Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 343–353
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

β_2-Adrenoceptor activation inhibits Shiga toxin2-induced apoptosis of renal tubular epithelial cells

Akio Nakamura[a,*], Akira Imaizumi[a], Yukishige Yanagawa[a], Ryo Niimi[a], Takao Kohsaka[b], Edward J. Johns[c]

[a]*Department of Paediatrics, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan*
[b]*Department of Immunology, National Children's Medical Centre, Tokyo 154, Japan*
[c]*Department of Physiology, University College Cork, Cork, Ireland*

Received 13 August 2002; accepted 9 December 2003

Abstract

Apoptosis is regulated by several pathways, such as caspases, mitogen activated protein kinase (MAPK) and cAMP/cAMP-dependent protein kinase A (PKA) cascade. This study investigated the effect of β_2-adrenoceptor activation on Shiga toxin (Stx)2-induced apoptosis in renal tubular cells and the contribution of these signalling pathways. Cultured human adenocarcinoma-derived tubular cells were exposed to Stx2 (64 pg/mL) for 2–24 hr following the addition of the β_2-adrenoceptor agonist (terbutaline) to the incubation medium. Stx2-induced apoptosis and its amelioration by β_2-adrenoceptor activation was confirmed using DNA degradation assays and by flow cytometry for annexin V, mitochondrial membrane potential and caspase(-3 and -7) activity. Exposure of cells to Stx2 for 24 hr increased the DNA fragmentation to $11.6 \pm 0.9\%$, compared to $3.3 \pm 0.2\%$ in control cells ($P < 0.05$) but was decreased to approximately 5–7% ($P < 0.05$) in the presence of terbutaline. Furthermore, Stx2-stimulated apoptosis, detected by TUNEL, annexin V and mitochondrial potential, was inhibited by terbutaline ($P < 0.05$) which was prevented by cAMP-PKA inhibitors and a β_2-adrenoceptor antagonist. However, inhibition of Stx2-mediated caspase activity by terbutaline was partially blocked by cAMP-PKA inhibitors. On the other hand, p38MAPK inhibition by terbutaline prevented Stx2-induced apoptosis and caspase activity through a cAMP-independent pathway via β_2-adrenoceptor. These data indicate that β_2-adrenoceptor activation can inhibit Stx2-induced apoptosis of the cells, which may be caused by a reduction in caspase activity through cAMP-PKA activation and the p38MAPK pathway.

Keywords: MAPK; Caspase; HUS; cAMP-PKA; Renal tubule

1. Introduction

HUS is characterised by renal failure, thrombocytopaenia and haemolytic anaemia and is often induced by Stx-producing strains of *Escherichia coli* [1,2]. The most extensive tissue damage in HUS occurs in the kidney and reports have indicated that renal tubular impairment is a contributor to the development of HUS [3–5]. Importantly, the receptor sites for Stx binding in normal kidney sections are most prominent in the renal cortex [3], probably in the distal tubule. These findings imply that the renal dysfunction associated with HUS may also be the consequence of Stx-induced cytotoxicity and apoptosis of the tubular cells [6,7].

Stx induces an apoptotic signal transduction cascade associated with enhanced expression of Bax in epithelial cells and the Stx-stimulated cell death was blocked by overexpression of Bcl-2 [8]. Zhu *et al*. [9] observed that the β_2-adrenoceptor agonist, clenbuterol, not only increased Bcl-2 expression but also decreased Bax expression in a rat model of forebrain ischemia. Thus, these findings suggested the possibility that β_2-adrenoceptor activation could have a pronounced anti-apoptotic effect.

* Corresponding author. Tel.: +81-3-3964-1211, Ext. 1480; fax: +81-3-3579-8212.
E-mail address: akio@med.teikyo-u.ac.jp (A. Nakamura).

Abbreviations: HUS, Haemolytic Uraemic Syndrome; Stx, Shiga toxin; MAPK, mitogen activated protein kinase; JNK, c-Jun N-terminal protein kinase; DMEM, Dulbecco's modified Eagles medium; PD, PD098059; SB, SB203580; ICI, ICI 118,551; KT, KT5720; FCS, foetal calf serum; FITC, fluorescent isothiocyanate; dbcAMP, dibutyryl cyclic AMP; PARP, poly ADP-ribose polymerase; Gb3, globotriaosylceramide.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(03)00256-9

However, the mechanisms underlying the action of β_2-adrenoceptor stimulation on Stx-induced apoptosis still remain unresolved and unclear.

β_2-Adrenoceptors couple to adenyl cyclases to raise intracellular levels of cAMP [10], suggesting that cAMP-PKA pathway could inhibit the Stx2-induced apoptosis. However, the action of cAMP-PKA pathway on cell apoptosis is also quite controversial. It has been reported that elevation of cAMP-PKA activity negatively regulates apoptosis in intestinal epithelial [11] and vascular smooth muscle cells [12]. In contrast, some investigators have shown that elevated levels of intracellular cAMP resulted in an induction of apoptotic cells [13–15]. Xia *et al*. [16] reported that cell survival and induction of cell death were controlled by opposing actions of the p42/p44MAPK and JNK-p38MAPK pathways. This was supported by evidence that an elevation of cAMP either blocks or stimulates cell proliferation by a mechanism which involves PKA-mediated Raf-1 kinase proliferation [17]. Therefore, it was suggested that cAMP-elevating agents determined Stx-induced apoptosis in either a positive or negative way by modulating the balance between p42/p44MAPK and JNK-p38MAPK pathways. Indeed, we have shown that Stx2 activated both p42/p44MAPK and p38MAPK pathways in renal tubular cells, which were attenuated by β_2-adrenoceptor agonists [18]. β_2-Adrenoceptor activation may regulate the Stx2-induced apoptosis through the MAPK cascade. However, it remains unclear whether the MAPK pathways were able to regulate the action of β_2-adrenoceptor activation on the Stx2-induced apoptosis in these renal tubular cells.

The objectives of the present study were, firstly, to determine the effectiveness of the β_2-adrenoceptor agonist in blocking the Stx2-induced apoptosis; secondly, to investigate possible mechanisms mediating these actions. This represented an attempt to clarify the possible roles of the cAMP-PKA cascade, MAPK pathway, and the so-called death caspase(-3 and -7) in the anti-apoptotic effect of β_2-adrenoceptor activation on the Stx2-triggered apoptosis in adenocarcinoma-derived (ACHN) cells, which are an *in vitro* model of human renal tubular epithelial cells.

2. Materials and methods

2.1. Reagents

Stx2 was prepared as described previously [19]. DMEM, glutamine, kanamycin, HEPES, PD, SB were obtained from COSMO BIO Corp. ICI and KT were obtained from Funakoshi Co. Anti-β_2-adrenoceptor antibody was obtained from Santa Cruz Biotechnology Inc. H-89 was purchased from Seikagaku Co. FCS was purchased from Dainippon Pharm. Co. Isotope ([gamma-^{33}P]ATP) was purchased from Amersham Japan. Unless otherwise stated, reagents were obtained from Sigma Chemical Co.

2.2. Culture conditions

The ACHN cell line was obtained from the American Type Culture Collection (ATCC CRL 1611). The cells express the renal tubular marker CD24 as well as CD77, the receptor for Stx [6] and were further identified by means of immunostaining using labelling with β_2-adrenoceptor antibody-FITC conjugate. The cells were maintained at 37° in 5% CO_2 in DMEM containing 10% FCS, 0.225% $NaHCO_3$, 0.1 mM non-essential amino acids, and 0.03% glutamine and were cultured on 25-cm^2 tissue culture flask and grown until approximately 80% confluent. After the dishes were washed with PBS to remove the nonadherent cells, the cells were placed in the medium for 2 hr prior to the Stx2 challenge (64 pg/mL).

2.3. Experimental conditions

For the apoptosis, caspase, and MAPK assays, the cells were incubated over 24 hr with 10% FCS-DMEM after the Stx2 (64 pg/mL) challenge either in the absence or presence of the β_2-adrenoceptor agonist, terbutaline, an analogue of cAMP, dbcAMP, the β_2-adrenoceptor antagonist, ICI, and/or the cAMP-PKA inhibitor, H-89 or KT. The agonists or antagonists were added 10 or 20 min before the Stx2 challenge, respectively. To examine the influence of MAPK inhibitors on the apoptosis, caspase, and MAPK activity in the cells, a p42/p44MAPK inhibitor, PD or a p38MAPK inhibitor, SB was added 20 min before the Stx2 challenge. Staurosporine (1.5×10^{-6} M) was used as a positive control. TNF-α was used as a p38MAPK activator.

2.4. Agarose gel visualization of DNA fragmentation

This process was detected using an apoptosis ladder detection kit (Wako Pure Chemical Industries, Ltd.). The cells were incubated for 24 hr and collected into a 1.5 mL microcentrifuge tube and spun at 200 *g* for 5 min. Pellets were resuspended in an enzyme reaction solution, enzyme activator solution, RNase solution, and protein digestion enzyme solution according to the manufacturer's recommendations. Following this, DNA extraction solution was added and samples were incubated at room temperature for more than 10 min. Samples were then loaded onto 1.5% agarose gels and run at 100 V for approximately 30 min in TAE buffer. Gels were soaked for 1 hr in a shaker bath and stained with SYBR Green nucleic acid reagent to enhance the visibility of the DNA bands. Imaging and analysis of the DNA ladder separated by electrophoresis was performed using the fluorescence imaging analyzer (FluorImager SI, Molecular Dynamics-Japan).

2.5. DNA fragmentation assay

The cells were incubated for 24 hr and scraped into a lysis buffer containing 10 mM Tris–HCl, 10 mM EDTA

and 0.5% Triton X-100. After a 30-min incubation at 4°, samples were spun at 17,000 *g* for 15 min. Pellets were resuspended in lysis buffer and 1 M perchloric acid, and then heated at 70° for 20 min. To each supernatant, pellet, and blank sample, 3% diphenylamine solution (dissolved in glacial acetic acid and concentrated sulphuric acid) was added and incubated for 24 hr at 30°. The level of DNA fragmentation in the samples was determined by spectrophotometric measurements at wavelengths of 600 nm (U-3310, Hitachi Instrument Inc.). %DNA fragmentation was determined in accordance with the following equation: OD^{600} supernatant / [OD^{600} supernatant + OD^{600} pellet] × 100.

2.6. TUNEL assay

The TUNEL assay was performed using the Apoptosis Screening Kit*wako* (Wako Pure Chemical Industries, Ltd.) which allows quantitative colorimetric analysis of a large number of cell samples using a 96-well plate. In brief, cells (10^6) collected from 25-cm^2 tissue culture flask were transferred to 96-well plates and then incubated for 4, 16, 24 hr after the Stx2 (64 pg/mL) challenge. The plate was centrifuged at 400 *g* (4°) and then 200 μL per well of the fixation solution and 100 μL per well of the permeability solution were added in the prescribed manner. After the cells were washed with PBS, Terminal deoxynucleotidyl transferase (TdT) labelling buffer (50 μL per well) plus TdT enzyme was added into each sample and incubated at 37° for 30 min. After washing the samples five times with PBS, 100 μL per well of chromogenic substrate was added to the samples. Absorption was read at 490 nm on a microplate reader (Labsystems Multiscan, Dainippon Parmacy Co.).

2.7. Annexin V assay

This assay used a MEBCYTO apoptosis kit (MBL International Co.). Cells were incubated for 4 hr and washed with PBS, removed from the culture dish with Trypsin/EDTA, centrifuged at 200 *g* (room temperature) and resuspended in Binding buffer. Samples were added to annexin V-FITC and incubated at room temperature for 15 min in the dark. Detection of apoptotic cells (annexin V-FITC-positive) was performed using a flow cytometry assay (Fluorescence-activated cell sorting calibur; Becton Dickinson) in combination with Cell Quest software (Becton Dickinson).

2.8. Mitochondrial membrane potential

The changes in the mitochondrial membrane potential were detected using a MitoCapture Apoptosis Detection kit (MBL International Co.). Cells were incubated for 4 hr and washed with PBS, removed from the culture dish with Trypsin/EDTA, centrifuged at 500 *g* (room temperature) and resuspended in diluted MitoCapture reagent in the kit. Samples were added to pre-warmed Incubation Buffer from the kit. MitoCapture is a cationic dye that fluoresces in apoptotic cells and the fluorescent signals were analysed by flow cytometry using the FITC channel.

2.9. Intracellular caspase activity

Caspase activity was detected using PhiPhiLux-G1D2 assay kit (Oncoimmunin). Cells were incubated for 4 hr and washed with PBS, removed from the culture dish with Trypsin/EDTA, centrifuged at 200 *g* (room temperature) for 5 min and resuspended with 50 μL of 10 μM substrate solution. After incubation for 1 hr at 37° avoiding direct light, the samples were diluted with 1 mL of ice-cold flow cytometry dilution buffer and filtered through a nylon mesh to remove all aggregates and/or debris. Flow cytometry analysis using the FITC channel was performed within 90 min of the end of the incubation period.

2.10. Measurement of the PKA activity

The cells were lysed by the addition of 500 μL ice-cold buffer (20 mM 3-[*N*-morpholino]propanesulfonic acid, pH 7.2, 125 mM KCl, 5 mM DTT, 2% (v/v) Triton X-100). The contents of each flask were centrifuged at 12,000 *g* (4°) for 5 min to precipitate cell debris and the resulting supernatants were used immediately as the enzyme source. PKA activity was assayed using the Pep Tag kit for non-radioactive detection of PKA (Promega Corp.). The phosphorylation of the coloured peptides supplied with the kit was used to measure kinase activity and quantitation of the phosphorylated peptide was detected by spectrophotometer (Labosystems Multiscan, Dainippon Pharmacy Co.). The data are expressed as units of kinase activity in the reaction. One unit of kinase activity is defined as the number of nanomoles of phosphate transferred to a substrate per minute per millilitre.

2.11. Assay of MAPK (p42/p44, p38)

MAPK activities were assayed using the MAPK (p42/p44) assay kit (Amersham Japan) and p38MAPK assay kit (Stratagene). The assay system is based upon the MAPK catalysed transfer of the gamma-phosphate group of adenosine-5′-triphosphate to a peptide which is selective for MAPK. Cells were lysed in 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 10 μg/mL aprotinin (pH 7.4) and then were precipitated at 25,000 *g* (4°) for 20 min. The supernatant was retained which contained the cytoplasmic MAPK. The reaction was initiated by the addition of [gamma-^{33}P]ATP and the incorporation of ^{33}P into the peptide at 30° for 10–30 min incubation was assayed as a MAPK activity in a scintillation counter.

2.12. Statistics

Statistical analysis was undertaken using ANOVA followed by a Bonferroni and Dunnet or using non-parametric analysis with Kruskal–Wallis test followed by the Mann–Whitney *U* test for multiple comparisons. Results were expressed as mean ± standard error (SE) of the mean.

3. Results

3.1. Analysis of DNA by gel electrophoresis and quantification of DNA degradation

Agarose gel electrophoresis (Fig. 1A) demonstrated that DNA degradation was apparent in the ACHN cells following the 24 hr Stx2 challenge which was attenuated by cAMP-PKA activation via β_2-adrenoceptors. Staurosporine-induced DNA laddering was used as a positive control and little fragmentation of DNA was found in the untreated control ACHN cells. DNA degradation induced by the 24 hr exposure to Stx2 was attenuated by addition of terbutaline (10^{-7} M) or dbcAMP (10^{-5} and 10^{-6} M). The terbutaline inhibition of Stx2-induced DNA degradation at 24 hr was completely abolished by H-89 (5×10^{-6} M), KT (10^{-5} M), or ICI (10^{-6} M). Exposure of the cells to terbutaline (10^{-7} M) or dbcAMP (10^{-5} M) alone caused little degradation of DNA. Agarose gel electrophoresis (Fig. 1B) demonstrated that the addition of terbutaline (10^{-7} to 10^{-9} M) dose-dependently inhibited the DNA degradation after the 24 hr Stx2 challenge. Furthermore, Fig. 1 shows that the addition of PD (10^{-6} M) did not block DNA degradation caused by the 24 hr exposure to Stx2 while the addition of SB (10^{-6} M) was able to prevent the degradation. Untreated control cells and cells exposed to terbutaline (10^{-7} M), PD (10^{-6} M), or SB (10^{-6} M) alone had a little degradation of DNA. The characteristic apoptotic DNA ladder as shown in Fig. 1A, was quantitated by the diphenylamine reaction method (Fig. 2A). Staurosporine as a positive control caused DNA fragmentation of 13.0 ± 1.2%. Twenty-four hours exposure to Stx2 increased DNA fragmentation in the cells

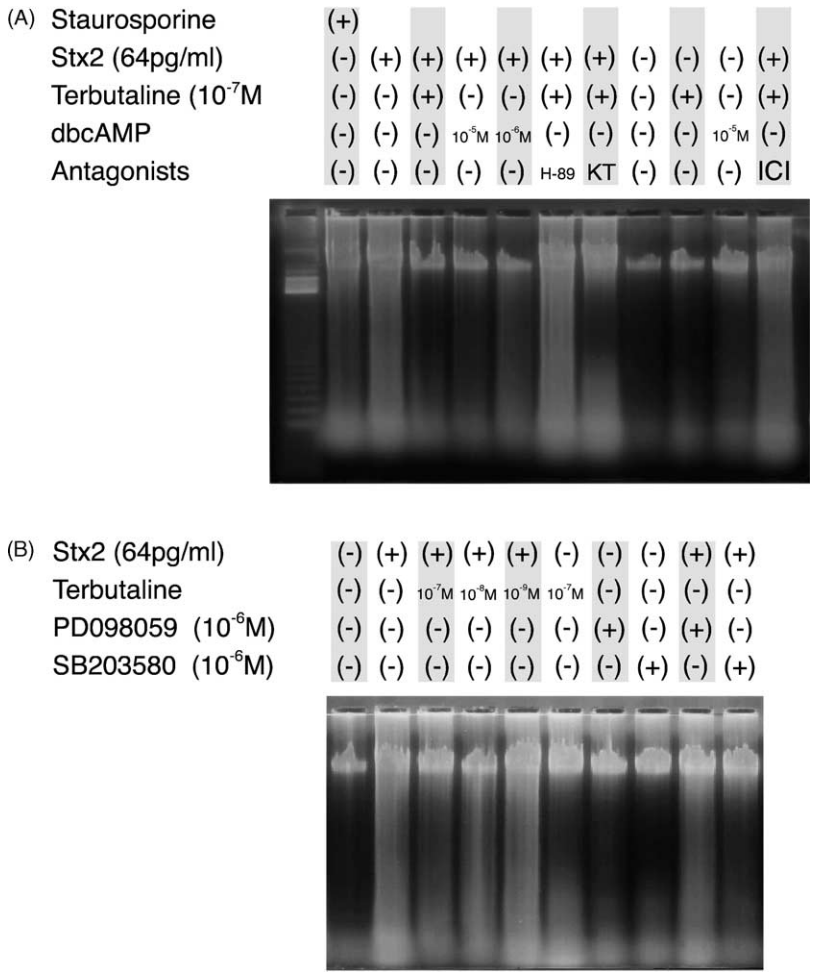

Fig. 1. DNA gel electrophoresis demonstrating apoptosis in ACHN cells. (A) The effect of cAMP-PKA activation via β_2-adrenoceptor on apoptosis after exposure to Stx2 (64 pg/mL) for 24 hr. Size marker is a 123 bp ladder. Shown is one representative of three. (B) The effects of different concentrations of the β_2-adrenoceptor agonist (terbutaline) and inhibitors (PD, SB) of MAPK (p42/p44, p38) pathway on apoptosis after exposure to Stx2 (64 pg/mL) for 24 hr. Shown is one representative of three.

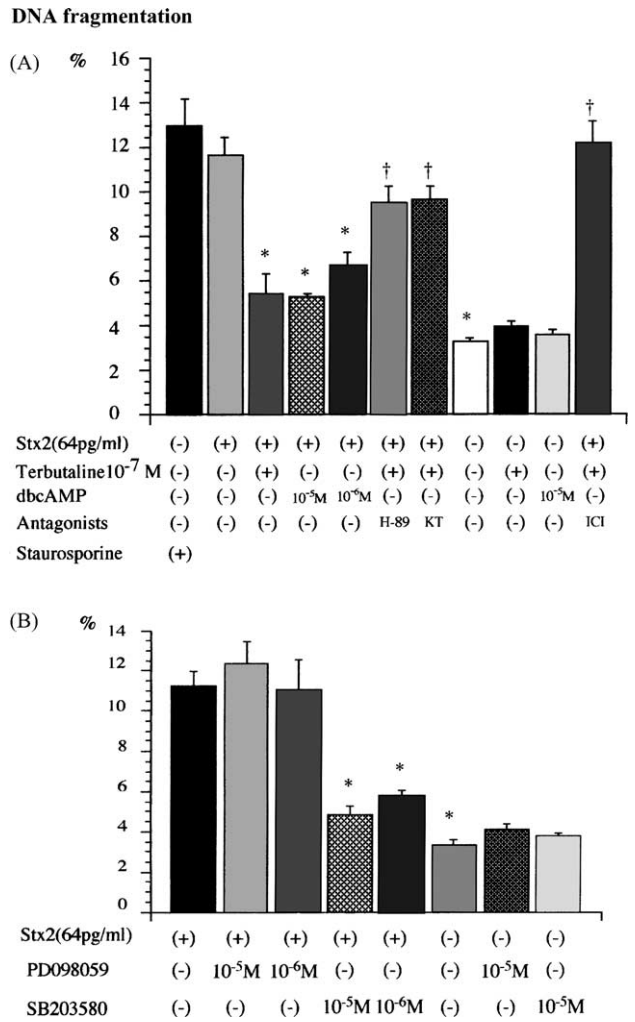

Fig. 2. Apoptosis in ACHN cells assessed using the %DNA fragmentation assay. The role of the cAMP-PKA pathway via β_2-adrenoceptors (A) and MAPK (B) pathway in the Stx2 (64 pg/mL)-stimulated apoptosis was evaluated using %DNA fragmentation. Data are the mean ± SE from three to four experiments. $^{*}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL). $^{\dagger}P < 0.05$ vs. cells exposed with Stx2 plus terbutaline (10^{-7} M). %DNA fragmentation was determined by spectrophotometric measurement.

to 11.6 ± 0.9%, compared to 3.3 ± 0.2% in control cells ($P < 0.05$). However, the addition of terbutaline (10^{-7} M) or dbcAMP (10^{-5} and 10^{-6} M) significantly decreased Stx2-induced DNA fragmentation to roughly 5–7% ($P < 0.05$). It was apparent that the inhibitory action of terbutaline on Stx2-mediated DNA fragmentation was blocked in the presence of H-89 (5×10^{-6} M), KT (10^{-5} M), or ICI (10^{-6} M). In the absence of Stx2, terbutaline (10^{-7} M) or dbcAMP (10^{-5} M) alone did not alter the amount of DNA fragmentation. The characteristic apoptotic DNA ladder as shown in Fig. 1B, was quantitated by the diphenylamine reaction method (Fig. 2B). The data of Fig. 2B indicated that %DNA fragmentation after the 24 hr Stx2 challenge was significantly attenuated by SB in a dose-dependent manner, but not by PD, suggesting that the Stx2-induced apoptosis was regulated by the p38MAPK cascade.

3.2. Analysis of DNA degradation by TUNEL

The effect of terbutaline on the apoptosis was further confirmed by the TUNEL assay. Figure 3A shows the time course of the effect of terbutaline on Stx2-mediated apoptosis detected by the TUNEL method. After 16 hr of exposure to Stx2 (64 pg/mL), there was a sharp increase in the level of TUNEL positive cells, indicative of apoptosis, of some 10-fold, which was maintained at 24 hr. Terbutaline (10^{-7} to 10^{-9} M) significantly suppressed these responses in a dose-dependent manner ($P < 0.05$). In Fig. 3B, it can be seen that the degree of Stx2 (64 pg/mL)-induced apoptosis at 16 hr was suppressed in a dose-dependent way by 10^{-5} to 10^{-6} M dbcAMP ($P < 0.05$). The inhibitory effect of terbutaline on Stx2-mediated apoptosis was blocked in the presence of H-89 (5×10^{-6} M), KT (10^{-5} M), or ICI (10^{-6} M). Terbutaline (10^{-7} M) or dbcAMP (10^{-5} M) alone had no effect on the level of TUNEL positive cells.

3.3. Estimation of cell apoptosis by annexin V assay

To extend these results to the level of the single cell, apoptosis of cells was evaluated by the annexin V assay and distinguished from necrosis using a PI staining flow cytometry methodology. Figure 4A shows that terbutaline (10^{-7} M) or dbcAMP (10^{-5} to 10^{-6} M) decreased the percentage of Stx2-mediated apoptosis cells 4 hr after the Stx2 challenge in a concentration-related fashion. Moreover, the inhibitory action of terbutaline (10^{-7} M) was blocked by H-89 (5×10^{-6} M), KT (10^{-5} M), or ICI (10^{-6} M). Incubation of the cells with terbutaline (10^{-7} M) or dbcAMP (10^{-5} M) alone did not alter the percentage of apoptotic cells. The data of Fig. 4B indicated that annexin V-FITC-positive cells after the 24 hr Stx2 challenge were significantly attenuated by SB in a dose-dependent manner, but not by PD.

3.4. Estimation of cell apoptosis by mitochondrial membrane potential assay

To investigate the apoptotic signals initiated by Stx2 (64 pg/mL) and the anti-apoptotic effect of terbutaline, we estimated the mitochondrial membrane potential using the uptake of the mitochondrial specific dye, Mitocapture. After incubation for 4 hr, Stx2, or staurosporine as a positive control, increased the percentage of FITC-positive (Fig. 5A). Moreover, the addition of terbutaline (10^{-7} M) or dbcAMP (10^{-5} to 10^{-6} M) significantly suppressed FITC-positive cell stimulation by Stx2 in a dose-dependent manner and the inhibitory action of terbutaline (10^{-7} M) was completely blocked by H-89 (5×10^{-6} M), KT (10^{-5} M), or ICI (10^{-6} M). Terbutaline (10^{-7} M) or dbcAMP (10^{-5} M) alone did not change mitochondrial membrane potential in the cells. The data of Fig. 5B indicated that Mitocapture-FITC-positive cells after the

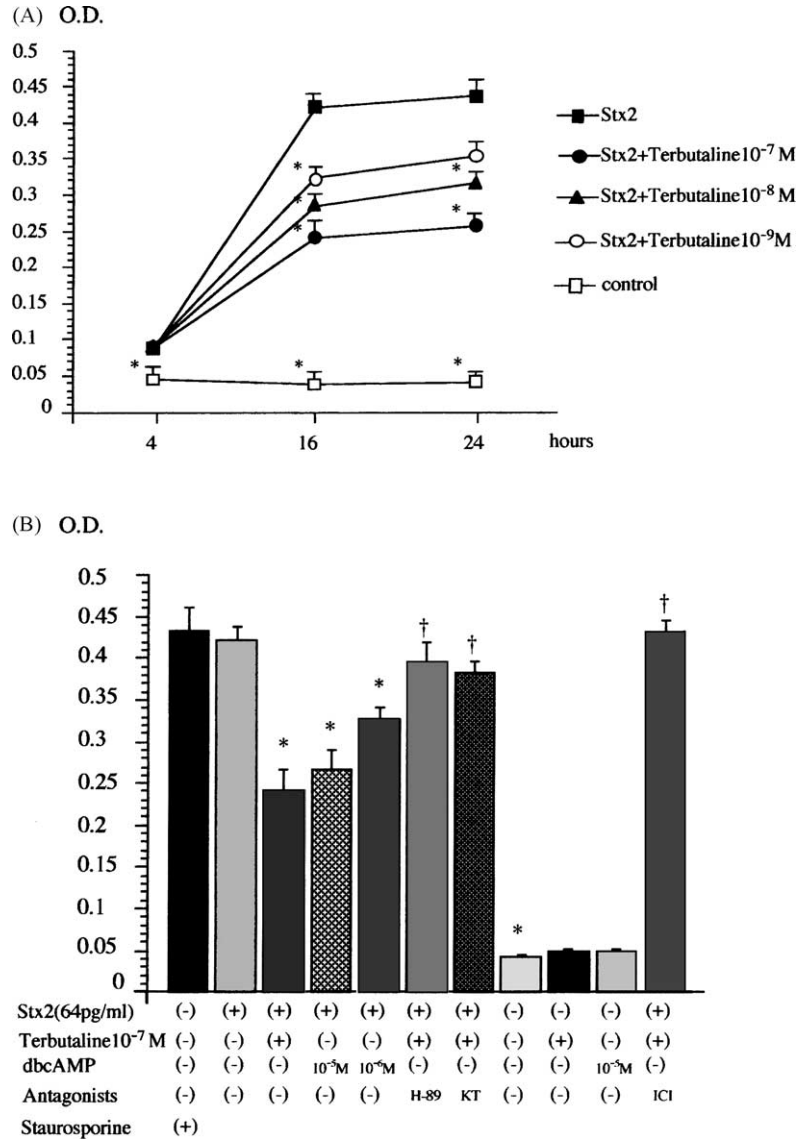

Fig. 3. Apoptosis in ACHN cells assessed using the TUNEL assay. (A) Time course changes of Stx2 (64 pg/mL)-induced apoptosis in the cells following the administration of terbutaline: 10^{-7} to 10^{-9} M. (B) The effect of the cAMP-PKA pathway via β_2-adrenoceptors on TUNEL positive cells after 16 hr exposure to Stx2. The optical density (OD) of TUNEL positive cells was determined by spectrophotometric measurements at 490 nm. Data are the mean ± SE from three experiments. $^{*}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL). $^{\dagger}P < 0.05$ vs. cells exposed to Stx2 plus terbutaline (10^{-7} M). Details are in Section 2.

24 hr Stx2 challenge were significantly attenuated by SB in a dose-dependent manner, but not by PD.

3.5. *Effect of β_2-adrenoceptor activation on caspase activity*

Next, we measured caspase activity in ACHN cells using PhiPhiLux-G1D2. This cell-permeable fluorescent substrate for caspase emits increased fluorescence when it is proteolysed within its PARP cleavage site sequence (GDEVDGID). Since caspase-3 and -7 appear to be primarily responsible for PARP cleavage during apoptosis, the increase in fluorescence should mainly reflect the activities of caspase-3 and -7. In Fig. 6A, Stx2-mediated caspase activity was significantly inhibited by terbutaline (10^{-7} M, $P < 0.05$) and dbcAMP (10^{-5}, 10^{-6} M, both $P < 0.05$). Although the terbutaline (10^{-7} M) suppression of caspase activity was blocked by H-89 (5×10^{-6} M), KT (10^{-5} M), or ICI (10^{-6} M), the magnitude of the block by H-89 (26% inhibition, $P < 0.05$) or KT (29% inhibition, $P < 0.05$) was small and the caspase activity was still inhibited significantly by terbutaline (10^{-7} M). Importantly, each treatment with H-89 (5×10^{-6} M) or KT (10^{-5} M) completely suppressed the terbutaline (10^{-7} M)-induced PKA activity in the cells (Fig. 7). Moreover, Fig. 7 demonstrated that terbutaline (10^{-7} and 10^{-8} M) can stimulate PKA activity in the cells whereas Stx2 (64 pg/mL) was unable to stimulate PKA activity. Thus, the inhibitory effect of terbutaline on caspase activity involved both cAMP-PKA-dependent and -independent pathways. Neither terbutaline (10^{-7} M) nor dbcAMP (10^{-5} M) alone altered the caspase activity over the incubation period. On the other hand, because

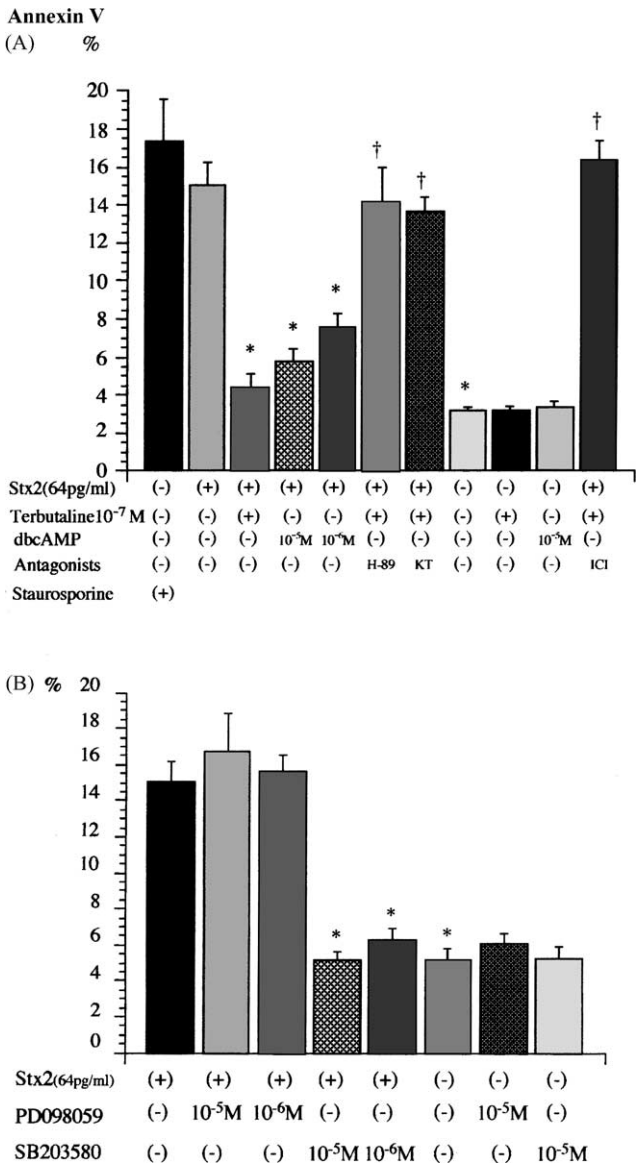

Fig. 4. Apoptosis in ACHN cells assessed using annexin V assay. The effects of cAMP-PKA pathway via β_2-adrenoceptors (A) or MAPK (p42/p44, p38) pathway (B) on apoptosis after 4 hr (A) or 24 hr (B) exposure to Stx2 (64 pg/mL) were measured with annexin V assay. The number of apoptotic cells was determined by flow cytometry using annexin V-FITC-positive cells, as indicated in Section 2. Data are the mean ± SE from three experiments. $^{*}P < 0.05$ vs. cells exposed to Stx2. $^{\dagger}P < 0.05$ vs. cells exposed to Stx2 plus terbutaline (10^{-7} M).

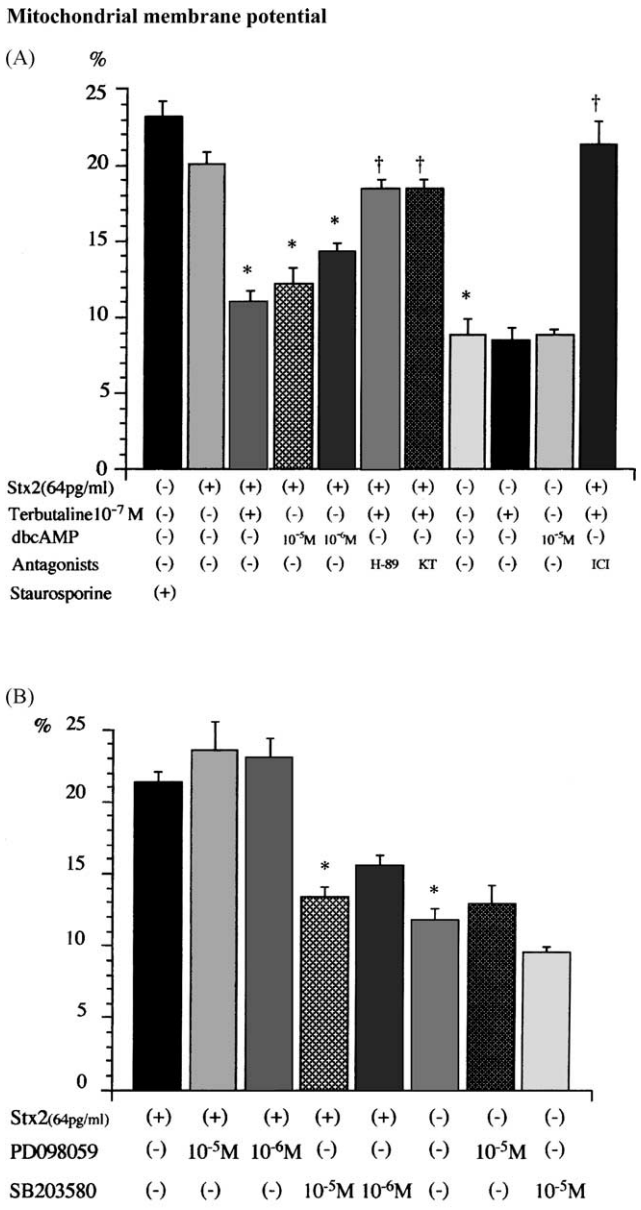

Fig. 5. Apoptosis in ACHN cells assessed using mitochondrial membrane potential assay. The effects of cAMP-PKA pathway via β_2-adrenoceptors (A) or MAPK (p42/p44, p38) pathway (B) on apoptosis after 4 hr (A) or 24 hr (B) exposure to Stx2 (64 pg/mL) were measured using mitochondrial membrane potential assay. The number of apoptotic cells was determined by flow cytometry using Mitocapture-FITC-positive cells, as indicated in Section 2. Data are the mean ± SE from three experiments. $^{*}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL). $^{\dagger}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL) plus terbutaline (10^{-7} M).

the caspase activity was significantly suppressed by SB (Fig. 6B), but not by PD, caspase(-3 and -7) activity was also dependent on p38MAPK pathways.

3.6. Effect of Stx2 stimulation and β_2-adrenoceptor activation on MAPK activity

Figure 8A illustrated the action of terbutaline (10^{-7} M) or PD (10^{-6} M) on Stx2-mediated p42/p44MAPK activity in the cells. The MAPK activity was stimulated by the Stx2 challenge and reached a peak level at 20 min [18]. In the presence of terbutaline or PD, the peak levels of Stx2-induced p42/p44MAPK activity were suppressed significantly. Figure 8B presented the effect of terbutaline (10^{-7} M), SB (10^{-6} M) and/or TNF-α (1 ng/mL) on Stx2-induced p38MAPK activity. p38MAPK activity was stimulated by the Stx2 challenge and reached a peal level at 60 min [18]. The peak level of Stx2-induced p38MAPK was significantly suppressed by terbutaline and SB. Moreover, the addition of TNF-α (1 ng/mL), a p38MAPK activator, blocked the inhibitory effect of

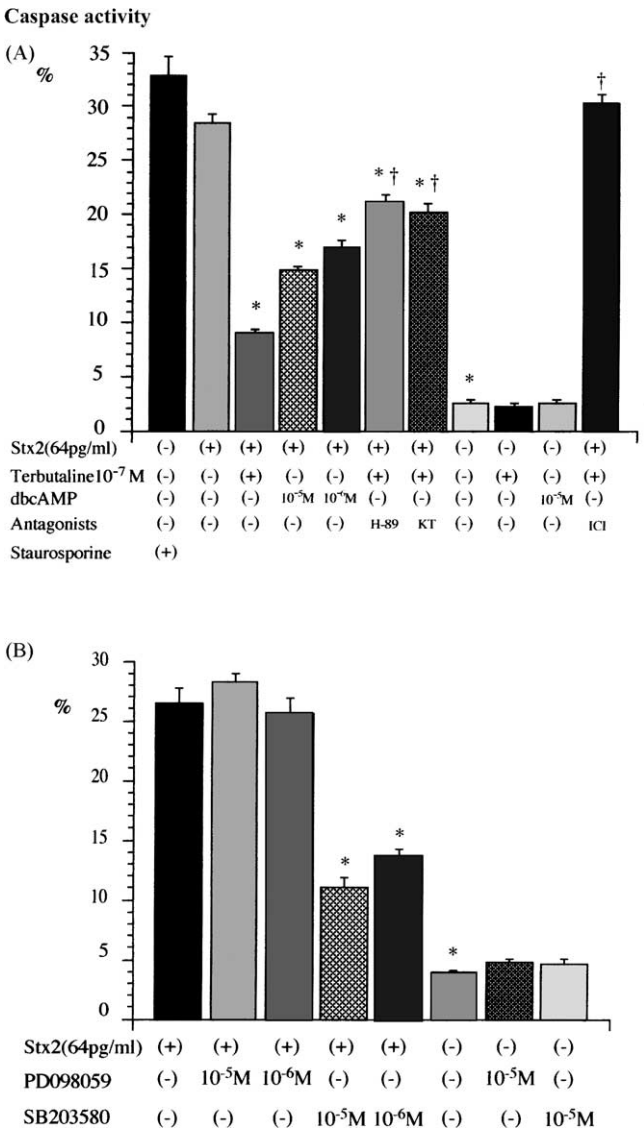

Fig. 6. The effects of cAMP-PKA pathway via β_2-adrenoceptors (A) or MAPK (p42/p44, p38) pathway (B) on caspase(-3 and -7) activity on apoptosis after 4 hr (A) or 24 hr (B) exposure to Stx2 (64 pg/mL) were assessed using the PhiPhiLax-G1D2. Caspase activity was determined by flow cytometry using PhiPhiLax-G1D2-FITC-positive cells, as indicated in Section 2. Data are the mean ± SE from five experiments. $^{*}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL). $^{\dagger}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL) plus terbutaline (10^{-7} M).

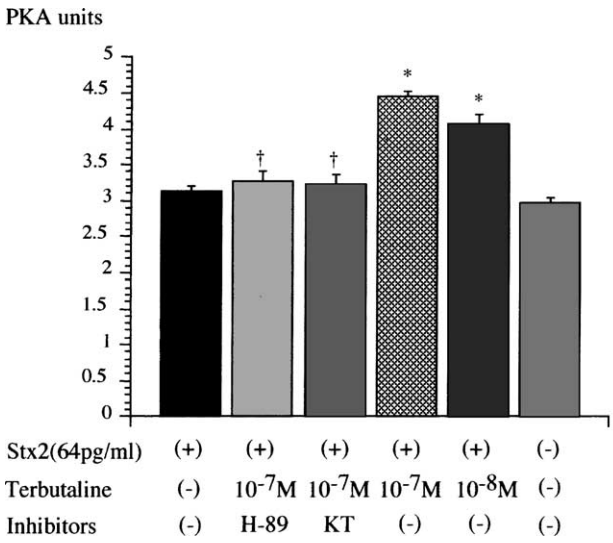

Fig. 7. Interactions of terbutaline (10^{-7}, 10^{-8} M) with the cAMP-PKA inhibitors (H-89, KT) on the PKA activity units of ACHN cells after 4 hr exposure to Stx2. PKA activity units were determined by spectrophotometry, as indicated in Section 2. Data are the mean ± SE from three experiments. $^{*}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL). $^{\dagger}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL) plus terbutaline (10^{-7} M).

terbutaline on Stx2-induced p38MAPK activity. These findings suggested that PD (10^{-6} M) and SB (10^{-6} M) have been applied in an effective concentration range in this study and TNF-α played an important role in Stx2-induced p38MAPK activity.

4. Discussion

The important finding arising from this study was that, on the basis of functional (flow cytometry using FITC-conjugated annexin V and mitochondrial potential) and molecular (DNA fragmentation and TUNEL) criteria, up to 24 hr after the initial exposure to Stx2, the β_2-adrenoceptor agonist was able to prevent Stx2-mediated ACHN cell apoptosis through the cAMP-PKA pathway and the apoptosis was attenuated by inhibition of the p38MAPK pathway. Figure 9 depicts these findings and summarizes the known and discussed effects of β_2-adrenoceptor and the possible interaction with Stx-2 in the ACHN cells.

Stx consists of an A subunit of 32 kDa associated with five B subunits, each of 7.5 kDa. The B subunits bind specifically to the cell surface Gb3 receptor, also known as CD77, and facilitates the entry of the holotoxin into susceptible cells [20]. ACHN cells express the receptor for Stx, Gb3/CD77 and binding to the CD77 antigen induces cell cytotoxicity/apoptosis [21]. The present study showed that Stx2 binding to Gb3 caused activation of MAPK (p42/p44) and terbutaline suppressed both MAPK pathways. It has to be recognized that the cAMP-PKA pathway did not inhibit Stx2-activated p38MAPK or JNK while an elevation of cAMP, via β_2-adrenoceptors, resulted in a suppression of the Stx2-induced p42/p44MAPK activity [18]. Because suppression of p42/p44MAPK by PD did not influence the Stx2-induced apoptosis, as shown in the present study, it is clear that the MAPK family (p42/p44, p38, JNK) did not contribute to the regulation of Stx2-induced apoptosis through a cAMP-PKA pathway. The question arises as to what mechanisms are involved in the anti-apoptotic effect of β_2-adrenoceptor agonist mediated through the cAMP-PKA pathway. This was partially answered in the present study by showing that caspase activity was significantly suppressed by an elevation in cAMP. Thus, this would suggest that the inhibitory effect of cAMP-PKA activation, via β_2-adrenoceptor stimulation,

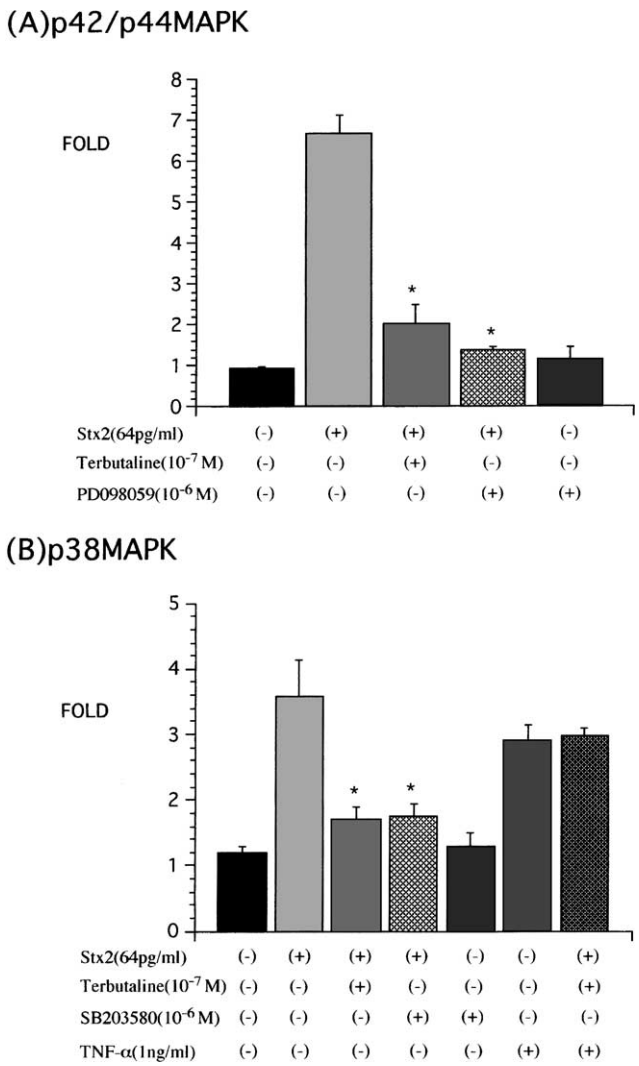

Fig. 8. MAPK activation in ACHN cells. (A) 64 pg/mL Stx2 increased p42/p44MAPK activity, but the response was suppressed by terbutaline (10^{-7} M) or PD (10^{-6} M). (B) 64 pg/mL Stx2 increased p38MAPK activity, but the response was suppressed by terbutaline (10^{-7} M) and SB (10^{-6} M). TNF-α (1 ng/mL), as a p38MAPK activator, was added into the culture together with Stx2. The inhibitory effect of terbutaline on Stx2-induced p38MAPK activation was significantly prevented by TNF-α. Data are the mean ± SE from three experiments. $^{*}P < 0.05$ vs. cells exposed to Stx2 (64 pg/mL).

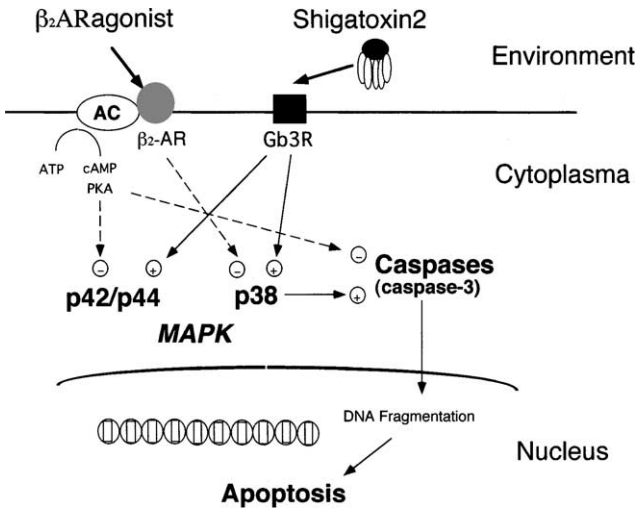

Fig. 9. Proposed model of pathways mediating apoptosis in ACHN cells exposed to Stx2 and/or β_2-adrenoceptor (β_2-AR) agonist. After binding of holotoxin to Gb3 receptor (Gb3R) in renal tubular cells, there is an increase in MAPK (p42/p44, p38) activity. The elevation of p38MAPK could result in activation of caspase cascade (caspase-3), thereby signaling cells to undergo apoptosis. On the other hand, β_2-adrenoceptors couple to adenyl cyclases to raise intracellular level of cAMP, which inhibits Stx2-induced caspase-3 activation. Moreover, β_2-adrenoceptor agonist suppressed p38MAPK activation with subsequent caspase-3 inhibition through a cAMP-independent mechanism. AC: adenyl cyclases. (⟶) Activation; (– – ⟶) inhibition.

on Stx2-mediated apoptosis was exerted through the activation of caspase(-3 and -7), which was independent of the MAPK family.

There is increasing evidence that Stx can induce apoptosis in human cells by a mechanism that possibly involves caspases. Jones *et al.* [8] reported that Stx produced by *E. coli* signal Gb3-expressing epithelial cells to undergo apoptosis in association with enhanced Bax expression, thereby resulting in activation of the caspase cascade. Kiyokawa *et al.* [22] also indicated that caspases-3, -7, -8 were activated following Stx treatment and suggested that Stx1-mediated apoptosis of Burkitt's lymphoma cells utilized a similar caspase cascade to that involved in Fas-mediated apoptosis. Importantly, caspase-3 activation was correlated with internucleosomal DNA fragmentation and chromatic condensation of the Stx-treated cells and the inhibition of caspase activation prevented Stx-stimulated apoptosis [23]. In addition, previous work in our laboratory [24] has observed that Stx2 stimulated caspase-3 in ACHN cells. Thus, it was suggested that caspase-3 plays an essential role as the effector molecules in the apoptotic process in this study. An additional observation that arises from this study is that Stx2-induced caspases were depressed by β_2-adrenoceptor activation. Previous investigators [25–27] have reported that cAMP was able to inhibit the proteolytic cleavage of caspase-3 in murine macrophage [25], human osteoblast [26], and human neutrophil [27], suggesting that a cAMP elevating agent could attenuate programmed cell death. These pieces of evidence suggest that Stx2-activated caspase-3 was blocked by β_2-adrenoceptor agonist which activated the cAMP-PKA pathway.

On the other hand, p38MAPK was found to be involved in the suppression of Stx2-induced caspase-3 by a cAMP-independent pathway. It is well known that the MAPK family plays an important role in the determination of cell survival or death [16]. The present study showed that inhibition of p38MAPK pathway by terbutaline correlated with blockade of Stx2-mediated apoptosis in the cells. Recently, much emphasis has been placed on the role of the p38MAPK in cellular stress signals for the apoptotic machinery [28,29]. Particularly, the activation of p38MAPK together with the inactivation of p42/p44MAPK is a critical step in cell apoptosis. Ikeda *et al.* [30] reported that

activation of p38MAPK was involved in Stx-induced cell death. We also indicated that the p38MAPK pathway contributed to the action of Stx2 stimulation on apoptotic factors, such as caspase-3 and TNF-α [24], and the inhibition of p38MAPK resulted in the decrease of Stx2-stimulated apoptotic parameters (%DNA fragmentation, annexin V, and mitochondrial membrane potential) and caspase activity in the present study. Together these studies suggested that the suppression of p38MAPK pathways by β_2-adrenoceptor activation via a cAMP-independent pathway appears to be part of a protective response against Stx2-induced cell apoptosis.

Suppression of TNF-α by β_2-adrenoceptor activation also plays an important role in inhibition of Stx2-induced cell apoptosis. TNF-α has been shown to enhance Stx-mediated apoptosis in ACHN cells [6] and β_2-adrenoceptor activation was able to suppress the Stx-induced TNF-α release and gene expression through p38MAPK pathway [18]. Importantly, addition of TNF-α blocked the effect of β_2-adrenoceptor activation to prevent the rise in p38MAPK, as shown in the present study. Therefore, an inhibitory action of the β_2-adrenoceptor agonist on TNF-α production could also contribute to the protection against Stx2-induced apoptosis through the p38MAPK pathway.

The present data suggested the idea of an involvement of the cAMP-PKA pathway via β_2-adrenoceptors in preventing the Stx2-induced apoptosis. However, a different response of apoptosis on exposure to cAMP elevating agents has been reported in other cell types. Mühl *et al.* [31] found that activation of the cAMP signalling pathway by dbcAMP or β_2-adrenoceptor agonists resulted in induction of apoptosis in mesangial cells. Gu *et al.* [32] also showed that cell death of thymocytes could be induced after stimulation of β_2-adrenoceptors or by addition of exogenous cAMP. However, the apoptotic action observed in these studies required very high doses of β_2-adrenoceptor agonists (10^{-3} to 2.5×10^{-5} M) or dbcAMP (10^{-3} M). Although the physiological or pathophysiological importance of an effect seen with these high doses was unclear, these reports suggested the possibility of a biphasic effect of β_2-adrenoceptor activation as shown in our previous report [33] which may be applicable to the action of β_2-adrenoceptor activation on cell apoptosis. Furthermore, it was also recognized that the origin of the cell or tissue could have determined whether an induction or inhibition of apoptosis might be caused by the cAMP-elevating agents. For example, cAMP-PKA activation induced apoptosis in myeloid [34], thymocyte [15] and embryonic epithelial cells [35]. By contrast, cAMP inhibited apoptosis in neutrophils and nerve tissue [36,37]. In any case, the action of cAMP-PKA via β_2-adrenoceptor activation on cell apoptosis is thought to be dependent on a number of factors. In summary, we found that the β_2-adrenoceptor agonist suppressed Stx2-induced caspases, which, in turn, was correlated with inhibition of Stx2-induced apoptosis. The inhibitory effect was regulated by both cAMP-PKA pathway and p38MAPK cascade.

Acknowledgments

This study was supported by grants from the Human Science Foundation in Japan.

References

[1] Fong JS, De Chadarevian JP, Kaplan BS. Hemolytic Uremic Syndrome. Current concepts and management. Pediatr Clin N Am 1982;29:835–56.
[2] Kaplan BS, Meyers KE, Schulman SL. The pathogenesis and treatment of Hemolytic Uremic Syndrome. J Am Soc Nephrol 1998;8:1126–33.
[3] Lingwood CA. Verotoxin-binding in human renal sections. Nephron 1994;66:21–8.
[4] Richardson SE, Karmali MA, Becker LE, Smith CR. The histopathology of the hemolytic uremic syndrome associated with verocytotoxin-producing *Escherichia coli* infection. Human Pathol 1988;19:1102–8.
[5] Inward C, Howie A, Fitspatrick M, Rafaat F, Milford D, Taylor CM. Renal histopathology in fetal cases of diarrhea-associated hemolytic uremic syndrome. Pediatr Nephrol 1997;11:556–9.
[6] Taguchi T, Uchida H, Kiyokawa N, Mori T, Sato N, Horie H, Takeda T, Fujimoto J. Verotoxins induce apoptosis in human renal tubular epithelium derived cells. Kidney Int 1998;53:1681–8.
[7] Kiyokawa N, Taguchi T, Mori T, Uchida H, Sato N, Takeda T, Fujimoto J. Induction of apoptosis in normal human renal tubular epithelial cells by *Escherichia coli* shiga toxin 1 and 2. J Infect Dis 1998;178:178–84.
[8] Jones NL, Islur A, Haq R, Mascarenhas M, Karmali MA, Perdue MH, Zanke BW, Sherman PM. *Escherichia coli* Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. Am J Physiol Gastrointest Liver Physiol 2000;278:G811–9.
[9] Zhu Y, Prehn JHM, Culmsee C, Krieglstein J. The β_2-adrenoceptor agonist clenbuterol modulates Bcl-2, Bcl-xl and Bax protein expression following transient forebrain ischemia. Neuroscience 1999;90:1255–63.
[10] Verghese MW, Synyderman R. Hormonal activation of adenylate cyclase in macrophage membranes is regulated by guanine nucleotides. J Immunol 1983;130:869–73.
[11] Drucker DJ, Ehrlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proc Natl Acad Sci USA 1996;93:7911–6.
[12] Orlov SN, Thorin-Trescases N, Dulin NO, Dam T-V, Fortuno MA, Tremblay J, Hamet P. Activation of cAMP signaling transiently inhibits apoptosis in vascular smooth muscle cells in a site upstream of caspase-3. Cell Death Differ 1999;6:661–72.
[13] Gjertsen BT, Cressey LI, Ruchaud S, Houge G, Lanotte M, Doskeland SO. Multiple apoptotic death types triggered through activation of separate pathways by cAMP and inhibitors of protein phosphatases in one (IPC leukemia) cell line. J Cell Sci 1994;107:3363–77.
[14] Aharoni D, Dantes A, Oren M, Amsterdam A. cAMP-mediated signals as determinants for apoptosis in primary granulosa cells. Exp Cell Res 1995;218:271–82.
[15] McConkey DJ, Orrenius S, Jondal M. Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. J Immunol 1990;145:1227–30.
[16] Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.
[17] Pfeilschifter J, Huwiler A, Briner VA. Proliferative mechanisms in kidney cells. Curr Opin Nephrol Hypertens 1995;4:98–103.
[18] Nakamura A, Johns EJ, Imaizumi A, Yanagawa Y, Kohsaka T. Activation of β_2-adrenoceptor prevents shiga toxin-induced tumour necrosis factor production. J Am Soc Nephrol 2001;12:2288–99.
[19] Oku Y, Yutsudo T, Hirayama T, O'Brien AD, Takeda Y. Purification and some properties of a Vero toxin from a human strain of *Escherichia coli*

that is immunologically related to Shiga-like toxin II (VT2). Microb Pathogen 1989;6:113–22.
[20] Donohue-Rolfe A, Keusch GT, Edson C, Thorley-Lawson D, Jacewicz M. Pathogenesis of shigella diarrhea. IX. Simplified high yield purification of Shigella toxin and characterization of subunit-specific monoclonal and polyclonal antibodies. J Exp Med 1984;160:1767–91.
[21] Van Setten PA, Van Hinsbergh VWM, Van der Velden TJAN, Van de Kar NCAJ, Vermeer M, Mahan JD, Asmann KJM, Van den Heuvel LPWJ, Monnens LAH. Effect of TNF alpha on verocytotoxin cytotoxicity in purified human glomerular microvascular endothelial cells. Kidney Int 1997;51:1245–56.
[22] Kiyokawa N, Mori T, Taguchi T, Saito M, Mimori K, Suzuki T, Sekino T, Sato N, Nakajima H, Katagiri YU, Takeda T, Fujimoto J. Activation of the caspase cascade during Stx1-induced apoptosis in Burkitt's lymphoma cells. J Cell Biochem 2001;81:128–42.
[23] Kojio S, Zhang H, Ohmura M, Gondaira F, Kobayashi N, Yamamoto T. Caspase-3 activation and apoptosis induction coupled with the retrograde transport of shiga toxin: inhibition by brefeldin A. FEMS Immunol Med Microbiol 2000;29:275–81.
[24] Nakamura A, Kohsaka T, Imaizumi A, Yanagawa Y, Johns EJ. β_2-Adrenoceptor activation protects against shiga toxin-induced apoptosis via p38 mitogen-activated protein kinase [abstract]. J Am Soc Nephrol 2000;11:A3195.
[25] von Knethen A, Brune B. Attenuation of macrophage apoptosis by the cAMP-signaling system. Mol Cell Biochem 2000;212:35–43.
[26] Chae HJ, Chae SW, An NH, Kim JH, Kim CW, Yoo SK, Kim HH, Lee ZH, Kim HR. Cyclic-AMP inhibits nitric oxide-induced apoptosis in human osteoblast: the regulation of caspase-3, -6, -9 and the release of cytochrome *c* in nitric oxide-induced apoptosis by cAMP. Biol Pharm Bull 2001;24:453–60.
[27] Martin MC, Dransfield I, Haslett C, Rossi AG. Cyclic AMP regulation of neutrophil apoptosis occurs via a novel protein kinase A-independent signaling pathway. J Biol Chem 2001;276:45041–50.
[28] Valladares A, Alvarez AM, Ventura JJ, Roncero C, Benito M, Porras A. p38 mitogen-activated protein kinase mediates tumor necrosis factor-alpha-induced apoptosis in rat fetal brown adipocytes. Endocrinology 2000;141:4383–95.
[29] Walton KM, DiRocco R, Bartlett BA, Koury E, Marcy VR, Jarvis B, Schaefer EM, Bhat RV. Activation of p38MAPK in microglia after ischemia. J Neurochem 1998;70:1764–7.
[30] Ikeda M, Gunji Y, Yamasaki S, Takeda Y. Shiga toxin activates p38 MAP kinase through cellular Ca(2+) increase in Vero cells. FEBS Lett 2000;485:94–8.
[31] Mühl H, Nitsch D, Sandau K, Brune B, Varga Z, Pfeilschifter J. Apoptosis is triggered by the cyclic AMP signalling pathway in renal mesangial cells. FEBS Lett 1996;382:271–5.
[32] Gu C, Ma YC, Benjamin J, Littman D, Chao MV, Huang XY. Apoptotic signaling through the beta-adrenergic receptor. A new Gs effector pathway. J Biol Chem 2000;275:20726–33.
[33] Nakamura A, Johns EJ, Imaizumi A, Yanagawa Y, Kohsaka T. Modulation of interleukin-6 by β_2-adrenoceptor in endotoxin-stimulated renal macrophage cells. Kidney Int 1999;56:839–49.
[34] Lanotte M, Riviere JB, Hermouet S, Houge G, Vintermyr OK, Gjertsen BT, Doskeland SO. Programmed cell death (apoptosis) is induced rapidly and with positive cooperativity by activation of cyclic adenosinemonophosphate-kinase I in a myeloid leukemia cell line. J Cell Physiol 1991;146:73–80.
[35] Pratt RM, Martin GR. Epithelial cell death and cyclic AMP increase during palatal development. Proc Natl Acad Sci USA 1975;72:874–7.
[36] Rossi AG, Cousin JM, Dransfield I, Lawson MF, Chilvers ER, Haslett C. Agents that elevate cAMP inhibit human neutrophil apoptosis. Biochem Biophys Res Commun 1995;217:892–9.
[37] Edwards SN, Buckmaster AE, Tolkovsky AM. The death programme in cultured sympathetic neurones can be suppressed at the posttranslational level by nerve growth factor, cyclic AMP, and depolarization. J Neurochem 1991;57:2140–3.