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Effect of nitric oxide and nitroxide SOD-mimic on the recovery of isolated rat heart following ischemia and reperfusion

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Abstract

Nitric oxide synthesized from L-arginine in cells has important salutary physiological roles, but can also exert deleterious effects. Nitric oxide (NO) can ameliorate post-ischemic reperfusion myocardial injury, yet formation from NO and $O_2^{\bullet -}$ of peroxynitrite and its downstream toxic products, such as $^{\bullet}OH$, $^{\bullet}NO_2$ and $CO_3^{\bullet -}$, can ultimately exacerbate reperfusion damage. Nitroxide stable radicals, such as 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TPL), unlike SOD, readily penetrate cells and catalytically remove intracellular $O_2^{\bullet -}$. Hence, nitroxides by virtue of catalytic removal of $O_2^{\bullet -}$ would be expected to diminish the adverse effect of NO and lower post-ischemic reperfusion cardiac damage. We show that post-ischemic recovery of hemodynamic functions of isolated perfused rat hearts treated with L-arginine or TPL alone did not differ from that of the control hearts. However, the recovery of hearts treated with the combined regimen of L-arginine and TPL was significantly improved, e.g. the Work Index = (left ventricular developed pressure × heart rate) recovered to 92 ± 1.6% (L-arginine and TPL) vs. 59.4 ± 5.4% (Control), 60 ± 2.9% (L-arginine) and 53.3 ± 4.3% (TPL) of the pre-ischemic value; mean ± SEM, N = 10, $P < 0.001$. The enhanced recovery of hemodynamic function of hearts treated with L-arginine and TPL was accompanied by an increased recovery of oxygen consumption during the reperfusion. The combined regimen of L-arginine and TPL reduces the negative effects of NO by either inhibiting the production of $ONOO^-$ or through reaction with $CO_3^{\bullet -}$ and $^{\bullet}NO_2$ radicals formed during the decomposition of peroxynitrite in the presence of bicarbonate, thus promoting cardioprotection following post-ischemic reperfusion.

Keywords: Langendorff; Peroxynitrite; Carbonate radical; Superoxide; Dityrosine; Kinetics; Nitroxide; Ischemia; Reperfusion

1. Introduction

Release of radical and non-radical deleterious species during myocardial post-ischemic reperfusion contributes to endothelial damage by impairing production of vasoactive substances such as NO, increasing direct regulatory injury to myocardial contractility and augmenting platelet aggregation [1–4]. At the cellular level reactive species formed upon reperfusion initiate various pathophysiological processes [5,6]. NO, which is produced from O_2 and L-arginine by a Ca^{2+}-dependent NO synthase (NOS) in various tissues including the myocardium [7], can have both beneficial and harmful effects [8]. For instance NO can function beneficially as a vasodilator, but can inhibit enzyme activity by reacting with functional groups or co-factors, mainly thiols and iron ions [9,10]. Experiments, performed with isolated rat hearts, have shown that L-arginine and several NO-donors (e.g. GEA-3162) can decrease the damage resulting from post-ischemic reperfusion (I/R) [11–15]. On the other hand, NO and its reaction products also have negative effects on I/R [16,17]. Substances, which release NO, e.g. nitroprusside, were found to exert negative effects on the Frank–Starling response, thereby lowering cardiac output [17], whereas radical scavengers positively affect recovery of ischemic heart upon reperfusion [18,19]. NG-monomethyl-L-arginine, an inhibitor of NOS, was found to inhibit the production of peroxynitrite and improve the recovery of the isolated heart following I/R [20].

Upon the onset of reperfusion, the increased flux of $O_2^{\bullet -}$ radicals, which react with NO at a diffusion-controlled

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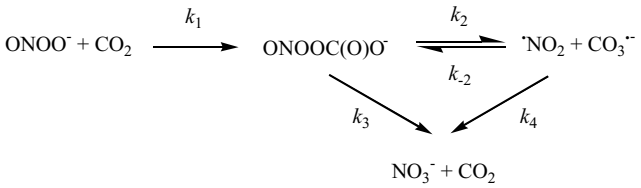

Scheme 1. Reactions (1)–(4) describe the mechanism for the interaction of peroxynitrite with CO_2.

rate, enhances the formation of $ONOO^-$ [21]. Peroxynitrite ions are quite stable but decompose upon protonation (such as during ischemia) through homolysis along the weak O–O bond ($k_{obs} = 1.2\ s^{-1}$, 25°) yielding about 28% $^{\bullet}OH$ and $^{\bullet}NO_2$ free radicals, while the remainder collapses to nitrate in the solvent cage [21,22]. Peroxynitrite reacts relatively fast with CO_2 ($k_1 = (2.9 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$ at 24° [23]). Therefore, in biological systems where high concentrations of CO_2 prevail most of the peroxynitrite is scavenged by CO_2. Presumably CO_2 reacts with NO to form $ONOOC(O)O^-$, where upon it undergoes homolysis to yield about 33% $^{\bullet}NO_2$ and $CO_3^{\bullet -}$ free radicals (Scheme 1) [24].

The carbonate radical is less reactive but much more selective than the hydroxyl radical and, therefore, potentially more toxic. Since the Krebs–Henseleit solution used for perfusion of isolated heart contains 25 mM of $NaHCO_3$ and is saturated with 95% O_2 and 5% CO_2, the carbonate and the nitrogen dioxide radicals that are formed within the heart might exert a deleterious effect.

The cell-permeable nitroxides, which catalytically remove $O_2^{\bullet -}$ [25] and detoxify redox-active metal ions [26], have been previously found to inhibit I/R injury in several experimental models [27,28]. Zeltcer *et al.* [27,29] showed that TPL prevented metal-induced exacerbation of I/R injury, by rendering transition metal ions redox-inactive thus preempting Fenton chemistry rather than by catalyzing the dismutation of $O_2^{\bullet -}$. The present study tested the effect of TPL and NO on the recovery of isolated rat heart. Using pulse radiolysis for studying the kinetics of TPL we found that TPL effectively scavenges carbonate radical. We suggest that the nitroxide attenuates the negative effects of NO and improves post-ischemic cardiac recovery by detoxification of carbonate radicals.

2. Materials and methods

2.1. Materials

L-Tyrosine, 4-hydtoxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TPL), $CuCl_2$, sodium nitrilotriacetate (NTA), L-arginine, and chelating resin Chelex 100 (iminodiacetic acid) were obtained from Sigma Chemical Co. The phosphate buffers are generally contaminated with redox-active transition metals such as copper and iron. Since the metals exacerbate the I/R injury the results might greatly depend on the source and purity of the buffers [30]. To avoid artifactual effects all the buffers have been treated with Chelex 100 to remove metals and, unless otherwise stated, a known and defined concentration of copper has been included in the buffer [27,29]. Solution of KH_2PO_4 (16.3 g/100 mL distilled MilliQ treated water) was added to 10 g of Chelex 100 and stirred over night at 4°. The slurry was filtered and the filtrate was autoclaved and stored at 4° for use in the KH solution.

2.2. Langendorff perfusion system

Male Sprague–Dawley rats weighing 280 ± 20 g were used for perfusion experiments in accord with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Sodium heparin (500 U) was injected into the peritoneum of the rats, which were subsequently anesthetized with nembutal (30 mg per animal). The hearts were immediately removed and placed in heparinized ice-cold saline solution. The aorta was cannulated to a Langendorff perfusion apparatus and the pulmonary artery was cut open to provide drainage.

Retrograde aortic perfusion was maintained with Krebs–Henseleit (KH) solution: 118 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11.1 mM glucose [31]. In many studies the metal content and particularly the trace fraction of redox-active "catalytic" metal ions in the buffer were neither defined nor controlled and can possibly exacerbate the I/R damage [30]. Therefore, $Cu(NTA)_2$ (10 μM final concentration) was added to KH_2PO_4 solution to maintain a known metal concentration in the system [27,29]. Unless otherwise stated, the KH used for perfusion contained 0.3 mM L-tyrosine, which renders copper redox-inactive and serves as a marker for carbonate radical [20]. KH was saturated with 95% oxygen and 5% CO_2. Aortic perfusion was maintained at 37° and a pressure of 90 cm H_2O (66.2 mmHg).

2.3. Experimental protocol

Each group contained 10 hearts. All hearts underwent 25 min of perfusion with KH, 20 min of no-flow global ischemia (at 37°) followed by 45 min of KH reperfusion (Control). In the second group 1 mM L-arginine was included in the KH solution during the last 5 min of pre-ischemic perfusion and throughout the reperfusion period. In the third group 0.1 mM TPL was included throughout the reperfusion. The fourth group was treated by a combination of the treatments of groups 2 and 3. Thus, L-arginine was added during the last 5 min of pre-ischemic perfusion and thereafter throughout the reperfusion period, whereas TPL was included throughout the reperfusion [15].

2.4. Hemodynamic parameters

A latex balloon tipped catheter was inserted through a small cut in the left atrium and advanced through the mitral valve into the left ventricle. The balloon was connected through a pressure transducer to a recording system (Hewlett Packard 7758B). The balloon was inflated with water and equilibrated to give an end-diastolic pressure of 0 mmHg. Left ventricular systolic and diastolic pressures and time derivatives of pressure were measured during contraction ($+dP/dt$) and relaxation ($-dP/dt$). Left ventricular developed pressure (LVDP) was calculated from the difference between the systolic and diastolic pressures. Coronary flow was measured by collecting the coronary effluent drained through the pulmonary artery in the pre-ischemic and reperfusion period. If during pre-ischemic perfusion an arrhythmia developed, thrombus formed or LVDP was less than 60 mmHg, then that particular heart was excluded from the study.

2.5. Cardiac consumption of oxygen

Samples of effluent were drained from the aorta and pulmonary artery at the end of the pre-ischemic perfusion and at the end of the reperfusion periods. Oxygen tensions were measured using a blood gas analyzer (AVL 993). Oxygen consumption was calculated according to the formula by Neely *et al.* [32]. Recovery for each parameter during reperfusion at any given time was compared with the respective pre-ischemic value and expressed as a percentage of that value. At the end of reperfusion, hearts were dried in an oven (90°) and dry mass determined.

2.6. Dityrosine released into coronary effluent

Peroxynitrite and its downstream active species have been previously shown to give rise to dityrosine [33,34], which is fluorescent. The dityrosine that accumulated in the coronary effluent was assessed by fluorescence intensity ($\lambda_{ex} = 320$ and $\lambda_{em} = 410$ nm [20]) at room temperature using the Kontron Instruments SFM 25 Spectrofluorometer. The pulmonary artery effluent was collected during the pre-ischemia, first 2 min of post-ischemia (each sample collected during 15 s) and afterwards at 5, 10, 20, 30, and 45 min of reperfusion. The results were presented as relative fluorescence intensity in comparison to that measured in pre-ischemic perfusion.

2.7. Pulse radiolysis

To study a rapid reaction as that of carbonate radical with TPL, a fast kinetics methodology was required. Pulse radiolysis experiments were carried out with a Varian 7715 linear accelerator with 5-MeV electron pulses of 0.5 μs and 200 mA. A 200 W Xe–Hg lamp produced the analyzing light. A cut-off filter, which does not transmit light below 400 nm, was used for all measurements in a 4-cm Spectrosil cell (room temperature) using three light passes. The kinetics of the reaction of $CO_3^{\bullet -}$ radical with TPL were studied upon pulse-irradiation of N_2O-saturated solutions containing 0.1 M sodium carbonate and 0.05–1 mM nitroxide at pH 10.7 or 2 mM nitrite and 4 mM phosphate buffer at pH 7.0, respectively.

2.8. Statistical analysis

All data are presented as mean ± SEM. Kruskal–Wallis non-parametric ANOVA test with Dunn's *post hoc* test were used to compare values measured for the different experimental groups. Statistical differences having $P < 0.05$ were considered to be significant.

3. Results

3.1. Recovery of hemodynamic function and oxygen consumption

Fig. 1 presents the extent of recovery within the four groups studied of several hemodynamic parameters, including left ventricular developed pressure (LVDP), pressure derivative at maximal contraction ($+dP/dt$) and coronary flow (CF) at the end of 45 min of reperfusion. The values measured for the three groups Control, L-arginine, and TPL did not differ from each other but significantly differed ($^*P < 0.05$) from that of the L-arginine and TPL group. Hence, the recovery of the group receiving both L-arginine and TPL was significantly higher than that of the

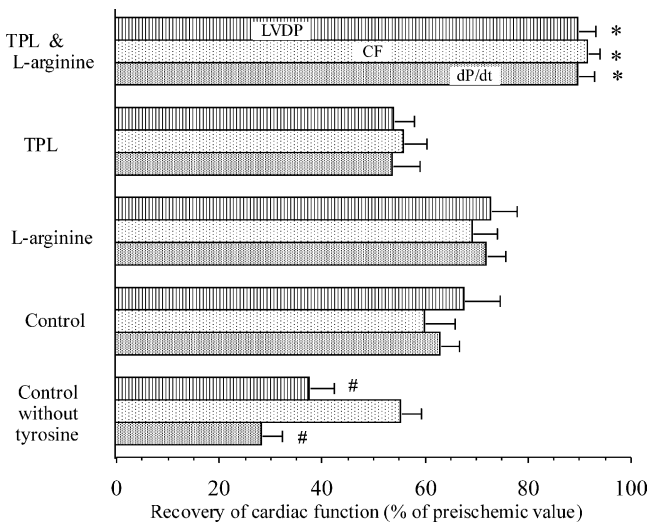

Fig. 1. Recovery of cardiac function following reperfusion. Four experimental groups of 10 hearts each were studied: Control; L-arginine; TPL; and L-arginine and TPL. The hemodynamic parameters: left ventricular developed pressure (LVDP), coronary flow (CF), and pressure derivative at maximal contraction ($+dP/dt$) are presented as percentage of their pre-ischemic values (mean ± SEM), which were: LVDP, 160±10.8 mmHg; CF, 10.7 ± 1 mL min^{-1} and $+dP/dt$, 3290 ± 220 mmHg s^{-1}.

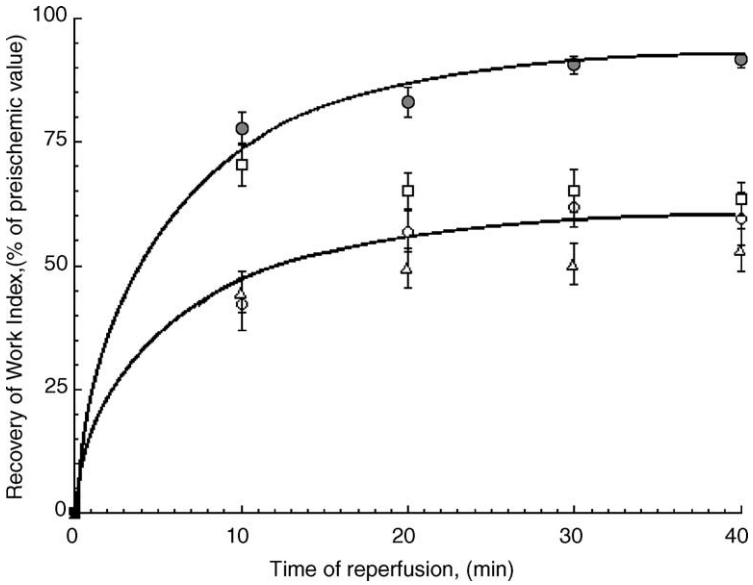

Fig. 2. The time-dependent recovery of cardiac Work Index. The relative values of Work Index = LVDP × HR, during reperfusion (mean ± SEM) presented as percentage of its pre-ischemic value, which was 32,300 ± 2900 mmHg × beat per minute. Four experimental groups of 10 hearts each were studied: Control (○); L-arginine (□); TPL (△); L-arginine and TPL (●). The values measured (mean ± SEM) for the L-arginine plus TPL group were significantly different, $P < 0.05$, from all other three groups.

other groups. The respective heart rate for all experimental groups did not differ significantly from each other (data not shown). An additional control group in which tyrosine was omitted from the perfusate was included in the experiments. In this group the post-ischemic recovery of the heart was less (Fig. 1), which indicates that Cu(II) ions increase the I/R injury in the absence of tyrosine, as previously found [27,29].

The recovery of the Work Index = LVDP × HR during the entire reperfusion is presented in Fig. 2. The lower solid line curve describes the kinetics of recovery for all three experimental groups: Control, L-arginine, and TPL. The high Work Index measured at time = 10 min can be considered more as experimental scattering rather than a genuine increase in recovery that later fades away. The kinetics of recovery of the Work Index measured for hearts treated with L-arginine and TPL are significantly different from the other three groups, which indicates improved recovery.

The recovery of myocardial oxygen consumption ratio (post-ischemic/pre-ischemic) was significantly higher in the L-arginine and TPL group than in the other three experimental groups: Control, L-arginine and TPL groups (mean ± SEM, $P < 0.001$, Fig. 3). The extent of recovery did not significant change amongst the three groups.

3.2. Dityrosine in coronary effluent

The relative fluorescence of the dityrosine released during the reperfusion is presented in Fig. 4. The lowest level of dityrosine in the coronary effluent was measured for hearts treated with L-arginine and TPL ($P < 0.01$ vs. Control, L-arginine and TPL), whereas the highest was measured for hearts treated with L-arginine alone. In addition, most of dityrosine release occurred during the first 3 min of reperfusion (Fig. 4).

3.3. Kinetics

The kinetics of the reaction of $CO_3^{\bullet -}$ or $^{\bullet}NO_2$ with TPL were studied upon pulse-irradiation of N_2O-saturated solutions containing 0.1 M sodium carbonate and 0.05–1 mM nitroxide at pH 10.7 or 2 mM nitrite and 4 mM phosphate buffer at pH 7.0, respectively. Under these conditions the following reactions take place (given in parentheses are the radiation-chemical yields of the species, defined as the

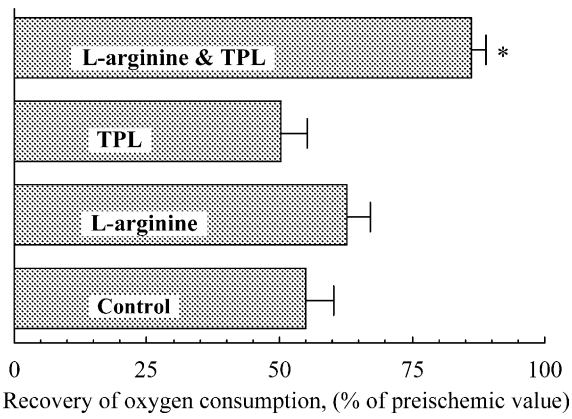

Fig. 3. Recovery of the cardiac consumption of oxygen following reperfusion. The values (mean ± SEM for 10 hearts of each experimental group) are presented as percentage of the pre-ischemic value, which was 1.9 ± 0.2 mmol O_2/h per gram dry weight. The values measured for the three groups Control, L-arginine and TPL did not differ from each other, but significantly differed ($^{*}P < 0.05$) from that of the L-arginine plus TPL group.

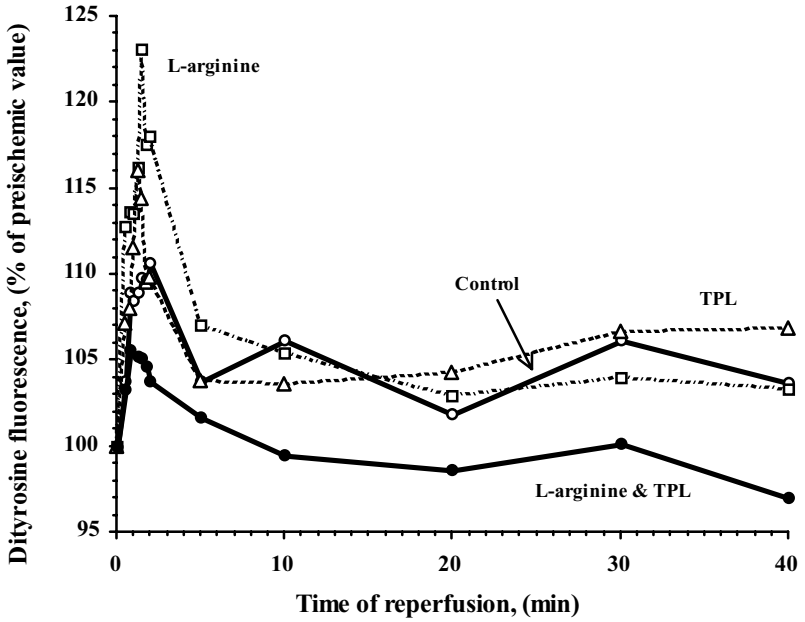

Fig. 4. Time dependence of peroxynitrite formation upon reperfusion. The accumulation of dityrosine in the coronary effluent during reperfusion has been followed by measuring its fluorescence (Ex, 320 nm; Em, 410 nm). The relative fluorescence intensity of dityrosine is presented as percentage of its pre-ischemic value. Each point represents the mean of values measured for 10 hearts. Control (○, solid line); L-arginine (□, dashed-dotted line); TPL (△, dashed line); L-arginine and TPL (●, dotted line). The error bars are not presented in order to avoid obscuring the figure.

number of species produced by 100 eV of energy absorbed):

$$H_2O \xrightarrow{\gamma} e_{aq}^{-}(2.6), {}^{\bullet}OH(2.7), H^{\bullet}(0.6), H_3O^{+}(2.6), H_2O_2(0.72) \quad (5)$$

$$e_{aq}^{-} + N_2O \rightarrow N_2 + OH^{-} + {}^{\bullet}OH \quad (6)$$

$${}^{\bullet}OH + CO_3^{2-} \rightarrow CO_3^{\bullet-} + OH^{-} \quad (7)$$

$$CO_3^{\bullet-} + TPL \rightarrow \text{products} \quad (8)$$

$${}^{\bullet}OH + NO_2^{-} \rightarrow {}^{\bullet}NO_2 + OH^{-} \quad (9)$$

$${}^{\bullet}NO_2 + TPL \rightarrow \text{products} \quad (10)$$

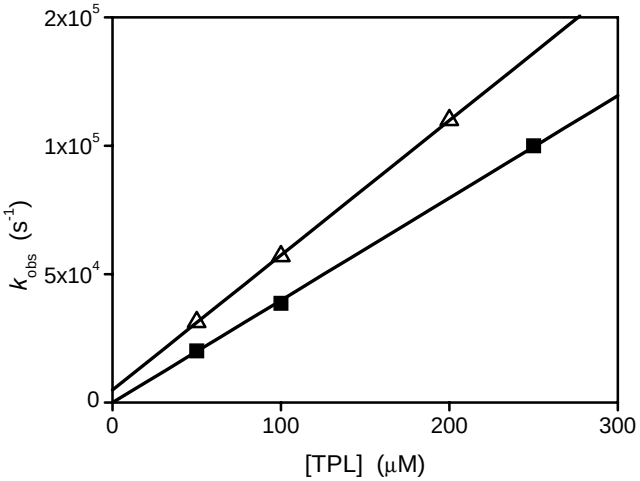

Fig. 5. Dependence of the observed first-order rate constant for the decay of $CO_3^{\bullet-}$ (■) or ${}^{\bullet}NO_2$ (△) on the concentration of TPL. All solutions where saturated with N_2O and contained 0.1 M sodium carbonate (pH 10.7) or 2 mM nitrite and 4 mM phosphate buffer (pH 7.0), respectively. The dose rate was 13.7 and 30 Gy per pulse, respectively.

The decays of $CO_3^{\bullet-}$ or ${}^{\bullet}NO_2$ were followed at 600 nm ($\varepsilon_{600} = 1860\ M^{-1}\ cm^{-1}$) or at 400 nm ($\varepsilon_{400} = 200\ M^{-1}\ cm^{-1}$), respectively, in the presence of excess concentrations of TPL and were found to obey first-order kinetics. Plots of k_{obs} were linearly dependent on the concentration of the TPL (Fig. 5), and from the slopes of the lines we determined $k_8 = (4.0 \pm 0.1) \times 10^8\ M^{-1}\ s^{-1}$ and $k_{10} = (5.3 \pm 0.1) \times 10^8\ M^{-1}\ s^{-1}$. The latter values suggest that TPL is an efficient scavenger of $CO_3^{\bullet-}$ and ${}^{\bullet}NO_2$ radicals.

4. Discussion

The involvement of NO in a system is generally tested by employing NOS-inhibitors or NO-releasing agents. In the present study cardiac NOS in hearts perfused with a buffer that contained L-arginine were anticipated to produce greater flux of NO and consequently yield more peroxynitrite. The I/R-induced injury in isolated hearts treated with L-arginine was accompanied by an increase in dityrosine release into the effluent. Obviously, the experimental model of the perfused isolated rat heart greatly differs from the *in vivo* situation, since the continuous washout of the heart, perfused only with buffer, leaves it practically devoid of L-arginine.

Particularly, under low concentration of L-arginine, inducible NOS can catalyze the production of superoxide [35–37]. Consequently, the supplementation of the perfusate with L-arginine is expected to provide protection both through an increased production of NO and a decrease of superoxide formation from NOS source. The present results demonstrate that a combination of L-arginine and

TPL, but not each of them given separately, significantly enhanced the recovery of post-ischemic hemodynamic and metabolic cardiac function. Also, the lowest level of dityrosine was measured in hearts treated with L-arginine and TPL. The failure of L-arginine to improve the recovery of the heart following I/R indicates that in the present experimental model the potential adverse effect of elevated NO production counterbalanced its beneficial activity.

Nitroxides have been previously found to provide protection against I/R injury [38]. Likewise, it has been demonstrated that iron and copper ions potentiate I/R injury, whereas nitroxide prevented the *metal-induced exacerbation* of I/R injury alone [27,29]. In the previously reported systems, TPL did not function as a SOD-mimic but rather acted to preempt the metal driven Fenton-like reaction [27,29]. Under the present experimental conditions (Fig. 1), tyrosine most probably coordinates the copper ions and inhibits Fenton-like chemistry. The latter suggestion is substantiated by previous finding that copper–tyrosine complex injected in mice effectively inhibited lipid peroxidation [39]. As seen in Figs. 1–3, TPL provided protection only when L-arginine was included in the perfusate, i.e. in the present model system TPL protected against metal-independent damage.

Dityrosine and 3-nitrotyrosine (3-NT) are formed when peroxynitrite reacts with CO_2 in the presence of tyrosine through reactions (11)–(14) and Scheme 1 [40]:

$$\mathrm{TyrOH} + \mathrm{CO_3^{\bullet -}} \rightarrow \mathrm{TyrO^{\bullet}} + \mathrm{HCO_3^{-}}, \quad k_{11} = 4.5 \times 10^7\ \mathrm{M^{-1}\,s^{-1}} \tag{11}$$

$$2\mathrm{TyrO^{\bullet}} \rightarrow \text{dityrosine}, \quad 2k_{12} = 4.5 \times 10^8\ \mathrm{M^{-1}\,s^{-1}} \tag{12}$$

$$\mathrm{^{\bullet}NO_2} + \mathrm{TyrOH} \rightarrow \mathrm{NO_2^{-}} + \mathrm{TyrO^{\bullet}} + \mathrm{H^{+}}, \quad k_{13} = 3.2 \times 10^5\ \mathrm{M^{-1}\,s^{-1}}(\mathrm{pH}\,7.4) \tag{13}$$

$$\mathrm{^{\bullet}NO_2} + \mathrm{TyrO^{\bullet}} \rightarrow \text{3-NT} + \text{products}, \quad k_{14} = 3 \times 10^9\ \mathrm{M^{-1}\,s^{-1}} \tag{14}$$

It has been also shown that the yield of dityrosine is highly dependent on the flux of peroxynitrite, i.e. the yield increases at the expense of 3-NT when the flux of peroxynitrite decreases [40]. In the present study, the increase in dityrosine was observed only in the first minutes of the reperfusion, thus confirming previous findings of oxygen-derived radical burst early upon reperfusion [41,42].

Since NOS in the perfused heart, does not function at its V_{max} unless sufficient L-arginine is provided, hearts supplemented with L-arginine yielded higher amount of dityrosine early in the reperfusion (Fig. 4). The increase in dityrosine reflected greater flux of NO, which in the presence of $O_2^{\bullet -}$ yields peroxynitrite and consequently $CO_3^{\bullet -}$ and $^{\bullet}NO_2$. Hence, TPL can destroy $O_2^{\bullet -}$, and thus prevent the production of peroxynitrite and/or scavenge $CO_3^{\bullet -}$ (reaction (8)) and $^{\bullet}NO_2$ (reaction (10)) and thus inhibit both the formation of dityrosine and 3-NT. A plausible explanation for the protection provided by TPL for hearts treated with L-arginine would be the catalytic removal of superoxide and consequent preemption of peroxynitrite production. It is not simple to define the activity of TPL under our experimental conditions as catalytic effect. The question of ''catalyst vs. scavenger'' obviously involves *also* the definition of native SOD as a catalyst. In enzymatic systems the substrate is generally present at a much higher concentration than that of the enzyme. Conversely, the cellular concentration of native SOD, which is at the range of 0.1–10 μM, is about 1,000,000-fold *higher* that the cellular steady state concentration of its substrate, namely superoxide radical. In other words at any given moment about 999,999 out of a 1,000,000 SOD molecules are practically idle. Consequently, no K_M value could have ever been determined for SOD. Can one define SOD, under such conditions, as a catalyst? The answer is given by the difference between the *production flux* and the *steady-state concentration* of superoxide radicals. The cellular concentration of SOD, which removes superoxide without being depleted, is far lower than the integral over time of the production flux of superoxide in the system. For these reasons SOD can be considered as a catalyst. The same argument holds for nitroxide SOD-mimics [43]. However, considering the TPL concentration in the present system (0.1 mM) and its catalytic rate constant for superoxide dismutation (about $10^6\ \mathrm{M^{-1}\,s^{-1}}$ [44]) the nitroxide might not effectively compete with NO ($(3.5-6.7) \times 10^9\ \mathrm{M^{-1}\,s^{-1}}$ [21]) for the superoxide. Although the values k_8[TPL] and k_{10}[TPL] are higher than k_{11}[TyrOH] and k_{13}[TyrOH], whereas TPL completely prevented the formation of 3-NT, the present results are not sufficient to specify whether TPL protected by catalyzing the dismutation of $O_2^{\bullet -}$ or through their reaction with $CO_3^{\bullet -}$ and $^{\bullet}NO_2$.

5. Conclusions

The recovery of cardiac hemodynamic parameters treated with L-arginine and TPL correlate well with the metabolic function, i.e. the decrease in the yield of dityrosine and the improved recovery of oxygen consumption. The protective effect of the combined regimen of L-arginine and TPL can be explained as follows: the increased production of NO, together with the increased flux of $O_2^{\bullet -}$ upon the onset of reperfusion enhances the generation of peroxynitrite. The harmful effect of peroxynitrite counterbalances or even overshadows the beneficial activity of NO and accounts for the failure of L-arginine to provide protection. However, when L-arginine and TPL are included together in the perfusate, further protection is observed. Unlike native SOD that can remove superoxide and lower the extracellular production of peroxynitrite, the nitroxide can also eliminate the radical products of peroxynitrite formed both extra- and intracellularly. In the present study TPL protects either by catalyzing

the dismutation of $O_2^{\bullet -}$ or through its reaction with $CO_3^{\bullet -}$ and $^{\bullet}NO_2$ radicals generated from peroxynitrite. The inability of SOD and catalase to reach the intracellular compartment and to improve the recovery of cardiac function is consistent with the notion that the decrease in $O_2^{\bullet -}$ flux and therefore in $ONOO^-$ concentration within the extracellular compartment does not protect cardiac tissue. The ability of TPL to reach the intracellular compartment, most likely, results in its beneficial post-ischemic effect.

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