DIDS Attenuates Staurosporine-induced Cardiomyocyte Apoptosis by PI3K/Akt Signaling Pathway: Activation of eNOS/NO and Inhibition of Bax Translocation

An-Heng Liu^{1,2}, Ya-Nan Cao¹, Hong-Tao Liu³, Wei-Wei Zhang¹, Yan Liu¹, Tang-Wang Shi¹, Guo-Liang Jia² and Xiao-Ming Wang¹

¹Department of Geriatrics, ²Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an, ³Department of Anesthesiology, First affiliated hospital, China Medical University, Shenyang

Key Words

Cardiomyocyte • Apoptosis • DIDS • PI3K/Akt • Signal transduction

Abstract

Aims: 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), a non-selective chloride channel blocker, has been shown to prevent cell apoptosis, however, the underlying mechanisms remain undefined, thus the present study was to explore whether phosphatidylinositol 3'-kinase (PI3K)/Akt and its downstream molecules are involved in the cytoprotective effect of DIDS. **Methods:** Neonatal rat cardiomyocytes were exposed to staurosporine (STS) in the presence or absence of DIDS. Cell viability, apoptosis and expressions of Akt, phospho-Akt (p-Akt), eNOS, phospho-eNOS (p-eNOS), Bcl-2/Bax and nitric oxide (NO) production were determined, and Bax translocation was assessed by double immunofluorescence labeling and Western blotting. **Results:** DIDS markedly improved cell viability and exerted an anti-apoptotic effect on STS-exposed cardiomyocytes. DIDS resulted in a 2.1-fold increase of p-Akt over control levels, prevented the reduction in eNOS expression and phospho-eNOS levels induced

by STS and significantly increased NO production (all $P < 0.01$ vs. STS alone). Treatment with LY294002, a selective PI3K inhibitor, abolished DIDS-induced increases in p-Akt, eNOS, p-eNOS and NO production, and completely abrogated the DIDS-induced anti-apoptotic effect ($P < 0.01$). Treatment with L-NAME, a non-selective NOS inhibitor similarly inhibited the increased NO but only partly abolished protective effects of DIDS ($P < 0.05$). In addition, DIDS effectively inhibited STS-induced Bax translocation to mitochondria, which was also reversed by LY294002. **Conclusion:** DIDS protects cardiomyocytes against STS-induced apoptosis via activating PI3K/Akt signaling pathway, including increasing eNOS phosphorylation and the subsequent NO production and inhibiting Bax translocation.

Copyright © 2008 S. Karger AG, Basel

Introduction

Apoptosis contributes to a variety of heart diseases such as myocardial infarction, cardiomyopathy, progressive pump-failure, arrhythmias as well as cardiac remodeling, and is also a major contributor to ischemia-reperfusion injury [1, 2]. The role of apoptosis in

cardiovascular diseases is becoming increasingly clear, and inhibition of apoptosis is emerging as a potential therapeutic strategy for various forms of cardiovascular diseases.

4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) is a non-selective chloride channel blocker, and has been reported to block multiple types of Cl⁻ transport pathways, including volume-sensitive outwardly rectifying (VSOR) Cl⁻ channel, an important candidate for cell apoptosis [3-5]. More importantly, staurosporine (STS)-induced apoptotic death of salmonid cells can be rescued by DIDS [6], and in U937, HeLa, NG108-15, and PC12 cells, DIDS has also been reported to reduce apoptosis induced by STS and tumor necrosis factor (TNF) [7]. Similarly, recent experimental results suggested that DIDS rescued cardiomyocytes from multiple other apoptotic stimuli, such as doxorubicin [8] and ischemia-reperfusion [9]. However to date, the molecular mechanism by which DIDS exerts its cardioprotective effect remains to be clarified.

The well-characterized phosphatidylinositol 3'-kinase (PI3K)/Akt signaling pathway delivers important anti-apoptotic signals in cell survival. Akt has been reported to play a critical role in the homeostasis of the cardiomyocytes [10] and has been demonstrated to inhibit both the mitochondrion- and receptor-mediated pathways of apoptosis [11]. A recent experiment reported the involvement of the PI3K/Akt signaling pathway in the cytoprotection of DIDS on the rabbit cardiomyocytes treated with doxorubicin [8], similar to a previous study on MDCK cells treated with STS [12].

Tremendous efforts have been made to identify the physiological substrates of Akt, which has been found to phosphorylate and regulate the function of many intracellular proteins involved in cell survival. eNOS is expressed and distributed in various cell types in the cardiovascular system far beyond their initially identified cell types and locations. Akt-mediated phosphorylation of eNOS may effectively modulate the enzyme activity and was shown to markedly affect cellular viability and diverse functions [13]. An explosion of information has recently emerged highlighting the role of mitochondria in the life and death of cells. The mitochondrion-mediated apoptosis is regulated by members of the Bcl-2 family, which contains both pro- and anti-apoptotic molecules. Indeed, the ratio of these two subsets helps to determine, in part, the susceptibility of cells to a certain death signal [14, 15]. However, it remains unclear whether PI3K/Akt and its downstream molecules are involved in the cytoprotective effect of DIDS.

Therefore, the present study was aimed to investigate the effect of DIDS on STS-induced cardiomyocyte apoptosis, and most importantly, to explore the signaling pathway involved.

Materials and Methods

Materials

All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) unless indicated otherwise. L-NAME was from Calbiochem (San Diego, CA, USA). Antibodies against Akt, phospho-Akt (Ser-473), eNOS, phospho-eNOS (Ser1177), Bax and Bcl-2 were obtained from Cell Signaling Technology (Beverly, MA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), MitoTracker DeepRed 633 and Collagenase II were purchased from Invitrogen (Carlsbad, CA, USA). Nitric oxide (NO) Assay Kit was from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). ECL reagent and bicinchoninic acid (BCA) protein Assay Kit were from Pierce (Rockford, IL, USA).

Primary neonatal rat cardiomyocyte cultures and experimental protocol

Care and use of animals in the present study were in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and were approved by the Fourth Military Medical University Committee on Animal Care. Primary cultures of neonatal rat cardiomyocytes were prepared as followings. Briefly, the hearts from 1 day-old Sprague-Dawley rats were isolated and placed in ice-cold D-Hank's buffer. The hearts were cleaned and minced with scissors into 1-2 mm fragments, and were then enzymatically digested 6-7 times with 5-7 min each time in HEPES containing 0.1% collagenase. The liberated cells from each digestion were collected in DMEM supplemented with 10% heat-inactivated FBS and centrifuged, and then were re-suspended in DMEM with 10% FBS, penicillin (100 U/ml) and streptomycin (0.1 mg/L). The cell suspension was filtered through a 100-μm diameter nylon mesh and the cells were plated onto a 10-cm Petri dish for 1 h cultivation (37°C in a 5% CO₂ incubator) for purification. After that, cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. 5-bromo-2'-deoxyuridine (BrdU; 100 μmol/L) was added during the first 48 h to inhibit proliferation of non-myocytes. We routinely obtained contractile, myocyte-enriched cells. The cells were allowed to grow in culture medium for 72 h before use. STS is a potent mitochondrial apoptotic inducer and was used to induce apoptosis in the present study, a parallel group was simultaneously treated with DIDS (250 μmol/L). To further examine the role of the PI3K/Akt pathway, another two groups were pretreated with the specific PI3K inhibitor LY294002 (20 μmol/L) or a non-specific eNOS inhibitor L-NAME (100 μmol/L) for 60 min before other additions and kept the same concentration during following studies. Cardiomyocytes cultured in normal medium served as a control group.

Cell viability assay

Cell viability was assessed by the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells seeded on 96-well culture plates at 1.2×10^5 cells/well were incubated with the test compounds for indicated time period. Following exposure, 20 μ L of the MTT solution (5 mg/ml) was added into each well with the final concentration of 0.5 mg/ml, and then the plates were incubated for an additional 2 h. After removing the medium, DMSO (150 μ L) was added into each well and the plate was incubated for 10 min at 37°C. The results were spectrophotometrically determined at 490 nm using a spectrophotometer (SpectraMax 190, Molecular Device, USA).

Caspase-3 activity

Caspase-3 activity was assayed using the caspase-3 Assay Kit/Fluorimetric by following the manufacturer's instruction. To exclude the involvement of other related proteases, the difference between fluorescence in the absence and presence of the specific inhibitor of caspase-3 was observed. The fluorogenic substrate (labeled with the fluorochrome 7-amino 4-methyl coumarin) for caspase-3 (Ac-DEVD-AMC) and the tetrapeptide inhibitor of caspase-3 (Ac-DEVD-CHO) were provided in the Caspase Assay System (Promega, Madison, WI, USA).

Determination of cardiomyocyte apoptosis

Cell shape of cardiomyocytes was observed under a phase-contrast microscope. To detect nuclear morphological changes accompanying apoptosis, the cultured cardiomyocytes were fixed with 4% formaldehyde/PBS for 10 min at room temperature. After gently rinsing with PBS, the cells were exposed to Hoechst-33258 (5 μ g/ml in PBS) and incubated for 10 min at room temperature. After rinsing with PBS again, the cells were examined under a fluorescence microscope.

For assessing DNA fragmentation (DNA ladder), cardiomyocytes were trypsinized and washed with PBS, and then incubated with mammalian cell lysis buffer (0.1% SDS, 10 mmol/L EDTA, 10 mmol/L Tris, pH=8.0) containing 20 μ g/ml heat-inactivated RNase A at 37°C for 1 h, and with 0.5 mg/ml proteinase K for another 60 min. Cell lysates were incubated at 50°C for 2 h and isopropanol was added. After ethanol precipitation, 8 μ g of DNA sample was separated by electrophoresis on 2% agarose gel containing ethidium bromide (0.5 μ g/ml). DNA ladder formation, a "hallmark" for cell apoptosis, was visualized under an ultraviolet light and photographed for permanent records.

Western blotting

Protein was extracted from the cultured cardiomyocytes and protein concentration was determined with BCA protein assay. Equal amounts of denatured protein samples (50-100 μ g protein/lane) were separated by electrophoresis on SDS-PAGE and transferred onto PVDF membranes in a semidry blotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% defatted milk in TBS for 1 h at 37°C, immunoblots were probed with primary antibodies overnight at 4°C followed by incubation with the corresponding HRP-

conjugated IgG antibody for 1 h at 37°C. The blots were developed with an enhanced chemiluminescence detection kit and the blot densities were analyzed with LabImage software. Beta-actin was used as the internal loading control.

To measure the Bax at subcellular level, the cultured cardiomyocytes were washed with PBS, scraped from the culture dishes and centrifuged at 750 g for 5 min. The cells were resuspended in 150 μ L of cold lysis buffer (at pH 7.5, in mmol/L, 250 sucrose, 20 HEPES, 10 KCl, 1 MgCl₂, 1 EDTA, 1 EGTA, 1 dithiothreitol) containing 1% protease inhibitor cocktail, and were incubated for 5 min on ice. The cells were homogenized with several strokes with a pestle and then the suspension was centrifuged at 750 g for 10 min at 4°C to precipitate the nuclei. The supernatant was centrifuged at 12,000 g for 10 min at 4°C to collect the mitochondrial fraction. The pellet was resuspended into 30 μ L of cold lysis buffer (at pH 7.5, in mmol/L, 20 Tris, 100 NaCl, 1% Triton X-100) supplemented with 1% protease inhibitor cocktail. The supernatant was further centrifuged at 100,000 g for 60 min at 4°C, and the resultant supernatant was collected as the cytosolic fraction [16, 17].

Determination of NO production

Total NO production (NO_x) in culture medium was determined by measuring the concentration of nitrite, a stable metabolite of NO, with a modified Griess reaction method. Briefly, culture medium (100 μ L) was taken and mixed with an equal volume of modified Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2% phosphoric acid). After 10 min of incubation at room temperature, the concentration of the resultant chromophore was spectrophotometrically determined at 540 nm using a spectrophotometer (SpectraMax 190, Molecular Device, USA). The nitrite concentrations in the samples were calculated from freshly prepared nitrite standard curves made from sodium nitrite with the same culture medium.

Immunofluorescence labeling

To determine the subcellular localization of Bax, both the treated and untreated cardiomyocytes were stained with MitoTracker DeepRed 633 (250 nmol/L) at 37°C for 25 min. The cells were then fixed in 3.5% paraformaldehyde for 15 min and permeabilized with 0.2% TritonX-100 in PBS for 5 min. The cells were blocked for 60 min in the blocking buffer (10% goat serum in PBS), followed by incubation with an anti-Bax antibody in blocking buffer overnight at 4°C. After rinsing several times with PBS, the cells were incubated with the goat anti-rabbit IgG (H+L) FITC-labeled secondary antibody for an additional 30 min. Labeled cells were rinsed with PBS again and cells were visualized with an Olympus FV1000 confocal fluorescence microscope.

Statistical analysis

All values in the text and figures are presented as mean $\pm$ SEM. All data were subjected to one-way ANOVA followed by the Bonferroni correction for post hoc *t* tests. For all analyses *P*-values were two-sided and differences were considered to be statistically significant with a *P*-value of <0.05.

Fig. 1. Effects of DIDS on STS-induced loss of cell viability in cardiomyocytes determined by MTT assay. Con: normal culture condition. S: cells treated with 4 $\mu\text{mol/L}$ STS for 8 h; S+D: cells treated with STS (4 $\mu\text{mol/L}$) plus DIDS (250 $\mu\text{mol/L}$) for 8 h; S+D+LY: cells pretreated with LY294002 (20 $\mu\text{mol/L}$) for 1 h before and during the co-application of STS and DIDS; S+D+L-N: cells pretreated with L-NAME (100 $\mu\text{mol/L}$) for 1 h before and during the co-application of STS and DIDS. Results were expressed as percentages of control and presented as mean $\pm$ SEM (vertical bar) ($n=12$) of 3 independent experiments. * $P<0.01$ vs. Con; # $P<0.01$ vs. S; ** $P<0.01$, ## $P<0.05$ vs. S+D.

Fig. 2. Effects of DIDS on morphological features of STS-treated rat cardiomyocytes. Control: cells cultured in normal condition; STS: treated with 4 $\mu\text{mol/L}$ STS for 8 h; STS+DIDS: cells treated with STS (4 $\mu\text{mol/L}$) plus DIDS (250 $\mu\text{mol/L}$) for 8 h. Top panels: representative phase-contrast photomicrographs. Bottom panels: representative fluorescence photomicrographs of cells stained with Hoechst-33258. Each photograph is a representative of 3 independent observations. Scale bar: 50 μm .

Results

DIDS inhibited STS-induced cardiomyocyte apoptosis

Exposure of cardiomyocytes to 4 $\mu\text{mol/L}$ STS for 8 h resulted in a significant decrease in cell viability ($42.9 \pm 3.4\%$, $P<0.01$ vs. control) (Fig. 1) and clear morphological alterations such as rounding up of the cells and a loss of cell-cell contacts (Fig. 2, top). Moreover, automatism usually observed in the primary cultured cardiomyocytes was markedly weakened or abolished when cells were treated with STS for 8 h, accompanied by typical apoptotic changes, such as segmented and irregularly shaped nuclei (Fig. 2, bottom), a significant increase of caspase-3 activity

(Fig. 3A) and obvious DNA ladder formation (Fig. 3B).

The impact of DIDS on STS-induced alterations in cardiomyocytes was investigated. As shown in Fig. 1, STS-induced cell death was significantly reduced ($77.4 \pm 6.8\%$, $P<0.01$ vs. STS) and cell shape changes were largely prevented in the presence of DIDS (250 $\mu\text{mol/L}$) (Fig. 2, top). DIDS prevented the alterations in cellular and nuclear morphology (Fig. 2, bottom), reduced the activation of caspase-3 by 60% (Fig. 3A) and DNA ladder formation (Fig. 3B). These results provide direct evidences that DIDS could protect cardiomyocytes against apoptosis induced by STS, consistent with previous reports [18, 19].

Fig. 3. Effects of DIDS on caspase-3 activity and DNA fragmentation in cardiomyocytes treated with STS for 8 h. (A) caspase-3 activity. Data (normalized with the control value) represented the mean $\pm$ SEM (vertical bar) ($n=8$) of 3 independent experiments. Con: control; S: STS; D: DIDS; LY: LY294002; L-N: L-NAME. * $P<0.01$ vs. Con; # $P<0.01$, vs. S; ** $P<0.01$, ## $P<0.05$ vs. S+D. (B) DNA laddering. Data are representative of 3 independent experiments.

DIDS treatment activated Akt

Western blotting results demonstrated no significant changes of Akt level among the Control, STS and STS plus DIDS groups (not shown). Akt phosphorylation (p-Akt) has been demonstrated to reflect Akt activation. As summarized in Fig. 4A, there is a relatively low level of p-Akt in normal cardiomyocytes. The levels of p-Akt were not clearly changed after challenging with STS for 8 h. However, in the presence of DIDS, Akt was significantly activated, resulting in a 2.1-fold increase of p-Akt in STS-exposed cardiomyocytes ($P<0.01$ vs. STS). Time-matched application of DIDS alone (direct application of DIDS to control cells) did not significantly influence p-Akt levels (Fig. 4B).

In addition, the effect of DIDS on Akt activity was completely abolished by treatment with LY294002 (20 μ mol/L), a specific PI3K blocker ($P<0.01$ vs. STS+DIDS). Treatment with LY294002 also completely abolished the protective effects of DIDS on STS-induced cardiomyocyte apoptosis. Specifically, cell viability was significantly diminished ($32.0 \pm 5.3\%$ compared to the control, $P<0.01$ vs. STS+DIDS) (Fig. 1). Furthermore, the inhibition of DNA ladder formation and caspase-3 activation were also reversed by LY294002 (Figs. 3A and 3B). These data indicate that the activation of PI3K/Akt pathway contributes to the cytoprotective effect of DIDS on STS-exposed cardiomyocytes.

DIDS treatment resulted in increases of eNOS protein, eNOS phosphorylation and NOx

eNOS is a novel substrate for Akt. Phosphorylation of eNOS by Akt and the increased NOx are important for its anti-apoptotic effect. We therefore attempted to determine whether eNOS phosphorylation (p-eNOS) and NOx contribute to the protective effects of DIDS. As illustrated in Fig. 5, cardiomyocytes treated with STS for 8 h, presented a significant decrease in eNOS and p-eNOS levels ($51.4 \pm 4.5\%$, $43.9 \pm 3.5\%$ of those in control cells respectively, $P<0.01$ vs. control). Most importantly, DIDS partly, but significantly reversed the STS-induced reduction in eNOS and eNOS phosphorylation (both

Fig. 4. Effect of DIDS on p-Akt levels in cardiomyocytes treated with STS for 8 h and its modification by LY294002 (A) and a direct effect of DIDS on p-Akt levels in cardiomyocytes for the indicated time period (B). Insets: Representative blots. Data obtained from quantitative densitometry were presented as mean $\pm$ SEM (vertical bar) of 5 independent experiments. Con: control; S: STS; D: DIDS; LY: LY294002; L-N: L-NAME. * $P<0.01$ vs. S; # $P<0.01$ vs. S+D.

Fig. 5. Effect of DIDS on eNOS (A) and p-eNOS levels (B) in cardiomyocytes treated with STS. Insets: Representative blots. Data obtained from quantitative densitometry were presented as mean $\pm$ SEM (vertical bars) of 5 independent experiments. Con: control; S: STS; D: DIDS; LY: LY294002; L-N: L-NAME. * P <0.01 vs. Con; # P <0.01 vs. S; ** P <0.01 vs. S+D.

P <0.01 vs. STS). To explore whether the increases in eNOS and p-eNOS were related to PI3K/Akt pathway, cardiomyocytes were treated with LY294002 for 1 h before and during the incubation with STS plus DIDS. The results showed that treatment with LY294002 completely abrogated the increase of p-eNOS (P <0.01 vs. STS+DIDS), and also abolished DIDS-induced increase in eNOS expression (P <0.01 vs. STS+DIDS). In contrast, pretreatment with L-NAME (100 μ mol/L), a non-specific eNOS inhibitor, for 1 h before and during the co-application of STS and DIDS, had no effect on eNOS and p-eNOS levels.

In line with the enhanced eNOS phosphorylation, DIDS also markedly reversed the STS-induced reduction in NO_x content, which was effectively blocked by LY294002. Similar to LY294002, L-NAME also completely inhibited the increased NO_x (Fig. 6). Most interestingly, unlike LY294002, L-NAME only partly abolished the cytoprotective effects of DIDS, as shown by cell viability (Fig. 1), caspase-3 activity (Fig. 3A) and DNA ladder formation (Fig. 3B). This indicates that the protective effect of DIDS is not exclusively related to the enhancement of NO_x formation via the PI3K/Akt pathway, but also related to other targets modulated by PI3K/Akt.

DIDS suppressed Bax translocation from cytosol to mitochondria

As aforementioned, the balance of Bcl-2/Bax levels determines, in part, cell fate to apoptotic stimuli and previous studies have demonstrated that Bax translocation is involved in the process of STS-induced apoptosis [20]. These prompted us first to investigate whether DIDS exerts its anti-apoptotic effect through regulating Bcl-2/Bax levels and our results demonstrated that neither STS nor DIDS altered Bcl-2/Bax levels (not shown).

By double immunofluorescence labeling technique, we found that in normal cardiomyocytes, a diffuse localization of Bax is primarily found in the cytoplasm. In contrast, exposure of cells to STS resulted in Bax translocation from cytoplasm to mitochondria as revealed by the localization of Bax at mitochondria visualized by a specific mitochondrial probe. Interestingly, treatment with

Fig. 6. NO_x concentration in cardiomyocytes culture medium upon different treatments. Results were expressed as mean $\pm$ SEM (vertical bar) (n =12) of 3 independent experiments. NO_x: nitric oxide production; Con: control; S: STS; D: DIDS; LY: LY294002; L-N: L-NAME. * P <0.01 vs. Con; # P <0.01, vs. S; ** P <0.01 vs. S+D.

DIDS significantly inhibited STS-induced Bax translocation (Fig. 7). In order to know whether inhibition

Fig. 7. Representative photomicrographs of double immunofluorescence labeling of DIDS on STS-induced Bax translocation from cytosol to mitochondria. Fluorescence microscopic visualization of Bax in cardiomyocytes (top panels); Mitochondria stained with MitoTracker (middle panels); Overlay images show Bax and MitoTracker fluorescence (bottom panels). Control: vehicle treatment; STS: 8 h after incubation with STS; STS+DIDS: 8 h after incubation with STS plus DIDS; STS+DIDS+LY: cells pretreated with LY294002 for 1 h before and during the co-application of STS and DIDS. Scale bar: 80 μ m.

Fig. 8. Western blotting observations of DIDS on Bax translocation from cytosol to mitochondria induced by STS. (A) The cytosolic fractions; (B) the mitochondrial fractions. Insets: Representative blots. Data obtained from quantitative densitometry were presented as mean $\pm$ SEM (vertical bar) of 5 independent experiments. Con: control; S: STS; D: DIDS; LY: LY294002. * P <0.01 vs. Con; # P <0.01 vs. S; ** P <0.01 vs. S+D.

of Bax translocation was related to PI3K/Akt signaling pathway, we pretreated cardiomyocytes with LY294002 for 1 h before and during the co-application of STS and DIDS. The results showed that LY294002 effectively blocked the effects of DIDS and restored the STS-induced Bax translocation.

The immunofluorescence findings were confirmed by Western blotting. Fig. 8 compared the content of Bax in the cytosolic and mitochondrial fractions. After exposing cardiomyocytes to STS, the mitochondrial Bax content was increased whereas cytosolic Bax content decreased (P <0.01 vs. control). An increase in mitochondrial Bax level accompanied by a concomitant decrease in cytosolic Bax level suggests increased Bax translocation. When cells were simultaneously treated with DIDS, the content of Bax in the mitochondria did not significantly increase, and Bax only slightly decreased in the cytosolic fraction compared to the control cells, but significant differences were found as compared with STS-treated cardiomyocytes (P <0.01 vs. STS). The effect of DIDS on Bax translocation was similarly reversed by LY294002.

Immunofluorescence staining and Western blotting results provide direct evidence that DIDS prevents STS-induced Bax translocation to mitochondria, a cytoprotective effect that may be inter-related with PI3K/Akt pathway.

Discussion

Several important observations were made in our present study. First, we demonstrated that DIDS prevents apoptosis of primary neonatal rat cardiomyocytes exposed to STS in a PI3K-Akt-pathway-dependent manner. Second, phosphorylation of eNOS by Akt with a subsequent increase in NO_x, partly but significantly contributes to the anti-apoptotic effect of DIDS. In addition, we have provided evidence that DIDS inhibits STS-induced Bax translocation from cytosol to mitochondria, which may also be related to PI3K/Akt anti-apoptotic signaling.

Apoptosis can either be physiological or pathophysiological and this form of cell death is defined by a distinct set of both biochemical and morphological characteristics including cell volume decrease, caspase activation, chromatin condensation, DNA laddering, and cell fragmentation [21]. STS is a potent mitochondrial apoptotic inducer. The present study on cardiomyocytes showed that STS resulted in a significant decrease in cell viability accompanied by typical apoptotic characteristics and these apoptotic events were prominently suppressed by DIDS, which is consistent with previous reports [18, 19]. Several studies demonstrated various Cl⁻ transport pathways including the VSOR Cl⁻ channel which is closely related to cell volume changes, are involved in the development of apoptosis [5, 22] and DIDS was found to exert its cytoprotection by preventing the early-phase apoptotic cell shrinkage via inhibition of Cl⁻ fluxes activated by apoptotic stimuli, either by blocking Cl⁻/HCO₃⁻ exchangers or Cl⁻ channels [5, 23]. However, several recent studies demonstrated that the rescue of DIDS on cardiomyocyte apoptosis is mainly mediated by inhibition of chloride channels, because a bicarbonate-free condition which minimizes the function of Cl⁻/HCO₃⁻ exchangers failed to affect the cytoprotective effect of DIDS on STS-induced apoptosis in rat cardiomyocytes [18], as well as STS-induced [19] and ischemia-reperfusion-induced apoptosis in mouse cardiomyocytes [9].

An important issue is how DIDS delivered its pro-survival effect in cell apoptosis. Among numerous signaling pathways participating in regulation of cell survival, the PI3K/Akt signaling plays a crucial role in limiting apoptosis and is involved in multiple pro-survival pathways [24]. Upon activation, this signaling pathway diverges, with Akt promoting many of the anabolic processes, including glucose uptake and transport, glycogen and protein synthesis, as well as phosphorylation of numerous protein substrates, such as Bad, caspase-9, apoptosis signal-

regulating kinase 1 (ASK1), and Forkhead transcription factors [10]. These molecules phosphorylated by Akt may reduce cell apoptotic death by inhibiting caspase activity, releasing anti-apoptotic molecule, and inhibiting pro-apoptotic molecule activation. Recent data also supported that activated Akt also translocates to the nucleus, where it is presumably involved in the regulation of gene expression and mediates inhibition of apoptosis in enhancing cardiomyocyte survival [25]. The effect of p-Akt changes in response to STS stimulation is still controversial. Our present study indicated the levels of p-Akt were not clearly changed after challenging with STS, in line with previous studies [26, 27]. Most importantly, our results provided evidence that DIDS could effectively upregulate p-Akt in the presence of STS, in line with a previous investigation [12], and significantly reversed the STS-induced apoptosis of cardiomyocytes. The protective effects of DIDS were completely abolished by inhibition of PI3K/Akt, consistent with a recent study that DIDS prevents apoptosis and contractile dysfunction induced by doxorubicin in freshly isolated adult rabbit cardiomyocytes through PI3K/Akt pathway [8]. Furthermore, no apparent changes were found in p-Akt levels with direct DIDS application, indicating DIDS did not directly activate the PI3K/Akt pathway. As for the interrelation between PI3K/Akt activation and chloride fluxes inhibition during cytoprotection, it should be stressed that the literature is limited to the best of our knowledge, and we postulate activated PI3K/Akt signaling pathway may be secondary to the inhibition of chloride fluxes for the cytoprotection afforded by DIDS could be completely blocked by LY294002, and then both may act synergistically to promote cell survival.

How activation of Akt increases cardiomyocyte resistance to apoptosis is incompletely understood. Because Akt diverges into multiple signaling cascades, its effect is versatile and it promotes cell survival by inhibiting apoptosis at multiple points. Strong evidence exists that eNOS functions as a cardiovascular protective molecule and there is a compelling report that eNOS is a novel target of Akt, which phosphorylates eNOS on a critical serine 1177 in a PI3K/Akt dependent manner [13]. Phosphorylation of serine 1177 is proposed to result in a conformational change thereby increasing eNOS activity. More importantly, the regulation of NO synthesis in the heart by eNOS phosphorylation potentially has been represented as a critical final common pathway to explain the benefit of several effective treatments for numerous cardiovascular diseases [28, 29]. Several biochemical and cellular mechanisms have been suggested to elucidate

the apoptosis-inhibitory effects of NO. NO has been shown to nitrosate the downstream apoptotic machinery acting either at the level of the executioner caspase-3, 6, 7 or upstream of these events to inhibit initiator caspases such as caspase-8, 9 [30]. Furthermore, NO has been implicated in suppression of apoptosis by inhibiting caspase-dependent Bcl-2 cleavage, and consequently, the release of mitochondrial cytochrome c [31]. In addition, it also acts as a scavenger of reactive radical species, preventing cell damage from myocardial ischemia-reperfusion [32]. Our present study showed that DIDS caused a nearly complete reversal of both the STS-induced reduction in eNOS expression and eNOS phosphorylation, both of which were also found to be sensitive to the PI3K inhibitor LY294002. These results were also confirmed by measuring NO production. Apart from suggesting that the promoted eNOS phosphorylation and increased NO production were PI3K/Akt dependent, our data also indicated that the activated eNOS and increased NO production were not the only factors responsible for the protective effects of DIDS, which prompts us to explore other potential molecular mechanisms.

It is worth mentioning that several studies have shown that NO stimulates anion fluxes which are sensitive to DIDS [33, 34], at the same time, other studies suggest that NO inhibits chloride fluxes [35, 36], which may be cell-type dependent or there may be an interesting bidirectional action due to different experiment conditions. In our present study, we observed activated PI3K/Akt with subsequent enhanced NO in response to DIDS, which is a classic chloride channel blocker; therefore, we presumed that the enhanced NO and chloride transport inhibition may act synergistically to protect cell viability.

Cardiomyocytes require large energy supply to maintain normal function and mitochondria comprise about 30% of the total intracellular volume within a cardiomyocyte [37, 38], which may put cardiomyocytes at a higher risk of involuntary induction of apoptosis. Many investigations have been reported on the activation of the mitochondrial apoptotic pathway in different cardiac models, and mitochondrion-initiated apoptosis of cardiac cells appears to be a significant contributor to cell death [39]. As Bcl-2 family members act upstream of

irreversible cellular damage to regulate the mitochondrial pathway to apoptosis, they play a pivotal role in deciding whether a cell will live or die. Bax resides primarily in cytoplasm in healthy cells. In response to apoptotic stimuli, Bax translocation from the cytosol to the mitochondria is believed as a key step in mitochondrion-initiated apoptosis, associated with loss of mitochondrial membrane potential and release of cytochrome c [14]. A pro-survival factor Bcl-2 antagonizes the pro-apoptotic activities of Bax by inhibiting Bax translocation from the cytosol to the mitochondria. Thus, we explored whether DIDS exerts its pro-survival effect through altering Bcl-2/Bax balance. Our findings showed for the first time that DIDS significantly inhibited Bax translocation from cytosol to mitochondria in response to PI3K/Akt activation. This novel result indicated that DIDS inhibits cell apoptosis by multiple mechanisms and that PI3K activity may be necessary for retaining Bax in the cytoplasm. Akt mediates the effects of PI3K in promoting cell survival and it was previously reported that expression of active Akt inhibits Bax translocation to mitochondria induced by STS [40]. However, the present study did not dissect the downstream pathway of PI3K on Bax translocation and it remains to be fully elucidated.

In summary, the current study demonstrated that DIDS could protect cardiomyocytes against STS-induced apoptosis, and more importantly, provided the first evidence that DIDS exerted its anti-apoptotic effect via activating PI3K/Akt signaling pathway, specifically, phosphorylating eNOS, increasing NO production and inhibiting Bax translocation.

Acknowledgements

The research was supported by the National Natural Science Foundation of China (NSFC, Nos. 30570758, 30770847) and Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (SRF for ROCS, SEM. No. HG2904). The authors are grateful to Dr. Xin L. Ma (the Department of Emergency Medicine, Thomas Jefferson University, Philadelphia, USA) for commenting on the manuscript.

References

- 1 Bennett MR: Apoptosis in the cardiovascular system. *Heart* 2002;87:480-487.
- 2 Kang PM, Izumo S: Apoptosis in heart: basic mechanisms and implications in cardiovascular diseases. *Trends Mol Med* 2003;9:177-182.
- 3 Gong W, Xu H, Shimizu T, Morishima S, Tanabe S, Tachibe T, Uchida S, Sasaki S, Okada Y: CIC-3-independent, PKC-dependent activity of volume-sensitive

- Cl channel in mouse ventricular cardiomyocytes. *Cell Physiol Biochem* 2004;14:213-224.
- 4 Okada Y, Shimizu T, Maeno E, Tanabe S, Wang X, Takahashi N: Volume-sensitive chloride channels involved in apoptotic volume decrease and cell death. *J Membr Biol* 2006;209:21-29.
- 5 Shimizu T, Numata T, Okada Y: A role of reactive oxygen species in apoptotic activation of volume-sensitive Cl(-) channel. *Proc Natl Acad Sci U S A* 2004;101:6770-6773.
- 6 Krumschnabel G, Maehr T, Nawaz M, Schwarzbach PJ, Manz C: Staurosporine-induced cell death in salmonid cells: the role of apoptotic volume decrease, ion fluxes and MAP kinase signaling. *Apoptosis* 2007;12:1755-1768.
- 7 Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y: Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci U S A* 2000;97:9487-9492.
- 8 d'Anglemont de Tassigny A, Berdeaux A, Souktani R, Henry P, Ghaleh B: The volume-sensitive chloride channel inhibitors prevent both contractile dysfunction and apoptosis induced by doxorubicin through PI(3)kinase, Akt and Erk 1/2. *Eur J Heart Fail* 2008;10:39-46.
- 9 Wang X, Takahashi N, Uramoto H, Okada Y: Chloride channel inhibition prevents ROS-dependent apoptosis induced by ischemia-reperfusion in mouse cardiomyocytes. *Cell Physiol Biochem* 2005;16:147-154.
- 10 Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, Ma XL: Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. *Circulation* 2002;105:1497-1502.
- 11 Sugden PH, Clerk A: Akt like a woman: gender differences in susceptibility to cardiovascular disease. *Circ Res* 2001;88:975-977.
- 12 Araki T, Hayashi M, Watanabe N, Kanuka H, Yoshino J, Miura M, Saruta T: Down-regulation of Mcl-1 by inhibition of the PI3-K/Akt pathway is required for cell shrinkage-dependent cell death. *Biochem Biophys Res Commun* 2002;290:1275-1281.
- 13 Kukreja RC, Xi L: eNOS phosphorylation: a pivotal molecular switch in vasodilation and cardioprotection? *J Mol Cell Cardiol* 2007;42:280-282.
- 14 Gustafsson AB, Gottlieb RA: Bcl-2 family members and apoptosis, taken to heart. *Am J Physiol Cell Physiol* 2007;292:C45-51.
- 15 Matsuzaki H, Tamatani M, Mitsuda N, Namikawa K, Kiyama H, Miyake S, Tohyama M: Activation of Akt kinase inhibits apoptosis and changes in Bcl-2 and Bax expression induced by nitric oxide in primary hippocampal neurons. *J Neurochem* 1999;73:2037-2046.
- 16 Kim BJ, Ryu SW, Song BJ: JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translocation and to apoptosis of human hepatoma HepG2 cells. *J Biol Chem* 2006;281:21256-21265.
- 17 Yao LL, Wang YG, Cai WJ, Yao T, Zhu YC: Survivin mediates the anti-apoptotic effect of delta-opioid receptor stimulation in cardiomyocytes. *J Cell Sci* 2007;120:895-907.
- 18 Tanabe S, Wang X, Takahashi N, Uramoto H, Okada Y: HCO(3)(-)-independent rescue from apoptosis by stilbene derivatives in rat cardiomyocytes. *FEBS Lett* 2005;579:517-522.
- 19 Takahashi N, Wang X, Tanabe S, Uramoto H, Jishage K, Uchida S, Sasaki S, Okada Y: CIC-3-independent sensitivity of apoptosis to Cl- channel blockers in mouse cardiomyocytes. *Cell Physiol Biochem* 2005;15:263-270.
- 20 Hou Q, Hsu YT: Bax translocates from cytosol to mitochondria in cardiac cells during apoptosis: development of a GFP-Bax-stable H9c2 cell line for apoptosis analysis. *Am J Physiol Heart Circ Physiol* 2005;289:H477-487.
- 21 Saraste A, Pulkki K: Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res* 2000;45:528-537.
- 22 Porcelli AM, Ghelli A, Zanna C, Valente P, Ferroni S, Rugolo M: Apoptosis induced by staurosporine in ECV304 cells requires cell shrinkage and upregulation of Cl-conductance. *Cell Death Differ* 2004;11:655-662.
- 23 Malekova L, Tomaskova J, Novakova M, Stefanik P, Kopacek J, Lakatos B, Pastorekova S, Krizanova O, Breier A, Ondrias K: Inhibitory effect of DIDS, NPPB, and phloretin on intracellular chloride channels. *Pflugers Arch* 2007;455:349-357.
- 24 Sugden PH: Ras, Akt, and mechanotransduction in the cardiac myocyte. *Circ Res* 2003;93:1179-1192.
- 25 Shiraishi I, Melendez J, Ahn Y, Skavdahl M, Murphy E, Welch S, Schaefer E, Walsh K, Rosenzweig A, Torella D, Nurzynska D, Kajstura J, Leri A, Anversa P, Sussman MA: Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. *Circ Res* 2004;94:884-891.
- 26 Zhu Y, Hoell P, Ahlemeyer B, Kriegelstein J: PTEN: a crucial mediator of mitochondria-dependent apoptosis. *Apoptosis* 2006;11:197-207.
- 27 Hill MM, Andjelkovic M, Brazil DP, Ferrari S, Fabbro D, Hemmings BA: Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase. *J Biol Chem* 2001;276:25643-25646.
- 28 Di Napoli P, Antonio Taccardi A, Grilli A, Spina R, Felaco M, Barsotti A, De Caterina R: Simvastatin reduces reperfusion injury by modulating nitric oxide synthase expression: an ex vivo study in isolated working rat hearts. *Cardiovasc Res* 2001;51:283-293.
- 29 Seddon M, Shah AM, Casadei B: Cardiomyocytes as effectors of nitric oxide signalling. *Cardiovasc Res* 2007;75:315-326.
- 30 Kim YM, Bombeck CA, Billiar TR: Nitric oxide as a bifunctional regulator of apoptosis. *Circ Res* 1999;84:253-256.
- 31 Kim YM, Kim TH, Seol DW, Talanian RV, Billiar TR: Nitric oxide suppression of apoptosis occurs in association with an inhibition of Bcl-2 cleavage and cytochrome c release. *J Biol Chem* 1998;273:31437-31441.
- 32 Li CQ, Wogan GN: Nitric oxide as a modulator of apoptosis. *Cancer Lett* 2005;226:1-15.
- 33 Kamosinska B, Radomski MW, Duszyk M, Radomski A, Man SF: Nitric oxide activates chloride currents in human lung epithelial cells. *Am J Physiol* 1997;272:L1098-1104.
- 34 Duta V, Duta F, Puttagunta L, Befus AD, Duszyk M: Regulation of basolateral Cl (-) channels in airway epithelial cells: the role of nitric oxide. *J Membr Biol* 2006;213:165-174.
- 35 Zhang Y, Vogalis F, Goyal RK: Nitric oxide suppresses a Ca(2+)-stimulated Cl-current in smooth muscle cells of opossum esophagus. *Am J Physiol* 1998;274:G886-890.
- 36 Plato CF, Stoos BA, Wang D, Garvin JL: Endogenous nitric oxide inhibits chloride transport in the thick ascending limb. *Am J Physiol* 1999;276:F159-163.
- 37 Barth E, Stammler G, Speiser B, Schaper J: Ultrastructural quantitation of mitochondria and myofilaments in cardiac muscle from 10 different animal species including man. *J Mol Cell Cardiol* 1992;24:669-681.
- 38 Haunstetter A, Izumo S: Future perspectives and potential implications of cardiac myocyte apoptosis. *Cardiovasc Res* 2000;45:795-801.
- 39 O'Rourke B: Apoptosis: rekindling the mitochondrial fire. *Circ Res* 1999;85:880-883.
- 40 Tsuruta F, Masuyama N, Gotoh Y: The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria. *J Biol Chem* 2002;277:14040-14047.