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Beneficial effects of ACE-inhibition with zofenopril on plaque formation and low-density lipoprotein oxidation in watanabe heritable hyperlipidemic rabbits

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Abstract

The effects of angiotensin-converting enzyme (ACE)-inhibition with zofenopril on the development of atherosclerosis and low-density lipoprotein (LDL) oxidation were determined in Watanabe Heritable Hyperlipidemic (WHHL) rabbits. Rabbits received either placebo (n = 6) or 0.5 mg/kg/day of zofenopril (n = 6). After 6 weeks of treatment, the computer-assisted analysis revealed that zofenopril reduced the aortic and common carotid corrected cumulative lesion area by 34% and 39%, respectively ($p < 0.05$ vs placebo-treated group). The intimal/medial ratio of the largest fatty streaks was 0.426 ± 0.158 in the zofenopril-treated group and 0.875 ± 0.238 in the placebo-treated group ($p < 0.05$). Furthermore, we found in the zofenopril-treated group smaller lesions with an intimal/medial ratio of zofenopril also reduced plasmatic LDL oxidation, as shown by significant reduction of malondialdehyde content ($p < 0.01$) and relative agarose gel mobility ($p < 0.05$), as well as by the prolongation of the lag-time ($p < 0.05$). Compared to zofenopril-treated rabbits, arterial sections of the placebo-group had significant increase in the intimal presence of macrophages-derived foam cells ($p < 0.05$), ox-LDL ($p < 0.01$), and native LDL ($p < 0.01$) detected by immunocytochemistry with RAM-11, MDA2 and NP1533975 monoclonal antibodies, respectively. To investigate the amount of platelet accumulation in the atherosclerotic plaque we also measured platelet-associated radioactivity. Autologous platelets were labeled with ^{111}Indium-oxine and injected intravenously. After 2 hours, WHHL were sacrificed and arterial sections were counted for platelet-associated radioactivity. In the placebo-treated group, platelet radioactivity was 0.52 ± 0.12 equivalent of radioactivity per mg of tissue in the common carotid and 0.25 ± 0.18 in the abdominal aorta; in contrast, rabbits treated by zofenopril had 0.20 ± 0.12 in the common carotid and 0.06 ± 0.01 in the abdominal aorta. These data indicate that ACE-inhibition with zofenopril has antiatherosclerotic and antioxidant effects in WHHL-rabbits. Our results also shows that these effects could be linked to a reduced wall-associated platelet deposition at the site of atherosclerotic lesions.

Keywords: Zofenopril; WHHL rabbit; LDL oxidation; Platelet; Antioxidants; Atherogenesis

1. Introduction

Atherogenesis is a complex disease involving an important cellular response to damaging stimuli; this process becomes clinically evident when ulcerating vascular lesions develop and provide a site for thrombosis, inflammation, and vessel obstruction (Fuster et al., 1992; Fuster, 1998; Kinlay et al., 1998; Loscalzo, 1992; Luscher, 1995; Ross, 1999). Thus, by asking questions about arterial inflammation (Ross, 1999) and the pathophysiological origin of fatty streak formation (Napoli et al., 1997a; Napoli et al., 1999a,b), we might gain new insights into the pathogenesis of the atherosclerotic disease in humans.

Much evidence now exists that oxidized low-density lipoprotein (ox-LDL) is taken up by macrophages more avidly than the native form of LDL in vivo by an entire

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family of scavenger receptors (reviewed in Witztum and Steinberg, 1991; Steinberg, 1997). The most recent version of this mechanism emphasizes that this has been shown to occur very early in the human development inducing foam cell accumulation and promoting atherogenesis (Napoli et al., 1997a; Napoli et al., 1999a). Antioxidants, or other drugs with antioxidant effects, may therefore be beneficial, in particular if they interfere with events on the luminal endothelial surface or with the interactions between the coagulation system and the arterial wall.

In patients with atherosclerosis or hyperlipidemia or both, coronary endothelial dysfunction is usually present and affects vasomotor tone, platelet reactivity, thrombosis, fibrinolysis, and regulation of inflammatory cells (Fuster et al., 1992; Fuster, 1998; Kinlay et al., 1998; Loscalzo, 1992; Luscher, 1995; Ross, 1999). New evidence suggests an interaction among hyperlipidemia, activation of the renin angiotensin-system and atherosclerotic-related diseases (Pitt, 1997). Angiotensin II alters the binding of native LDL to its receptor and increases endothelial uptake of LDL, indicating a potential therapeutic use of angiotensin-converting enzyme (ACE)-inhibitors in patients with atherosclerosis and/or hyperlipidemia (Pitt, 1997). ACE-inhibitors are predicted to act as free radical scavengers, in particular those with a sulfhydryl group (Chopra et al., 1992; Mak et al., 1990). Moreover, these drugs seem to exert both antiatherosclerotic and antioxidative effects in apolipoprotein E-deficient mice (Hayek et al., 1998); antiatherosclerotic properties in hamsters (Kowala et al., 1998), atherosclerotic minipigs (Charpiot et al., 1993), and cynomologus monkeys (Aberg and Ferrer, 1990); and reduction of the arterial expression of NF-kappaB-dependent proinflammatory factors in the rabbit (Hernandez-Presa et al., 1998).

ACE-inhibitors have been also shown to interfere with platelet function (Pitt, 1997). Specifically, the ACE-inhibitor fosinopril has been shown to reduce ADP-induced platelet aggregation in hypertensive patients (Keidar et al., 1996) and to decrease platelet response to thrombin receptor agonist SFLRRN-NH2 in monkeys (Hale et al, 1998). In doing so, these drugs may interfere with platelet accumulation at the site of atherosclerotic lesions.

The ACE-inhibitor zofenopril is a sulfur containing pro-drug, which, after bio-conversion to its active free-sulfhydryl containing form, has five-fold more potent anti-hypertensive effect than the ACE-inhibitor captopril (Chopra et al., 1992; DeFelice and Kostis, 1987; Mak et al., 1990). To assess the effects of the ACE-inhibition with zofenopril on plaque formation and LDL oxidation, we used the Watanabe Heritable Hyperlipidemic Rabbit (WHHL), an animal model of human homozygous familial hypercholesterolemia (Tanzawa et al., 1980). In this model, the absence of functional LDL receptors leads to decreased clearance of lipoproteins containing apoproteins B or E (apo B, apo E) or both and to consequent overproduction of LDL with massive hyperlipidemia and, ultimately, atherosclerosis (Buja et al., 1983).

The goal of the study was to compare the size and composition of atherosclerotic plaques, the effects both on plasma LDL oxidation and oxidation-specific epitopes of ox-LDL directly in the arterial wall, and platelet accumulation at the site of lesions in WHHL rabbits treated chronically with the ACE-inhibitor zofenopril or placebo.

2. Materials and methods

2.1. Experimental animals

Homozygous WHHL rabbits 3-months old were purchased from Harlan-Nossan (Milan, Italy). At this age, WHHL rabbits show a rapid increase in atherosclerotic lesion formation. We used animals of both genders because there are no gender-related differences in atherosclerosis in WHHL rabbits (Shiomi et al., 1992). Rabbits received either placebo (n = 6) or 0.5 mg/kg/day of zofenopril by gavage (n = 6) for additional 6 weeks. The dose used in the present study was chosen on the basis of preliminary experiments done to exclude marked hypotensive effects in the WHHL rabbit (data not shown) and is well within the range of doses used to show efficacy by oral administration in experimental models and in humans. In particular, oral doses ranging between 0.5–1 and 0.3–2.2 mg/kg cause inhibition of pressure response in rats and dogs, respectively (DeForrest et al., 1989; Sun and Mendelsohn, 1991). In patients, zofenopril in the range of 30–60 mg/day induces a prompt reduction of blood pressure in mild to moderate essential hypertension (Lacourciere and Provencher, 1989) and positive effects on mortality and morbility after myocardial infarction (Ambrosioni et al., 1995). Lipoprotein cholesterol levels and tryglicerides in the blood were evaluated immunoenzymatically (Boehringer-Mannheim, Italia). Serum ACE-activity was assayed according to Holmquist at al. (1979) by measuring the reduction in the absorbance at 340 nm which results from ACE-induced hydrolysis of furylacryloylphenylglycemic to furylacryloylhemyalanine and glycylglycine. Rabbits took all of their regular diet and water ad libitum during the experiment.

The animals were managed in accordance with the Guidelines of the American Physiological Society together with the strict observance of the rules established by the Institutional Animal Care and Use Committee of the Federico II University of Naples (Prof. G. Cirino, local coordinator) and the laws of the Italian Ministries of Health and University and Scientific Research (M.U.R.S.T.; Rome, Italy).

2.2. Evaluation of oxidation-induced oxidative parameters in circulating LDL

Plasma was obtained from blood samples of the ear artery immediately prior to sacrifice. LDL was isolated by two-consecutive steps of discontinuous density ultracentrifugation in a KBr gradient, as previously described in detail (Napoli et al., 1997b). A Sephacryl S-300 column (5 × 0.9 cm, equilibrated with 150 mM NaCl-PBS, 1 mM EDTA) was used to desalt and remove low molecular weight components from the samples. LDL was used within a few hours to prevent spontaneous peroxidation (Napoli, 1996; Napoli et al., 1997b; Corso et al., 1997). LDL purity was checked by both agarose, under non-denaturing conditions, and SDS-polyacrylamide gel electrophoresis (PAGE), performed on a 5–16% linear gradient slab gel (Napoli et al., 1995; Napoli et al., 1996; Napoli et al., 1997c). Protein content was measured as described by Lowry et al. (1951), using bovine serum albumin as a standard.

LDL (100 μg/ml) was incubated for 12 h at 37°C, in the presence of xanthine (2 mM, final concentration) and xanthine oxidase (100 mU/ml, salicylate-free, from bovine milk, specific activity 1 U/mg of protein) in 0.150 M NaCl-0.01 M sodium phosphate, pH 7.4, as previously described in detail (Ambrosio et al., 1994; Napoli et al., 1995; Napoli et al, 1997d). In parallel experiments, superoxide radical production by xanthine-xanthine oxidase system was monitored by following the reduction of cytochrome-c (1.2 mM) at 550 nm in a double-beam spectrophotometer (Uvikon 810, Kontron, Zurich, Switzerland). This reaction yields both superoxide radicals and hydrogen peroxide, which in turn may produce hydroxyl radicals in the presence of trace amounts of iron or other transition metals inducing a mild form of ox-LDL (Napoli, 1996; Napoli et al., 1997d). This system generates about 20 nmoles per min^{-1} per ml^{-1} of superoxide radicals and about 40 nmoles per min^{-1} per ml^{-1} of hydrogen peroxide at peak activity (i.e., 90 s), which in turn progressively declines within 6 min (Ambrosio et al., 1994; Napoli et al., 1995).

To measure the susceptibility of LDL to oxidation, we determined the length of the lagphase preceding the onset of rapid oxidation in LDL (lag-time), as previously described (Napoli et al., 1995; Napoli et al., 1997b,c,e). In this method, originally described by Esterbauer et al. (1989), the 234 nm absorption results from the conversion of PUFA with isolated double bonds into lipid hydroperoxides with conjugated double bonds. Thus, from the diene versus time profile the duration of the lag phase can be deduced, and it was assumed that the length of the lag phase may be an index for its oxidation resistance. LDL peroxidation was also evaluated from the amount of malondialdehyde (MDA) produced. This latter compound is an end-product of peroxidation of unsaturated fatty acids and is a widely used marker of lipid oxidation. MDA content was assayed by the thiobarbituric acid method, modified as previously described (Ambrosio et al., 1994; Napoli et al., 1995). The amplification of peroxidation during this assay was prevented by adding the chain-breaking butylate hydroxytoluene (10 μM final concentration) to the sample before the thiobarbituric reagents are added. This to reduce artifacts due to variations in sample lipid content and/or antioxidant concentration and possible iron contamination of reagents (Napoli, 1996).

2.3. Morphometric assessment of lesions and immunocitochemistry of lesion components

At the end of the period of drug administration, rabbits were anesthetized with intravenous sodium pentobarbital (25 mg/kg of body weight). The common carotid artery and the aorta were dissected, cleaned of adherent fat and fascia, cut open, washed thoroughly with cold sterile phosphate-buffered saline (PBS) containing 2 mM EDTA, and placed in ice-cold PBS containing 50 μM butylated hydroxytoluene, 0.001% aprotinin, 50 mM EDTA, and 0.008% chloramphenicol, equilibrated with nitrogen, as previously described (Napoli et al., 1997a; Napoli et al., 1999a). Each arterial segment was then divided into two parts. One of these was immersed in OTC medium, flash frozen in liquid nitrogen. Seven μm-thick sections were prepared with a cryotome for morphometric determination of lipid-rich lesions. The second part of each arterial segment was fixed in buffered 10% formalin, paraffin embedded; 12 to 15 serial sections (57-μm thick) were prepared for immunocytochemistry.

Thirty cryo-sections from both arteries were stained with oil red-O and counterstained with hematoxylin. We traced contours of the lumen and lesion, and then measured at the common carotid artery and abdominal aorta the surface lesion area. In particular, using computer-assisted image analysis (Napoli et al., 1997a; Napoli et al., 1999a), we determined the presence of oil red O-positive intimal lipid accumulations, the cumulative area of all lipid accumulations per section, and the intimal-media ratio of lesions. To permit a direct comparison of lesion formation between arteries of different size (carotid and abdominal aorta), data were then corrected by dividing the cumulative lesion area by the average circumference of the aorta and the common carotid artery at each site of measurement. We excluded vacuoles and lacunae that were considered artifacts from the analysis of extracellular lipid deposits.

Lesional components were semi-quantitatively evaluated by immunocytochemical staining that was performed as previously described in detail (Napoli et al., 1997a; Napoli et al., 1999a). Briefly, duplicate serial sections of the fixed and paraffin embedded arterial segments were stained with the following antibodies: MDA2, a murine monoclonal antibodies against malondialdehyde (MDA)-lysine epitopes of ox-LDL, RAM-

11, a monoclonal antibody against rabbit monocyte/macrophages-derived foam cells (Axcel Accurate, Westbury, NY), and NP1533975, a mouse monoclonal antibody (IgG_1) to the native form of apolipoprotein B of LDL (Boehringer Mannheim, Italia). Antibodies were used at a dilution of 1:500. Epitopes recognized by the primary antibody were detected by an avidin-biotin-peroxidase method, as previously described in detail (Napoli et al., 1997a; Napoli et al., 1999a). The area of each lesional component was determined. The percent of lesional components were calculated by dividing area of each lesional component by the overall area of the atherosclerotic lesion. Results were corrected by the ratio between the cumulative lesion area/the average circumference of the vessel at each site of measurement (Napoli et al., 1999a).

2.4. Platelet experiments

Blood was obtained from a marginal ear vein and platelets were labeled with ^{111}Indiumoxine, as previously described in detail (Itoh et al., 1996; Liu et al., 1994). Briefly, blood (9 ml) was collected into 1 ml of 3.8% (w/v) trisodium citrate and centrifuged to 200 × g (10 min) to obtain platelet-rich plasma (PRP). Platelets were then washed by centrifugation (640 × g for 15 min) in Ca^{++} free Tyrode solution containing PGE1 (300 ng/ml) and gently resuspended and incubated for 1.5 min at 37°C with 25–50 μCi of ^{111}Indium-oxine. After further centrifugation (640 × g for 15 min), the supernatant was discarded and platelet pellet resuspended and injected intravenously into the recipient rabbit (Liu et al., 1994). After 2 hours, WHHL rabbits were killed and the aortic and common carotid arterial sections from standardized atherosclerotic areas were obtained. Control values were obtained at the site of uninvolved vessel. The radioactivity in arterial samples was determine in a γ-counter (Packard, Cobra B5002 model), together with 0.5 ml duplicate samples of blood and expressed as the blood-corrected values of radioactivity per mg (wet weight) of arterial tissues (Itoh et al, 1996; Liu et al., 1994). To assess free ^{111}Indium circulating in the blood, at the end of experiments plasma samples were also prepared and counted in the γ-counter (Itoh et al, 1996; Liu et al., 1994).

2.5. Chemicals

Acrylamide, SDS and other electrophoresis grade reagents were purchased from Bio-Rad (Richmond, VA, U.S.A.). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cholesterol content of LDL was measured by enzymatic assay method using a commercial kit (Cholesterol 50, Sigma Chemical Co.) according to the manufacturer's instructions. Zofenopril was obtained from the Department of Pharmasynthesis of the Preclinical Pharmacology Unit, A. Menarini, Florence.

2.6. Statistical analysis

Data are presented as mean ± standard deviation or SEM. Differences between treated groups and controls were primarily tested by one-way ANOVA followed by the Bonferroni's corrected t-test or by Kruskal-Wallis non-parametric analysis of variance. The t-student umpaired test was used for ^{111}Indium-oxine platelet experiments. A value of $p < 0.05$ was considered as significant.

3. Results

To determine the effect of ACE-inhibition with zofenopril on the development of atherosclerotic plaque, WHHL rabbits were treated with 0.5 mg/kg/day of zofenopril for a period of six weeks. At the end of the treatment period, blood was collected for plasmatic determinations and LDL oxidation. Then animals were sacrificed and arterial sections were examined for plaque formation, presence of oxidation-specific epitopes formed during the oxidation of LDL in the arterial wall, and ^{111}Indium-oxine platelet-accumulation at the site of atherosclerotic lesions.

3.1. Plasmatic determinations and LDL-oxidation

As expected, administration of zofenopril to the WHHL rabbit reduced the plasma ACEactivity from 898 ± 38 U/L in the placebo-treated group to 435 ± 48 U/L in the zofenopril-treated group ($p < 0.01$). The lipid profile was evaluated in all WHHL rabbits at the beginning of the study. Total triglyceride was 428.9 ± 157 mg/dl, total cholesterol was 793.3 ± 171.6 mg/dl, VLDLcholesterol was 206.5 ± 142 mg/dl, HDL-cholesterol was 15.9 ± 12 mg/dl, and LDL-cholesterol was 390.8 ± 95 mg/dl. The whole lipid profile was not significantly affected by the administration of zofenopril (in the zofenopri-treated group total triglyceride was 431.6 ± 150 mg/dl, total cholesterol was 902.6 ± 185.2 mg/dl, VLDL-cholesterol was 200.3 ± 153 mg/dl, HDL-cholesterol was 16.0 ± 13 mg/dl, and LDL-cholesterol was 393.9 ± 99 mg/dl, p = NS vs baseline values for all parameters). Then, we analyzed the susceptibility of LDL from the WHHL rabbit treated with zofenopril or placebo to undergo oxidative modification (lag time) and other oxidative parameters (malondialdehyde and agarose gel mobility) in the presence of oxygen radicals generated through the xanthine-xanthine oxidase reaction. Results are summarized in Table 1. On the basis of the data, we concluded that ACE-inhibition with zofenopril increased the resistance of plasmatic LDL to oxidative stress, as shown by significant reduction of malondialdehyde content ($p < 0.01$ vs placebo) and relative agarose gel mobility ($p < 0.05$ vs placebo), as well as by the prolongation of the lag-time ($p < 0.05$ vs placebo).

Table 1
The susceptibility of plasma low-density lipoprotein (LDL) derived from placebo- and zofenopril-treated Watanabe Heritable Hyperlipidemic (WHHL) rabbits

	Placebo-treated group	Zofenopril-treated group	p<
MDA (nmoles/mg prot)	25 ± 5	14 ± 4	0.01
Lag-Time (min)	100 ± 21	76 ± 12	0.05
REL (mm)	1.8 ± 0.2	1.4 ± 0.1	0.05

LDL (100 μg/ml) derived from placebo- and zofenopril-treated rabbits was incubated for 12 h at 37°C, in the presence of xanthine (2 mM, final concentration) and xanthine oxidase (100 mU/ml, salicylate-free, from bovine milk, specific activity 1 U/mg of protein) in 0.150 M NaCl-0.01 M sodium phosphate, at pH 7.4. Then, the amount of malondialdehyde (MDA), the lag-time, and the relative agarose gel mobility (REM) were evaluated.

3.2. Morphometric assessment of lesions and immunocitochemistry

Data of atherosclerotic lesions were corrected for the vascular size (by dividing the cumulative lesion areas by the vascular circumference) (Fig. 1A). Morphometric analysis of oil red O-stained sections of arteries from the control placebo-group revealed extensive occurrence of intimal lipid accumulations in aortas and carotids (Fig. 1A). The thickness of the atherosclerotic lesions was assessed by the intimal/medial ratio. This ratio of the largest fatty streaks was 0.875 ± 0.238 in the placebo-treated group. In contrast, the average intimal thickening was significantly decreased in the zofenopril-treated group both in abdominal aorta and common carotid ($p < 0.05$ vs placebo-treated group) (Fig. 1A). Furthermore, we found in the zofenopril-treated group smaller lesions with an intimal/medial ratio of 0.426 ± 0.158. Fig. 2 shows a typical oil red O- pattern of a carotid segment from a placebo-treated WHHL rabbit (upper panel) compared to that of zofenopril-treated WHHL rabbit (lower panel). Thus, this study illustrates that zofenopril reduced both the cumulative lesion area and the severity (intimal/medial ratio) of atherosclerotic lesions compared to the placebo. Interestingly, the lagtime, the index of resistance to LDL oxidation, was inversely correlated to the cumulative lesion area in the zofenopril-treated group ($r = -0.69$, $p < 0.001$), whereas both malondialdehyde content and relative agarose gel mobility (formed after 12 h exposure of LDL to oxidation) correlated positively with the cumulative lesion area ($r = 0.72$ and $r = 0.79$, $p < 0.001$, and $p < 0.0005$, respectively). Thus, the extent of plasmatic LDL oxidation well correlated with the extent of atherosclerosis in the WHHL rabbit.

In addition to the sections used for morphometric determinations, 15 paraffin-embedded serial sections of each arterial segment were immunostained and assessed for the intimal presence of the "oxidation-specific" epitopes (MDA2 monoclonal antibody), macrophagesfoam cells (RAM-11 monoclonal antibody), and native apolipoprotein B of LDL (NP1533975 monoclonal antibody). Results are shown as percent of positive sections for immunostaining in Fig. 1B. No significant differences in relative lesion composition between aorta and carotid arteries were seen. The zofenopril-treated group contained significantly fewer intimal macrophages, native LDL and ox-LDL than those of

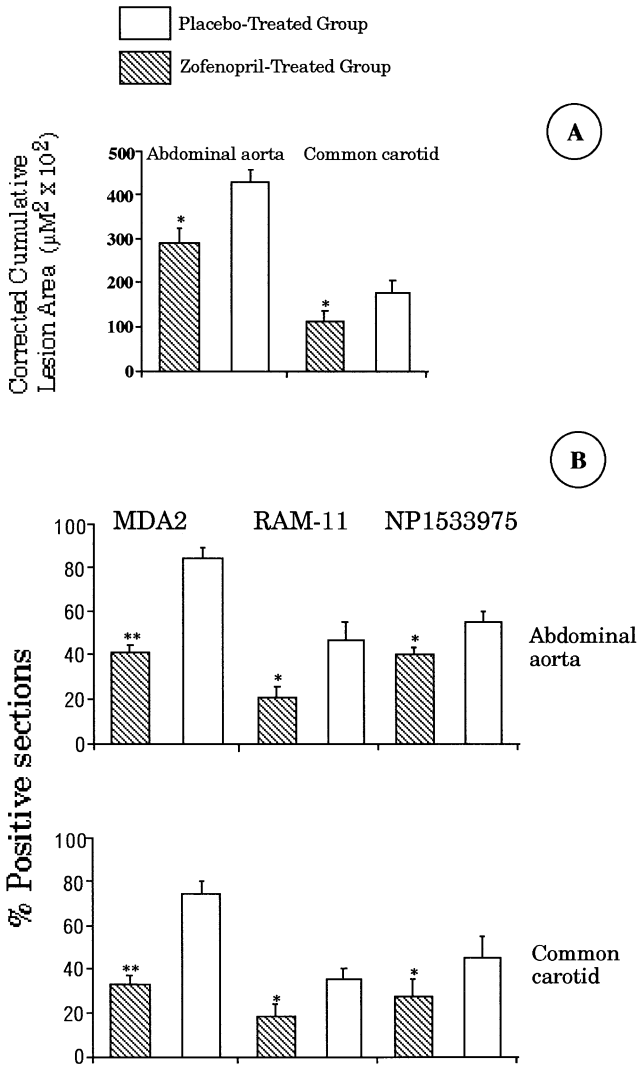

Fig. 1. (A) The effects of zofenopril on the atherosclerotic lesion area in Watanabe Heritable Hyperlipidemic (WHHL) rabbits. After 6 weeks of treatment with zofenopril (0.5 mg/kg/day) or placebo, the rabbits were killed and the arterial sections were stained with oil red O and hematoxylin, and the corrected cumulative lesion area was assessed by the computer-assisted image analysis. (See section 2, Materials and methods for further details.) Results are expressed as the mean ± SEM of the lesions of 6 animals from each group. (B) Analysis of lesion composition detected by immunohistochemistry in the arterial sections of the WHHL rabbits. The "oxidation-specific" epitopes (ox-LDL) were detected by the MDA2 monoclonal antibody, macrophages-foam cells by the RAM-11 monoclonal antibody, and the epitopes of apolipoprotein B of LDL by the NP1533975 monoclonal antibody. Results are shown as the mean ± SEM of the percent of positive sections for immunostaining. (See Section 2, Materials and methods for further details.)

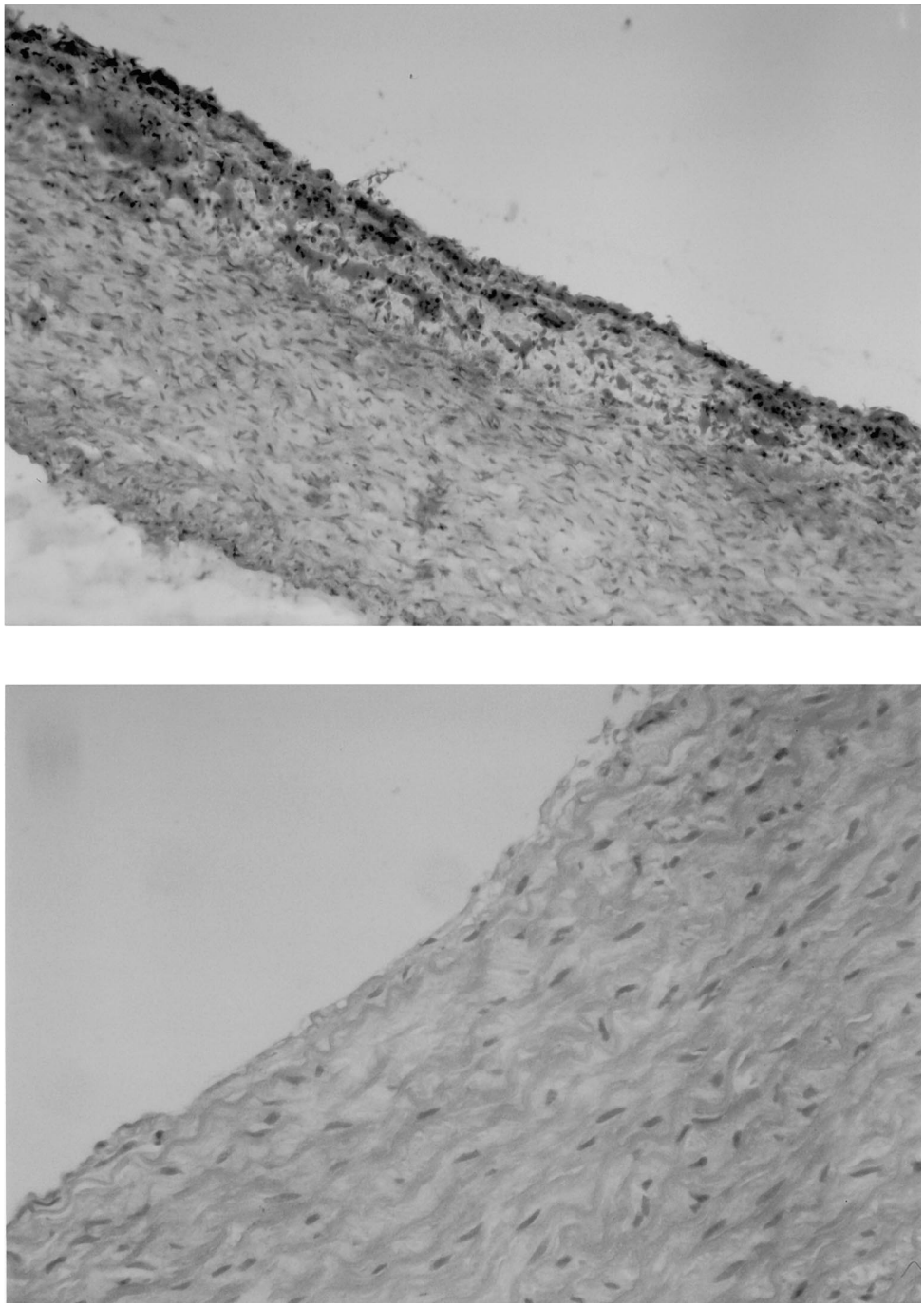

Fig. 2. Presence of intimal lipid accumulation in common carotid segments of Watanabe Heritable Hyperlipidemic (WHHL) rabbits. Frozen sections were prepared and stained with oil red O, as described in Section 2, Materials and methods. Representative examples are shown here. Atherosclerotic lesion containing substantial amounts of lipids in a WHHL rabbit artery from the placebo-treated group (top), compared to the only adaptative intimal thickening of a WHHL rabbit from the zofenopril-treated group (bottom).

the placebo group (Fig. 1B, *$p < 0.05$ and **$p < 0.01$ vs placebo-group). The lag-time, the index of resistance to LDL oxidation, was inversely correlated to the presence of oxidation-specific epitopes of ox-LDL in the arterial wall of the common carotid ($r = -0.78$, $p < 0.0001$) as well as in the abdominal aorta ($r = -0.84$, $p < 0.001$). Thus, the extent of plasmatic LDL oxidation also well correlated with the arterial formation of the oxidation-specific epitopes of ox-LDL in the WHHL rabbit. Fig. 3 shows a typical MDA2 staining pattern of an abdominal aorta segment from a placebo-treated WHHL rabbit (upper panel) compared to that of a zofenopril-treated WHHL rabbit (lower panel).

3.3. ^{111}*Indium-labelled platelet accumulation in vivo*

Following prolonged treatment with zofenopril, the ^{111}Indium-oxine platelet-associated radioactivity, at the site of atherosclerotic lesions, was reduced both in

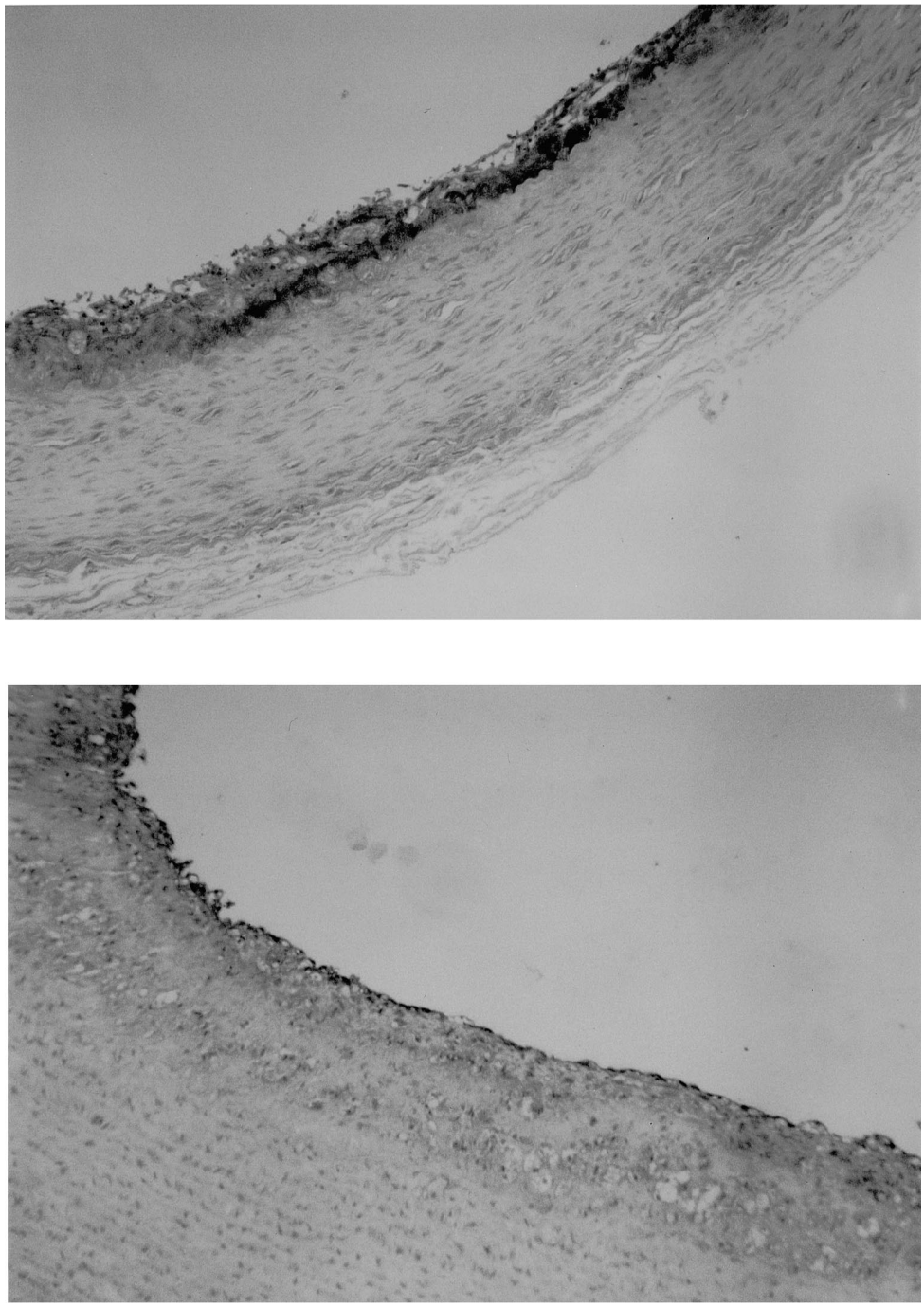

Fig. 3. Presence of oxidation-specific epitopes of ox-LDL in the arterial wall of the abdominal aorta. Arterial sections were prepared as described in Methods and immunostained with the avidin-biotin peroxidase method. Epitopes recognized by the primary antibody are brown; the nuclei are counterstained with hematoxilin. A typical example of a section of the abdominal aorta from the segment from a Watanabe Heritable Hyperlipidemic (WHHL) rabbit of the placebo-treated group (top) and of the WHHL group treated with 0.5 mg/kg/day zofenopril (bottom), immunostained with MDA2, a monoclonal antibody against MDA-lysine, an oxidation-specific epitope of ox-LDL. In the placebo-treated (top) but not in zofenopril-treated rabbit (bottom) a diffuse cellular and extracellular presence of oxidation-specific epitopes can be noted.

the abdominal aorta and the common carotid artery compared to that in the placebo-treated group (Fig. 4). Statistically significant inhibition of zofenopril of platelet accumulation was found in the carotid artery ($*p < 0.05$). Although the difference in the aorta was quite impressive it did not reach statistical significance ($p = 0.327$). Control values at the site of uninvolved vessel were 50- to 75-fold lower than those achieved at the site of atherosclerotic lesions (data not shown).

4. Discussion

Following prolonged treatment of WHHL rabbits with the ACE-inhibitor zofenopril, we demonstrated

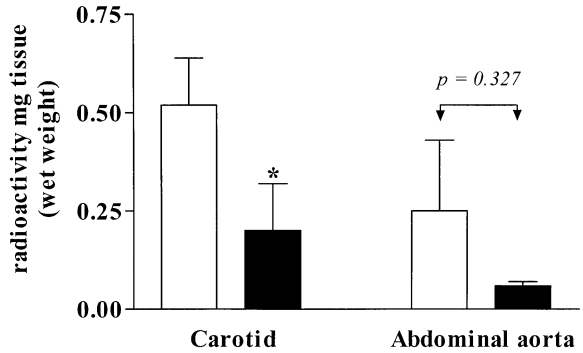

Fig. 4. ^{111}Indium-oxine platelet-associated radioactivity (radioactivity equivalents per mg of tissue, see Section 2, Materials and methods), at the site of atherosclerotic lesions, was reduced both in the abdominal aorta and the common carotid artery of the zofenopril-treated group (black bars) compared to that reached in the placebo-treated group (white bars) (*$p < 0.05$ vs placebo-treated group).

not only reduced atherogenesis but also a decrease in plasmatic LDL oxidation, measured by several parameters, and formation of oxidation-specific epitopes of ox-LDL in the arterial wall, together with reduced platelet accumulation at the site of atherosclerotic lesions. In the examination of plaque components, the content of macrophages-derived foam cells and native LDL also decreased in atherosclerotic lesions. These effects occurred independently of changes in plasmatic lipid profile or levels.

The mechanisms of the antiatherogenic effects of ACE-inhibitors are poorly understood. Reduction in arterial pressure may be a factor for their antiatherogenic effect, but some studies have demonstrated that these drugs attenuate atherogenesis without affecting blood pressure (Hayek et al., 1998; Kowala et al., 1998; Charpiot et al., 1993). The activation of the bradykinin/prostacyclin/nitric oxide pathway by the ACE-inhibitor captopril might play a role in its antiatherogenic effect (Linz et al., 1995). More importantly, zofenopril, as well as other ACEinhibitors significantly reduced serum ACE levels—an effect that could be directly involved for reducing atherogenesis by inhibiting angiotensin II-dependent effects, such as smooth-muscle cell proliferation, vasoconstriction, chemotaxis of monocytes, the alteration of the binding of LDL to its receptor, the increases endothelial uptake of native LDL, and the increased oxidative stress (reviewed in Pitt, 1997).

The present study shows for the first time that ACE-inhibition with zofenopril reduced the presence of oxidation-specific epitopes of ox-LDL in the arterial intimal wall. This effect was associated with the parallel reduction of plasmatic LDL oxidation seen also in another recent study (Hayek et al., 1998). A reduction of the expression of NF-kappaB-dependent proinflammatory factors by ACE-inhibitors has also been reported (Hernandez-Presa et al., 1998). These lines of evidence strongly suggest that beneficial effects of zofenopril on plaque formation may depend on its antioxidant effect. In fact, LDL oxidation is demonstrated to exert a potent atherogenic stimulus in experimental models and in humans (Witztum and Steinberg, 1991; Steinberg, 1997; Napoli et al., 1997a; Ross, 1999). Our observations are also consistent with the potent antioxidant effects of the sulfhydryl-containing ACE inhibitor zofenopril seen in several in vitro experiments, and similar to those of the potent chain-breaking antioxidant vitamin E (Chopra et al., 1992; Mak et al., 1990). Sulfhydryl compounds are a major class of protective agents against oxygen radicals generated by radiation (Simic, 1988). These drugs can neutralize oxygen radicals by either a hydrogen atom donating or electron transferring reaction (Simic, 1988; Mak et al., 1990). The mechanism of oxygen radical repair mediated by sulfhydryl compounds may involve carbon-centered (e.g., LOO°, °OH) radicals. It also appears that the protective effects of the sulfhydryl-agents correlate with their direct hydroxyl radical (°OH) scavenging abilities than with their antiperoxidative potency (Simic, 1988; Mak et al., 1990). In this regard, the xanthine/xanthine oxidase reaction, used in the present study to oxidize LDL, generates both superoxide radicals and hydrogen peroxide, which in turn may produce hydroxyl radicals in the presence of trace amounts of iron or other transition metals. Thus, the protective effects of zofenopril on LDL oxidation (and possibly on the presence of arterial oxidationspecific epitopes generated on ox-LDL) may depend, at least in part, by scavenging of the hydroxyl radical-induced oxidation. Further experiments with ACE-inhibitors without the protective sulfhydryl group may corroborate this hypothesis.

In WHHL rabbits, an early study demonstrated that captopril may exert beneficial effets on atherogenesis (Chobanian et al., 1990). We have extended those findings with a systematic computer-assisted image analysis of atherogenesis, and have provided new insights into the mechanisms involved in the phenomenon (i.e., LDL oxidation and platelet accumulation). In fact, we also found that in the zofenopril-treated group there was a reduced platelet-associated radioactivity, at the site of atherosclerotic lesions compared to the of placebo-treated group. Substantial evidence indicates that platelet activation and adherence to the wall may promote atherogenesis. Morphological analysis of evolving atherosclerotic lesions after infusion of autologous thrombi into pulmonary circulation showed that phagocytiosed platelets may serve as a source of lipids in fatty streaks (Chandler and Hand, 1961). Platelet deposition could support macrophage-derived foam cell formation, as has been shown using cultured aortic smooth muscle cells (Kruth, 1985; Ross, 1999).

More direct evidence of platelet role has been obtained by depleting platelets. In fact, platelet depletion dramatically reduces the mitogenic response to mech-

anical injury in the arterial wall by reducing the local release of critical cytokines and growth factors (Fuster et al., 1992; Fuster, 1998; Kinlay et al., 1998; Loscalzo, 1992; Luscher, 1995; Ross, 1999). Thus, activated platelets release their granules, which contain cytokines, adhesion molecules (integrins), and various growth factors, together with thrombin, they might contribute to migration and proliferation of intimal smooth muscle cells and monocytes (Bombeli et al., 1998; Ross, 1999). The von Willenbrand factor, the polymeric plasma glycoprotein important for platelet adhesion to the injured vessel wall, particularly at high shear stress rates, is most important for the development of acute occlusive thrombosis in atherosclerotic individuals and supports the development of a mixed microthrombus on a preexistent occlusive thrombus (Nichols et al., 1990; Ross, 1999). Interestingly, ox-LDL also contains low concentrations of acetylhydrolase, the enzyme that catabolizes the platelet-activating factor and increased rate of LDL oxidation also promotes platelet aggregation and its degranulation (Ambrosio et al., 1994). The reduction of platelet-associated radioactivity at the site of lesions and decreased LDL oxidation induced by the ACE-inhibitor zofenopril, therefor, may produce several beneficial effects on atherosclerotic initiation and progression.

In conclusion, prolonged treatment with the ACE-inhibitor zofenopril attenuates the development of atherosclerotic lesions in the WHHL rabbit. This activity may be related to its plasmatic and intimal antioxidant effect, and to interfering with platelet accumulation at the site of atherosclerotic lesions. These findings are particularly relevant since hypercholesterolemia is important in approximately 50% of patients with atherosclerotic-related diseases and other factors need to be taken into consideration in the prevention of atherogenesis (Witztum and Steinberg, 1991; Braunwald, 1997; Ross, 1999).

5. Summary

The goal of the study was to investigate the size and composition of atherosclerotic plaques, the effects both on plasma low-density lipoprotein (LDL) oxidation and oxidation-specific epitopes on ox-LDL in the arterial wall, and platelet accumulation at the site of lesions in Watanabe Heritable Hyperlipidemic (WHHL) rabbits treated chronically with the ACE-inhibitor zofenopril compared to the placebo. Rabbits received either placebo (n = 6) or 0.5 mg/kg/day of zofenopril (n = 6) for 6 weeks. Computer-assisted analysis revealed that zofenopril significantly reduced the extent of atherosclerosis compared to the placebo. Zofenopril also reduced plasmatic LDL oxidation. In analogy, arterial sections of the placebo-group had much greater increase in the intimal presence of macrophages-foam cells, ox-LDL, and native LDL detected by immunocytochemistry with RAM-11, MDA2 and NP1533975 monoclonal antibodies, respectively. To investigate the amount of platelet deposition in the atherosclerotic plaque, we also determined ^{111}Indium-oxine platelet-associated radioactivity. In the placebo-treated group, arterial associated radioactivity was greater than in rabbits treated with zofenopril. These data demonstrate that ACE-inhibition with zofenopril has antiatherosclerotic and antioxidant effects in WHHL-rabbits.

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