Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4680–4684, June 1991
Medical Sciences

Cardiac reperfusion damage prevented by a nitroxide free radical

(ischemia/spin label/hydroxyl radical/superoxide dismutase mimic/heart)

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Communicated by Morris E. Friedkin, February 26, 1991

ABSTRACT **Experimental evidence is presented that directly links ischemia/reperfusion injury to the formation of oxygen-derived free radicals. 2,2,6,6-Tetramethylpiperidine-*N*-oxyl (TEMPO)—a stable nitroxide radical that disproportionates superoxide radicals and oxidizes reduced metal ions required for OH· formation—was tested for its ability to prevent reperfusion damage in the isolated rat heart subjected to regional ischemia. Severe reperfusion arrhythmia—ventricular fibrillation and ventricular tachycardia—were prominent in control hearts, and their duration was significantly reduced by the presence of 0.4 or 1 mM TEMPO. TEMPO also repressed both postischemic release of lactate dehydrogenase and OH· formation. TEMPO slowed the heart rate, but compensatory pacing did not alter the dramatic effect of the nitroxide on reperfusion arrhythmia. TEMPO was partially protective when introduced at the end of ischemia but had no effect when added 1 min into reperfusion. It was concluded that both reperfusion arrhythmia and cell damage were directly related to oxidative damage incurred during the critical first minute of reperfusion. TEMPO strongly protected against reperfusion injury by preventing the formation of OH· and not by decreasing heart rate or by direct suppression of arrhythmia.**

Considerable evidence suggests that oxygen-derived free radicals are involved in ischemia/reperfusion injury (as reviewed in refs. 1 and 2). Increased postischemic susceptibility to oxygen radical damage results from the build-up of a strongly reducing environment during ischemia, along with a decreased antioxidant defense capacity (ref. 1 and references therein). The mechanism of tissue damage involves the stepwise reduction of molecular oxygen to the relative inactive superoxide and hydrogen peroxide, with subsequent metal ion-catalyzed formation of the highly reactive hydroxyl radical, the ultimate tissue toxicant (3–7). Alternative pathways involving HOCl (6) or the cupryl or ferryl ions (8) have been suggested.

Therapeutic strategies have attempted to reduce free radical damage either by intervening in their formation process or by scavenging free radicals already formed. Varying degrees of protection have been obtained with (*i*) OH· scavengers (9–11) and spin traps (12, 13), (*ii*) chelators of iron and copper (11, 14, 15), (*iii*) antioxidant enzymes such as superoxide dismutase and catalase (9–11), and (*iv*) redox-metal displacement by zinc (16, 17). The practical application of these compounds is limited, however, by their toxicity and side effects, their solubility, persistence and cell penetration, the requirement for preischemic loading of the tissue, and the incomplete protection they provide even in the best cases. Evidence presented here demonstrates unequivocally that ischemia/reperfusion tissue damage is caused by OH· and can be prevented.

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Nitroxide spin labels exhibit superoxide dismutase-mimicking properties (18–21), oxidize reduced metal ions (21), and react with carbon-centered free radicals (22). Such activities suggest a prime role for nitroxides in the prevention of biological free radical damage. Furthermore, the low toxicity of most nitroxides (23) would make them good candidates for *in vivo* use. So far nitroxide spin labels have been shown to prevent free radical damage dramatically in cell cultures (18, 19, 21, 24). However, protective effects of the nitroxide radicals have not been investigated in intact organs or in whole animals.

In the present study, 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO)—a stable nitroxide free radical with antioxidant properties—was used to probe the events involved in reperfusion injury and postischemic free radical formation in isolated rat hearts. This model conveniently expresses ischemia/reperfusion injury in readily measurable cardiac arrhythmia. While the precise causative mechanism of the arrhythmia is not known, free radicals have been proposed as an initiating agent (1, 9, 11, 13).

It was observed that TEMPO decreased oxygen radical formation and protected against postischemic reperfusion arrhythmia and cell damage, provided the nitroxide was present at the time of reperfusion.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (250–350 g) were obtained from Charles River Breeding Laboratories or Taconic Farms. All rats were allowed at least 3 days of in-house acclimatization with ad libitum access to standard laboratory food and water.

Reagents. TEMPO was obtained from Aldrich. Sodium salicylate and 2,5-dihydroxybenzoic acid (DHBA) were from Sigma. All other chemicals were either of HPLC or reagent grade and were obtained from standard sources. Distilled deionized water was used throughout.

Experimental Conditions. Rat hearts were obtained and perfused as described (16), according to the method of Langendorff (25). The perfusate was a modified Krebs–Henseleit (KH) buffer (118 mM NaCl/6.1 mM KCl/3.0 mM $CaCl_2$/0.5 mM Na_2EDTA/1.2 mM $MgSO_4$/25 mM $NaHCO_3$/1.0 mM NaH_2PO_4/11.1 mM glucose). The perfusate was gassed with 95% O_2/5% CO_2 for oxygenation and to maintain pH at 7.4. TEMPO (0.4 or 1 mM) was added to the KH buffer from a 4 M stock solution containing 40% (vol/vol) ethanol. Control experiments were performed to determine the effect of 0.01% (≈2 mM) ethanol—the highest concentration added with TEMPO. For assessment of OH· formation, KH buffer was supplemented with 0.1 mM sodium salicylate, previously shown not to affect cardiac function (26). All perfusion

Abbreviations: TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; LDH, lactate dehydrogenase; DHBA, 2,5-dihydroxybenzoate; VF, ventricular fibrillation; VT, ventricular tachycardia; NSR, normal sinus rhythm; bpm, beats per min.

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buffers were prepared the day of the experiment and were filtered through a 0.2-μm filter. Cardiac electrical activity and hemodynamic function were monitored as described (16, 26).

Experimental Protocols. Two models of cardiac ischemia/reperfusion injury were used: (*i*) the regional ischemia model using reversible occlusion of the left anterior descending coronary artery (16), and (*ii*) the normothermic global ischemia model involving cessation of flow to the heart (26). In regional ischemia experiments, an initial 10-min equilibration period with KH buffer was followed by a 10-min perfusion with the test buffer, 10 min of regional ischemia, and 5 min of reperfusion. Cardiac rhythm was monitored continuously. In an otherwise identical experiment, heart rate was controlled by ventricular pacing (Medtronic model 5320 rapid atrial pulse generator) at a rate of 330 beats per min (bpm) during equilibration and 310 bpm during ischemia. Pacing was discontinued 30 sec before initiation of reperfusion. In some experiments, hearts were perfused with KH, and TEMPO was introduced 30 sec before (R − 30) or 60 sec after (R + 60) the end of ischemia.

In the normothermic global ischemia model, an initial 10-min equilibration period with KH buffer was followed by an additional 10-min perfusion with the test buffer, 10 min of "no-flow" normothermic global ischemia, and 30 min of reperfusion. Pulmonary artery effluent samples were collected and analyzed for lactate dehydrogenase (LDH) activity and DHBA content.

Classification of Arrhythmia. A heart was considered to be in ventricular fibrillation (VF) if an irregular undulating baseline was present on the electrocardiogram. Ventricular tachycardia (VT) was defined as three or more consecutive premature ventricular contractions. This classification included repetitive monomorphic VT and ventricular flutter, which is difficult to differentiate from rapid VT. An alternative definition of sustained VT as 30 or more premature ventricular contractions or VT deteriorating into VF did not alter the quantitation of VT appreciably, and thus the two terms are used interchangeably. A heart was considered to be in normal sinus rhythm (NSR) if normal sinus complexes occurring in a regular rhythm were present on the electrocardiogram. This included supraventricular tachycardia as well as sinus bradycardia. A fourth classification group included other arrhythmias that did not fit into the three major groups, such as individual premature ventricular contractions, couplets, bigeminy, trigeminy, etc.

Cell Damage. Release of intracellular enzymes was used as an indicator of cell damage. The pulmonary artery effluent was assayed for LDH activity by the unit system of Racker, as described by Bergmeyer *et al.* (27).

OH$^{\cdot}$ Formation. OH$^{\cdot}$ formation was determined by the formation of DHBA isomers from salicylate. DHBA was separated by HPLC and quantitated by electrochemical detection as originally described by Floyd *et al.* (28) and modified by Powell and Hall (26).

Statistical Analysis. Statistical analysis for all parameters of cardiac function was performed as described (16). DHBA measurements at seven points in time (including the equilibration value as time 0) were analyzed by using a logarithmic transformation of the raw data, based on an observed linear association between mean DHBA and its standard deviation. The data were analyzed by using a repeated measures analysis of covariance, in which the "within" factor was time, which ranged from time 1 through time 6, and the covariate was the DHBA value at time 0.

RESULTS

Effect of TEMPO on Ischemia/Reperfusion Damage. The effect of TEMPO on reperfusion arrhythmia was examined in the isolated rat heart. Isolated hearts were equilibrated with the perfusion buffer, subjected to 10 min of regional ischemia, and reperfused. Four treatments were compared: KH buffer, KH buffer with 0.4 or 1 mM TEMPO, and KH buffer with 0.01% (≈2 mM) ethanol—as provided with the highest concentration of TEMPO. The occurrence of reperfusion arrhythmias was monitored (Fig. 1). In control hearts, perfused with KH buffer, 80% of the 5-min reperfusion period was spent in severe cardiac arrhythmia (VF and VT), and only 20% was spent in NSR. TEMPO was dramatically protective, limiting the duration of the severe reperfusion arrhythmia, and extending the duration of NSR to 65% at 0.4 mM and 90% at 1 mM TEMPO. Ethanol alone had a small effect on the duration of severe reperfusion arrhythmia, extending NSR to 35%. While the duration of severe reperfusion arrhythmia was decreased by TEMPO in a dose-dependent manner, the incidence of arrhythmia was significantly reduced only by the highest TEMPO concentration (Table 1).

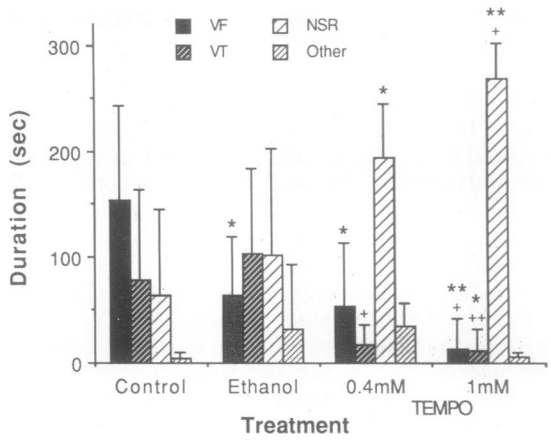

FIG. 1. Effect of TEMPO on the duration of reperfusion arrhythmia. Isolated rat hearts were perfused with either KH buffer alone (control), KH buffer plus 0.01% ethanol, or KH buffer plus TEMPO (0.4 or 1 mM). All hearts were equilibrated for 10 min and subjected to 10 min of regional ischemia followed by 5 min of reperfusion, during which time cardiac rhythms were quantitated. The values depicted represent the mean (±SD) of 8–10 experiments. *, $P < 0.05$; **, $P < 0.01$ (*t* test) when compared to control. +, $P < 0.05$; ++, $P < 0.01$ (*t* test) when compared to ethanol.

The incidence of NSR at the conclusion of a 5-min reperfusion is presented in Fig. 2. TEMPO, but not ethanol, supported the spontaneous reversion of hearts to NSR.

The assessment of cardiac function during the equilibration phase showed that TEMPO consistently affected the heart rate, but not other functional parameters. In the presence of 0.4 and 1 mM TEMPO, the heart rate decreased ≈15% and ≈30%, respectively, during the 10-min equilibration period. Ethanol alone had no effect on the hemodynamic parameters.

Table 1. Incidence of reperfusion arrhythmia after regional ischemia

Treatment	VF	VT	NSR
Control	11/11	11/11	6/11
Ethanol (0.01%)	8/8	8/8	6/8
TEMPO (0.4 mM)	9/9	9/9	9/9
TEMPO (1 mM)	2/10**	4/10*	10/10
Pacing			
Control	10/10	10/10	3/10
TEMPO (1 mM)	8/10	10/10	10/10*
Delayed TEMPO			
R − 30 sec	8/8	8/8	8/8
R + 60 sec	8/8	8/8	6/8

Significance vs. control and ethanol (Fisher's exact test): *, $P < 0.05$; **, $P < 0.01$.

Effect of TEMPO on Reperfusion Arrhythmia in Paced Hearts. The severity of cardiac ischemia/reperfusion injury has been related to the heart rate during ischemia—the higher rates resulting in increased damage (29, 30). The possibility that TEMPO protected through its negative chronotropy was investigated by pacing all hearts uniformly during the equilibration and ischemic phases. Pacing was discontinued 30 sec before reperfusion to allow the observation of reperfusion arrhythmia. The duration of severe arrhythmia and NSR in paced control and TEMPO-treated hearts (Fig. 3) was essentially the same as in their unpaced counterparts (Fig. 1). In the control group a shift was noted from VF to VT, the latter being the predominant arrhythmia in the paced hearts. In the TEMPO-treated group the incidence of arrhythmia was changed by pacing, with more paced hearts having very short and spontaneously reverting episodes of VF (Table 1).

As in unpaced hearts, the final rhythm differed in paced control and TEMPO-treated hearts (Fig. 2). All hearts treated with 1 mM TEMPO spontaneously reverted to NSR, compared to only 30% of the controls ($P < 0.05$). The protection by TEMPO was thus not related to its negative chronotropy.

Effect of Postischemic TEMPO Introduction. It is conceivable that TEMPO functions as an antiarrhythmic drug, suppressing the arrhythmia directly. This was investigated by introducing TEMPO after the arrhythmia had developed, 1 min into reperfusion (R + 60). Alternatively, TEMPO was introduced 30 sec before reperfusion (R − 30), to be present at the time of reintroduction of oxygen, but not preloaded in the tissue. The patterns of arrhythmia (Fig. 4) and the final rhythm (Fig. 2) in the R + 60 group were practically identical to the control group. Partial protection, roughly equivalent to that obtained with 0.4 mM TEMPO, was observed in the R − 30 group. Thus, TEMPO was not directly antiarrhythmic, and its presence during the first minute of reperfusion was essential for its protective effect.

Release of LDH and OH· Formation. The biochemical indicators of cellular damage (LDH release) and OH· production (DHBA formation from salicylate) were determined in globally ischemic hearts. The involvement of the whole heart in the ischemia/reperfusion cycle gave higher concentrations of the indicators in the effluent perfusate (and therefore more accurate detection), and eliminated variability in the amount of tissue affected by regional ischemia. Hearts were subjected to 10 min of no-flow global ischemia and were then reperfused. The effluent perfusion buffer was collected and assayed for LDH activity and for DHBA. Preischemic LDH release [0.59 unit·min^{-1}·g^{-1} (dry weight)] was not significantly affected by TEMPO [0.88 unit·min^{-1}·g^{-1} (dry weight)] or ethanol [0.85 unit·min^{-1}·g^{-1} (dry weight)]. The postischemic release of LDH from control and ethanol-treated hearts was increased ≈5-fold over the equilibration period (Fig. 5). The presence of 1 mM TEMPO limited the postischemic increase in LDH release to 3-fold. The significance of this trend was obscured by variability. The presence of TEMPO and ethanol did not interfere with the assay for LDH activity.

DHBA was observed in the effluent from all hearts, during both equilibration and reperfusion. The mean log DHBA changed significantly with time ($P < 0.045$) in all groups. In KH-perfused hearts, postischemic DHBA production was increased up to 80% over preischemic levels (Fig. 6). The presence of 1 mM TEMPO decreased the preischemic DHBA formation almost to half the control value. This concentration of TEMPO also completely abolished the postischemic increase in DHBA formation, with all reperfusion values below the already low equilibration level. Ethanol alone did not affect preischemic DHBA formation but limited the postis-

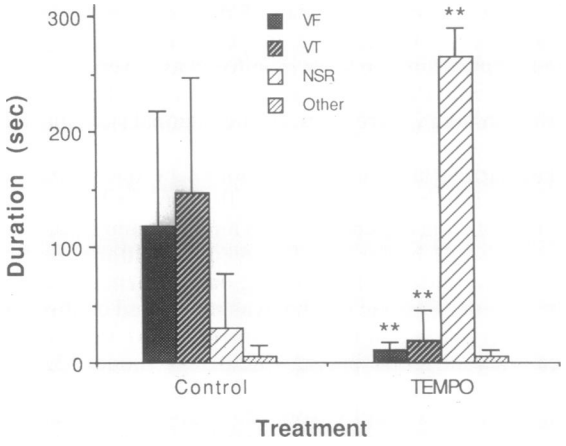

FIG. 3. The effect of TEMPO on reperfusion arrhythmia in paced hearts. Isolated rat hearts, perfused with KH buffer with or without 1 mM TEMPO as described in Fig. 1, were paced at 330 bpm during equilibration and at 310 bpm during ischemia. Pacing was discontinued 30 sec before reperfusion and the duration of cardiac rhythms was quantitated. The values depicted represent the mean (±SD) of 10 experiments. **, $P < 0.01$ (t test) when compared with control.

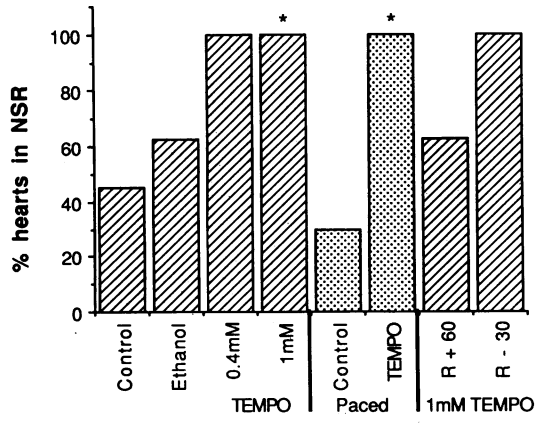

FIG. 2. Incidence of NSR after 5 min of regional ischemia. Hearts were treated as described in Figs. 1, 3, and 4. The incidence of NSR at the end of the 5-min reperfusion period is presented as a percentage of hearts tested in each experimental group. *, $P < 0.05$ (Fisher's exact test) when compared to control.

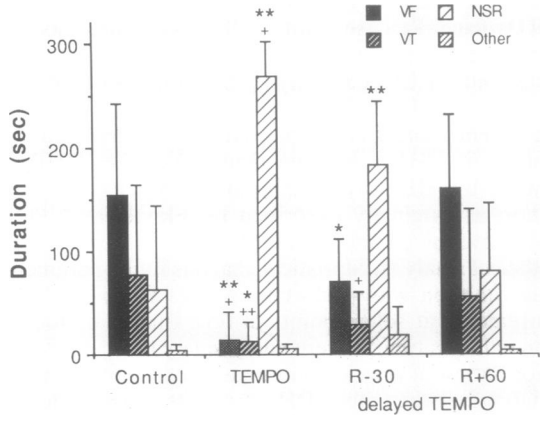

FIG. 4. Effect of postischemic TEMPO delivery on reperfusion arrhythmia. Isolated rat hearts were equilibrated with KH buffer, subjected to 10 min of regional ischemia, and reperfused. TEMPO (1 mM) was introduced either at the start of equilibration (TEMPO), 30 sec before (R − 30), or 60 sec after (R + 60) the start of reperfusion. Cardiac rhythms were quantitated during the 5-min reperfusion period. The values depicted represent the mean (±SD) of 8–11 experiments. *, $P < 0.05$; **, $P < 0.01$ (t test) when compared to control. +, $P < 0.05$; ++, $P < 0.01$ (t test) when compared to ethanol.

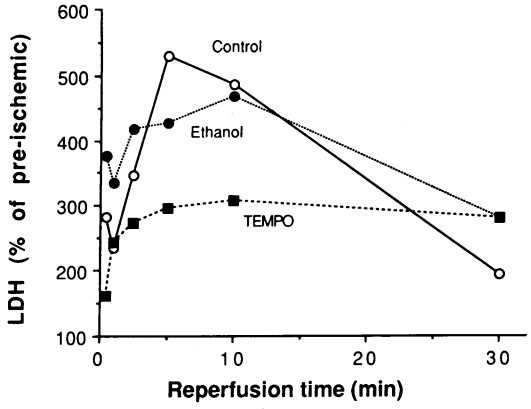

FIG. 5. Effect of TEMPO on release of LDH from postischemic rat hearts. Hearts were equilibrated 10 min with KH buffer, KH plus 1 mM TEMPO, or KH plus 0.01% ethanol, subjected to 10 min of normothermic no-flow global ischemia, and reperfused. The pulmonary effluent was assayed for LDH activity at various times during equilibration and reperfusion. The results are presented as a percentage of the preischemic (end of equilibration) LDH activity. The values depicted represent the mean of six to eight experiments.

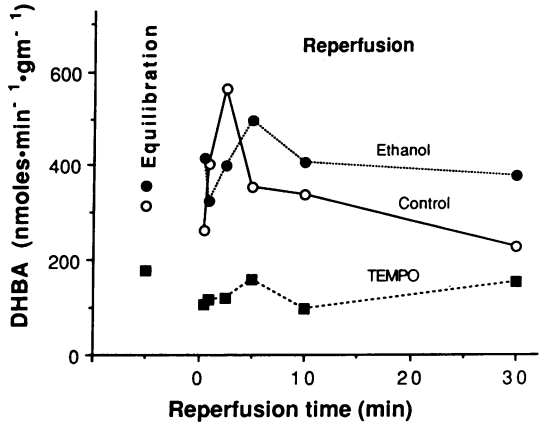

FIG. 6. Effect of TEMPO on OH• production. Hearts were treated as described in Fig. 5, with the inclusion of 0.1 mM salicylate in the buffers. The pulmonary effluent was assayed for DHBA at the indicated times. The values depicted represent the mean of six experiments. The statistical analysis is presented in *Results*.

chemic increase in DHBA formation to 40% (not significantly different from control). The difference between the TEMPO-treated group and both the control and ethanol-treated group was highly significant ($P < 0.003$).

DISCUSSION

The present investigation demonstrates that the development of arrhythmia and cardiac cell damage in the reperfused isolated rat heart correlate with OH• formation. TEMPO—a stable nitroxide free radical—protects against reperfusion arrhythmia and cell damage in the postischemic heart by preventing OH• formation, provided it is present during the critical first minute of reperfusion.

The ability of TEMPO to protect against reperfusion arrhythmia was dramatic and consistent by all criteria used: duration, incidence, and the spontaneously developed final rhythm after 5 min of reperfusion. This protective effect was dose dependent and not attributable to the ethanol provided with the TEMPO. A side effect of the treatment was a drop in heart rate, reaching 30% at 1 mM TEMPO. It has previously been reported that the severity of reperfusion damage is related to the heart rate during the ischemic period (29, 30). However, the elimination of this difference by pacing the control and TEMPO-treated hearts at a set rate during equilibration and ischemia did not alter the protective effect of TEMPO, indicating that the protection was not derived from the negative chronotropic effect. Pacing diminished the variation in incidence and duration of reperfusion arrhythmia within test groups, as seen in the more uniform occurrence of very short episodes of VF in paced TEMPO-treated hearts and lower standard deviations in the duration of the arrhythmia (incidence, 8/10; average duration for 10 hearts, 10.3 ± 8.1 sec), as opposed to rare but prolonged episodes in the parallel unpaced group (incidence, 2/10; average duration for all 10 hearts, 13.7 ± 28.2 sec).

The suppressive effect of TEMPO on reperfusion arrhythmia could be due to either a direct antiarrhythmic activity, protection from ischemic damage incurred by the deprivation of oxygen, or repression of a reperfusion-related event such as free radical damage. The latter has been strongly implied in the causation of ischemia/reperfusion damage (1, 2). The lack of protection by TEMPO introduced 1 min after the onset of reperfusion, at a time when arrhythmia had already developed, indicated that TEMPO was unable to directly repress arrhythmia. The nitroxide thus appeared to act on the cause of the arrhythmia, rather than on subsequent damage. The partial protection observed with TEMPO introduced toward the end of the ischemic period, thus made available to the ischemic part of the hearts only at reperfusion, may be interpreted two ways: (*i*) cell damage is incurred both by anoxia and by reoxygenation, and only the latter is prevented by TEMPO; (*ii*) all cell damage is incurred by reoxygenation but the protection provided by TEMPO introduced at reperfusion was incomplete. In either case, the effect of TEMPO introduced at the onset of reperfusion and the lack of effect of TEMPO introduced 1 min later pinpoints the critical event in the causation of reperfusion damage to the first minute of reperfusion.

TEMPO equilibrates rapidly between the extra- and intracellular compartments (31). The partial protection provided by TEMPO introduced at the start of reperfusion suggests that the equilibration of oxygen between the extra- and intracellular compartments is faster than that of TEMPO, allowing complete reoxygenation before the maximal intracellular TEMPO concentration is attained. This suggestion assumes that reperfusion damage in the Langendorff heart is an intracellular event, a point supported by the low and variable protection obtained with externally supplied superoxide dismutase and catalase (9–11).

Cell damage, as evidenced by the leakage of LDH from heart cells, was decreased by the presence of TEMPO but not by ethanol. This correlated well with the protection provided by the nitroxide against the development of arrhythmia and suggests a common origin for these two manifestations of ischemia/reperfusion damage.

The formation of DHBA in control hearts increased during reperfusion, as previously reported for 20- and 40-min ischemia (26), albeit at lower levels, indicating an accelerated postischemic production of OH•. TEMPO decreased the preischemic background level of DHBA almost to half the value seen in control hearts and completely prevented the postischemic surge in OH• production. This is consistent with its functions as a superoxide dismutase mimic and an oxidant of reduced redox-active metal ions—both functions acting to prevent OH• formation (18–21). The decrease in OH• production in the presence of TEMPO correlated well with the prevention of reperfusion arrhythmia and the decreased leakage of LDH—supporting a causative relationship between free radical formation and reperfusion injury.

The TEMPO-dependent protection of isolated rat hearts from free radical damage extends the reported effect of

nitroxides in bacteria and mammalian cell cultures (18, 19, 21, 24) to whole organs. The dramatic protective activity, high efficiency, and apparent rapid cell penetration of TEMPO, along with low toxicity, suggest that a clinical application of nitroxides may be feasible. TEMPO has a distinct advantage in its rapid equilibration in the tissue, thus maintaining a high degree of activity even when introduced at the onset of reperfusion. This contrasts with other approaches, which have generally required preloading into tissues, rendering the treatment effective only in anticipated ischemia. Limitations on the biological use of TEMPO may be imposed by its negative chronotropy observed in this study and an anesthetic effect previously reported (32). Nevertheless, the present observations point to a very promising family of antioxidant agents, which may include compounds with the same protective properties as TEMPO but without its undesired side effects.

The authors thank Miss Donna Hall for excellent technical assistance and Martin Lesser, Ph.D., and Marge Goldstein, M.Ph., of the division of Biostatistics at North Shore University Hospital, for assistance in statistical analysis of the data. We appreciate the assistance of Mrs. Chava Bondy, Baruch Berkowits, Ph.D., and Mr. John Neglia of Medtronic, Inc., in providing the pacemaker for this study. This study was supported by American Heart Association Grant-in-Aid 890731 and National Institutes of Health Grants S07RR05924 (Biomedical Research Support Grant) and AM-1238.

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