A Pyrroline Derivative of Mexiletine Offers Marked Protection against Ischemia/Reperfusion-Induced Myocardial Contractile Dysfunction

HAIQUAN LI, KAI Y. XU, LAN ZHOU, TAMAS KALAI, JAY L. ZWEIER, KALMAN HIDEG, and PERIANNAN KUPPUSAMY

Division of Cardiology, Department of Medicine, Johns Hopkins University, School of Medicine, Baltimore, Maryland (H.L., K.Y.X., L.Z., J.L.Z., P.K.); and Institute of Organic and Medicinal Chemistry, University of Pécs, Pécs, Hungary (T.K., K.H.)

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A combination of reversible inhibition of Na\(^+\)-ATPase activity during ischemia and site-targeted antioxidative effect upon reperfusion seems to contribute to this cardioprotection.

Cardiac ischemia leading to secondary myocardial infarction is among the most common causes of morbidity and mortality. Chemical or surgical interventions allow the recovery of the ischemic myocardium by restoration of blood flow or reperfusion. This reperfusion, however, is known to be associated with ventricular arrhythmias and myocardial dysfunction that can lead to severe cardiac impairment and cell death (Manning et al., 1984; Pogwizd and Corr, 1986; Forman et al., 1990; Yamada et al., 1990). Reactive oxygen species (ROS) such as superoxide anion (O\(_2^-\)), hydrogen peroxide (H\(_2\)O\(_2\)), hydroxyl radical (\(\cdot\)OH), and singlet oxygen (\(\cdot\)O\(_2\)) have been implicated as important factors in the pathogenesis of cellular injury in the postischemic heart (Zweier et al., 1989; Kukreja et al., 1991; Halliwell et al., 1992; Esteban et al., 1993). These species have been shown to mediate the contractile dysfunction observed during reperfusion and may be implicated in reoxygenation injury (Hearse and Tosaki, 1988; Tosaki et al., 1990; Horton and White, 1995). ROS affect membrane ion exchanges, in particular the transport of cations such as Na\(^+\), K\(^+\), and Ca\(^{2+}\), across cell membranes. Some examples of ROS-induced ion-exchange modifications include the following: a decrease of Ca\(^{2+}\) current in guinea pig ventricular myocytes through a modification of L-type calcium channel (Guerra et al., 1996); a decrease in the activity of membrane transport proteins such as Na\(^+\), K\(^+\)-ATPase in pig cardiomyocytes (Shao et al., 1995); inhibition of the ATP-binding site of sarcolemmal Na\(^+\),K\(^+\)-ATPase (Xu et al., 1997b); inhibition of Na\(^+\),H\(^+\) transport in bovine pulmonary endothelial cells (Cutai and Parks, 1994); a reduction in sarcoplasmic reticulum Ca\(^{2+}\)-ATPase activity (Rowe et al., 1983; Krause et al., 1989; Xu et al., 1997a); a decrease in Ca\(^{2+}\) uptake in isolated sarcoplasmic reticulum (Okabe et al., 1983); inhibition of isolated sarcolemmal Na\(^+\),K\(^+\)-ATPase by MEX-NH; and inhibition of Na\(^+\)-lactate dehydrogenase by MEX-NH.

ABBREVIATIONS: ROS, reactive oxygen species; MEX-NH, 1-(2,6-dimethylphenoxy)-[2-N-(2,5-dihydro-3-methyl-2,2,5,5-tetramethyl-1H-pyrrolo)-aminopropane; MEX-NO, 1-(2,6-dimethylphenoxy)-[2-N-(1-oxyl-2,5-dihydro-3-methyl-2,2,5,5-tetramethyl-1H-pyrrolo)-aminopropane; LDH, lactate dehydrogenase; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; LVDP, left ventricular end-diastolic pressure; MEX, 1-(2,6-dimethylphenoxy)-2-aminopropane; CF, coronary flow; LVSP, left ventricular systolic pressure; HR, heart rate; SL, sarcolemmal; EPR, electron paramagnetic resonance; LVDP, left ventricular developed pressure; RPP, rate-pressure product.
ATPase vesicles by singlet oxygen (Vinnikova et al., 1992) and calcium overloading in isolated rat ventricular myocytes (Josephson et al., 1991).

Over the years, a variety of therapeutic approaches to detoxify these oxidants and protect the myocardium have been investigated. This detoxification can be provided by enzymatic or nonenzymatic mechanisms. Enzymatic antioxidants include superoxide dismutase, catalase, and glutathione peroxidase that catalytically break down oxidants, whereas nonenzymatic antioxidants are primarily reducing agents such as vitamin C and vitamin E that can scavenge oxidants by H atom donation in a stoichiometric manner (Halliwell and Gutteridge, 1989; Tosaki et al., 1993). In most cases the defense provided by the enzymatic and nonenzymatic antioxidants is adequate. However, in acute situations, such as the ischemia/reperfusion-induced oxidative injury, exogenous antioxidants are necessary to minimize the damage. Because the targets and sites of such damage vary depending upon the kind of oxidative insult, successful treatment of the damage requires proper identification of the target sites and appropriate site-targeted antioxidant strategy. Desired features in an effective agent that would protect against ischemia/reperfusion-induced damages to the heart include 1) ability to localize in subcellular compartments, 2) ability to reduce or limit ischemic injury, 3) ability to react and scavenge a wide range of reactive species produced on reperfusion, and 4) ability to be efficiently regenerated to the active form.

Derivatives of sterically hindered five-membered cyclic amines without hydrogen atom at the α-positions (for example, 2,2,5,5-tetramethylpyrrolines) possess all the desired properties and are shown to be efficient antioxidants in a variety of in vitro and in vivo conditions (Hankovszky et al., 1986; Twomey et al., 1997; Krishna et al., 1998). We have recently reported the cardioprotective effect of a pyrrole derivative that showed both antiarrhythmic and antioxidative properties in rat hearts (Shankar et al., 2000). In this article we report the efficacy and mechanism of a new 2,2,5,5-tetramethylpyrrole derivative of mexiletine, MEX-NH, in protecting against ischemia/reperfusion-induced cardiac dysfunction. It is observed that the MEX-NH provides marked protection against ischemia/reperfusion-induced contractile dysfunction in isolated hearts and that the mechanism of protection appears to be a combination of reversible inhibition of Na⁺/K⁺-ATPase activity during ischemia and site-targeted antioxidative effect upon reperfusion.

### Materials and Methods

**Chemicals.** The compounds (Fig. 1) 1-(2,6-dimethylphenoxo)-1-[2-N-(2,5-dihydro-3-methyl-2,2,5,5-tetramethyl-1H-pyrrol)]aminopropane (HO-2434, abbreviated hereafter as MEX-NH) and 1-(2,6-dimethylphenoxo)-1-[2-N-(1-oxyl-2,5-dihydro-3-methyl-2,2,5,5-tetramethyl-1H-pyrrol)]aminopropane (HO-2433, abbreviated hereafter as MEX-NO), were synthesized from a modification of the procedures reported previously (Hankovszky et al., 1986). Complete synthesis and characterization of MEX-NH and MEX-NO and other derivatives will be published. The components of the modified Krebs’ buffer solution, lactate dehydrogenase (LDH) diagnostic kit, and magnesium-ATP were purchased from Sigma (St. Louis, MO). [3H]Ouabain was obtained from American Biotechnology (Picatway, NJ). The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Dojindo Laboratories (Kumamoto, Japan).

**Isolated Heart Perfusion.** Female Sprague-Dawley retired-breeder rats (Harlan Company, Boston, MA) of weight 300 ± 30 g were used. All experiments were carefully conducted in compliance with the National Institutes of Health Guidelines for the Use of Laboratory Animals. After complete anesthesia (65 mg/kg pentobarbital i.p.) the heart was excised and the ascending aorta was rapidly cannulated. Retrograde perfusion was initiated by the method of Langendorf at a constant pressure of 80 mm Hg using modified Krebs-bicarbonate-buffered perfusate containing 17 mM glucose, 120 mM NaCl, 25 mM NaHCO₃, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, and 0.5 mM EDTA. All perfusates were routinely filtered through two 1.2-μm Millipore filters and bubbled with 95% O₂, 5% CO₂ gas mixture at 37°C. A side arm in the perfusion line located just proximal to the aortic cannula allowed infusion of the drug solutions. The drugs were infused at a dilution of 1:20, with respect to the coronary flow, using a Harvard infusion pump (Harvard Apparatus, Holliston, MA). Contractile function of the heart was measured using a fluid-filled latex balloon inserted into the left ventricular cavity through the atrioventricular valve. The balloon was connected via a hydraulic line to a Spectramed P23XL pressure transducer with pressures amplified to a Gould four-channel strip chart recorder as well as to a personal computer equipped with MacLab data acquisition software. The balloon volume was adjusted to achieve an initial left ventricular end-diastolic pressure (LVEDP) of 8 to 12 mm Hg and all subsequent measurements were performed at the same balloon volume. The coronary flow was measured by a T106 Transonic flowmeter just proximal to the aortic cannula.

Studies were performed in three groups as follows: 1) control, 2) MEX-NH (25 μM), and 3) MEX (25 μM). Studies were also performed in additional groups using different dosages of MEX-NH (10, 25, 50, and 100 μM). At least seven hearts were studied per group.

**LDH Assay.** LDH leakage in the reperfused myocardium was assayed from effluents collected at regular intervals during reperfusion. The amount of LDH in the effluent was measured spectrophotometrically using Sigma diagnostics LDH kit reagents, which consisted of 0.2 mg of NADH, 0.1 mol/l phosphate buffer, pH 7.5, and 2.27 mM sodium pyruvate. Effluents were collected from hearts treated with 1) control, 2) MEX, and 3) MEX-NH. Typically, an aliquot containing 1 ml of the effluent was mixed with 0.2 mg of reduced NADH, followed by 1.9 ml of phosphate buffer. After a period of 20 min at 25°C, 0.1 ml of sodium pyruvate solution was added. The mixture was immediately transferred to a 1-ml quartz cuvette and absorbance was measured at 340 nm at 30-s intervals for 3 min using a Hewlett-Packard 8452A diode-array spectrophotometer. The activity of LDH was calculated using standard procedures and expressed as effluent LDH units per milliliter.

**Ischemia/Reperfusion and Drug Infusion Protocol.** After an equilibrium period of 15 min to allow for functional stabilization, baseline values of coronary flow (CF), LVEDP, left ventricular systolic pressure (LVSP), and heart rate (HR) were measured. The hearts were then subjected to a 1-min preconditioning, controlled infu-
sion of drug/saline through a side arm, at 5% of the coronary flow rate, to achieve a final drug concentration of 25 μM in the perfusate. Subsequently, the hearts were subjected to 30 min of global, no-flow ischemia, followed by 45 min of reperfusion without the drug. In some cases during the first 5 min of reperfusion, the hearts were also subjected to a controlled infusion of the drug at the same concentration and flow rate, as in the preischemic state. After the first 5 min, reperfusion was continued with the Krebs’ buffer alone for the remaining 40 min. Serial measurements of cardiac contractile function and coronary flow were performed during the period of reperfusion. Coronary effluents were collected in 2-min intervals up to 10 min and then every 5 min up to 45 min of reperfusion to perform the LDH assay.

Measurement of Cardiac Sarcolemmal (SL) Na⁺,K⁺-ATPase Activity. Cardiac SL Na⁺,K⁺-ATPase was isolated from Sprague-Dawley rat heart muscle using a protocol based on the published methods (Jones, 1988; Watanabe et al., 1988). The enzymatic activity was defined as the strophanthidin-sensitive hydrolysis of magnesium-ATP in the presence of saponin as described previously (Kyte et al., 1987). Briefly, SL Na⁺,K⁺-ATPase (29 μg/ml) was premixed for 12 min at room temperature with saponin (0.5%). The assay reaction was initiated by adding magnesium-ATP (3 mM) and stopped after 30 min at 37°C by adding 0.75 ml of quench solution and 0.02 ml of developer (Kyte, 1971). The color was allowed to develop for 30 min at room temperature, and phosphate was then determined at 700 nm with a spectrophotometer. An incubation mixture without the enzyme served as a blank to correct for the time-dependent evolution of phosphate in strong acid. To have an accurate comparison, cardiac SL vesicles were isolated from individual rat hearts after ischemia/reperfusion. The purified enzyme was preferred instead of whole heart homogenates to have higher specific activity of the Na⁺,K⁺-ATPase enzyme.

Determination of [3H]Ouabain Binding to Cardiac Na⁺,K⁺-ATPase. [3H]Ouabain-labeling reaction was performed with or without 25 μM MEX-NH. The standard reaction contained the Na⁺,K⁺-ATPase (100 μg/ml), magnesium-ATP (3 mM), Na⁺ (100 mM), and [3H]Ouabain (80 nM) in the presence or absence of 100 mM K⁺. The reaction mixture was incubated with [3H]Ouabain for 30 min at 37°C. The reaction was stopped by pelleting the sample at 14,000 rpm for 30 min at room temperature. The pellet was washed three times with buffer, and then dissolved in 100 μl of 10% SDS solution. The radioactivity was determined by a beta-scintillation counter.

Statistical Analysis. Data are presented as mean ± S.E. Comparisons between groups were made by a one-way ANOVA designed for repeated measures. A confidence level of more than 95% was considered statistically significant (P < .05).

Results

Effect of MEX-NH on ‘OH Radicals. MEX-NH is structurally characterized as a derivative of the antiarrhythmic compound MEX with a sterically hindered five-membered cyclic amine. Recently, we have shown that such derivatization in some known antiarrhythmic agents also results in additional or enhanced antioxidative properties in a variety of in vitro and in vivo conditions (Hankovszky et al., 1986; Twomey et al., 1997; Krishna et al., 1998; Shankar et al., 2000). To investigate the antioxidative capability of MEX-NH, we performed in vitro experiments using hydroxyl radicals (‘OH) as the oxidant. The ‘OH radicals were generated using the Fenton couple, Fe³⁺-nitroliotriacetic acid/H₂O₂ and measured by spin-trapping electron paramagnetic resonance (EPR) spectroscopy in presence of MEX-NH. Figure 2 shows the time course plot of the ‘OH radical adduct signal intensity in the absence and presence of 100 μM MEX-NH. It is observed that addition of MEX-NH to the system decreased the intensity of the ‘OH adduct signal, suggesting that MEX-NH is capable of scavenging ‘OH radicals.

Autodation of MEX-NH in the Heart. In biological tissues, MEX-NH undergoes autodation to form the corresponding nitroxide MEX-NO. MEX-NO is a paramagnetic molecule and hence can be directly detected by EPR spectroscopy. A saline solution of 25 μM MEX-NH did not show any EPR signal (Fig. 3A). However, effluents collected from hearts perfused with 25 μM MEX-NH showed a triplet EPR spectrum as shown in Fig. 3B. This triplet spectrum was identified to be from the nitroxide metabolite MEX-NO by comparison with the spectrum from an authentic sample (Fig. 3C).

Effect of MEX-NH on the Recovery of Cardiac Contractile Function. After 15 min of equilibration, hearts were perfused with 25 μM concentration of MEX-NH, MEX, or buffer for 1 min and then subjected to 30 min of global ischemia followed by 45 min of reperfusion. LVSP, LVEDP, HR, and CF were measured at preischemic and during 45 min of reperfusion. In all the hearts the preischemic LVEDP value was adjusted to 10 ± 2 mm Hg. The other preischemic baseline values were as follows: LVSP, 160 ± 42 mm Hg; HR, 320 ± 24 bpm; and CF, 16 ± 5 ml/min. The LVPD was computed as the difference between LVSP and LVEDP. The rate-pressure product (RPP) was obtained as a product of HR and LVPD. The functional and coronary flow data obtained during the reperfusion were expressed as percentage of their corresponding preischemic baseline values. Figure 4 shows the recovery of LVDP, RPP, and CF as a function of reperfusion time for hearts treated with the compounds. The percentage recovery of LVPD in the hearts infused with MEX-NH was significantly higher (P < .001) compared with that of control (buffer-perfused) or MEX-treated hearts. However, the recovery of hearts treated with MEX was not significant (P > .05) compared with that of control hearts. Recoveries of 96.3 ± 2.7 and 19.9 ± 2.7% of LVPD were observed, respectively, for MEX-NH and MEX compared with 13.7 ± 1.0% for control after 45 min of reperfusion (Fig. 5).
Hearts treated with MEX-NH showed a recovery of 78.8 ± 4.0% of RPP and 74.6 ± 5.0% of CF at the end of 45 min of reperfusion compared with 10.2 ± 0.5% of RPP and 29.1 ± 3.9% of CF for control. The results show that MEX-NH treatment provided almost complete recovery of function in the hearts subjected to 30 min of ischemia. Also no significant differences were observed in the recoveries of RPP or CF between the MEX-treated and control hearts.

To further evaluate whether the cardioprotective action of MEX-NH occurs during ischemic or during reperfusion phase, additional groups of hearts were treated with MEX-NH, either only during the first 5 min of reperfusion or 1 min before the onset of ischemia. The LVDP, RPP, and CF at 45 min of reperfusion in hearts that received MEX-NH for 1 min before ischemia showed enhanced recovery of contractile function compared with the hearts that received the drug only during reperfusion (Fig. 5). However, no significant differences were observed in the recoveries of coronary flow in these hearts.

**Dosage Effect of MEX-NH on the Recovery of Contractile Function.** To further evaluate the dose-dependent effect of MEX-NH on the recovery of contractile function, hearts were treated with 10, 25, 50, or 100 μM concentrations of MEX-NH for 1 min before the onset of ischemia. After 30 min of global ischemia the hearts were reperfused for 45 min, during which time the contractile function was continuously measured. Figure 6 shows the recovery at the end of 45 min of reperfusion as a function of preischemic infusion dose of MEX-NH. The recoveries of LVDP, RPP, and CF were observed to increase in a dose-dependent manner with maximum protection seen at 25 μM MEX-NH and reaching a plateau thereafter. The results suggest that 10 μM concentration of the drug is capable of protecting the heart up to 80% of ischemia/reperfusion-induced contractile dysfunction, whereas concentrations higher than 25 μM may attenuate the beneficial effects of the drug.

**Effect of MEX-NH and MEX on Ischemia/Reperfusion-Induced Cell Injury.** The cellular damage caused by the ischemia/reperfusion-induced alterations of membrane integrity was estimated by measuring the leakage of cyto-
also performed on additional groups of hearts infused with 25 MEX-NH, or Krebs' buffer for 1 min before ischemia. Measurements were collected and assayed for LDH as described under Materials and Methods. Effluents from the perfused hearts were collected for 15 min after beginning the infusion (Fig. 8). An immediate drop in the heart rate was observed during the infusion period. However, the rate recovered quickly thereafter during continued perfusion with the normal perfusate. The heart rate returned to control levels in about 15 min after the infusion of the drug. The measured LVDP during the first few minutes of infusion showed a large variation, presumably due to the drop in the heart rate during this period. The LVDP, however, returned to the control level in about the same period as the restoration of the heart rate.

**Effect of MEX-NH on the Cardiac SL Na⁺,K⁺-ATPase Activity in the Ischemia/Reperfusion Hearts.** The structure of MEX-NH has a lipophilic aromatic group at one end and a hydrophilic amino group at the other end separated by an alkyl chain. This particular design has been shown to enable preferential partitioning into the aqueous-lipid membrane interface (Subczynski et al., 1998). This property may result in enhanced membrane activity, leading to blockage of ion channels or pumps, thus offering ischemic protection. To investigate whether MEX-NH can protect Na⁺,K⁺-ATPase function of cardiac myocytes against ischemia/reperfusion-induced inactivation, hearts subjected to ischemia/reperfusion were assayed for Na⁺,K⁺-ATPase activity. Figure 9 shows the cardiac SL Na⁺,K⁺-ATPase activity in hearts treated with MEX-NH at various stages of the ischemia/reperfusion protocol. After 30 min of ischemia, there was 35 ± 5.3% inhibition of cardiac SL Na⁺,K⁺-ATPase activity, as shown in Fig. 9. No significant further injury was seen on the enzyme function after 45 min of reperfusion, suggesting that the Na⁺,K⁺-ATPase injury occurred during global ischemia. In contrast, when hearts were pretreated with 25 μM MEX-NH, complete protection of the Na⁺,K⁺-ATPase activity was observed. There was no loss of enzymatic activity in both ischemia and ischemia/reperfusion conditions (Fig. 9). These results clearly demonstrate that MEX-NH offers almost complete protection of Na⁺,K⁺-ATPase activity against ischemia-induced inactivation.

**In Vitro Measurements of the Effect of MEX-NH on the Inhibition of Na⁺,K⁺-ATPase Activity.** To investigate the mechanism of the protective effect of MEX-NH, the activity of Na⁺,K⁺-ATPase was measured in presence of MEX-NH. The enzyme was incubated with various concentrations of MEX-NH and its activity measured as described under Materials and Methods. Figure 10 shows that no inhibition on Na⁺,K⁺-ATPase was detected at low concentrations (up to 50 μM) of MEX-NH, however a dose-dependent inhibition was observed at higher concentrations. The half-maximal inhibition of Na⁺,K⁺-ATPase was achieved at 520 μM MEX-NH and a complete inactivation of the enzyme occurred in the presence of 1 mM MEX-NH.

To further investigate the possible MEX-NH-binding site on the Na⁺,K⁺-ATPase enzyme, [3H]ouabain-binding studies were performed in the presence and absence of MEX-NH.
Figure 11 shows that MEX-NH competes with $[^{3}H]$ouabain-binding site of Na$^{+}$,K$^{+}$-ATPase, reducing the labeling to 27 ± 4%. The results suggest that MEX-NH may bind at the ouabain-binding site of the Na$^{+}$,K$^{+}$-ATPase. Moreover, because the K$^{+}$ ion antagonizes the ouabain binding (Kukreja et al., 1990; Xu, 1992), this suggests that the binding of MEX-NH may be near the K$^{+}$-binding site as well. Taken together, these results suggest that the protective effect of the MEX-NH may be due at least in part to a reversible binding at the ouabain-binding site of the Na$^{+}$,K$^{+}$-ATPase. This in turn may induce a conformational change of the

Fig. 6. Effect of different dosages of MEX-NH on the recovery of cardiac contractile function in hearts subjected to ischemia and reperfusion. Perfused rat hearts were subjected to 30 min of global no-flow ischemia, followed by 45 min of reperfusion. Hearts were infused with different doses of MEX-NH (0–100 μM) for 1 min before ischemia. The contractile function and coronary flow at 45 min of reperfusion are expressed as a percentage of the corresponding preischemic baseline values. Data are plotted as mean ± S.E.

Fig. 7. Time course of LDH release in the reperfused hearts. Myocardial injury was assessed by measurement of the amount of LDH released from reperfused heart effluents. Rat hearts were subjected to 30 min of global no-flow ischemia, followed by 45 min of reperfusion. Hearts were infused with 25 μM MEX-NH (●, n = 7), MEX (○, n = 7), or Krebs’ buffer (△, n = 7). Effluents from the heart were collected at different times during the reperfusion and assayed for LDH as described under Materials and Methods. Data are plotted as mean ± S.E.

Fig. 8. Effect of MEX-NH on perfused isolated heart function. Hearts were infused with MEX-NH (0–50 μM) in perfusate for 1 min and contractile functions (heart rate and developed pressure) were continuously measured for 15 min. The data (n = 6) are expressed as a percentage of the preischemic baseline. Data are plotted as mean ± S.E.
Cardiac SL Na\(^+\),K\(^+\)-ATPase was isolated from rat heart muscle after 30-min ischemia, or 30-min ischemia and 45-min reperfusion in the presence or absence of MEX-NH. Data represent the mean ± S.E. of six independent experiments. * significantly different (P < .001) from the control group. The Na\(^+\),K\(^+\)-ATPase activity in hearts subjected to 30-min ischemia and 45-min reperfusion was completely protected by 25 μM MEX-NH.

Fig. 9. Protective effect of MEX-NH on the Na\(^+\),K\(^+\)-ATPase activity. Cardiac SL Na\(^+\),K\(^+\)-ATPase was isolated from rat heart muscle after 30-min ischemia, or 30-min ischemia and 45-min reperfusion in the presence or absence of MEX-NH. Data represent the mean ± S.E. of six independent experiments. * significantly different (P < .001) from the control group. The Na\(^+\),K\(^+\)-ATPase activity in hearts subjected to 30-min ischemia and 45-min reperfusion was completely protected by 25 μM MEX-NH.

Fig. 10. Concentration-dependent inactivation of Na\(^+\),K\(^+\)-ATPase by MEX-NH. The enzyme activity was measured as a function of MEX-NH concentration as described under Materials and Methods. The results show that no inhibition was detected in the presence of 25 or 50 μM MEX-NH. Nevertheless, Na\(^+\),K\(^+\)-ATPase was inactivated to 15, 39, 58, and 100% in the presence of 0.2, 0.4, 0.6, 0.8, and 1 mM MEX-NH, respectively.

Discussion

The present study establishes that the compound MEX-NH provides marked protection against ischemia/reperfusion-induced contractile dysfunction in isolated hearts. The mechanism of protection appears to be a combination of reversible inhibition of Na\(^+\)/K\(^+\)-ATPase activity during ischemia and possibly site-targeted antioxidative effect during ischemia and reperfusion.

The events leading to cardiac contractile dysfunction have been well established. Ischemia rapidly produces acidosis in cardiac cells, possibly due to accumulation of inorganic phosphate produced by ATP breakdown, and/or lactate and acidosis produced by anaerobic metabolism (Dennis et al., 1991). This may lead to stimulation of Na\(^+\)/H\(^+\) exchange, resulting in an excessive Na\(^+\) overload in ischemic or anoxic cardiac cells (Meng and Pierce, 1991). The ischemia-induced Na\(^+\) overload may enhance Ca\(^2+\) overload in the cell by stimulating the Na\(^+\)/Ca\(^2+\) exchange mechanism (Tani and Neely, 1989) or by increasing cell membrane permeability, when oxygen is replenished (Crake and Poole-Wilson, 1990). The resulting increase in intracellular Ca\(^2+\) may exert deleterious effects on the mechanical function and biochemical activities of cardiac cells (Crake and Poole-Wilson, 1990; Bagchi et al., 1997). These sequences may eventually lead to cardiac contractile dysfunction. The intracellular calcium overload can also set off a cascade of events, including the formation of reactive oxygen species, promoting arachidonic acid metabolism and converting xanthine dehydrogenase into xanthine oxidase. Particularly, the generation of highly reactive oxidants and oxygen-derived free radicals, including the superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen during ischemia/reperfusion has been implicated as an important factor in the pathogenesis of cellular injury in the postischemic heart. Thus, agents that can control the transport of the essential ions across the cell membrane during an ischemic event and/or scavenge the deleterious species generated during reoxygenation are effective in protecting ischemia/reperfusion-induced contractile dysfunction.

The class Ib antiarrhythmic agent MEX acts by inhibiting cardiac sodium channels, and hence calcium influx in cells via decreasing reverse operation of the Na\(^+\)/Ca\(^2+\) exchanger. MEX has been shown to enhance posts ischemic functional recovery and biochemical dysfunction of perfused hearts (Kamiyama et al., 1995). Treatment of perfused hearts with 10 to 100 μM MEX during preischemia resulted in an enhance-
ment of postischemic contractile recovery, a suppression of changes in tissue Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\) contents and an attenuation of the release of creatine kinase and ATP metabolites in an almost concentration-dependent manner. It has been suggested that the mechanism underlying the protective actions of MEX was the prevention of Na\(^{+}\) overload and accompanying Ca\(^{2+}\) overload in cardiac cells (Kamiyama et al., 1995). It has recently been shown that MEX can also function as an antioxidant by inhibiting hydroxyl radical-mediated lipid peroxidation in brain membranes (Demirpence et al., 1999). Although the electrophysiological mechanism of MEX has been well established, the extent of the antioxidative mechanism in postischemic myocardial protection is still not clear.

The particular design of the MEX-NH molecular structure (Fig. 1), having a lipophilic/antiarrhythmic head group and a hydrophilic/antioxidant tail group, may offer a dual role to its cardioprotective action: both by inhibiting Ca\(^{2+}\) influx, and by providing site-targeted antioxidant protection. The MEX-NH contains a five-membered sterically hindered pyrroline moiety that is biologically converted to a nitroxide (MEX-NO, Fig. 1). The nitroxides are an important class of antioxidants that have been shown to inhibit lipid peroxidation by functioning as a chain-breaking antioxidant (Cighetti et al., 1997). The inhibition is comparable to that of α-tocopherol, which is a lipid-soluble antioxidant known to work in fatty areas such as the lipids, suggesting that a good affinity for cell membranes increases the lipid peroxidation inhibitory effect. The nitroxides also offer intracellular protection against oxidative damage by their ability to oxidize reduced metal ions, such as iron and copper. The oxidation of reduced metal ions will preempt the Fenton reaction and prevent the formation of secondary ‘OH radicals. We have recently reported that a pyrrole derivative, having a structural similarity to MEX-NH, was metabolized in vivo to form the corresponding nitroxide derivative and offered membrane-targeted antioxidant protection against myocardial postischemic reperfusion injury (Shankar et al., 2000).

Cardiac sarcolemmal Na\(^{+}\),K\(^{+}\)-ATPase plays a crucial role in the active transport of Na\(^{+}\) outside the cell in exchange for K\(^{+}\). The Na\(^{+}\),K\(^{+}\)-ATPase functions in the presence of ATP. During cardiac ischemia due to a shortage of ATP, the function of the sodium pump would be limited or inhibited, leading to a cascade of ionic imbalances. On postischemic reperfusion, an irreversible inhibition of the Na\(^{+}\),K\(^{+}\)-ATPase pump was observed. This irreversible injury to the pump was suggested to be due to the generation of the reactive oxygen species upon reoxygenation. In vitro studies have shown that hydroxyl radicals directly attack the ATP-binding site of the cardiac SL Na\(^{+}\),K\(^{+}\)-ATPase from rat heart muscle (Kukreja et al., 1990; Xu et al., 1997a). The present study showing the ability of MEX-NH to scavenge hydroxyl radicals suggests that MEX-NH may also play a significant role in the detoxification of hydroxyl radical-mediated Na\(^{+}\),K\(^{+}\)-ATPase injury during reperfusion. In addition, the particular abilities of the compound for preferential partitioning and accumulation in to the membrane (Subczynski et al., 1998), and binding to the ouabain-binding site on the Na\(^{+}\),K\(^{+}\)-ATPase enzyme may involve a site-targeted detoxification mechanism of membrane protection.

The present investigation clearly demonstrates that MEX-NH is capable of providing marked protection against ischemia/reperfusion injury in isolated rat hearts. The protective effect could be due to a reduction in the severity of ischemic damage caused by the deprivation of oxygen (ischemic protection), or scavenging of toxic oxidative species that cause damage during reperfusion, or a combination of both. The observation that hearts pretreated with MEX-NH for 1 min before ischemia showed a better recovery of contractile function compared with post-treated (5 min, beginning reperfusion) hearts (Fig. 5) suggests that the ischemic protection may be the predominant mechanism in this model. Although significant protections of the contractile function and coronary flow have been observed in the post-treated hearts, the magnitude of protection is far below to that of pretreated hearts. Although the compound can also function as an antioxidant and hence protect the heart against reperfusion-mediated oxidative injury, the beneficial effect seems to be offset by its effect on the heart rate (Fig. 8). Furthermore, the antioxidative protection during the reperfusion phase may require pre-equilibration or localization of molecule at the site of injury.

The hearts treated with MEX-NH showed enhanced recovery of coronary flow, which may suggest the involvement of endothelial function, possibly by enhanced release of nitric oxide during reperfusion. Measurement of nitric oxide generation in the reperfused hearts, treated with MEX-NH, may provide further evidence as to whether the drug also protects the enzyme nitric-oxide synthase against ischemic injury.

In conclusion, MEX-NH has a dual effect toward ischemia/reperfusion-induced contractile dysfunction and injury in heart, both by protecting Na\(^{+}\),K\(^{+}\)-ATPase enzyme activity and by functioning as a site-targeted antioxidant against reactive oxygen species-induced lipid peroxidation of the cell membrane. Thus, MEX-NH is highly effective at preventing postischemic injury.

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References


Send reprint requests to: Periannan Kuppusamy, Ph.D., Division of Cardiology, Johns Hopkins University School of Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224. E-mail: kuppu@welch.jhu.edu