CIC-3-independent Sensitivity of Apoptosis to Cl⁻ Channel Blockers in Mouse Cardiomyocytes

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Key Words

Apoptosis • Chloride channel • CIC-3 • Cardiomyocyte

Abstract

It has been shown that Cl⁻/HCO₃⁻ exchangers and Cl⁻ channels, both of which are sensitive to stilbene derivatives, have essential roles in the mechanism of apoptosis induction. Staurosporine-induced apoptosis in neonatal mouse cardiomyocytes was prevented by a stilbene derivative, DIDS. To clarify whether Cl⁻/HCO₃⁻ exchangers or Cl⁻ channels are targets of DIDS and whether CIC-3 is involved in the apoptotic process, staurosporine-induced reduction of cell viability, DNA laddering and caspase-3 activation were examined in cultured mouse ventricular myocytes derived from wild-type and CIC-3-deficient mice. Staurosporine-induced apoptosis and its DIDS sensitivity in ambient HCO₃⁻-free conditions in which operation of Cl⁻/HCO₃⁻ exchangers is minimized were indistinguishable from when HCO₃⁻ was present. Apoptosis was also prevented by application of a non-stilbene-derivative Cl⁻ channel blocker, NPPB, which cannot block Cl⁻/HCO₃⁻ exchangers. Cardiomyocytes derived from

CIC-3-deficient mice similarly underwent apoptosis after exposure to staurosporine; moreover, apoptosis was prevented by application of DIDS or NPPB. Thus, we conclude that in cardiomyocytes, apoptosis is critically dependent on operation not of Cl⁻/HCO₃⁻ exchangers but of Cl⁻ channels which are distinct from CIC-3.

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Introduction

Apoptosis is a fundamental biological process occurring in embryogenesis, organ development, tissue homeostasis, and immune system maturation [1, 2]. When the pro-apoptotic machinery is not functional because of loss-of-function mutations or disruption of its regulatory system, abnormal morphogenesis and uncontrollable cell proliferation occur, resulting in disease and/or lethality [3]. Therefore, apoptosis is indispensable for multicellular organisms. On the other hand, too much or too little apoptosis has been implicated in various pathological conditions and diseases [4]. Apoptosis of cardiomyocytes is a critical problem in heart injury, occurring not only in ischemia/reperfusion in animal models [5] but also in

human acute myocardial infarction [6].

In cultured cardiomyocytes, apoptosis can be induced by various stresses such as chemical treatment (e.g. with staurosporine [7] or anticancer drugs [8]) and ischemia/reperfusion (hypoxia/reoxygenation) [9, 10]. During apoptosis, cells generally undergo shrinkage [11] that occurs even before activation of caspases begins [12, 13]. During this early-phase shrinkage, called "apoptotic volume decrease (AVD)", volume-regulatory Cl^- and K^+ channels are activated, leading to water egress driven by KCl efflux [13, 14]. It was suggested that activation of these channels is essential for the induction of apoptosis, because blockage of the channels reduces apoptotic cell death in many cell types [12-18]. Indeed, direct electrophysiological evidence has been provided indicating that the activation of a Cl^- channel is associated with the apoptotic process in many cell types [18-22]. On the other hand, the critical involvement of a $\text{Cl}^-/\text{HCO}_3^-$ exchanger (anion exchanger: AE) has been demonstrated in HeLa cells [23], endothelial cells [24, 25] and cardiomyocytes [25].

A stilbene derivative, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), has been shown to prevent apoptotic cell death in rat cardiomyocytes exposed to a mitochondrion-mediated apoptosis inducer (staurosporine: STS) [25] and in epithelial, lymphoid and neuronal cells exposed to STS or a death receptor-mediated apoptosis inducer (TNF α or Fas ligand) [12]. The stilbene derivative is an effective blocker of both $\text{Cl}^-/\text{HCO}_3^-$ exchangers and Cl^- channels. Thus, in the present study, we first addressed the question of whether the main target of the DIDS inhibitory effect on STS-induced cardiomyocyte apoptosis is the anion exchanger or a Cl^- channel.

Recently, we have demonstrated that STS induces the normotonic activation of an outwardly rectifying Cl^- current, the properties of which are essentially identical to those of the volume-sensitive outwardly rectifying (VSOR) anion channel, in HeLa cells [22]. Duan et al. [26] showed that normotonic activation of a DIDS-sensitive, VSOR-like Cl^- current is induced by expression of cardiac CIC-3. Although recent studies using CIC-3 knock-out mice demonstrated that this protein itself is not the VSOR anion channel [27-29], it is possible that CIC-3 is a target of DIDS that affects the VSOR-like Cl^- conductance associated with induction of apoptosis in STS-treated cardiomyocytes. Thus, the second aim of this study is to test this possibility using CIC-3 knock-out mice.

Materials and Methods

Neonatal mouse cardiomyocytes

Mice with a homozygous disruption of the *Cicn3* gene and wild-type mice were obtained as previously described [29]. Primary cultures of neonatal mouse cardiomyocytes were prepared by a modification of a protocol employed in our previous study [29]. In brief, beating hearts were harvested from <24-h old wild-type or CIC-3-deficient mice and immediately placed in Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution (HBSS). The hearts were minced and subjected to trypsin digestion (0.01% in HBSS) for 16-18 h at 4°C. The trypsin digestion was terminated by addition of trypsin inhibitor (SIGMA, St. Louis, MO). The hearts were further digested with collagenase (type-I collagenase, 150 U/ml; Worthington, Lakewood, OH) at 37°C in a shaking bath for 45 min. Cell clumps were filtered through a 70- μm nylon filter, centrifuged, and washed with M199 medium. Cell pellets were resuspended in M199 medium supplemented with 10% fetal bovine serum. For cell volume assays, the cells were instantly used. For cell viability, DNA ladder and caspase assays, cells were cultured in the serum-containing M199 medium supplemented with 0.1 mM bromodeoxyuridine (SIGMA) after plating onto collagen-coated wells at a density of 1.2×10^5 /well in 96-well plates or 8.0×10^5 /well in 24-well plates, and they were used after maintaining for 4 days at 37°C in the presence of 5% CO_2 in a humidified incubator.

Staurosporine treatment and cell viability assays

To induce apoptosis, the cells were incubated at 37°C in DMEM culture medium containing STS (SIGMA) at various concentrations. In order to minimize the contribution of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger, STS was applied under HCO_3^- -free conditions in which Leibovitz's L-15 medium (Gibco Invitrogen) was used instead of DMEM, and cells were cultivated in a 37°C incubator equilibrated with an atmosphere of normal air. After STS treatment for 4 h or other indicated times, the cells were washed twice with culture medium that did not contain STS and then cultured for 4 h at 37°C. DIDS (SIGMA), 4-acetamido-4'-isothiocyanostilbene (SITS; SIGMA), or 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; Tocris, Cookson Ltd., Bristol, UK) was added at the indicated concentration during treatment with STS. To estimate total cell viability, mitochondrial dehydrogenase activity was measured by a colorimetric MTT assay using the Cell Counting Kit-8 (DOJIN, Kumamoto, Japan) according to the manufacturer's protocol, as previously described [12].

Apoptosis assays

Internucleosomal DNA fragmentation and caspase-3 activity were observed as previously described [12]. Briefly, cardiomyocytes cultured in 24-well plates were digested and incubated in mammalian cell lysis buffer (0.1% SDS, 10 mM EDTA, 10 mM Tris, pH 8.0) containing 20 $\mu\text{g}/\text{ml}$ RNase (Nacalai Tesuque, Kyoto, Japan) at 37°C for 1 h and then 0.5 mg/ml proteinase K (Nacalai Tesuque) at 37°C for 1 h. The chromosomal DNA was analyzed by agarose gel electrophoresis (2%)

Fig. 1. Apoptosis induction by staurosporine (STS) in mouse cardiomyocytes. A. concentration-dependent effects of STS on cell viability (top panel) and DNA laddering (bottom panel) detected 4 h after STS treatment. B. Time dependence of STS effects on cell viability (top) and DNA laddering (bottom); cells were incubated in STS-free culture medium for indicated times after 4-h exposure to 4 μ M STS. The cell viability data represent the means $\pm$ S.E.M. (vertical bar) of 6 experiments. *, significantly different ($p < 0.05$) from the control data (treated with vehicle (0.4% DMSO) alone). The DNA laddering data are representative of three independent experiments.

Fig. 2. Morphological features of STS-induced apoptosis in mouse cardiomyocytes. A. Control cells cultured for 4 h after 4-h treatment with vehicle (0.4% DMSO) alone. B. Cells cultured for 4 h after 4-h treatment with 4 μ M STS (+ vehicle). Top, phase-contrast photomicrographs. Bottom, fluorescence photomicrographs of cells stained with Hoechst 33345.

followed by staining with ethidium bromide (SIGMA). Caspase-3 activity was measured by using a fluorometric assay. To exclude the involvement of other related proteases, the difference in fluorescence when the specific inhibitor of caspase-3 was absent compared to when it was present was observed.

The fluorogenic substrate for caspase-3 (Ac-DEVD-AMC), which was labeled with the fluorochrome 7-amino-4-methyl coumarin (AMC), and the tetrapeptide inhibitor of caspase-3 (Ac-DEVD-CHO) were provided in the CaspASE Assay System (PROMEGA, Madison, WI). To detect nuclear morphological

changes accompanying apoptosis, cultured cardiomyocytes were stained with Hoechst 33324 (Molecular Probes, Eugene, OR) at 50 ng/ml for 10 min, after fixation with 4% formaldehyde for 10 min at room temperature. After rinsing the cells in PBS, cells were observed under a fluorescence microscope and photographed using a DP-50 cooled-CCD camera system (OLYMPUS, Tokyo, Japan). To observe the early phase of apoptotic cell shrinkage, that is the apoptotic volume decrease (AVD) [12], the mean cell volume of freshly isolated cardiomyocytes was measured before and during 30-min application of STS (4 μ M) or DMSO (a vehicle control) in the absence or presence of DIDS (250 μ M or NPPB (100 μ M) by an electronic sizing technique with a Coulter-type cell size analyzer (SYSMEX, Kobe, Japan).

Results

Cardiomyocyte apoptosis induced by staurosporine

STS has long been used in vitro as an initiator of apoptosis in many different cell types including rat cardiomyocytes in primary culture [7, 25], although the exact mechanism of its action is still unclear. In mouse cardiomyocytes in primary culture, reduction of cell viability was observed by the MTT assay when the cells were incubated for 4 h after 4-h exposure to 1 to 16 μ M STS, as shown in Fig. 1A (top panel). Internucleosomal fragmentation of genomic DNA was also induced by STS in a concentration-dependent manner, although STS-induced DNA laddering was not apparent at a concentration lower than 1 μ M (Fig. 1A, bottom panel). STS-induced apoptosis was dependent on the time of exposure to STS, as shown in Fig. 1B. Significant reduction of cell viability was observed 4 h after STS treatment for ≥ 1 h, whereas DNA laddering was detected 4 h after STS treatment for ≥ 4 h. Thus, we adopted a protocol of 4-h culture after 4-h treatment with STS as the standard for the following experiments.

Microscopic observations of STS-treated mouse cardiomyocytes demonstrated morphological features characteristic of apoptotic cell death, as shown in Fig. 2. Compared to control cells treated with DMSO alone (A), STS-treated cardiomyocytes were shrunken and retracted. Significant AVD was consistently observed 30 min after exposure to STS, and the relative cell volume was 93.8% ($n=8$, $p<0.05$ vs. the control without STS treatment). When cells stained with Hoechst 33324 were observed under a fluorescence microscope, it was found that the number of cells with smaller or fragmented nuclei had been increased by the STS treatment (B).

Effects of DIDS on STS-induced cardiomyocyte apoptosis in the presence or absence of ambient HCO_3^-

A stilbene derivative, DIDS, inhibited STS-induced apoptosis in mouse cardiomyocytes (Fig. 3A), as previously observed in rat cardiomyocytes [25]. Cell viability reduction and DNA laddering induced by STS were both inhibited by 250 μ M DIDS which was applied during exposure to STS (top and middle panels). The STS treatment also induced activation of caspase-3, and this was significantly suppressed by DIDS (bottom panel). STS-induced AVD was significantly prevented by DIDS. In the presence of 250 μ M DIDS, the relative cell volume of STS-treated cells was 97.8% ($n=8$, $p<0.05$ vs. STS treatment in the absence of DIDS).

Based on the DIDS effects, Fujita et al. [25] suggested that the $\text{Cl}^-/\text{HCO}_3^-$ exchanger is involved in STS-induced apoptosis in rat cardiomyocytes and endothelial cells. To test this in mouse cardiomyocytes, we observed the effects of removal of ambient HCO_3^- , which should minimize the activity of HCO_3^- -dependent transporters such as the $\text{Cl}^-/\text{HCO}_3^-$ exchanger and the $\text{Na}^+/\text{HCO}_3^-$ cotransporter. Surprisingly, results similar to those in experiments with HCO_3^- present were obtained for STS-induced apoptosis and its suppression by DIDS in mouse cardiomyocytes treated under HCO_3^- -free conditions, as shown in Fig. 3B. Both cell viability and caspase-3 activity in cells treated with STS and/or DIDS in experiments performed with ambient HCO_3^- present (A) or absent (B) were statistically indistinguishable.

Effects of NPPB on STS-induced cardiomyocyte apoptosis

Since DIDS is known to be a blocker not only of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger but also of various types of Cl^- channel, we next examined the effects of another Cl^- channel blocker. As shown in Fig. 4, NPPB, a carboxylate analog which is a non-specific blocker of Cl^- channels but cannot block $\text{Cl}^-/\text{HCO}_3^-$ exchangers [30], effectively suppressed STS-induced cell viability reduction, DNA laddering and caspase-3 activation. STS-induced AVD also became less prominent in the presence of 100 μ M NPPB. The relative cell volume was 96.9% ($n=8$, $p<0.05$ vs. STS treatment in the absence of NPPB). These data suggest that STS-induced apoptosis is critically dependent on the activity of a Cl^- channel sensitive to DIDS and NPPB.

Fig. 3. Effects of DIDS on STS-induced apoptosis in mouse cardiomyocytes in the presence (A) and absence (B) of ambient HCO_3^- . Top, middle and bottom panels show the data of cell viability, DNA laddering and relative caspase-3 activity data, respectively. The data shown in the top and bottom panels represent the means $\pm$ S.E.M. of 6 and 5 experiments, respectively, and the data shown in the middle panel are representative of three independent experiments. * and #, significantly different ($p < 0.05$) from the control data (+ vehicle) and from the data with STS (+ vehicle) alone, respectively.

Lack of involvement of the ClC-3 Cl^- channel in STS-induced cardiomyocyte apoptosis

Since ClC-3 -associated Cl^- currents have been reported to be sensitive to DIDS and NPPB [26, 31, 32], the effects of DIDS and NPPB on STS-induced apoptosis were examined in cardiomyocytes prepared from ClC-3 null-mutant mice. As shown in Fig. 5, STS induced cell viability reduction, DNA laddering and caspase-3 activation in ClC-3 -deficient cardiomyocytes. Values for cell viability and caspase-3 activity observed in ClC-3 -deficient cells treated with STS in the absence or presence of DIDS or NPPB were not statistically different from values obtained in ClC-3 -expressing cells (Figs. 3 and 4). These data indicate that the DIDS- and NPPB-sensitive anion channel involved in STS-induced apoptosis is distinct from ClC-3 .

Discussion

The $\text{Cl}^-/\text{HCO}_3^-$ exchanger has been reported to play a critical role in the mechanism of apoptosis induction by STS in rat cardiomyocytes [25] and in HeLa cells [23]. In the present study, however, STS was found to induce apoptosis under HCO_3^- -free conditions as effectively as when HCO_3^- was present in mouse cardiomyocytes. Furthermore, DIDS could inhibit STS-induced apoptosis even in the absence of ambient HCO_3^- . These data suggest that the effect of DIDS occurs via blockage of a Cl^- channel but not the $\text{Cl}^-/\text{HCO}_3^-$ exchanger. Indeed, a carboxylate analog Cl^- channel blocker (NPPB), which cannot block the $\text{Cl}^-/\text{HCO}_3^-$ exchanger [30], was found to markedly block STS-induced apoptosis in mouse cardiomyocytes.

Fig. 4. Effects of NPPB on STS-induced apoptosis in mouse cardiomyocytes. Top, middle and bottom panels as well as the number of experiments, * and # are the same as in Fig. 3A.

In many other cell types, it has been reported that apoptosis is blocked by a number of blockers of Cl^- channels [12-14, 17-25, 33-37] and K^+ channels [12-16]. Our recent studies [12-14] provided evidence that KCl efflux induced by activation of volume-regulatory K^+ and Cl^- channels is responsible for the early-phase apoptotic volume decrease (AVD) which is one of the prerequisite steps of the apoptosis process [12-15, 38]. In HeLa cells we have shown that actual activation of Cl^- current is associated with the induction of apoptosis by stimulators

Fig. 5. Effects of DIDS and NPPB on STS-induced apoptosis in ClC-3 null-mutant mouse cardiomyocytes. Top, middle and bottom panels as well as the number of experiments, * and # are the same as in Fig. 3A.

of both death receptor- and mitochondrion-mediated apoptosis [22].

The biophysical and pharmacological properties of apoptosis-associated Cl^- channels, including sensitivity to DIDS and NPPB, and sensitivity to volume are identical to those typical of the VSOR anion channel [22]. Although ClC-3 was previously proposed to be identical to the VSOR anion channel [26], STS-induced apoptosis and its sensitivity to DIDS and NPPB in ClC-3 -expressing and ClC-3 -deficient cardiomyocytes were found to be

essentially indistinguishable. Therefore, it is clear that apoptosis-associated Cl^- channels, presumably VSOR anion channels, are independent of ClC-3 in mouse cardiomyocytes. This inference is in good agreement with recent reports that the activity of the VSOR anion channel is independent of the molecular expression of ClC-3 in pancreatic acinar and hepatic cells [27] as well as in parotid acinar cells [28] and cardiomyocytes [29].

The role of apoptosis in cardiac disorders including ischemia-reperfusion injury, acute myocardial infarction, cardiomyopathy and myocarditis has been addressed previously [5, 39-42]. Thus, to treat cardiac disorders, the mechanism of apoptosis induction could be targeted for pharmacological intervention [43]. Also, it is noteworthy that, as Mizoguchi et al. [44] demonstrated, apoptotic cell death induced by ischemia-reperfusion during heart transplantation could be prominently blocked by DIDS and NPPB. The induction of AVD, which

involves activation of volume-regulatory K^+ and Cl^- channels, is an early event preceding cytochrome c release, caspase activation and internucleosomal DNA fragmentation [12-14]. Therefore, blockers of K^+ or Cl^- channels essential to AVD may serve as quick-acting medicines which can exert their actions on the plasma membrane where these channels exist, for cardiac disorders in which apoptosis is involved.

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