subunits on HeLa cells. Quantification of Bcl-2 and BAX expression analysis was performed using Gel Doc software. Ratios (between Stx-induced gene levels and control levels that had not been treated with rStx, rStxA, or rStxB) shown are the mean ± SEM for triplicate experiments. Values are the mean ± SEM. *P values < 0.05, **P values < 0.01, and ***P values < 0.001versus control.

regulate their interactions with other Bcl-2 family members. Bax and Bak induce mitochondrial permeabilization and release cell death-promoting proteins.[4,23] The cytoplasmic Ca2+ wave is another factor that can modulate Bcl-2 and Bcl-xl and can be antagonized by Bax and Bak as pro-apoptotic Bcl-2 family members.[4]

Subunit B is responsible for binding to the cell surface receptor. Subunit A is essential for the endocytosis process as it has been shown to stimulate the clathrin-dependent uptake of the toxin.[24] Moreover, both the holotoxin and the subunit B induce phosphorylation and tyrosine kinase activation, which can then phosphorylate the clathrin heavy chain.[25] The stimulating cells with Stx also increased the formation of clathrin-coated pits, which is considered as a mechanism by which the toxin enhances its uptake.[26] Induction of apoptosis in HeLa cells by Stx 1 and StxA enzymatic activity was shown in 2003.[27] In 2009, it was shown that Bcl-2 induction is sufficient by StxB retrograde transport, and StxA enzymatic activity is not required for Bcl-2 induction.[16] The pro-survival of Bcl-2 functions has remained to be fully characterized. However, these

functions may involve the association of Bcl-2 with the other pro-apoptotic members of the BCL-2 family of proteins, such as Bax and Bak, at the mitochondrial membrane.

On the other hand, the apoptosis induction by the signaling cascade activation results from Stx with a massive influx of extracellular Ca++ ions followed by increased intracellular cAMP concentrations and activation of PKA. Also, the StxB subunits apoptosis induction via PKA or PKC was demonstrated.[16] In this study, PK inhibitor activation by rStx, rStxA, and rStxB treatment was compared in HeLa and Vero cells. However, KT5720 (PKA), KT5823 (PKG), and GF (PKC) inhibitor activation were only significant in HeLa cells. Conversely, KN62 (CaM kinase) was affected by rStx treatment in Vero cells. It was also shown that the signaling pathways induced by rStx, rStxA, and rStxB treatments could be different. rStx and its subunit treatment in HeLa cells significantly detected the PKA, PKC, and PKG signaling pathways. Kinase activation by rStx and its subunits was dependent on the CaM kinase-signaling pathway in Vero cells.

In the epithelial cell line of HEp-2, treatment with Stx 1 enhanced the expression of the pro-apoptotic Bax while pro-apoptotic Bak and anti-apoptotic Bcl-2 expression were not altered. It has been shown that purified B-subunits do not initiate HEp-2 cell apoptosis effectively. It suggests that Stx can trigger apoptosis by the pro- and anti-apoptotic balance of Bcl-2 protein members. The activation of anti-apoptosis Bcl-2 by Stx and its subunits was also demonstrated. Stx and its subunits did not show anti-apoptosis Bcl-xl induction. However, pro-apoptotic Bax activation by the rStxB was prominent. However, the method we conducted in this study is a simple way to identify signaling pathways using gene expression that can be complemented by the delta-delta or quantitative RT-PCR method.

Conclusion

The toxic properties of Stx and the cell-targeting abilities of its subunits could be exploited for medical purposes.[28,29] Several studies have demonstrated that Stxs can target and kill cancer cells *in vitro* and *in vivo*. Stxs can induce apoptosis in cancer cells and evading apoptosis.[30,31] Therefore, a long-term effect of the toxin in several cell types blocks the protein synthesis and induces apoptosis.[4] However, Stxs are potent inhibitors of protein synthesis with CD50s for many cell types *in vitro*. Moreover, the cytotoxic action of Stxs on Vero cell lines makes them referred to as Vero toxins or Vero cytotoxins.[32,33] Apoptosis induction by the toxins may independently act on their protein biosynthesis-inhibitory.[2] However, Stxs inhibition of protein biosynthesis is an important co-factor for the induction of apoptosis.

This study addressed a molecular insight into rStx subunit signaling cell induction. Stx 1 exhibits cytotoxic activity in

different mammalian cells; therefore, purified recombinant subunits of Stx 1 activate signaling pathways and induce apoptosis in mammalian cells. Our observations suggest that Bcl-2 induction could be stimulated by rStx and its subunits. While the expression of pro-apoptotic protein Bax could be significantly stimulated in HeLa cell by rStxB.

In conclusion**,** the signaling effect of rStx and its subunits in HeLa and Vero cells was evaluated. PKA, PKG, and PKC PK signaling were induced in HeLa cells. However, CaM kinase induction was significant in Vero cells. Moreover, rStxB induced the Bax pro-apoptotic factor in the HeLa cell. Stx signaling could be used in targeted cancer therapy to block cell damage. In this report, a different signaling pathway of protein kinase activation was detected in Vero and HeLa cells to induce cell death by rStx and its subunits. Further investigations are required to evaluate the signaling pathway in different cell targets.

Conflicts of interest

There are no conflicts of interest.

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