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VASCULAR BIOLOGY – HEMODYNAMICS – HYPERTENSION

The antioxidant N-acetylcysteine prevents accelerated atherosclerosis in uremic apolipoprotein E knockout mice

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Background. Cardiovascular disease is the most frequent cause of mortality in chronic renal failure (CRF). Therefore, it is important to identify appropriate treatment measures. The antioxidant N-acetylcysteine (NAC) has been shown to reduce cardiovascular events in hemodialysis patients. Here we examine a possible direct effect of NAC supplementation on uremia-enhanced atherosclerosis in apolipoprotein E–deficient ($apoE^{-/-}$) mice.

Methods. Uremia was induced surgically in 8-week-old female $apoE^{-/-}$ mice. Two weeks after creation of CRF mice were randomized to receive either NAC (daily oral gavage with 200 mg/kg for 8 weeks) or placebo. They were compared to a control group of sham-operated $apoE^{-/-}$ mice receiving placebo. After 8 weeks of treatment, the mice were sacrificed, and the cross-section surface area of atherosclerotic plaques was measured in aortic root and descending aorta.

Results. At 10 weeks following surgery, atherosclerotic lesions were significantly larger in uremic $apoE^{-/-}$ mice than in nonuremic controls. This accelerated atherosclerosis was associated with an increase in aortic nitrotyrosine expression and collagen plaque content. NAC treatment inhibited the progression of atherosclerotic lesions and plaque collagen content compared with placebo treatment. In addition, plaques from NAC-treated uremic animals showed a significant decrease in nitrotyrosine expression whereas the degree of macrophage infiltration was comparable in both uremic groups. There was no difference in mean arterial blood pressure between the three groups.

Conclusion. We show for the first time that the antioxidant NAC is capable of reducing atheroma progression, in an animal model of uremia-enhanced atherosclerosis, probably via a decrease in oxidative stress.

Key words: antioxidants, atherosclerosis, chronic renal failure, oxidative stress.

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Chronic renal failure (CRF) patients are at greater risk of developing atherosclerosis than patients with normal kidney function [1]. Consequently, the mortality rate in such patients is substantially higher than in the general population, and deaths are mainly attributable to cardiovascular disease [2, 3]. Advanced age, dyslipidemia, hypertension, glucose intolerance, and cigarette smoking, which are well-known causes in the general population, only partly explain the increased incidence of cardiovascular complications. Uremic patients are exposed to a number of additional factors. Thus, they suffer from a dysregulation of the immune system and exhibit an excessive generation of reactive oxygen species (ROS). Increased oxidative stress induced by ROS is associated with atherosclerosis and cardiovascular morbidity and mortality in general population and in CRF patients [4, 5]. Therefore, antioxidant administration appears to be promising approach. However, a causal relationship between oxidative stress and cardiovascular disease has not yet been firmly established in CRF patients [5]. Published data are limited with respect to the possible success of interventional trials with antioxidants aimed at reducing cardiovascular disease in these patients [6, 7].

Recently, independent results from three research groups provided evidence that nephrectomy-induced uremia accelerated atherosclerosis in apolipoprotein E-deficient ($apoE^{-/-}$) mice [8–10]. This new uremic mouse model provides a useful tool to analyze different cellular and molecular mechanisms underlying atherosclerosis in the settings of CRF and to test possible new therapeutic strategies. The finding of increased aorta nitrotyrosine expression in uremic $apoE^{-/-}$ mice [9, 10] points to enhanced oxidative stress. However, the exact mechanisms underlying the accelerated progression of atherosclerotic lesions in uremic arteries remain to be clarified.

N-acetylcysteine (NAC), a thiol-containing antioxidant, is currently used therapeutically in several disorders related to oxidative stress such as chronic bronchitis and acetaminophen poisoning. It has also been shown to protect renal function in patients with acute

and CRF [11, 12]. NAC exerts direct and indirect antioxidant activity due to its sulfhydryl group [13, 14]. Moreover, NAC releases cysteine after deacetylation, which in turn increases the formation of reduced glutathione sulfhydryl (GSH) within the intracellular pool of antioxidant molecules [15]. GSH in turn can react with peroxynitrite to form *S*-nitrosothiols, which may prevent the accumulation of peroxynitrite toward the range of toxic levels and protect against nitrosative stress [16]. GSH represents one of the most important natural antioxidant defense systems that decrease early in the course of CRF and progresses with its degree of severity [17]. NAC successfully reduces plasma malondialdehyde levels [18] and homocysteine concentration [19], and improves pulse pressure and endothelial function in CRF patients [19]. Finally, NAC has been recently shown to reduce cardiovascular events in chronic hemodialysis patients [7]. However, whether this positive effect was related to a direct action of NAC on atherosclerosis progression is unknown.

The present study was designed to test the hypothesis that chronic attenuation of oxidative stress by NAC has a direct impact on uremia-enchanced atherosclerosis in apoE$^{-/-}$ mice

METHODS

Animals

Experiments were performed in female apoE$^{-/-}$ mice, which were obtained from Charles Rivers Breeding Laboratories (Wilmington, MA, USA) and then bred at the Necker medical faculty (Paris, France). All mice were backcrossed for 10 generations to the C57BL/6J genetic background. The mice were housed in polycarbonate cages in pathogen-free, temperature-controlled (25°C) facility, with a strict 12-hour light-dark cycle, and were given free access to water and standard chow diet (Harlan Teklad Global Diets, Gannat, France). The components of the diet as listed by the manufacturer were 5.7% fat, 18.9% protein, 72.3% carbohydrates, 1.01% calcium, 0.65% phosphorus, 0.2% magnesium, and 1540 IU/kg vitamin D_3. After sexual maturation at 4 weeks of age, the mice were separated by gender and housed in groups up to five.

Creation of uremia

Uremia was induced by a two-step surgical procedure according to Gagnon and Ansari [20], as previously described [8]. Briefly, at age of 8 weeks, the right kidney was exposed and electrocoagulation of the entire surface was performed except for a 2 mm of intact tissue around the hilum. Special care was taken not to manipulate with the ureter and to preserve the suprarenal gland. At 10 weeks of age, left kidney was removed after double ligation of the renal blood vessels and the ureter. Control animals underwent sham operatation, that is, both kidneys were decapsulated. All surgical procedures were done under ketamine/xylazine anesthesia (100 mg/kg and 20 mg/kg) and 5-0 silk sutures were used throughout.

Experimental protocol

Blood samples were taken 2 weeks after nephrectomy and animals from the uremia group with a serum urea level > 20 mmol/L (normal urea level 8 to 10 mmol/L) were randomized to two subgroups: subgroup 1 was treated with NAC (Zambon Group, Milan, Italy), whereas subgroup 2 received placebo. NAC was dissolved in distilled water and administered daily by oral gavage at a dose of 200 mg/kg for 8 weeks. NAC dosage was determined according to previous reports of NAC administration to nonuremic mice [21]. Control animals received distilled water only.

Biochemical analysis

Blood samples were obtained from mice at baseline and then at monthly intervals until the end of the study. Serum urea, total cholesterol, and triglycerides were measured using Hitachi 917 autoanalyzer (Roche, Meylan, France). Plasma urea is a more sensitive marker of uremia than creatinine in mice, as the picric acid analysis (Jaffé method) tends to overestimate creatinine levels due to the presence of interfering substances [22]. Urine was taken prior to sacrifice and urinary protein and creatinine concentration was measured as previously described [23].

Preparation of the aortic sinus

Each mouse was anesthetized and whole blood was collected via cardiac puncture. The aortic tree was perfused with 20 mL phosphate-buffered saline (PBS) via 26 G cannula inserted in the left ventricle, allowing unrestricted reflux from an incision into the right atrium. To further minimize possible adhesion of contaminating nonarterial cells to the arterial wall, adjacent lung tissue was removed from the exterior of the aorta prior to perfusion.

The following procedure of organ dissection and preparation was done as reported previously [8].

Preparation of the descending aorta

The aorta and its main branches were dissected from the left subclavian artery to the iliac bifurcation. The adventitia was removed as much as possible in situ to prevent misinterpretation resulting from Sudan staining of the vessel. The aorta was cut in two (thoracic and abdominal part) using the level of the diaphragm as cutting point. The remaining branches were then cut off and the aorta (from the heart to approximately 5 mm beyond the iliac bifurcation) was removed and fixed with

4% paraformaldehyde for further investigations. After a minimum of 24 hours for initial fixation the aortas were opened longitudinally. The primary incision followed the inner side of the thoracic part and the inner curvature of the arch. To obtain a flat preparation for imaging, a second incision was made along the outer curvature of the arch. The aortas were then stained with Oil red O and a third "cleaning" was performed, by carefully dissecting the remaining adventitial tissues from the outer wall of the aorta. All procedures were made under dissecting microscope.

Quantification of atherosclerotic lesions

Serial 10 μm sections of the aortic sinus with valves (60 to 80 per mouse) were cut in a cryostat starting from the appearance of the first valve (point zero). Of every tenth sections, one was kept for detection of lipid deposition and stained with Oil red O, while remaining sections were used for other staining and immunocytochemistry analyses. The Oil red O stained cryosections (slide 1) were quantified at 2.5 × magnification. The image was captured on a microcomputer equipped with Histolab software (Microvision Instruments, Evry, France) and analyzed by computerized image analysis.

Evaluation of the atherosclerotic plaque area of the entire aorta opened longitudinally was made by the "en face" method [24] using the same image analysis system. The extent of atherosclerosis was expressed as the percent of surface area of the entire aorta covered by lesions. The acquisition of images and analysis of lesions were performed in blinded fashion.

Quantification of monocyte-macrophage (MOMA) infiltration, collagen content, and nitrotyrosine expression in aortic lesions

MOMA infiltration and collagen content were quantified as reported previously [8].

For nitrotyrosine analysis aortic sections were first fixed in room temperature acetone for 10 minutes and placed in bath of wash buffer. The sections were then preincubated in peroxidase blocking solution (Dako Cytomation, Trappes, France) for 5 minutes before incubation for 15 minutes at room temperature with biotinylated nitrotyrosine monoclonal mouse antibody (Cayman Chemical, SpiBio, Massy, France). Antibodies were used in diluents with background reducing components (Dako). After repeat rinsing with Tris buffer, the sections were treated with peroxidase-labeled streptavidin (Dako) for 15 minutes followed by reaction with diaminobenzidine/hydrogen peroxidase as chromogen substrate which results in a brown-colored precipitate at the antigen site. Positive controls were obtained by pretreating the sections with peroxynitrite generated in situ with mixture solution (vol/vol) of sodium nitrite (1 mmol/L) and hydrogen peroxide (1 mmol/L). Negative controls included omission of the primary antibody. At the end, the sections were counterstained with hematoxylin.

Blood pressure measurements

We measured mean arterial blood pressure at the day of sacrifice using direct intra-arterial recording as previously described [8].

Statistical analysis

Data were analyzed by analysis of variance (ANOVA), chi-square, linear regression analyses, and Student unpaired *t* tests, as appropriate. Results were expressed as means ± SEM. Differences between groups were considered significant when $P < 0.05$.

RESULTS

Body weight and serum biochemistry

Body weight was comparable for the three study groups (Table 1). Compared to the nonuremic control group, uremic placebo group showed a significant increase in serum urea, total cholesterol, and triglycerides (Fig. 1). Such an increase was also observed in uremic apoE$^{-/-}$ mice receiving NAC treatment (Fig. 1). No significant difference in serum urea or lipid levels was found between NAC- and placebo-treated uremic mice. Urinary protein/creatinine ratio excretion was comparable between the three groups (Table 1). Similarly, mean arterial blood pressure did not differ (Table 1).

Atheromatous lesions of aorta

Mice of uremic placebo group showed a significant increase in atherosclerotic surface area in the aortic sinus compared with the sham operated control group (Figs. 2A and 3A). Likewise, total aortic plaque area fraction in descending aorta was markedly larger in uremic compared with control mice. Evaluation of specific areas within the aorta indicated that this increase in atherosclerosis was observed in uremic animals in both thoracic (Figs. 2B and 3B) and abdominal aortic regions (data not shown). NAC treatment of uremic mice led to a significant reduction of the lesion surface area in the aortic root, as compared to placebo-treated uremic mice (Figs. 2A and 3A). In line with the aortic root findings, treatment with NAC also resulted in a significant reduction of lesion surface area in both thoracic aorta (Figs. 2B and 3B) and abdominal aorta (data not shown). There were no significant correlation between serum urea or cholesterol levels on the one hand and the atherosclerotic plaque area on the other hand.

Table 1. Characteristics of the mice at the end of the study

	Control ($N = 9$)	Uremic ($N = 10$)	Uremic + N-acetylcysteine ($N = 11$)
Body weight *g*	22.9 ± 0.3	21.8 ± 0.6	22.6 ± 0.3
Mean arterial pressure *mm Hg*[a]	67.5	73.7	68.7
Macrophage infiltration% *of lesions*	15.0 ± 3.4	18.2 ± 2.5	13.1 ± 1.6
Collagen content% *of lesions*	22.4 ± 2.9	36.9 ± 2.1[b,c]	26.7 ± 2.4
Nitrotyrosine expression% *of animals*	12.5	87.5[d]	27.3
Protein/creatinine ratio *g/mmol/L*	0.37 ± 0.04	0.35 ± 0.05	0.37 ± 0.15

Values are means ± SEM.
[a] $N = 4$ for the three groups; [b] $P < 0.01$ vs. control group; [c] $P < 0.05$ vs. uremic + N-acetylcysteine group; [d] $P < 0.02$ vs. control group or uremic + N-acetylcysteine group

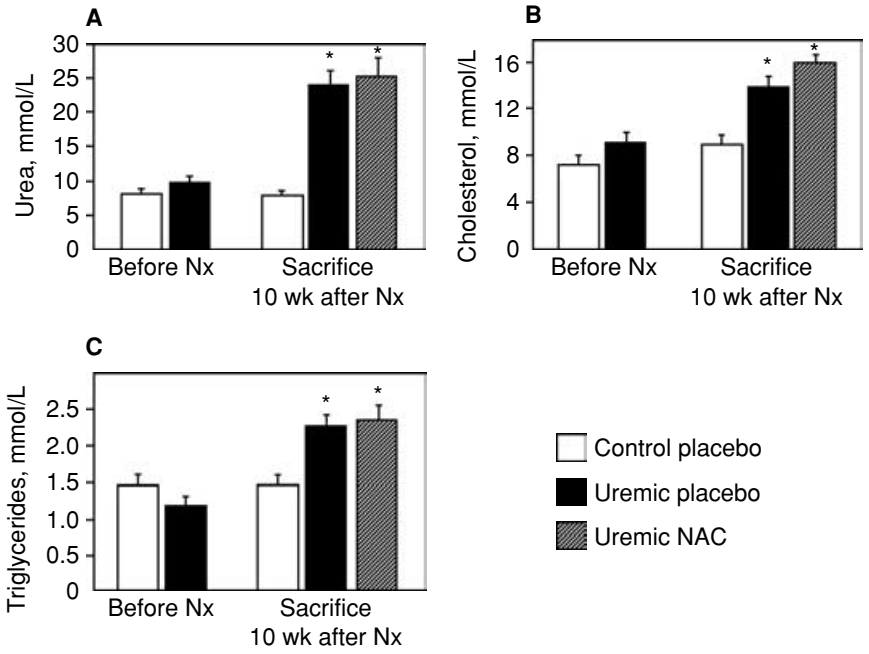

Fig. 1. Serum concentrations of urea (*A*), total cholesterol (*B*), and triglycerides (*C*) on the day of nephrectomy before introducing N-acetylcysteine (NAC) treatment and on the day of sacrifice (10 weeks later). Note that only two groups of mice existed at time of nephrectomy (sham and electrocoagulated mice, respectively), that is, before randomization to the three treatment groups. Number of animals as shown in Table 1 except for tryglycerides ($N = 5$) for the three groups. Nx is left total nephrectomy. $P < 0.01$ analysis of variance (ANOVA) between groups at sacrifice $^*P < 0.01$ Fischer's post hoc least significant difference (PLDS) test between chronic renal failure (CRF) and control mice at sacrifice Values are means ± SEM.

Total collagen content, nitrotyrosine, and MOMA infiltration

To investigate whether NAC had any impact on plaque composition in uremic mice, detailed histologic studies analyzing lesion nitrotyrosine expression, macrophage infiltration, and collagen content were performed. Collagen content was increased in the lesions of uremic apoE$^{-/-}$ mice as compared to control animals (Table 1) (Fig. 4A and B). NAC treatment significantly reduced collagen content to levels that were similar to the ones observed in control animals (Table 1) (Fig. 4C). Furthermore, uremic mice showed an incease in nitrotyrosine expression in the aorta compared to nonuremic control mice (Table 1) (Fig. 4D and E). Such an increase was observed in the atherosclerotic plaques and also in the aortic medial layer in the uremic animals (data not shown). NAC treatment in uremic mice significantly prevented the observed up-regulation (Table 1) (Fig. 4F). The percentage of lesion cross-section area occupied by macrophages, as revealed by MOMA-2 staining, was comparable between the three groups (Table 1).

DISCUSSION

In the present study, we showed that the administration of the antioxidant NAC led to a reduction of atheromatous lesion progression in an animal model of uremia-enhanced atherosclerosis. This reduction was associated with a decrease of plaque collagen content and aortic nitrotyrosine expression, but not with macrophage infiltration.

The induction of uremia in apoE$^{-/-}$ mice actually led to significant plaque progression in all aortic segments studied, and this enhancement could be prevented by NAC treatment. Our finding could serve as a pathogenetic explanation for the recent observation by Tepel et al [7] who showed that chronic dialysis patients given a NAC supplement for $14^1/_2$ months had a better outcome in terms

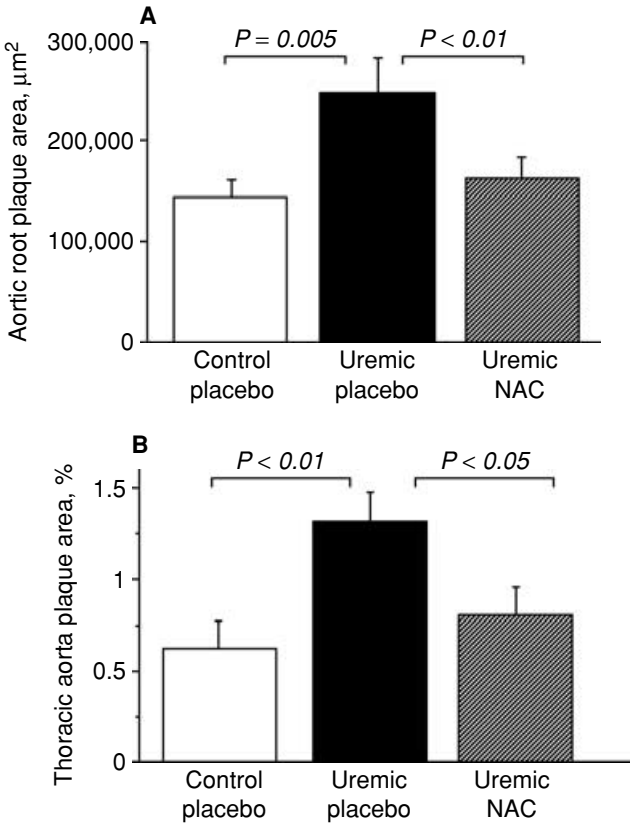

Fig. 2. Effect of 8-week N-acetylcysteine (NAC) treatment vs. placebo on atherosclerotic plaque surface area in aortic root (*A*) and thoracic aorta (*B*) in female uremic apolipoprotein E-deficient (apo $E^{-/-}$) mice. NAC prevented uremia-accelerated atherosclerosis in both aortic regions. Control placebo ($N = 8$), uremic placebo ($N = 7$), and uremic NAC ($N = 11$). Values are means ± SEM

of combined cardiovascular events than placebo-treated dialysis patients.

The beneficial effects of NAC in uremia-enhanced atherosclerosis development might be explained by an interference with oxidative stress. Evidence from reliable oxidative stress markers present in the plasma of CRF patients indicates that CRF is a pro-oxidant state [4, 5]. The results of the present study and previous studies by others [9, 10] in uremic mouse models are in agreement with this hypothesis. Oxidative damage to endothelial cells is postulated to be of prime importance in the development of fatty streaks, the early lesion of atherosclerosis [25]. In the present setting, the NAC effect was associated with decreased nitrotyrosine expression in the aorta of uremic mice. This finding further supports the hypothesis linking oxidative stress and atherosclerosis in CRF, and points to the possible importance of nitrosative-oxidative stress in the accelerated atherosclerosis of CRF apo$E^{-/-}$ mice. Nitrotyrosine is an indirect marker of peroxynitrite generation that results from the reaction between nitric oxide and superoxide. Peroxynitrite further sustains oxidative injury to the endothelium and reduces nitric oxide availability. Nitrotyrosine has been shown to be present in proteins in a variety of clinical conditions, including atherosclerotic lesions in human coronary arteries, postischemic heart, and placenta during preeclampsia [26–28]. NAC, by increasing the formation of GSH [15], can protect against nitrosative stress by favoring the reaction between GSH and peroxynitrite to form *S*-nitrosothiols [16]. Of note, GSH deficiency appears early in the course of CRF and progresses with its degree of severity [17]. Additional effects of NAC include direct inactivation of ROS and HOCl by conjugation or reduction, forming NAC radicals [13]. However, the effects of NAC on chlorinated stress is less likely to explain its atherosclerosis protective effects in mice, since it has been shown that, on the opposite, an increase myeloperoxidase-generated reactive intermediates has a protective role in murine atherosclerosis [29]. However, a possible protective vascular action of NAC on chlorinated stress in CRF patients cannot be excluded, and could certainly explain the better cardiovascular outcome in terms of combined events in the study of Tepel et al [7]. Indeed, in dialysis patients, chlorinated stress appears to prevail since we and others did not observe an increase of plasma nitrotyrosine levels, whereas high concentrations of plasma chlorinated markers were present [30, 31]. These observations are in agreement with possible differences between murine and human atherosclerosis development with regard to the involvement of chlorinated stress.

The total serum cholesterol concentration was higher in CRF mice than in controls. High cholesterol is a well-identified risk factor in the atherosclerotic process and surely contributed to the development of accelerated atherosclerosis in our experimental model. However, in the present study NAC treatment did not affect serum cholesterol. Thus, it appears that mechanisms other than a reduction of serum cholesterol were involved in the observed antiatherosclerotic effect of NAC.

Increased production and deposition of extracelular matrix proteins, including collagens, are a hallmark of fibrosis. Extracellular matrix collagen is the primary component of atherosclerotic plaque structure. In the present study the atheromatous lesions of uremic animals exhibited a marked increase in collagen content although there was no evidence of increased infiltration by inflammatory cells, which is in agreement with our previous report [8]. NAC treatment significantly reduced the collagen content, suggesting that oxidative stress may be involved in uremia-stimulated collagen plaque production. In line with this contention, Liu et al [32] showed that NAC abrogated transforming growth factor (TGF)-stimulated collagen production in fibroblasts. NAC also blocked homocysteine-induced collagen expression in human vascular smooth muscle cells [33]. Similarly, it has been shown that treatment with the antioxidant

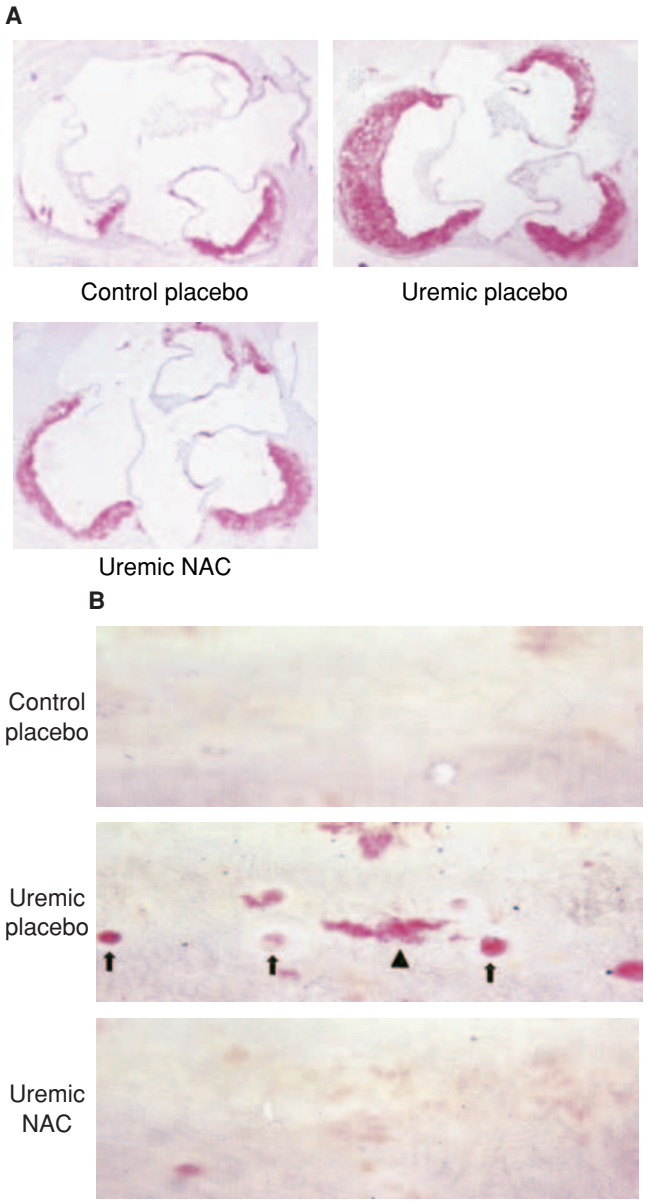

Fig. 3. Representative findings of atherosclerosis progression in aortic root and thoracic aorta in the three study groups. (*A*) Increased Red oil O–positive lipid staining in aortic root of placebo-treated uremic mice, compared with nonuremic control mice and N-acetylcysteine (NAC)-treated uremic animals, respectively. (*B*) Red oil O–positive lipid staining in several different arterial and arteriolar ostia of the descending thoracic aorta (arrows) and more diffuse wall deposits of the aortic wall of placebo-treated uremic mice (arrowhead). No such lipid staining is seen in control placebo and NAC-treated uremic mice, respectively.

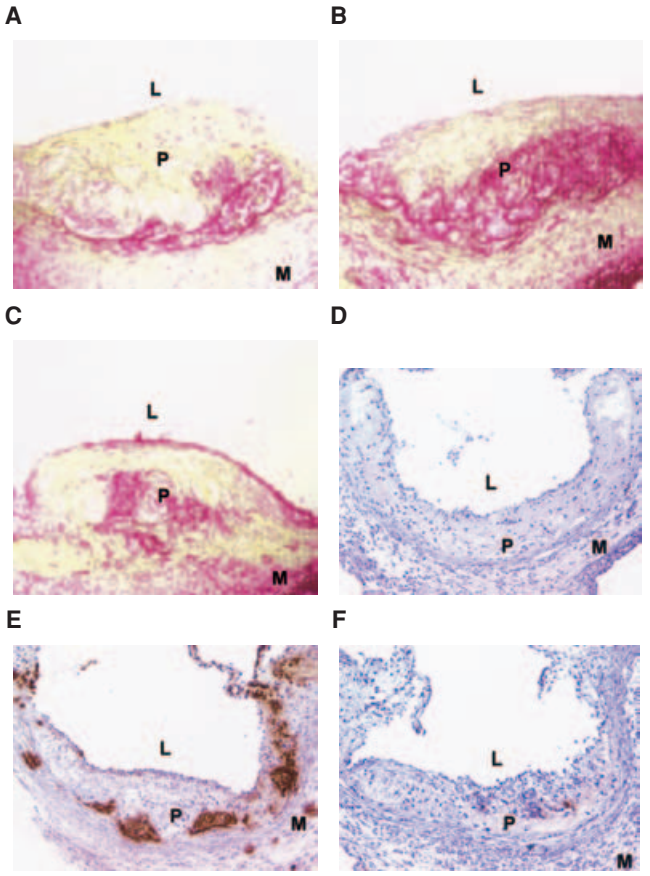

Fig. 4. Representative immunohistologic findings in aortic plaques of control, uremic and N-acetylcysteine (NAC)-treated uremic female apolipoprotein E-deficient (apo $E^{-/-}$) mice. The collagen plaque content (Sirius red staining) in uremic placebo mice (*B*) was significantly increased compared with control nonuremic mice (*A*) and was reduced by NAC (*C*). Nitrotyrosine protein expression (brown). Control nonuremic mice (*D*). Note strong staining for nitrotyrosine in the center of the plaque in uremic placebo mice (*E*). NAC treatment in uremic mice completely prevented the observed up-regulation (*F*) (magnification ×10). Abbreviations are: M, media; P, plaque; L, lumen.

alpha-tocopherol prevented myocardial fibrosis and cardiomyocyte/capillary mismatch in rats with CRF [34].

There are certain limitations to the interpretation of the present study. No animal model mimics human atherosclerosis perfectly. We also have to point out that the dose of NAC used in the present study was far greater than that used in clinical practice. The reason for choosing such a high dose is that mice and man differ notably with respect to the rate at which they produce free radicals. The rate of free radical generation per unit body weight is several-fold higher in small animals, reflecting a higher metabolic rate [35]. In addition, hypercholesterolemia per se promotes oxidation by still unclear mechanisms [36], and the degree of hypercholesterolemia in apo$E^{-/-}$ mice far exceeds that seen in human subjects. Furthermore, it is not known whether NAC would have similar effects on atherosclerosis progression in nonuremic subjects. Several trials showed that antioxidants did not reduce the risk of fatal or nonfatal infarctions in an unselected popoulation with established atherosclerosis [35, 37]. However, it would seem resonable that a population under high oxidative stress such as CRF patients might benefit the most from antioxidant intervention.

There is an increasing body of evidence in favor of an involvement of ROS in accelerated atherosclerosis in

CRF. Two recent reports suggested that antioxidant therapy might be beneficial in reducing cardiovascular events in this patient population [6, 7]. The present study lends further support to the contention that NAC treatment has a direct positive impact on uremia-enchanced atherosclerosis.

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