Inhibition of the Renin–Angiotensin System Abolishes the Proatherogenic Effect of Uremia in Apolipoprotein E–Deficient Mice

Susanne Bro, Christoph J. Binder, Joseph L. Witztum, Klaus Olgaard, Lars B. Nielsen

Objective—Uremia accelerates atherosclerosis in apolipoprotein E–deficient (apoE−/−) mice. We examined whether this effect may be preventable by pharmacological blockade of the renin–angiotensin system (RAS).

Methods and Results—Uremia was induced in apoE−/− mice by 5/6 nephrectomy (NX). Treatment with the angiotensin converting enzyme inhibitor enalapril (2 or 12 mg/kg/d) from week 4 to 36 after NX reduced the aortic plaque area fraction from 0.23±0.02 (n=20) in untreated mice to 0.11±0.01 (n=21) and 0.08±0.01 (n=23), respectively (P<0.0001); the aortic plaque area fraction was 0.09±0.01 (n=22) in sham-operated controls. Enalapril from week 20 to 44 after NX also retarded the progression of atherosclerosis. Plasma levels of soluble intercellular adhesion molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 (sVCAM-1) and concentrations of IgM antibodies against oxidized low density lipoprotein (OxLDL) increased after NX (P<0.01). Enalapril (12 mg/kg/d) attenuated these increases (P<0.05) and reduced aortic expression of vascular cell adhesion molecule (VCAM)-1 mRNA (P<0.05). Atherosclerosis in NX mice was also reduced by losartan (an angiotensin II receptor-blocker), but not when blood pressure was lowered with hydralazine (a non–RAS-dependent vasodilator).

Conclusion—The results suggest that inhibition of RAS abolishes the proatherogenic effect of uremia independent of its blood pressure-lowering effect, possibly because of antiinflammatory and antioxidative mechanisms. (Arterioscler Thromb Vasc Biol. 2007;27:1080-1086.)

Key Words: renal failure ■ atherosclerosis ■ blood pressure ■ oxidized low density lipoprotein antibodies ■ ICAM-1 ■ VCAM-1 ■ angiotensin converting enzyme inhibitor ■ angiotensin II receptor antagonist

Although renal dysfunction is often accompanied by dyslipidemia, hypertension, and diabetes, the high prevalence of cardiovascular disease in patients with renal disease cannot be explained by the classical risk factors alone.1,2 In addition, patients with renal failure seemingly respond differently than the general population to treatment for cardiovascular disease, eg, there was no effect of statin treatment on cardiovascular disease outcomes in hemodialysis patients with type 2 diabetes,3 but a reduction of cardiovascular disease in a heterogenous population of hemodialysis patients with pre-existing cardiovascular disease treated with vitamin E.4 Uremia confers markedly accelerated formation of atherosclerotic lesions in apolipoprotein E–deficient (apoE−/−) mice5–8 which are qualitatively similar to those in mice with normal kidney function, ie, with intimal accumulation of macrophage-derived foam cells and cholesteryl esters.7 The mechanisms for the proatherogenic effect of uremia are, however, unknown.

Uremic patients show increased plasma concentrations of various markers of oxidative stress and inflammation.9–11 Moreover, atherosclerotic lesions in uremic mice display pronounced accumulation of nitrotyrosine5,6 and increased expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1).7 Oxidative stress leading to formation of oxidized LDL (OxLDL) appears to play a pivotal role in the development of atherosclerosis.12 Hence, OxLDL has important proatherogenic effects in the vasculature including endothelial damage and accelerated foam cell formation.13 The formation of oxidized neoepitopes in LDL can elicit an immune response with formation of antibodies against OxLDL.14 The potential role of these antibodies in the atherogenic process, however, is complex and remains unresolved.14,15 The cause of inflammation and increased oxidative stress in uremia may relate to activation of the renin angiotensin system (RAS) with formation of angiotensin II. Angiotensin II increases the oxidation of LDL by macrophages via a lipoxygenase-dependent pathway.16 Interestingly, treatment with angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor antagonists have previously been reported to be antiinflammatory17 and to inhibit LDL oxida-
Effect of Chronic Uremia and Enalapril Treatment on Body Wt, Blood Hemoglobin, Plasma Cholesterol, and Soluble Adhesion Molecules in ApoE−/− Mice (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Enalapril</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sh</td>
<td>NX</td>
</tr>
<tr>
<td>n</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>Enalapril, mg/kg/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>32.6±0.8</td>
<td>27.1±0.5</td>
</tr>
<tr>
<td>B-hemoglobin, mmol/L</td>
<td>9.5±0.2</td>
<td>7.3±0.3</td>
</tr>
<tr>
<td>P-urea, mmol/L</td>
<td>10.7±0.3</td>
<td>32.5±4.5</td>
</tr>
<tr>
<td>P-creatinine, mmol/L</td>
<td>0.014±0.001</td>
<td>0.034±0.005</td>
</tr>
<tr>
<td>P-calcium, mmol/L</td>
<td>2.52±0.05</td>
<td>2.98±0.08d</td>
</tr>
<tr>
<td>P-phosphate, mmol/L</td>
<td>2.50±0.08</td>
<td>3.18±0.33</td>
</tr>
<tr>
<td>P-Ca×P, mmol/L²</td>
<td>6.31±0.27</td>
<td>9.72±1.26b</td>
</tr>
<tr>
<td>P-cholesterol, mmol/L</td>
<td>14.12±0.66</td>
<td>16.43±0.78a</td>
</tr>
<tr>
<td>B-glucose*, mmol/L</td>
<td>4.7±0.4</td>
<td>5.5±0.3</td>
</tr>
<tr>
<td>P- sICAM-1, ng/mL</td>
<td>374±26</td>
<td>492±18e</td>
</tr>
<tr>
<td>P- sVCAM-1, ng/mL</td>
<td>679±32</td>
<td>896±64a</td>
</tr>
</tbody>
</table>

Values are mean±SEM at 36 weeks after 5/6 nephrectomy (NX) or sham-operation (Sh). Enalapril was administered at the indicated doses from 4 weeks after NX. *Blood glucose was only measured in 12 mice per group. NA indicates not available. Kruskal–Wallis test: P<0.05 for all variables, except for blood glucose (NS); P<0.05, P<0.001, P<0.0001 vs untreated Sh. P<0.05, P<0.001 vs untreated NX. P<0.05, P<0.01, P<0.001 vs NX mice treated with enalapril 2 mg/kg/d.

Induction of uremia
Moderate uremia was induced by surgical 5/6 nephrectomy (NX) in apoE−/− mice (please see supplemental materials, available online at http://atvb.ahajournals.org).

Blood pressure and Atherosclerosis Measurements
Systolic blood pressure (BP) was measured with a tail-cuff system. Atherosclerosis was determined by digital image analysis (please see supplemental materials).

Biochemistry
Plasma biochemistry analyses were performed as described in the supplemental materials. Aortic ICAM-1 and VCAM-1 mRNA were quantified with real-time polymerase chain reaction (PCR) (please see supplemental materials).

Experimental Design

**Experiment 1**
To study the effect of ACE inhibition on the development of atherosclerosis in uremic apoE−/− mice, treatment with the ACE inhibitor enalapril (2 or 12 mg/kg/d) was started 4 weeks after 5/6 nephrectomy (NX) (n=21 and n=23, respectively); control mice received no medication (n=21). Sham-operated mice were allocated to treatment with enalapril 12 mg/kg/d (n=24) or no medication (n=23). Aortas were removed 36 weeks after NX.

**Experiment 2**
To examine the effect of ACE inhibition on progression of established atherosclerosis, NX apoE−/− mice were treated with enalapril (2 mg/d/kg for the initial 2 weeks, 7 mg/kg/d for the following 2 weeks, and 12 mg/kg/d for 20 weeks) (n=7) or no medication (n=6) from 20 weeks after NX. Aortas were removed 44 weeks after NX.

**Experiment 3**
To compare the effect of ACE inhibition, blockade of the angiotensin II receptor, and blood pressure lowering with a non-RAS dependent vasodilator on uremic atherosclerosis in apoE−/− mice, treatment was started 1 week after NX with enalapril (12 mg/kg/d; n=13), losartan (30 mg/kg/d; n=15), hydralazine (55 mg/kg/d; n=7), or no medication (n=10). Sham-operated mice (n=6) received no medication. Aortas were removed 16 weeks after NX or sham-operation.

**Experiment 4**
To examine the effect of ACE inhibition on formation of antibodies against OxLDL in acute uremia, NX apoE−/− mice were treated with enalapril (12 mg/kg/d; n=16) or received no medication (n=14) from the day of NX (ie, removal of the left kidney in the second operation). Plasma for antibody titer determinations and aortas for RNA isolation were collected 2 weeks after NX.

**Experiment 5**
To assess the effect of uremia on titers of antibodies against OxLDL in normocholesterolemic mice, plasma from C57Bl/6J wild-type mice was collected 2 and 12 weeks after NX (n=22) or sham-operation (n=11).

Results

**Effect of Uremia on Plasma Biochemistry, Body Weight, Blood Pressure, and Plasma ACE Activity**
Subtotal 5/6 nephrectomy (NX) rendered both apoE−/− and wild-type mice moderately uremic with 2.2- to 3.0-fold increases of plasma urea concentrations (Experiment 1, Ta-
ble; Experiment 3, supplemental Table I; Experiment 5, supplemental Table II). Accordingly, NX also had significant effects on blood hemoglobin, plasma calcium phosphate product, plasma cholesterol concentration, and body weight, but not on the blood glucose concentration (Experiment 1, Table). As previously seen,7 the plasma concentrations of sICAM-1 and sVCAM-1 were significantly elevated in NX compared with sham-operated mice (Experiment 1, Table).

The mean systolic arterial BP was slightly increased in NX compared with sham-operated mice 2 weeks after surgery (119±1, n=65 versus 113±1 mm Hg, n=47, P<0.01), but was similar in NX (114±2 mm Hg) and sham-operated mice (112±1 mm Hg) at 36 weeks (Experiment 1, Figure 1A). The plasma ACE activity was increased in NX compared with sham-operated mice at 8 weeks (671±16 versus 569±19 U/L, P<0.001) and at 36 weeks (Experiment 1, Figure 1B) after surgery. Plasma ACE activity decreased with age both in NX and sham-operated mice.

Figure 1. Effects of chronic uremia and enalapril treatment on systolic blood pressure and plasma ACE activity (Experiment 1). Blood pressure (A) and plasma ACE activity (B) were measured at 36 weeks after 5/6 nephrectomy (NX) or sham operation in apoE−/− mice. Enalapril administration was started 4 weeks after NX or sham operation. ○, Sham-operated mice treated with 0 or 12 mg/kg/d of enalapril (n=23 and 24, respectively); ●, uremic mice treated with 0, 2 or 12 mg/kg/d of enalapril (n=21, 21 and 23, respectively). Each point represents data from an individual mouse.

Effect of RAS Inhibition on the Development of Atherosclerosis in Uremic Mice

In Experiment 1, the aortic plaque area fraction was 0.23±0.02, n=20 in NX mice versus 0.09±0.01, n=22 in sham-operated mice (P<0.0001), when the mice received no medication and aortas were removed 36 weeks after surgery (Experiment 1, Figure 1). In NX mice, enalapril 2 and 12 mg/kg/d reduced the aortic plaque area fraction to 0.11±0.01, n=21 and 0.08±0.01, n=23, respectively (P<0.0001 compared with NX mice receiving no medication). In sham-operated mice, enalapril 12 mg/kg/d reduced the aortic plaque area fraction to 0.06±0.01, n=24 (P<0.01 compared with sham-operated mice receiving no medication).

In Experiment 2, we assessed whether enalapril inhibited the progression of established lesions by commencing treatment 20 weeks after NX. Enalapril was administered at increasing doses, ie, 2 mg/kg/d for the initial 2 weeks. At the end of the study, BP was lower in the enalapril-treated than in the no medication group (91±4 versus 117±6 mm Hg, P<0.01). There was no difference in plasma urea concentrations between the enalapril-treated and control NX mice (20.5±1.2 versus 20.8±4.3 mmol/L). The enalapril-treated NX mice had a significantly smaller aortic plaque area fraction (0.22±0.03, n=7) compared with NX mice receiving no medication (0.32±0.02, n=6, P<0.01) (Experiment 2, supplemental Figure II).

In Experiment 3, we compared the effect of enalapril, losartan, and non-RAS dependent BP lowering with hydralazine on atherosclerosis in NX apoE−/− mice. In NX apoE−/− mice, enalapril and losartan reduced the aortic plaque area fraction to a similar extent from 0.021±0.003, n=10 in NX mice receiving no medication to 0.009±0.002, n=13 (P<0.01) and 0.014±0.001, n=15 (P<0.05), respectively (Experiment 3, Figure 4). The aortic plaque area fraction was similar to that in mice receiving no medication in hydralazine-treated NX apoE−/− mice (0.029±0.007, n=7).

We measured oxidized phospholipid (EO6) epitopes present and apoB100 (OxLDL-EO6) to assess whether the immune response was associated with increased plasma levels of OxLDL. The plasma OxLDL-EO6 concentrations were ~50% higher in NX compared with sham-operated mice (3092±363, n=10 versus 2088±101 RLUs, n=6, P<0.05; Experiment 3, supplemental Figure I, available online at http://atvb.ahajournals.org).

Effect of Uremia on Formation of Antibodies Against Oxidized LDL

Two weeks after NX, the plasma titers of IgM and IgG antibodies reacting with malondialdehyde modified (MDA)-LDL and Cu2+ oxidized (CuOx)-LDL displayed pronounced increases (Experiment 1, Figure 3). Importantly, sham operations did not affect the titers of IgM antibodies or anti–MDA-LDL IgG antibodies, although a slight increase in the titers of anti–CuOx-LDL IgG antibodies was observed. The total IgM concentration also increased after NX (data not shown). Importantly, however, the ratios between IgM antibodies reacting with MDA-LDL and CuOx-LDL versus total IgM increased 1.7-fold (P<0.05) and 2.4-fold (P<0.01) after NX, respectively, and both were significantly higher in NX versus sham-operated mice.

Thirty-six weeks after surgery, the plasma titers of IgM and IgG antibodies reacting with CuOx-LDL remained significantly higher in NX compared with sham-operated mice (Experiment 1, supplemental Table III). At this time point, the plasma titers of IgM antibodies reacting with MDA-LDL were also increased in NX mice, whereas the titers of IgG antibodies reacting with MDA-LDL did not differ significantly between the 2 groups. Interestingly, in normocholesterolemic wild-type mice (Experiment 5, supplemental Table II), the antibody response to OxLDL on the induction of uremia by NX was similar to that in apoE−/− mice.
The mean systolic arterial BP was reduced to a similar extent in NX mice treated with either enalapril, losartan, or hydralazine (Experiment 3, supplemental Table I). Plasma urea and cholesterol concentrations were higher in enalapril-treated as compared with untreated NX mice \((P < 0.05)\), whereas body weight and plasma urea concentration were lower in hydralazine-treated compared with enalapril-treated NX mice \((P < 0.01)\).

**Effect of ACE Inhibition on Measures of Uremia, Blood Pressure, Plasma ACE Activity and Soluble Adhesion Molecules, and Aortic mRNA Expression of ICAM-1 and VCAM-1**

Enalapril (12 mg/kg/d) did not affect measures of uremia (Experiment 1, Table). However, despite similar concentrations of plasma urea at randomization (data not shown), NX mice treated with 2 mg/kg/d of enalapril from 4 weeks after surgery appeared slightly less uremic than NX mice treated with 12 mg/kg/d and the NX mice that received no medication. Nevertheless, in NX mice, enalapril 2 and 12 mg/kg/d reduced the plasma ACE activity and the systolic arterial BP dose dependently (Experiment 1, Figure 1). Enalapril (12 mg/kg/d) also reduced the plasma ACE activity and the systolic arterial BP in sham-operated mice.

Enalapril (12 mg/kg/d) reduced the plasma concentrations of sICAM-1 and sVCAM-1 in NX mice \((P < 0.01\) and \(P < 0.05\), but not in sham-operated mice at 36 weeks (Experiment 1, Table). Moreover, enalapril (12 mg/kg/d) reduced the aortic mRNA expression of VCAM-1 in NX mice \((P < 0.05)\), when treatment was initiated immediately after NX and aortas were removed 2 weeks later (Experiment 4, Figure 5A).

**Effect of RAS Inhibition on Formation of Antibodies Against Oxidized LDL**

When enalapril-treatment (12 mg/kg/d) was commenced 4 weeks after NX it only led to lower plasma titers of IgG antibodies against CuOx-LDL but did not affect IgG antibod-
ies against MDA-LDL nor IgM antibodies against CuOx- or MDA-LDL (Experiment 1, supplemental Table III). Even when treatment was started 1 week after the induction of uremia (Experiment 3), neither enalapril (12 mg/kg/d), nor losartan (30 mg/kg/d) affected plasma titers of IgM or IgG antibodies against MDA-LDL or CuOx-LDL as measured 15 weeks after initiation of drug treatment (data not shown). Also, RAS inhibition did not affect plasma levels of OxLDL-E06 (supplemental Figure I). We therefore conducted Experiment 4 where enalapril treatment was started immediately after NX, to see whether enalapril would prevent the acute effect of uremia on IgM antibody titers, which was evident already 2 weeks after NX. In that study, enalapril (12 mg/kg/d) reduced the titers of IgM antibodies against CuOx-LDL and MDA-LDL after 2 weeks of treatment (Experiment 4, Figure 5B), but did not affect the titers of IgG antibodies against CuOx-LDL or MDA-LDL (data not shown).

Discussion

As in previous studies, NX conferred markedly increased lesion areas in aortas of apoE\(^{-/-}\) mice. The results strongly support the notion that RAS plays a pivotal role in the accelerated growth of atherosclerotic lesions in NX mice. Firstly, the effect of NX on atherosclerosis was essentially eliminated by 12 mg/kg/d of enalapril when the treatment was started 4 weeks after NX, ie, before or at a very early stage of lesion development. Secondly, when enalapril treatment was initiated 20 weeks after NX and commenced for 24 weeks the mean aortic plaque area fraction was reduced from 32% to 22%. We suspect that lesion formation was extensive when treatment was started at 20 weeks, because we previously found that NX mice had a mean aortic plaque area fraction of 27% after 22 weeks. Thirdly, enalapril and losartan both reduced aortic atherosclerosis in NX apoE\(^{-/-}\) mice. Thus, RAS blockade effectively abolishes the effect of moderate uremia both on lesion initiation and on progressive growth of preestablished atherosclerotic lesions in apoE\(^{-/-}\) mice.

Hypertension is highly prevalent in patients with chronic renal failure, and BP-lowering drugs reduce the risk of vascular events even in nonhypertensive individuals with high-risk disorders, including chronic renal failure. The NX apoE\(^{-/-}\) mice develop extensive atherosclerosis, even though BP is essentially normal: notwithstanding careful training of the mice before measurements, we only detected a small increase of the BP at 2 weeks, but not at 36 weeks after NX. The effect of enalapril on atherosclerosis in NX mice was dose-dependently associated with a lowering of the BP in uremic mice. However, although hydralazine was as effective as enalapril and losartan in lowering the BP, it did not reduce atherosclerosis in NX apoE\(^{-/-}\) mice. Thus, the results suggest that the antiatherosclerotic effect of enalapril and losartan is, at least in part, related to BP-unrelated effects of RAS inhibition. This conclusion is in agreement with the BP-independent reduction of atherosclerosis by losartan in unilaterally nephrectomized apoE\(^{-/-}\) mice. RAS blockade has a number of potentially antiatherogenic effects, eg, reduction of expression of adhesion molecules and chemokines, subintimal macrophage infiltration and macrophage-mediated oxidation of LDL, deactivating of NF-\(\kappa\)B, upregulation of peroxisome proliferator-activated receptors, and increase of vascular NO release. Indeed, enalapril reduced the aortic expression of VCAM-1 mRNA and the plasma concentrations of sVCAM-1 and sICAM-1, suggesting that reduced inflammation in the arterial wall contributes to the antiatherogenic effect of RAS blockade in NX apoE\(^{-/-}\) mice.

Even though chronic uremia attenuates the function of the immune system, some studies of patients with chronic uremia have seen increased plasma titers of antibodies against OxLDL, and the present results strongly suggest that acute uremia, albeit moderate in the NX mouse model, leads to a rapid immune response against OxLDL, as indicated by

![Figure 3. Effects of chronic uremia and enalapril on the development of aortic atherosclerosis in apoE\(^{-/-}\) mice (Experiment 1). Aortic atherosclerosis was measured at 36 weeks after 5/6 nephrectomy (NX) or sham operation in apoE\(^{-/-}\) mice. Enalapril administration was started 4 weeks after NX or sham operation. ○, Sham-operated mice treated with 0 or 12 mg/kg/d of enalapril (n=22 and 24, respectively); ●, uremic mice treated with 0, 2, or 12 mg/kg/d of enalapril (n=20, 21, and 23, respectively). Each point represents data from an individual mouse.

![Figure 4. Effect of treatment with enalapril, losartan, and hydralazine on atherosclerosis in uremic apoE\(^{-/-}\) mice (Experiment 3). Aortic atherosclerosis at 16 weeks after NX or sham-operation (Sh) in apoE\(^{-/-}\) mice. Drugs were administered from 1 week after 5/6 nephrectomy (NX) and continued for 15 weeks. Sh, sham-operated mice receiving no treatment (n=6); NX, uremic mice receiving no treatment (n=10); NX+E, uremic mice treated with enalapril 12 mg/kg/d (n=13); NX+L, uremic mice treated with losartan 30 mg/kg/d (n=15); NX+H, uremic mice treated with hydralazine 55 mg/kg/d (n=7). Values are mean±SEM. NS=not significant.](http://atvb.ahajournals.org/)

Hypertension is highly prevalent in patients with chronic renal failure,21 and BP-lowering drugs reduce the risk of vascular events even in nonhypertensive individuals with high-risk disorders, including chronic renal failure.20,22–24 The NX apoE\(^{-/-}\) mice develop extensive atherosclerosis, even though BP is essentially normal: notwithstanding careful training of the mice before measurements, we only detected a small increase of the BP at 2 weeks, but not at 36 weeks after NX. The effect of enalapril on atherosclerosis in NX mice was dose-dependently associated with a lowering of the BP in uremic mice. However, although hydralazine was as effective as enalapril and losartan in lowering the BP, it did not reduce atherosclerosis in NX apoE\(^{-/-}\) mice. Thus, the results suggest that the antiatherosclerotic effect of enalapril and losartan is, at least in part, related to BP-unrelated effects of RAS inhibition. This conclusion is in agreement with the BP-independent reduction of atherosclerosis by losartan in unilaterally nephrectomized apoE\(^{-/-}\) mice. RAS blockade has a number of potentially antiatherogenic effects, eg, reduction of expression of adhesion molecules and chemokines, subintimal macrophage infiltration and macrophage-mediated oxidation of LDL, deactivating of NF-\(\kappa\)B, upregulation of peroxisome proliferator-activated receptors, and increase of vascular NO release. Indeed, enalapril reduced the aortic expression of VCAM-1 mRNA and the plasma concentrations of sVCAM-1 and sICAM-1, suggesting that reduced inflammation in the arterial wall contributes to the antiatherogenic effect of RAS blockade in NX apoE\(^{-/-}\) mice.

Even though chronic uremia attenuates the function of the immune system, some studies of patients with chronic uremia have seen increased plasma titers of antibodies against OxLDL,30,31 and the present results strongly suggest that acute uremia, albeit moderate in the NX mouse model, leads to a rapid immune response against OxLDL, as indicated by
marked increases of titers of IgM antibodies against OxLDL 2 weeks after NX. Of note, this effect is not dependent on hypercholesterolemia, because we observed similar increases of OxLDL antibodies in normocholesterolemic wild-type NX mice. Moreover, although total IgM levels were increased 2 weeks after NX, the ratio of OxLDL-specific IgM to total IgM was always higher and there was no difference in total IgM levels between sham-operated and NX mice after 16 weeks. Thus, uremia induces an immune response specific for epitopes of OxLDL.

The formation of antibodies against OxLDL 2 weeks after NX likely reflects increased generation of OxLDL. Indeed, uremia is associated with increased production of reactive oxygen species,9,32 and reduced levels of antioxidants,9,32 which promote the generation of OxLDL. Hence, the circulating plasma levels of OxLDL-EO6 were increased in NX mice.

The current data suggest that activation of RAS may contribute to the acute immune response against OxLDL in uremia. Thus, the plasma ACE activity was elevated in the NX mice and the acute rise in titers of IgM antibodies against MDA-LDL and CuOx-LDL was attenuated when enalapril treatment was started immediately after induction of uremia. When enalapril treatment was started 1 week (Experiment 3) or 4 weeks (Experiment 1) after induction of uremia, however, there was no effect of enalapril on the IgM titers. This suggests that the blunting of the IgM response by ACE inhibition in NX apoE−/− mice depends on starting treatment immediately when uremia is introduced.

The plasma OxLDL-EO6 levels in NX apoE−/− mice were not reduced by enalapril. There are, however, multiple epitopes in OxLDL besides EO6. Thus, further studies are needed to resolve how enalapril might affect OxLDL formation in uremia.

Increased oxidative stress is a known proatherogenic stimulus,13,33 but it is not clear whether the induced antibodies themselves have pro- or antiatherogenic properties. In LDL receptor deficient mice, titers of antibodies against OxLDL are positively associated with atherosclerosis.34 Nevertheless, vaccination with OxLDL protects animal models against atherosclerosis.35–37 The findings in the present study imply that the antiatherogenic effect of enalapril is not reflected by an effect on antibody titers when started after uremia has developed. They also suggest that the antibodies against modified LDL themselves are not causing the accelerated lesion formation in NX mice.

In summary, the present study showed that RAS blockade with enalapril or losartan almost completely prevented the otherwise accelerated formation of atherosclerotic lesions in uremic apoE−/− mice. This was, at least partly, independent of BP-lowering and possibly reflects antiinflammatory and antioxidative effects.
Acknowledgments
We thank Kirsten Bang, Karen Rasmussen, Hanne Kjaergaard, Elizabeth Miller, Sonja Kapiotis, Tina Axen, and Kirsten Hansen for technical assistance at various stages of this project.

Sources of Funding
The Danish Medical Research Council, The Danish Heart Foundation, The Copenhagen Hospital Corporation Research Council, The Danish Kidney Foundation, and The Helen and Ejnar Bjornernow Foundation supported this study.

Disclosures
None.

References


Inhibition of the Renin-Angiotensin System Abolishes the Proatherogenic Effect of Uremia in Apolipoprotein E-Deficient Mice

Susanne Bro, Christoph J. Binder, Joseph L. Witztum, Klaus Olgaard and Lars B. Nielsen

Arterioscler Thromb Vasc Biol. 2007;27:1080-1086; originally published online March 8, 2007; doi: 10.1161/ATVBAHA.107.139634

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/5/1080

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/03/09/ATVBAHA.107.139634.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIALS AND METHODS

Animals

Male C57Bl/6J wild-type and apoE-/- mice on a C57BL/6 background (Taconic M&B Laboratory Animals and Services for Research, Ry, Denmark) were kept (five mice per cage) on a 12-hour light/dark cycle in a temperature-controlled room at 21 °C to 23 °C with free access to water and a standard mouse diet containing 22.5 % protein, 5 % fat, 48 % carbohydrates, 0.9 % calcium, 0.7 % phosphorus, and 600 IU/kg of vitamin D₃ (Altromin 1314, Altromin, Lage, Germany). At 8-10 weeks of age, the mice were randomly allocated to 5/6 nephrectomy (NX) or sham operation (Sh). The experiments were performed according to the principles stated in the Danish law on animal experiments, and approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

Surgical procedures

Moderate uremia was induced by a 2-step surgical procedure¹. Briefly, the upper and lower poles of the right kidney were resected. Two weeks later the entire left kidney was removed. Control mice underwent sham-operations at both time points. Anesthesia was achieved with a mixture of fentanyl 0.079 mg/ml, fluanisone 2.5 mg/ml, and midazolam 1.25 mg/ml (Hypnorm/Dormicum) (8-10 µl/g body weight, subcutaneously). Buprenorphine (0.1 µg/g body weight, subcutaneously twice daily for 3 days) was used as analgesia after surgery. At the end of the study, each mouse was anesthetized and the circulation was perfused with 0.9 % NaCl (0 °C) through the left ventricle. The aorta from the heart to the iliac arteries was removed, freed of connective tissue under a dissection microscope, opened longitudinally, and placed between a...
microscope slide and a cover slip. The intimal surface was scanned with an Agfa Snapscan e50 flatbed scanner (Agfa-Gevaert, Glostrup, Denmark). Aortic total area and lesion area were determined by digital image analysis with the Multi-Analyst/PC version 1.1 software from Bio-Rad Laboratories (Hercules, CA, USA)\textsuperscript{1,2}.

**Drugs**

Enalapril maleate used in experiment 1, 2, and 4 was an unrestricted gift from A/S GEA, Hvidovre, Denmark (batch no. 30002911), whereas that used in Experiment 3 was from Sigma Aldrich (Brondby, Denmark) (batch no. 064K1219). Losartan potassium was a gift from Merck & Co., Rahway, NJ (lot no. RLN-201). Hydralazine hydrochloride was from Sigma-Aldrich (batch no. 025K1412). Drugs were administered to the mice in the drinking water; the dosages were calculated assuming a water intake of 100 ml/kg/d and the drug solutions were freshly prepared twice per week.

**Biochemistry**

Blood from the retro-orbital venous plexus was collected in heparinized microtubes (Capiject; Terumo, Medical, Elkton, MD). Whole blood hemoglobin was determined using an OSM3 hemoximeter (Radiometer, Denmark) and blood glucose was determined with a Glucometer Elite (Bayer Diagnostics Manufacturing Ltd., Kyoto Daiichi Kagaku Co. Ltd., Kyoto, Japan). Plasma was separated by centrifugation at \( 2000 \times g \) for 10 min at 4 °C and stored at \(-80^\circ C\). Plasma urea, creatinine, total calcium, phosphate, and total cholesterol were measured with a Modular Automatic analyzer (Roche A/S, Hvidovre, Denmark) using reagents from Roche
Plasma concentrations of soluble (s) ICAM-1 and VCAM-1 were measured with monoclonal antibody-based sandwich ELISA kits (catalog no. MVC00 and MIC100, R&D Systems Europe, Abingdon, Oxon, UK). The intra-assay coefficients of variation were < 5 % for both ELISA assays.

Plasma ACE activity was measured after centrifugation of the plasma samples at 20.000 × g at 4 ºC for 45 min with a kit from Trinity Biotech Sigma Clinical Chemistry, Bray, Ireland (Product No. 305-10). The intra-assay coefficient of variation was < 5 %.

Antibody titers against OxLDL were determined by chemiluminescent enzyme immunoassays, as described. Oxidation of LDL leads to formation of multiple neo-epitopes, e.g., different antibodies can react with malondialdehyde modified LDL (MDA-LDL) and/or Cu²⁺-oxidized LDL (CuOx)-LDL. MDA-LDL and CuOx-LDL were prepared from freshly isolated human LDL. Fifty µl of CuOx-LDL or MDA-LDL at 5 µg/ml in PBS (containing 0.27 mM EDTA) were coated onto white, round bottomed High Binding Microfluor microtitration plates (Dynex Technologies, Chantilly, VA, USA) overnight at 4 ºC. After washing with PBS, antigen-coated wells were blocked with PBS containing 1 % BSA (BSA-PBS) for 30 min. Wells were washed again and 50 µl aliquots of murine plasma at a 1:200 dilution in BSA-PBS were incubated for 60 min at 4 ºC. After further washing, the amount of murine antibodies bound was detected, using specific secondary alkaline phosphatase-labeled antibodies (Sigma) diluted in TBS buffer (containing 150 mM NaCl, 50 mM Tris base, 0.27 mM EDTA, and 2 % BSA). Wells were washed again, and then 25 µl of a 50 % solution of LumiPhos 530 (Lumigen Inc., Southfield, MI, USA) was incubated for 90 min at room temperature in darkness and antibody binding was measured in relative light units (RLUs) in 100 ms using a Luminometer (Dynex Technologies). Antibody titers in plasma of mice treated with enalapril from the day of NX and
of normocholesterolemic mice were measured at a 1:100 dilution and measured using a different Luminometer (Victor2, Perkin Elmer). Plasma levels of oxidized phospholipid (EO6) epitopes present on apoB-100 (OxLDL-EO6) were measured as described\(^6,7\).

Isolation of aortic RNA and real-time PCR quantification with a LightCycler (Roche) of ICAM-1, VCAM-1, and GAPDH mRNA were done as previously described\(^1\). The primers for GAPDH were (mGAPDH 33: 5´-ggtgctgagtatgtcgtgga-3´) and (mGAPDH 53: 5´-gtggttcacacccatcaca-3´). All mRNA quantifications were done twice in separate runs. The inter-assay coefficients of variations for ICAM-1 and VCAM-1 mRNA quantifications were 10.3 % and 14.0 %, respectively.

**Blood pressure**

Systolic blood pressure (BP) was measured with a tail-cuff system (BP 2000; Visitech Systems, Apex, NC) that uses a photoelectric sensor to detect the blood flow in the tail\(^8\). In Experiment 1, the mice were familiarized to the procedure during four consecutive days before BP recordings on the fifth day. In each mouse, at least one set of 10 measurements with nine or more successful readings was obtained. In Experiment 3, the mice were familiarized to the procedure with repeated BP measurements throughout the experiment. Reported BP at the end of this study was the mean of 10 measurements per mouse per day on 5 consecutive days. The accuracy of measurements was secured by regular calibration of the pressure transducer. The average intra-individual variability in the BP measurement was 6.1 %.
Statistical analyses

Group means were compared with Kruskal-Wallis, Mann-Whitney, or Wilcoxon’s tests unless otherwise indicated. Data are presented as mean ± SEM, with \( n \) indicating the number of mice studied.
References


Supplemental Table I. Effects of uremia and treatment with enalapril, losartan, and hydralazine on blood pressure, heart rate, body weight, and plasma indices of uremia in apoE-/- mice (Experiment 3)

<table>
<thead>
<tr>
<th></th>
<th>Sh</th>
<th>NX</th>
<th>NX+E</th>
<th>NX+L</th>
<th>NX+H</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>6</td>
<td>10</td>
<td>13</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td><strong>BP (mmHg)</strong></td>
<td>121±2</td>
<td>117±1</td>
<td>104±2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>104±1</td>
<td>103±3</td>
</tr>
<tr>
<td><strong>Heart rate (bpm)</strong></td>
<td>648±14</td>
<td>696±20</td>
<td>670±15</td>
<td>673±17</td>
<td>653±7</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>31.3±0.7</td>
<td>28.3±1.3</td>
<td>28.4±0.5</td>
<td>27.4±0.7</td>
<td>23.4±0.5&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>P-urea (mmol/L)</strong></td>
<td>10.8±0.3</td>
<td>26.2±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.8±5.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.4±1.8</td>
<td>19.3±2.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>P-creatinine (mmol/L)</strong></td>
<td>0.007±0.001</td>
<td>0.024±0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.031±0.004</td>
<td>0.035±0.002</td>
<td>0.016±0.001&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>P-cholesterol (mmol/L)</strong></td>
<td>10.55±0.77</td>
<td>12.76±0.85</td>
<td>17.76±1.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.09±0.91</td>
<td>16.70±1.07</td>
</tr>
</tbody>
</table>

Values are mean ± SEM at 16 weeks after 5/6 nephrectomy (NX) or sham-operation (Sh). Enalapril 12 mg/kg/d (NX+E), losartan 30 mg/kg/d (NX+L) or hydralazine 55 mg/kg/d (NX+H) were administered from 1 week after NX. Kruskal-Wallis test: P < 0.001 for all variables, except for heart rate (NS). <sup>a</sup>P < 0.001 vs. untreated Sh. <sup>b</sup>P < 0.05 , <sup>c</sup>P < 0.01 vs. untreated NX. <sup>d</sup>P < 0.01 , <sup>e</sup>P < 0.001 vs. enalapril-treated NX.
Supplemental Table II. Effect of uremia on the formation of antibodies against oxidized LDL in normocholesterolemic wild-type mice (Experiment 5)

<table>
<thead>
<tr>
<th></th>
<th>2 weeks</th>
<th></th>
<th>12 weeks</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sh</td>
<td>NX</td>
<td>Sh</td>
<td>NX</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>22</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>P-urea (mmol/L)</td>
<td>NA</td>
<td>NA</td>
<td>10.9±0.7</td>
<td>24.2±1.6a</td>
</tr>
<tr>
<td>CuOx-LDL, IgM</td>
<td>19946±3788</td>
<td>99266±11653a</td>
<td>24716±5418</td>
<td>91538±11326a</td>
</tr>
<tr>
<td>MDA-LDL, IgM</td>
<td>44528±6796</td>
<td>127036±8912a</td>
<td>71621±13744</td>
<td>134881±7661b</td>
</tr>
<tr>
<td>CuOx-LDL, IgG</td>
<td>997±147</td>
<td>1821±230c</td>
<td>1394±333</td>
<td>3989±1069</td>
</tr>
<tr>
<td>MDA-LDL, IgG</td>
<td>5680±1035</td>
<td>12063±1843c</td>
<td>6371±921</td>
<td>18915±4105c</td>
</tr>
</tbody>
</table>

Values are mean ± SEM at 2 and 12 weeks after 5/6 nephrectomy (NX) or sham-operation (Sh) in normocholesterolemic C57Bl/6J mice. Antibody titers are in RLUs. NA: not available. aP < 0.0001, bP < 0.001, cP < 0.05 vs. Sh.
Supplemental Table III. Effects of chronic uremia and enalapril treatment on the formation of antibodies against oxidized LDL in apoE-/- mice (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Enalapril (12 mg/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sh</td>
<td>NX</td>
</tr>
<tr>
<td>n</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>CuOx-LDL, IgM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5983±818</td>
<td>10597±1671a</td>
</tr>
<tr>
<td>MDA-LDL, IgM</td>
<td>14537±1351</td>
<td>20641±1131a</td>
</tr>
<tr>
<td>CuOx-LDL, IgG</td>
<td>786±243</td>
<td>1607±513a</td>
</tr>
<tr>
<td>MDA-LDL, IgG</td>
<td>1363±158</td>
<td>2403±758</td>
</tr>
</tbody>
</table>

Values are mean ± SEM at 36 weeks after 5/6 nephrectomy (NX) or sham-operation (Sh). Antibody titers are in RLUs. Enalapril was administered from 4 weeks after NX. Kruskal-Wallis test: P < 0.05 for all variables, except for plasma MDA-LDL, IgG titers (NS).

aP < 0.01 vs. untreated Sh. bP < 0.05 vs. untreated NX.
Supplemental Figure I

Effect of chronic uremia and treatment with enalapril, losartan, and hydralazine on the level of oxidized phospholipid (EO6) epitopes on circulating apoB-100 in uremic apoE-/- mice (Experiment 3).

Plasma levels of oxidized phospholipid (EO6) epitopes on circulating apoB-100 (OxLDL-EO6) at 16 weeks after 5/6 nephrectomy (NX) or sham-operation (Sh) in apoE-/- mice. Drugs were administered from 1 week after NX and continued for 15 weeks. Sh, sham-operated mice receiving no treatment (n = 6); NX, uremic mice receiving no treatment (n = 10); NX+E, uremic mice treated with enalapril 12 mg/kg/d (n = 13); NX+L, uremic mice treated with losartan 30 mg/kg/d (n = 15); NX+H, uremic mice treated with hydralazine 55 mg/kg/d (n = 7). Values are mean ± SEM. The indicated P-values are from unpaired t-tests with Welch’s correction. NS = not significant.
Supplemental Figure II

Effect of enalapril on plasma ACE activity and progression of aortic atherosclerosis in apoE-/-mice with chronic uremia (Experiment 2).

Plasma ACE activity (A) was measured at 2, 4 and 6 weeks after start of enalapril treatment or no medication. Aortic atherosclerosis (B) was measured at 44 weeks after 5/6 nephrectomy. Each point represents data from an individual mouse. ●, Uremic (NX) mice receiving no enalapril treatment from week 20 to 44 after NX (n = 6); ▲, Uremic mice treated with increasing doses of enalapril (NX+enalapril), i.e., 2 mg/kg/day for the initial 2 weeks, 7 mg/kg/day for the following 2 weeks, and 12 mg/kg/day for 20 weeks from week 20 to week 44 after 5/6 nephrectomy (n = 7).