Vascular dysfunction and myocardial contractility in the JCR:LA-corpulent rat

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Received 23 November 1999; accepted 18 February 2000

Abstract

Objective: The JCR:LA-corpulent rat is a unique animal model of human vascular disease that exhibits a profound insulin resistance, vasculopathy, and cardiovascular dysfunction. We tested the hypothesis that the defects affect endothelial and smooth muscle function of the coronary microcirculation as well as cardiac contractility. Coronary, myocardial and aortic function were assessed in obese (homozygous for the cp gene, cp/cp) and lean (heterozygous or homozygous normal, +/?) littermates aged 7 and 18 weeks. Methods: Coronary endothelial relaxation was examined in isolated perfused hearts by determining the effect of bradykinin (0.1–1000 nmol l^-1) on coronary perfusion pressure (CPP), myocardial mechanical function was evaluated in terms of left-ventricular developed pressure (LVDevP), and aortic relaxation with the endothelium-dependent agonist, A23187 (1–1000 nmol l^-1). Results: In rats aged 7 weeks, bradykinin reduced CPP from 133±1 mmHg to 43±1 mmHg (−67%) in lean rats, but only to 64±3 mmHg (−52%) in corpulent rats (n=6, P<0.05). Similar differences were found in rats aged 18 weeks (n=8). Inhibition of NO synthase with N-nitro-L-arginine (L-NNA; 0.2 mmol l^-1), impaired, and tetrahydrobiopterin (0.1 mmol l^-1), a NO synthase cofactor, restored relaxation in cp/cp rats. Spermine/NO equally reduced CPP in both groups (−58%). Mechanical function was similar in lean and corpulent rats, aortic endothelial relaxation was attenuated by ~30% and aortic smooth muscle function was normal (7 weeks) or improved (18 weeks) in the cp/cp genotype. Conclusion: These results suggest that (i) there is a specific impairment of NO-mediated relaxation of the coronary resistance vessels in the JCR:LA-corpulent rat that is not associated with impaired baseline myocardial contractility, and (ii) exogenous tetrahydrobiopterin reversed the relaxation defects that are part of the vascular complications typical for the insulin resistance syndrome.

Keywords: Diabetes; Endothelial function; Microcirculation; Nitric oxide; Ventricular function

1. Introduction

The insulin resistance syndrome, a common metabolic disorder of the adult population in Western cultures, is associated with a cluster of cardiovascular abnormalities such as hypertension, dyslipidemia and atherosclerosis [1]. The relationship between the syndrome and cardiovascular disease is not completely understood, but may involve abnormalities in arterial wall function. One prominent feature of abnormal arterial vasomotion is defective endothelium-dependent relaxation in insulin resistance states such as hypertension and non-insulin-dependent diabetes mellitus (NIDDM). In humans, endothelial dysfunction is frequently studied in terms of the blood flow response to local injection of endothelium-dependent agonists, whereas vascular smooth muscle function is tested with endothelium-independent agents such as the classical nitrovasodilators [2]. Such studies have shown that the leg blood flow response to intrafemoral methacholine which generates NO via activation of endothelial NO synthase [3]...
was considerably lower in obese insulin-resistant subjects or in subjects with NIDDM, compared with non-diabetic control subjects [4]. Similar differences were obtained in the forearm, even after adjustment for obesity [5]. Elevation of circulating free fatty acids to levels seen in insulin-resistant subjects can impair endothelial function [6] and maneuvers enforcing NO activity can impair it. Therefore, the pathogenesis of endothelial dysfunction in NIDDM is currently believed to be a consequence of the effect of dyslipoproteinemia and of hyperoxidative stress on the formation, action and disposal of NO by diverse molecular mechanisms [7]. Clearly, endothelial dysfunction appears to be an integral aspect of the syndrome, independently of hyperglycemia. In contrast to endothelial dysfunction, vascular smooth muscle function as tested with the endothelium-independent agents nitroprusside or nitroglycerin has been mostly normal.

The insulin resistance syndrome is also an independent predictor of ischemic heart disease [8] and diabetes-related cardiomyopathy [9], but neither the coronary conductance nor microvessels can be conveniently studied in humans. However, ex vivo studies of the intact coronary circulation and isolated vessels from an insulin-resistant rat model allow assessments of coronary microvascular and conductance vessel function. These studies were undertaken in the JCR:LA-corpulent (JCR:LA-cp) rat, a unique animal model that exhibits all aspects of the metabolic syndrome as it occurs in humans [10]. In this model, animals homozygous for the autosomal recessive cp gene (cp/cp) are hyperphagous and become obese and insulin-resistant, whereas homozygous normal (+/+ or heterozygous (+/c) animals are lean and do not develop metabolic alterations. Animals of both genotypes are normotensive. The cp mutation has recently been shown to create a stop codon in the extracellular domain of the leptin receptor with consequent loss of receptors [11]. The male cp/cp animals spontaneously develop atherosclerotic disease by middle age as well as myocardial lesions, consistent with an ischemic origin [12].

We have previously characterized aortic function in cp/cp rats and found evidence for a specific impairment of endothelium-dependent relaxation which appeared to be limited to that mediated by muscarinic receptors [13]. We hypothesized that besides affecting endothelial function in conductance vessels, hyperinsulinemia would impair coronary resistance vessel and NO-mediated cardiac function. Therefore, we have characterized coronary microvessel function and myocardial contractility in obese and lean rats using endothelium-dependent and -independent agents. Additionally, we specifically tested the hypothesis that the endothelial dysfunction resulted from an aberrant NO synthase reaction at the level of the essential cofactor, tetrahydrobiopterin (H₂biopterin) [14]. To this end, coronary endothelium-dependent dilation was determined in the presence of exogenous H₂biopterin and cofactor levels were measured in plasma.

2. Methods

2.1. Animals

Male rats of the JCR:LA-cp strain were bred in our breeding colony using standard husbandry techniques and a formal system of outbreeding [15]. The young rats can be identified either as homozygous for the cp gene (cp/cp) and obese, or lean and homozygous/heterozygous normal (+/+ or +/c, a 2:1 mixture referred to as +/? in this paper) at 3 weeks of age when they are weaned. Male cp/cp and +/? rats aged 7 or 18 weeks were used in this study (in one protocol cp/cp rats aged 37 weeks were used). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Heart perfusion

Hearts were perfused retrogradely (Langendorff mode) at a rate of 9.0 ml min⁻¹ g⁻¹ heart wet weight with a modified Krebs–Henseleit bicarbonate buffer as previously described [16]. Cardiac parameters were monitored continuously and included heart rate, coronary perfusion pressure (CPP), left-ventricular developed pressure (LVDevP; difference between left-ventricular peak systolic pressure and end-diastolic pressure), maximal rate of rise of LVDevP (dP/dt) and left-ventricular end-diastolic pressure (LVEDP).

2.3. Coronary function

Coronary flow was set at 15 ml min⁻¹ g⁻¹ resulting in a CPP of 130–135 mmHg. Coronary endothelium-dependent relaxation was tested with bradykinin given as bolus injections through a sideline, resulting in concentrations of 0.1–1000 nmol l⁻¹. Maximal relaxation was reached 3–4 min after injection. Doses were given in cumulative manner. After the last dose, bradykinin was washed out for 20 min and CPP returned to ~133 mmHg. To test the NO-dependence of the relaxation, L-NNA (0.2 mmol l⁻¹) was applied over 20 min and the bradykinin dose–response curve was repeated in the continued presence of L-NNA. Because L-NNA constricted coronary vessels, coronary flow was decreased to ~10 ml min⁻¹ to maintain the baseline CPP at ~130 mmHg. Finally, after washout of L-NNA and bradykinin for 20 min, a single bolus dose of spermine/NO (final concentration 0.1 mmol l⁻¹) was added to the perfusion buffer to test coronary smooth muscle function. In a separate protocol, indomethacin (10 μmol l⁻¹) was used to test for a role of cyclo-oxygenase products. The NO synthase cofactor, H₂biopterin (0.1 mmol l⁻¹) was used in hearts from cp/cp rats aged 37 weeks to test whether an aberrant NO synthase reaction is involved in the impaired relaxation response to bradykinin.
2.4. Relaxation of aortic rings

The thoracic aorta was cleaned of connective tissue and cut into rings ~3-mm long with endothelium present. The rings were suspended in tissue baths containing 5 ml Krebs–Henseleit solution maintained at 37°C, pH 7.4 and gassed with carbogen and tension was recorded isometrically. Basal tension before addition of agonist was 2 g. Experimental groups consisted of 5 animals aged 7 and 8 animals aged 18 weeks, both +/? and cp/cp. Four to five aortic rings were prepared from animals aged 7, and 6–8 rings from animals aged 18 weeks. The preparations were studied after 90–120 min of equilibration, with changes of bath fluid every 30 min. Tissues were precontracted with U 46619 (100 nmol l⁻¹) and angiotensin II (5 μmol l⁻¹) which gave a maximal tension of ~5 g. When the contraction had reached a stable plateau, the relaxation to the endothelium-dependent agonist, calcium ionophore A 23187 (1–1000 nmol l⁻¹) was tested. After the last concentration, tissues were washed (~30 min), contracted again with U 46619+angiotensin II and treated with L-NNA (0.2 mmol l⁻¹) to inhibit NO synthase, and the concentration–response curve of A 23187 was repeated. Smooth muscle function was examined by cumulative addition of spermine/NO complex (10 nmol l⁻¹–100 μmol l⁻¹) or the ATP-sensitive K⁺-channel opener rilmakalim (3 nmol l⁻¹–100 μmol l⁻¹). To test the relaxant effect of rilmakalim, two modes of precontraction were used, i.e. U 46619+angiotensin II (as in all other cases), or K⁺ (40 mmol l⁻¹). All experiments were performed in the presence of indomethacin (10 μmol l⁻¹) to block the formation of vasoactive prostanoids by cyclo-oxygenases.

2.5. Assay for NO synthase activity in cell homogenates

Left ventricular tissue was homogenized in 50 mmol l⁻¹ tetraethyl ammonium containing 1% β-mercaptoethanol, 10 mmol l⁻¹ CHAPS [((3-chol-amidopropyl)dimethylammonio)-1-propanesulfonate] and 0.5 mmol l⁻¹ EDTA using a glass homogeniser. Aortas were finely cut using a scalpel and suspended in the same buffer. After 3 cycles of freeze–thawing followed by centrifugation at 10,000 g, the protein concentration in the supernatant was determined using the Bradford assay. NO synthase activity was determined by the conversion of 3H-L-arginine to 3H-L-citrulline [17].

2.6. Determination of biopterin in plasma

Plasma samples (120 μl) were filtered (0.22-μm cellulose acetate centrifuge tube filters from Szabo, Vienna, Austria). Tetrahydro- and dihydrobiopterin were oxidized to biopterin by treatment with KI/I₂ under acidic conditions as described [18]. Fifty-μl samples were injected onto a 250X4 mm C₁₈ reversed-phase column (LiChro- spher 100 RP-18, 5-μm particle size, Merck, Vienna, Austria), eluted with 20 mmol l⁻¹ sodium phosphate buffer, pH 3, containing 5% (v/v) methanol, at 0.7 ml min⁻¹ and detected by fluorescence spectrophotometry (Hitachi F 1050; extinction 350 nm, emission 440 nm). Calibration curves were recorded with authentic tetrahydrobiopterin (10–500 nmol l⁻¹).

2.7. Data analysis and calculations

Coronary relaxation was calculated as reduction in CPP from the maximal pressure (130–135 mmHg), and relaxation of aortic rings as reduction in tone from the maximal induced tone (4–6 g) and expressed as a percentage. Individual concentration–response curves were fitted to a Hill-type model giving estimates of agonist potency (EC₅₀) and efficacy (Eₘₐₓ). Significance was assumed at P<0.05. Data are reported as arithmetic mean±S.E.M. (hemo-dynamic parameters and Eₘₐₓ values) or geometric mean±95% confidence interval (EC₅₀) [19]. All concentrations are expressed as final concentrations in the coronary circulation or organ bath.

3. Results

3.1. Experimental groups

The body weight of cp/cp rats was significantly higher than that of lean controls in all age groups: 246±2 vs. 183±3 g (7 weeks, n=6) and 574±5 vs. 357±7 g (18 weeks, n=8; P<0.05 in both cases). However, the corresponding heart weights were not different between groups: 0.69±0.025 vs. 0.78±0.018 g (7 weeks) and 1.23±0.01 vs. 1.18±0.02 g (18 weeks). Systolic arterial blood pressure was measured in animals aged 14–16 weeks and was not different between groups (134±1.5 vs. 138±1.6 mmHg, n=9). All animals showed similar heart rate, LVDevP and CPP irrespective of age and experimental group (Table 1).

3.2. Coronary circulation

The coronary relaxant effect of bradykinin in hearts from rats aged 7 weeks is shown in Fig. 1. The agonist was less effective in corpulent rats (maximal relaxation: ~52±3%; EC₅₀: 2.8±1.5 mmol l⁻¹) than in lean controls (~67±1%; EC₅₀: 0.48±0.04 mmol l⁻¹; P<0.05). In the presence of L-NNA to block NO synthase, the effect of bradykinin was uniformly antagonized in both experimental groups (maximal relaxation: ~29%; EC₅₀: 4.8±1.7 mmol l⁻¹ in +/? and 8.8±1.2 mmol l⁻¹ in cp/cp animals; N.S.). The effect of the endothelium-independent agonist spermine/NO (0.1 mmol l⁻¹) was similar in both experimental groups (~59±2 and ~57±2% of maximum; N.S.; Fig. 1).
Table 1
Hemodynamic parameters in lean (+/?) and obese (cp/cp) rats aged 7 or 18 weeks

|                      | Heart rate (beats min⁻¹) | LVDevP (mmHg) | CPP (mmHg) |
|----------------------|--------------------------|---------------|------------|
| +/? rats, 7 weeks    |                          |               |            |
| Baseline (6)         | 318 ± 7                  | 80 ± 1        | 132 ± 1    |
| + L-NNA (6)          | 312 ± 6                  | 64 ± 1*       | 132 ± 1    |
| cp/cp rats, 7 weeks  |                          |               |            |
| Baseline (6)         | 321 ± 5                  | 81 ± 2        | 132 ± 1    |
| + L-NNA (6)          | 313 ± 4                  | 64 ± 1*       | 131 ± 2    |
| +/? rats, 18 weeks   |                          |               |            |
| Baseline (8)         | 310 ± 5                  | 81 ± 1        | 131 ± 1    |
| + L-NNA (8)          | 299 ± 5                  | 61 ± 1*       | 132 ± 1    |
| cp/cp rats, 18 weeks |                          |               |            |
| Baseline (8)         | 301 ± 6                  | 81 ± 2        | 132 ± 1    |
| + L-NNA (8)          | 291 ± 6                  | 62 ± 2*       | 132 ± 1    |

LVDevP, left ventricular developed pressure; CPP, coronary perfusion pressure. Data are means ± S.E.M. of the number of hearts given in parentheses.
+/?, lean rats; cp/cp, obese rats.* P < 0.05 vs. corresponding baseline.

The corresponding effects of bradykinin in hearts from rats aged 18 weeks are shown in Fig. 2. Again, both the maximal effect and the potency of the agonist was lower in corpulent rats (−44 ± 1% of baseline; EC₅₀ 1.4 ± 0.24 nmol L⁻¹) than in lean controls (−62 ± 1% of maximum; EC₅₀: 0.46 ± 0.05 nmol L⁻¹; P < 0.05). In the presence of L-NNA, the difference in Eₘₐₓ was abolished (26 ± 2% vs. 29 ± 2%; N.S.), but the EC₅₀ values were still different in this age group (4.2 ± 0.6 and 10.0 ± 1.6 nmol L⁻¹; P < 0.05). As in the younger rats, spermine/NO (0.1 mmol L⁻¹) was similarly effective in both experimental groups (Fig. 2).

We also tested whether products of cyclo-oxygenase were involved in the impaired relaxation response in cp/cp rats. However, indomethacin (10 μmol L⁻¹) had no effect on the reduction of CPP by bradykinin, both in control and cp/cp hearts (n = 5; not shown).

The effect of the natural cofactor for NO synthase, H₂biotin (0.1 mmol L⁻¹) on bradykinin-induced coronary relaxation was tested in obese rats aged 37 weeks (Fig. 3). In these hearts, agonist function was similarly depressed as in cp/cp hearts aged 18 weeks (EC₅₀: 1.7 ± 0.53 nmol L⁻¹; CPP reduction −42 ± 3%). The cofactor restored agonist potency to the level observed in lean rats aged 18 weeks (EC₅₀: 0.35 ± 0.05 nmol L⁻¹) and significantly increased its maximal relaxant effect (−55 ± 2%; compare Figs. 2 and 3).

3.3. Myocardial function

The effect of bradykinin on LVDevP is shown in Fig. 4.
Baseline LVDevP was 80±81 mmHg in both experimental groups and was reduced to 64±2 mmHg by L-NNA (P<0.05). Bradykinin weakly, but significantly, reduced contractility in concentration-dependent fashion in lean and corpulent rats, both in the absence and presence of L-NNA. A positive inotropic effect was never observed. Virtually identical results were obtained in rats aged 18 weeks: LVDevP was reduced from 80±1 mmHg (baseline) to 61±1 mmHg (at 1 µmol l⁻¹ bradykinin) in the absence of L-NNA, and from 61±2 mmHg (baseline) to 47±2 mmHg in the presence of L-NNA. Indomethacin had no effect on the bradykinin-induced reduction of LVDevP in any group. Comparable results as for LVDevP were obtained for the second contractility parameter, dP/dt (not shown).

3.4. NO synthase activity

NOS activity was determined in terms of formation of [³H]citrulline in aorta and left ventricle. In +/? tissue, the rates of formation were 8.4±1.1 and 3.4±0.5 pmol mg⁻¹ min⁻¹; in cp/cp tissues they were 10.6±0.5 and 3.6±1.4 pmol mg⁻¹ min⁻¹ (n=3 in each case, N.S. +/? vs. cp/cp; P<0.05 aorta vs. ventricle).

3.5. Plasma biopterin levels

As a surrogate for determinations in vascular tissue, total biopterin levels were determined in plasma using a sensitive HPLC method. Similar levels were found in lean and obese rats aged 18 weeks (40.3±2.84 and 41.9±3.20 nmol l⁻¹; P<0.05, n=8).

3.6. Aorta

Aortic ring relaxation was studied after submaximal stable contraction using the organ bath setup. The calcium ionophore A 23187 relaxed tissues from cp/cp and +/? animals with similar potency (no difference in EC₅₀ values), but the maximum relaxation response was less (P<0.05) in the obese genotype of both age groups (Table 2). L-NNA reduced relaxation to ≤20% and abolished the difference in Eₘₐₓ between genotypes (N.S. cp/cp vs. +/?).

Fig. 5 shows the relaxation of aortic rings in response to the endothelium-independent agent spermine/NO. In both age groups, the agonist was similarly effective in relaxing rings from lean and corpulent rats (N.S. for E₅₀ and maximal relaxation), but the tissues from rats aged 18 weeks showed a greater relaxation (Eₘₐₓ~60%) than those from the younger rats (Eₘₐₓ~37%; P<0.05; Table 2).

Finally, to determine whether hyperpolarization-induced relaxation was affected in cp/cp rats, the relaxant potency of rilmakalim, a K⁺ channel agonist was tested in aortic rings from rats aged 18 weeks following contraction with U 46619+angiotensin II or K⁺ depolarization (Fig. 6). After either precontraction, the agonist was similarly effective in both groups (~51% and ~50% relaxation, respectively; N.S. +/? vs. cp/cp) but, as expected, the respective EC₅₀ values were much higher after K⁺ depolarization than receptor-mediated contraction (Table 2).
Table 2
Relaxant responses of aortic rings

|          | 7 weeks |                  |                  | 18 weeks |                  |                  |
|----------|---------|------------------|------------------|----------|------------------|------------------|
|          | n       | Maximal response | $EC_{50}$ (mol l$^{-1}$) | n       | Maximal response | $EC_{50}$ (mol l$^{-1}$) |
|          | (%) relaxation |                  |                  | (%) relaxation |                  |                  |
| A 23187  |         |                  |                  |          |                  |                  |
| +/-?, Vehicle | 20     | 28±1.0           | 7.9 (2.1–32)×10$^{-8}$ | 28     | 34±0.3           | 7.4 (5.4–10)×10$^{-8}$ |
| +/-?, t-NNA | 27     | 30±1.0           | 3.2 (3.2–5.8)×10$^{-7}$ | 26     | 4×2.0$^b$        | 2.0 (1.4–2.8)×10$^{-7}$ |
| cp/cp, Vehicle | 21     | 20±0.4$^a$       | 4.5 (3.7–5.4)×10$^{-8}$ | 30     | 23×0.7$^a$       | 6.6 (3.1–14)×10$^{-8}$ |
| cp/cp, t-NNA | 23     | 3±0.1$^b$        | 2.2 (1.1–4.4)×10$^{-7}$ | 28     | 3×0.2$^b$        | 2.2 (1.3–3.5)×10$^{-7}$ |
| Spermine/NO |         |                  |                  |          |                  |                  |
| +/-?, Vehicle | 23     | 38±0.8           | 4.0 (5.3–5.6)×10$^{-8}$ | 15     | 62×1.0$^c$       | 5.8 (3.0–11)×10$^{-7}$ |
| cp/cp, Vehicle | 19     | 36±1.5           | 5.4 (3.8–7.6)×10$^{-8}$ | 16     | 57±1.0$^c$       | 1.3 (0.9–1.9)×10$^{-6}$ |

Rilmakalim

|          |         |                  |                  |          |                  |                  |
|----------|---------|------------------|------------------|----------|------------------|------------------|
| +/-?, K$^+$ contraction | 22     | N.D.             |                  | 22       | N.D.             |                  |
| +/-?, U 46619+ang. II contr. | 17     | 51±0.4           |                  | 18       | 50±1.8           |                  |
| cp/cp, K$^+$ contraction | 22     |                  |                  |          |                  |                  |
| cp/cp, U 46619+ang. II contr. | 18     |                  |                  |          |                  |                  |

$^a$P<0.05 +/-? vs. cp/cp within age group.

$^b$P<0.05 t-NNA vs. vehicle within age group.

$^c$P<0.05 between age groups.

cp/cp indicates rats homozygous for the corpulent (cp) gene; +/-?, homozygous normal rats. The rilmakalim curves were obtained after precontraction with K$^+$ (40 mmol l$^{-1}$) or U 46619 (100 nmol l$^{-1}$)+angiotensin II (ang. II, 5 μmol l$^{-1}$). Values are arithmetic mean±S.E.M. (maximal response) and geometric mean±95% confidence interval ($EC_{50}$ values), respectively. n refers to the number of tissues; these were derived from 4 to 5 hearts (animals aged 7 weeks) and 6–7 hearts (animals aged 18 weeks), respectively. N.D., not determinable.

4. Discussion

The JCR:LA-corpulent rat is a unique genetic animal strain that closely resembles the features of humans with early stage type II diabetes characterized by insulin resistance and hyperinsulinemia. Animals of the cp/cp strain become detectably obese at 3 weeks of age, mild hyperinsulinemia is present at 4 weeks and develops rapidly, and at 12 weeks of age there is no insulin-mediated glucose uptake or turnover. The profound peripheral insulin resistance in cp/cp rats leads to the...
diversion of glucose to triglyceride synthesis and marked VLDL hyperlipidemia [10]. Our major finding was a specific impairment of NO-mediated relaxation of the coronary resistance vessels that is completely reversed by exogenous H₄biop. Furthermore, we have shown that endothelium-dependent but not endothelium-independent aortic relaxation was reduced, and myocardial contractility was unaffected, in these relatively young obese animals.

We examined the contractility of the hearts in terms of LVDevP and dP/dt measurements and found matching values in both groups of hearts. Endogenous NO exerted a strong positive inotropic effect as evident from the reduction of LVDevP in the presence of l-NNA (−27%) whereas NO generated by bradykinin depressed myocardial function. Because experiments were done in the presence of indomethacin, endothelial mediators generated by stimulated cyclo-oxygenase activity can be excluded. These findings are reminiscent of recent evidence supporting a biphasic effect of NO/cGMP on myocardial pump function [20]. The complete lack of a positive inotropic effect of bradykinin at low concentrations was unexpected and not investigated further. It appears that the striking myocardial lesions in cp/cp rats that we observed previously in cp/cp rats aged 39 weeks [12] are without consequence to normoxic left-ventricular function in animals up to 18 weeks of age, whereas older animals exhibit an increased sensitivity to ischemic myocardial injury [21]. In streptozotocin-diabetic rats, basal ventricular performance and the responsiveness to β-adrenergic stimulation was suppressed, and inhibition of NO synthase with Ν⁴-nitro-l-arginine methyl ester restored hemodynamic function to the level of non-diabetic hearts [22]. Cardiodepression was attributed to products of inducible NO synthase in this model, whereas, apparently, no such induction occurs in the cp/cp rat heart in which dysfunction is related to hyperinsulinemia and hypertriglyceridemia [10,15].

The intact coronary circulation of cp/cp rats showed a strikingly impaired relaxation response to bradykinin that was fully expressed at 7 weeks of age. The impairment was clearly due to a reduced activity of the NO/cGMP pathway because l-NNA inhibited the agonist’s effect to the same extent in both experimental groups, whereas the cyclo-oxygenase pathway was not involved, as is evident from the lack of effect of indomethacin. The impairment is consistent with the 2-fold elevation of activity, and 3-fold elevation of content, of phosphodiesterase-3A in the aorta of cp/cp rats [23]. An important component of the underlying pathophysiological mechanism was revealed when hearts from cp/cp rats were perfused with H₄biop which promptly resulted in restoration of bradykinin-induced coronary relaxation. H₄biop is an allosteric activator of NO synthase that is needed to direct the electron flow to l-arginine, thus coupling NADPH oxidation to NO synthesis [24]. In the presence of suboptimal levels of H₄biop, the NO synthase reaction results in the generation of superoxide anions rather than NO, followed by production of hydrogen peroxide and/or peroxynitrite [14,25]. These biochemical investigations support the concept that an uncoupled NO synthase is a source of reactive oxygen metabolites that may play an important role in the endothelial dysfunction and oxidative vascular injury described in several diseases, including diabetic endothelial dysfunction [26,27]. The substantially reduced relaxation observed in the coronary microvasculature of cp/cp rats in the present study, and its alleviation by H₄biop, is strong evidence for a dysfunctional endothelial NO synthase enzyme in the obese, insulin-resistant state. However, in view of the complexity of the NO synthase reaction, the mechanism responsible for the dysfunction is more difficult to discern. A reduced formation of intracellular H₄biop due to impaired activity of the key enzyme, cyclohydrolase, hastened degradation or a reduced activity of the cofactor could all play a role. We speculated that a reduced formation might translate to reduced plasma cofactor levels in cp/cp rats, but this was not the case. Because similar plasma levels of H₄biop do not necessarily indicate similar intracellular cofactor levels, the simplest explanation of our data assumes a reduced intracellular activity of H₄biop. Alternatively, availability of the substrate l-arginine may be limiting NO synthase activity. However, under normal conditions, both the concentration of l-arginine in blood and the intracellular l-arginine concentration are far greater than the Κₐ of NO synthase for l-arginine, suggesting that substrate availability is unlikely to be a limiting factor (see Discussion in [28]). We found similar rates of citrulline formation in two different tissues (myocardium and aorta) of lean and corpulent rats, indicating that equal amounts of NO synthase enzyme were expressed in both groups. Finally, still other factors essential to the NO synthase reaction including NADPH may be limiting or dysfunctional in the present model.

We have tested previously whether increased degradation of NO in obese rats might result from increased formation of reactive oxygen species. Pro布col treatment did not decrease intimal lesions, although it was cardioprotective [12]. When exposed to copper oxidation stress in vitro, acetylcholine-mediated relaxation is less impaired in mesenteric rings from obese cp/cp animals than from the lean littersmates (O’Brien et al., unpubl. observations). Thus, there is no evidence that reactive oxygen species are responsible for the abnormal behavior of the vessels from the cp/cp genotype.

The responses of conductance vessels to vasodilators have varied in different experimental models of diabetes, and only limited studies have been performed in human subjects. In a previous investigation in male cp/cp rats aged 6 months, aortic rings from cp/cp rats, precontracted with norepinephrine, responded more weakly to acetylcholine than control vessels, whereas A 23187 and sodium nitrite were similarly active [13]. We have also found that both aorta and mesenteric resistance vessels from cp/cp
rats have an enhanced contractile response to both norepinephrine and phenylephrine [29,30]. This is consistent with the hyperproliferative and migratory characteristics of the vascular smooth muscle cells of the cp/cp rat that are associated with the hyperinsulinemia [31,32]. In the present study, we have precontracted the aortic rings with U 46619 and angiotensin II, a combination that gives a considerably more forceful contraction than that used in our studies with norepinephrine and phenylephrine contraction [29,30]. We have used the calcium ionophore A 23187 to study the endothelium-dependent relaxation, and spermine/NO, an NO-donor releasing the agonist at a controlled and reliable rate [33], to probe smooth muscle function. We found no evidence for differences in A 23187 potency between genotypes (similar EC_{50} values), but maximal relaxation was ~30% (relative value) weaker in aortas from obese than lean rats, suggesting that defects in endothelial function start to appear around this age (≤18 weeks), whereas smooth muscle function was unimpaired. On the contrary, in the older animals (18 weeks), spermine/NO generated a greater maximal relaxation than was the case in the younger animals (7 weeks), suggesting heightened effectiveness of the NO/cGMP pathway, but within age groups there was no difference between genotypes (Table 2). Likewise, rilmakalim, a K^{+} channel opener, was as effective in cp/cp as in +/+ aortic rings. Thus, aortic smooth muscle relaxation is normal or even improved in this obese animal model. The contrasting hypercontractility in response to norepinephrine by mesenteric, and possibly coronary vessels, found in other studies [13,29,30] suggests that early damage to the endothelium may have substantial effects on the underlying smooth muscle cells. This can lead both to vasospasm, the development of intimal lesions, and impaired fibrinolysis, all precursors of ischemic events.

5. Conclusion

The present study further supports the notion that abnormalities of vascular wall function may play a role in the development of vascular abnormalities seen in insulin resistance. The progressive loss of endothelium-dependent coronary dilator function in this genetic model of the disease appears to be due to a defective NO generation that can be alleviated by exogenous addition of H_{2}biopterin, a cofactor essential to the NO synthase reaction. Additional studies are necessary, including comparative measurements of H_{2}biopterin and NO synthase products in vascular tissues of obese and lean rats to ascertain whether H_{2}biopterin supplementation might be a viable therapeutic option to treat vascular dysfunction in insulin resistance. The evidence suggests that the dysfunction of the vascular smooth muscle cells of the cp/cp rat, while a prominent part of the disease state, may represent alterations secondary to endothelial damage by hyperinsulinemia.

Acknowledgements

The authors thank Dr. Penelope Andrew for determinations of NO synthase activity, Hoechst Marion Roussel, Frankfurt/Main (Germany) for a sample of rilmakalim and the Austrian Research Fund (FWF) for financial support (project nos. 12934 and 13013, to F.B.). Silvia Pfeiffer is a recipient of an Austrian Academy of Sciences APART fellowship (APART 7198). The skillful technical assistance of Mrs. B. Oberer is also acknowledged.

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