Circulating oxidized low-density lipoprotein is increased in hypertension

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ABSTRACT

Oxidized low-density lipoprotein (OxLDL) and autoantibodies to OxLDL (aOxLDL) are implicated in the development of atherosclerosis. The objective of this study was to determine the importance of these factors in hypertension, a major risk factor for atherosclerosis. Samples were obtained from 111 men with established hypertension (diastolic pressure > 95 mmHg) from the Swedish component of an ongoing hypertension study (European Lacidipine study on Atherosclerosis, ELSA) and from 75 normotensive control men, who were from a Swedish population-screening programme (diastolic pressure < 80 mmHg). The presence of carotid atherosclerosis and the intima-media thicknesses were determined by ultrasonography. A monoclonal antibody to OxLDL, EO6, was used to determine oxidation epitopes in LDL. aOxLDL of IgG and IgM subclass were tested by ELISA against OxLDL. Hypertensive men had increased OxLDL levels compared with normotensives (P = 0.002), whereas autoantibodies tested were largely similar between groups. There was no association between the antibodies tested, or OxLDL and carotid atherosclerosis. Age was not associated with OxLDL or aOxLDL measurements. Taken together, our findings indicate that OxLDL is elevated in hypertensive men, which may predispose to atherosclerosis in hypertension. In contrast, aOxLDL levels were unchanged and the role of aOxLDL may depend on disease stage and/or type.

INTRODUCTION

Hypertension and atherosclerosis are associated, although the underlying mechanisms that cause this association are not completely known, and it is possible that each of the two conditions predispose to and/or aggravate the other [1]. Once established, atherosclerosis is an inflammatory disease, where immune-competent cells are present and proinflammatory cytokines are produced in significant amounts in lesions [2,3]. We have demonstrated recently [4–7] that immunological alterations are present in borderline hypertension. In contrast, relatively little is known about the role of the immune system in frank hypertension, although differences in immune function, such as decreased T-cell responses, defective complement function and enhanced immunoglobulin levels, have been described [8,9]. Increased oxidative stress is also a feature of hypertension [10]. Low-density lipoprotein (LDL) is generally believed to be important in the development of atherosclerosis,

Key words: antibody, atherosclerosis, hypertension, oxidized low-density lipoprotein.

Abbreviations: ABS, adult bovine serum; apoB, apolipoprotein B; BMI, body mass index; CVD, cardiovascular disease; ELSA, European Lacidipine Atherosclerosis Study; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OxLDL, oxidized LDL; aOxLDL, autoantibodies to OxLDL; PAF, platelet-activating factor; RLU, relative light units; SLE, systemic lupus erythematosus.

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and its atherogenicity is likely to depend on modification, especially by oxidation [2]. Oxidized LDL (OxLDL) has inhibitory and/or toxic effects on endothelial cells and also on monocytes and T-cells, especially at higher concentrations [11–14]. In contrast, at lower concentrations and after low grade oxidation, OxLDL stimulates endothelial adhesiveness [15–17] and promotes T-cell and monocyte activation [13,15]. OxLDL is taken up by macrophages in the artery wall, which develop into foam cells, and is therefore generally believed to be atherogenic [2].

Autoantibodies to OxLDL (aOxLDL) exist in healthy individuals, but their exact role during different stages in the development of atherosclerosis and cardiovascular disease (CVD) is not clear. The level of aOxLDL is elevated in established atherosclerosis and/or CVD [18–20]. In contrast, we have demonstrated recently [6] that plasma levels of aOxLDL, but not OxLDL, are decreased in early CVD as in patients with borderline hypertension.

In the present study, we sought to investigate the role of OxLDL and aOxLDL in hypertension, using men with overt hypertension from the European Lacedipine Atherosclerosis Study (ELSA) study [21,22]. We now report that OxLDL is increased in hypertensive compared with normotensive men. The implications of these findings are discussed.

METHODS

Study group

Serum samples were obtained from 111 male subjects with established hypertension (diastolic pressure >95 mmHg) prior to their entry into the Swedish component of ELSA [21–23] and following a 4 week washout period with no medication to minimize the effects of treatment on the measured parameters. Inclusion criteria for the study were an age between 45 and 75 years, a diastolic blood pressure between 150 and 210 mmHg, and a systolic blood pressure between 95 and 115 mmHg and a systolic blood pressure at first visit of 95 mmHg prior to their entry into the Swedish population-screening programme [24].

The study was approved by the Ethics Committee of the Karolinska Hospital and was conducted in accordance with the Helsinki Declaration. All subjects gave informed consent.

Blood lipids and blood pressure were determined as described previously [23–25]. The procedure for carotid ultrasound determinations in the ELSA study have been reported elsewhere [6,26].

All subjects were investigated according to the same schedule. Blood samples for analyses of metabolic and inflammatory variables and blood pressure were obtained from 75 normotensive controls (diastolic pressure <80 mmHg) prior to their entry into the Swedish population-screening programme [24]. In contrast, at lower concentrations and after low grade oxidation, OxLDL stimulates endothelial adhesiveness [15–17] and promotes T-cell and monocyte activation [13,15]. OxLDL is taken up by macrophages in the artery wall, which develop into foam cells, and is therefore generally believed to be atherogenic [2].

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Determination of OxLDL epitopes

The EO6 epitope concentration on apolipoprotein B (apoB) 100-containing particles was measured by a chemiluminescent modification of a previously described assay [27–29]. This sandwich assay utilizes an anti-human apoB-100 (Sigma) or anti-human IgG (A-3150; Sigma) or anti-human IgM (A-3275; Sigma) sera diluted 1:9000 and 1:7000 respectively with PBS, at 37°C for 2 h. After four washes, 100 µl of substrate [Sigma 104 phosphatase substrate; 5 mg tablet in 5 ml of diethanolamine buffer (1% MgCl₂ in 50 mM carbonate/bicarbonate buffer, pH 9.6)] was added. The plates were incubated at room temperature for 30 min and read in an ELISA Multiskan Plus spectrophotometer (Labsystems, Helsinki, Finland) at 405 nm. Each determination was done in triplicate. All samples were measured in a single assay and the coefficient of variation was 10–15%.

Determination of aOxLDL by ELISA

IgG and IgM subclasses aOxLDL were determined by ELISA essentially as described previously [6]. OxLDL was diluted to 2 µg/ml in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6), and 100 µl/well was used to coat ELISA plates (Costar 2581; Cambridge, MA, U.S.A.). The plates were kept at 4°C overnight, washed four times with PBS containing 0.05% Tween-20, and then blocked with 20% (v/v) adult bovine serum (ABS) in PBS (20% ABS/PBS) for 2 h at room temperature. The plates were then incubated with 100 µl of serum diluted 1:20 in PBS at room temperature. After three washes with PBS, the plates were incubated with 50 µl/ml alkaline phosphatase-conjugated goat anti-human IgG (A-3150; Sigma) or anti-human IgM (A-3275; Sigma), diluted 1:9000 and 1:7000 respectively with PBS, at 37°C for 2 h. After four washes, 100 µl of substrate [Sigma 104 phosphatase substrate; 5 mg tablet in 5 ml of diethanolamine buffer (1% MgCl₂ in 50 mM carbonate/bicarbonate buffer, pH 9.6)] was added. The plates were incubated at room temperature for 30 min and read in an ELISA Multiskan Plus spectrophotometer (Labsystems, Helsinki, Finland) at 405 nm. Each determination was done in triplicate. All samples were measured in a single assay and the coefficient of variation was 10–15%.
well an equal number of apoB-containing particles are bound. This has been verified by demonstrating that biotinylated MB24, another apoB-specific antibody that binds to a distinct apoB epitope from that of MB47 [31], binds equally to each well.

After incubation, the plates were washed as above and 10 µg/ml biotin-labelled EO6 in BSA/washing buffer was added to the plates (50 µl/well) and incubated overnight at 4 °C. After washing the plates as above, a 1:10,000 dilution of avidin-conjugated alkaline phosphatase (NeurAvidin, alkaline phosphatase conjugated; Pierce, Rockford, IL, U.S.A.) in BSA/washing buffer containing 1 mM MgCl₂ and 1 mM ZnCl₂ was added to the plates (50 µl/well) and incubated for 1 h at room temperature. The plates were then washed four times with washing buffer, and 50 % Lumi-Phos 530 (Lumigen Inc., Southfield, MI, U.S.A.) in distilled water was added to the plates (30 µl/well) and incubated for 1.5 h at room temperature in the dark. Chemiluminescence was read on a MLX microtitre plate luminometer (Dynex Technologies Inc.). Data are expressed in relative light units (RLU) measured over 100 ms. All samples were measured in a single assay and the intra-assay coefficients of variation of low and high standards were 6–8 %. Data are expressed as absolute amounts of EO6 bound/well (in RLU).

Statistical methods

Variables were tested for normality. Groups with skewed variables were compared using the non-parametric Mann–Whitney’s U test, normally distributed data were compared using the Student’s t test and categorical variables were compared with the χ² test. Logistic regression analysis (Stat View software) was used with blood pressure status (hypertension yes/no) as the dependent variable and oxLDL and related parameters and possible confounders as independent variables. Data are presented as means ± S.E.M. (parametric) or medians and inter-quartile ranges (non-parametric), as appropriate. The level of statistical significance was set at a value of P ≤ 0.05.

RESULTS

Characteristics of the study cohort

Baseline characteristics for the normotensive and established hypertensive subject groups are shown in Table 1. Serum levels of IgG (9.8 ± 2.3 compared with 18.8 ± 8.0 g/l; P < 0.001) and IgA (2.1 ± 0.12 compared with 3.7 ± 2.3 g/l; P < 0.001) were higher in subjects with established hypertension compared with normotensive controls, whereas levels of IgM were equivalent (0.99 ± 0.46 compared with 0.92 ± 0.93 g/l).

Body mass index (BMI), plasma cholesterol and triacylglycerols (triglycerides) were significantly raised (P < 0.005) in subjects with established hypertension, whereas the difference between glucose, LDL and high-density lipoprotein (HDL) levels between normotensive and hypertensive individuals did not reach statistical significance (Table 1). As a group, subjects with established hypertension were significantly older than the normotensive controls (P < 0.0001). When age was taken into account using logistic regression analysis, triacylglycerols still differed significantly between groups (P = 0.01), which was not the case with cholesterol (P = 0.17).

There were more smokers in the normotensive group (37 % compared with 17 % in the hypertensive group). One potential reason for this difference is that subjects with hypertension are advised by their physicians to stop smoking. However, levels of OxLDL or aOxLDL in smokers and non-smokers were not significantly different (results not shown).

OxLDL and aOxLDL levels

Hypertensive men had 62 % higher mean OxLDL levels compared with normotensive controls, a difference that was highly significant (Table 2). OxLDL levels were not correlated with intima-media thickness measurements,
blood lipids or with any of the blood pressure determinations (results not shown).

The levels of IgG or IgM subclass aOxLDL did not differ between the hypertensive and normotensive men (Table 2).

There was no significant association between aOxLDL and determinations of either diastolic or systolic blood pressure or pulse pressure in hypertensives (results not shown).

In hypertensive men, atherosclerosis, as determined by carotid intima-media thickness, was not associated with IgG or IgM subclass aOxLDL levels. If antibody levels were considered ‘positive’ they still were not associated with any of the intima-media thickness measurements.

 Plasma cholesterol, LDL, HDL, triacylglycerols or OxLDL levels were not associated with the antibodies tested (results not shown).

The relationships of OxLDL and antibody levels with age, smoking behaviour, plasma cholesterol, plasma triacylglycerols, glucose and BMI were evaluated further using logistic regression analysis. Since glucose was not significantly different between normotensive and hypertensive men, this parameter was not included in the logistic regression model. As noted, the hypertensive men were significantly older than normotensive men, but this did not influence the difference between OxLDL levels \( (P = 0.003 \text{ whether or not age was included in the model}) \). Likewise, if smoking behaviour, plasma cholesterol, BMI and plasma triacylglycerols were also included in the statistical model in addition to age, OxLDL levels were still highly significantly increased in hypertensive men \( (P = 0.008) \) with \( \beta = 1.8 \) compared with \( -1.5 \) in the univariate analysis. Also, BMI remained significantly higher in the hypertensive group in this model as with age and smoking \( (P < 0.001, P < 0.001 \) and \( P = 0.04 \) respectively).

The finding that the \( \beta \) values from multivariate analyses was similar to the univariate analysis for OxLDL levels indicates that OxLDL was independent of group differences in these parameters.

**DISCUSSION**

The main finding in the present study is that oxidized phosphorylcholine-containing phospholipids, detected by the monoclonal antibody EO6 [30], are present in enhanced levels in the circulation in apoB-containing lipoproteins in hypertensive men compared with normotensive controls.

Although the EO6 epitope may represent only one epitope among many that form when LDL undergoes oxidation, the oxidized phospholipids recognized by EO6 are implicated in atherogenesis, since they stimulate monocyte–endothelial interactions [27]. The epitope recognized by EO6 is an oxidized phospholipid with platelet-activating-factor (PAF)-like properties, since its proinflammatory effects are abolished by a PAF-receptor antagonist [27]. However, it is not known if inhibition of PAF may ameliorate the proatherogenic effects of hypertension or if such treatment could be beneficial in CVD or atherosclerosis in general, although one animal study supports this notion [32].

Our present finding is consistent with previous reports demonstrating the presence of enhanced oxidative stress in hypertension [10], although the underlying mechanisms remain to be fully clarified.

Systemic lupus erythematosus (SLE) is an autoimmune disease associated with a strongly enhanced risk of CVD and, consistent with the findings in the present report, we have demonstrated recently [28] that OxLDL levels, as determined by the same technique, were also raised in SLE patients with CVD compared with age-matched SLE patients without CVD.

Further studies will be necessary to determine where the enhanced content of oxidized phospholipids found on plasma LDL is derived. Although OxLDL in general is believed to be atherogenic, the role of aOxLDL is far from clear in cardiovascular disease. aOxLDL increase with progression of lesion formation and decrease in parallel with decreases in lesion formation in animal models [33–35]. In humans, aOxLDL are associated in many studies with the presence of CVD [18,36], and aOxLDL levels are raised in patients with SLE and cardiovascular disease [28]. On the other hand, we have demonstrated recently [37] that aOxLDL are decreased in borderline hypertension, which presumably represents a state of early human CVD. Consistent with this, a negative association between IgM subclass aOxLDL and atherosclerosis in healthy individuals was reported [37]. Available evidence from animal experiments demonstrate that immunization of experimental animals with OxLDL, leading to enhanced aOxLDL levels, inhibits the development of atherosclerosis [38,39]. Thus, in addition to serving as ‘markers’ of disease activity, it is therefore possible that these antibodies are protective at early stages of disease, but later may serve to be adverse. It is also possible that aOxLDL at very high levels are atherogenic, in parallel with closely related anti-phospholipid antibodies, which at very high levels predispose to arterial disease [40,41].

Little is known about the role of aOxLDL in overt hypertension, although one study [42] indicates that aOxLDL are enhanced in late stage hypertension. In the present study of patients with moderate hypertension, we found that the aOxLDL were present in similar levels in hypertensive men compared with normotensive controls and that they were not associated with atherosclerosis. Hypertension thus differs from borderline hypertension in this respect. We [6] and others [37] have indicated that, at an early stage, a low level of aOxLDL may predispose to an increased risk of cardiovascular disease, including...
both atherosclerosis and hypertension. At a later stage of developing CVD, as in established hypertension, aOxLDL may play a different role with rising aOxLDL levels.

In contrast with the situation in borderline hypertension, serum levels of total IgG and IgA were elevated in men with established hypertension. This is consistent with early reports [43] indicating that established, but not mild, hypertension is characterized by hyper-gammaglobulinemia, although knowledge about what role increased immunoglobulin levels may play in hypertension is surprisingly scarce and this phenomenon clearly deserves further study.

Taken together, our present data indicate that hypertension is characterized by enhanced levels of OxLDL, as determined by monoclonal EO6, which detects biologically active oxidized phospholipid epitopes. Antibodies against epitopes of OxLDL were not elevated, but instead were unchanged or decreased compared with controls. In contrast, in our earlier study [6], OxLDL was not elevated in borderline hypertension and aOxLDL were decreased. These findings suggest that treatment against the raised OxLDL levels either with potent antioxidants or other compounds, such as PAF inhibitors, could be a therapeutic possibility in hypertension that deserves further study.

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REFERENCES

8 Fu, M. L. (1995) Do immune system changes have a role in hypertension? J. Hypertens. 13, 1259–1265

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