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Inhibition of nitric oxide generation unmasks vascular dysfunction in insulin-resistant, obese JCR:LA-*cp* rats

[1,2]Joyce D. McKendrick, [2,3]Eduardo Salas, [4]Gregory P. Dubé, [2,3]Jesus Murat, [1]James C. Russell & [2,5]Marek W. Radomski

Departments of [1]Surgery and [2]Pharmacology, University of Alberta, Edmonton, Canada, T6G 2H7

1 The effects of nitric oxide (NO) on vascular reactivity and platelet function in the obese (*cp*/*cp*) and lean (+/?) JCR:LA-*cp* rats were investigated.

2 Phenylephrine (PE; 0.1 nM – 10 μM) induced contraction of isolated aortic rings in both genotypes (*cp*/*cp* and +/?) of JCR:LA-*cp* rats. The sensitivity to contraction with PE was enhanced in *cp*/*cp* compared with +/? rings. Rings from both genotypes showed an increased contraction upon removal of the endothelium.

3 Acetylcholine (ACh; 0.1 nM – 10 μM)-induced endothelium-dependent relaxation of rings was not significantly different in the two genotypes. Both were inhibited to a similar extent by N^G-nitro-L-arginine methyl ester (L-NAME; 0.01 – 1 mM) when administered *in vitro*.

4 The nitric oxide synthase (NOS) inhibitor (L-NAME; 0.3, 1 or 3 mg ml^{-1}, p.o.) when administered *in vivo* increased blood pressure in *cp*/*cp* rats but not in +/? rats.

5 L-NAME resulted in greater inhibition of ACh-induced relaxation in *cp*/*cp* rings compared with +/? rings.

6 L-NAME treatment *in vivo* caused a decrease in cyclic GMP and NOS activity in rings from *cp*/*cp* but not +/? rats.

7 The NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP; 0.1 nM – 10 μM)-induced relaxation of rings from +/? rats, an effect enhanced by the treatment with L-NAME *in vivo*.

8 Oral administration of L-NAME did not enhance the vasorelaxant effect of SNAP on rings of aorta from *cp*/*cp* animals.

9 Platelet aggregation and NOS activity were similar in both genotypes and were not modified by oral administration of L-NAME.

10 These results show that unimpaired generation of NO is crucial for maintenance of vascular tone particularly under conditions of vascular insult exemplified by insulin resistance, obesity and dyslipidemia detected in *cp*/*cp* rats.

Keywords: Nitric oxide; JCR:LA-*cp* rat; insulin resistance; vascular smooth muscle; N^G-nitro-L-arginine methyl ester; endothelium; platelets

Introduction

A large component of the vascular control of blood pressure and blood flow is due to the release of nitric oxide (NO) from the vascular endothelium. Nitric oxide is synthesized from either of the guanidino nitrogens of the amino acid, L-arginine, by nitric oxide synthase (NOS: Palmer *et al*., 1988). There are at least three distinct isoforms of NOS, endothelial (eNOS), neuronal (nNOS) and an isoform expressed by immunological stimuli (iNOS). The eNOS and nNOS isoforms require calcium/calmodulin for their activities and are expressed under normal conditions (Bredt *et al*., 1990; Sessa *et al*., 1992). The iNOS is calcium- independent as the calmodulin domain binds tightly to the enzyme and is not destabilized by calcium removal (Cho *et al*., 1992). The activity of NOS can be competitively inhibited by L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME: Rees *et al*., 1989, 1990; Moore *et al*., 1990).

The changes in the expression and activity of NOS have been linked to the pathogenesis of various vascular disorders (Radomski & Salas, 1995; Sowers & Epstein, 1995). Indeed, endothelium-dependent vasodilatation that depends, in part, on intact activity of NOS, is reduced in human and experimental atherosclerosis and in diabetes (Andrews *et al*., 1987; Bossaller *et al*., 1987; Chester *et al*., 1990; Calver *et al*., 1992; Anderson *et al*., 1995; Liao *et al*., 1995). In human pathology, diabetes is an important risk factor for atherosclerosis (Sowers & Epstein, 1995).

In this study, we have examined the JCR:LA-*cp* rat. The JCR:LA-*cp* rat is an interesting model of vascular complications of insulin-resistance and glucose intolerance showing hyperlipidaemia and vasculopathy including intimal and myocardial lesions (Russell *et al*., 1990; Russell, 1995). The *cp* gene of this animal was first isolated by Koletsky (1973; 1975). Animals homozygous for the *cp* gene (*cp*/*cp*) become obese, insulin resistant and hyperlipidaemic (Russell, 1995). Corpulent males develop atherosclerotic and ischaemic myocardial lesions (Russell *et al*., 1990). Male JCR:LA-*cp* rats demonstrate several of the attributes that are typical of the obese/diabetic/hypertensive/dyslipidemic syndrome observed in man (syndrome X: Defronzo & Ferrannini, 1991). On the other hand, heterozygous (*cp*/+) and homozygous normal(+/+) are lean and metabolically normal. Lean rats, as bred, are 2:1 *cp*/+ and +/+ and are characterized as +/? (Russell *et al*., 1990; 1995).

The central role of NO metabolism in vascular function suggests that dysfunction of this regulation may contribute to

[3] Present address: Division of Research and Development, Lacer Labs., Sardenya 350, Barcelona, Spain.
[4] Present address: Eli Lilly Inc., Cardiovascular Research, Lilly Corporate Center, Indianapolis, Indiana, 46285, U.S.A.
[5] Author for correspondence.

the vasculopathy of the insulin resistant state. The aim of this study was to examine the generation and actions of NO in the vasculature and platelets of insulin resistant, atherosclerosis-prone JCR:LA-cp rats and compare the vascular responses with those of apparently healthy animals (+/?). As long term inhibition of NO synthesis promotes the development of vascular disease in rabbits (Naruse *et al*., 1994; Bryant *et al*., 1995; Zanachi *et al*., 1995; Henrion *et al*., 1996), we have also treated the JCR:LA-*cp* rats with the NOS inhibitor, L-NAME. Finally, we investigated the effects of L-NAME on vascular reactivity of aortic rings from these rats.

Methods

Animals

The rats used were bred in the established colony of JCR:LA-*cp* rats in our laboratory, by use of breeding methods and conditions previously described (Russell *et al*., 1995). All care and treatment of the animals was in accordance with the guidelines of the Canadian Council on Animal Care and subject to prior institutional review and approval.

Blood pressure measurements

JCR:LA-*cp* rats (12 weeks of age) were anaesthetized with halothane. All rats were anaesthetized with the same setting on our Halothane vaporiser and the time under anaesthetic before measurement of blood pressure was similar in all rats (6–7 min). Blood pressure was measured by inserting a needle into the femoral artery of rats. The needle was attached to a pressure analyser (Beckman, type 4-327-c) and meter, and blood pressure was expressed in mm Hg. Blood pressure was measured in control rats and rats treated *in vivo* with L-NAME for 4 weeks at doses of 0.3, 1 and 3 mg ml^{-1} in the drinking water.

Preparation of rat aortic rings

The thoracic aorta was excised, trimmed of adhering fat and connective tissue and cut into four 3 mm long transverse rings by a razor blade slicing device. The endothelium was removed from two rings by gently rubbing the intimal surface with a moist stick. The remaining aorta was frozen in liquid nitrogen and stored at $-80°C$ until used for gunanosine3′:5′-cyclic monphosphate (cyclic GMP) and citrulline assays.

Tension recordings

The aortic rings were mounted on stainless-steel hooks under 1.5 g resting tension in 20 ml organ baths and bathed at 37°C in Krebs solution containing (mM): NaCl 116, KCl 5.4, $CaCl_2$ 1.2, $MgCl_2$ 2, Na_2PO_4 1.2, glucose 10 and $NaHCO_3$ 19 and gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically with Grass FTO3C transducers and displayed on Digi-Med (Micro-Med, Louisville, KY, USA), tissue force analyser (model 210) which was linked to an IBM compatible computer. This computer ran the program DMSI 210/4. The tissues were allowed to equilibrate for 45 min before experiments were begun and during this time resting tension was re-adjusted to 1.5 g, as required and the tissues were washed every 15 min.

Organ bath protocol

Basal release of NO by rat aortic rings was measured by the endothelium-dependent depression of phenylephrine (PE)-induced vasoconstriction (Martin *et al*., 1986). In these experiments, the contractile response to PE of endothelium-containing and -denuded rings of rat aorta were measured. The concentration-response curves to PE (0.1 nM – 100 μM) were analysed.

The release of NO was measured by the relaxation of endothelium-containing rings to acetylcholine (ACh; 10 nM – 10 μM), pre-contracted with PE at the EC_{80} concentrations. The contribution of NO to ACh-induced relaxation was further investigated with L-NAME (0.01–1 mM) which was added 10 min before the addition of PE. The effects of exogenous NO on the reactivity of both endothelium-containing and -denuded rings were investigated by use of an NO donor (S-nitroso-N-acetyl-DL-penicillamine, SNAP; 10 nM – 10 μM) following precontraction of rings with PE.

In vivo *experiments*

To investigate the effects of inhibition of NO generation on the contractility of vasculature *in vivo* the rats were treated with L-NAME (0.3, 1 and 3 mg ml^{-1}), which was dissolved in the drinking water and given *ad libitum*. The water was changed weekly. There was no significant difference in the relative water intake between *cp*/*cp* and +/? rats (76.0 ± 4.2 and 66.0 ± 3.7 ml kg^{-1}, respectively).

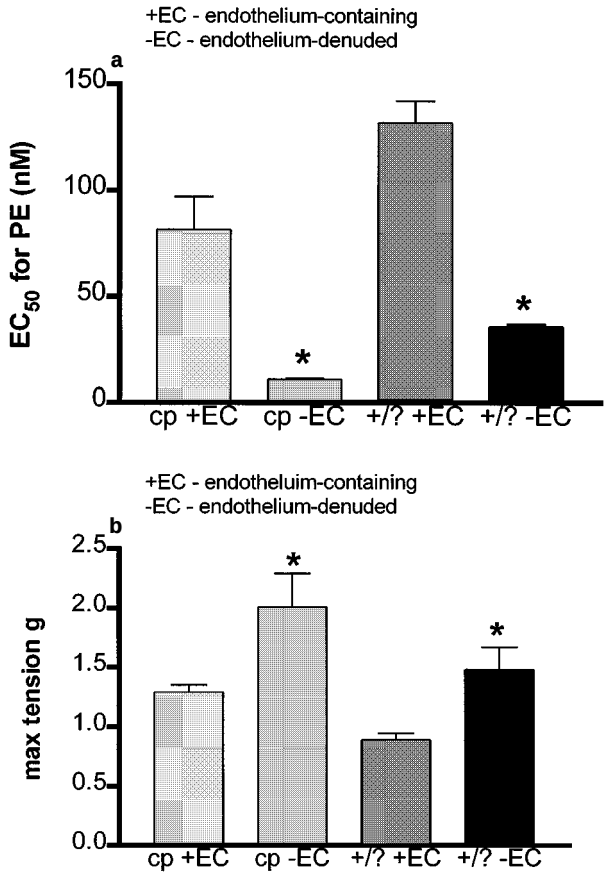

Figure 1 Contractile effects of phenylephrine (PE) on aortic rings from JCR:LA-*cp* rats. (a) EC_{50} values for contraction-induced by PE. (b) Maximum contraction generated by PE. Each column is the mean ± s.e.mean of 10–20 observations. $^{*}P<0.05$, indicates a significant difference from the respective endothelium-containing (+EC) rings.

Platelet aggregation, NOS and cyclic GMP formation

JCR:LA-*cp* rats were anaesthetized and blood collected into polycarbonate tubes containing tri-sodium citrate (3.15% w:v, 1 part of citrate and 9 parts of blood). Platelet aggregation was measured in whole blood with a whole blood platelet-ionized calcium lumi-aggregometer (Chrono-Log, Havertown, P.A., U.S.A.). Blood (490 μl diluted in 490 μl physiological saline) was stirred at 1000 r.p.m. and incubated at 37°C for 3 min before the addition of collagen (2–20 μg ml^{-1}). Platelet aggregation was then studied for 3 min and measured with an Aggro-link computer data processing system.

Whole blood was centrifuged at 240 *g* for 20 min at room temperature, platelet-rich plasma was collected and recentrifuged at 500 *g* for 10 min to obtain platelets. Platelet samples were frozen at −80°C until assayed for cyclic GMP and citrulline.

Citrulline and cylic GMP assays

Aorta was homogenized by crushing and sonication in 450 μl of homogenizing buffer (Tris HCl/base 50 mM; sucrose 320 mM, dithiothreitol 1 mM, leupeptin 10 μg ml^{-1}, soybean trypsin inhibitor 10 μg ml^{-1} and aprotinin 2 μg ml^{-1}). The suspension was then centrifuged at 10 000 *g* for 20 min at 4°C. The resultant supernatant was kept for cyclic GMP, protein analysis and the citrulline assay. Platelets were homogenized by freezing in liquid nitrogen and rapid thawing at 37°C in 450 μl homogenizing buffer. The suspension was then centrifuged at 10 000 *g* for 20 min at 4°C. The supernatant was kept for cyclic GMP, protein analysis and the citrulline assay.

Citrulline assay was performed on homogenized aorta and platelets as described previously (Radomski *et al*., 1993). Briefly, 40 μl aliquots of homogenized sample was added to tubes containing 100 μl assay buffer and either L-NMMA 1 mM, EGTA 1 mM or saline. Assay buffer contained KH_2PO_4 50 mM, $MgCl_2$ 1 mM, $CaCl_2$ 0.2 mM, valine 50 mM, L-citrulline 1 mM, L-arginine 20 μM, dithiothreitol 1.5 mM, tetrahydrobiopterin 0.01 mM, FAD 1.3 mM, FMN 3.8 mM and NADPH 3.4 mM. The assay buffer also contained 100 μl of L[U-^{14}C]-arginine (Amersham) per 10 ml of buffer. The samples were incubated at 37°C for 20 min. After this time the reaction was stopped and the unreacted arginine removed by the addition of 1 ml of AG 50W-X8 resin (200–400 mesh, Bio-Rad). The resin was allowed to sediment for 45 min, afterwards 500 μl of the supernatant was taken from each tube and counted for the presence of ^{14}C-bound radioactivity by liquid scintillation. Citrulline synthesis was related to the total protein content or the number of platelets in the sample.

The cyclic GMP measurements were made with a cyclic GMP enzyme immunoassay (Amersham) in the presence of 100 μM 3-isobutyl-1-methylxanthine. Cyclic GMP content was

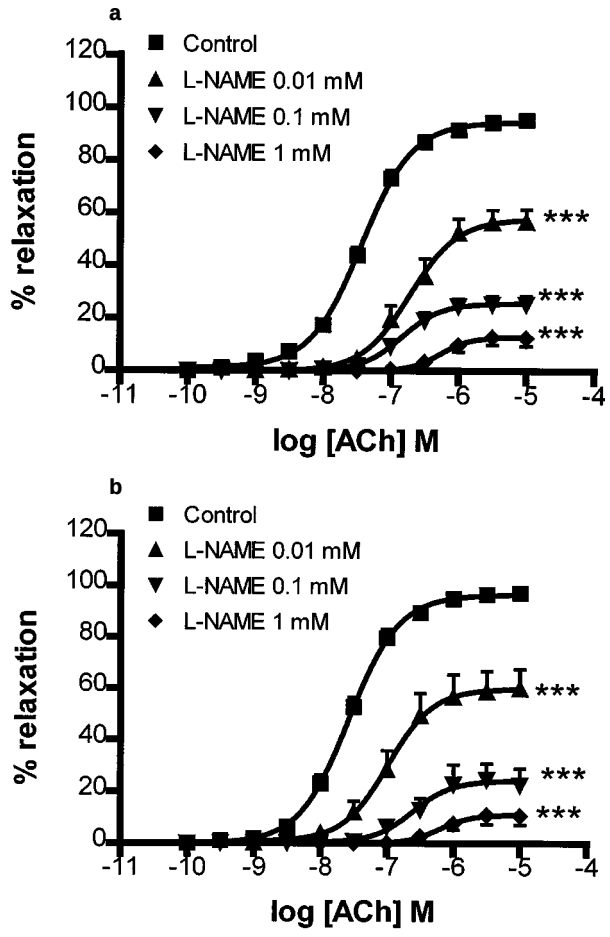

Figure 2 Concentration-response curves showing acetylcholine (ACh)-induced relaxation of phenylephrine precontracted aortic rings from JCR:LA-*cp* rats and the ability of L-NAME to block this relaxation; (a) *cp*/*cp* rats and (b) +/? rats. Each point is the mean of 3 observations; vertical lines show s.e.mean ***$P<0.005$, indicates a significant difference from control.

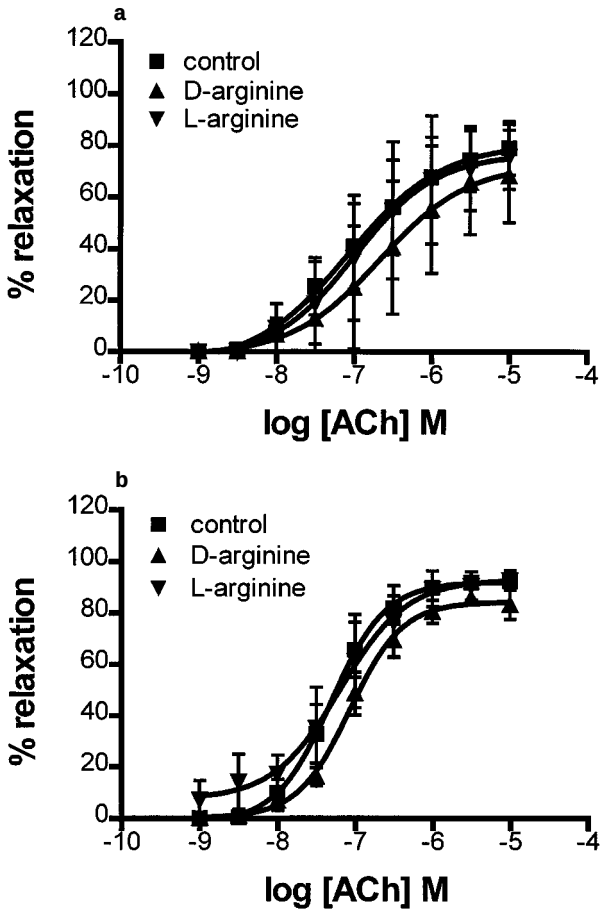

Figure 3 Concentration-response curves showing acetylcholine (ACh)-induced relaxation of phenylephrine precontracted aortic rings from JCR:LA-*cp* rats and the effects of L-arginine and D-arginine (1 mM) on this relaxation. (a) *cp*/*cp* rats and (b) +/? rats. Each point is the mean of 3 observations; vertical lines show s.e.mean.

related to the total protein content or the number of platelets in the sample.

Reagents

Acetylcholine bromide, D-arginine, L-arginine, N^G-nitro-L-arginine methyl ester hydrochloride, phenylephrine hydrochloride, 3-isobutyl-1-methylxanthine were obtained from Sigma and S-nitroso-N-acetyl-DL-penicillamine and N^G-monomethyl-L-arginine were kindly provided by Dr D. Rees (Glaxo-Wellcome).

Statistical analysis

Results are expressed as the mean ± s.e. mean. The statistical package used was Instat (Graphpad, San Diego, CA, USA). The data were compared by one-way analysis of variance and when significant differences were found the Tukey-Kramer multiple comparison test was then used. A probability of 0.05 or less was considered as statistically significant.

Results

Contractility of rat aortic rings: in vitro

Phenylephrine induced a contraction in both *cp/cp* and +/? rat aortic rings (Figure 1). The maximal contraction generated in *cp/cp* aortic rings was significantly greater than the contraction produced in +/? aortic rings (Figure 1; 1.29 ± 0.06 g and 0.89 ± 0.05 g, respectively, $n = 10-20$). The removal of the endothelium significantly increased the contraction in both genotypes. However, denuded *cp/cp* rat aortic rings contracted significantly more strongly than denuded +/? rings (Figure 1; 2.0 ± 0.3 g and 1.5 ± 0.2 g, respectively, $n = 6-10$).

Acetylcholine produced 100% relaxation of endothelium-containing rings, precontracted with PE, in both *cp/cp* and +/? animals (Figure 2a and b). L-NAME (0.01 – 1 mM) added to the organ bath resulted in a significant inhibition of ACh-induced relaxation. This inhibition was similar in both *cp/cp* and +/? rat aortic rings (Figure 2a and b).

Addition of L-arginine or D-arginine (1 mM) exerted no significant effect on ACh-induced relaxation (Figure 3).

Blood pressure

The mean blood pressure of *cp/cp* rats was similar to that of +/? rats (Figure 4). *In vivo* administration of L-NAME (0.3, 1 and 3 mg ml^{-1}) significantly raised blood pressure in *cp/cp* rats but exerted no significant effect on +/? rats (Figure 4).

Effect of oral L-NAME on contractility of rat aortic rings: ex vivo

The treatment of rats with L-NAME (3 mg ml^{-1}) significantly increased the contraction produced by PE in *cp/cp* endothe-

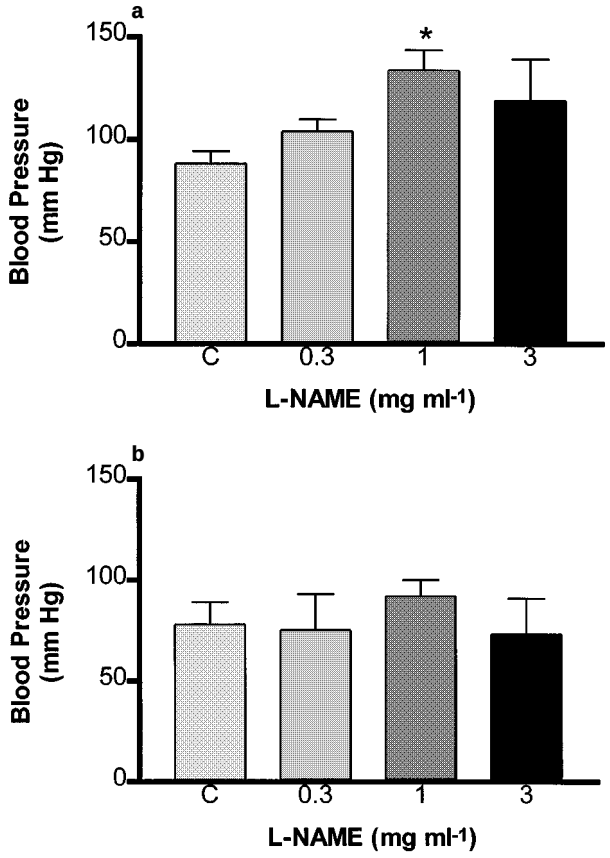

Figure 4 Mean blood pressure measurements of JCR:LA-*cp* rats treated with L-NAME (0.3, 1 and 3 mg ml^{-1}). (a) *cp/cp* rats and (b) +/? rats. Each column is the mean ± s.e.mean of 3 observations. $*P < 0.05$, indicates a significant difference from control (C).

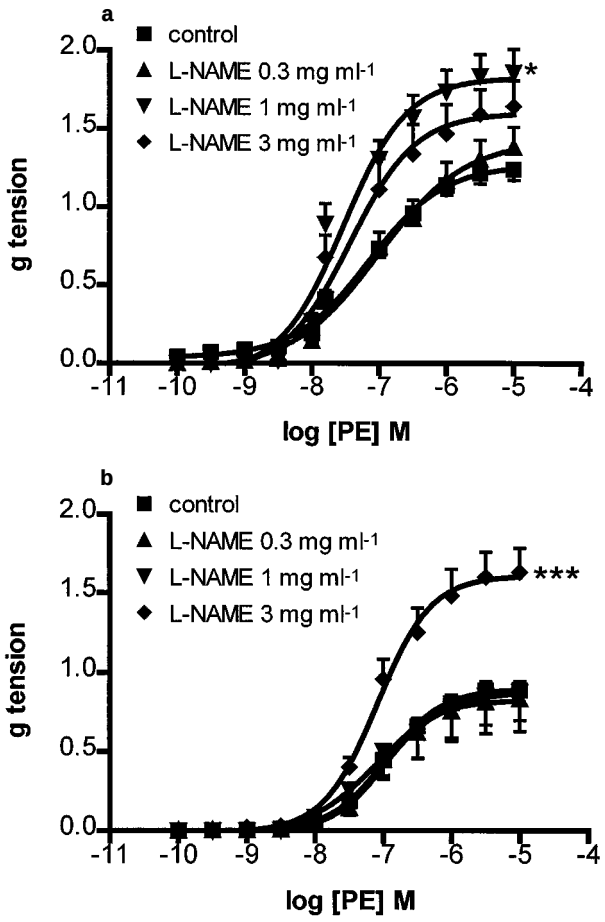

Figure 5 Concentration-response curves showing the contractile effects of phenylephrine on aortic rings from JCR:LA-*cp* rats and the ability of N^G-nitro-L-arginine methyl ester (L-NAME) to increase this contraction. (a) endothelium-containing rings of rat aorta from *cp/cp* rats and (b) endothelium-containing rings of rat aorta from +/? rats. Each point is the mean of 3 observations; vertical lines show s.e.mean. $*P < 0.05$ and $***P < 0.005$, indicate a significant difference from respective control.

lium-containing rat aortic rings (Figure 5a). L-NAME (0.3 and 1 mg ml^{-1}) exerted no significant effect on PE-induced contraction. However, L-NAME (3 mg ml^{-1}) increased the contraction produced by PE in endothelium-containing aortic rings from +/? rats. The inhibitor exerted no significant effect on the contractility of endothelium-denuded rings of aorta from both *cp*/*cp* and +/? rats (data not shown).

L-NAME caused significantly greater inhibition of ACh-induced relaxation in *cp*/*cp* rat aortic rings compared with +/? rings (L-NAME at 3 mg ml^{-1} resulted in 75.7±5.5 and 41.3±8.1 % maximal inhibition, respectively; Figure 6).

The NO donor, SNAP induced concentration-dependent relaxation of rings pre-contracted with PE (Figure 7). The sensitivity (expressed as EC_{50}) of *cp*/*cp* rat aortic rings to SNAP was similar to that of rings from +/? animals. The removal of endothelium resulted in increased sensitivity of rings from both genotypes to SNAP. The treatment with L-NAME resulted in increased sensitivity of rings from +/? rats to SNAP, an effect that was not detected in the *cp*/*cp* genotype (Figure 7).

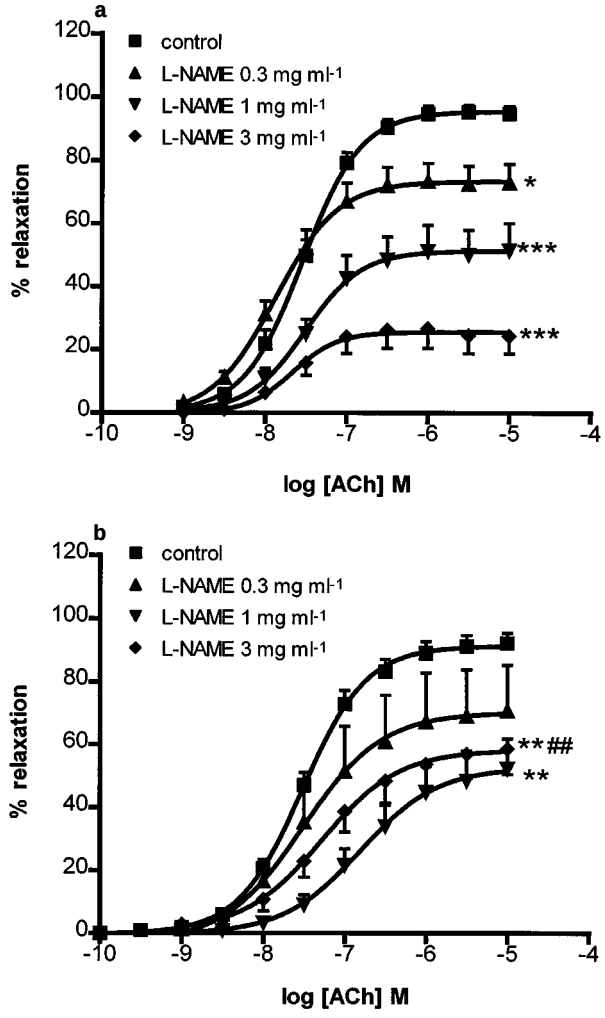

Figure 6 Concentration-response curves showing the acetylcholine (ACh)-induced relaxation of phenylephrine precontracted aortic rings from JCR:LA-*cp* rats and the ability of N^G-nitro-L-arginine methyl ester (L-NAME) to block this relaxation; (a) *cp*/*cp* rats and (b) +/? rats. Each point is the mean of 3 observations; vertical lines show s.e.mean. *$P<0.05$, **$P<0.01$ and ***$P<0.005$ indicate a significant difference from control. ##$P<0.01$, indicates a significant difference from *cp*/*cp* rings treated with the same concentration of L-NAME.

Effect of oral L-NAME on cyclic GMP and NOS activity in aorta

Nitric oxide synthase activity was similar in aorta of both genotypes. L-NAME treatment reduced NOS activity in *cp*/*cp* (Figure 8a) but not in +/? (Figure 8c) rat aorta.

Cyclic GMP content was similar in *cp*/*cp* and +/? rats. *In vivo* administration of L-NAME (0.3, 1 and 3 mg ml^{-1}) significantly reduced cyclic GMP content in *cp*/*cp* rats (Figure 8b) but exerted no significant effect on +/? rats (Figure 8d).

Effect of oral L-NAME on aggregation and cyclic GMP levels in platelets

Platelet aggregation in whole blood induced by collagen was similar in *cp*/*cp* and +/? rats (Figure 9a and c). *In vivo* administration of L-NAME (0.3, 1 and 3 mg ml^{-1}) exerted no significant effect on platelet aggregation in either genotype.

The platelet cyclic GMP levels were similar between *cp*/*cp* and +/? rats and L-NAME (0.3, 1 and 3 mg ml^{-1}) exerted no significant effect on cyclic GMP levels (Figure 9b and d).

Discussion

We have investigated the generation and actions of NO and its effects on vascular contractility and platelet function in JCR:LA-*cp* rats. Since basal release of NO is known to

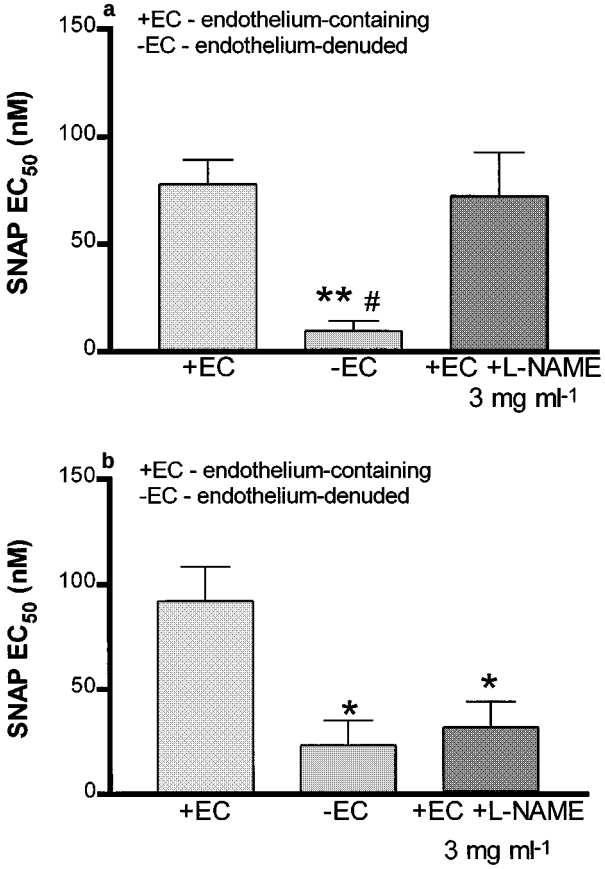

Figure 7 The effect of endothelial removal and NOS inhibition by L-NAME (3 mg ml^{-1}) on the sensitivity of rings from *cp*/*cp* (a) and +/? (b) rats to SNAP. Each column is the mean±s.e. mean of 3 observations. *$P<0.05$ and **$P<0.01$, indicate a significant difference from +EC and #$P<0.05$, indicates a significant difference from +EC +L-NAME (3 mg ml^{-1}).

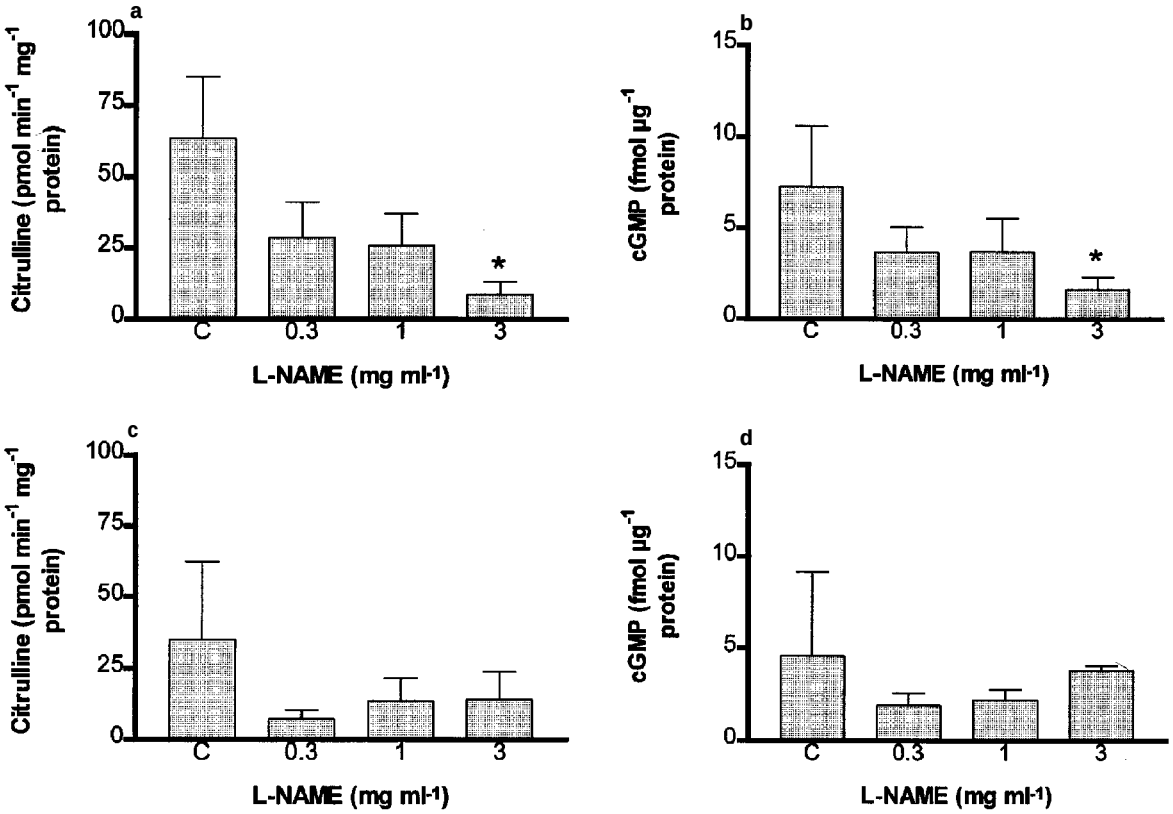

Figure 8 The activity of NOS and cyclic GMP (cGMP) content of aorta from JCR:LA-*cp* rats treated with L-NAME (0.3, 1 and 3 mg ml^{-1}). (a) NOS activity measured by the citrulline assay in *cp*/*cp* aorta; (b) cyclic GMP content in *cp*/*cp* aorta; (c) NOS activity measured by the citrulline assay in +/? aorta (d) cyclic GMP content in +/? aorta. Each column is the mean ± s.e. mean of 3 observations. $*P<0.05$, indicates a significant difference from control (C).

depress PE-induced contraction in endothelium-containing rings (Martin *et al*., 1986) and stimulated release of NO accounts for a significant part of ACh-induced relaxation in rat aorta (Furchgott & Zawadzki, 1980; Rees *et al*., 1989), we have used these tests to study the release and actions of NO in JCR:LA-*cp* rats.

The experiments with PE (a selective α_1 adrenoceptor agonist) showed that the rings obtained from *cp*/*cp* rats are more sensitive to the contractile action of this agonist than to those from +/? animals. The difference could not be attributed to an altered basal release of NO since this effect persisted following endothelial removal. In both genotypes, ACh-induced relaxation and the inhibition of this effect with L-NAME were similar. These results demonstrated that a differential sensitivity of aortic rings to PE between +/? and *cp*/*cp* rats was not due to alterations in NO generation by endothelial cells but to changes in the α_1 adrenoceptor and/or transduction mechanism operated by this compound. This effect is likely to occur at the vascular smooth muscle cells and is consistent with our recent observations that smooth muscle cells from *cp*/*cp* rats are hyper-proliferative and hyper-responsive to cytokines, including insulin-like growth factor (Absher *et al*., 1997). These findings are in agreement with other studies that have also shown that in vascular disease states in rats, such as spontaneous hypertension (Brown *et al*., 1994) and streptozotocin-induced diabetes mellitus (Chang & Stevens, 1992), the sensitivity of isolated vasculature to contracting agents is increased.

There is evidence that the long-lasting inhibition of NOS in vascular disorders including atherosclerosis (Naruse *et al*., 1994; Bryant *et al*., 1995; Zanchi *et al*., 1995; Henrion *et al*., 1996) accelerates the cellular lesion associated with these pathologies. We have, therefore, investigated the effects of such treatment on the vascular reactivity measured by PE-induced contraction and ACh-induced relaxation of aortic rings from *cp*/*cp* and +/? rats.

The treatment with L-NAME significantly increased mean blood pressure in *cp*/*cp* but not +/? rats. This is consistent with the *ex vivo* data showing that L-NAME reduced ACh-induced relaxation to a greater extent in *cp*/*cp* compared to +/? rats. Moreover, the activity of NOS and the bioactivity of NO as assessed by the citrulline assay and cyclic GMP content respectively, decreased only in *cp*/*cp* animals. It is likely that differential inhibition of NOS by L-NAME accounts for the increased blood pressure in *cp*/*cp* rats compared to +/? animals. In the +/? animals the weaker inhibitory effect of L-NAME is likely to be counteracted by the action of other physiological vasodilators, resulting in the maintenance of blood pressure at the physiological level.

A number of factors, including L-NAME intake, pharmacokinetics, the presence of endogenous inhibitors of NOS, changes in NOS activation and NO bioactivity, could account for the functional defect detected in *cp*/*cp* rats. This result is unlikely to be due to a difference in drug intake, as when water consumption and body weight are taken into consideration both groups received the same amount of L-NAME day^{-1} kg^{-1} body weight. Another possibility is that the pharmacokinetics of L-NAME are altered in *cp*/*cp* rats. N^G-nitro-L-arginine methyl ester (L-NAME) is a pro-drug that is metabolized to its active form, N^G-nitro-L-arginine (L-NOARG) in the blood and endothelial cells (Krejcy *et al*., 1993; Pfeiffer *et al*., 1996) and this activation could be

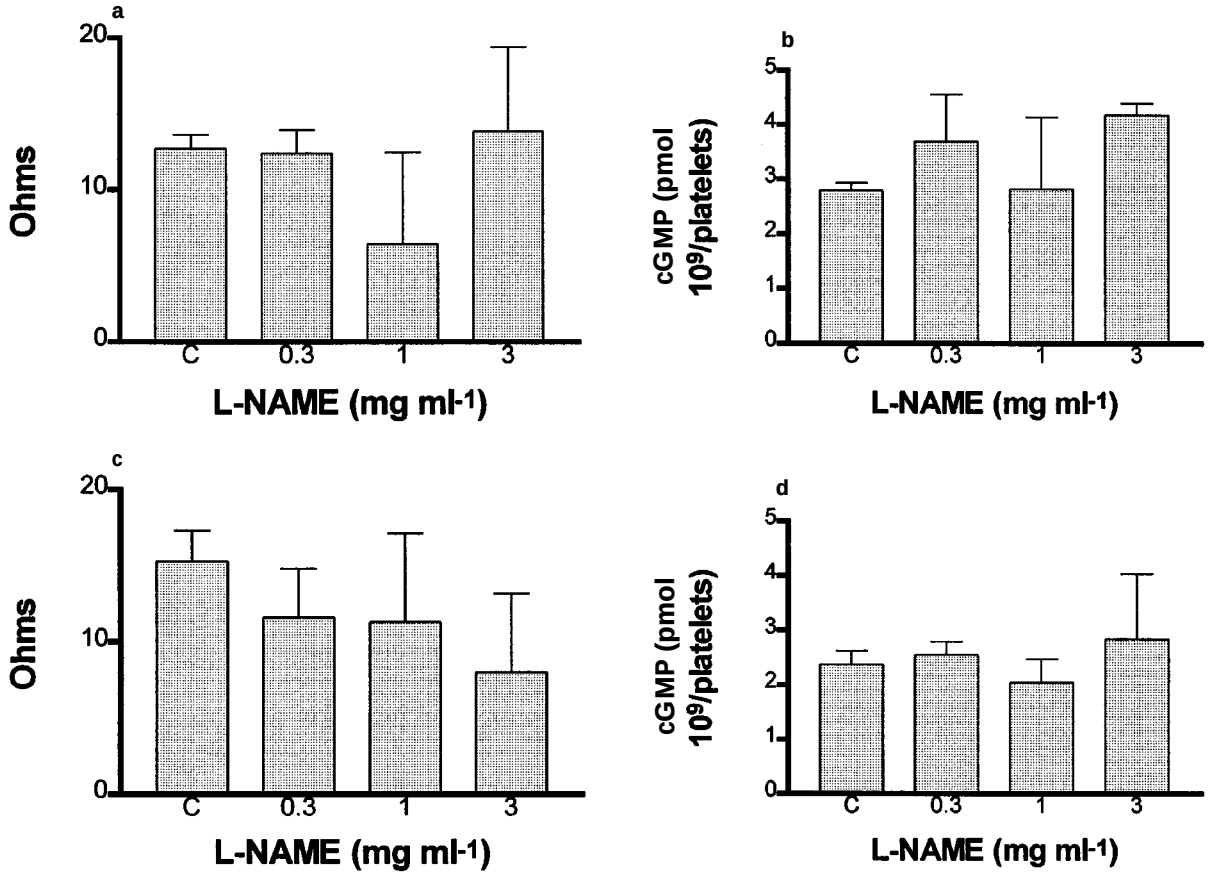

Figure 9 Whole blood platelet aggregation and cyclic GMP content of platelets from JCR:LA-*cp* rats treated with L-NAME (0.3, 1 and 3 mg ml^{-1}). (a) Platelet aggregation from *cp*/*cp* rats; (b) cyclic GMP content of platelets from *cp*/*cp* rats; (c) platelet aggregation from +/? rats; (d) cyclic GMP content of platelets from +/? rats. Each column is the mean±s.e.mean of 3 observations.

increased in *cp*/*cp* rats. Since L-NAME, when administered *in vitro*, was equipotent in inhibiting endothelium-dependent relaxation of rings, changes in endothelial bioactivation of L-NAME are unlikely to account for a differential sensitivity of rats to this inhibitor.

In vivo, activation of NOS depends mainly on the bioavailability of intracellular L-arginine and on the presence of factors that can affect the expression and activity of NOS. In patients with insulin-dependent diabetes mellitus, NO generation does not appear to be limited by L-arginine bioavailability (MacAllister *et al*., 1995). However, this may be the case in rats with streptozotocin-induced diabetes (Pieper & Peltier, 1995) as administration of L-arginine has been shown to restore endothelium-dependent relaxation that is impaired in this cytotoxic model of insulin-dependent diabetes. In *cp*/*cp* and +/? rats, administration of L-arginine did not affect endothelium-dependent relaxation, suggesting that an adequate amount of L-arginine is available for NOS in these animals.

A marked very low density lipoprotein (VLDL) hyperlipidaemia is a characteristic feature of obese *cp*/*cp* but not +/? rats (Russell, 1995). Low-density lipoproteins down-regulate the expression and activity of NOS (Chen *et al*., 1996) and increase the levels of the endogenous inhibitor of NOS, N^G,N^G-dimethylarginine (Yu *et al*., 1994). It is, therefore, likely that these metabolic alterations sensitize NOS to the inhibitory effect of L-NAME. Thus, dyslipidemia present in the *cp*/*cp* rats could contribute to increased sensitivity of NOS in these animals to the inhibition by L-NAME.

Interestingly, inhibition of NOS did not increase the sensitivity of *cp*/*cp* smooth muscle cells to the vasorelaxant actions of exogenous NO. In Wistar rats, mechanical removal of endothelium, or pharmacological removal of the endothelium through administration of NOS inhibitors, enhances the sensitivity of smooth muscle cells to NO donors (Moncada *et al*., 1991). This may be explained by sensitization of the soluble guanylate cyclase to exogenous NO following inhibition of endogenous NO. We observed a similar phenomenon in +/? but not in *cp*/*cp* rats.

We also investigated the function and metabolism of NO in platelets. Vascular complications of insulin-dependent diabetes mellitus can cause increased platelet activation and decrease bioactivity of NO (Amado *et al*., 1993). Insulin is known to inhibit platelet aggregation and this effect depends upon stimulation of platelet NOS and the subsequent increase in cyclic GMP levels (Trovati *et al*., 1997). Recently, Michimata and colleagues (1996) have shown that activation of the soluble guanylate cyclase is decreased in male patients suffering from NIDDM. We have failed to detect changes in platelet function in *cp*/*cp* rats. However, increased activity of plasminogen activator inhibitor type-1 (PAI-1), has been demonstrated in these animals, supporting the notion that *cp*/*cp* animals may be susceptible to thrombus formation (Schneider *et al*., 1998).

The results of our experiments emphasize the importance of NO for maintenance of vascular homeostasis, particularly under conditions of vascular insult exemplified by insulin

resistance, obesity and dyslipidaemia detected in *cp*/*cp* rats. The presenting functional defect appears to be in the vascular endothelium and smooth muscle cells and may result from hyperlipaedemia and/or hyperinsulinaemia.

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References

ABSHER, P.M., SCHNEIDER, D.J., BALDER, L.C., RUSSELL. J.C. & SOBEL, B.E. (1997). Increased proliferation of explanted vascular smooth muscle cells: a marker of atherogenesis. *Atherosclerosis,* **131,** 187 – 197.

AMADO, J.A., SALAS, E., BOTANA, M.A. POVEDA, J.J. & BERRAZUETA, J.R. (1993). Low levels of intraplatelet cGMP in IDDM. *Diabetes Care,* **16,** 809 – 811.

ANDERSON, T.D., GERHARD, M.D., MEDREDITH, I.T., CHARBONNEAU, F., DELAGANGE, D., CREAGER, M.A., SELWYN, A.P & GANZ, P. (1995). Systemic nature of endothelial dysfunction in atherosclerosis. *Am. J. Cardiol.,* **75,** 71B – 74B.

ANDREWS, H.E., BRUCKDORFER, K.R., DUNN, R.C. & JACOBS, M. (1987). Low-density lipoproteins inhibit endothelium-dependent relaxation in rabbit aorta. *Nature,* **327,** 237 – 239.

BOSSALLER, C., HABIB, G.B., YAMAMOTO, H., WILLIAMS, C., WELLS, S. & HENRY, P. (1987). Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5′-monophosphate formation in atherosclerotic human coronary artery and rabbit aorta. *J. Clin. Invest.,* **79,** 170 – 174.

BREDT, D.S., HWANG, P.M. & SNYDER, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature,* **347,** 768 – 770.

BROWN, L., AMOS, G. & MILLER, B. (1994). Disease-induced changes in a-adrenoceptor-mediated cardiac and vascular response in rats. *Clin. Exper. Pharmacol. Physiol.,* **21,** 721 – 728.

BRYANT, C.E., ALLOCOCK, G.H. & WARNER, T.D. (1995). Comparison of the effects of chronic and acute administration of N^G-nitro-L-arginine methyl ester to the rat on inhibition of nitric oxide-mediated responses. *Br. J. Pharmacol.,* **114,** 1673 – 1679.

CALVER, A., COLLIER, J. & VALLANCE, P. (1992). Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *J. Clin. Invest.,* **90,** 2548 – 2554.

CHANG, K.S.K, & STEVENS, W.C. (1992). Endothelium-dependent increase in vascular sensitivity to phenylephrine in long-term streptozotocin diabetic rat aorta. *Br. J. Pharmacol.,* **107,** 983 – 990.

CHEN, L.Y., MEHTA, P. & MEHTA, J.L. (1996). Oxidized LDL decreases L-arginine uptake and nitric oxide synthase protein expression in human platelets: relevance of the effect of oxidized LDL on platelet function. *Circulation,* **93,** 1740 – 1746.

CHESTER, A.H., O'NEIL, G.S., MONCADA, S., TADJKARIMI, S. & YACOUB, M.H. (1990). Low basal and stimulated release of nitric oxide in atherosclerotic epicardial coronary arteries. *Lancet,* **336,** 897 – 900.

CHO, H.J., XIE,, Q., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D. & NATHAN, C. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.,* **176,** 599 – 604.

DEFRONZO, R.A. & FERRANNINI, E. (1991). A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care,* **14,** 173 – 194.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature,* **288,** 373 – 376.

HENRION, D., DOWELL, F.J., LEVY, B.I. & MICHEL, J-B. (1996). *In vitro* alteration of aortic vascular reactivity in hypertension induced by chronic N^G-nitro-L-arginine methyl ester. *Hypertension,* **28,** 361 – 366.

KOLETSKY, S. (1973). Obese spontaneously hypertensive rats: a model for the study of atherosclerosis. *Exp. Mol. Pathol.,* **19,** 53 – 60.

KOLETSKY, S. (1975). Pathologic findings and laboratory data in a new strain of obese hypertensive rats. *Am. J. Pathol.,* **80,** 129 – 142.

KREJCY, K., SCHWARZACHER, S. & RABERGER, G. (1993). Distribution and metabolism of N^G-nitro-L-arginine and N^G-nitro-L-arginine methy ester in canine blood *in vitro*. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **347,** 342 – 345.

LIAO, J.K., SHIN, W,S., LEE W.Y. & CLARK, S.L. (1995). Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem.,* **270,** 319 – 24.

MACALLISTER, R.J., CALVER. A.L., COLLIER, J., EDWARDS, C.M., HERREROS, B., NUSSEY, S.S. & VALLANCE P. (1995). Vascular and hormonal responses to arginine: provision of substrate for nitric oxide or non-specific effect?. *Clin. Sci.,* **89,** 183 – 190.

MARTIN, W., FURCHGOTT, R.F., VILLANI, G.M. & JOTHIANANDAN, D. (1986). Depression of contractile response in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.,* **237,** 529 – 537.

MICHIMATA, T., MURAKAMI, M. & IRIUCHIJIMA, T. (1996). Nitric oxide-dependent soluble guanylate cyclase activity is decreased in platelets from male NIDDM patients. *Life Sci.,* **59,** 1463 – 1471.

MONCADA, S., REES, D.D., SCHULZ, R. & PALMER, R.M.J., (1991). Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 2166 – 2170.

MOORE, P.K., AL-SAWAYEH, O.A., CHNG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-Nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilation *in vitro*. *Br. J. Pharmacol.,* **99,** 408 – 412.

NARUSE, K., SHIMIZU, K., MURAMATSU, M., TOKI, Y., MIYAZAKI, Y., OKUMURA, K., HASHIMOTO, H. & ITO, T. (1994). Long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta: PGH_2 does not contribute to impaired endothelium-dependent relaxation. *Arterioscler. Thromb.,* **14,** 746 – 752.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S., (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature,* **333,** 664 – 666.

PFEIFFER, S., LEOPOLD, E., SCHMIDT, K., BRUNNER, F. & MAYER B. (1996). Inhibition of nitric oxide synthesis by N^G-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, N^G-nitro-L-arginine. *Br. J. Pharmacol.,* **118,** 1433 – 1440.

PIEPER, G.M & PELTIER, B.A. (1995). Amelioration by L-arginine of a dysfunctional arginine/nitric oxide pathway in diabetic endothelium. *J. Cardiovasc. Pharmacol.,* **25,** 397 – 403.

RADOMSKI, M.W. & SALAS, E. (1995). Nitric oxide - biological mediator, modulator and factor of injury: its role in the pathogenesis of atherosclerosis. *Atherosclerosis,* **118,** S69 – S80.

RADOMSKI, M.W., VALLANCE, P.J., WHITELY, G., FOXWELL, N. & MONCADA, S. (1993). Modulation of platelet adhesion to human vascular endothelium by the constitutive and cytokine-inducible nitric oxide synthases. *Cardiovasc. Res.,* **27,** 1380 – 1382.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acd. Sci. U.S.A.,* **86,** 3375 – 3378.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.,* **101,** 746 – 752.

RUSSELL, J.C. (1995). The atherosclerosis-prone JCR:LA-corpulent rat. *In Atherosclerosis X: Proceedings of the 10th International Symposium on Atherosclerosis*. ed. Woodford, F.P., Davignon, J. & Sniderman, A. pp. 121 – 125. Amsterdam: Elsevier.

RUSSELL, J.C., AMY, R.M., GRAHAM, S.E., DOLPHIN, P.J., WOOD, G.O. & BARTANA, J. (1995). Inhibition of atherosclerosis and myocardial lesions in the JCR:LA-*cp* rat by β,β'-tetramethylhexadecanedioic acid (MEDICA 16). *Atherosceler. Thromb. Vasc. Biol.,* **15,** 918 – 923.

RUSSELL, J.C., AMY, R.M., MICHAELIS, O.E., MCCUNE, S.M. & ABRAHAM, A.A. (1990). Myocardial disease in the corpulent strains of rats. *In Frontiers in Diabetes Research: Lessons From Animal Models III*. ed. Shafrir, E. pp. 402 – 407. London: Smith-Gordon

SCHNEIDER, D.J., ASHER, P.M., NEIMANE, D., RUSSELL, J.C. & SOBEL, B.E. (1998). Fibrinolysis and atherogenesis in the JCR:LA-*cp* rat in relation to insulin and triglyceride concentration in blood. *Diabetologia,* in press.

SESSA, W.C., HARRISON, J.K., BARBER, C.M., ZENG, D., DURIEUX, M.E., D'ANGELO, D., LYNCH, K.R. & PEACH, M.J. (1992). Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J. Biol. Chem.,* **267,** 15274–15276.

SOWERS, J.R. & EPSTEIN, M. (1995). Diabetes mellitus and associated hypertension, vascular disease, and nephropathy: an update. *Hypertension,* **26,** 869–879.

TROVATI, M., ANFOSSI, G., MASSUCCO, P., MATTIELLO, L., COSTAMAGNA, C., PIRETTO, V., MULARONI, E., CAVALOT, F., BOSIA, A. & GHIGO, D. (1997). Insulin stimulates nitric oxide synthesis in human platelets and, through nitric oxide, increases platelet concentrations of both guanosine-3′, 5′-cyclic monophosphate and adenosine-3′, 5′-cyclic monophosphate. *Diabetes,* **46,** 742–749.

YU, X-J., LI, Y-J. & XIONG, Y. (1994). Increase of an endogenous inhibitor of nitric oxide synthesis in serum of high cholesterol fed rabbits. *Life Sci.,* **54,** 753–758.

ZANCHI, A., AUBERT, J.F., BRUNNER, H.R. & WAEBER, B. (1995). Vascular acetylcholine response during chronic NO synthase inhibition *in vivo* versus *in vitro*. *Cardio. Res.,* **30,** 122–129.

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