N-Hydroxy-pyrroline Modification of Verapamil Exhibits Antioxidant Protection of the Heart against Ischemia/Reperfusion-Induced Cardiac Dysfunction without Compromising Its Calcium Antagonistic Activity

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ABSTRACT

Any clinical intervention (e.g., coronary angioplasty, thrombolysis) used to reintroduce blood flow to an ischemic region of the myocardium is accompanied by a complex enzymatic cascade of reactions resulting in severe injury to the heart, termed myocardial ischemia/reperfusion (I/R) injury. In this study, we evaluated the ability of H-3010 (1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole-3-carboxylic acid (2-(3,4-dimethoxyphenyl)-5-(2-(3,4-dimethoxyphenyl)-ethyl)-methylamino)-2-isopropylpentyl)-amide), a pyrroline modification of verapamil (2-(3,4-dimethoxyphenyl)-5-(2-(3,4-dimethoxyphenyl)-ethyl)-methyl-amino)-2-(1-methyllyl)pentanenitrile), to protect the heart against I/R-mediated injury. Isolated perfused rat hearts pretreated with verapamil and H-3010 were subjected to 30 min of global no-flow ischemia followed by 45 min of reperfusion. The recovery (expressed as a percentage of preischemic baseline) in contractile function (left ventricular developed pressure) of hearts subjected to I/R was significantly higher in hearts treated with H-3010 at 5 μM (51.0 ± 6.4%) as well as at 50 μM (75.1 ± 7.4%) as compared with verapamil at 5 μM (32.2 ± 3.7%) or untreated control hearts (18.1 ± 2.8%). Creatine kinase release was significantly attenuated in hearts treated with H-3010 (45.7 ± 4.5 U/liter) as compared with untreated controls (131.5 ± 6.4 U/liter). Similar trends were also observed for lactate dehydrogenase release as well. A marked reduction in percent area of infarction was observed in the H-3010 group (11.7 ± 1.6%) compared with verapamil (25.1 ± 2.9%) and control (41.3 ± 1.9%) groups. Additional in vitro studies showed a marked decrease in reactive oxygen species generation with H-3010. In conclusion, our data clearly demonstrated that the verapamil derivative, H-3010, significantly decreased I/R-induced cardiac dysfunction. This can be attributed to the combined benefits of the pyrroline moiety (antioxidant) and the parent verapamil component (antiarrhythmic) in the protection of the heart from I/R-induced injury.

Verapamil is a class IV antiarrhythmic agent and a potent calcium channel blocker. It is used in the treatment of hypertension, angina pectoris, and atrial fibrillation. Verapamil’s primary mechanism of action is via L-type calcium channel blockade, which prevents excessive calcium influx into cardiomyocytes as well as smooth muscle cells of the coronary vasculature. Calcium channel blockade also occurs in cells of the specialized sinoatrial and atrioventricular nodes and is responsible for verapamil’s negative chronotropic effects. Furthermore, prevention of calcium influx into the cells of the myocardium accounts for verapamil’s pronounced negative inotropic effects. In addition to its beneficial effects in the treatment of hypertension and atrial arrhythmias, verapamil...
has also been reported to provide modest protection against myocardial ischemic injury (da Luz et al., 1980; Kloner and Braunwald, 1987).

Although verapamil’s protective mechanisms against ischemia/reperfusion (I/R)-mediated injury is not well understood, suggested mechanisms for its protective role against I/R-mediated injury include its significant negative inotropic effects, which prevents cellular calcium overload, thereby inhibiting the activation of many proteins that contribute to I/R injury (Miller and Stoclet, 1985). Calcium overload contributes to I/R injury through a number of mechanisms, including the promotion of reactive oxygen species at the onset of reperfusion. Calcium overload also elicits a number of other detrimental effects on the functional recovery of the heart after I/R injury; thus, some studies have suggested that its attenuation with the pretreatment of verapamil may help to mitigate postreperfusion myocardial damage (Przyklenk and Kloner, 1988; Przyklenk et al., 1989). In addition, the blockage of calcium influx into the smooth muscle cells of the coronary vasculature prevents excessive vasoconstriction after I/R injury. This permits adequate blood flow to the coronary arteries supplying the myocardium and thus improves collateral blood flow (Gerritsen et al., 1987). Verapamil has also been shown to reduce ischemia-induced conduction delay (Nakaya, 1981).

Although the etiology of I/R injury is complex, it has been shown that the generation of reactive oxygen species (ROS) plays a critical role in the cellular oxidative damage that occurs during I/R injury (Ambrosio et al., 1993; Griendling and FitzGerald, 2003). A number of studies have measured the profound generation of free radicals that occurs during I/R injury (Arroyo et al., 1987; Garlick et al., 1987; Bolli et al., 1988) and have found that ROS generation peaks immediately after the onset of aerobic reperfusion but can persist for as long as 3 h in some experimental models (Bolli et al., 1988). ROS produce detrimental effects on myocardial cellular membrane proteins, cellular DNA, and mitochondria of cardiac cells. ROS also have the ability to induce cellular apoptosis and necrosis, which contribute to the overall cardiac dysfunction after I/R injury.

Because free radical scavengers (e.g., antioxidants) have the ability to protect the heart from oxidative damage resulting from the formation of ROS during I/R injury (Burton, 1985; Ambrosio et al., 1987; Menasche et al., 1987), we hypothesized that the addition of an antioxidant moiety to the verapamil molecule would provide an additional benefit of scavenging toxic free radicals that are formed at the onset of reperfusion. We modified the verapamil molecule with a heterocyclic nitroxide-precursor, 2,2,5,5-tetramethylpyrroline group in H-3010 (Fig. 1). The nitroxide precursor group transforms into its nitroxide form in tissue and is thereby able to protect cells and tissues from extra- and intracellular oxidative damage (Krishna et al., 1996).

Thus, the new verapamil derivative with its nitroxide precursor, hereafter referred to as H-3010 (Fig. 1), was hypothesized to exhibit the combined benefits of both verapamil and the antioxidant nitroxide in the prevention of I/R injury. We evaluated the cardioprotective ability of H-3010 in comparison with that of verapamil in an isolated perfused heart model. The results demonstrated that H-3010 significantly mitigated I/R-induced cardiac dysfunction by protecting the heart from I/R injury through its antiarrhythmic and antioxidant properties.

Materials and Methods

Chemicals. Verapamil and H-3010 were synthesized as reported (B. Bognár, R. Mandal, M. Khan, T. Kállai, O. H. Hankovsky, P. Kuppusamy, and K. Hideg, manuscript in preparation). Stock solutions of verapamil and its analogs were freshly prepared in dimethyl sulfoxide. Superoxide dismutase (SOD), dihydroethidium (DHE), 5,4-aminopyridine, insulin, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were analytical grade or higher purchased from Sigma-Aldrich, unless otherwise noted.

Isolated Heart Preparation. The experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of Ohio State University and conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23). All hearts were perfused and tested using a modified Langendorff isolated heart apparatus set-up. Male Sprague-Dawley rats (350–400 g) were anesthetized i.p. with 60 mg/kg Nembutal sodium pentobarbital and heparinized with 500 IU/kg heparin. Access to the heart was gained surgically via bilateral midaxial thoracotomy. Hearts were then rapidly excised and removed from the thoracic cavity and placed into ice-cold Krebs-Henseleit buffer to arrest residual contractions. The aorta was subsequently cannulated to the perfusion apparatus. Hearts were immediately retrogradely perfused through the aorta with a modified Krebs-Henseleit buffer containing the following: 120 mM NaCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 1.2 mM KHPO₄, 1.2 mM CaCl₂, and 11 mM glucose. The perfusion solution was maintained at 37°C by a heated water jacket. Carbogen (95% oxygen, 5% carbon dioxide) was continuously bubbled throughout the perfusate to ensure oxygen saturation of the perfusate throughout the course of the experiment.
Myocardial ischemia from the time of occlusion of the heart from the thoracic cavity to the reintroduction of aerobic perfusion was limited to no more than 30 s to prevent any ischemic preconditioning.

A small fluid-filled latex balloon was inserted into the left ventricle via the left atrium. The balloon was inflated with water to mimic a preload with an end diastolic pressure of 8 to 12 mm Hg. Analog input of pressure fluctuations within the balloon, as a result of left ventricular contractions, was converted to a digital signal using a pressure transducer. The hemodynamic data were monitored and recorded using data acquisition software (PC PowerLab with Chart 5 software; ADI Instruments, Colorado Springs, CO). A single in-line flowmeter probe was used to measure the volume of perfusate delivered to the heart (Transonic System, Ithaca, NY). Hemodynamic parameters including left ventricular developed pressure (LVDP) (calculated as the difference between left ventricular systolic and diastolic pressures), rate pressure product (RPP) (calculated as heart rate × LVDP), and coronary flow (CF) were continuously measured and recorded.

**I/R Experimental Protocol.** In all the experiments, isolated hearts were subjected to 15 min of perfusion for stabilization of hemodynamic parameters. In treated groups, drug was administered via a side-arm infusion at various concentrations for 1 min at a controlled infusion rate of 1 ml/min using an infusion apparatus (Harvard Apparatus, Holliston, MA). Immediately after drug infusion, a global no-flow ischemia was induced using an overhead shut-off valve and was allowed to persist for 30 min. Aerobic perfusion was then subsequently reintroduced, and hemodynamic data were obtained for 45 min into reperfusion. Temperature was maintained at 37°C throughout the course of the experiment.

**LDH and CK Assay.** Myocardial tissue damage was assessed by determining the amount of LDH and CK in the coronary effluents collected both before ischemia and during reperfusion. The level of LDH and CK in the coronary effluents was determined using commercially available kits: LDH Assay Kit (Sigma Diagnostics) and CK Assay Kit (Catachem, Bridgeport, CT). The rate of change in absorbance of reduced nicotinamide adenine dinucleotide (NADH) was determined by measuring at 340 nm for 5 min at 25°C on a Varian Cary 50 spectrophotometer (Varian, Inc, Palo Alto, CA). The enzyme levels were calculated using the molar extinction coefficient of reduced nicotinamide adenine dinucleotide (ε = 6.22 M/cm).

**Measurement of Myocardial Infarct Size.** Myocardial infarct size was measured using triphenyltetrazolium chloride (TTC) staining. TTC crosses the cell membrane and is readily oxidized by a number of dehydrogenases and cofactors present in viable tissue to form a dark red formazan pigment. The nonviable necrotic tissue, with its nitroxide metabolite is paramagnetic and hence can be detected after 7 min of superfusion, based on time to reach steady-state signal.

**TTC was used to elicit IC₅₀, at voltages from -40 to +50 mV (10-mV increments). IC₅₀ recordings began 3 min after patch rupture and recordings with verapamil and analogs (5 μM) were obtained after 7 min of superfusion, based on time to reach steady-state inhibition in initial pilot studies.**

**Measurement of Superoxide Generation.** Superoxide generation in the myocardial tissue subjected to I/R was determined using DHE fluorescence (Miller et al., 1998). The cell-permeable DHE is oxidized to fluorescent hydroxyethidium by superoxide, which is then intercalated into DNA. Since it has been reported that the superoxide generation peaks during the first 15 min of reperfusion, we measured the DHE fluorescence at 15 min of reperfusion. After 15 min of reperfusion, hearts were placed in cold PBS buffer and then embedded in ornithine carbamyl transferase for cryosectioning. The frozen segments were cut into 5-μm-thick transverse sections that were then placed on glass slides. DHE (10 μM) was topically applied to each tissue section. The slides were incubated in a light-protected chamber at 37°C for 30 min. Images of the tissue sections were obtained using a fluorescence microscope (Nikon) with a rhodamine filter. Fluorescence intensity, which positively correlates with the amount of superoxide generation, was determined in the myocardial tissue using MetaMorph software.

**Electron Paramagnetic Resonance Spectroscopy.** Electron paramagnetic resonance (EPR) spectroscopy was used to quantitatively determine the relative superoxide scavenging ability of verapamil and H-3010 in vitro. Superoxide radicals were generated using xanthine (0.2 mM) and xanthine oxidase (0.02 U/ml) in PBS solution at 37°C, pH 7.4. Also present in the reaction mixture were the EPR spin-trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO; 1 mM) and diethylenetriaminepentaacetate (0.1 mM). Superoxide generated by the xanthine-xanthine oxidase system was captured by DEPMPO to form a stable DEPMPO adduct, which was then subsequently detected via X-band (9.8 GHz) EPR spectroscopy 10 min after the onset of the reaction. In separate experimental groups, verapamil (1 mM) and H-3010 (1 mM) were added to reaction mixtures, and the attenuation of EPR signal from the DEPMPO adduct was observed and recorded.

**Data Analysis.** The statistical significance of the results was evaluated using analysis of variance and standard Student’s t test analysis. All values were expressed as mean ± S.D. Consideration for significance was such that p < 0.05.

**Results**

**Redox-Cycling and Radical-Scavenging Properties of H-3010 in Vitro.** The pyrroline-N-hydroxyl group of H-3010, introduced as a structural modification to verapamil, has been designed to confer antioxidant capabilities to the compound. We first wanted to establish the redox conversion properties of H-3010 in biological milieu and study its free radical-scavenging properties. In biological systems, as well as in aerobic solutions, H-3010 is expected to undergo a one-electron oxidation to its corresponding nitroxide form and establish equilibrium between its hydroxylamine and nitroxide forms in solution (Fig. 1). Unlike H-3010’s parent form, its nitroxide metabolite is paramagnetic and hence can...
be conveniently measured by EPR spectrometry. Figure 2A shows the EPR spectrum obtained from a 10 μM solution of H-3010 in aerated PBS, pH 7.4. A similar spectrum was obtained from H-3010 (10 μM) incubated with freshly isolated canine cardiomyocytes (5 × 10^6 cells/ml) for 30 min under aerated conditions (Fig. 2B). The three-line (triplet) spectrum is characteristic of nitroxide radicals as verified by an authentic nitroxide spectrum (Fig. 2C). The concentration of the nitroxide was approximately 0.4 μM, corresponding to 4% H-3010. The signal intensity of H-3010 in the presence of cells was comparable with that in cell-free solution, suggesting the occurrence of redox cycling and establishment of equilibrium between the species.

Because H-3010 undergoes redox-cycling in solutions to maintain a steady level of nitroxide, and nitroxides are well known to be scavengers of oxygen radicals, we next determined the superoxide radical-scavenging ability of H-3010 by using spin-trapping EPR spectroscopy. Superoxide radicals, generated using xanthine/xanthine-oxidase system, were detected as DEPMPO adducts. Verapamil or H-3010 was used to compete with 1 mM DEPMPO for the superoxide ions. As shown in Fig. 3, the SOD-inhibitable superoxide accumulation, measured as DEPMPO-OOH signal intensity, was significantly less in the presence of as low as 50 μM H-3010 compared with control or verapamil (1 mM), suggesting that H-3010 can scavenge superoxide radicals with a rate comparable with that of a DEPMPO reaction with superoxide.

**H-3010 Blocks Calcium Channel as Effectively as Verapamil.** To determine whether the calcium channel-blocking property of verapamil was retained by the structurally modified compound, we studied the effect of H-3010 on the inhibition of calcium transport in freshly isolated canine left ventricular myocytes using the whole-cell patch-clamp technique. The results demonstrated that H-3010 at 5 μM concentration significantly (p < 0.01) inhibited the calcium current (I_{Ca,L}) in the ventricular myocytes (Fig. 4). Furthermore, the calcium channel-blocking efficacy of H-3010 was comparable with that of verapamil at the same concentration. The results clearly demonstrated that the calcium channel-blocking property of verapamil was not significantly compromised by the structural modification introduced in H-3010.

**Effect of H-3010 on the Contractile Function of Heart.** The effect of H-3010 on the contractile function of nonischemic hearts was evaluated. The drugs (verapamil and H-3010) were delivered to the heart via a side-arm infusion for 1 min, and the contractile functions were monitored for 30 min.

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Fig. 2. EPR spectra showing the metabolic conversion of H-3010 to nitroxide in cells. A, H-3010 (10 μM) incubated in aerated PBS for 30 min. B, canine cardiomyocytes (5 × 10^6 cells/ml) incubated with H-3010 (10 μM) for 30 min. C, authentic nitroxide (tempol, 0.5 μM) in aerated PBS. The concentration of nitroxide, as estimated from spectrum (B), was 0.32 μM or 3.2% of H-3010.

Fig. 3. Superoxide-scavenging ability of H-3010. A continuous flux of superoxide was generated by xanthine (0.2 mM)/xanthine oxidase (0.02 U/ml) and detected as DEPMPO-superoxide adduct (DEPMPO-OOH) by X-band EPR spectroscopy. The measurement was done at 6 min after mixing the superoxide-generating system with DEPMPO (1 mM) in the presence of verapamil (1 mM), H-3010 at concentrations as indicated, or SOD (500 U/ml). A, representative EPR spectra acquired at 6 min after adding the reagents are presented. The spectra show an admixture of DEPMPO-OOH (multiplet) and the one-electron oxidized form of H-3010 (nitroxide, ~3% of H-3010, triplet). The nitroxide spectra are displayed amplitude-truncated at fields indicated by an asterisk. B, amounts of SOD-inhibitable superoxide, quantified as DEPMPO-superoxide adduct in the presence of verapamil or different concentrations of H-3010, are expressed as a percentage of control (X/XO only). Data represent mean ± S.D. (n = 3). *, p < 0.05 versus control.
min, while the heart was continuously perfused with drug-free perfusate. The results, as shown in Fig. 5, showed a sharp drop in LVDP with the infusion of verapamil (5 μM), which was attributed to verapamil’s negative inotropic effect. A similar drop was observed with H-3010 at 5 or 50 μM concentrations; however, the effect was significantly less at 5 μM. The heart rate (HR) data showed a negative chronotropic effect induced by verapamil (5 μM), whereas H-3010 (5 μM) showed a negligibly small influence on the heart rate. In all cases, both LVDP and HR returned to their preinfusion baseline values within 30 min, indicating that neither verapamil nor H-3010 had any deleterious effect on the postinfusion cardiac functions.

**H-3010 Decreases I/R-Induced Cardiac Contractile Dysfunction.** Hearts were subjected to 15 min of perfusion for stabilization of hemodynamic parameters followed by 30 min of no-flow global ischemia. Aerobic perfusion was then subsequently reintroduced. Verapamil or H-3010 was delivered to the heart via a side-arm infusion for 1 min before the induction of global ischemia. The optimal concentration of the verapamil that could be administered without loss of functional recovery was 5 μM, whereas that of H-3010 was 50 μM or higher without significant loss of cardiac function. Therefore, we used the 5 μM dose of verapamil, and we also used 5 μM as well as 50 μM concentrations of H-3010 to study the dose-dependent response. Hemodynamic and contractile data were obtained for 45 min into reperfusion. LVDP, RPP, and CF values were obtained and expressed as percentages of their preischemic baseline values: CF, 17 ± 3 ml/min; LVDP, 110 ± 15 mm Hg; and HR, 290 ± 35 beats per min. Hearts treated with the verapamil showed a significant (p < 0.05) improvement in the recovery of contractile functions as compared with control hearts (Fig. 6). Furthermore,

**Fig. 4.** Verapamil and H-3010 inhibit L-type calcium current (I_{Ca-L}) in isolated ventricular myocytes. A, representative peak I_{Ca-L} recorded in response to a depolarizing step to 0 mV from a holding potential of −50 mV; recorded at baseline and after superfusion with the drugs in myocytes of similar capacitances. The arrow to the left of the raw traces indicates the zero current line. B, data (mean ± S.D.) peak I_{Ca-L}. To avoid potential variability due to cell size, data were normalized to cell size and measured as cell capacitance. *, p < 0.01 versus baseline. The results indicate that H-3010 is as effective as verapamil in inhibiting L-type calcium current in ventricular cardiomyocytes.

**Fig. 5.** Effect of verapamil and H-3010 on the contractile functions of perfused hearts. Hearts were infused with verapamil or H-3010 for 1 min, whereas the HR and developed pressure (LVDP) were measured continuously for 30 min postinfusion. Values are expressed as mean ± S.D. (n = 3). *, p < 0.01 as compared with verapamil or H-3010 at 50 μM; **, p < 0.01 as compared with verapamil. Verapamil or H-3010 induced a sudden drop in HR and LVDP that recovered on subsequent perfusion without the drug. The results reveal that H-3010 induces significantly less inotropic and chronotropic effects compared with verapamil.

**Fig. 6.** Pretreatment of hearts with verapamil or H-3010 attenuated the I/R-induced cardiac dysfunction. Hearts were infused with verapamil (5 μM), H-3010 (5 μM), or H-3010 (50 μM) for 1 min before 30 min of global ischemia immediately followed by 45 min of reperfusion at 37°C. Data show the recovery of CF (A), LVDP (B), and RPP (C) at the end of 45 min of reperfusion. The results are expressed as percentages (mean ± S.D., n = 4) of preischemic baseline values. *, p < 0.05 versus control; **, p < 0.05 versus control or verapamil group. ***, p < 0.01 versus control, verapamil, or H-3010 (5 μM) group.
the H-3010 showed a significantly \((p < 0.01)\) better recovery as compared with control as well as verapamil-treated hearts. H-3010 showed significant contractile recovery at both 5 and 50 \(\mu M\) concentrations beyond that of verapamil-treated hearts. The results demonstrated that H-3010 significantly reduced I/R-mediated cardiac dysfunction as compared with verapamil.

**H-3010 Inhibits I/R-Induced CK and LDH Release in the Heart.** CK and LDH are key enzymes found in myocardial tissue, and their release into the coronary effluent is indicative of myocardial tissue damage due to I/R injury. Both CK and LDH release was found to peak at approximately 15 min into reperfusion (Khan et al., 2006). Thus, all collections of the coronary effluent for CK/LDH analysis were obtained at 15 min after the onset of reperfusion. Untreated (control) hearts showed high CK activity in coronary effluent collected at 15 min into reperfusion (Fig. 7A). The CK activity was significantly diminished in verapamil- or H-3010-treated hearts as compared with untreated hearts. Similar results were observed for LDH release into the coronary effluent (Fig. 7B). LDH activity was high in the effluents of untreated control hearts and significantly decreased in hearts treated with verapamil or H-3010. Furthermore, the H-3010 showed significantly decreased LDH activity as compared with verapamil. Both CK and LDH release in hearts treated with H-3010 were greatly reduced beyond that of control hearts as well as hearts treated with verapamil, suggesting that H-3010 provided additional protection against I/R-mediated injury beyond that of verapamil.

**H-3010 Attenuates I/R-Induced Myocardial Infarction.** Myocardial infarct size was measured using TTC staining. Hearts were subjected to 30 min of no-flow global ischemia at 37°C followed by 120 min of reperfusion to allow greater accuracy and optimal contrast between the necrotic tissue area and the area at risk. Left ventricular infarction, expressed as a percentage of the total area at risk, was found to be significantly decreased in hearts treated with verapamil and H-3010 (Fig. 8). H-3010 showed a significant reduction in infarct size beyond that of the verapamil.

**H-3010 Suppresses I/R-Induced Superoxide in the Heart.** The superoxide generation in hearts subjected to I/R was measured by hydroethidium (HE) fluorescence. Transverse sections of the heart were stained with DHE, which was converted to fluorescent HE by superoxide. Since it was previously reported that superoxide generation occurs in the reperfused hearts typically during the first 15 min of reperfusion, we performed the HE fluorescence measurements at 15 min of reperfusion. The fluorescence intensity of HE was significantly higher in untreated (control) hearts subjected to 30 min of ischemia followed by 15 min of reperfusion (Fig. 9). In contrast, the fluorescence intensity in hearts pretreated with H-3010 was significantly \((p < 0.05)\) attenuated as compared with control. Hearts treated with verapamil showed no significant difference as compared with control. In a separate set of experiments, hearts were infused with tempol (100 \(\mu M\)), a known free radical scavenger, for 1 min before ischemia. The results indicated that tempol significantly attenuated the I/R-induced superoxide generation. The results further suggested that H-3010 significantly suppressed the superoxide level in the reperfused hearts.

**Discussion**

The results of the present study clearly demonstrated that the modified verapamil compound, H-3010, significantly improved the recovery and contractile function of hearts subjected to ischemia/reperfusion injury. Overall, the beneficial effect of H-3010 was significantly higher than that observed with verapamil. Some previous studies have investigated the...
effects of the coadministration of superoxide scavengers with calcium channel antagonists and have reported some improvement in postsischemic cardiac function (Villari et al., 1993). The novelty of this study lies in the direct chemical addition of an antioxidant pronitroxyl group to the verapamil molecule. Thus, H-3010, as a single compound affords a multidimensional strategy to attenuate both the ischemic as well as the oxidative stress endured by the heart during I/R. Furthermore, another advantage of having the antioxidant moiety directly attached to the verapamil compound allows H-3010 to deliver a degree of site-specific antioxidant action as a result of the avid binding of H-3010 to calcium channels throughout the ischemic heart where oxidative damage is of particular concern. H-3010 can thereby exert a site-specific effect offering a therapeutic strategy that is more efficacious than the administration of verapamil and a superoxide scavenging nitroxide separately.

H-3010 was capable of redox cycling and superoxide scavenging. Superoxide generation in hearts was determined by hydrothidine fluorescence. Unfixed cryosections of hearts after reperfusion (15 min) were incubated with dihydroethidium (10 μM) at 37°C under dark for 30 min and determined by fluorescence microscopy. A, representative photographs from triplicate experiments are shown. B, mean fluorescence intensity after deducting the baseline values of preischemic control hearts. Data are represented as mean ± S.D. *p < 0.05 versus control (I/R).

**Fig. 9.** Effect of verapamil or H-3010 on I/R-induced superoxide generation. Superoxide generation in hearts was determined by hydrothidine fluorescence. Unfixed cryosections of hearts after reperfusion (15 min) were incubated with dihydroethidium (10 μM) at 37°C under dark for 30 min and determined by fluorescence microscopy. A, representative photographs from triplicate experiments are shown. B, mean fluorescence intensity after deducting the baseline values of preischemic control hearts. Data are represented as mean ± S.D. *p < 0.05 versus control (I/R).

**Cardioprotection by a Verapamil Derivative**

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al., 2000; Li et al., 2002), and ionizing irradiation (Hahn et al., 1992). This antioxidant activity can be attributed to the scavenging of oxygen-centered radicals (Shankar et al., 2000; Goldstein et al., 2006). In addition to direct superoxide scavenging, nitroxides have also been shown to attenuate the formation of other reactive oxygen and nitrogen species. Nitroxides have been reported to prevent the formation of OH-free radicals from H₂O₂ by not only superoxide scavenging but also by superoxide-independent mechanisms (Samuni et al., 1991). The stable nitroxide tempol was shown to reoxidize Fe²⁺ to Fe³⁺, thus preventing its participation in OH- generation via the Fenton reaction (Zhang et al., 1998). It has also been reported that nitroxides have ability to prevent the propagation of free radical chain reactions by secondary radicals such as R, RO-, and ROO• (Gerlock et al., 1990; Shankar et al., 2000).

Structure-activity relationship studies of verapamil analogs have shown that the aromatic rings, nitrite group, and tertiary amine are critical to its function, whereas the isopropyl group and the substituents of aminoethyl aromatic ring are less important for its calcium antagonistic activity (Mannhold et al., 1981, 1987). H-3010 contains a sterically hindered N-hydroxy-pyrroline extension at the nitrite terminal of verapamil (Fig. 1). The N-hydroxy-pyrrolines and their one-electron oxidized metabolites (nitroxides) are known for their nonenzymatic multifunctional antioxidant properties (Samuni et al., 2004). The sterically hindered hydroxylamines can also act as proton-donating antioxidants (Krishna et al., 1998). The in vitro results, shown in Figs. 2 and 3, clearly established that the retention of redox-cycling and superoxide-scavenging capabilities of the pyrroline group in H-3010. The N-hydroxy compound is in equilibrium with 4% nitroxide in the presence of cells under aerated conditions. Thus, the observed superoxide scavenging of H-3010 is attributed to both the reduced and oxidized forms of the pyrroline group.

Whole-cell patch-clamp experiments showed a significant inhibition of calcium current in ventricular myocytes treated with H-3010 (Fig. 4). The inhibition by H-3010 was comparable with that of verapamil at the same concentration. Thus, the calcium channel-blocking ability of verapamil was not compromised by the structural modification. Although the calcium channel-blocking efficacy of H-3010 in isolated cardiomyocytes was intact, there were substantial differences in the inotropic/chronotropic effects as compared with verapamil. At the 5 μM dose, H-3010 showed negligible chronotropic effect as compared with a ~80% decrease in heart rate induced by verapamil (Fig. 5). Even a 10-fold increase in the dose of H-3010 was not sufficient to match the effect of verapamil on the initial heart rate. H-3010 also showed a similar difference in the inotropic effect as compared with verapamil. It is interesting to note that, although the calcium channel-blocking ability of H-3010 was comparable with that of verapamil (Fig. 4), the negative chronotropic and inotropic effects of H-3010 were significantly less compared with those of verapamil. This difference could be attributed to the protective effect of the N-hydroxy-pyrroline moiety. Although additional work is required to establish the actual mechanism, the less pronounced chronotropic/inotropic effects of H-3010 on the heart enabled us to study the protective effect of H-3010 at a higher dose (50 μM). In contrast, hearts
treated with 50 μM verapamil showed toxicity due to its more pronounced negative chronotropic/inoetric effects.

H-3010 exhibited a dose-dependent increase in the recovery of posts ischemic contractility. The recovery of contractility was also significantly higher than verapamil at the same dose. In addition, the recovery in flow observed with the treatment of verapamil in agreement with previously published reports (Alanen et al., 1984). The CK and LDH activity in the coronary effluents showed a significant reduction of cellular leak of these enzymes as compared with verapamil; however, the effect seemed to be further attenuated at a higher dose of H-3010. The extent of infarction developed throughout the course of prolonged reperfusion was also reflected by the net effects of the contractile, hemodynamic, and biochemical parameters in the hearts. Taken together, the ex vivo results clearly demonstrated that H-3010 was more effective than verapamil in the restoration of cardiac function of hearts subjected to I/R-induced injury.

We had previously reported the cardioprotective role of pyrroline modifications against I/R-induced injury and contractile dysfunction (Shankar et al., 2000). We recently reported that the anti-ischemic drug trimetazidine showed significant cardioprotection against I/R-induced injury when the molecule was modified with a number of structurally similar pyrroline antioxidant moieties (Kutala et al., 2006). Interestingly, we had found that the pyrroline moieties most successful in minimizing I/R-induced injury were those containing a secondary amine (N-H) group as opposed to structurally similar moieties containing nitroxides (NO) or hydroxylamines (NOH) as the primary reactive group. Unlike the N-hydroxy function, the secondary amine group has to undergo two-electron oxidation to generate nitroxide. However, the amino form itself has been shown to scavenge oxygen free radicals, thereby providing antioxidant protection during reperfusion. The protective effects of the trimetazidine derivatives appear to stem from multiple mechanisms: radical-scavenging property (antioxidant activity) of the nitroxide precursor, prosurvival Akt activity, and the anti-ischemic effect of trimetazidine itself (Kutala et al., 2006).

Cardiac anomalies such as ventricular tachycardia or ventricular fibrillation are believed to have their etiology in part in the oxidative stress induced by ischemia/reperfusion (Woodward and Zakaria, 1985; Hearse and Tosaki, 1987; Manning et al., 1988). H-3010, with its protrinitoxide and verapamil groups, may possess the ability to attenuate the occurrence of reperfusion arrhythmias via multiple mechanisms. Free radical scavengers such as nitroxides have been shown to ameliorate the severity and extent of cardiac arrhythmias indirectly by scavenging free radicals that would otherwise contribute to reperfusion arrhythmias (Gelvan et al., 1991). For example, 2,2,6,6-tetramethylpiperidine-N-oxide, a stable nitroxide, was shown to protect perfused rat hearts against free radical-induced arrhythmia, but it did not prevent arrhythmic occurrence via its negative chronotropic effects or any other direct suppressive mechanism of cardiac arrhythmia. In fact, the study demonstrated that TEMPO's antiarrhythmic effects occur by its presence in the first 1 min of reperfusion when free radical generation is at its peak, indicating that its antiarrhythmic effects are due to its antioxidant properties (Gelvan et al., 1991). It has been suggested that verapamil provides protection against I/R injury, at least in part through this mechanism of negative chronotropy, and thus prevents severe arrhythmias that occur at the onset of reperfusion. Thus, in reference to H-3010, we see that the suppression of reperfusion arrhythmia could be a result of the scavenging of free radicals by the attached N-hydroxy-pyrroline moiety and the negative chronotropic effects resulting from the verapamil portion of H-3010 leading to a reduction in ischemia/reperfusion-induced incidence of abnormal impulse conduction. Hence, we observe the unique ability of H-3010 to exert a multidynamic mechanism by which it can prevent deleterious cardiac arrhythmias following ischemic insult.

In conclusion, the improved efficacy seen with the use of H-3010 in the protection against I/R-mediated contractile dysfunction and tissue injury is possibly attributed to the combined effects of the calcium channel-blocking ability of the verapamil group and its protrinitoxide antioxidant addendum. Multidimensional therapeutic strategies such as those seen with H-3010 can lead to a significant reduction in cardiac dysfunction following ischemia/reperfusion injury and thus vastly improve the chances of cardiac recovery.

References


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