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Role of Nitric Oxide in the Reperfusion Induced Injury in Hyperthyroid Rat Hearts

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We recently reported that hyperthyroidism affects the heart response to ischemia/reperfusion. A significant tachycardia during reperfusion was associated with an increase in the oxidative stress of hearts from T_3-treated animals. In the present study we checked the possible role of nitric oxide (NO) in this major stress induced by the hyperthyroid state. We compared the functional recovery from ischemia/reperfusion of Langendorff preparations from euthyroid (E) and hyperthyroid (H, ten daily intraperitoneal injections of T_3, 10 μg/100 g body weight) rats, in the presence and in the absence of 0.2 mM N^{ω}-nitro-L-arginine (L-NNA). At the end of the ischemia/reperfusion protocol (10 min preischemic perfusion, 20 min global ischemia, 30 min reperfusion) lipid peroxidation, antioxidant capacity (C_A) and susceptibility to *in vitro* oxidative stress were determined on heart homogenates. The main effect of hyperthyroidism on the reperfusion functional response was confirmed to be a strong tachycardic response (154% recovery at 25 min reperfusion) accompanied by a low recovery in both left ventricular diastolic pressure (LVDP) and left ventricular dP/dt_{max}. This functional response was associated with a reduction in C_A and an increase in both lipid peroxidation and susceptibility to oxidative stress. Perfusion of hearts with L-NNA *per se* had small but significant negative chronotropic and positive inotropic effects on preischemic performance of euthyroid rat hearts only. More importantly, L-NNA perfusion completely blocked the reperfusion tachycardic response in the hyperthyroid rats. Concomitantly, myocardium oxidative state (lipid peroxidation, C_A and *in vitro* susceptibility to oxidative stress) of L-NNA perfused hearts was similar to that of E animals. These results suggest that the higher reperfusion-induced injury occurring in hyperthyroid animals is associated with overproduction of nitric oxide.

Keywords: Nitric oxide, hyperthyroidism, ischemia/reperfusion, heart injury, oxidative stress, nitric oxide synthase

Nitric oxide (NO) has been reported to function as both protective- and injury-producing factor in tissue ischemia/reperfusion. Evidence of an *in vivo* protective effect has been supplied by inhibition of leukocyte accumulation by NO-generating agents.[1,2] Mechanisms of a direct role of NO in protecting from ischemia/reperfusion injury are less clear.[2] A role of NO in the injury production, instead, has been suggested to result from the formation of reactive NO species, particularly peroxinitrite.[2,3] It is likely that the levels of both reactive oxygen species (ROS) and

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NO, combined with the antioxidant defence systems, have a major role in determining if NO would have defensive or harmful effects. In this respect, any condition that would induce an increase in both oxidative stress and NO production would favor NO-induced injury. Reperfusion combined with hyperthyroidism would be one such condition.

Hyperthyroid state in mammals is characterized by an increase of oxygen consumption associated with higher electron flux in mitochondrial respiratory chain,[4] a condition that may increase generation of ROS, such as superoxide radical (O_2^-) and/or hydrogen peroxide.[5] Hyperthyroidism accelerates free radical production,[6] and alters the antioxidant defence system in skeletal and heart muscle.[7] Lipid peroxidation increases in the heart of hyperthyroid rats[8,9] and we have shown that this increase is associated with a decrease in the heart antioxidant capacity and a higher susceptibility to *in vitro* oxidative stress.[9] Overproduction of nitric oxide during reperfusion has been implicated in myocardial ischemia/reperfusion injury. Regional ischemia in an *in vivo* model significantly stimulated both superoxide and NO production, the last associated with an increase in both total nitric oxide synthase (NOS) and iNOS activity.[10] Protective effects of NOS inhibitors on lipid peroxidation following ischemia/reperfusion[11] and post-hypoxia reoxygenation injury[12] have also been reported. Cooperative effects of overproduction of free radicals and nitric oxide have been implicated in atherosclerosis,[13] hypoxia/reoxygenation of cultured neurons,[14] lipid peroxidation and reperfusion injury in the heart.[3]

Previously, we have shown that the heart from hyperthyroid rats displays a significant tachycardic response, associated with an impaired inotropic recovery.[15] This increased myocardial contractile dysfunction during reperfusion was imputed to an increase of myocardial oxidative stress, as indicated by the protective effect of vitamin E.[15] In the present study we tested the possibility of involvement of NO in such hyperthyroidism-related tachycardic response during reperfusion of Langendorff preparations, by perfusing hearts from both euthyroid and hyperthyroid animals with N^{ω}-nitro-L-arginine (L-NNA), a specific NOS inhibitor.

MATERIALS AND METHODS

Animals, Preparation Setup and Equipment

Male Wistar rats (60 days old) were used in the experiments. The animals, purchased at weaning from Nossan (Correzzana, Italy), were housed in separate cages at $24 \pm 1°C$, with an artificial lighting cycle (LD 8–20 h). All animals were provided with water *ad libitum* and a commercial rat chow diet (Nossan). From day 50, animals were randomly assigned to one of two groups: E, euthyroid rats ($N = 12$, mean weight ± SEM, 239 ± 6 g); H, rats made hyperthyroid by treatment with daily intraperitoneal injections of T_3 (10 μg/100 g body weight) for 10 days ($N = 13$, mean weight ± SEM, 214 ± 3 g). Hearts from each of the animal groups were randomly assigned to two subgroups, according to their *in vitro* perfusion with basal saline or with L-NNA, as described below. The resulting four heart groups were E_c (controls from euthyroid rats, $N = 6$), H_c (controls from hyperthyroid rats, $N = 7$), $E_{L\text{-}NNA}$ (L-NNA treated from euthyroid rats, $N = 6$) and $H_{L\text{-}NNA}$ (L-NNA treated from hyperthyroid rats, $N = 6$).

Animals were anesthetized by intraperitoneal injection of chloral hydrate (40 mg/100 g body weight) combined with ether and subjected to electrocardiographic registration. After heparinization, a rapid thoracotomy was performed and the aorta cannulated retrogradely. The hearts were excised and flushed to get rid of blood for 1 min with Krebs–Henseleit (KH) buffer containing (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, EDTA 0.5, glucose 11, pH 7.4, and gassed with 5% CO_2 in O_2. The hearts were then perfused with the same buffer at

37°C under a pressure of 70 mmHg according to Langendorff. After stabilization and 15 min perfusion, hearts were subjected to global normothermic ischemia for 20 min and then reperfused for 25 min with KH oxygenated buffer. $E_{L\text{-}NNA}$ and $H_{L\text{-}NNA}$ heart perfusion was shifted from KH to KH containing 0.2 mM L-NNA 5 min before ischemia. Functional performance was determined just before ischemia and during reperfusion at 5 min intervals. In the $E_{L\text{-}NNA}$ and $H_{L\text{-}NNA}$ hearts the performance was also determined immediately before shifting to L-NNA perfusion. As functional performance during reperfusion usually reached its maximum between 15 and 20 min after the onset of reperfusion, the hemodynamic parameter values measured at 25 min reperfusion were considered as a measure of the functional recovery during reperfusion.[15]

Aortic pressure was recorded via a sidearm connected with a pressure transducer. Left ventricular pressure was measured using a Latex intraventricular balloon connected with a pressure transducer. Signals were acquired via an analogical-digital device and computer analyzed to get mean aortic pressure, left ventricular developed pressure (LVDP), left ventricular dP/dt_{max} and heart rate. Coronary effluent was collected and weighed for determination of coronary flow.

At the end of the perfusion protocol heart great vessels, valves, and atria were trimmed away and the ventricles were cut open, rinsed free of liquid, and weighed. Homogenates (20% w/v) were prepared with a Potter–Elvehjem homogenizer set at a standard velocity (500 rpm) for 2 min in 125 mmol/l KCl, 15 mmol/l Tris, pH 7.4. Tissue homogenates were used for analytical procedures.

Analytical Procedure

All chemicals used (Sigma Chimica, Milano, Italy) were of the highest grade available. The extent of peroxidative reactions was determined measuring thiobarbituric acid reactive substances (TBARS)[16] and the hydroperoxides (HP) according to Heath and Tappel.[17] The vitamin E content was determined using a high-performance liquid chromatography procedure.[18] Heart homogenate antioxidant capacity and response to oxidative stress were determined by using reagents and instruments of the commercially available Amerlite System (Johnson & Johnson, Cinisello Balsamo, Italy). Response to oxidative stress was determined as previously described.[19] In short, samples of 10% (w/v) homogenates were obtained by diluting the 20% homogenates with an equal volume of 0.2% Lubrol in 15 mmol/l Tris, pH 8.5. Further dilutions of samples up to a tissue concentration of 0.002% were prepared with 15 mmol/l Tris, pH 8.5. The assays were performed in microtiter plates. Enhanced chemiluminescent reactions were initiated by addition of 250 µl of the reaction mixture to 25 µl of the samples. To obtain the reagent mixture, an Amerlite Signal Reagent Tablet, containing excess of substrate (sodium perborate) and signal generating reagents (sodium benzoate, indophenol and luminol) was dissolved just before the reaction in the Amerlite Signal Reagent Buffer (pH 8.6). The plates were incubated at 37°C for 30 s under continuous shaking and then transferred to a luminescence analyser (Amerlite Analiser). The emission values were expressed as percent of the emission of a standard (25 µl of 22 ng/ml horseradish peroxidase) and were fitted to dose-response curves using the statistical facilities of a graphic program (FigP, Biosoft, Cambridge, UK).

The determination of the overall antioxidant capacity (C_A) was performed according to Venditti *et al.*[20] Briefly, the ability of the 10% homogenates (w/v) in 0.1% Lubrol to quench the light emission from a peroxidase-induced luminescent reaction was compared with that of solutions of a known antioxidant. Because the highest reproducibility in the results was demonstrated to occur with desferrioxamine as antioxidant,[20] this substance was used to determine the activity scale and C_A of tissue homogenate was

expressed as an equivalent concentration of desferrioxamine.

Statistical Analysis

The results are presented as mean values ± SEM. Effects of L-NNA on preischemic performance were tested with paired Student's *t*-test. The effects of animal treatment and L-NNA perfusion on functional recovery and oxidative stress indexes were analyzed by two way ANOVA followed by the Scheffé multiple comparison *post hoc* test to determine the statistical significance of differences between group means. Only the comparisons between T_3-treated vs. T_3-untreated or between L-NNA-perfused vs. controls perfused groups were taken into consideration. The comparisons $H_{L\text{-}NNA}$ vs. E and H vs. $E_{L\text{-}NNA}$ were not considered for their scarce relevance to the aims of this study. Probability values $(P) < 0.05$ were considered significant.

RESULTS

The hyperthyroid state of T_3-treated animals was reflected in their higher heart/body weight ratios ($E = 2.86 \pm 0.06$ mg/g; $H = 3.81 \pm 0.07$ mg/g, $p < 0.05$, unpaired Student's *t*-test) and *in vivo* heart rate ($E = 442 \pm 7$ beats/min, $H = 543 \pm 8$ beats/ min).

Basal preischemic coronary flow was significantly higher in the hearts from T_3-treated animals ($E = 11.9 \pm 1.3$ ml/min; $H = 8.98 \pm 0.6$ ml/min), while coronary flow expressed per g of tissue was not significantly different ($E = 13.3 \pm 1.0$ ml/min/g; $H = 14.4 \pm 1.3$ ml/min/g). Preischemic ventricular performance was also significantly affected by T_3 treatment of animals. Hyperthyroid animals displayed a higher intrinsic heart rate ($E = 269 \pm 15$ beats/min; $H = 326 \pm 21$ beats/min) and lower LVDP ($E = 88.3 \pm 4.6$ torr; $H = 61.3 \pm 5.3$ torr) and dP/dt_{max} ($E = 1086 \pm 59$ torr/s; $H = 789 \pm 94$ torr/s) values than euthyroid animals.

Effect of L-NNA on Preischemic Performance

Figure 1 displays the effects of 0.2 mM L-NNA on preischemic coronary flow and cardiac performance. A slight but significant vasoconstriction was observed in both euthyroid and hyperthyroid animals. The NOS inhibitor significantly affected ventricular performance of hearts from euthyroid animals. L-NNA induced a slight bradycardia accompanied by an increase in both LVDP and dP/dt_{max}. Interestingly, these inotropic and chronotropic effects of L-NNA were absent in the hearts from the hyperthyroid animals.

Functional Recovery from Ischemia/Reperfusion

Percent recovery values from 20 min ischemia of coronary flow and ventricular performance are reported in Figure 2. The main effect of T_3 treatment was a significant, strong tachycardia during reperfusion, with a heart rate, which was 154% than the preischemic value at 25 min reperfusion (Figure 2, control – dashed bars). This tachycardia was accompanied by a recovery in ventricular contractility (LVDP and dP/dt_{max}) significantly lower than in the euthyroid hearts. Control hyperthyroid hearts also displayed a significant low recovery of coronary flow: as shown in left-upper panel of Figure 2, less than 50% recovery was observed after 25 min reperfusion, against a 75% recovery for euthyroid hearts.

Perfusion with L-NNA did not affect recovery of coronary flow and heart rate in the euthyroid rat hearts, whilst it affected heart inotropism during reperfusion, as shown by the significant higher dP/dt_{max} recovery (Figure 2, +L-NNA, open bars). The tachycardic response to reperfusion of T_3-treated animals was abolished by L-NNA perfusion (Figure 2, +L-NNA, dashed bars). As a consequence, LVDP and dP/dt_{max} in the $H_{L\text{-}NNA}$ hearts were close to that of euthyroid hearts. The perfusion with L-NNA also increased significantly coronary flow recovery of hyperthyroid hearts up to the euthyroid level.

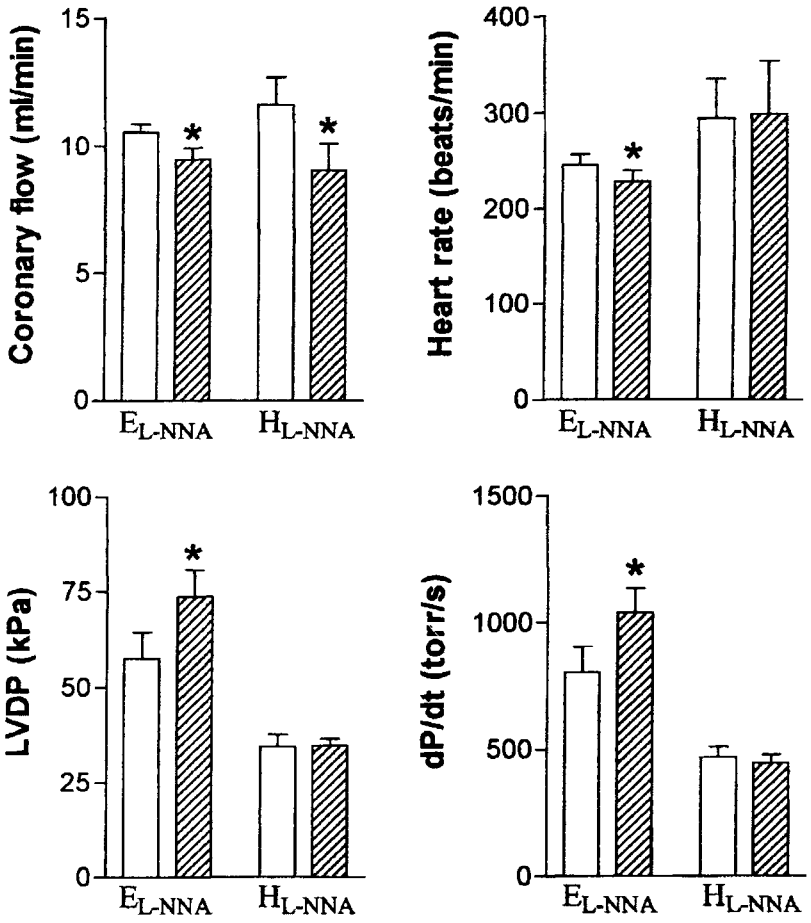

FIGURE 1 Effects of L-NNA on the preischemic coronary flow and left ventricular performance of Langendorff preparations from euthyroid ($E_{L\text{-}NNA}$) and hyperthyroid ($H_{L\text{-}NNA}$) animals. Coronary flow, heart rate, LVDP and LV$dP/dt_{max}$ were measured before (open bars: control perfusion) and 5 min after (dashed bars: L-NNA perfusion) switching of perfusion from basal saline (KH) to KH containing 0.2 mM L-NNA. Data are mean ± SEM of 6 (E) and 6 (H). Asterisks indicate a significant effect of L-NNA (paired *t*-test, $P < 0.05$). Control values were not significantly different from the preischemic values measured in the E and H groups (see Figure 2 legend).

Myocardial Antioxidant State and Lipid Peroxidation

While T_3 treatment did not affect tissue Vitamin E content, as measured at the end of the ischemia/reperfusion protocol, it significantly affected both total C_A and lipid peroxidation. C_A was lower in the H hearts than in the E hearts, while both TBARS and hydroperoxides were significantly higher (Table I).

L-NNA perfusion significantly reduced oxidative damage of myocardium following ischemia/reperfusion in both euthyroid and hyperthyroid rats. As shown in Table I, C_A was higher and lipid peroxidation was lower in the L-NNA perfused hearts than in the control perfused hearts.

Response of Tissue Homogenate to Oxidative Stress *In Vitro*

The response to *in vitro* oxidative stress of myocardium homogenates obtained from the hearts at the end of the ischemia/reperfusion protocol was

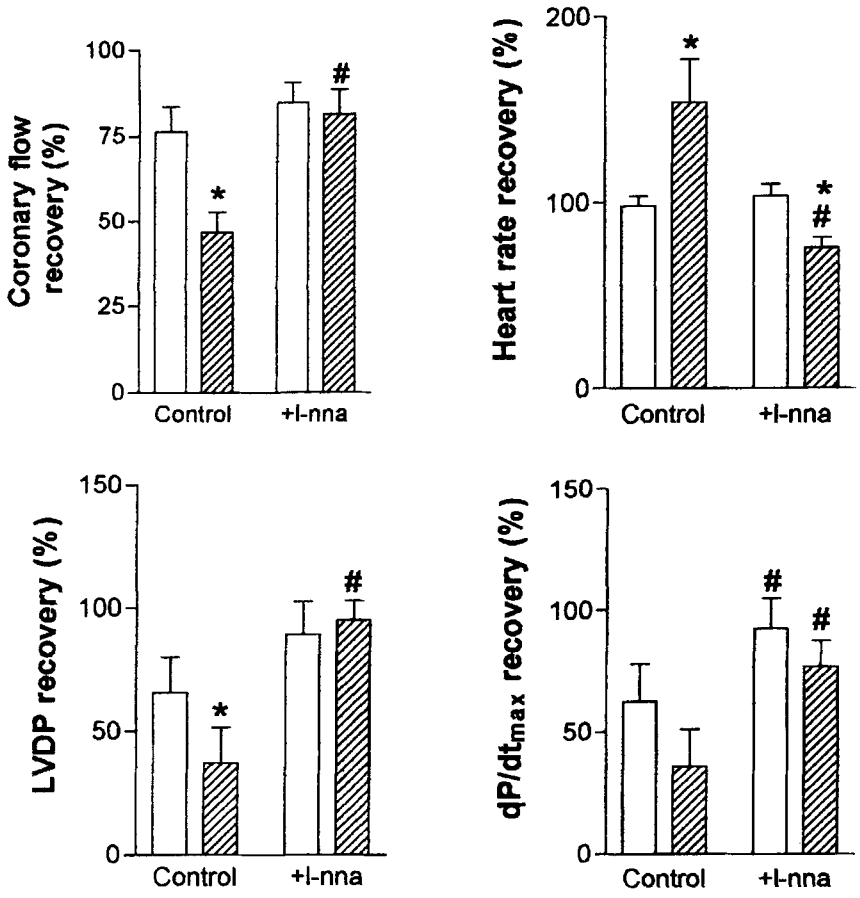

FIGURE 2 Reperfusion percent recovery of coronary flow, heart rate, LVDP and LV$dP/dt_{max}$ following 20 min ischemia in the experimental heart groups. Recovery was measured at 25 min reperfusion. Data are mean ± SEM. Preischemic values for L-NNA untreated hearts were: (E, open) coronary flow (9.26 ± 0.607 ml/min); heart rate (292 ± 16 beats/min); LVDP (92.37 ± 7.12 torr); dP/dt_{max} (1224 ± 112 torr/s); (H, dashed) coronary flow (10.38 ± 1.7 ml/min); heart rate (333 ± 18 beats/min); LVDP (65.02 ± 8.4 torr); dP/dt_{max} (714.93 ± 101 torr/s). Preischemic values for L-NNA treated hearts are in Figure I, dashed bars. Asterisks indicate a significant effect of T_3 treatment of animals; # indicate a significant effect of L-NNA perfusion of hearts (two way ANOVA plus Sheffé *post hoc*, $P < 0.05$).

tested in order to evaluate the effect of ischemia/reperfusion on the myocardium ability to face further oxidative stress. Table II reports the parameters describing the relationship between light emission (E) and homogenate concentration (C, g/100 ml) in a batch where the tissue was stressed with sodium perborate and in presence of a luminescent system sensitive to $^{\bullet}OH$ production.[21,22] Such relationship is biphasic (Figure 3), and fits to the following equation: $E = a \times C / \exp(b \times C)$. The coefficient a depends from tissue concentrations of iron ligands, catalyzing $^{\bullet}OH$ production, while b from tissue antioxidants, so that the maximal light emission ($E_{max} = a/e \times b$) is an index of tissue susceptibility to oxidative stress.[21,22] As shown by the E_{max} values reported in Table II, hyperthyroidism significantly reduced the ability of tissue homogenate from hearts submitted to ischemia/reperfusion to face *in vitro* oxidative stress. The higher E_{max} of the H groups was consequent to an increase in the a value, and a decrease in the b value. Interestingly, the perfusion of both euthyroid and hyperthyroid hearts with 0.2 mM L-NNA significantly reduced

TABLE I Effect of ischemia/reperfusion on Vitamin E (nmol/g wet mass), antioxidant capacity (C_A, expressed as equivalent concentration of desferrioxamine, mmol/l) and lipid peroxidation (malondialdehyde, MDA, nmol/g wet mass, and hydroperoxide, HP, nmol NADPH/g wet mass) in the isolated rat heart

Group	Vitamin E	C_A	Lipid peroxidation	
			MDA	HP
E	57.42 ± 2.53	0.57 ± 0.03	33.57 ± 3.27	0.82 ± 0.02
H	64.25 ± 3.89	0.44 ± 0.09^a	50.82 ± 3.28^a	0.96 ± 0.07^a
$E_{L\text{-}NNA}$	56.70 ± 0.76	0.73 ± 0.05^b	23.15 ± 0.84	0.80 ± 0.06
$H_{L\text{-}NNA}$	55.08 ± 5.17	0.79 ± 0.10^b	28.00 ± 0.75	0.84 ± 0.07

Values are the mean ± SEM. [a]Significant difference for T_3-treated animals vs. T_3-untreated animals ($P < 0.05$). [b]Significant difference for L-NNA-treated vs. L-NNA-untreated animals ($P < 0.05$).

TABLE II Effect of ischemia–reperfusion on the parameters characterizing light emission from homogenates of rat heart stressed with sodium perborate

Group	Parameters		
	a	b	E_{max}
E	5.42 ± 0.52	0.42 ± 0.05	4.94 ± 0.82
H	7.81 ± 0.50^a	0.32 ± 0.02	8.87 ± 0.23^a
$E_{L\text{-}NNA}$	3.35 ± 0.36^b	0.32 ± 0.03	3.99 ± 0.45^b
$H_{L\text{-}NNA}$	3.49 ± 0.47^b	0.33 ± 0.02	3.81 ± 0.50^b

Values are the mean ± SEM. The relation between light emission and heart homogenate is described by the equation: $E = a \times C/\exp(b \times C)$. Emission maximum ($E_{max}$) is given by $a/e \times b$. [a]Significant difference for T_3-treated animals vs. T_3-untreated animals ($P < 0.05$). [b]Significant difference for L-NNA-treated vs. L-NNA-untreated hearts ($P < 0.05$).

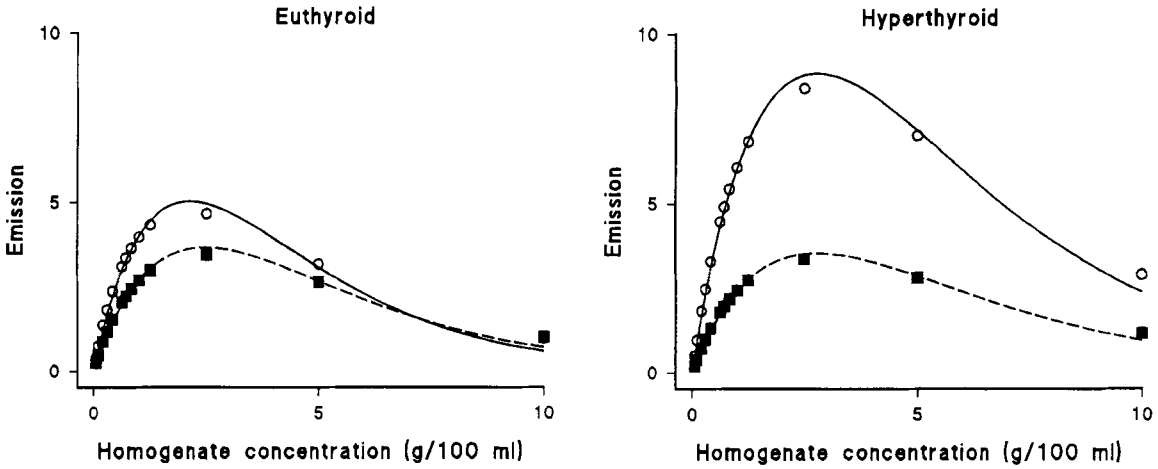

FIGURE 3 Effect of ischemia/reperfusion on *in vitro* response to oxidative stress of heart from euthyroid and hyperthyroid rats, perfused with control Ringer (solid lines) or with 0.2 mM L-NNA (dashed line). The tissue susceptibility to the stress was evaluated by determining the variations, with concentration of homogenates, of light emission from a luminescent reaction. Emission values are given as percentages of an arbitrary standard (44 ng/ml peroxidase). Curves are computed from the experimental data according to the equation: $E = a \times C/\exp(b \times C)$. Representative curves are drawn. See Table II for mean values of a, b and E_{max}.

the oxidative stress sensitivity of heart homogenates by reducing the a value.

DISCUSSION

In a recent study[15] we have reported that hearts from hyperthyroid animals displayed a significant tachycardia during reperfusion following 20 min global normothermic ischemia. This arrhythmic dysfunction induced by reperfusion was associated with a significant reduction of overall antioxidant capacity and vitamin E level, and an increase of lipid peroxidation, which states a T_3-induced oxidative stress. The fact that the tachycardic response during reperfusion of hyperthyroid hearts was due to a major oxidative stress was confirmed by the protective effect obtained by treatment of hyperthyroid animals with vitamin E.

In the present study we present strong evidence that the higher oxidative stress inducing tachycardia during reperfusion in the hyperthyroid rats was associated with NOS activity with possible overproduction of nitric oxide. The perfusion of hearts with the NOS inhibitor L-NNA allowed to prevent the tachycardic response, as well as all the changes in the oxidative stress indexes tested, and associated with reperfusion in hyperthyroid rat hearts.

Preischemic Effects of L-NNA

L-NNA *per se* induced vasoconstriction in both euthyroid and hyperthyroid hearts. This was in agreement with the widely described effects of NOS inhibitors on coronary resistance,[23–25] and indicates the presence of tonic release of nitric oxide in the preparation. Ventricular performance of euthyroid animals was affected by L-NNA perfusion (both LVDP and dP/dt_{max} were increased by the inhibitor) according to the known depressing effects of NO on heart contractility.[26] L-NNA also induced a slight bradycardia in the euthyroid rat hearts. Although *in vivo* NO donors induce tachycardia[27] and NOS inhibitors bradycardia,[28] these effects are considered the consequence of a baroreceptor reflex secondary to systemic pressure changes.[29,30] However, slight direct effects of NO on the heart, as suggested by our result, cannot be ruled out. Interestingly, these effects on myocardium were absent in the hyperthyroid animals. At the moment there is no straightforward explanation for this result. As the depressive inotropic effect of NO has been reported to be mediated by alteration in myofilament Ca^{2+} sensitivity,[26] it is possible that this effect is counteracted in the hyperthyroid state by the induction of a high ATPase activity myosin.[31]

NO and Reperfusion Injury

Our results show that L-NNA perfusion improves recovery of ventricular inotropism during reperfusion in the euthyroid rat hearts, adding to the increasing experimental evidence which has implicated nitric oxide overproduction in reperfusion injury.[10–12,32] This role of NO may involve its effects on myocardial mitochondria. NO decreases myocardial oxygen consumption and reduces ATP synthesis by reversible binding to cytochrome oxidase.[33] The inhibition of respiratory chain results in a greater superoxide anion production[34] and NO-derived species cause further damage of various mitochondrial proteins, such as the complexes I and II of the respiratory chain.[35] Furthermore, the oxidative stress induced by increased production of oxygen reactive species enhances the products of peroxidative reaction, such as 4-hydroxy-2-nonenal, that is involved in deactivation of functional proteins.[36]

The main result of the present study is the protective effect of L-NNA perfusion from the reperfusion-induced tachycardia of hyperthyroid rat hearts. This protective effect of L-NNA suggests that a main role is played by nitric oxide in such tachycardic response. This role may involve a direct effect of NO overproduction or NO interaction with free radicals, overproduced under hyperthyroidism.

A direct induction of tachycardia by NO during reperfusion implies stimulation of NOS in the hyperthyroid animals. Although Fernandez *et al.*[37] have recently reported that hyperthyroidism induces a significant increase in rat liver NOS, there is no evidence of NOS activity enhancement in the heart of hyperthyroid animals. On the other hand, ischemia/reperfusion *per se* seems to be a sufficient stimulus for NOS activity, both constitutive and inducible.[10] Moreover, it seems unlikely that NO displays substantial tachycardic effects, considering that in the present study L-NNA did not display chronotropic effects on the spontaneous preischemic heart rate of Langendorff preparations from the H group, and only slight bradycardic effects on preparations from the E group. In agreement with this conclusion, NO does not evoke chronotropic effect in

rat atrial preparations,[38] and is known to have cardioprotectant antiarrhythmic effects during reperfusion.[39]

In contrast, a mechanism involving NO interaction with free radicals appears more likely to occur. NO is a relatively unreactive free radical that may react with superoxide, producing the more dangerous peroxynitrite.[2] Hyperthyroidism induces free radicals overproduction combined with depletion of antioxidant defences.[5,9] Moreover, free radicals have a well-known role in the reperfusion-induced ventricular arrhythmias.[40] The potentially synergistic effects of simultaneous overproduction of NO and superoxide is suggested by the fact that half-life of NO and the relaxation of aorta rings by NO are enhanced by reduction in the concentration of superoxide radicals with SOD or SOD mimics.[41] The interaction between free radicals and nitric oxide has been reported to enhance both lipid peroxidation and reperfusion injury.[3]

Another mechanism, implying NOS activation but not NO overproduction, may involve an increased depletion of BH_4 (biopterin, a NOS cofactor) consequent to ROS overproduction in the hyperthyroid animals. Free radical dependent depletion of BH_4 has been reported to occur during ischemia/reperfusion.[42] It is known that when L-arginine or BH_4 are limiting, NOS utilizes oxygen as a terminal electron acceptor, consuming NADPH in a way which is uncoupled with NO production, and resulting in superoxide anion.[2] The combination of NOS stimulation by I/R, ROS overproduction by both I/R and hyperthyroidism and BH_4 inactivation by ROS, could amplify superoxide production with consequent major functional injury. Uncoupling of L-arginine metabolism from NO synthesis, with superoxide production, has been involved also in the low density lipoprotein induced dysfunction of endothelium, a primary defect in the premature development of atherosclerosis.[13]

The hypothesis of a major oxidative stress consequent to concomitant hyperthyroidism and I/R is supported by the effects of L-NNA perfusion on the oxidative state of hearts determined at the end of the ischemia/reperfusion protocol. L-NNA perfusion significantly protected from the higher lipid peroxidation in the hyperthyroid rat hearts following I/R, and this result was associated with a higher C_A and lower sensitivity to *in vitro* oxidative stress. In particular, the high value of the parameter *a* found in absence of L-NNA, indicates that the high susceptibility of heart homogenates to oxidative challenge depends mainly on a greater capacity to produce $^{\bullet}OH$ radicals.[9] It is unlikely that in the presence of NO the tissue content of substances catalyzing Fenton reaction is modified. The above higher susceptibility may depend, instead, on the lower capacity of such substances to cage the $^{\bullet}OH$ radicals formed at the point of metal binding.[43,44] Therefore, we can envisage that in the presence of NO overproduction, and particularly under hyperthyroidism, there is a relatively lower amount of cellular system effective in trapping the produced free radicals. This view is supported by the fact that hemoprotein oxidation associated with increased oxidative stress can lead to heme ring disruption and iron release.[22]

In conclusion, the results presented in the present study indicate that the concomitance of phenomena or conditions that increase the potential for oxidative stress may have synergistic effects and enhance the probability of heart dysfunction and injury. In particular, the concurrence of a higher oxidative stress associated with hyperthyroidism and ischemia/reperfusion seems to increase the potential for an injury producing role of nitric oxide.

References

[1] I. Kurose, R. Wolf, M.B. Grishman and D.N. Granger (1994) Modulation of ischemia/reperfusion-induced microvascular dysfunction by nitric oxide. *Circulation Research*, **74**, 376–382.

[2] S.S. Gross and M.S. Wolin (1995) Nitric oxide: pathophysiological mechanisms. *Annual Review of Physiology*, **57**, 737–769.

[3] G.L. Squadrito and W.A. Pryor (1998) Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radical Biology and Medicine*, **25**, 392–403.

[4] H.I. Schwartz and J.H. Oppenheimer (1978) Physiologic and biochemical actions of thyroid hormone. *Pharmacology and Therapeutics*, **3**, 349–376.

[5] V. Fernandez and L.A. Videla (1993) Influence of hyperthyroidism on superoxide radical and hydrogen peroxide production by rat liver submitochondrial particles. *Free Radical Research Communications*, **18**, 329–335.

[6] A. Swaroop and T. Ramasarma (1985) Heat exposure and hypothyroid conditions decrease hydrogen peroxide generation in liver mitochondria. *Biochemical Journal*, **226**, 403–408.

[7] K. Asayama and K. Kato (1990) Oxidative muscular injury and its relevance to hyperthyroidism. *Free Radical Biology and Medicine*, **8**, 293–303.

[8] K. Asayama, K. Dobashi, H. Hayashibe and K. Kato (1989) Vitamin E protects against thyroxine-induced acceleration of lipid peroxidation in cardiac and skeletal muscles in rats. *Journal of Nutritional Science and Vitaminology*, **35**, 407–418.

[9] P. Venditti, M. Balestrieri, S. Di Meo and T. De Leo (1997) Effect of thyroid state on lipid peroxidation, antioxidant defences, and susceptibility to oxidative stress in rat tissues. *Journal of Endocrinology*, **155**, 151–157.

[10] P. Liu, C.E. Hock, R. Nagele and P.Y.-K. Wong (1997) Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. *American Journal of Physiology*, **272**, H2327–H2336.

[11] B.C. Yang and J.L. Mehta (1997) Inhibition of nitric oxide dose does not affect reperfusion-induced myocardial injury, but it prevents lipid peroxidation in the isolated rat heart. *Life Science*, **61**, 229–236.

[12] G. Matheis, M.P. Sherman, G.D. Buckberg, D.M. Haybron, H.H. Young and L.J. Ignarro (1992) Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. *American Journal of Physiology*, **262**, H616–H620.

[13] K.A. Pritchard, L. Groszek, D.M. Smalley, W.C. Sessa, M. Wu, P. Villalon, M.S. Wolin and M.B. Stemerman (1995) Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circulation Research*, **77**, 510–518.

[14] C. Cazevieille, A. Muller, F. Meynier and C. Bonne (1993) Superoxide and nitric oxide cooperation in hypoxia/reoxygenation-induced neuron injury. *Free Radical Biology and Medicine*, **14**, 389–395.

[15] P. Venditti, P. Masullo, C. Agnisola and S. Di Meo (1999) Effect of vitamin E on the response to ischemia–reperfusion of Langendorff heart preparations from hyperthyroid rats. *Life Science* (in press).

[16] J.A. Buege and S.D. Aust (1978) Microsomal lipid peroxidation. *Methods in Enzymology*, **52**, 302–310.

[17] R.L. Heath and A.L. Tappel (1976) A new sensitive assay for the measurement of hydroperoxides. *Analitical Biochemistry*, **76**, 184–191.

[18] J.K. Lang, K. Gohil and L. Packer (1986) Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. *Analitical Biochemistry*, **157**, 106–116.

[19] S. Di Meo, P. Venditti, M.C. Piro and T. De Leo (1995) Enhanced luminescence study of liver homogenate response to oxidative stress. *Archives of Physiology and Biochemistry*, **103**, 187–195.

[20] P. Venditti, S. Di Meo, P. De Martino Rosaroll and T. De Leo (1995) Determination by enhanced luminescence technique of liver antioxidant capacity. *Archives of Physiology and Biochemistry*, **103**, 484–491.

[21] S. Di Meo, P. Venditti and T. De Leo (1996) Tissue protection against oxidative stress. *Experientia*, **52**, 786–794.

[22] P. Venditti, T. De Leo and S. Di Meo (1999) Determination of tissue susceptibility to oxidative stress by enhanced luminescence technique. *Methods in Enzymology*, **300**, 245–252.

[23] R.E.A. Smith, R.M.J. Palmer, C.A. Bucknall and S. Moncada (1992) Role of nitric oxide synthesis in the regulation of coronary vascular tone in the isolated perfused rabbit heart. *Cardiovascular Research*, **26**, 508–512.

[24] W.M. Chilian, L. Kuo, D.V. DeFily, C.J. Jones and M.J. Davis (1993) Endothelial regulation of coronary microvascular tone under physiological and pathophysiological conditions. *European Heart Journal*, **14**, Suppl. I, 55–59.

[25] C.J. Jones, D.V. DeFily, J.L. Patterson and W.M. Chilian (1993) Endothelium-dependent relaxation competes with alpha 1- and alpha 2-adrenergic constriction in the canine epicardial coronary microcirculation. *Circulation*, **87**, 1264–1274.

[26] R.A. Kelly, J.-L. Balligand and T.W. Smith (1996) Nitric oxide and cardiac function. *Circulation Research*, **79**, 363–380.

[27] H. Bohn, P.A. Martorana and K. Schonafinger (1992) Cardiovascular effects of the new nitric oxide donor, pirsidomine. Hemodynamic profile and tolerance studies in anesthetized and conscious dogs. *European Journal of Pharmacology*, **220**, 71–78.

[28] S.M. Gardiner, A.M. Compton, P.A. Kemp and T. Bennett (1990) Regional and cardiac haemodynamic effects of NG-nitro-L-arginine methyl ester in conscious, Long Evans rats. *British Journal of Pharmacology*, **101**, 625–631.

[29] X.Y. Wang and C.C. Pang (1993) Functional integrity of the central and sympathetic nervous systems is a prerequisite for pressor and tachycardic effects of diphenyleneiodonium, a novel inhibitor of nitric oxide synthase. *Journal of Pharmacology and Experimental Therapeutics*, **265**, 263–272.

[30] M.L. Nurminen, A. Ylikorkala and H. Vapaatalo (1997) Central inhibition of nitric oxide synthesis increases blood pressure and heart rate in anesthetized rats. *Methods and Findings in Experimental Clinical Pharmacology*, **19**, 35–41.

[31] D. Seiden, M. Srivatsan and P.A. Navidad (1989) Changes in myosin isozyme expression during cardiac hypertrophy in hyperthyroid rabbits. *Acta Anatomica*, **135**, 222–230.

[32] L. Yu, P.E. Gengaro, M. Niederberger, T.J. Burke and R.W. Schrier (1994) Nitric oxide: a mediator in rat tubular hypoxia/reoxygenation injury. *Proceedings of National Academy of Sciences of USA*, **91**, 1691–1695.

[33] A. Cassina and R. Radi (1996) Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Archives of Biochemistry and Biophysics*, **328**, 309–316.

[34] J.J. Poderoso, J.G. Peralta, C.L. Lisdero, M.C. Carreras, M. Radisic, F. Schopfer, E. Cadenas and A. Boveris (1998) Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. *American Journal of Physiology*, **274**, C112–C119.

[35] T.L. Vanden Hoek, L.B. Becker, Z. Shao, C. Li and P.T. Schumacker (1998) Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *Journal of Biological Chemistry*, **273**, 18 092–18 098.

[36] J.J. Chen, H. Bertrand and B.P. Yu (1995) Inhibition of adenine nucleotide translocator by lipid peroxidation products. *Free Radical Biology and Medicine*, **19**, 583–590.

[37] V. Fernandez, P. Cornejo, G. Tapia and L.A. Videla (1997) Influence of hyperthyroidism on the activity of liver nitric oxide synthase in the rat. *Nitric Oxide. Biology and Chemistry*, **1**, 463–468.

[38] R.H. Kennedy, K.K. Hicks, J.E. Brian Jr. and E. Seifen (1994) Nitric oxide has no chronotropic effect in right atria isolated from rat heart *European Journal of Pharmacology*, **255**, 149–156.

[39] R. Pabla and M.J. Curtis (1995) Effects of NO modulation on cardiac arrhythmias in the rat isolated heart. *Circulation Research*, **77**, 984–992.

[40] P.R. Hansen (1995) Myocardial reperfusion injury: experimental evidence and clinical relevance. *European Heart Journal*, **16**, 734–740.

[41] T.P. Kasten, S.L. Settle, T.P. Misko, D.P. Riley, R.H. Weiss, M.G. Currie and G.A. Nickols (1995) Potentiation of nitric oxide-mediated vascular relaxation by SC52608, a superoxide dismutase mimic. *Proceedings of Society for Experimental Biology and Medicine*, **208**, 170–177.

[42] C.P. Tiefenbacher, W.M. Chilian, M. Mitchell and D.V. DeFily (1996) Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. *Circulation*, **94**, 1423–1429.

[43] J.M.C. Gutteridge (1986) Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Letters*, **20**, 291–295.

[44] E.R. Stadtman (1993) Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annual Review of Biochemistry*, **62**, 797–821.