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EXPERIMENTAL STUDIES

Antiapoptotic Effect of Nicorandil Mediated by Mitochondrial ATP-Sensitive Potassium Channels in Cultured Cardiac Myocytes

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OBJECTIVES We examined whether nicorandil, a clinically useful drug for the treatment of ischemic syndromes, inhibits myocardial apoptosis.

BACKGROUND Nicorandil has been reported to have a cardioprotective action through activation of mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channels. Based on our recent observation that mitoK$_{ATP}$ channel activation has a remarkable antiapoptotic effect in cultured cardiac cells, we hypothesized that the protective effects of nicorandil may be at least partially due to an antiapoptotic effect.

METHODS Cultured neonatal rat cardiac myocytes were exposed to hydrogen peroxide to induce apoptosis. Effects of nicorandil were evaluated using a number of apoptotic markers.

RESULTS Exposure to 100 μM hydrogen peroxide resulted in apoptotic cell death as shown by TUNEL positivity, cytochrome *c* translocation, caspase-3 activation and dissipation of mitochondrial inner membrane potential ($\Delta\Psi_m$). Nicorandil (100 μM) suppressed all of these markers of apoptosis. Notably, nicorandil prevented $\Delta\Psi_m$ depolarization in a concentration-dependent manner ($EC_{50} \sim 40$ μM, with saturation by 100 μM), as shown by fluorescence-activated cell sorter analysis of cells stained with a fluorescent $\Delta\Psi_m$-indicator, tetramethylrhodamine ethyl ester (TMRE). Time-lapse confocal microscopy of individual cells loaded with TMRE shows that nicorandil suppresses $\Delta\Psi_m$ loss. Subcellular calcein localization revealed inhibition of the mitochondrial permeability transition by nicorandil. These protective effects of nicorandil were blocked by the mitoK$_{ATP}$ channel antagonist 5-hydroxydecanoate.

CONCLUSIONS Our findings identify nicorandil as an inhibitor of apoptosis induced by oxidative stress in cardiac myocytes, and confirm the critical role of mitoK$_{ATP}$ channels in inhibiting apoptosis. (J Am Coll Cardiol 2002;40:803–10) © 2002 by the American College of Cardiology Foundation

Nicorandil is a drug with both nitrate-like and ATP-sensitive potassium (K_{ATP}) channel activating properties (1) and is currently used clinically for the treatment of ischemic heart disease. By virtue of this dual mechanism of action, the drug acts as a balanced coronary and peripheral vasodilator, reducing both preload and afterload. This drug has been reported to have an infarct size limiting effect in a number of studies that use animal models of ischemia/reperfusion (2–4). In agreement with these findings, clinical studies have documented beneficial effects of nicorandil in patients undergoing angioplasty (5) as well as cardiopulmonary bypass surgery (6). Moreover, adjunctive administration of nicorandil in patients with acute myocardial infarction resulted in better functional and clinical outcome (7,8). The cardioprotective effects of nicorandil are at least partially mediated by selective activation of K_{ATP} channels in the mitochondrial inner membrane (mitoK$_{ATP}$ channels) (9,10).

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We have recently found that activation of mitoK$_{ATP}$ channels inhibits oxidative stress-induced cellular apoptosis in cultured neonatal rat cardiac myocytes, suggesting a direct mechanistic link between mitoK$_{ATP}$ channels and programmed cell death (11). Since apoptosis substantially contributes to cellular demise and subsequent functional loss in a number of cardiovascular diseases (12), we conjectured that the cardioprotective effects of nicorandil may be, at least in part, attributable to an antiapoptotic effect. Here, we demonstrate that nicorandil prevents apoptosis induced by oxidative stress in isolated neonatal rat cardiac myocytes, providing mechanistic insights of cardioprotection afforded by nicorandil.

METHODS

All procedures were performed in accordance with the Johns Hopkins University animal care guidelines, which conform to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institute of Health.

Primary culture of neonatal rat cardiac myocytes. Cardiac ventricular myocytes were prepared from 1 to 2-day-old Sprague-Dawley rats and cultured as described (11). In brief, the hearts were removed, and the ventricles were minced into small fragments, which were digested by

Abbreviations and Acronyms

$\Delta\Psi_m$	= mitochondrial inner membrane potential
5-HD	= 5-hydroxydecanoate
FACS	= fluorescence-activated cell sorter
K_{ATP}	= adenosine triphosphate-sensitive potassium
mitoK_{ATP}	= mitochondrial adenosine triphosphate-sensitive potassium
PTP	= (mitochondrial) permeability transition pore
TMRE	= tetramethylrhodamine ethyl ester
TUNEL	= terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

trypsin dissociation. The dissociated cells were preplated for 1 h to enrich the culture with myocytes. The nonadherent myocytes were then plated in plating medium consisting of Dulbecco's Modified Eagle Medium (Mediatech) supplemented with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and 2 μg/ml vitamin B12. The final myocyte cultures contained >90% cardiac myocytes at partial confluence. The cells were maintained at 37°C in the presence of 5% CO_2 in a humidified incubator. Bromodeoxyuridine (0.1 mM) was included in the medium for the first 3 days after plating to inhibit fibroblast growth. Cultures were then placed in serum-free DMEM containing vitamin B12, transferrin and insulin, 24 h prior to the drug treatment.

Experimental protocol. Neonatal rat cardiac myocytes in primary culture were randomly assigned to one of four experimental groups: 1) control group; 2) incubation with 100 μM hydrogen peroxide (H_2O_2); 3) pretreatment with 100 μM nicorandil for 20 min, followed by 100 μM H_2O_2; 4) pretreatment with 100 μM nicorandil and 500 μM of mitoK_{ATP} channel blocker, 5-hydroxydecanoate (5-HD) for 20 min, followed by 100 μM H_2O_2. At the beginning of the experiment, culture media were replaced with fresh serum-free DMEM containing those drugs, and cells were exposed to those drugs during the entire experimental period. Nicorandil is a gift from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. The TUNEL staining was performed according to the manufacturer's protocol (Roche, Indianapolis, Indiana). Fluorescein labels incorporated in nucleotide polymers were detected by laser scanning confocal microscopy at an excitation wavelength of 488 nm (argon laser).

Immunofluorescence staining. Immunofluorescence staining was carried out as described previously (11). In brief, cells were plated on multiwell culture slides, treated with drugs as indicated, fixed in an ice-cold 1:1 mixture of methanol/acetone, blocked with 10% normal goat serum and 0.075% saponin in PBS, and incubated with primary antibody dissolved in blocking solution at a dilution of 1:100. For cytochrome *c* staining, mouse monoclonal anti-cytochrome *c* antibody (Pharmingen; 6H2.Ba4) was used; for caspase-3 staining, rabbit polyclonal antiserum raised against the activated form of caspase-3 (Pharmingen), which recognizes only the processed 20 kDa subunit of cleaved caspase-3, was used. After washing with PBS, cells were incubated with secondary antibody consisting of Alexa Fluor 546 goat antimouse IgG, $F(ab')_2$ (Molecular Probes, diluted 1:100) or Alexa Fluor 546 goat anti-rabbit IgG, $F(ab')_2$ (Molecular Probes, diluted 1:100, Eugene, Oregon) respectively. Finally, cells were counterstained with the DNA binding dye DAPI (5 μM, Molecular Probes). Cells were examined by a laser scanning confocal microscope (Zeiss, LSM 410, Thornwood, New York), using 40× water-immersion lens and 2× optical zoom. Alexa 546 was excited and visualized by a helium/neon laser (543 nm), and DAPI by an ultraviolet laser (351 nm).

Caspase-3 activity assay. Caspase-3 activity was measured by detection of the cleavage of a colorimetric caspase-3 substrate, N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline, using an assay kit, ApoAlert CPP32 (Clontech, Palo Alto, California). The assay was performed according to the manufacturer's protocol at 4, 8, and 16 h of stimulation by each drug. In brief, lysates from cells were incubated in a reaction mixture including N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline as a substrate of caspase-3-like proteases for 1 h at 37°C. The levels of the resulting proteolytic fragment p-nitroanilide were measured as optical density at 405 nm. The relative activity was standardized by protein concentration, which was determined using Bradford protein assay reagent (Bio-Rad, Hercules, California).

Assessment of mitochondrial membrane potential ($\Delta\Psi_m$). Loss of $\Delta\Psi_m$ was assessed using either fluorescence-activated cell sorter (FACS) analysis or time-lapse laser scanning confocal microscopy of cells stained with tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) (13). Cells were incubated with 100 ng/ml TMRE for at least 15 min in 37°C before starting drug treatment.

For FACS analysis, cells were harvested by trypsinization at the end of experimental protocols, and analyzed by FACScan (20,000 cells/sample). The fluorescence intensity of TMRE was monitored at 582 nm (FL-2). The FACS data were analyzed using analysis software (WinMDI, http://facs/scripps.edu/software.html).

For time-lapse confocal analysis of $\Delta\Psi_m$, cells plated on 35 mm dishes were maintained at 37°C using a heater platform (Warner Instrument, Hamden, Connecticut) installed on a microscope stage, and were placed in phenol-red-free DMEM (Life-Tech, Carlsbad, California) supplemented with 50 nM TMRE and 25 mM HEPES, in order to avoid pH changes in the non–CO_2-equilibrated environment. After the desired temperature was reached, time-lapse confocal microscopy was started with a 5 min interval, using 20× objective lens. The TMRE was excited using a 543 nm line of a helium/neon laser. Fifty cells were randomly selected in each scan by drawing regions around individual cells, and red fluorescence intensity was sequentially monitored.

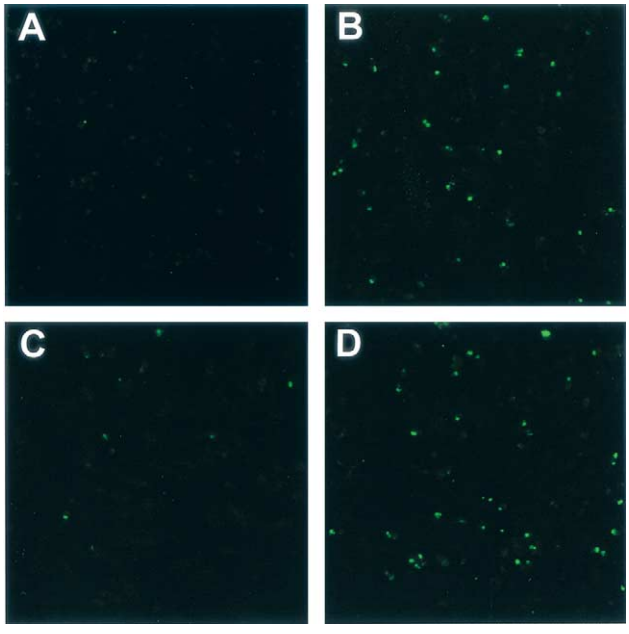

Figure 1. The TUNEL staining in neonatal rat cardiac myocytes. **(A)** Control cells. **(B)** Cells exposed to 100 μM H_2O_2 for 16 h. **(C)** Cells pretreated with 100 μM nicorandil followed by 100 μM H_2O_2 for 16 h. **(D)** Cells pretreated with 100 μM nicorandil and 500 μM 5-hydroxydecanoate, followed by 100 μM H_2O_2 for 16 h.

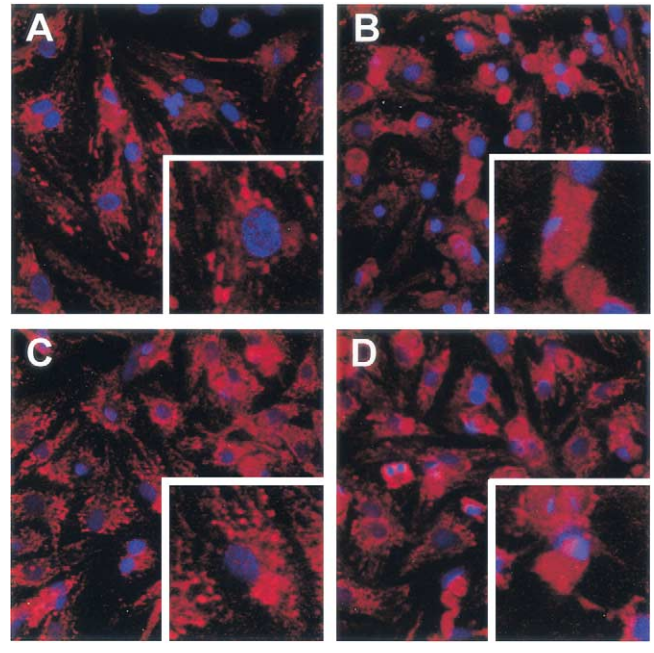

Figure 2. Immunofluorescent staining for cytochrome *c* in neonatal rat cardiac myocytes. Blue fluorescent dye DAPI demonstrates the nuclear morphology. **(A)** Control cells. **(B)** Cells exposed to 100 μM H_2O_2 for 16 h. **(C)** Cells pretreated with 100 μM nicorandil followed by 100 μM H_2O_2 for 16 h. **(D)** Cells pretreated with 100 μM nicorandil and 500 μM 5-hydroxydecanoate, followed by 100 μM H_2O_2 for 16 h. Each panel contains an inset to demonstrate high power image.

Assessment of mitochondrial permeability transition pore (PTP). To monitor the opening of PTP, cultured cardiac myocytes were loaded with calcein acetomethoxy ester (calcein-AM) (Molecular Probes), as previously described (14) with minor modifications. Briefly, cells loaded with 100 nM of TMRE (37°C, 15 min), were further loaded for 15 min with 2 μM calcein-AM at room temperature in the presence of 4 mM of cobalt chloride ($CoCl_2$) to quench cytosolic and nuclear calcein loading. After washing calcein-AM and $CoCl_2$, cells were kept at 37°C using a heater platform installed on a microscope stage. Calcein was excited with 488 nm line of an argon laser.

Statistical analysis. Statistical analysis of changes in caspase-3 activity over the time course and that of the percentage of cells with high TMRE fluorescence were performed using one-way analysis of variance with Fisher's least significant difference as the post-hoc test. A level of $p < 0.05$ was accepted as statistically significant.

RESULTS

Figure 1A to 1D demonstrates TUNEL staining in each experimental group. The TUNEL staining detects nuclear DNA strand breaks, which occur during the terminal phase of apoptosis. Control cells exhibited sparse TUNEL-positive nuclei (Fig. 1A), but exposure to 100 μM H_2O_2 for 16 h increased the number of TUNEL-positive nuclei, which can be seen as bright spots (Fig. 1B), indicating enhanced apoptosis under the treatment with H_2O_2. There are obviously fewer TUNEL-positive nuclei in the nicorandil-treated group (Fig. 1C), despite the similar density of cells compared with H_2O_2 group. This protective effect of nicorandil was blocked by the selective mitoK_{ATP} channel antagonist 5-HD (Fig. 1D), suggesting that this protective effect of nicorandil is mediated by mitoK_{ATP} channel opening.

Figure 2A to 2D demonstrates cytochrome *c* immunofluorescence. Cytochrome *c*, a member of the electron transport chain, has been revealed to play a critical role in the initiation of apoptosis; the translocation of cytochrome *c* from mitochondria to the cytoplasm in response to a variety of stress subsequently causes cleavage and activation of the apoptosis-executioner proteases, caspases (15,16). As shown in Figure 2A, the distribution of cytochrome *c* is punctate, suggesting mitochondrial localization, which was further confirmed with colocalization with a specific mitochondrial marker dye, MitoTracker (Molecular Probes) (data not shown). Incubation with 100 μM H_2O_2 for 16 h induced cytochrome *c* translocation to the cytoplasm, resulting in a homogeneous distribution (Fig. 2B). Pretreatment with nicorandil inhibited the translocation of cytochrome *c* (Fig. 2C), while the addition of 500 μM 5-HD cancelled the effect of nicorandil (Fig. 2D).

Figure 3A to 3D demonstrates the immunofluorescent staining for caspase-3, another key contributor to the execution of apoptosis (17). In this experiment, polyclonal antiserum that recognizes only the active form of the enzyme was used. Therefore, cells that have activated caspase-3 are recognized by the antibody and stained as red.

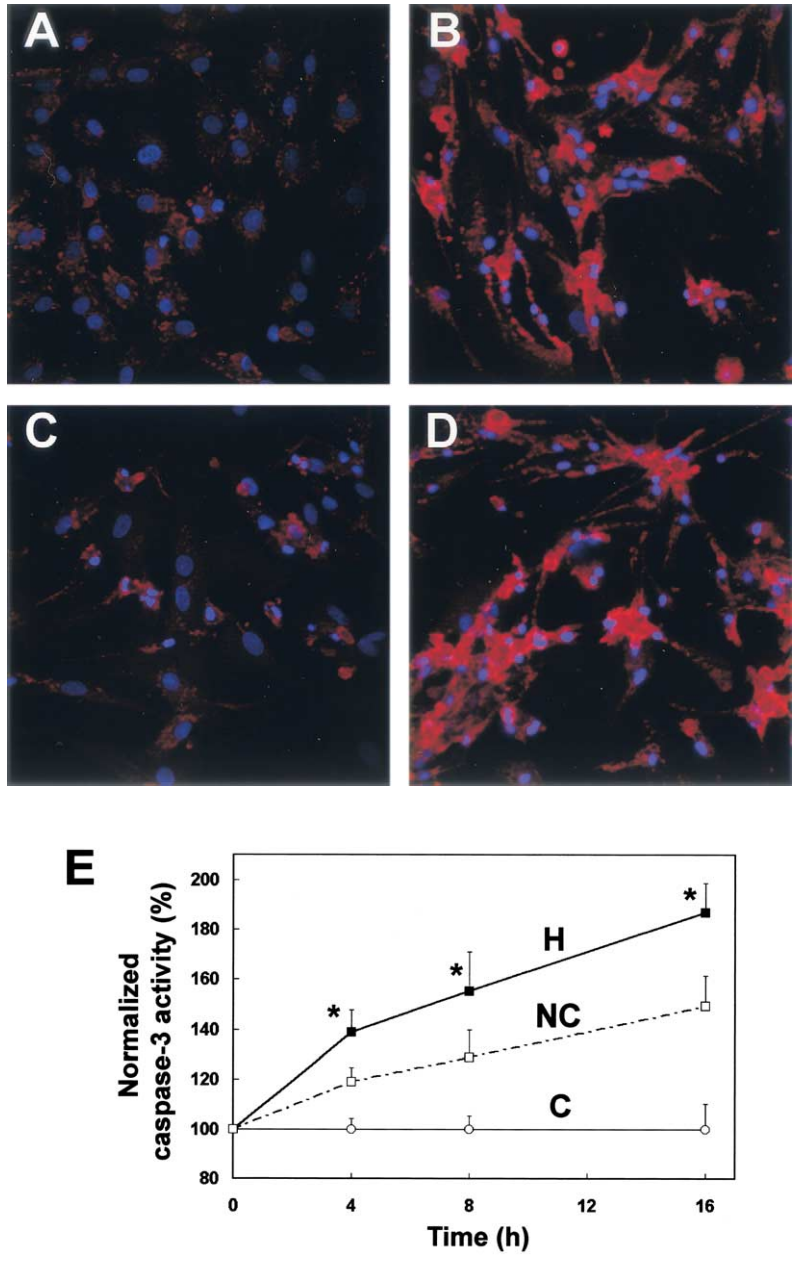

Figure 3. Immunofluorescent staining for active form of caspase-3 in neonatal rat cardiac myocytes. Red fluorescence represents 20-kDa active form of caspase-3. Blue fluorescent dye DAPI demonstrates the nuclear morphology. **(A)** Control cells. **(B)** Cells exposed to 100 μM H_2O_2 for 16 h. **(C)** Cells pretreated with 100 μM nicorandil followed by 100 μM H_2O_2 for 16 h. **(D)** Cells pretreated with 100 μM nicorandil and 500 μM 5-hydroxydecanoate, followed by 100 μM H_2O_2 for 16 h. **(E)** Time-dependent changes in caspase-3 activity measured by detection of the cleavage of a colorimetric caspase-3 substrate. Each data point is expressed as a relative ratio against the control value of the same time points (n = 3 for each data point). Control value in each time point is defined as 100%. There are no actual data for 0 h. C = control **(open circle)**; H = H_2O_2 100 μM **(closed square)**; NC = H_2O_2 + nicorandil 100 μM **(open square)**. Data are mean ± SEM. *$p < 0.05$ vs. C or NC.

In control, cells showed little cytosolic fluorescence (Fig. 3A), but incubation with 100 μM H_2O_2 for 16 h increased the red signal in the cytosol (Fig. 3B). Pretreatment with 100 μM nicorandil inhibited the H_2O_2-induced activation of caspase-3 (Fig. 3C), while addition of 500 μM 5-HD abrogated the effect of nicorandil (Fig. 3D). Caspase-3 activity in each group was further quantified using colorimetric caspase-3 substrate (Fig. 3E). Exposure to 100 μM H_2O_2 resulted in time-dependent increase in caspase-3 activity. Pretreatment with nicorandil significantly inhibited caspase-3 activation at all time points.

Next, we measured $\Delta\Psi_m$ as an early apoptotic marker in all experimental groups, using FACS analysis of cells stained with a fluorescent $\Delta\Psi_m$ indicator, TMRE. This cationic dye accumulates in mitochondria driven by the negative mitochondrial matrix potential under the normal conditions. Depolarization of $\Delta\Psi_m$ is represented by the loss of TMRE fluorescence. Figure 4A shows histograms of

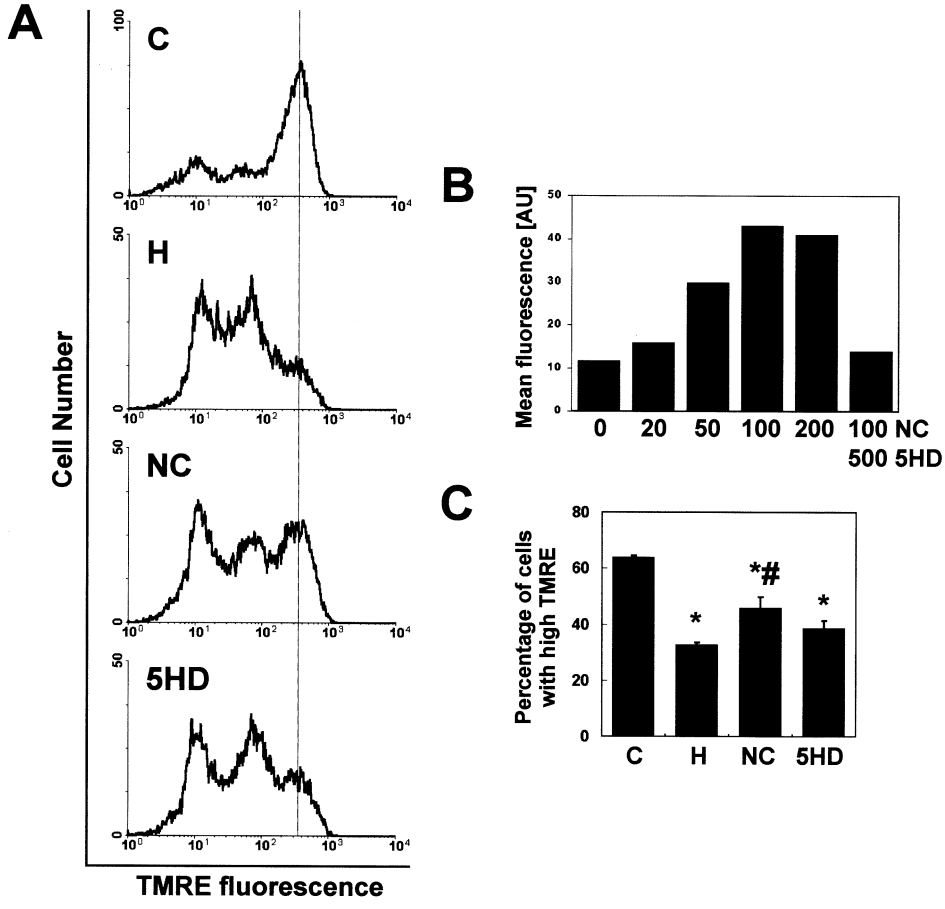

Figure 4. Mitochondrial membrane potential ($\Delta\Psi_m$) in neonatal rat cardiac myocytes assessed by fluorescence-activated cell sorter (FACS) analysis of cells stained with a red fluorescent $\Delta\Psi_m$ indicator, tetramethylrhodamine ethyl ester (TMRE). **(A)** The histograms of FL-2 channel (red fluorescence) are shown. C = control; H = 100 μM H_2O_2 for 2 h; NC = pretreatment with 100 μM nicorandil followed by 100 μM H_2O_2 for 2 h; 5HD = pretreatment with 100 μM nicorandil and 500 μM 5-hydroxydecanoate, followed by 100 μM H_2O_2 for 2 h. In all of the histograms the position of the major population of control cells were indicated by **vertical dashed line**. Results are representative data from at least four independent experiments. **(B)** Mean values of TMRE fluorescence obtained from FL-2 histograms of FACS analysis. **Bars, from left to right,** show data from the following: **bar 1** = H_2O_2; **bar 2** = H_2O_2 + nicorandil 20 μM; **bar 3** = H_2O_2 + nicorandil 50 μM; **bar 4** = H_2O_2 + nicorandil 100 μM; **bar 5** = H_2O_2 + nicorandil 200 μM; **bar 6** = H_2O_2 + nicorandil 100 μM + 5-HD 500 μM. The concentration of H_2O_2 was 100 μM for all groups. The value of control group was 203. **(C)** Summarized data of the percentage of cells that maintain high ($>10^2$) TMRE fluorescence (n = 4 for all groups). Data are mean ± SEM. *$p < 0.05$ vs. C group. #$p < 0.05$ vs. H group.

TMRE fluorescence in the various experimental groups. The majority of cells in the control group (Fig. 4A, panel C) belonged to a population with high TMRE fluorescence level (indicated by vertical dashed line). Exposure to H_2O_2 shifted the predominant population to lower TMRE fluorescence (panel H). Nicorandil protected against H_2O_2-induced loss of $\Delta\Psi_m$, preserving a sizable population of cells with a normal $\Delta\Psi_m$ level (panel NC). The protective effect of diazoxide was abolished by 500 μM 5-HD, shifting cells into populations with low $\Delta\Psi_m$ level (panel 5HD). Similar experiments were further carried out for various concentrations of nicorandil, and the mean fluorescence intensities from those histograms were obtained (Fig. 4B). This result clearly shows the positive concentration-response relationship of nicorandil. The $\Delta\Psi_m$-preserving effect of nicorandil saturates at 100 μM and EC_{50} is 40 μM. This value is comparable to that known to activate mitoK_{ATP} channels (10). In addition, 5-HD alone had no effect on $\Delta\Psi_m$ in the absence of nicorandil and it did not promote any of the apoptotic markers examined here compared with the control group (data not shown), and its incubation together with H_2O_2 did not further aggravate those markers compared with the H_2O_2-treated drug-free group. Figure 4C summarizes pooled data showing the percentage of cells, which retain a high TMRE fluorescence (defined as $>10^2$ in this analysis) in various experimental groups. These data indicate that nicorandil is effective in limiting the extent of $\Delta\Psi_m$ depolarization due to H_2O_2, and that the protection is 5-HD-sensitive.

To examine the time-dependent changes of $\Delta\Psi_m$ on a single-cell basis, we used time-lapse confocal analysis of cardiac myocytes loaded with TMRE. Images were taken every 5 min over the entire time-course of 90 min. The majority of cells underwent $\Delta\Psi_m$ changes within 90 min, and there were no further changes of signals afterwards. In Figure 5A to 5D, representative confocal images of cardiac myocytes at selected time points are shown. Throughout the period of observation (90 min), TMRE fluorescence inten-

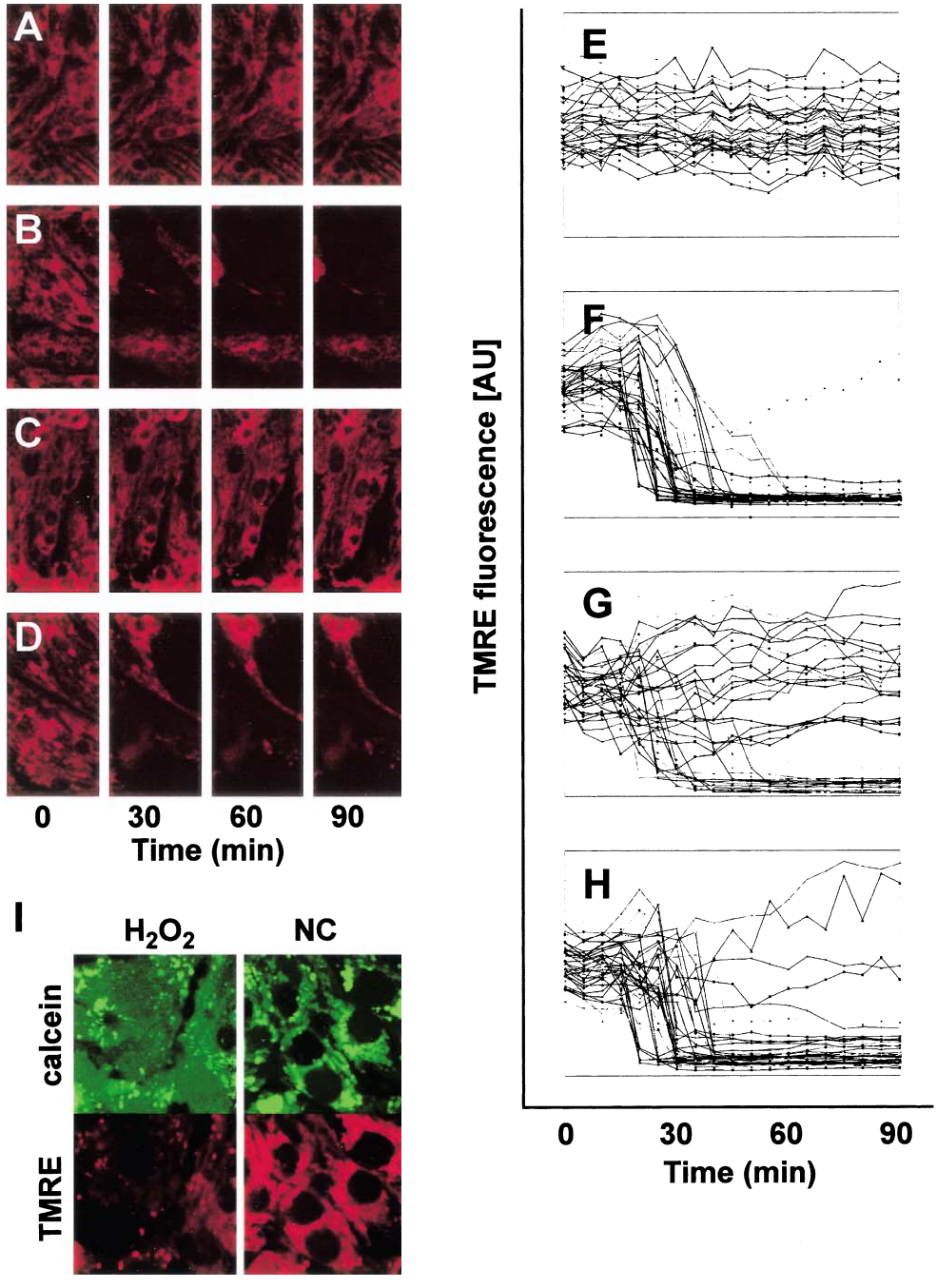

Figure 5. **(A to D)** Time-lapse analysis of the loss of mitochondrial inner membrane potential in neonatal rat cardiac myocytes. Cells were subjected to time-scan for 90 min at 5-min interval. Images from selected time points are shown. **(A)** Control cells. **(B)** Cells exposed to 100 μM H_2O_2. **(C)** Cells pretreated with 100 μM nicorandil followed by 100 μM H_2O_2. **(D)** Cells pretreated with 100 μM nicorandil and 500 μM 5-hydroxydecanoate (5-HD), followed by 100 μM H_2O_2. **(E to H)** Time course of red fluorescence in each individual cell which was prospectively selected at the beginning of the scan (E = control cells; F = H_2O_2 100 μM; G = H_2O_2 100 μM + nicorandil 100 μM; H = H_2O_2 100 μM + nicorandil 100 μM + 5-HD 500 μM). All of 50 cells are shown. **(I)** Confocal images of cardiac myocytes co-loaded with tetramethylrhodamine ethyl ester (TMRE) **(red)** and a fluorescent mitochondrial permeability transition pore indicator, calcein **(green)**. **(Left panel)** Cells exposed to 100 μM H_2O_2 for 1 h. **(Right panel)** Cells pretreated with 100 μM nicorandil followed by 100 μM H_2O_2 for 1 h.

sities of control cells remained unchanged (Fig. 5A). In contrast, cells treated with 100 μM H_2O_2 progressively lost TMRE fluorescence, indicating irreversible dissipation of $\Delta\Psi_m$ (Fig. 5B). Nicorandil remarkably inhibited this catastrophic loss of $\Delta\Psi_m$, and the majority of cells maintained intact $\Delta\Psi_m$ (Fig. 5C). This protective effect of nicorandil was abrogated by 5-HD (Fig. 5D). From these image sequences, 50 cells were randomly and prospectively selected in each group, and the mean values of red fluorescence intensity from each cell are plotted in Figure 5E to 5H. Exposure to H_2O_2 caused catastrophic loss of $\Delta\Psi_m$ in most of the cells (Fig. 5F). The duration of $\Delta\Psi_m$ loss of each cell

was abrupt (5 to 10 min) and complete, regardless of the time elapsed since the H_2O_2 was applied. Nicorandil decreased the number of cells undergoing $\Delta\Psi_m$ loss (Fig. 5G), and its protective effect was negated by 5-HD (Fig. 5H). However, nicorandil did not change the duration of $\Delta\Psi_m$ loss in those cells that did lose their inner membrane potential.

Such an abrupt and irreversible dissipation of $\Delta\Psi_m$ may result from opening of PTP (18,19), a pathway which contributes not only to apoptosis but also to necrosis (20,21). Therefore, we investigated if the effects of nicorandil on preservation of $\Delta\Psi_m$ are related to the inhibition of PTP opening. Myocytes were co-loaded with TMRE and the green fluorescent indicator calcein. Fluorescent calcein concentrates in the mitochondrial matrix, such that the loss of punctate calcein fluorescence can be used to detect the opening of the PTP in situ (14). Exposure to H_2O_2 resulted in loss of TMRE and concomitant massive translocation of calcein fluorescence from the mitochondrial matrix into the cytoplasm, indicating that the abrupt loss of $\Delta\Psi_m$ is mediated by PTP opening (Fig. 5I, left panel). Pretreatment with nicorandil effectively suppressed the PTP opening as demonstrated by preserved TMRE fluorescence and maintained punctate localization of calcein in the mitochondria (Fig. 5I, right panel).

DISCUSSION

Nicorandil as a cardioprotective agent. Nicorandil has attracted keen interest due to its well-documented cardioprotective actions both in vivo and in vitro. A number of studies in animal models of ischemia/reperfusion indicate that nicorandil has cardioprotective effects, as evidenced by the preservation of cardiac function or infarct size reduction (2–4). Meanwhile, studies in patients undergoing percutaneous transluminal coronary angioplasty have shown that the administration of nicorandil reduces ST-segment elevation during ischemia (5). Similar beneficial effects of nicorandil in patients undergoing coronary bypass surgery have been described as well (6). The drug also improved the results of exercise tolerance tests relative to placebo in early randomized, double blind, placebo-controlled trials (22). In randomized, double-blind comparative studies in patients with angina pectoris, nicorandil has demonstrated equivalent efficacy, as measured by exercise tolerance testing, to isosorbide dinitrate and mononitrate, metoprolol, propranolol, atenolol, diltiazem, amlodipine and nifedipine (23,24). Moreover, adjunctive administration of nicorandil can improve regional left ventricular wall motion in patients with acute myocardial infarction, and result in better clinical outcomes as assessed by the incidence of malignant arrhythmia or congestive heart failure (7,8).

We have previously reported that the mitoK$_{ATP}$ opening plays a major role in cardioprotection afforded by nicorandil (10). We and other investigators have suggested a paradigm whereby activation of mitoK$_{ATP}$ channels is a significant mechanism of preconditioning-associated cytoprotection (9,25). Diazoxide, the archetypical agonist of mitoK$_{ATP}$ channels, has recently been found to suppress apoptosis (11). Thus, there is good reason to wonder whether nicorandil may likewise function as an inhibition of apoptosis, a property, which might rationalize its cardioprotective effects.

More recently, large-scale randomized clinical trial has been started to test the effects of nicorandil on the incidence of cardiovascular events in patients with chronic effort angina (Impact of Nicorandil in Angina: IONA study) (26). This study is currently underway, but preliminary data presented at the American Heart Association Scientific Sessions in 2001 demonstrated that nicorandil was associated with a significant risk reduction in coronary heart disease death, non–fatal myocardial infarction and unplanned hospitalization. Despite those remarkable effects against coronary heart disease and widespread use of the drug in Japan and Europe, nicorandil is not presently available in the U.S. As a result of this clinical trial as well as a number of experimental studies including ours, the status of this drug in the U.S. might have to be reconsidered.

MitoK$_{ATP}$ channels and apoptosis. Apoptosis is a tightly regulated mode of cell death, which has been revealed to play a role in the pathogenesis of a number of human diseases. It accounts for at least part of cell death or functional organ failure in acute myocardial infarction or congestive heart failure (12). We have discovered that the activation of mitoK$_{ATP}$ channels inhibits oxidative stress-induced apoptosis in isolated cardiac myocytes (11). Unfortunately, the precise molecular mechanisms by which mitoK$_{ATP}$ channel opening protects against apoptosis remain elusive. However, as evidenced in this and our previous study (11), mitoK$_{ATP}$ acting drugs not only suppress nuclear breakdown, which is a terminal phenotype in the entire apoptosis pathway, but also inhibit early apoptotic events, such as cytochrome *c* release or $\Delta\Psi_m$ depolarization. The way nicorandil inhibits $\Delta\Psi_m$ loss resembles that of diazoxide (11); nicorandil not only decreased the number of cells undergoing dissipation of $\Delta\Psi_m$, but also delayed the onset of $\Delta\Psi_m$ loss, whereas it did not change the duration of $\Delta\Psi_m$ loss in the minority of cells that were not protected. These observations suggest that nicorandil could modulate the initiation process of $\Delta\Psi_m$ loss, but not the dissipating process itself.

We have more recently found that the activation of mitoK$_{ATP}$ channels prevented calcium overload in mitochondrial matrix induced by metabolic inhibition (27). Mitochondrial matrix calcium overload is a well-documented trigger of PTP (28). Therefore, the prevention of PTP might be a functional consequence of mitoK$_{ATP}$ channel activation. Supporting this hypothesis, we demonstrated that nicorandil prevented the opening of PTP induced by H_2O_2 using a fluorescent PTP indicator, calcein (Fig. 5I). The prevention of PTP may be a critical mecha-

nism by which mitoK_{ATP} channel opening elicits preservation of mitochondrial integrity and consequent protection of cellular function.

Clinical implications. Apoptotic cell death is observed in pathological situations of the heart such as acute myocardial infarction (29) or congestive heart failure (30). Since cardiac myocytes are terminally differentiated and are refractory to cell cycle entry, apoptotic cell death in the myocardium likely result in a cumulative decrease in cell number and progressive functional loss (12). Therefore, understanding how cardiac myocytes undergo apoptosis under a variety of cellular stress is of primary importance for developing rational therapeutic strategies that may prevent myocyte death after injury or disease. In this study, we demonstrate the antiapoptotic property of a clinically used antianginal drug, nicorandil. Recognition of nicorandil's antiapoptotic effect suggests that this agent may have potential clinical benefit not only in ischemic syndromes, but also in the broad category of cardiovascular diseases in which apoptosis is involved.

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