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Endothelium Dependent and Independent Relaxation of Aortic Rings from Watanabe Heritable Hyperlipidemic Rabbits after Exposure to a Free Radical Generating System

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The effects of the xanthine oxidase/hypoxanthine free radical generating system on endothelium dependent and independent relaxation were compared in aortic rings from New Zealand white rabbits and heterozygous Watanabe heritable hyperlipidemic (WHHL) rabbits with mild atherosclerosis. Studies were carried out in young (3 months) and mature (18 months) animals. Plasma cholesterol levels were significantly higher in both 3 and 18 month WHHL animals. Endothelium independent relaxation to SNP did not differ between groups. However, the attenuation of relaxation to carbachol after xanthine oxidase/hypoxanthine treatment tended to be less in WHHL. This reached significance at 18 but not 3 months. We propose that this could be due to increases in levels of endogenous scavenger enzymes in these WHHL rabbits.

Keywords: Watanabe heritable hyperlipidemic rabbits, free radical damage, endothelium

INTRODUCTION

The likelihood of a cardiovascular accident occurring is related to the number of risk factors present.[1] Atherosclerosis is exacerbated by hypertension and diabetes.[2,3] It has been suggested that shared response mechanisms may explain the synergistic effects of hypertension in enhancing atherosclerosis.[4] Enhanced production of reactive oxygen species/free radicals (ROS/FR) and subsequent endothelial damage may be a key factor linking a number of risk factors. Increased ROS/FR production has been reported in hypertension, hypercholesterolemia and diabetics.[5–7] ROS are also present in cigarette smoke and other environmental pollutants.[8] ROS have been shown to impair endothelial function in *in vitro* systems.[9] Decreased endothelium relaxation has been extensively reported in human atherosclerotic disease and in animal models of hypertension, hypercholesterolemia and diabetics.[10–13]

The aim of this study was to examine the hypothesis that pre-existing endothelial dysfunction

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will render the vasculature more susceptible to additional free radical insult.

The effects of hypoxanthine/xanthine oxidase free radical generating system on vascular responses in rings of thoracic aorta from New Zealand white rabbits and a strain of genetically hyperlipidemic rabbits – the Watanabe heritable hyperlipidemic rabbit were compared. This system was chosen for investigation of possible interactions between risk factors as we have extensive experience showing impaired vascular responses in Watanabe heritable hyperlipidemic rabbits and using the hypoxanthine/xanthine oxidase system independently. In particular, we wanted to investigate whether exposure to free radicals would have greater effects on vascular responses in vessels from WHHL in which there was evidence for prior existence of both functional and histological changes.[14]

METHODS

1. Animals

Four groups of animals were studied: 3–4 and 18–24 months old New Zealand white (NZW) rabbits and age matched Watanabe heritable hyperlipidemic (WHHL) rabbits from the colony bred at Stobhill Hospital. Equal numbers of male and female rabbits were used. All WHHL rabbits had a blood sample taken at weaning, animals with cholesterol levels between 75 and 200 mg/dl were selected for study. These animals were assumed to be heterozygous for the LDL receptor gene. Such animals have previously been shown to exhibit small but significant changes in vascular function compared to NZW rabbits consistent with early atheroma.[14]

2. Cholesterol Measurement

Blood for cholesterol measurement was taken from a marginal ear vein prior to weaning in WHHL animals and before sacrifice in both WHHL and NZW animals. Plasma cholesterol was measured enzymatically using the Boehringer Mannheim cholesterol C-system.

3. Vascular Responses

Rabbits were killed with an overdose of sodium pentobarbitone (50 mg/kg), administered intravenously in the marginal ear vein. The thoracic aorta was immediately dissected out and immersed in ice cold Krebs bicarbonate buffer. The thoracic aorta was trimmed free of fat and adhering connective tissue. Two mm wide transverse rings were cut and suspended between two stainless steel hooks in a 10 ml organ bath, filled with Krebs buffer at 37°C, continuously gassed with 95% O_2, 5% CO_2. The composition of the Krebs buffer was as follows: (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, CaEDTA 0.05, glucose 11.1, pH 7.4. 17β oestradiol (10^{-5} M), cocaine hydrochloride (10^{-5} M) and indomethacin (10^{-5} M) were added to the Krebs buffer to block extraneuronal and neuronal adrenergic uptake and prostaglandin synthesis, respectively.[15,16] Addition of the uptake blockers stabilised contractile responses. Addition of oestrogen did not modify relaxation under our experimental conditions. Isometric tension was recorded using a force transducer (Grass model FT03), connected to a chart recorder (Grass Polygraph model 7B). Each ring was set individually at the optimal point of its length tension relationship as determined by repeated exposure to phenylephrine (10^{-7} M). Tissues were then allowed to equilibrate for 1 h. Cumulative concentration curves to phenylephrine (10^{-8}–10^{-5} M) were obtained. The baths were washed out and the tissues were allowed to re-equilibrate, they were then recontracted to between 50 and 70% of the maximum contraction to phenylephrine as determined from the full concentration response curve. Once a plateau contraction was established cumulative concentration response curves to carbachol or sodium nitroprusside (SNP) (10^{-8}–10^{-5} M) were obtained.

Drugs were then washed out and the tissue exposed to a xanthine oxidase/hypoxanthine (XO/HX) free radical generating system for 30 min.[9] To initiate free radical generation HX 250 μM, EDTA 37.5 μM and $FeCl_3$ 25 μM were added to the baths followed by XO 20 mU/ml. This theoretically could result in generation of superoxide and hydroxyl radicals and hydrogen peroxide. However, previous work suggests that hydrogen peroxide is the primary species mediating effects on vascular responses under our experimental conditions, although superoxide may also contribute.[9] After 30 min the tissues were washed and allowed to equilibrate for 10 min before re-examining responses to carbachol or SNP as before.

In parallel some rings were not exposed to xanthine oxidase/hypoxanthine but were incubated in buffer for 30 min to allow for changes in responses (not related to exposure to the free radical generating system) occurring over the time of the experiment.

Materials

The following materials were supplied by Sigma Chemicals Co. (UK): Carbamyl choline chloride, L-phenylephrine hydrochloride, hypoxanthine, indomethacin, 17β oestradiol, xanthine oxidase from milk (0.17 U/mg). Sodium nitroprusside was supplied by CP Pharmaceuticals (Wrexham). Cocaine hydrochloride was obtained from the pharmacy at the Western Infirmary, Glasgow. All concentrations are expressed as final concentrations (M or U/ml) in the bath media (10 ml Krebs bicarbonate buffer). New Zealand white rabbits were obtained from Interfauna Wyton Huntingdon, UK.

Analysis of Results

The dose response curves for relaxation to carbachol and SNP were characterised by the maximum relaxation (E_{max}) and the concentration which produced 50% of the maximum response (EC_{50}). These values were calculated for each agonist in individual animals pre- and post-intervention (vehicle or XO/HX) using Microsoft Excel. E_{max} and EC_{50} pre- and post-treatment were then compared using either students t-test or ANOVA. Bonferroni corrections were used when multiple comparisons were made.

In the text and figures results are given as the mean ± SE. The number of animals in each group varied between 6 and 10.

RESULTS

Plasma Cholesterol Levels

Plasma cholesterol levels at weaning were 96 ± 35 mg/dl and 100 ± 35 mg/dl in the 3–4 and 18–24 month WHHL rabbit groups respectively. At study the corresponding levels were 88 ± 54 mg/dl and 69 ± 65 mg/dl. The fall in plasma cholesterol in the older group was significant, occurring in all but one animal studied. Plasma cholesterol levels were below 56 mg/dl in all New Zealand white rabbits used for the study, with no overlap between the groups.

Relaxation to Carbachol

Vehicle treatment caused an increase in the EC_{50} for relaxation in the 3 months NZW animals but had no other significant effects on relaxation to carbachol (Figures 1 and 2). After XO/HX treatment the maximum relaxation was reduced and the EC_{50} increased in all groups of animals. The attenuation of responses tended to be greater in NZW than WHHL animal groups. At 3 months relaxation was reduced by 52 ± 7% in NZW and 37 ± 7% in the WHHL group. In older groups (18–24 months) relaxation was reduced by 44 ± 7% and 18 ± 3% for NZW and WHHL animals respectively. This difference between NZW and WHHL rabbits reached significance at 18 months ($p = 0.009$ CI −43.8, −8.5) but not at 3 months ($p = 0.18$ CI −7.5, 36.6) (Figures 1 and 2).

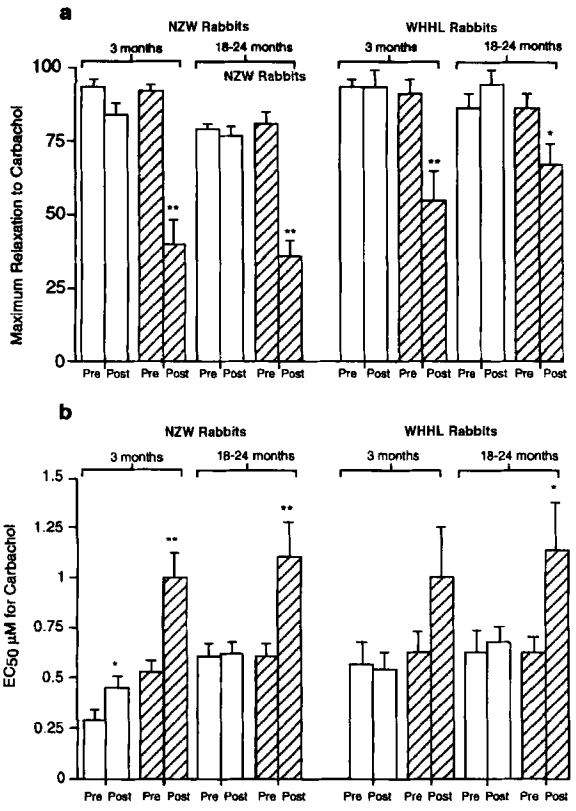

FIGURE 1 The effect of treatment with the XO/HX free radical generating system on E_{max} (a) and EC_{50} (b) for relaxation to carbachol in aortic rings from NZW and WHHL rabbits. □ Vehicle; ▨ XO/HX. $^{*}p < 0.05$; $^{**}p < 0.01$.

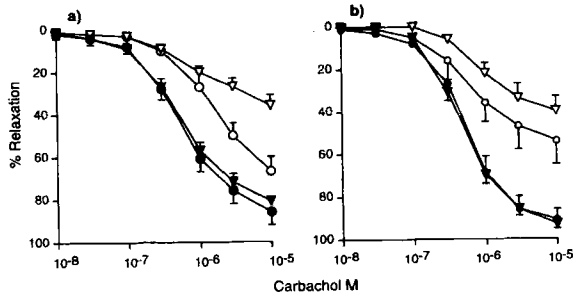

FIGURE 2 Full dose response curves showing the effect of treatment with the XO/HX free radical generating system on relaxation to carbachol in aortic rings from NZW and WHHL rabbits. (a) 18–24 month old animals; (b) 3–4 month old animals; ● Pre-treatment WHHL animals; ○ Post-treatment WHHL animals; ▼ Pre-treatment NZW animals; ▽ Post-treatment NZW animals.

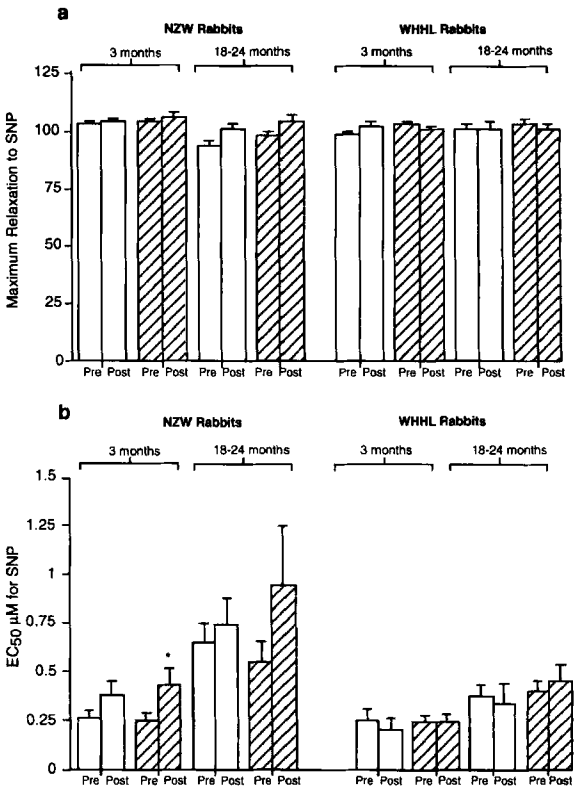

FIGURE 3 The effect of treatment with the XO/HX free radical generating system on E_{max} (a) and EC_{50} (b) for relaxation to SNP in aortic rings from NZW and WHHL rabbits. □ Vehicle; ▨ XO/HX. $^{*}p < 0.05$.

Relaxation to SNP

Neither vehicle nor XO/HX treatment had any significant effect on responses to SNP in WHHL rabbits. In NZW rabbits no significant reduction in the maximum relaxation to SNP was observed in any group. However, EC_{50} values tended to increase both with vehicle and XO/HX treatment (Figure 3). This reached significance in the 3 months group treated with XO/HX.

DISCUSSION

There is much evidence suggesting synergy between risk factors for atherosclerotic disease.[2,3] It has been suggested that this may be

related to shared mechanisms. Increased FR/ROS production and impaired endothelial function are common findings among risk factors. We were interested to see whether the *in vitro* effects of a free radical generating system on endothelial function were additive or synergistic when related to the pretreatment status of the endothelium. To do this we compared the effects of the XO/HX FR generating system on rings of thoracic aorta from heterozygous WHHL and NZW rabbits. Heterozygous WHHL were studied in preference to homozygous WHHL as we wanted to use a model of mild to moderate atherosclerosis such as is commonly present in man. Homozygous WHHL have grossly elevated cholesterol levels in the range 400–1200 mg/dl. Abnormalities in vascular function develop rapidly. By 3 months of age relaxation to carbachol is only about 75% of that observed in normocholesterolemic rabbits, by 9 months relaxation is further reduced to approximately 20%. At this age all animals show quantifiable areas of atheromatous plaque, medial smooth muscle cells are changing orientation and foam cells, collagen and protoglycons are present in the media. In contrast, in heterozygous animals cholesterol levels rarely exceed 200 mg/dl. At 9 months of age only minimal areas of true atheromatous plaque are present although lipid deposition and foam cells may be observed in the media. Responses to carbachol are on average reduced by about 20%.[14] The decrease in endothelium dependent relaxation in the homozygous WHHL is so great that it would have been difficult to examine the effects of further insults to the endothelium on top of those already present. However, in the heterozygous animals we believed it would be possible to detect either additive or synergistic effects between hypercholesterolemia and free radical induced changes in endothelial function. To our surprise the endothelium dependent attenuation of relaxation to carbachol observed after XO/HX treatment in rings of thoracic aorta from WHHL tended to be less than in rings from NZW rabbits. This reached significance in the older but not the younger age group. One possible explanation is that levels of endogenous free radical scavenging systems which would protect the tissue from the effects of the XO/HX system are increased in WHHL animals. Endogenous production and/or metabolism of free radicals may well differ between WHHL and NZW rabbits. Increased superoxide production has been reported by several groups in hypercholesterolemic animals and man[17–19] as well as in some models of hypertension.[20–22] In many cases elevations in superoxide production are accompanied by compensatory increases in nitric oxide production and/or levels of antioxidant enzymes.[22–24] Levels of glutathione peroxidase and superoxide dismutase have been reported to be increased in aortae from cholesterol fed hypercholesterolemic rabbits.[25,26] Similarly Lapenna *et al.*, 1992[27] observed increased superoxide dismutase, catalase and glutathione transferase activities in hearts of hypercholesterolemic rabbits. In these studies the increased enzyme activities were a consequence of cholesterol feeding. In our WHHL rabbits a difference in enzyme levels could be inborn or result from the higher circulating levels of cholesterol in these animals. Comparison of results from the younger and older groups would support the later hypothesis as it was in the older group that differences between NZW and WHHL became significant.

Another possibility is that endogenous inhibitors of xanthine oxidase are present in the vessels from WHHL animals. However, the greater difference in the response to carbachol between strains in the older animals is more consistent with induction of free radical scavenging systems. Moreover, White *et al.*, 1996[28] have suggested that circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits.

When we examined endothelium independent relaxation using SNP a significant increase in EC_{50} for relaxation was observed in rings from the young NZW animals after XO/HX treatment. This is consistent with previous findings.[29]

In contrast, XO/HX treatment had no effect on responses to SNP in tissues from WHHL animals, which suggests that in these WHHL rabbits both endothelium dependent and independent relaxation may be less susceptible to FR induced modification.

EC_{50} values for both carbachol and SNP induced relaxation tended to be greater after vehicle treatment in NZW but not WHHL animals. This increase is probably due to deterioration of the preparation with time. This could also be explained by higher levels of endogenous FR/ROS scavenging systems in WHHL rabbit tissues protecting the tissues in this case from ROS generated in the organ bath during the course of the experiments.

Differences in the effects of the FR generating system on tissue from WHHL and NZW animals could also have been related to the initial response to agonists. However, in this series of experiments pretreatment dose response curves were similar in the two strains. In previous studies we had shown a small but significant attenuation in relaxation to carbachol in rings from WHHL animals in addition to structural changes in the endothelium.[14] Plasma cholesterol levels of the WHHL rabbits were similar in both studies and the reason for the slightly reduced response to carbachol in the previous but not this study is not obvious. It would have been possible to examine the effects of XO/HX treatment on vessels from homozygous WHHL rabbits with more severe endothelial dysfunction. However, particularly in older animals severe atheroma is present and responses to carbachol are greatly reduced. As XO/HX treatment can also cause a reduction in relaxation to carbachol of 50% or more it might have been difficult to observe interactions between XO/HX and hypercholesterolemia induced changes in endothelial dependent relaxation in these animals. Moreover plasma cholesterol levels in the homozygous WHHL rabbits generally exceeded 300 mg/dl. Such severe hypercholesterolemia is only observed in man in rare cases of familial hypercholesterolemia. Ma *et al.*, 1997[30] reported an increased susceptibility to oxidative stress and reduced levels of glutathione in NZW rabbits fed 1% cholesterol for 8 weeks. This dietary manipulation normally leads to gross elevations in plasma cholesterol similar to those observed in homozygous WHHL rabbits.[31] It appears that under these conditions endogenous antioxidants and free radical scavenging systems were unable to compensate for the additional oxidative stress.

In summary, our heterozygous WHHL with only mild atherosclerosis are less susceptible to impairment of both endothelium dependent and independent relaxation by the XO/HX free radical generating system than NZW rabbits. We hypothesise that this may be due to increased levels of endogenous free radical scavenging enzymes.

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