

N,N,N',N'-Tetrakis(2-pyridylmethyl)-ethylenediamine Improves Myocardial Protection against Ischemia by Modulation of Intracellular Ca\(^{2+}\) Homeostasis

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ABSTRACT

N,N,N',N'-Tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), a transition-metal chelator, was recently found to protect against myocardial ischemia-reperfusion injury. The goals of this study were to investigate the in vivo antiarrhythmic and antifibrillatory potential of TPEN in rats and guinea pigs and to study the in vitro effects of TPEN on calcium homeostasis in cultured newborn rat cardiac cells in normoxia and hypoxia. We demonstrated on an in vivo rat model of ischemia-reperfusion that TPEN abolishes ventricular fibrillation incidence and mortality and decreases the incidence and duration of ventricular tachycardia. To elucidate the mechanism of cardioprotection by TPEN, contraction, synchronization, and intracellular calcium level were examined in vitro. We have shown for the first time that TPEN prevented the increase in intracellular Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_i\)) caused by hypoxia and abolished [Ca\(^{2+}\)]\(_i\), elevation caused by high extracellular Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_o\)) or by caffeine. Addition of TPEN returned synchronized beating of cardiomyocytes desynchronized by [Ca\(^{2+}\)]\(_o\), elevation. To discover the mechanism by which TPEN reduces [Ca\(^{2+}\)]\(_i\), in cardiomyocytes, the cells were treated with thapsigargin, which inhibits Ca\(^{2+}\) uptake into the sarcoplasmic reticulum (SR). TPEN successfully reduced [Ca\(^{2+}\)]\(_i\), elevated by thapsigargin, indicating that TPEN did not sequester Ca\(^{2+}\) in the SR. However, TPEN did not reduce [Ca\(^{2+}\)]\(_i\) in the Na\(^+\)-free medium in which the Na\(^+\)/Ca\(^{2+}\) exchanger was inhibited. Taken together, the results show that activation of sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger by TPEN increases Ca\(^{2+}\) extrusion from the cytoplasm of cardiomyocytes, preventing cytosolic Ca\(^{2+}\) overload, which explains the beneficial effects of TPEN on postischemic cardiac status.

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ABBREVIATIONS: TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine; ES, extra-systoles; VT, ventricular tachycardia; VF, ventricular fibrillation; SR, sarcoplasmic reticulum; SERCA2a, SR Ca\(^{2+}\)-ATPase; LAD, left anterior descending; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; PI, propidium iodide; DASPML, 4-(4-(dimethylamino styryl)-N-methylpyridinium iodide; RPP, rate-pressure product; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger.
has been hypothesized as a mechanism of cardioprotection by TPEN. Increase of [Ca\(^{2+}\)] is one of the most important factors of tissue injury caused by oxygen deprivation (Piper et al., 1993). [Ca\(^{2+}\)]\(_{i}\) elevation in cardiac cells occurs through three ways: via the Ca\(^{2+}\) channels (Schröder et al., 1998), via the reverse mode of the Na\(^+/Ca^{2+}\) exchanger (Dipa et al., 1999), and on Ca\(^{2+}\) release from sarcoplasmic reticulum (SR) stores. Because the affinity of TPEN for calcium is low, it may be not sufficient to prevent an overload in calcium even by direct binding of calcium in calcium stores of the SR. Therefore, it may be assumed that TPEN activates Ca\(^{2+}\) channels and pumps to remove calcium from the cytosol. TPEN may activate them by heavy metal ion chelation as an indirect effect (McNulty and Taylor, 1999) or by activation of signal transduction via G protein-coupled receptors (Webster et al., 2003). The effect of TPEN on [Ca\(^{2+}\)]\(_{i}\) has already been demonstrated in noncardiac cells. TPEN significantly reduced the level of free Ca\(^{2+}\) in the endoplasmic reticulum and increased the activity of calcium-dependent calcium channels in glandular cells (Liu and Ambudkar, 2001) and in bovine adrenal chromaffin cells (Powis and Zerbes, 2002). Beneficial effects of TPEN in cells are found in a narrow concentration range, between 100 nM and 2 \(\mu\)M (Kim et al., 1999).

The aim of this study was to demonstrate the antiarrhythmic and antifibrillatory effects of TPEN in vivo and to investigate the mechanism by which TPEN protects cardiomyocytes from hypoxic damage. Prevention of Ca\(^{2+}\) overload following hypoxia by TPEN was verified by in vitro study. It is proposed that TPEN activates SR Ca\(^{2+}\)-ATPase (SERCA2a) or Na\(^+/Ca^{2+}\) exchanger to remove surplus Ca\(^{2+}\) and thereby protect the cardiac cells from injury caused by Ca\(^{2+}\) overload during hypoxia.

Materials and Methods

Experiments in Vivo

Animal Preparation. This study complied with Israeli legislation regarding experimental procedures on laboratory animals. Adult male Sprague-Dawley rats \((n = 38)\) weighing 270 to 330 g (supplied by Harlan, Indianapolis, IN) were housed in pairs in an environmentally controlled room, with food and water available ad libitum. The rats were anesthetized by i.p. administration of 40 mg/kg pentobarbital sodium in a volume of 1 ml/kg. Tracheostomy and cannulation were performed, and the animals were artificially ventilated (Harvard rodent ventilator, model 683) using 1 ml of room air per 100 g b.w.t. at 54 to 56 cycles/min. A catheter filled with heparinized saline (50 IU/ml) was inserted into the left main carotid artery for arterial pressure measurement using a pressure transducer (UFI, model 1050 bp). The right jugular vein was cannulated for drug administration. ECG (lead II) was monitored with three subcutaneous limb electrodes and recorded along with the arterial pressure using an MP100 data acquisition system (Biopac Systems Inc., Santa Barbara, CA).

Adult male Dunkin Hartley guinea pigs \((n = 10)\) weighing 500 to 700 g were used (Harlan). The preparation procedure included anesthesia, tracheotomy, catheterization of both jugular veins, and ECG recording and was similar to that described above. The guinea pigs were ventilated with 0.8 to 1 ml of room air per 100 g b.w.t. at 45 cycles/min.

Ischemia-Reperfusion Induced Arrhythmias in Rats

Preventive Model. The rat’s chest was opened by a horizontal cut through the ribs by a rib spreader. The rat was allowed to stabilize for 2 min before the administration of the drug. TPEN dissolved in EtOH was infused at 100 \(\mu\)l/min in the following concentrations: 0.075 mg/ml \((n = 11)\), 0.3 mg/ml \((n = 12)\), and vehicle solution −5% EtOH in saline \((n = 11)\). The final TPEN doses were 0.5 and 2 mg/kg.

An additional 10 mg/kg (1.5 mg/ml) TPEN dose was tested \((n = 5)\), but these experiments were discontinued due to the toxicity of TPEN at this dose. Arterial pressure and heart rate data were collected from four rats that received 10 mg/kg TPEN, without opening their chests. TPEN was administered for 9 to 11 min before the occlusion period, depending on body weight, and administration continued during the 10-min occlusion period. During infusion, a suture was made around the left anterior descending (LAD) coronary artery using a 5-0 silk thread, which was fed through a plastic tube so that the tightening of the tube with the use of tight clips occluded the artery. After 10 min of myocardial ischemia, the ligature was released, the drug infusion was stopped, and reperfusion was initiated. The incidence of arrhythmia was recorded during ischemia and reperfusion in accordance with the Lambeth convention, as ES, salvos, bigeminy, VT, and VF (Walker et al., 1988). The onset and termination times of arrhythmia were also recorded.

Curative Model. In this model, the same procedure as in the preventive paradigm was used with the following alterations. The LAD was first occluded, and then the drug (the same formulation) was administered by manual injection during the 4th and 5th min of the ischemic period. Two animal groups were tested: 2 mg/kg TPEN \((n = 7)\) and control rats \((n = 7)\).

Experiments in Vitro

Solutions. Standard Tyrode’s solution, pH 7.4, contained 136.9 mM NaCl, 2.68 mM KCl, 0.42 mM NaH\(_{2}\)PO\(_4\), 11.9 mM NaHCO\(_3\), 1.05 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), and 5.55 mM glucose. Tyrode-choline solution, pH 7.4, contained: 10 mM NaCl, 126.9 mM choline chloride, 2.68 mM KCl, 0.42 mM NaH\(_{2}\)PO\(_4\), 11.9 mM NaHCO\(_3\), 1.05 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), and 5.55 mM glucose. Buffer Na\(^{+}\)-free solution, pH 7.4, contained: 136.9 mM choline chloride, 2.68 mM KCl, 0.42 mM NaH\(_{2}\)PO\(_4\), 1.05 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 11 mM HEPES, and 5.55 mM glucose. Standard phosphate-buffered saline (PBS) solution, pH 7.4, contained: 135 mM NaCl, 8.09 mM Na\(_{2}\)HPO\(_4\), 1.47 mM K\(_{2}\)HPO\(_4\), 2.68 mM KCl, 0.9 mM CaCl\(_2\), 0.48 mM MgCl\(_2\), and 5.55 mM glucose. In Ca\(^{2+}\)-free medium, PBS solution was without CaCl\(_2\) and MgCl\(_2\).

Preparation of Rat Heart Cell Cultures. The rat hearts (1–2 days old) were sterilely removed and washed three times in Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS to remove excess blood cells. The hearts were minced to small fragments and then again in a proteolytic enzyme-RBD solution prepared from a fig tree extract as described previously (Brik and Shainberg, 1990; Shneyveys et al., 2001). The RDB was diluted 1:200 in PBS at 25°C for a few cycles of 10 min each. The supernatant suspension containing dissociated cells, to which medium containing 10% horse serum was added, was centrifuged at 500g for 5 min. After centrifugation, the supernatant phase was discarded, and cells were resuspended in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated horse serum and 0.5% chick embryo extract. The suspension of cells was diluted to 1 × 10\(^6\) cells/ml, and 1.5 ml was placed in 35-mm collagen/gelatin-coated plastic culture dishes or on collagen/gelatin-coated coverslips. Cultures were incubated in a humidified atmosphere of 5% CO\(_2\) and 95% air at 37°C. A confluent monolayer, which exhibits spontaneous beating, was obtained in 2 to 3 days. The growth medium was replaced every 3 days. The experiments were performed on these cardiomyocyte cultures after 3 to 5 days in vitro.

Hypoxia. Cardiomyocytes from neonatal rat hearts were cultured and exposed to hypoxia for 90 min with glucose-free PBS (1 ml/dish) in a closed chamber by replacement of air with N\(_2\) (100%) as previously described (Safran et al., 2001). After 90 min of hypoxia, the pO\(_2\) in the PBS was <0.4 torr. Oxygen levels in the N\(_2\) atmosphere were below 1%. Continuous monitoring of intracellular [Ca\(^{2+}\)]\(_{i}\) during hypoxia was performed in a special barrier well, where cells were
protected from oxygen by a laminar counterflow layer of the inert gas argon (Ar 100%). The coverslips with cultured cells were placed at the bottom of the chamber. This chamber was mounted on a specially modified Zeiss inverted epifluorescence microscope (Carl Zeiss GmbH, Jena, Germany) as previously described (Stern et al., 1988). Hypoxic stress injury was characterized by lactate dehydrogenase (LDH) released from cells and by the propidium iodide (PI) staining method.

Experiments on rat cardiomyocyte cultures were performed in three groups. The first group was treated with TPEN (dissolved in DMSO) for 15 min before and during hypoxia. The second group was untreated and underwent hypoxia for various time periods. The third group was the control group, which was untreated and remained in normoxia.

**LDH Activity.** After hypoxia, LDH activity was determined using LDH-L kit (Thermo Trace, Melbourne, Australia) in the cardiac cell medium (PBS). The product of the enzyme activity was measured in a spectrophotometer at a wavelength of 340 nm at 30°C.

**PI and Hoechst measurement.** Cultured cardiomyocytes were incubated with PI (5 μg/ml) (Molecular Probes, Eugene, OR) in PBS for 30 min at 37°C to stain nucleus/DNA red of dead cells, washed with PBS twice, and the fluorescence was measured with excitation at 485 nm and emission at 635 nm. Afterward cells were labeled with Hoechst 33342 (Molecular Probes) to stain the nucleus/DNA blue of all cells.

**Mitochondrial activity.** Living cardiomyocytes grown on coverslips were exposed to 4-(4-(dimethylaminostyryl)-N-methylpyridinium iodide (DASPMI) (Sigma-Aldrich, St. Louis, MO), dissolved in PBS at a final concentration of 10 μg/ml, for 10 min at a temperature of 37°C. The coverslips were then washed and mounted on the hypoxic chamber containing dye-free medium. DASPMI fluorescence was excited at 460 nm, and emission was measured at a wavelength of 540 nm as previously described (Shneyvays et al., 2002). The signal intensity was quantified by using densitometry analysis software ImageJ (National Institutes of Health, Bethesda, MD).

**Measurement of contractility.** Culture dishes were placed in a specially designed Plexiglas chamber at 37°C. The chamber was placed on the stage of an inverted phase contrast microscope (Olympus, Tokyo, Japan). Measurement of the contractions of the cardiomyocytes at baseline and in response to interventions was conducted using a video motion detector system as described in detail by Zangen and Shainberg (1997). Analogue tracing was recorded using an oscilloscope joined through a specially designed interface to an IBM computer, and kinetic data were analyzed by the Microsoft Excel program.

**Intracellular Ca²⁺ measurements.** Intracellular free calcium ([Ca²⁺][], free intracellular calcium) from an individual cell was measured during hypoxia by using the indicator Indo-1 under an epifluorescence microscope. The ratio metric method for the experiments has been described previously (Shneyvays et al., 2001). Cardiomyocytes were incubated with 3 μM Indo-1/AM and 1.5 μM pluronic acid for 30 min in glucose-enriched PBS at 25°C. Indo-1 was excited at 355 nm, and the emitted light was then split by a dichroic mirror to two photomultipliers (Hamamatsu Corporation, Bridgewater, NJ) with input filters at 405:490 as described previously (Shneyvays et al., 2004).

**Statistical analysis.** In each experimental group, in vivo measurements of onset and termination times, heart rate, and arterial pressure and in vitro results are expressed as mean ± S.D. n represents the number of experiments. The incidence of VF and death are shown as a percentage of total animals tested and compared by means of the χ² method. In vitro data were analyzed by analysis of variance with application of a post hoc Tukey-Kramer test. p < 0.05 was accepted as indicating statistical significance. Each experiment was repeated at least three times and performed each time on three separate dishes per treatment.

**Results**

**Antiarhythmic and Anti-fibrillatory Effects of TPEN**

Ischemia-reperfusion was performed on two rat models: the preventive paradigm model, where animals were infused with TPEN before the LAD occlusions and the curative model, where TPEN was given during LAD occlusion.

TPEN in concentrations of 0.5 mg/kg had no effect on hemodynamics (heart rate and arterial pressure). Treatment with 2 mg/kg TPEN caused a small but significant decline in the heart rate throughout the whole experiment compared with nontreated animals, and a decrease in mean arterial pressure was seen only during the early occlusion period (Fig. 1, A and B). TPEN (10 mg/kg) had a severe hemodynamic effect, which was exemplified by bradycardia and a decrease in arterial pressure but was not lethal. This hemodynamic effect was reduced after about 20 min following termination of drug infusion (data not shown).

In this experiment, rate-pressure product (RPP) was calculated from heart rate and arterial pressure (Fig. 1C). In the 2 mg/kg treatment group, decrease of RPP comparatively with baseline (before occlusion) was due to depression of myocardial contractility. On the other hand, TPEN in a dose of 2 mg/kg decreases RPP significantly in each experimental period relative to the vehicle group because the antiarrhythmic effect of TPEN is possibly due to decrease of myocardial oxygen demand.

Results from the preventive treatment model are shown in Table 1. Within the experimental control group, the ischemia-reperfusion procedure resulted in cardiac arrest in the middle of the ischemic period in 2 of 11 rats. The remaining animals displayed arrhythmia following LAD occlusion and reperfusion, whereas most of them experienced reversible or irreversible VF periods. All nontreated rats demonstrated long VT intervals during both the occlusion and the reperfusion periods.

With 0.5 mg/kg TPEN, VF occurred in only 1 of the 11 group members (p < 0.05 versus nontreated rats), and two rats died during the ischemia-reperfusion period (Table 1). TPEN (0.5 mg/kg) significantly decreased the incidence and duration of VT during the ischemic period but not during the reperfusion period (Table 1; Fig. 2). The incidence and duration of other kinds of arrhythmia (ES, salvos, and bigeminy) were not significantly affected in either TPEN-treated or nontreated animals.

TPEN (2 mg/kg) abolished VF incidence and mortality when compared with the control group (p < 0.05). TPEN significantly decreased both the incidence and duration of VT.
A. Heart rate

B. Blood pressure

C. Rate-pressure product (RPP)

Fig. 1. The hemodynamic effect of TPEN throughout the experimental procedure of ischemia and reperfusion in a preventive paradigm. Animals were infused with TPEN for 20 min (10 min before and 10 min during LAD occlusion). Both heart rate and arterial pressure data were calculated during periods of sinus rhythm only. Results are presented as mean ± S.D. Significance: *, \( p < 0.05 \); **, \( p < 0.005 \) versus vehicle. BPM, beats per minute.
During the occlusion period in the 2 mg/kg treatment group, and the duration of VT in the reperfusion period was also significantly shortened (Table 1; Fig. 2). TPEN (10 mg/kg) completely abolished all ischemia and reperfusion arrhythmias (data not shown) but had a side effect of severe hypotension and bradycardia.

Within the nontreated group, the first arrhythmic incidents were single ES or short salvos periods that occurred 5 \pm 0.2 min from the moment of LAD occlusion. Arrhythmic incidents occurred throughout the ischemic period. Reperfusion-induced arrhythmias began seconds from blood reflow in all the experimental groups and lasted for 7.5 \pm 1.3 min in the control animals. As seen in Fig. 3, both 0.5 and 2 mg/kg TPEN treatments significantly decreased the ischemia-induced arrhythmia, and 2 mg/kg TPEN reduced reperfusion-induced arrhythmias ($p < 0.001$).

**Acute Treatment in Rats.** As shown in Table 2, 2 mg/kg TPEN decreased the incidence of VF and death when compared with nontreated animals, but the results did not reach statistical significance. TPEN-treated rats showed a significant decrease in VT duration during the ischemic period, but no significant difference appeared during the reperfusion period, in contrast to the preventive treatment.

**Effects of TPEN on Cardiomyocyte Cultures Subjected to Hypoxia**

**TPEN Prevents LDH Leakage.** To verify the cardioprotective ability of TPEN, cardiac cells grown in vitro were subjected to hypoxia. The cells were pretreated with TPEN for 15 min before and during various periods of hypoxia. LDH release from cardiomyocytes increased in a time-dependent manner with hypoxia duration ranging from 1 h to 135 min (Fig. 4A). However, in cells treated with 1 \mu M TPEN before and during hypoxia, the amount of LDH released was reduced 2-fold (from 161 \pm 4% to 86 \pm 10% after 1 h of hypoxia).

### Table 1

Incidence of malignant arrhythmia in TPEN-treated rats (preventive paradigm)

| Incidence of ischemia-reperfusion-induced malignant arrhythmias and mortality in TPEN-treated animals in a preventive paradigm. Animals were infused with TPEN for 20 min (10 min prior to LAD occlusion and 10 min during occlusion). The mortality incidence refers to the whole control group, whereas the VT and VF incidence refers to 9 arrhythmic rats of the total of 11 control rats. |
|---|---|---|---|
| | Vehicle ($n = 11$) | 0.5 mg/kg TPEN ($n = 11$) | 2 mg/kg TPEN ($n = 12$) |
| VT (Occlusion) | 9/9 | 64 (7/11)*a | 25 (3/12)*a |
| VT (Reperfusion) | 9/9 | 91 (10/11) | 100 (12/12) |
| VF (Occlusion) | 3/9 | 0 (0/11) | 0 (0/12)*a |
| VF (Reperfusion) | 78 (7/9) | 9 (1/11)*a | 0 (0/12)*a |
| Mortality (%) | 73 (8/11) | 18 (2/11) | 0 (0/12)*a |

*a Significance: $p < 0.05$ versus vehicle.
and in the same proportion after 135 min of hypoxia, Fig. 4A). Protective effect of TPEN was obtained also in a dose-dependent manner beginning at 100 nM TPEN (LDH leakage decreased from 527 ± 7% to 203 ± 14% during 105 min of hypoxia, Fig. 4, A and B). In the range of 0.1 to 3 μM TPEN, 1 μM was the most efficient in reducing LDH leakage especially during prolonged (120 min) hypoxia (Fig. 4B).

**TPEN Protects Cardiomyocytes from Necrosis and Decrease of Mitochondrial Activity Induced by Hypoxia.** PI staining was used as a measure of hypoxic damage and compared with Hoechst staining. Entry of PI to the cells indicates irreversible damage because it reaches the nucleus only by penetrating damaged membranes (necrosis), whereas Hoechst stains the nucleus of all cells. In cultures that underwent 1.5 h of hypoxia (Fig. 5C), an increase of the fluorescent level to 394 ± 16% compared with control cells (Fig. 5A) was observed. In cells treated with 1 μM TPEN for 15 min before and during 1.5 h of hypoxia (Fig. 5D), the fluorescence was much lower (125 ± 13% of the control level, Fig. 5). Treatment with TPEN without hypoxia had no effect (Fig. 5B). This indicates that the plasma membrane of cells preincubated with TPEN was less damaged by hypoxia.

The accumulation of DASPMI dye in the mitochondrial matrix space is known to be dependent on mitochondrial inner membrane potential. Cardiomyocytes treated with 1 μM TPEN for 15 min before and during 90 min of hypoxia and cardiomyocytes subjected to 90 min of hypoxia without TPEN were stained in vitro with the fluorescent dye DASPMI to monitor the mitochondrial membrane potential. In cardiomyocytes in normoxia, the DASPMI fluorescence of mitochondrial patterns was revealed in the perinuclear and intermyofibrillar regions forming parallel longitudinal rows. In many cells, localization of the mitochondria followed the striation pattern of the myofibrils (Fig. 5E). In the subsarcolemmal region of these cells, the mitochondrial patterns were stretched and oriented longitudinally. Hypoxia for 90 min resulted in a decrease in the fluorescence intensity of the mitochondria, i.e., dissipation of their membrane potential and its collapse by many organelles (Fig. 5G). After hypoxia, the intermyofibrillar mitochondrial patterns disappeared almost completely and remained partially only in the perinuclear space. In the TPEN-treated cells (Fig. 5H) after hypoxia, mitochondrial membrane potential was maintained or slightly decreased in many cells. Addition of 1 μM TPEN alone to cardiomyocytes did not affect the distribution and intensity of DASPMI fluorescence (Fig. 5F).

**TPEN Protects the Cardiomyocytes from Ca2+ Overload during Hypoxia.** Because the increase of intracellular Ca2+ ([Ca2+]i) levels in cardiomyocytes is considered a major factor in causing hypoxic damage (Borgers et al., 1988), the ability of TPEN to reduce [Ca2+]i during hypoxia was elucidated with the fluorometric calcium probe Indo-1. Continuous monitoring of [Ca2+]i during hypoxia was realized in a special barrier well, where cells were protected from oxygen by a laminar countercflowing layer of the inert gas argon. Intracellular Ca2+ levels were measured every 5 min during hypoxia in nontreated cells and cells treated with TPEN (each measurement lasted 10 s). In untreated cells, a gradual increase in baseline (diastolic) Ca2+ levels (Rbas, baseline ratio 405:495) and a decrease of amplitude in [Ca2+]i transients were observed during hypoxia, up to cessation of Ca2+ oscillations (Fig. 6A). In cells pretreated with 1 μM TPEN, Ca2+ levels during hypoxia remained significantly lower than in nontreated cells (mean Rbas ± S.D., 1.35 ± 0.08 after 30 min of hypoxia with 1 μM TPEN, Fig. 6B, compared with 2.24 ± 0.17 after 30 min of hypoxia without TPEN, Fig. 6A), and Ca2+ transients were less interrupted.

The level of Ca2+ in cytosol was elevated during hypoxia by both entry of Ca2+ from the surroundings and release of Ca2+ from the SR. To clarify the mechanism of the delay in [Ca2+]i elevation caused by TPEN, we examined the effect of TPEN after [Ca2+]i elevation (in Tyrode medium) by inducing of Ca2+ release from the SR with caffeine, extracellular Ca2+ ([Ca2+]o) elevation, and inhibiting the SERCA2a with thapsigargin.

**TPEN Decreased Caffeine-Induced Calcium Level Elevation in the Cytosol. To verify whether TPEN regulates the amount of calcium in cytosol, myocytes were paced by electric stimulation for 20 s to ensure SR calcium release. After pacing was stopped, caffeine (10 mM) was rapidly applied. Addition of caffeine in this system caused Ca2+ release from SR. However, the high level of [Ca2+]i, was decreased during the next few minutes. Treatment with TPEN after [Ca2+]i elevation by caffeine caused a slow decrease in [Ca2+]i (Manoach et al., 1997). Application of TPEN when [Ca2+]i, was elevated induced a decrease of [Ca2+]i. In a total of six tests after elevation of [Ca2+]i from 1.8 to 3.8 mM, the level of intracellular [Ca2+]i, was 2.16 ± 0.14 (mean Rbas ± S.D.) versus 1.08 ± 0.06 in

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**TABLE 2**

| Incidence of ischemia-reperfusion-induced malignant arrhythmias and mortality in TPEN-treated animals in a curative paradigm. Animals were infused with TPEN for 2 min during the LAD occlusion period. |
| --- | --- |
| Vehicle (n = 7) | 2 mg/kg TPEN (n = 7) |
| VT duration, log (s) | 2.07 ± 0.22 | 2.14 ± 0.37 |
| VT incidence (%) | 100 (7/7) | 100 (7/7) |
| VF incidence (%) | 85.7 (6/7) | 28.5 (2/7) |
| Mortality (%) | 57 (4/7) | 28.5 (2/7) |

* Significance: p < 0.05 versus vehicle.
control cells. In such conditions, addition of 1 \( \mu \)M TPEN decreased baseline level of \([\text{Ca}^{2+}]_i\) to \(R_{bas} = 1.58 \pm 0.17\) (Fig. 7B).

These results suggest that 1 \( \mu \)M TPEN increased \(\text{Ca}^{2+}\) extrusion activity. Because of the low affinity of TPEN to \(\text{Ca}^{2+}\), its chelating effect may be neglected, and \(\text{Ca}^{2+}\) extrusion may be postulated either by SR calcium uptake or by activation of the \(\text{Na}^+\)/\(\text{Ca}^{2+}\) exchanger on the sarcolemma.

**TPEN Activates \(\text{Na}^+\)/\(\text{Ca}^{2+}\) Exchanger.** To test whether TPEN stimulated SR calcium uptake, we examined its effect in the presence of the SERCA2a inhibitor, thapsigargin. Blockade of SR \(\text{Ca}^{2+}\) uptake by administration of 5 \( \mu \)M
Thapsigargin in regular Tyrode medium significantly elevated intracellular \[^{\text{Ca}^{2+}}\] level (bas = 2.17 ± 0.19, n = 9) compared with control (bas = 1.03 ± 0.09, n = 9). The increased baseline level of \[^{\text{Ca}^{2+}}\] was significantly reduced after application of 1 \(\mu\)M TPEN (bas = 1.08 ± 0.12, n = 9), indicating that blockade of SR calcium uptake did not prevent the effect of TPEN. To examine the role of the \(^{\text{Na}^{+}}/{\text{Ca}^{2+}}\) exchanger in TPEN activity, we studied the \[^{\text{Ca}^{2+}}\] response in \(^{\text{Na}^{+}}\)-free medium. Obtained results show that 1 \(\mu\)M TPEN reduced the basal level of elevated \[^{\text{Ca}^{2+}}\] with thapsigargin, bas only to 1.56 ± 0.1 in low-\(^{\text{Na}^{+}}\) medium (20 mM), and did not change the \[^{\text{Ca}^{2+}}\] level in the \(^{\text{Na}^{+}}\)-free medium in which the \(^{\text{Na}^{+}}/{\text{Ca}^{2+}}\) exchanger was inhibited (n = 9, Fig. 7, C and D). These results show that TPEN reduces \[^{\text{Ca}^{2+}}\] overload by activation of \(^{\text{Na}^{+}}/{\text{Ca}^{2+}}\) exchanger to extrude \[^{\text{Ca}^{2+}}\] from the cytosol (Fig. 7, C and D).

**Effects of TPEN on Synchronization of Cardiomyocyte Contraction.** The cellular contraction activity of cultured cardiomyocytes was analyzed by a video technique previously described (Zangen and Shainberg, 1997; Shneyveys et al., 1998). The majority of the cultured cells exhibited synchronized spontaneous contractions (Fig. 8). Increase of \[^{\text{Ca}^{2+}}\] from 1.8 to 3.8 mM violated synchronized beating activity of cardiomyocytes up to total desynchronization with fibrillating contractions and termination of cell movement. Addition of 1 \(\mu\)M TPEN after \[^{\text{Ca}^{2+}}\] elevation rapidly restored the synchronized cell movement. Figure 8 demonstrated cardiomyocyte contraction of four different cells un-
der normal conditions, following an increase of [Ca\(^{2+}\)]\(_o\) to 3.8 mM and administration of 1 \(\mu\)M TPEN.

**Discussion**

TPEN is known as a cell-permeable metal ion chelator. In addition to binding Cu\(^{2+}\), Zn\(^{2+}\), and Fe\(^{2+}\) with a high affinity, TPEN binds Ca\(^{2+}\) and Mg\(^{2+}\) with a low affinity. Much research has focused on the use of specific and nonspecific transition metal ion chelators as cardioprotective agents in reperfusion-induced arrhythmias. Iron chelators such as deferoxamine and copper chelators such as bathocuproine and neocuproine (which is also an iron chelator) are the best studied groups so far. The cardioprotective properties of these compounds were shown in several studies both in vitro and in vivo (Ambrosio et al., 1987; Reddy et al., 1989; Appelbaum et al., 1990; Williams et al., 1991; Spencer et al., 1998; Shadid et al., 1999), but the mechanism of the protective effect is still not known.

**Possible Mechanisms of TPEN Cardioprotection.** TPEN has been found as a cardioprotective drug only in ex vivo models, using the Langendorff model (Appelbaum et al., 1990; Chevion, 1991; Karck et al., 1992; Ferdinandy et al., 1998). Due to the different properties of TPEN, it can most probably prevent cardiac damages by more than one
mechanism. TPEN can act as an antioxidant during reoxygenation because metals such as iron may convert weakly reactive molecules to strongly reactive molecules, and the chelator of metals could reduce damage caused by free radical production (Galey et al., 1998). On the other hand, the antiarrhythmic effects of TPEN and reduction of VT duration in the ischemic period, when oxygen deprivation leads to energetic exhaustion and Ca\(^{2+}\)/H\(^{+}\) overloading in the myocardium, could be the result of vasodilatation or modulation of heart contractility and oxygen consumption. The increase in intracellular level of Zn\(^{2+}\)/H\(^{+}\) under pathological conditions may be an important factor in hypoxic damage (Powell et al., 1994), and chelation of Zn\(^{2+}\)/H\(^{+}\) may be included in the cardioprotective mechanism of TPEN (Turan, 2003). Additionally, TPEN can prevent Ca\(^{2+}\) overload in cardiac cells by direct Ca\(^{2+}\) binding or by activating mechanisms of Ca\(^{2+}\) removal. The last option was examined for the first time in this work.

**Research on TPEN in Vivo.** This study was performed to verify the cardioprotective effects of TPEN in vivo and to investigate the mechanism of protection in vitro. Our results in vivo have shown that TPEN successfully improves cardiac recovery of perfused hearts after regional and prolonged ischemia. TPEN applied i.v. to anesthetized rats at the lowest dose used (0.5 mg/kg) displayed protection against fibrillation and ischemia-induced arrhythmias and partially protected against reperfusion-induced arrhythmia and mortality. At 2 mg/kg, TPEN provided overall cardiac protection for all the parameters measured. However, 10 mg/kg TPEN proved to have a hemodynamic toxic effect that accompanied the cardiac protection. The lethal dose of TPEN determined in this study, 30 to 40 mg/kg, is in accord with published data (Evangelou and Kalfakakou, 1993).

Two in vivo modes of treatment were employed here, a preventive paradigm in which the drug was infused prior and during the ischemic period, and a curative paradigm in which a bolus injection was given during the expected onset of arrhythmia in the ischemic period. In the preventive model, TPEN effectively reduced ischemia-reperfusion-induced arrhythmia and successfully decreased the mortality rate. The antiarrhythmic properties of TPEN were not fully sustained in the curative treatment model. It is possible that the ability of TPEN to prevent arrhythmia in the acute treatment paradigm decreased because of a requirement for additional time to penetrate into the cells.

**Research on TPEN in Vitro.** To elucidate the cellular mechanism of cardioprotection of TPEN, we designed and carried out an in vitro study on primary cardiac cell cultures. The results of in vitro LDH leakage and propidium iodide staining after hypoxia demonstrate that the treatment with 0.1 to 3 \(\mu\)M TPEN reduces damage of cardiomyocyte membranes in a dose-dependent manner. TPEN also successfully preserved the structure and energetic capability of mitochondria, which were significantly damaged during 90 min of hypoxia. In cells pretreated with 1 \(\mu\)M TPEN for 15 min before and during hypoxia, mitochondria appeared much more active than in nontreated cells subjected to hypoxia.

**TPEN Preserves Ca\(^{2+}\) Homeostasis.** In oxygen-deprived cardiomyocytes, Ca\(^{2+}\) inserted from the medium and from SR release accumulated in the cytosol and caused cell damage (Piper et al., 1993). Therefore, we tested the ability of TPEN to prevent cytosolic Ca\(^{2+}\) overload during hypoxia. As was shown in this study, 1 \(\mu\)M TPEN delayed the increase of
intracellular free calcium in cardiomyocytes caused by hypoxia (Fig. 6, A and B).

In previous studies, it was suggested that Ca$^{2+}$ regulation by TPEN occurs by direct binding of Ca$^{2+}$ in the calcium stores (endoplasmic reticulum). Very high concentrations of TPEN were used because of its low affinity to Ca$^{2+}$ ($K_d = 4.0 \times 10^{-8}$ M). Direct measurements of luminal free Ca$^{2+}$ in BHK-21 fibroblasts have demonstrated intraluminal binding of Ca$^{2+}$ by 200 $\mu$M TPEN (Hofer et al., 1998; Caroppo et al., 2003). In the SR of rat cardiomyocytes (Zucchi et al., 2001), the Ca$^{2+}$ level is also high, but these concentrations of TPEN are toxic for cardiac cells. TPEN (1 $\mu$M), which was found to protect cardiomyocytes from hypoxic damage, is unlikely to be sufficient to significantly affect the millimolar Ca$^{2+}$ concentration in the SR by binding Ca$^{2+}$. We suggest an alternative and more useful mechanism of Ca$^{2+}$ regulation by TPEN. We suppose that low concentration of TPEN may protect cardiomyocytes by regulation of calcium homeostasis in a nonbinding way. Prevention of Ca$^{2+}$ overload may be due to inhibition of Ca$^{2+}$ influx from the medium or efflux from SR or by activation of the extrusion systems. Other previous studies have shown that TPEN can strongly inhibit the L-type Ca$^{2+}$ channel current (Turan, 2003) and thereby inhibit Ca$^{2+}$ influx to the cells. As is known, in cardiac cells, Ca$^{2+}$ influx through dihydropyridine receptors is necessary for contractions, and Ca$^{2+}$-induced Ca$^{2+}$ release from SR is thought to be the dominant mechanism (Evangelou and Kalfakakou, 1993). However, inhibition of Ca$^{2+}$ influx may explain the prevention of intracellular calcium overload but cannot explain the decrease of Ca$^{2+}$ after Ca$^{2+}$ overload.

**TPEN Decreases [Ca$^{2+}$], Overload by Activation of Na$^{+}$/Ca$^{2+}$ Exchanger.** We examined the hypothesis that a decrease of cytosolic calcium by TPEN may occur due to removal of Ca$^{2+}$ by activation of SERCA2a or Na$^{+}$/Ca$^{2+}$ exchanger (NCX). We applied TPEN the moment after [Ca$^{2+}$], increase, so the role of L-type Ca$^{2+}$ channel current inhibition became irrelevant. We have shown that TPEN decreased [Ca$^{2+}$] in the cytosol after caffeine application, which not only induces Ca$^{2+}$ efflux from SR but also closed Ca$^{2+}$ influx to SR (Bassani et al., 1994). These data were confirmed by the direct inhibition of the SERCA2a by thapsigargin. The lowering of cytosolic [Ca$^{2+}$] by TPEN in the presence of the SERCA2a inhibitor thapsigargin suggests that SERCA2a is not mediated in decrease of [Ca$^{2+}$] in cytosol. However, TPEN did not decrease [Ca$^{2+}$], after elevation in the absence of Na$^{+}$ in the buffer medium, conditions which inhibit the NCX. These results suggest that TPEN prevents Ca$^{2+}$ overload via activation of the NCX.

Earlier in our laboratory, it was shown that elevation of extracellular [Ca$^{2+}$], from 1.9 to 3.9 mM increased [Ca$^{2+}$], and transformed the synchronized cell movement to an asynchronous one (Manoah et al., 1997). We used this model in our study and found that addition of 1 $\mu$M TPEN after elevation of [Ca$^{2+}$], returned the beating of cells to the normal rate and promoted synchronization of cell contractions. In conclusion, in vivo antiarrhythmic and antiarrhythmic effects of TPEN are demonstrated here. Given the cardioprotective properties of TPEN against ischemia-reperfusion-induced arrhythmias, therapeutically useful doses for i.v. administration are defined. In vitro results suggest that TPEN activates the NCX, but not SERCA2a, to work more effectively and in this way preserve homeostasis of Ca$^{2+}$ during hypoxia. TPEN protects cardiac cells against Ca$^{2+}$ overload damage during hypoxia by activating Ca$^{2+}$ surplus extrusion to the medium and not by regulating Ca$^{2+}$ influx to the SR. Thus, TPEN has considerable therapeutic potential for use in some surgical cardiovascular procedures. TPEN may be useful in the future as an antiarrhythmic and antiarrhythmic drug. TPEN has the potential to protect against Ca$^{2+}$ overload damage caused by ischemia and cardioactive drugs such as anticancer treatments (Alloatti et al., 1998; Shneyveys et al., 2001; Wang et al., 2001; Suter et al., 2004; Yeh et al., 2004). Additionally, this study opens a new direction for a therapeutic strategy by direct activation of the NCX forward mode to circumvent the problems of Ca$^{2+}$ overload in other excitable cells such as the central nervous system (Pettty and Wettstein, 1999).

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**References**


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