Chronic selective hypertriglyceridemia impairs endothelium-dependent vasodilatation in rats

Klaus Kusterer, Tilla Pohl, Hans-Peter Fortmeyer, Winfried März, Hubert Scharnagl, Anke Oldenburg, Sabine Angermüller, Ingrid Fleming, Klaus H. Usadel, Rudi Busse

Abstract

Objective/Methods: In order to investigate whether selective hypertriglyceridemia impairs endothelium-dependent vasodilatation in the rat hindlimb, rats were selectively bred to establish two strains, one with a pronounced hypertriglyceridemia (HT) and the other with normal plasma levels of triglycerides (LT). Results: Carotid arteries and aortae removed from 3, 6, 9 and 12 month old LT- and HT-rats exhibited a normal morphology. However, marked morphological differences were observed between vessels from 18–20 month old HT- and LT-rats. The endothelium-dependent vasodilator acetylcholine (2 to 50 mg/kg), administered into the iliac artery, elicited a concentration-dependent increase in hindlimb blood flow which was not different in 3, 6 and 9 month old LT- or HT-rats but was impaired in 12 and 18–20 month old HT-rats. In contrast the endothelium-independent vasodilator sodium nitroprusside enhanced blood flow in both strains to a similar extent. Neither administration of the nitric oxide (NO) synthase (NOS) substrate, L-arginine, nor the NOS inhibitor N nitro-L-arginine, affected the responsiveness to endothelium-dependent vasodilators in 12 month old HT-rats. These attenuated responses could not be attributed to a decrease in endothelial NOS expression as Western blot analysis revealed identical levels of this enzyme in the aortae and carotid arteries from LT- and HT-rats. Determination of superoxide anion (O2-) formation however, demonstrated a markedly elevated production of O2- in aortae from HT-rats. Conclusion: We conclude that chronic selective hypertriglyceridemia, an independent risk factor in the development and progression of atherosclerosis, leads to an endothelial dysfunction which is associated with an increased vascular O2- production and a subsequent decrease in bioavailable NO.

Keywords: Endothelium; Superoxide anion; Nitric oxide; Rat

1. Introduction

High serum cholesterol is regarded as one of the main causes of coronary atherosclerosis. Indeed several cholesterol lowering interventions have been reported to significantly reduce coronary heart disease [1–3], and feeding a cholesterol-rich diet to laboratory animals precipitates a progressive decrease in endothelium-dependent dilator responsiveness as well as the development of intimal lesions [4,5]. Apart from cholesterol, many clinical and population studies have related increased cardiovascular risk, i.e., the presence, extent and progression of coronary heart disease, to increases in plasma levels of triglyceride-rich lipoproteins [6].

Although the literature on the epidemiological association between plasma triglyceride levels and atherosclerosis is not completely consistent, the emerging trend is that high plasma triglyceride levels can be a significant predic-
tor of coronary heart disease [7]. This is particularly highlighted by the Monitored Atherosclerosis Regression Study (MARS), a 2 year trial of lovastatin monotherapy. In the latter study lovastatin induced marked lowering of total cholesterol levels and significantly decreased the average percent of stenosis of severe (>50%) lesions, while the progression of mild to moderate lesions (<50%) was accelerated [8]. Analysis of lipids and clinical characteristics indicated that following aggressive lowering of total plasma cholesterol levels, triglyceride-rich lipoproteins and very low density lipoprotein-associated apolipoprotein C-III were risk factors promoting the progression of mild to moderate lesions [9]. Subsequent studies have also identified a significant and independent association between plasma levels of triglycerides and the incidence of major coronary events [10]. Moreover, it would appear that high plasma levels of triglycerides are a greater risk among subjects with moderate to high plasma levels of low-density lipoprotein (LDL) cholesterol and low high-density lipoprotein (HDL) cholesterol [11,12].

Although the endothelium in atherosclerotic vessels is generally physically intact, a number of functional modifications exist such as impaired responsiveness to increases in blood flow as well as to endothelium-dependent vasodilators such as acetylcholine (ACh), bradykinin and substance P (for review see Ref. [13]). As these stimuli elicit the release of the endothelial autacoid nitric oxide (NO), which is synthesised from the amino acid L-arginine by the NO synthase (eNOS) [14], endothelial dysfunction has been attributed to a decrease in the production of NO. The nature of the endothelial dysfunction resulting in an apparent decrease in NO-mediated dilator responses is unknown although possibilities include the decreased expression of eNOS, an imbalance between the production of endothelium-derived constricting and relaxing factors, decreased substrate availability, production of an endogenous NOS inhibitor and overproduction of oxygen-derived free radicals. Experimental evidence has been provided to support almost all of these possibilities [13]. Whatever the reason for the apparent decrease in the production of bioactive NO, it is clear that attenuated NO-mediated responses can be detected prior to any appreciable intimal thickening and is already apparent in healthy subjects with a family history of atherosclerosis [15,16].

A serious potential bias in case-control studies is that they cannot determine whether elevated triglyceride levels are an independent risk factor and precede the onset of atherosclerosis or whether this elevation only occurs during the course of the disease process. In order to address this issue we selectively bred laboratory rats to establish two distinct strains displaying high (HT-rats) and low (LT-rats) plasma triglyceride levels but with only slightly elevated levels of cholesterol. We then investigated the effects of chronic hypertriglyceridaemia on the progression of changes in vascular morphology, endothelium-dependent vasodilatation and vascular free radical production. Moreover since supplementation with L-arginine has been reported to improve endothelium-dependent vasodilatation in hypercholesterolemic patients [17] as well as in animal models [18,19] we investigated the ability of L-arginine to restore endothelium-dependent vasodilator responsiveness.

2. Methods

2.1. Materials

ACh, sodium nitroprusside (SNP), and N^G^-nitro-L-arginine (L-NNa) were obtained from Sigma (Munich, Germany), L-arginine from Calbiochem (Bad Soden, Germany) and bradykinin from Bachem (Heidelberg, Germany).

2.2. Animals

A wide range of serum triglyceride levels were observed in an outbred strain of laboratory rats (Sprague Dawley). Breeding pairs with either very high or low serum triglyceride levels were selected from this stock and the same principle of selection served to determine breeding pairs in subsequent generations. Selection of breeding pairs demonstrating either extremely high or normal concentrations of triglycerides led to the establishment of two separate strains, one with high circulating levels of triglycerides (HT) and the other with normal lipoprotein levels (LT). The characteristics of the two strains were established by the third to sixth generations. The rats were housed in makron cages and had free access to a standard diet (Altromin® 1324, Lage/Lippe, Germany) and tap water. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.3. Endothelium-dependent vasodilatation

Groups of male HT- and LT-rats (ages: 3, 6, 9, 12 and 18–20 months) were anaesthetised with sodium pentobarbital i.p. (60 mg/kg body weight). The right carotid artery was cannulated for the continuous monitoring of blood pressure. After midline laparotomy the left iliac artery was cannulated and the tip of a polyethylene tubing was placed 2 mm proximal to the aortic bifurcation. A perivascular flow probe was positioned around the right iliac artery and blood flow was continuously measured with an ultrasonic flow meter (Transonic Systems, T206, New York, NY, USA). After a short stabilisation period (t=0 min) saline (0.9% NaCl, 0.0375 ml/min) was perfused via the cannulated left iliac artery. Following a further 10 min, vasoactive agents were added as a bolus to the perfusate, each treatment (ACh 2 μg/kg, 10 μg/kg and
50 µg/kg; SNP 100 µg/kg; total volume 50 µl) lasted for 90 s and 5 min were allowed between each treatment for equilibration.

In separate experiments vasodilator responsiveness to NaCl (50 µl), ACh (50 µg/kg), bradykinin (50 µg/kg) and SNP (100 µg/kg) was assessed in 18–20 month old HT- and LT-rats. In a second series L-arginine (10 mg/kg/min) was added to the NaCl infusion, and after 10 min, administration of the vasoactive agents was repeated. Thereafter, the rats were continuously infused with a solution containing the NOS inhibitor L-NNA (15 mg/kg/min) and following a 10 min equilibration period, vasodilator responsiveness was assessed for a third time. In preliminary experiments we observed no alteration in the responses to ACh, bradykinin or SNP upon repetitive application (up to four times).

2.4. Metabolic control parameters

At the end of each experiment blood was withdrawn from the aorta for determination of lipid and lipoprotein content. Cholesterol and triglycerides were measured enzymatically (CHOD–PAP and GPO–PAP respectively; Boehringer Mannheim). Lipoproteins were separated by preparative ultracentrifugation using the density limits 1.006–1.019 kg/l, 1.019–1.063 kg/l and 1.063–1.21 kg/l for very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL, and HDL. After separation, the lipoprotein fractions were analysed on the basis of their cholesterol and triglyceride contents.

2.5. Histology

The carotid artery and the aorta were fixed with a solution containing 5% glutaraldehyde in 100 mmol/l Pipes buffer (piperazine-N,N′-bis (2-ethanesulfonic acid) at pH 7.4 (60 min). The tissue was dehydrated in graded ethanol and embedded in Epon 812. Epon sections (1 µm) were stained with toluidine blue and evaluated for histological differences in a double blind manner.

2.6. Western blot analysis

Groups of male LT- and HT-rats (12 month old) were anaesthetised with sodium pentobarbital (60 mg/kg i.v.) and exsanguinated by cutting through both the aorta and vena cava. The aorta and carotid arteries were excised, cleaned of connective and adipose tissue and quickly frozen in liquid nitrogen. Crude protein extracts were subjected to polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad) as previously described [20]. Prestained molecular weight marker proteins (Bio-Rad) were used as standards for the sodium dodecyl sulfate (SDS)-PAGE. A Ponceau S staining was performed to verify the quality of the transfer and the equal amount of protein in each lane. eNOS protein was detected using a selective eNOS monoclonal antibody (Transduction Laboratories, Nottingham, UK) and was visualised by enhanced chemiluminescence using a commercially available kit (Amersham, Germany). The autoradiographs were analysed by scanning densitometry.

To reprobe Western blots the nitrocellulose membranes were incubated at 50°C for 30 min in a buffer containing Tris–HCl (67.5 mmol/l, pH 6.8); β-mercaptoethanol (100 mmol/l) and SDS (2%). After extensive washing in buffer containing Tris (50 mmol/l, pH 7.5) and NaCl (200 mmol/l), the filters were incubated in blocking buffer containing bovine serum albumin (3%), horse serum (10%), and subsequently with a monoclonal antibody against β-actin (Sigma, Munich, Germany).

2.7. Determination of superoxide anion formation

The thoracic aortae from groups of male (12 month old) LT- and HT-rats were cut into short segments (~10 mm) and inserted into a cuvette containing 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered Tyrode solution of the following composition (mmol/l): NaCl, 132; KCl, 4; CaCl₂, 1; MgCl₂, 0.5; HEPES, 9.5 and glucose; and containing bis-N-methyl acridinium nitrate (lucigenin; 250 µmol/l). The chemiluminescence emitted was monitored at 37°C using a biocounter (Lumac/3M Biocounter M2010, Abimed), under basal conditions and following agonist stimulation. The chemiluminescence signal was calibrated by monitoring the signal produced by known amounts of O₂ generated by xanthine–xanthine oxidase (50 µmol/l/0.3–10 mU) and O₂ formation calculated on the basis of the xanthine–xanthine oxidase calibration curve, the incubation time and the wet weight of the tissues. The chemiluminescence signal generated by the reaction of lucigenin with reactive oxygen species was specific for O₂ since no signal was detected in the presence of either hydrogen peroxide or authentic NO.

2.8. Statistical analysis

Data are expressed as the mean±SEM. Statistical differences between HT- and LT-rats were assessed by the unpaired Student’s t-test. Student’s t-test for paired samples was used for statistical analysis of flow changes between different time points. The statistical differences between proportions were tested with the Fisher–Yates exact test.

3. Results

3.1. Metabolic parameters

Plasma levels of lipids and lipoproteins were monitored in all of the animals studied. Both strains of rat exhibited
constant triglyceride and cholesterol levels over the 20 month experimental period. There was no age-dependent alteration in either triglyceride or cholesterol levels in LT-rats. In contrast there was a slight age-dependent variation in serum levels of cholesterol obtained from HT-rats. However, triglyceride and cholesterol levels in HT-rats were consistently elevated (five- to eightfold) over the values obtained in serum from LT-rats (Table 1).

A more detailed analysis of the lipoprotein profile was performed on serum from 20 month old rats (Table 2). In these animals there was a sixfold increase in serum triglycerides levels ($P<0.05$), and a small but significant elevation in total cholesterol ($P<0.05$). The increase in triglycerides was mainly attributable to an accumulation of triglycerides in the VLDL fraction as VLDL cholesterol and VLDL triglycerides were significantly increased in the HT-rats. IDL and LDL were, on the other hand, only moderately increased. As expected HDL cholesterol was lower in the HT- than in the LT-rats. The ratio of triglycerides to cholesterol in VLDL was more than twofold higher in the HT- than in the LT-rats, indicating that the composition of VLDL was also altered. Interestingly, HDL was also enriched in triglycerides at the expense of cholesterol in the HT-rats. Only a moderate increase in the ratio of triglycerides to cholesterol was seen in IDL and no enrichment in triglycerides was observed in the LDL fraction. After storing serum from HT-rats overnight at 4°C, small amounts of chylomicrons were visible. This was confirmed by lipoprotein electrophoresis which revealed lipoproteins remaining at the origin (data not shown). These changes in the plasma lipoprotein pattern observed in the HT-rats resembles type V hyperlipoproteinemia according to the Fredrickson classification in humans.

3.2. Histology

Carotid arteries and aortae removed from 3, 6, 9 and 12 month old LT- and HT-rats exhibited a normal morphology. Marked differences were however observed between 18–20 month old HT- and LT-rats (Fig. 1). Analysis of cross sections through the media of the carotid arteries (Fig. 1A) and aortae from LT-rats (Fig. 1C) revealed that smooth muscle cells formed a well-organised layer which was interspersed with concentric elastic sheets. In the media of the carotid arteries (Fig. 1B) and aortae (Fig. 1D) from HT-rats, the elastic sheets were markedly thinner and the smooth muscle cells appeared to lie separately in the ground substance. In addition some lipid deposits could be seen under the basal lamina. These histological changes were detected in six out of eight HT-rats ($P<0.05$) by two independent observers.

3.3. Endothelium-dependent vasodilatation

Administration of ACh (2 to 50 μg/kg) into the iliac artery elicited a concentration-dependent increase in hind-limb blood flow which was similar in 3, 6 and 9 month old LT- and HT-rats (data not shown). In 12 month old HT-rats however the ACh-induced increase in blood flow was significantly attenuated compared with that observed in age-matched LT-rats (Fig. 2). There was also a tendency for the endothelium-independent vasodilator response to

### Table 1
Comparison of triglyceride and cholesterol levels in serum from LT- and HT-rats over the 20 month experimental period

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<td>20 Months</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>76±5</td>
<td>617±130**</td>
<td>494±214*</td>
<td>493±309*</td>
<td>659±343*</td>
<td>468±193*</td>
<td>434±41**</td>
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<td>Cholesterol (mg/dl)</td>
<td>60±3</td>
<td>62.6±0.57</td>
<td>61±8</td>
<td>65±12</td>
<td>85±22</td>
<td>97±14*</td>
<td>14±94</td>
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<tr>
<td>Weight (g)</td>
<td>510±12</td>
<td>450±2.4</td>
<td>504±21</td>
<td>612±58</td>
<td>593±108</td>
<td>659±105</td>
<td>645±22</td>
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Data are presented as the mean±SEM of values obtained from eight animals in each group: * $P<0.05$, ** $P<0.01$ HT vs. LT. There was no age-dependent alteration in serum triglyceride and cholesterol levels in 3 to 20 month old LT-rats, therefore only the values for 20 month old animals are given.

### Table 2
Detailed analysis of the lipoprotein profile of serum from 20 month old LT- and HT-rats (triglycerides: TG, and cholesterol: C), showing that the increase in triglycerides was mainly attributable to an accumulation of triglycerides in the VLDL fraction

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<td></td>
<td>TG</td>
<td>C</td>
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<td>C</td>
<td>TG/C</td>
<td>TG</td>
<td>C</td>
<td>TG/C</td>
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<tr>
<td>Serum</td>
<td>57.5±5.7</td>
<td>68.3±11.7</td>
<td>–</td>
<td>357.1±161.4*</td>
<td>99.3±9.7*</td>
<td>–</td>
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<tr>
<td>VLDL (μg/dl)</td>
<td>27.1±2</td>
<td>3.0±0.7</td>
<td>9.1</td>
<td>450.6±148.0*</td>
<td>20.9±14.0*</td>
<td>21.6</td>
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<tr>
<td>IDL (mg/dl)</td>
<td>11.7±2.5</td>
<td>0.8±0.2</td>
<td>14.1</td>
<td>37.9±4.7*</td>
<td>1.5±0.5*</td>
<td>25.3</td>
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<td>LDL (mg/dl)</td>
<td>14.5±1.2</td>
<td>17.2±6.4</td>
<td>0.84</td>
<td>26.4±5.6*</td>
<td>39.2±14.0*</td>
<td>6.67</td>
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<tr>
<td>HDL (mg/dl)</td>
<td>4.2±1.2</td>
<td>47.3±4.6</td>
<td>0.09</td>
<td>12.8±3.5*</td>
<td>37.7±2.8*</td>
<td>0.34</td>
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Data are presented as the mean±SEM of values obtained from six animals in each group: * $P<0.05$ HT vs. LT.
SNP to be decreased in 12 month old HT- vs. LT-rats, however this effect failed to attain statistical significance.

To further characterise the impaired endothelium-dependent vasodilatation, the hind limbs of 18–20 month old HT- or LT-rats were infused with either saline solution (50 μl 0.9% NaCl administered over 90 sec), ACh (50 μg/kg), bradykinin (50 μg/kg) or SNP (100 μg/kg) in the absence and presence of l-arginine (10 mg/kg/min) or l-NNA (15 mg/kg/min). The two rat strains exhibited no significant differences in mean systemic blood pressure (LT/HT: 118±8/107±3 mmHg) or heart rate (296±11 and 263±12 beats/min). Pretreatment with l-arginine had no effect on mean blood pressure (LT/HT: 120±10/104±6 mmHg) whereas, l-NNA markedly increased mean blood pressure in both strains (LT/HT: 157±3/144±9 mmHg). The administration of ACh, bradykinin or SNP into the iliac artery failed to influence mean blood pressure. Heart rate was not affected by any of the treatment protocols.

Basal blood flow was approximately equal in LT- and HT-rats (LT/HT: 4.53±0.88/3.25±0.62 ml/min). In LT-rats ACh (50 μg/kg) enhanced blood flow by 9.4±0.9 ml/min from baseline, an increase which was significantly greater than that observed following the administration of ACh to HT-rats (5.5±0.8 ml/min from baseline; P<0.05). The endothelium-dependent vasodilator bradykinin (50 μg/kg) increased blood flow in both animal strains but the changes in flow observed in HT-rats were attenuated compared with those recorded in LT-rats (Fig. 3). In contrast the endothelium-independent vasodilator SNP enhanced blood flow in both animal strains to a similar
extent (Fig. 3). Pretreatment of LT-rats with L-arginine resulted in a modest increase in blood flow (5.0±0.90 ml/min) which, although not markedly different from basal flow, was significantly greater than the blood flow measured following L-arginine administration to HT-rats (3.18±0.74 ml/min, P<0.05). L-Arginine failed to potentiate either ACh or bradykinin-induced vasodilatation when compared to saline controls. The administration of ACh and bradykinin was associated with increases in blood flow in both LT- and HT-rats but the changes observed in HT-rats were notably less than those observed in LT-rats (Fig. 3). Infusion of L-NA decreased blood flow in LT-rats (from 4.53±0.88 to 2.40±0.57 ml/min) but had no effect on basal blood flow in HT-rats (3.25±0.62 before and 3.59±0.78 ml/min after administration of L-NA). Pretreatment with L-NA attenuated vasodilator responsiveness to ACh and bradykinin in LT-rats but failed to affect responses observed in the HT-strain (Fig. 3).

No significant difference was observed in the vasodilator responsiveness to SNP between the LT- and HT-rats after pretreatment with saline solution or L-NA. However, L-arginine slightly, but not significantly, improved responsiveness to SNP in LT- and HT-rats (Fig. 3).

3.4. eNOS expression in the aorta and carotid artery

To determine the effect of hypertriglyceridemia on eNOS expression, we compared eNOS protein levels in aortae and carotid segments from 12 month old LT- and HT-rats by Western blot analysis. No strain-related difference in eNOS protein content could be detected in any of the samples studied (Fig. 4A and B).

3.5. Superoxide anion production in LT- and HT-rats

O$_2^-$ production was assessed in endothelium-intact and -denuded segments of aortae removed from LT- and HT-rats. There was no difference in the vascular production of O$_2^-$ by either LT-rats or 3, 6 and 9 month old HT-rats (data not shown) but a significant increase in the production of vascular O$_2^-$ was observed in 12 and 20 month old animals. In endothelium-intact aortae from 12 month old LT-rats a basal production of O$_2^-$ was detected and was enhanced following stimulation with ACh (1 μmol/l; Fig. 5A). Removal of the endothelium failed to affect basal levels of O$_2^-$ but abolished the ACh-induced increase in its production (Fig. 5B). The basal O$_2^-$ production from both endothelium-denuded and -intact aortic segments from HT-rats was significantly greater than that observed in vessels from LT-rats (Fig. 5A and B). Neither the application of ACh to endothelium-intact segments nor the removal of the endothelium significantly affected O$_2^-$ production in these segments (Fig. 5B). Although ACh tended to increase O$_2^-$ production in endothelium-denuded segments from HT-rats, this effect just failed to attain statistical significance. The maximal phorbol ester-stimulated pro-
Fig. 3. Effect of L-arginine and N\textsuperscript{\textcircled{O}} nitro-L-arginine (L-NNA) on vasodilator responsiveness of the right iliac artery in 18–20 month old rats with low (LT, closed columns) and high (HT, crosshatched columns) plasma triglyceride levels. Blood flow in the right iliac artery was measured continuously by an ultrasonic flow meter and dilator responsiveness to NaCl, acetylcholine (ACh, 50 \textmu g/kg), bradykinin (Bk, 50 \textmu g/kg) and sodium nitroprusside (SNP, 100 \textmu g/kg) was assessed. The same protocol was repeated after pretreatment with L-arginine (10 mg/kg/min, infusion rate, 0.0375 ml/min) and L-NNA (15 mg/kg/min). The results are presented as the mean±SEM of data obtained using 5–8 different animals in each group \( * P<0.05, ** P<0.001; \) vs. responses observed in LT-rats; \( ^{\text{a}} P<0.05 \) vs. responses observed in the presence of L-arginine.

4. Discussion

By breeding pairs of Sprague Dawley rats displaying normal levels of serum cholesterol but extremely different concentrations of triglycerides we were able to establish two substrains, one with hypertriglyceridemia and the second with normal triglyceride levels. A similar approach has been used to establish a hypertriglyceridemic strain of Wistar rats [21,22], although in the latter studies triglyceridemia was “stimulated” by a diet high in sucrose. Apart from the difference in triglycerides significantly higher plasma levels of HDL cholesterol were detected in HT-rats. However, since a low plasma level of HDL cholesterol has been shown to be a significant independent risk factor for coronary heart disease [23,24] and increased HDL cholesterol levels are associated with a decreased cardiovascular risk [25], it is unlikely that the elevated HDL cholesterol in HT-rats can account for the observed changes in endothelial function. It is more likely that the effects observed in our model can be attributed to the extreme hypertriglyceridemia and not to the minor alterations in the other lipoprotein fractions.

Early fatty streaks characterised by macrophage infiltration of the intima, accumulation of lipid-filled cells and intermediate lesions characterised by the presence of foam cells, minimal coarse grained particles and heterogeneous extracellular lipid have been classified as type I and type II atherosclerotic lesions, respectively [26]. While the aortae and carotid arteries removed from 3 to 12 month old HT-rats appeared morphologically normal, histological investigation of vessels from 18–20 month old HT-rats revealed changes which were more reminiscent of the advanced type III lesions in which extracellular lipid pools disrupt the continuum of the smooth muscle cell layer [26]. Although a detailed analysis of the lipid content of this lesion was not performed this lesion appears markedly different to lesions induced by a cholesterol-rich diet.

In the present study selective hypertriglyceridemia was associated with an age-dependent impairment of agonist-induced, endothelium-dependent vasodilatation such that a
marked depression of the ACh-induced increase in hindlimb blood flow was apparent in 12 month old rats. When the vasodilator responsiveness to ACh, bradykinin and SNP was compared in 12 month old HT- and LT-rats a significantly attenuated response to the receptor-dependent agonists was observed in HT-rats. While the increase in blood flow elicited by the receptor-independent vasodilator SNP was similar in both rat strains between 3 and 9 months of age, a slight but statistically not significant attenuation of the SNP response was apparent in 12 month old HT-rats. Thus on the whole these findings fit well with observations in both patients and animal models of atherosclerosis in which an attenuated responsiveness to endothelium-dependent vasodilators but not endothelium-independent nitrovasodilators could be demonstrated [5,16,27,28]. Therefore, hypertriglyceridemia does not appear to attenuate vascular responsiveness by decreasing the sensitivity of vascular smooth muscle to NO. Inhibition of NOS using L-NNA decreased basal blood flow in LT-rats but had no effect on basal blood flow in HT-rats. Moreover, NOS inhibition attenuated the vasodilatation induced by bradykinin and ACh in LT-rats but only minimally affected the responses obtained in HT-rats. The residual vasodilatation observed following the application of bradykinin and ACh to LT- and HT-rats in the presence of L-NNA may be attributed to the synthesis and release of the two other potent endothelium-derived dilator autacoids; prostacyclin and the endothelium-derived hyperpolarizing factor [29]. The lack of effect of L-NNA in HT-rats implies that both the basal and the receptor-dependent agonist-induced production of NO is more or less abolished by hypertriglyceridemia. This phenomenon cannot be attributed to a decrease in the availability of the NOS substrate since the coadministration of enzyme saturating concentrations of L-arginine failed to improve either basal blood flow or vasodilator responsiveness in the HT-rats. Our findings are in contrast with reports from both animal and human studies in which acute administration of large intravenous doses of L-arginine has been shown to improve vascular responsiveness in hypercholesterolemia [17,18,30]. Interpretation of some of the clinical data is however complicated by reports that L-arginine exerts a

Fig. 4. Effect of hypertriglyceridemia on eNOS expression. (A) Western blots showing eNOS protein levels in aortae (upper panel) and carotid artery segments (lower panel) removed from two different low triglyceridemic (LT)- and high triglyceridemic (HT)-rats (12 months of age). Proteins were separated by SDS-PAGE and eNOS proteins were detected using a specific anti-eNOS antibody as described in Section 2.6. Porcine endothelial cell lysate (PAEC) was used as a positive control. (B) Statistical summary of eNOS/β actin expression in aortae and carotid arteries. The results presented are representative of experiments performed using eight separate animals in each group.

Fig. 5. Superoxide anion (O₂⁻) generation in aortae from LT- and HT-rats. The basal and acetylcholine (ACh; 1 μmol/l)-stimulated generation of O₂⁻ was assessed in (A) endothelium-intact and (B) endothelium-denuded aortic segments from LT- (open columns) and HT-rats (shaded columns) by lucigenin-enhanced chemiluminescence. Results are presented as the mean±SEM of four separate experiments; §§ P<0.01 vs. the respective basal value; * P<0.05, ** P<0.01 vs. values obtained in the LT group.
greater influence on the vascular response to ACh in healthy volunteers than in hypercholesterolemic patients [31–33]. Moreover, high doses of d-arginine have also been shown to induce vasodilatation [34], and augment endothelium-dependent responses [32]. Taken together with the findings that plasma levels of l-arginine were essentially normal in hypercholesterolemia [35], it would appear unlikely that the decreased availability of the NOS substrate can completely account for the impaired endothelial function in this condition. Some of the beneficial effects of l-arginine administration may however be related to decreased endothelial production of O$_2^-$ [36], competition with an endogenous NOS inhibitor [37], or effects on other cell types, e.g., decreased production of O$_2^-$ from circulating neutrophils [38,39].

Attenuated expression of eNOS is also not able to explain the blunted vasodilator responsiveness observed in HT-rats since levels of the enzyme were identical in carotid arteries removed from LT- and HT-rats. In a departure from the widely held belief that endothelial dysfunction can be attributed to the decreased production of endothelium-derived NO, recent evidence suggests that impaired endothelium-dependent relaxation in hypertension and atherosclerosis is due neither to decreased NOS activity nor to a deficiency in the availability of l-arginine, but to an augmented inactivation of NO by O$_2^-$ [13]. Since significant increases in O$_2^-$ production were recorded in aortae removed from HT-rats it seems probable that the decreased responsiveness to ACh and bradykinin may be attributed to the accelerated scavenging of NO rather than to its attenuated production. Our observation that vasodilator responses to SNP were not significantly impaired in HT-rats does not contradict this hypothesis as it is generally not the case that the amount of O$_2^-$ generated by the vascular wall is able to significantly affect the dilution induced by an exogenous NO donor. We have investigated this phenomenon in a number of models of “endothelial dysfunction” and found vascular responsiveness to NO donors to be attenuated only in experimental models in which a decrease in the expression of the soluble guanyyl cyclase in vascular smooth muscle cells could be demonstrated [40]. An enhanced vascular O$_2^-$ production has already been reported in a number of different pathophysiological conditions associated with endothelial dysfunction including hypercholesterolemia [41] and various models of hypertension [42–44]. This apparent increase in O$_2^-$ levels may reflect either a reduced O$_2^-$ scavenging capacity or an enhanced O$_2^-$ formation. In fact, experimental evidence has been provided to support both hypotheses. We have shown that although serum triglycerides are elevated in 3 month old HT-rats, endothelium-dependent vasodilator responsiveness was only attenuated in 12 and 20 month old animals and was therefore coincident with the measurable increases in O$_2^-$ production. Our data suggest that in 12 month old HT-rats the vascular wall (smooth muscle and infiltrating inflammatory cells) and not the endothelium is the source of the increased O$_2^-$ since endothelial removal failed to reduce O$_2^-$ formation. However, it cannot be ruled out at this stage that there may be an initial increase in O$_2^-$ production by the endothelium in the early stages of hypertriglyceridemia as demonstrated in other models of endothelial dysfunction [43,44].

The importance of the endothelium in the development of atherosclerosis was first highlighted by the observation that endothelial removal facilitated lesion development which led to the concept that the atherogenic process is initially a “response to injury” [45]. The original hypothesis, continuously modified in the light of accumulating evidence tends towards the proposal that atherogenesis is related to “endothelial dysfunction”, i.e., the shut down or inactivation of certain intrinsic antiatherogenic mechanisms. The idea that an altered functioning of the endothelium may act as an initiator of the atherosclerotic process has recently begun to gain momentum following reports that endothelial dysfunction can occur prior to any appreciable intimal thickening and is already apparent in healthy subjects with a family history of atherosclerosis [15,16]. Our observation that endothelial function in the hindlimb of HT-rats was impaired before noticeable changes in the morphology of the aorta and carotid arteries occurred, but was coincident with an increased vascular O$_2^-$ production, supports the hypothesis that an altered functioning of the endothelium is an early event in the atherosclerotic process. Moreover, attenuated vasodilator responses were evident in two out of eight HT-rats (20 month old) although the aorta and carotid arteries appeared morphologically normal, supporting the concept that endothelial dysfunction is not necessarily confined to vessels in which the atheroma develops. Indeed, endothelial dysfunction can be evidenced in vivo in resistance vessels of the coronary and peripheral circulations which themselves do not develop overt atherosclerosis [46].

In summary, we have demonstrated that a selective hypertriglyceridemia induces a progressive depression of endothelial vasodilator responsiveness which is manifest prior to the development of morphological changes. This endothelial dysfunction was associated with an apparent decrease in NO production, but no change in the expression of eNOS, and with a marked increase in vascular O$_2^-$ production. These observations underline the importance of hypertriglyceridemia as an independent risk factor in the development and progression of atherosclerosis and the importance of developing therapeutic strategies to lower plasma levels of both LDL cholesterol and triglyceride rich proteins.

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References


