Chloride Channel Inhibition Prevents ROS-dependent Apoptosis Induced by Ischemia-Reperfusion in Mouse Cardiomyocytes

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Key Words

Ischemia-reperfusion • Apoptosis • Chloride channel • ROS • Cardiomyocyte

Abstract

Apoptosis of cardiomyocytes following ischemia and reperfusion is of clinical importance. However, little is known about the mechanism by which it is induced. Recently, essential roles of a Cl⁻ channel whose activity triggers the apoptotic volume decrease and of reactive oxygen species (ROS) in activation of this channel have been identified in mitochondrion-mediated apoptosis. Therefore, in this study, involvement of Cl⁻ channels and ROS in apoptosis was studied in primary mouse cardiomyocyte cultures subjected to ischemia-reperfusion. Apoptotic cell death as measured by caspase-3 activation, chromatin condensation, DNA laddering, and cell viability reduction was observed tens of hours after reperfusion but never immediately after ischemia. A non-selective Cl⁻ channel blocker (DIDS or NPPB) rescued cells from apoptotic death when applied during the reperfusion, but not ischemia, period. Another blocker relatively specific to the volume-sensitive outwardly rectifying (VSOR) Cl⁻ channel (phloretin) was also effective in protecting ischemic cardiomyocytes from apoptosis induced by reperfusion. A profound increase in intracellular ROS was detected in

cardiomyocytes during the reperfusion, but not ischemia, period. Scavengers for ROS, H₂O₂ and superoxide all inhibited apoptosis induced by ischemia-reperfusion. Thus, it is concluded that the mechanism by which cardiomyocyte apoptosis is induced by ischemia-reperfusion involves VSOR Cl⁻ channel activity and intracellular ROS production.

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Introduction

Prolonged myocardial ischemia or hypoxia causes tissue injury and heart failure, and, paradoxically, early reperfusion or reoxygenation also causes cell death [1]. Ischemia-reperfusion or hypoxia-reoxygenation is important not only in a number of myocardial diseases but also in heart transplantation. Although the mechanisms of ischemia-reperfusion injury are not well-defined, it is known that apoptosis occurs concomitantly with necrosis in infarcted and reperfused myocardium [2, 3]. Apoptosis has thus attracted considerable attention as a potentially reversible cause of cardiac dysfunction. Apoptosis has also been detected in animal models of cardiac ischemia-reperfusion injury [4-7] and cultured cardiomyocytes undergoing hypoxia-reoxygenation [8, 9].

Apoptotic cells generally undergo shrinkage that starts before activation of caspases [10-12]. During this apoptotic volume decrease (AVD), which is a prerequisite step of apoptotic cell death [10], KCl efflux caused by activation of volume-regulatory K⁺ and Cl⁻ channels drives the egress of water [11-15]. Recently, the AVD-inducing Cl⁻ channel has been functionally identified to be a volume-sensitive outwardly rectifying (VSOR) Cl⁻ channel in HeLa cells stimulated with staurosporine, Fas ligand or TNF α [16]. Consistent with this, it was found that cardiomyocytes were rescued from staurosporine-induced apoptosis by a VSOR Cl⁻ channel blocker [17, 18]. Thus, the first aim of the present study is to address the questions as to whether a VSOR Cl⁻ channel blocker is effective in preventing apoptotic cell death associated with ischemia-reperfusion injury in mouse cardiomyocytes and, if effective, whether the target phase of the VSOR blocker is the ischemic or reperfusion period.

It has been recognized that reactive oxygen species (ROS) are involved in the pathogenesis of myocardial injury induced by ischemia-reperfusion [19-21]. Oxidative stress is known to be a major apoptotic stimulus in various cardiovascular diseases [22]. Cardiomyocyte apoptosis was actually shown to be suppressed by *in vivo* expression [23] or *in vitro* application [24] of antioxidants. Moreover, we have shown that ROS mediate the staurosporine-induced activation of the VSOR Cl⁻ channel in HeLa cells [16]. Thus, it is possible that ROS generation is causally involved in cardiomyocyte apoptosis induced by ischemia-reperfusion. The second purpose of this study is therefore to test this possibility in mouse cardiomyocytes and to determine the phase during which ROS act, the ischemic or reperfusion period.

Materials and Methods

Cardiomyocyte culture

Primary cultures of neonatal mouse cardiomyocyte were prepared as previously described [17]. Cells were plated onto collagen-coated wells at a density of 1.2×10^5 /well and 8.0×10^5 /well for 96- and 24-well plates, respectively, in DMEM supplemented with 10% fetal bovine serum containing bromodeoxyuridine (0.1 mM; SIGMA, St Luis, MO). The cells were maintained at 37°C in the presence of 5% CO₂ in a humidified incubator for 4 days before experiments.

Ischemia-reperfusion treatment and cell viability measurements

Cardiomyocytes were subjected to ischemic stress, as previously described [25]. In brief, cells of the ischemic group were incubated for given time periods (4-8 h) under hypoxic

conditions in a glucose-free solution, which contained 108 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, and 2.5 mM Tris (pH adjusted to 7.4 with NaHCO₃; osmolality adjusted to 300 mosmol/kg-H₂O with mannitol) and was bubbled with CO₂ for over 1 h before application. Hypoxic conditions were produced by placing the plates of cultured cardiomyocytes in an airtight incubator in which normal air was replaced with a gas mixture of 95% Ar/5% CO₂. The pO₂ of the ischemic solution in the culture dish was found to reach a level of <10 mmHg by a pO₂ probe (LICOX CMP: GMS, Mielkendorf, Germany). After ischemic incubation, the glucose-free solution was replaced with fresh normal medium and incubated in a CO₂ incubator for given time periods (24-96 h). In the non-ischemia group, the culture medium was replaced with fresh medium when ischemia and reperfusion started for the ischemia group, and the cells were cultured in a CO₂ incubator. A Cl⁻ channel blocker (DIDS, NPPB, or phloretin) or a ROS scavenger (tiron, NAC, or catalase) was added during either the ischemia or reperfusion period at the indicated concentrations. In order to minimize the contribution of Cl⁻/HCO₃⁻ exchanger activity, some experiments were performed under HCO₃⁻-free conditions using Leibovitz's L-15 medium (Invitrogen Corp., Carlsbad, CA) instead of DMEM, and cells were cultivated in a 37°C incubator equilibrated to a normal air atmosphere.

To estimate cell viability of the cardiomyocytes cultured in 96-well plates, a colorimetric MTT assay was performed using the Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to the manufacturer's protocol.

Apoptosis detection

Internucleosomal DNA fragmentation assays, caspase-3 activity measurements, and Hoechst staining were performed as previously described [10, 17]. Briefly, the DNA fragmentation was detected by DNA laddering in agarose electrophoresis. 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 μ g/ml RNase (Nacalai Tesque, Kyoto, Japan) at 37°C for 1 h and 0.5 mg/ml proteinase K (Nacalai Tesque) which was added following RNase treatment at 37°C for 1 h. The chromosomal DNA was analyzed by agarose gel electrophoresis (2%) followed by staining with ethidium bromide (EtBr; SIGMA). Caspase-3 activity was measured using the CaspACE Assay System (Promega, Madison, WI). To exclude the involvement of other related proteases, the difference between fluorescence in the absence and presence of a specific inhibitor of caspase-3 was observed. For Hoechst staining, the cultured cardiomyocytes were fixed with 4% formaldehyde/PBS for 10 min at room temperature. After several washes with PBS, the cells were exposed to Hoechst 33324 (50 ng/ml in PBS) and incubated for 10 min at room temperature. After rinsing with PBS, the cells were examined under a fluorescence microscope with an appropriate filter.

Measurements of ROS production

Intracellular ROS production was measured using 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) as previously described [16]. In brief, neonatal mouse cardiomyocytes cultured in 96-well plates were

Fig. 1. Effects of ischemia and reperfusion on morphological features of mouse cardiomyocytes. Control: cells cultured without ischemia-reperfusion treatment. 6 h-I: cells cultured under ischemic conditions for 6 h. 6 h-I/96 h-R: cells cultured under ischemic conditions for 6 h and then subjected to reperfusion for 96 h. A. Phase-contrast photomicrographs. B. Fluorescence photomicrographs of cells stained with Hoechst 33345. Each photograph is representative of 3 independent observations.

incubated for 30 min with normal culture medium containing 10 μ M carboxy- H_2 DCFDA and washed twice with isotonic solution containing 95 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 105 mM mannitol, and 5 mM HEPES (pH 7.3 and 310 mosmol/kg- H_2O). Fluorescence was measured by a plate reader (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Chemicals

Channel blockers, such as 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), 5-nitro-2-(3-phenylpropyl-amino)-benzoate (NPPB), and phloretin, as well as ROS scavengers, such as tiron, N-acetyl-cysteine (NAC) and catalase, were purchased from SIGMA or Tocris Cookson (Bristol, UK). Other chemicals were from Nacalai Tesque or SIGMA.

Statistical analysis

The data, presented as means $\pm$ SEM, were statistically analyzed using the unpaired *t*-test or the Welch *t*-test when variances were heterogeneous. Differences were considered significant when *p* was < 0.05.

Results

Cardiomyocyte apoptosis induced by ischemia-reperfusion

Although the number of non-adherent cardiomyocytes increased (Fig. 1A), Hoechst 33324 staining showed that no morphological change occurred in the nucleus immediately after ischemia for 6 h (Fig. 1B). However, prominent changes in cell morphology (especially shrinkage and chromatin condensation) were

observed when 6-h ischemia was followed by 96-h reperfusion (Fig. 1).

Viability of mouse cardiomyocytes monitored by the MTT assay was reduced to some extent immediately after 4- to 8-h exposure to ischemic stress (Fig. 2A). Marked reduction of cell viability was induced, in a time-dependent manner, by reperfusion, as shown in Fig. 2A. Internucleosomal cleavage of nuclear DNA (DNA laddering), which is a hallmark feature of apoptosis, was observed after 6-h ischemia followed by 96-h (but not 72-h) reperfusion and after 8-h ischemia followed by 72- or 96-h reperfusion (Fig. 2B), but was never observed immediately after 6- and 8-h ischemia (data not shown).

Cl⁻ channel involvement in cardiomyocyte apoptosis induced by ischemia-reperfusion

When applied during 6-h ischemia period, non-selective Cl^- channel blockers, DIDS and NPPB, failed to reduce cell death and DNA laddering observed after reperfusion for 96 h (Fig. 3A). In contrast, both the cell viability decrease and DNA laddering were significantly prevented by DIDS (50 or 200 μ M) and NPPB (50 or 100 μ M), when they were applied during the reperfusion period (Fig. 3B).

Since it is known that DIDS is a blocker not only of Cl^- channels but also of the Cl^-/HCO_3^- exchanger, the effects of removal of ambient HCO_3^- , which should minimize the contribution of the Cl^-/HCO_3^- exchanger, on the rescue by DIDS were observed. As summarized in Fig. 4A, the rescuing effect of DIDS applied for the

Fig. 2. Apoptosis induction by ischemia-reperfusion treatment in mouse cardiomyocytes. A. Time-dependent effects of ischemia for 4, 6 or 8 h (4 h-I, 6 h-I or 8 h-I) followed by reperfusion for 48, 72 or 96 h (48 h-R, 72 h-R or 96 h-R) on cell viability. Data represent the means $\pm$ S.E.M. (vertical bars) of 6 experiments. * and **, significantly different from the control data at $p < 0.05$ and $p < 0.01$, respectively. B. Effects of ischemia-reperfusion on DNA laddering. Data are representative of 3 independent experiments.

Fig. 3. Rescuing effects of Cl^- channel blockers added during the reperfusion, but not ischemia, period, as assessed by enhancement of cell viability (top panels) and reduction in DNA laddering (bottom panels) in mouse cardiomyocytes. A. Effects of Cl^- channel blockers (50 or 200 μM DIDS and 50 or 100 μM NPPB) added during the ischemia period for 6 h (6 h-I or I). B. Effects of Cl^- channel blockers added during the reperfusion period for 96 h (96 h-R or R). It is noted that DIDS or NPPB alone did not significantly affect cell viability. The cell viability data (normalized with the control value) represent the means $\pm$ S.E.M. (vertical bars) of 10 experiments. *, significantly different ($p < 0.05$) from the control data (treated with the vehicle, DMSO, alone). The DNA laddering data are representative of 3 independent experiments.

reperfusion period in the absence of HCO_3^- was indistinguishable from that when it was present. The rescuing effect of another Cl^- channel blocker, NPPB, which cannot block $\text{Cl}^-/\text{HCO}_3^-$ exchangers [26], was also not affected by removal of HCO_3^- (Fig. 4A). Profound activation of caspase-3 was observed 24 h after reperfusion in mouse cardiomyocytes in the presence or absence of HCO_3^- (Fig. 4B). DIDS or NPPB at 50 μM prominently suppressed reperfusion-induced activation of caspase-3, and this effect was not altered by removal of HCO_3^- (Fig. 4B). These data suggest that the activity of

some type of Cl^- channel during the reperfusion period has an essential role in the induction of apoptosis in ischemic cardiomyocytes.

Since the VSOR Cl^- channel is known to be activated in apoptotic cells [16], we next examined the effect of a low concentration of phloretin, which blocks the VSOR Cl^- channel without affecting cAMP- and Ca^{2+} -activated Cl^- channels [27]. At 30 μM , phloretin administered during the 96-h reperfusion period significantly diminished the cell viability loss observed after ischemia-reperfusion (Fig. 5A). Phloretin also abolished caspase-3 activation (Fig.

Fig. 4. HCO_3^- independence of rescuing effects of Cl^- channel blockers on ischemia-reperfusion-induced apoptosis in mouse cardiomyocytes. DIDS (200 μM) or NPPB (100 μM) was added during the reperfusion period in the presence or absence of HCO_3^- . A. Effects of Cl^- channel blockers on cell viability reduction induced by ischemia for 6 h (6 h-I or I) followed by reperfusion for 96 h (96 h-R or R). B. Effects of Cl^- channel blockers on caspase-3 activation induced by 6 h-I followed by reperfusion for 24 h (24 h-R or R). The data (normalized with the control value) represent the mean $\pm$ S.E.M. (vertical bars) of 8 experiments. * and **, significantly different from the control data (treated with the vehicle, DMSO, alone) at $p < 0.05$ and $p < 0.01$, respectively.

Fig. 5. Effects of phloretin (30 μM) added during the reperfusion period on cell viability reduction (A) and caspase-3 activation (B) induced by ischemia for 6 h (6 h-I or I) followed by reperfusion for 96 h (96 h-R or R) and 24 h (24 h-R or R), respectively, in mouse cardiomyocytes. It is noted that phloretin alone did not induce non-specific reduction of cell viability at this concentration. The data (normalized with the control value) represent the mean $\pm$ S.E.M. (vertical bars) of 6 experiments. * and **, significantly different from the control data (treated with the vehicle, DMSO, alone) at $p < 0.05$ and $p < 0.01$, respectively.

5B) and DNA laddering (data not shown) in cardiomyocytes subjected to ischemia-reperfusion. These results are evidence that the activity of the VSOR Cl^- channel during the reperfusion period plays an essential role in apoptosis induction in ischemic cardiomyocytes.

ROS involvement in cardiomyocyte apoptosis induced by ischemia-reperfusion

To assess a possible role of ROS in apoptosis induced by ischemia-reperfusion in cardiomyocytes, ROS production was measured during 8-h ischemia and following 96-h reperfusion by using carboxy- H_2DCFDA . As shown in Fig. 6 (filled circles) dramatic elevation of

the intracellular ROS level was observed 1 to 64 h after starting reperfusion. However, significant ROS production was never observed during the ischemia period of 8 h (Fig. 6: open circles). Essentially similar ROS production induced by ischemia-reperfusion was observed in the presence of 200 μM DIDS (data not shown).

To evaluate whether ROS play an essential role in the mechanism by which reperfusion induces apoptosis in ischemic cardiomyocytes, the effects of scavengers for ROS (tiron), superoxide (NAC) and H_2O_2 (catalase) added during the reperfusion period were observed. All scavengers significantly restored cell viability monitored by the MTT activity (Fig. 7A). Activation of caspase-3

Fig. 6. ROS production induced by ischemia (I)-reperfusion (R) in mouse cardiomyocytes. The data are normalized with the control value, which was measured immediately before ischemia treatment. Each symbol represents the mean $\pm$ S.E.M. (vertical bar) of 6 experiments. **, significantly different from the control data at $p < 0.01$.

induced by ischemia-reperfusion was largely inhibited by tiron and abolished by NAC or catalase (Fig. 7B). These data indicate that elevated ROS production during the reperfusion period is a causal factor for apoptotic death of cardiomyocytes subjected to ischemia-reperfusion.

Discussion

Although apoptosis is involved in normal physiological processes of animal development and tissue homeostasis [28], it also underlies pathological processes [29]. Apoptosis has been implicated in a variety of heart diseases such as myocardial infarction, cardiomyopathy, paroxysmal arrhythmias, fibromuscular dysplasia of the small coronary arteries, arrhythmogenic right ventricular dysplasia, and some form of myocarditis [30, 31]. Notably, it has been shown that apoptosis is a major contributor to myocardial cell death following ischemia-reperfusion injury [6]. In the present study, apoptotic death of mouse cardiomyocytes in primary culture subjected to ischemia-reperfusion was not observed immediately after ischemic treatment but prominent apoptosis could be observed after reperfusion. This observation is in good agreement with previous reports that reperfusion, but not ischemia, induces

Fig. 7. Rescuing effects of ROS scavengers on ischemia-reperfusion-treated mouse cardiomyocytes, as measured by enhancement of cell viability (A) and suppression of caspase-3 activity (B). A ROS scavenger (tiron, 1 mM), a superoxide scavenger (NAC, 2 mM) and a H_2O_2 scavenger (catalase, 200 U/ml) were added during the reperfusion period of 24 h (24 h-R or R) or 96 h (96 h-R or R) but not the ischemia period of 6 h (6 h-I or I). The data (normalized with the control value) represent the means $\pm$ S.E.M. (vertical bars) of 6 experiments. *, significantly different from the control data (treated with the vehicle, DMSO, alone) at $p < 0.05$.

apoptosis in rabbit adult cardiomyocytes *in vivo* [5] and in rat neonatal cardiomyocytes in primary culture [9].

Oxidative stress was shown to trigger cardiomyocyte apoptosis in myocardial infarction, ischemia-reperfusion injury, cardiomyopathy, atherosclerosis and heart failure

[32, 33]. In the present study, robust ROS production was observed during the reperfusion period for 1 to 64 h but not during the ischemia period. This result contrasts with a previous observation that ROS is produced before reperfusion in ischemic chick cardiomyocytes [34]. This difference may be due to the difference of animal species. It was also found in the present study that three different types of ROS scavenger were effective in preventing cardiomyocytes from undergoing apoptosis when any one of them was applied during the reperfusion, but not ischemia, period. Thus, ROS scavengers are potential clinical tools, which can be administered via blood vessels for protection against ischemia-reperfusion injury of the heart.

We have recently demonstrated that elevated ROS production is involved in activation of the VSOR Cl⁻ channel during mitochondrion-mediated apoptosis in human epithelial cells [16]. Our previous studies [17, 18] showed that staurosporine-induced cardiomyocyte apoptosis was prevented by DIDS and NPPB, which are known to block the VSOR Cl⁻ channel in mouse cardiomyocytes at submillimolar concentrations [35]. These results suggest the possibility that the activity of the VSOR Cl⁻ channel is involved in ischemia-reperfusion-induced apoptosis in mouse cardiomyocytes, which exhibit robust functional expression of the channel [35]. Indeed, the present study showed that DIDS and NPPB rescued mouse cardiomyocytes from ischemia-reperfusion-induced apoptosis when applied during the reperfusion, but not ischemia, period. Even under HCO₃⁻-free conditions, ischemia-reperfusion-induced apoptosis and its prevention by these Cl⁻ channel blockers were unaltered. Thus, it appears that the events of apoptosis and the action of Cl⁻ channel blockers are independent of the functional operation of the Cl⁻/HCO₃⁻ exchanger, although this anion exchanger was found to be upregulated by hypoxia in isolated rat ventricular myocytes [36]. Also,

the present study demonstrated that ischemia-reperfusion-induced apoptosis was rescued by application of a low concentration (30 μM) of phloretin, which was shown to block the VSOR Cl⁻ channel in mouse cardiomyocytes (X. Sun, T. Shimizu & Y. Okada, unpublished observations) and in other cells without affecting cAMP- and Ca²⁺-activated Cl⁻ channels [27]. These data suggest that electroconductive Cl⁻ efflux through VSOR Cl⁻ channels induces KCl efflux, which is associated with AVD, a critical event of apoptosis [10-16]. Supporting this hypothesis is the observation that a non-selective blocker of K⁺ channels (50 μM quinine or Ba²⁺) reduced apoptotic cell death in cardiomyocytes subjected to ischemia-reperfusion (X. Wang, N. Takahashi & Y. Okada, unpublished observations). There may be evidence that ischemic preconditioning of myocardium is also related to activation of the VSOR Cl⁻ channel [37, 38], although this has not been fully confirmed [39]. Here we have provided evidence that activation of the VSOR Cl⁻ channel during the reperfusion period is involved in the induction of apoptosis in cardiomyocytes subjected to ischemia-reperfusion. To treat cardiac disorders including ischemia-reperfusion injury, the mechanism of apoptosis induction could be targeted for pharmacological intervention [40]. Thus, the VSOR Cl⁻ channel would be a novel target for the development of drugs, which protect against myocardial ischemia-reperfusion injury.

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