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Evidence for superoxide anion generation in aortas of cholesterol-fed rabbits treated with L-arginine

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Abstract

The inducible form of nitric oxide synthase (iNOS) is present in advanced atherosclerotic lesions. The aim of the present paper was to compare the functionality of iNOS in rabbits fed a 0.3% cholesterol-diet for 24 weeks (Baseline), and 36 weeks, with L-arginine (L-Arg) or vehicle supplementation (Saline) for the last 12 weeks. *N*-iminoethyl-L-lysine (L-NIL; 10 μM), a selective inhibitor of iNOS, potentiated the contractions to phenylephrine in aortas from Baseline, Saline and L-Arg rabbits confirming the presence of a functional iNOS. In L-Arg rabbits, the contractions induced by L-NIL were less pronounced than those noted in Baseline and Saline rabbits; superoxide dismutase (150 U/ml) significantly increased the phenylephrine-induced contractions only in the L-Arg rabbits. In the presence of NADPH, aortas from L-Arg rabbits produced more superoxide anions than aortas from saline rabbits as evidenced by the lucigenin-enhanced chemiluminescence technique. In conclusion, our results show functional and biochemical evidence for an increased superoxide anion production in atherosclerotic aortas from hypercholesterolemic rabbits treated with L-Arg for 12 weeks. These data may thus help to explain the lack of beneficial effects of L-Arg on atherosclerosis progression in long-term experimental hypercholesterolemia as well as in severely atherosclerotic humans.

Keywords: Inducible nitric oxide synthase; L-NIL (*N*-iminoethyl-L-lysine); L-arginine; Atherosclerosis; (Rabbit); Superoxide anion

1. Introduction

Experimental and human atherosclerosis profoundly alters the contractile and relaxant properties of the arterial wall. Endothelium-dependent and endothelium-independent relaxations of isolated arteries of cholesterol-fed rabbits are progressively inhibited as the degree of lesions augments (Verbeuren et al., 1986). In addition, the contractile responses to alpha-adrenergic agonists are decreased while those to 5-hydroxytryptamine (5-HT) are initially augmented (Verbeuren et al., 1986; Henry and Yokoyama, 1980) to become decreased in advanced stages of atherosclerosis (Verbeuren et al., 1993). Non selective nitric oxide synthase (NOS) inhibitors potentiate the contractions of the atherosclerotic arteries to noradrenaline and 5-HT in the presence or absence of endothelium; this effect is reversed by the addition of L-arginine (L-Arg), suggesting the presence of a functional inducible nitric oxide synthase (iNOS) in atherosclerotic lesions (Simonet et al., 1993; Verbeuren et al., 1993), which has been confirmed by high levels of cGMP in aortas (Rupin et al., 1996) and by immuno-histochemical studies (Esaki et al., 1997; Behr-Roussel et al., 1999).

The way in which L-Arg influences the nitric oxide-system and alters the oxidant status of the atherosclerotic blood vessel wall is not well understood (Loscalzo, 2000). In vivo, administration of L-Arg was shown to improve vascular responses to endothelium-dependent relaxations both in cholesterol-fed animals and in humans (Drexler et al., 1991; Cooke et al., 1992; Böger et al., 1995) although such an effect may not be sustained (Wennmalm et al., 1995; Blum et al., 2000). In hypercholesterolemic rabbits treated with 1% cholesterol for 4 weeks followed by 0.5%

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cholesterol for 12 weeks, L-Arg supplementation for the last 12 weeks increased nitric oxide formation and reduced superoxide release as well as plaque progression (Böger et al., 1998). However, in rabbits treated with 0.3% cholesterol for 36 weeks, we showed that 12 weeks dietary L-Arg supplementation provoked only a slight improvement of endothelium-dependent relaxations, but failed to influence lesion progression and composition in thoracic aortas and coronary arteries (Behr-Roussel et al., 2000a).

The aim of the present paper was to investigate the effect of L-Arg treatment on the functionality of the iNOS in rabbits fed a 0.3% cholesterol-diet for 36 weeks. Therefore, the effects of the selective inhibitor of iNOS, L-NIL (*N*-iminoethyl-L-lysine, Moore et al., 1994), and those of superoxide dismutase and catalase were studied in vitro on atherosclerotic aortas of those rabbits. Direct evidence of oxygen free radical production was obtained in these atherosclerotic aortas and their source and nature have been investigated.

2. Material and methods

2.1. Induction of hypercholesterolemia and L-arginine supplementation

This study was in accordance with European Community Guidelines for the use of experimental animals and was approved by the ethical committee on Animal Experiments of the Servier Research Institute. The experiments were performed on 26 male New Zealand rabbits (Charles River, France). Two-month-old rabbits were fed a diet containing cholesterol for a period of 24 or 36 weeks. The final cholesterol content of the atherogenic diet averaged $0.30 \pm 0.02\%$. The protocol used for the development of the experimental atherosclerosis was identical to that described previously (Behr-Roussel et al., 2000a). After 24 weeks, atherosclerosis was evaluated in 9 rabbits (Baseline group) and 17 animals were maintained on the diet during 12 weeks. Nine of these rabbits were treated with saline (Saline group) and eight with 2.25% L-Arg in the drinking water (L-Arg group). After the 24- and 36-week periods, blood samples were prepared for the determination of plasma concentrations of total cholesterol and L-Arg.

2.2. Plasma L-arginine and cholesterol levels

Plasma concentrations of L-Arg and cholesterol levels were monitored as previously described (Bode-Böger et al., 1996).

2.3. Tissue preparation

Animals were anaesthetised with sodium pentobarbital (30 mg/kg, i.v.) and the thoracic aorta was removed and immediately placed in physiological salt solution (composition see Verbeuren et al., 1986). Rings (3 mm) with endothelium of these aortas were mounted in organ chambers filled with oxygenated salt solution in presence of the cyclooxygenase inhibitor indomethacin (10 μM), at 37 °C for isometric tension recording (Verbeuren et al., 1986, 1990). Then, in all segments, a contraction caused by potassium chloride (KCl; 60 mM) was initiated. After washing and return to the baseline, cumulative concentration–response curves to KCl (10 to 120 mM), noradrenaline (0.01 to 30 μM) and 5-HT (0.01 to 30 μM) were realized. In order to determine the tone-related basal nitric oxide release from aortic rings, vascular tone (30–50% of the contractile response from initial KCl) was induced with phenylephrine and the concentration-related contractile responses to L-NIL were assessed in the presence of saline, superoxide dismutase (150 U/ml) and superoxide dismutase plus catalase (1200 U/ml) added 15 min before L-NIL. A time-control curve to saline, superoxide dismutase and superoxide dismutase plus catalase were also realized.

2.4. Detection of superoxide anions in rings of rabbit aorta

Vascular superoxide anions ($\cdot O_2^-$) were measured in rings of rabbit aorta from Saline and L-Arg rabbits using lucigenin-enhanced chemiluminescence (Münzel et al., 2002; Tarpey and Fridovich, 2001). Briefly, aortas were cleaned of adherent adventitial tissue and cut into rings of 3 mm length. Tissues were equilibrated for 30 min at 37 °C in a modified Krebs–HEPES solution (NaCl 140 mM, KCl 5.4 mM, $CaCl_2$ 2.4 mM, $MgSO_4$ 0.7 mM, HEPES/Tris 20 mM, pH 7.4). Aortic rings were placed in a 24-well plate and incubated in Krebs–HEPES (30 min, 37 °C, 5% CO_2) in the presence or absence of either superoxide dismutase (copper–zinc superoxide dismutase from bovine erythrocytes, 250 U/ml), superoxide scavenger (Tiron, 10 mM), flavoprotein inhibitor diphenyleneiodonium chloride (DPI, 50 μM) or the superoxide mimetic (M40403, 100 μM). Immediately before recording chemiluminescence, 200 μM NADPH and 5 μM lucigenin were added. After a 15-min dark adaptation, photon emission was measured in a microtiterplate scintillation counter (Microbetajet, Packard) switched to the out-of-incidence mode and modified to maintain a temperature of 37 °C. Results were obtained as photon emission/s/mg dry weight. In response to NADPH, the Tiron-inhibitable photon emission was calculated as a measure of superoxide production.

2.5. Drugs

The following pharmacological agents were used: catalase, 5-hydroxytryptamine creatinine sulphate, indomethacin, noradrenaline bitartrate, phenylephrine hydrochloride, superoxide dismutase, Tiron, diphenyleneiodonium chloride, β-Nicotinamide adenine dinucleotide phosphate (NADPH), lucigenin (Sigma, St. Louis, MO, USA), L-NIL hydrochloride (Cayman Chemical, France) and M40403 (MetaPhore Pharmaceuticals, St. Louis, MO, USA). Stock

solutions of the drugs used were prepared no longer than 2 h before use by dissolving the compounds in distilled water, except for DPI and indomethacin that were dissolved in dimethylsulfoxide. For isolated organ experiments appropriate dilutions were prepared using distilled water and for photon emission determination dilutions were performed in a modified Krebs–HEPES.

2.6. Data analysis

Results are expressed as means ± S.E.M.; in all experiments, *n* equals the number of animals from which the blood vessels were taken. Concentration–response curves obtained with KCl, noradrenaline and 5-HT were analysed to determine the EC_{50} values (the concentration of the agonist inducing a half-maximal response). All estimates of the EC_{50} values were realised by linear interpolation. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Newman–Keuls complementary analysis. Comparison of superoxide anions produced by aortas from L-Arg and Saline rabbits was performed using unpaired Student's *t*-test. A value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Plasma L-arginine and cholesterol levels

The plasma L-Arg concentrations were significantly increased in the L-Arg group ($P \leq 0.05$, one-way ANOVA). The level of total cholesterol, triglycerides, and high-density lipoprotein cholesterol was summarised in Table 1. No difference was detected between the three groups.

3.2. Effect of the selective inducible NO synthase inhibitor (L-NIL) during phenylephrine-induced contraction

In the Baseline group, phenylephrine at 0.1 μM induced contractions with an amplitude of 3.2 ± 0.4 g that slowly decreased with time by 15 ± 5%. L-NIL (10 μM) increased the phenylephrine-induced contraction by 1.8 ± 0.4 g (Fig. 1). The presence of superoxide dismutase (150 U/ml) or superoxide dismutase plus catalase (1200 U/ml) did not significantly modify the phenylephrine-induced contraction (3.6 ± 0.4 and 3.9 ± 0.9 g, respectively, NS, one-way ANOVA) or their increase caused by L-NIL (Fig. 1).

In the Saline group, phenylephrine induced contractions that average 3.7 ± 0.7 g, a value comparable to that obtained in the Baseline group. L-NIL (10 μM) significantly increased these contractions by 1.8 ± 0.4 g (Fig. 1). As in the Baseline group, the presence of superoxide dismutase or superoxide dismutase plus catalase did not modify the phenylephrine-induced (2.7 ± 0.4 and 3.3 ± 0.5 g, respectively, NS, one-way ANOVA) or the L-NIL-induced responses (Fig. 1).

In the L-Arg group, the phenylephrine-induced contractions were significantly lower than those noted in the Baseline and Saline groups (2.0 ± 0.5 g, $P \leq 0.05$,). Also, the increase caused by 10 μM of L-NIL, 0.75 ± 0.2 g, was

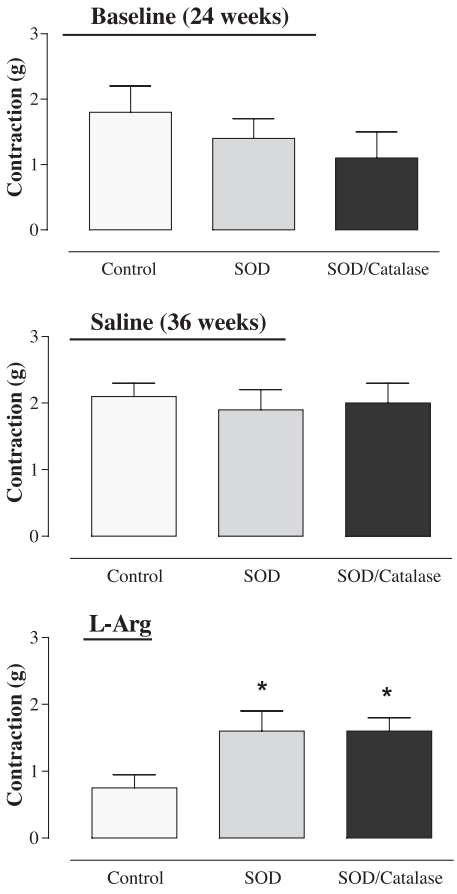

Fig. 1. The contractile effects of L-NIL (10 μM) were evaluated during phenylephrine-induced contractions in aortas of Baseline, Saline and L-Arg rabbits. These effects were not changed by the presence of superoxide dismutase (SOD, 150 U/ml) or that of superoxide dismutase plus catalase (SOD/Catalase, 1200 U/ml) in Baseline and Saline groups. In the L-Arg group, the contraction caused by L-NIL is less important, but is significantly increased by the presence of superoxide dismutase or SOD/Catalase (*$P \leq 0.05$, one-way ANOVA, $n = 8$ or 9). Data are shown as mean ± S.E.M.

Table 1
Plasma L-arginine and cholesterol levels of hypercholesterolemic rabbits from different groups

	24 Weeks	36 Weeks	
	Baseline ($n = 9$)	Saline ($n = 9$)	L-Arg ($n = 8$)
Plasma L-arginine concentrations (μM)	72.1 ± 11.5	96.2 ± 8.6	175.9 ± 26.4[a]
Total cholesterol (mM)	16.4 ± 1.7	18.7 ± 2.4	19.1 ± 1.8
Triglycerides (mM)	2.1 ± 0.5	1.1 ± 0.2	1.7 ± 0.3
HDL cholesterol (mM)	0.35 ± 0.04	0.39 ± 0.09	0.23 ± 0.03

Results are shown as means ± S.E.M. Plasma concentration of L-Arginine is significantly increased in the L-Arg group. No difference is observed on cholesterol levels between the three groups (NS, one-way ANOVA).

[a] $P \leqslant 0.05$ L-Arg vs. Baseline, one-way ANOVA.

significantly lower than that noted in the Baseline or the Saline groups ($P \leq 0.05$, Fig. 1). The presence of superoxide dismutase or superoxide dismutase plus catalase did not modify the phenylephrine-induced contractions (2.2 ± 0.5 and 1.4 ± 0.3 g, respectively, NS, one-way ANOVA), but significantly increased those induced by L-NIL (Fig. 1).

3.3. Contractile responses to KCL, noradrenaline and 5-HT in thoracic aortic rings

The maximal contractile responses and EC_{50} values of thoracic aorta from Baseline (24 weeks), Saline (24 weeks + 12 weeks saline) and L-Arg (24 weeks + 12 weeks L-Arg) groups of cholesterol fed rabbits in response to KCl, noradrenaline and 5-HT are shown in Table 2. The sensitivity of the contractility to each agonist is similar in the different groups of rabbits. However, the maximal contractions in response to KCl, noradrenaline or 5-HT are significantly decreased in the L-Arg group as compared to the Baseline or the Saline groups (Table 2).

3.4. Detection of superoxide anions in rings of rabbit aorta

Photon emission was determined by lucigenin-enhanced chemiluminescence in intact aortic rings from Saline or L-Arg rabbits (photons/s/mg dry weight). Basal production of photons was undetectable in both groups but greatly increased by NADPH (200 μM). The superoxide production was evaluated by Tiron-inhibitable photon generation (Fig. 2). In comparison to Saline aortas, the NADPH-dependent superoxide production was increased 1.7-fold in L-Arg aortas ($176 \pm 37.6\%$ vs. $100 \pm 17.9\%$ for L-Arg and Saline, respectively, $P \leq 0.05$, unpaired Student's *t*-test, $n = 5$).

To characterize the source and nature of vascular NADPH dependant photon emission, an inhibitor of flavin-containing enzyme (DPI), a scavenger of $\cdot O_2^-$ (Tiron), a cell permeant superoxide dismutase mimetic (M40403) and an exogenous source of copper–zinc superoxide dismutase were used (Fig. 3). Tiron decreased the photon emission by $86.3 \pm 4.3\%$ for saline and $89.0 \pm 2.4\%$ for L-Arg and M40403 by $91.7 \pm 2.8\%$ for saline and $92.3 \pm 2\%$ for L-Arg. Superoxide production was totally abolished by diphenylene iodonium (DPI) and exogenous Cu–Zn superoxide dismutase reduced the production of superoxide by $43.3 \pm 9.7\%$ for saline and $52.8 \pm 9.5\%$ for L-Arg rabbits.

Table 2
Effect of KCl, noradrenaline and 5-HT on thoracic aorta of hypercholesterolemic rabbits from different groups

	24 Weeks	36 Weeks	
	Baseline ($n = 9$)	Saline ($n = 9$)	L-Arg ($n = 8$)
Contraction (g)			
KCl (120 mM)	10.8 ± 1.4	9.9 ± 1.0	6.8 ± 1.4[a]
Noradrenaline (30 μM)	13.6 ± 1.9	12.3 ± 0.8	7.8 ± 0.8[a]
5-HT (30 μM)	7.2 ± 0.9	5.5 ± 0.7	4.1 ± 0.9[a]
EC_{50}			
KCl (mM)	22.4 ± 1.1	23.7 ± 1.1	21.2 ± 1.4
Noradrenaline (μM)	0.43 ± 0.14	0.33 ± 0.08	0.41 ± 0.11
5-HT (μM)	0.57 ± 0.13	0.52 ± 0.07	0.51 ± 0.09

Contractile responses and EC_{50} values to KCl, noradrenaline and 5-HT are shown as means ± S.E.M. Maximal contractile responses to KCl, noradrenaline and 5-HT of aortas from L-Arg-treated group are significantly decreased. No difference is observed on EC_{50} values between the three groups (NS, one-way ANOVA).

[a] $P < 0.05$ L-Arg vs. Baseline, one-way ANOVA.

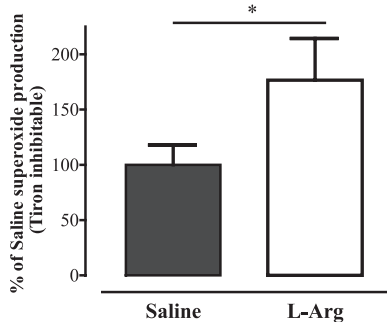

Fig. 2. Superoxide generation was determined in intact aortic rings of Saline and L-Arg rabbits by lucigenin-enhanced chemiluminescence (5 μM) in response to 200 μM NADPH. The superoxide production obtained by Tiron-inhibitable photon emission was increased in L-Arg compared to Saline Rabbits (% of saline superoxide production, $*P \leq 0.05$ unpaired *t*-test, $n = 5$). Bars represent mean ± S.E.M.

4. Discussion

The major goal of the present study was to analyse the effects of a 12 week L-Arginine treatment on the functionality of iNOS expressed in rabbit aortas with pre-existing atherosclerotic lesions induced by a cholesterol-rich diet. Previous studies illustrated that the cholesterol diet, given for 24 weeks, causes development of atherosclerotic lesions in thoracic aortas and in coronary arteries and also induces expression of iNOS (Behr-Roussel et al., 2000a). At this level of disease, endothelium-dependent relaxations are nearly abolished while those in response to nitroso-compounds are reduced (Behr-Roussel et al., 2000a). The L-arginine treatment was efficient in these hypercholesterolemic rabbits since plasma L-arginine levels were increased about two-fold (Table 1).

Since L-NIL is a selective iNOS inhibitor, we studied its effects in vitro at a concentration of 10 μM that did not interfere with eNOS activity (Behr-Roussel et al., 2000b). L-NIL caused significant contractions in phenylephrine-stimulated atherosclerotic thoracic aortas, confirming that after 24 and 36 weeks of cholesterol-rich diet, atherosclerotic lesions contain functional iNOS (Verbeuren et al., 1993). Since it has been shown that hypercholesterolemia can increase superoxide anion production (Ohara et al., 1993), we studied the effects of superoxide dismutase and superoxide dismutase plus catalase on the vasoreactivity of

the atherosclerotic aortas. Superoxide dismutase and superoxide dismutase plus catalase did not influence the contractile response of the atherosclerotic aortas to phenylephrine neither under control conditions nor in the presence of L-NIL suggesting that extracellular superoxide anions or hydrogen peroxide were not involved in these contractions. However, the fact that superoxide dismutase and catalase do not influence contractions does not mean that superoxide anions and/or hydrogen peroxide are not produced intracellularly.

Interestingly, in atherosclerotic aortas from rabbits treated for 12 weeks with L-Arg, phenylephrine causes less contraction than in the Saline rabbit aorta. This could indicate that in L-Arg aortas, a relaxing factor is produced or contractility is decreased. In the L-Arg group, the contraction to phenylephrine is increased with L-NIL, however, the increase is less than that noted in aortas from Baseline or Saline groups, indicating that less nitric oxide from iNOS is bioavailable. Contrary to the observations in the Baseline or Saline groups of rabbits, we found that the L-NIL contractions were potentiated by the addition of superoxide dismutase and superoxide dismutase plus catalase in the L-Arg treated rabbits, suggesting that nitric oxide but also extracellular O_2^- radicals participate to the altered responses to phenylephrine. The finding that superoxide dismutase became active in L-Arg treated rabbits when iNOS is blocked by L-NIL may suggest that more superoxide was generated that can diffuse to intravascular sites that are accessible to superoxide dismutase. In this context, it has been recently shown that O_2^- impair contractility in cultured aortic smooth muscle cells (Kimura et al., 2002). The fact that superoxide dismutase and superoxide dismutase plus catalase do not affect phenylephrine-induced contractions in the L-Arg rabbit in the absence of L-NIL might be due to the fact that O_2^- reacts three times faster with nitric oxide than with superoxide dismutase (Fukai et al., 2002) and thus the equilibrium between O_2^- and nitric oxide is not modified by superoxide dismutase. The other possible explanation is that scavenging of the O_2^- leaves more nitric oxide available for tissue relaxation. Indeed, when both nitric oxide and O_2^- are present in the tissue, they will cause formation of peroxynitrite, which has less vasodilator action than nitric oxide itself (Iesaki et al., 1999). The local bioavailability of nitric oxide from iNOS and that of superoxide anions appears to influence all contractile responses, since also contractions to KCl, noradrenaline and 5-HT are decreased in the L-Arg group. The exact source and nature of the superoxide anions produced in organ chamber experiments cannot be defined precisely. NADPH oxidase (Griendling et al., 2000) but also xanthine oxidase (White et al., 1996) or uncoupled NOS isoforms (Pritchard et al., 1995; Xia and Zweuer, 1997; Chen et al., 2003) could participate.

An important finding of the present study is that treatment of severely atherosclerotic rabbits with L-arginine leads to an increased superoxide anion production. Indeed, in response to NADPH, we found a higher superoxide anion production in L-Arg aortas than in Saline aortas. The dramatic decrease of photon emission observed with aortas from Saline and L-Arg rabbits in the presence of the radical scavenger Tiron and the superoxide dismutase mimetic M40403 demonstrates the specificity of our result. The complete decrease also found in the presence of the flavin-containing enzyme inhibitor DPI suggested that NADPH oxidase is the main source of superoxide anion production. Copper–zinc superoxide dismutase decreased the superoxide production by 50%, suggesting that half of the generation of superoxide anions was intracellular.

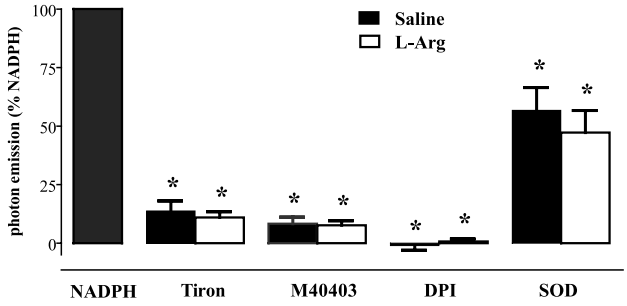

Fig. 3. Source of superoxide generation was determined from intact aortic rings of Saline and L-Arg rabbits in the absence or presence of superoxide scavenger (Tiron, 10 mM), cell permeant superoxide dismutase mimetic (M40403, 100 μM), flavoprotein inhibitor diphenylene iodonium chloride (DPI, 50 μM) and copper–zinc superoxide dismutase (SOD, 250 U/ml). Vessel rings were incubated with the compounds for 30 min before the addition of NADPH (200 μM) and lucigenin (5 μM). $*P \leq 0.05$, unpaired t-test vs. NADPH, $n = 5$. Bars represent mean ± S.E.M.

In conclusion, the present data show functional and biochemical evidence for an increased superoxide anion production in atherosclerotic aortas from hypercholesterolemic rabbits treated by L-Arg for 12 weeks in comparison to Saline rabbits. These results may help to explain the lack of beneficial effects of L-Arg on atherosclerosis progression reported in the long-term experimental model of atherosclerosis (Behr-Roussel et al., 2000a; Chen et al., 2003; Jeremy et al., 1996) as well as in severely atherosclerotic human (Quyyumi, 1998; Kobayashi et al., 1999; Blum et al., 2000; Oomen et al., 2000).

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