Carbamylated low-density lipoprotein induces endothelial dysfunction

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Aims	Cardiovascular events remain the leading cause of death in Western world. Atherosclerosis is the most common underlying complication driven by low-density lipoproteins (LDL) disturbing vascular integrity. Carbamylation of lysine residues, occurring primarily in the presence of chronic kidney disease (CKD), may affect functional properties of lipoproteins; however, its effect on endothelial function is unknown.
Methods and results	Low-density lipoprotein from healthy donors was isolated and carbamylated. Vascular reactivity after treatment with native LDL (nLDL) or carbamylated LDL (cLDL) was examined in organ chambers for isometric tension recording using aortic rings of wild-type or lectin-like-oxidized LDL receptor-1 (LOX-1) transgenic mice. Reactive oxygen species (ROS) and nitric oxide (NO) production were determined using electron spin resonance spectroscopy. The effect of LDL-carbamyl-lysine levels on cardiovascular outcomes was determined in patients with CKD during a median follow-up of 4.7 years. Carbamylated LDL impaired endothelium-dependent relaxation to acetylcholine or calcium-ionophore A23187, but not endothelium-independent relaxation to sodium nitroprusside. In contrast, nLDL had no effect. Carbamylated LDL enhanced aortic ROS production by activating NADPH-oxidase. Carbamylated LDL stimulated endothelial NO synthase (eNOS) uncoupling at least partially by promoting S-glutathionylation of eNOS. Carbamylated LDL-induced endothelial dysfunction was enhanced in LOX-1 transgenic mice. In patients with CKD, LDL-carbamyl-lysine levels were significant predictors for cardiovascular events and all-cause mortality.
Conclusions	Carbamylation of LDL induces endothelial dysfunction via LOX-1 activation and increased ROS production leading to eNOS uncoupling. This indicates a novel mechanism in the pathogenesis of atherosclerotic disease which may be pathogenic and prognostic in patients with CKD and high plasma levels of cLDL.
Keywords	Endothelial function • Lipoprotein • Carbamylation • Reactive oxygen species • Nitric oxide

Introduction

Cardiovascular disease is the leading cause of death in industrialized countries. In the USA, cardiac and cerebrovascular disease accounted for almost one-third of all deaths in 2006.[1] Although mortality declined during the last years, the morbidity associated with atherosclerosis and its consequences remains high, particularly in patients with diabetes and/or chronic kidney disease (CKD).[2,3]

Besides other cardiovascular risk factors such as hypertension, obesity, diabetes, and smoking,[4] hypercholesterolaemia is strongly

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associated with the incidence of cardiovascular events.[5] There is a growing body of evidence indicating that lipid-lowering therapy considerably reduces cardiovascular event rate after the occurrence of a first event as well as in primary prevention.[6,7] Despite of such beneficial effects, the incidence of cardiovascular events remains high. Notably, half of all the patients with a myocardial infarction display normal plasma lipid levels,[8] and biomarkers such as C-reactive protein seem to be stronger predictors of cardiovascular events than low-density lipoprotein (LDL)-cholesterol plasma levels.[9] Recent evidence suggests that not only the absolute LDL-cholesterol plasma level, but functional properties of the lipoproteins may play an important role in the pathogenesis of atherosclerosis.[10] It is well established that posttranslational modifications of LDL particles such as oxidation (oxLDL) crucially deteriorate its functional properties leading to a pro-atherogenic phenotype.[11,12] Plasma levels of oxLDL correlate well with the vulnerability of atherosclerotic lesions[13] and are strong predictors for cardiovascular events.[14,15]

Carbamylation of LDL-cholesterol was recently discovered as a novel modification of this lipoprotein in patients with CKD.[16,17] Urea-derived cyanate induces the carbamylation of lysine residues leading to the formation of ε-carbamyl-lysine (homocitrulline).[18] Additionally, myeloperoxidase catalysed lipoprotein carbamylation was identified as an alternative for cyanate-mediated protein carbamylation, which is particularly important in smokers.[19]

Recent data suggest that carbamylated LDL (cLDL) may promote the formation of atherosclerotic lesions.[20] However, the underlying mechanisms, in particular the role of cLDL in endothelial dysfunction as a first critical step in the pathogenesis of atherosclerosis, have not been well understood yet. We therefore conducted the present study to examine the effect of cLDL on endothelial function.

Methods

Detailed description of methods is presented on Supplementary material online.

Detection of reactive oxygen species and nitric oxide using electron spin resonance spectroscopy

Production of ROS in aortic rings, human aortic endothelial cells (HAECs), and whole blood was measured using electron spin resonance (ESR) spectroscopy, as described previously.[21] Details are described in Supplementary material online.

Statistics

All data are expressed as means ± SEM. Statistical comparisons were performed with Student's *t*-test for simple comparisons between two groups. For multiple comparisons, results were analysed by one-way ANOVA followed by Tukey's *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

In the clinical study, patients were stratified into two categories according to the median of carbamylated-lysine in LDL (≤ 28.1 or > 28.1 µg/mg LDL protein). Continuous data are presented as means ± SD when normally distributed or as median and inter-quartile range for variables with skewed distribution. Categorical data are presented as percentage. Statistical differences between continuous variables were determined using the one-way ANOVA or Kruskal−Wallis test or χ^2 test for categorical variables. To examine the effect of LDL-carbamyl-lysine

levels on all-cause mortality and cardiovascular events, Kaplan−Meier curves were built for LDL-carbamyl-lysine levels divided into two categories at median. Differences between both groups were analysed by using the Log-rank test. Moreover, Cox-proportional hazard models were built, including LDL-carbamyl-lysine levels in a crude model as well as adjusted for age, sex, diabetes mellitus, pre-existing coronary artery disease, hs-C reactive protein, and eGFR.

All *P*-values were adjusted for multiple comparisons where appropriate. All statistical calculations were carried out using the PRISM software (GraphPad Prism for MacOSX, 5.0d) or SPSS software (Version 20.0).

Results

Low-density lipoprotein carbamylation

Immediately after *ex vivo* carbamylation, the degree of carbamoyl-DL-lysine was determined. Carbamylation of LDL yielded 27.4 ± 0.9 nmol/mg carbamoyl-DL-lysine, whereas no carbamoyl-DL-lysine was detectable in native LDL preparations (*Figure 1A* and *B*). Neither cLDL nor LDL preparations contained relevant lipid peroxidation (*Figure 1C*).

Carbamylated low-density lipoprotein impairs endothelium-dependent relaxation

To determine the effect of LDL carbamylation on endothelial function, organ chamber experiments using aortic rings from wild-type (WT) C57BL/6J mice were performed. Treatment with cLDL inhibited acetylcholine-induced endothelium-dependent relaxation ($P = 0.002$), whereas nLDL did not affect the response ($P = 0.99$) (*Figure 2A*). Neither cLDL nor nLDL altered vascular contraction of aortic rings in the presence of norepinephrine (*Figure 2B*). Similar to acetylcholine, endothelium-dependent relaxation to the calcium-ionophore A23187 was impaired after cLDL treatment ($P < 0.001$; *Figure 2C*). In contrast, relaxations to sodium nitroprusside remained unaltered by cLDL ($P = 0.92$, *Figure 2D*), indicating that cLDL inhibits endothelium-dependent, but not endothelium-independent relaxations to both receptor-dependent and receptor-independent agonists.

Carbamylated low-density lipoprotein induces endothelial reactive oxygen species production

To gain further insights into the mechanism of cLDL-induced endothelial dysfunction (ED), we tested the vascular reactivity of aortic rings in the presence of indomethacin, an inhibitor of cyclooxygenase. Indomethacin did not alter the inhibitory effect of cLDL on vasodilation ruling out the involvement of vasoactive prostaglandins as a cause of the impaired endothelium-dependent relaxations (*Figure 3A*).

Enhanced production of reactive oxygen species (ROS) is known to reduce the bioavailability of endothelium-derived nitric oxide (NO) leading to ED.[22] Addition of the ROS scavengers PEG-SOD/PEG-catalase restored endothelium-dependent relaxation to acetylcholine in cLDL-treated aortic rings ($P < 0.001$; *Figure 3B*), indicating a pivotal role of ROS in cLDL-induced ED. Accordingly, in aortic rings, cLDL significantly induced aortic ROS production ($P < 0.01$), while nLDL had no effect ($P = 0.93$; *Figure 3C* and *D*).
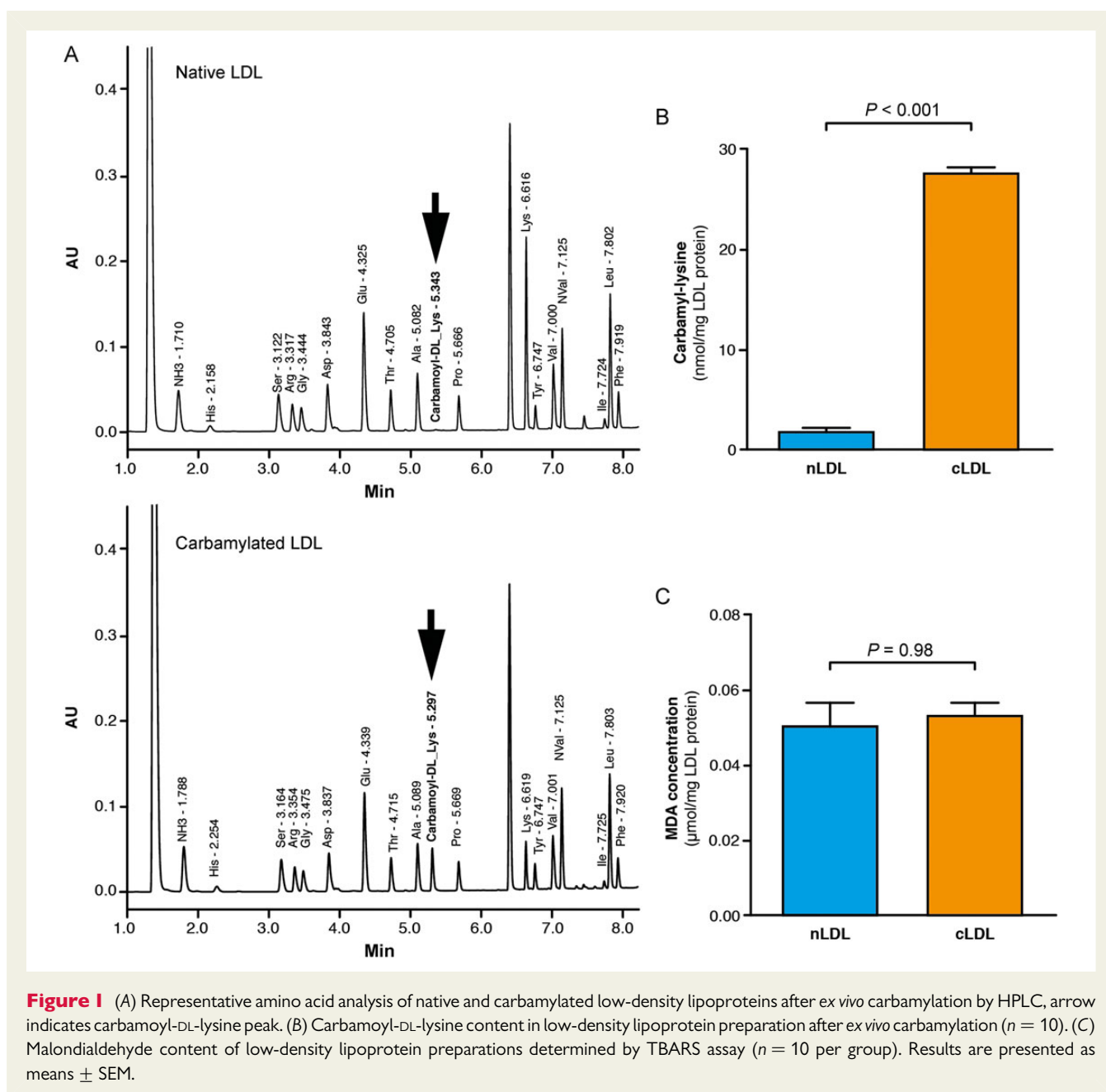
Figure 1 (*A*) Representative amino acid analysis of native and carbamylated low-density lipoproteins after *ex vivo* carbamylation by HPLC, arrow indicates carbamoyl-DL-lysine peak. (*B*) Carbamoyl-DL-lysine content in low-density lipoprotein preparation after *ex vivo* carbamylation (*n* = 10). (*C*) Malondialdehyde content of low-density lipoprotein preparations determined by TBARS assay (*n* = 10 per group). Results are presented as means ± SEM.

Carbamylated low-density lipoprotein induces uncoupling of endothelial nitric oxide synthase

Human aortic endothelial cells were incubated with cLDL to quantify endothelial NO production. Carbamylated LDL, but not nLDL, significantly reduced basal NO production in HAEC (*Figure 4A* and *B*) confirming the functional data of the organ chamber experiments.

Enzymatic activity of endothelial NO synthase (eNOS) is modulated by phosphorylation at different regulatory sites.[23] Carbamylated LDL treatment reduced eNOS phosphorylation at the activating residue Ser1177 and concomitantly enhanced phosphorylation of eNOS at the inhibitory site Thr495 (*Figure 4C–D*). Thus,

inhibition of endothelium-dependent relaxation through cLDL is in part mediated by a reduced activity of eNOS.

Since cLDL stimulated ROS production in aortic rings, we analysed whether the endothelium is the prevailing source for ROS production in response to the modified lipoproteins. Carbamylated LDL induced production of ROS in HAEC, whereas nLDL had no effect (*P* = 0.016, *Figure 4E*).

Besides its role as NO producing enzyme, eNOS can act as a source of ROS when its dimer is uncoupled.[24] Therefore, we quantified eNOS uncoupling using a western blot technique. Carbamylated LDL increased the ratio of monomeric to total eNOS compared with untreated cells 10 min after stimulation, while nLDL did not affect this ratio (*Figure 4F*).
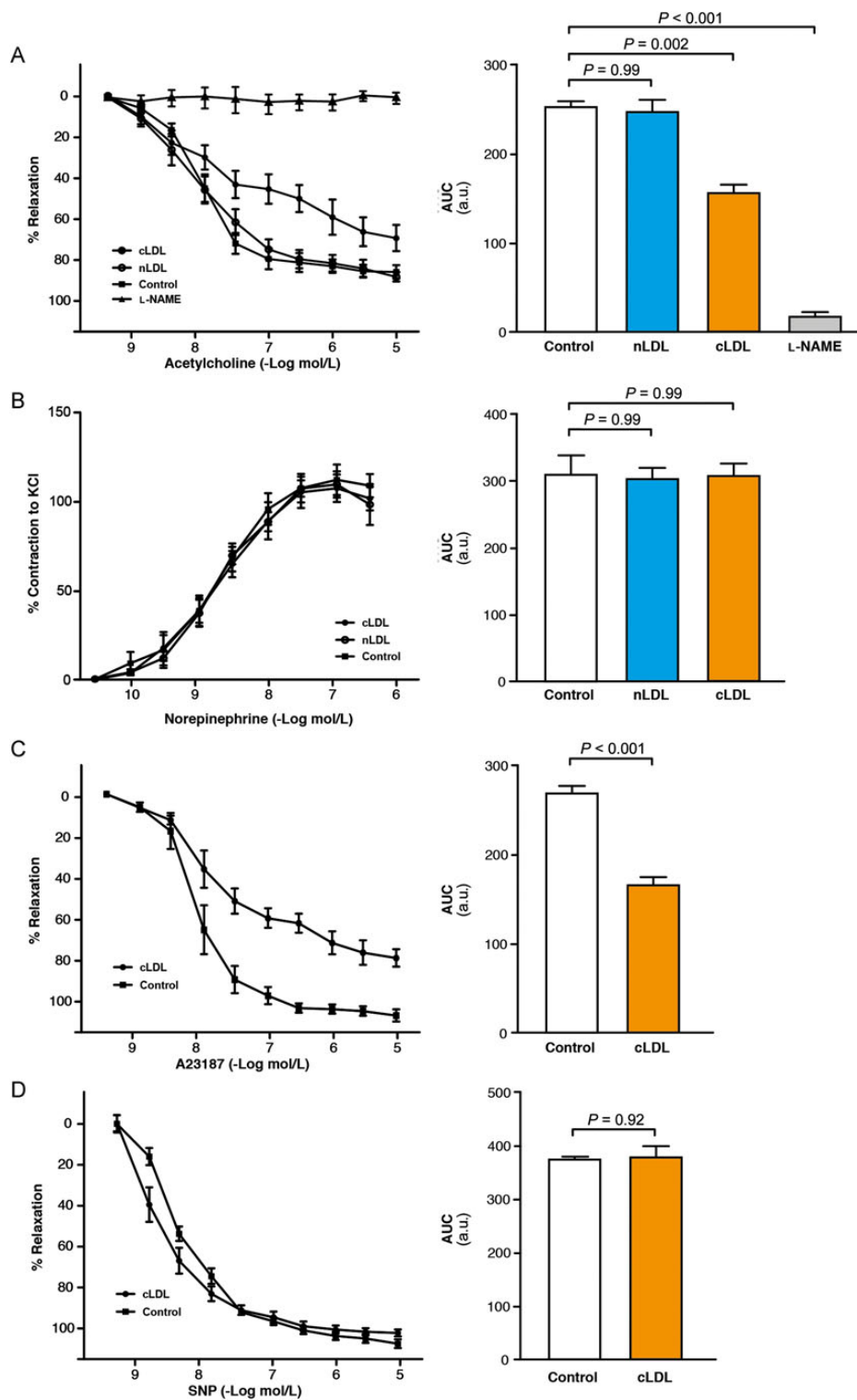
Figure 2 (*A*) Endothelium-dependent relaxations to acetylcholine in aortic rings treated with native low-density lipoprotein or carbamylated low-density lipoprotein (100 μg/mL; *n* = 8 per group) and calculated AUC. (*B*) Contractions to norepinephrin in aortic rings (*n* = 6 per group) and calculated AUC. (*C*) Endothelium-dependent relaxations to A23187 (*n* = 6 per group) and calculated AUC. (*D*) Endothelium-independent relaxations to sodium nitroprusside (*n* = 5 per group) and calculated AUC. Results are presented as means ± SE and *P*-values were adjusted for multiple comparisons where appropriate.
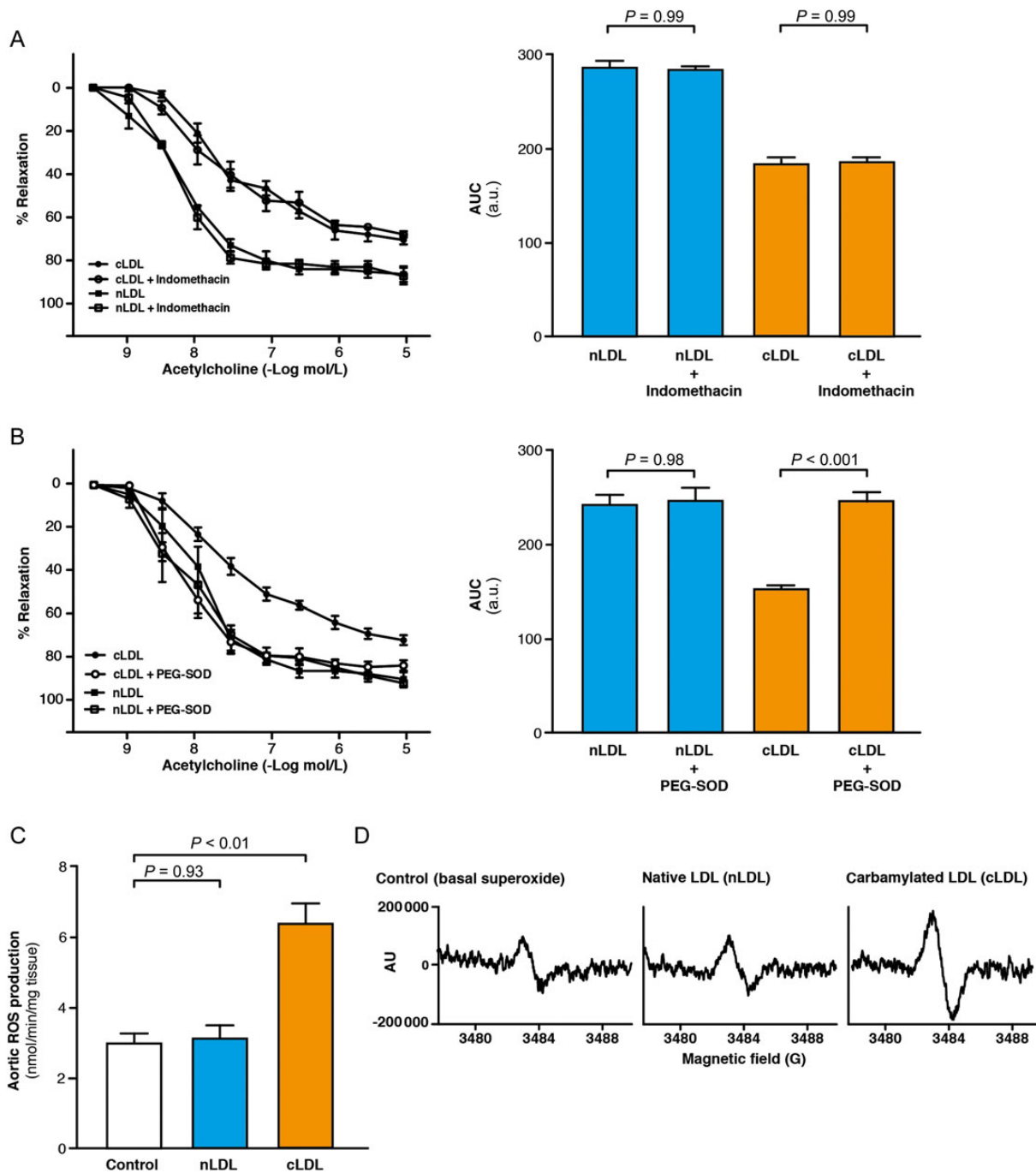
Figure 3 (*A*) Endothelium-dependent relaxations to acetylcholine in aortic rings ± indomethacin (*n* = 5 per group) and calculated AUC. (*B*) Endothelium-dependent relaxations to acetylcholine in aortic rings ± PEG-SOD/PEG-catalase (*n* = 7 per group) and calculated AUC. (*C*) Reactive oxygen species production in aortic rings treated with native low-density lipoprotein or carbamylated low-density lipoprotein (100 µg/mL, 1 h, *n* = 10 per group) determined by electron spin resonance spectroscopy. (*D*) Representative electron spin resonance spectra of reactive oxygen species production in aortic rings. Results are presented as means ± SE and *P*-values were adjusted for multiple comparisons where appropriate.

S-glutathionylation of eNOS may induce eNOS uncoupling.[25] Therefore, we visualized co-localization of *S*-glutathionylation and eNOS in HAEC treated with nLDL, cLDL, and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), an inhibitor of glutathione reductase, by immunofluorescence microcopy (*Figure 4G*).

Carbamylated LDL, but not nLDL, up-regulated endothelial *S*-glutathionylation, which co-localized with eNOS comparable with cells treated with BCNU. Thus, *S*-glutathionylation of eNOS may be at least partially responsible for cLDL-induced eNOS uncoupling.
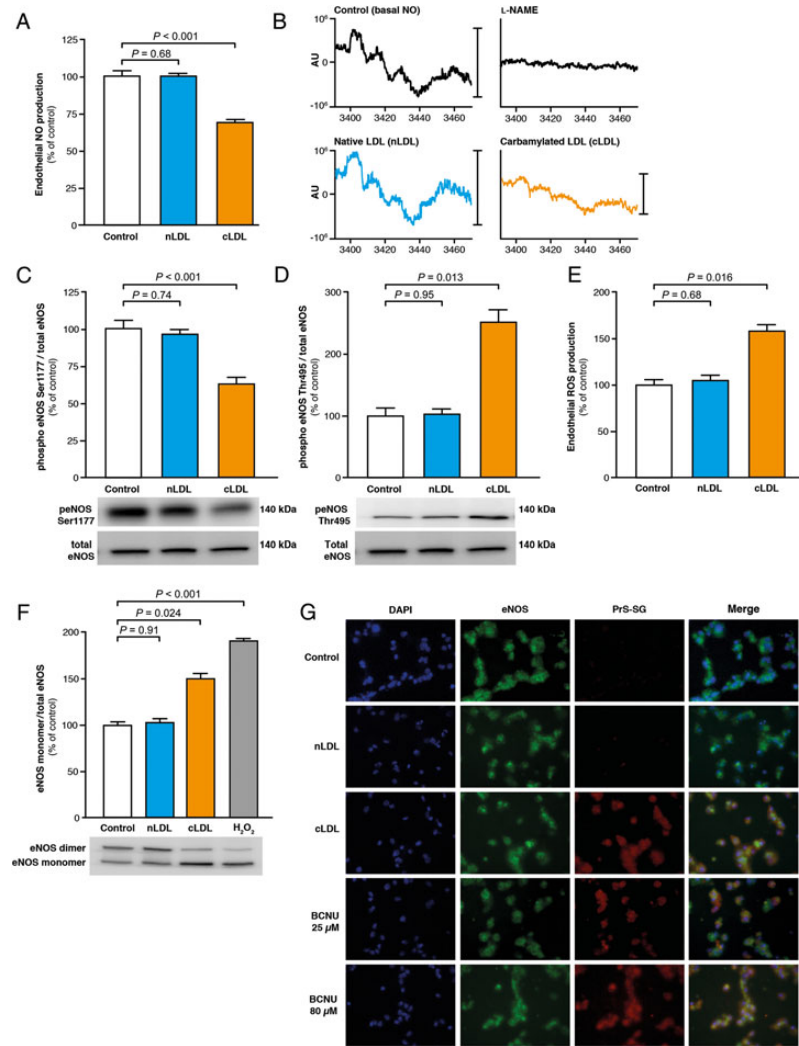
Figure 4 (*A*) Nitric oxide production in human aortic endothelial cells treated with native low-density lipoprotein or carbamylated low-density lipoprotein (100 μg/mL, 1 h, *n* = 4 per group) determined by electron spin resonance spectroscopy. (*B*) Representative electron spin resonance spectra of nitric oxide production in human aortic endothelial cells and human aortic endothelial cells treated with L-NAME (0.3 mM) as a control. (*C*) Endothelial nitric oxide synthase-activating phosphorylation at Ser1177 in human aortic endothelial cells treated with native low-density lipoprotein or carbamylated low-density lipoprotein (100 μg/mL, 10 min, *n* = 5 per group). (*D*) Endothelial nitric oxide synthase -inhibiting phosphorylation at Thr495 in human aortic endothelial cells treated with native low-density lipoprotein or carbamylated low-density lipoprotein (100 μg/mL, 10 min, *n* = 5 per group). (*E*) Reactive oxygen species production in human aortic endothelial cells treated with native low-density lipoprotein or carbamylated low-density lipoprotein (100 μg/mL, 1 h, *n* = 4 per group) determined by electron spin resonance spectroscopy. (*F*) Endothelial nitric oxide synthase uncoupling in human aortic endothelial cells treated with native low-density lipoprotein or carbamylated low-density lipoprotein (100 μg/mL, 10 min, *n* = 6 per group) determined by western blot analysis quantifying endothelial nitric oxide synthase monomer/total endothelial nitric oxide synthase, H_2O_2 (5 mmol/L) served as a control. (*G*) Immunofluorescent staining for endothelial nitric oxide synthase (green) and S-glutathionylation (red). Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). Overlay is performed in human aortic endothelial cells treated with native low-density lipoprotein, carbamylated low-density lipoprotein (100 μg/mL), or 1,3-bis(2-chloroethyl)-1-nitrosourea (25 and 80 μM) as a control for 4 h. Images are representative for three independent experiments. Results are presented as means ± SE and *P*-values were adjusted for multiple comparisons where appropriate.

Lectin-like-oxidized low-density lipoprotein receptor-1 mediates carbamylated low-density lipoprotein-induced ED

Recent evidence suggests that cLDL may interact with the endothelial lectin-like-oxidized LDL receptor-1 (LOX-1).[26] Lectin-like-oxidized LDL receptor-1 is known to mediate adverse endothelial effects of oxLDL and other agents by inducing oxidative stress.[27] To examine the impact of LOX-1 on cLDL-induced ED, transgenic mice over-expressing endothelial LOX-1 were generated (LOX-1/Tg) and organ chamber experiments using aortic rings from these mice were performed. Under control conditions, endothelium-dependent relaxation in response to acetylcholine did not differ between WT and LOX-1/Tg mice. In contrast, the inhibitory effect of cLDL on vasodilation was significantly enhanced in LOX-1/Tg mice when compared with WT control mice (*Figure 5A*).

To confirm LOX-1 as the receptor-mediating cLDL-induced ED, we quantified the effect of cLDL on endothelial NO production in the presence or absence of siRNA targeting LOX-1. Silencing of LOX-1 abrogated the inhibitory effect of cLDL on endothelial NO release ($P < 0.001$), whereas siRNA targeting LOX-1 did not change the effect of nLDL on endothelial NO production ($P = 0.16$; *Figure 5B*).

Accordingly, *in vivo*, 24 h after injection of cLDL or nLDL into WT and LOX-1/Tg mice, cLDL-induced ROS production in whole blood, and in aortic rings was further augmented in LOX-1/Tg mice when compared with WT animals (*Figures 5C* and *D*). These *in vivo* findings confirm our results obtained *in vitro* in HAEC and in *ex vivo*-treated aortic rings.

Lectin-like-oxidized low-density lipoprotein receptor-1 stimulated activation of NADPH-oxidase

We next aimed at determining the molecular mechanisms by which activation of LOX-1 by cLDL increases endothelial ROS production. Since LOX-1 activation has been shown to increase endothelial NADPH-oxidase activity,[28] we measured endothelial ROS production in response to cLDL in the presence of the NADPH-oxidase inhibitor diphenylene iodinium (DPI) or captopril. Indeed, cLDL-induced endothelial ROS production was almost completely prevented in the presence of DPI or captopril, indicating a crucial role of NADPH-oxidase for cLDL-induced ROS production (*Figure 6A*).

Moreover, we studied the role of different mitogen-activated protein kinases (MAPK), which have been documented as downstream factors of LOX-1,[28] in mediating the adverse endothelial effects of cLDL. In these experiments, we found that the inhibitor of p38-MAPK SB202190 significantly attenuated the effect of cLDL on endothelial ROS production (*Figure 6B*). We also observed that cLDL substantially increased phosphorylation of p38-MAPK in HAEC in a time-dependent manner (*Figure 6C*). These results indicate that the effects of cLDL on endothelial ROS production may at least in part be mediated by LOX-1-induced p38-phosphorylation.

It has been reported that several amino acids may attenuate carbamylation of albumin.[29] Therefore, we determined ROS of HAEC treated with LDL, which was carbamylated in the presence of distinct amino acids (*Figure 6D*). Notably, L-arginine, L-cysteine, and L-histidine present during incubation of LDL with potassium cyanate significantly reduced the effect of such prepared cLDL on endothelial ROS production.

Carbamylated low-density lipoprotein in patients with chronic kidney disease

To examine the relevance of these findings in patients, we quantified the number of post-translationally carbamylated-lysine residues in LDL isolated from healthy subjects and patients with CKD by using matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (*Figure 7A*). In *ex vivo* cLDL, which was used for the experiments in the present study, we could detect posttranslational carbamylation of 34 lysine residues. Notably, in CKD patients an average of 54 ± 4 lysine residues of LDL was carbamylated, whereas no carbamylated sites were detected in LDL isolated from healthy control subjects.

Notably, in HAEC treated with isolated LDL from the same patients and healthy controls, we observed a significant reduction of endothelial NO production after incubation with LDL from CKD patients ($P < 0.001$) when compared with LDL from healthy subjects (*Figure 7B*).

Finally, to examine whether carbamylation of LDL may affect CV outcome in patients with CKD, we isolated LDL from 96 patients with proven CKD and determined the concentration of carbamyl-lysine in the LDL fractions. The baseline characteristics of the study cohort are shown in Supplementary material online, *Table S1*. The cohort was divided into two categories at the median of LDL-carbamyl-lysine levels. Patients with LDL-carbamyl-lysine $>28.1 \mu g/mg$ LDL protein had a significantly higher all-cause mortality ($P = 0.002$, *Figure 7C*) as well as a significantly shorter cardiovascular events (CVE)-free survival ($P < 0.001$, *Figure 7D*). Notably, even after adjustments for potential confounders, LDL-carbamyl-lysine remained a powerful predictor for all-cause mortality and CVE in Cox-proportional hazard models (Supplementary material online, *Table S2*).

Discussion

This study demonstrates for the first time that cLDL induces endothelial dysfunction via LOX-1 activation leading to p38-MAPK and NADPH-oxidase activation, eNOS uncoupling, increased endothelial ROS production, reduced NO bioavailability, and finally impaired endothelium-dependent vasodilation. Moreover, in patients with CKD, carbamyl-lysine levels in LDL were associated with increased cardiovascular events and all-cause mortality.

Nitric oxide is a key regulator of endothelial function since the free radical does not only regulate vascular tone, but also prevents endothelial inflammatory and pro-coagulatory pathways.[30] Not surprisingly, endothelial dysfunction represents a crucial initial step in the pathogenesis of atherosclerosis and predicts future cardiovascular events.[31] Endothelial dysfunction is mainly characterized by a dysbalance between the production of NO and that of ROS resulting in a vicious circle, as ROS react with NO to form peroxynitrite leading to a further reduction in NO bioavailability and posttranscriptional modification of cellular proteins.[30,31]
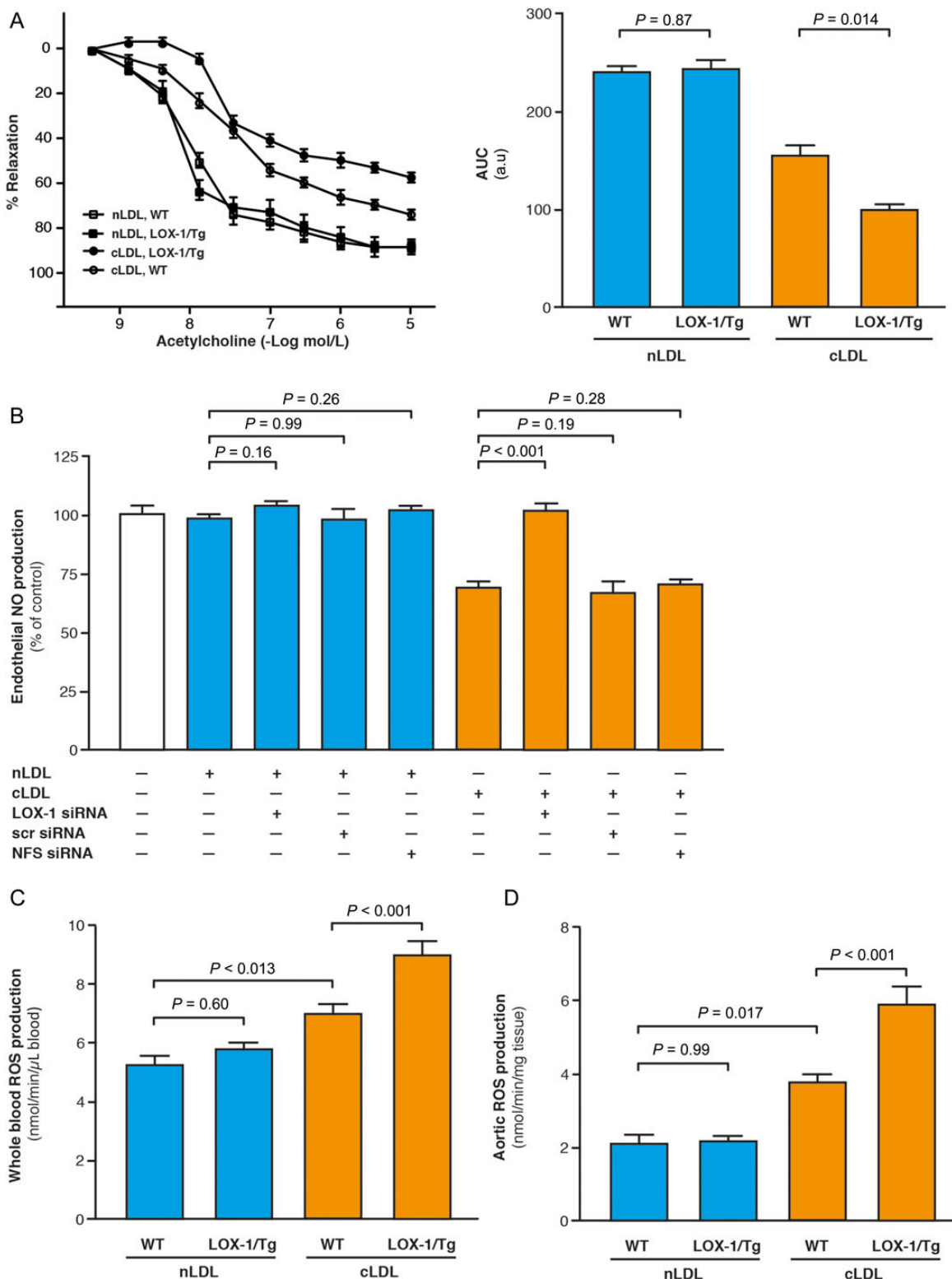
Figure 5 (*A*) Endothelial-dependent relaxations to acetylcholine in aortic rings from wild-type and endothelial lectin-like-oxidized low-density lipoprotein receptor-1/Tg mice (*n* = 6 per group) and calculated AUC. (*B*) Nitric oxide production in human aortic endothelial cells stimulated with native low-density lipoprotein or carbamylated low-density lipoprotein after silencing of lectin-like-oxidized low-density lipoprotein receptor-1 (*n* = 6 per group). NFS, nanoparticle forming solution. Reactive oxygen species production in whole blood (*C*) and aortic rings (*D*) of wild-type and lectin-like-oxidized low-density lipoprotein receptor-1/Tg mice 24 h after injection of native low-density lipoprotein or carbamylated low-density lipoprotein (15 mg/kg, *n* = 4 per group) determined by electron spin resonance spectroscopy. Results are presented as mean ± SE and *P*-values were adjusted for multiple comparisons where appropriate.
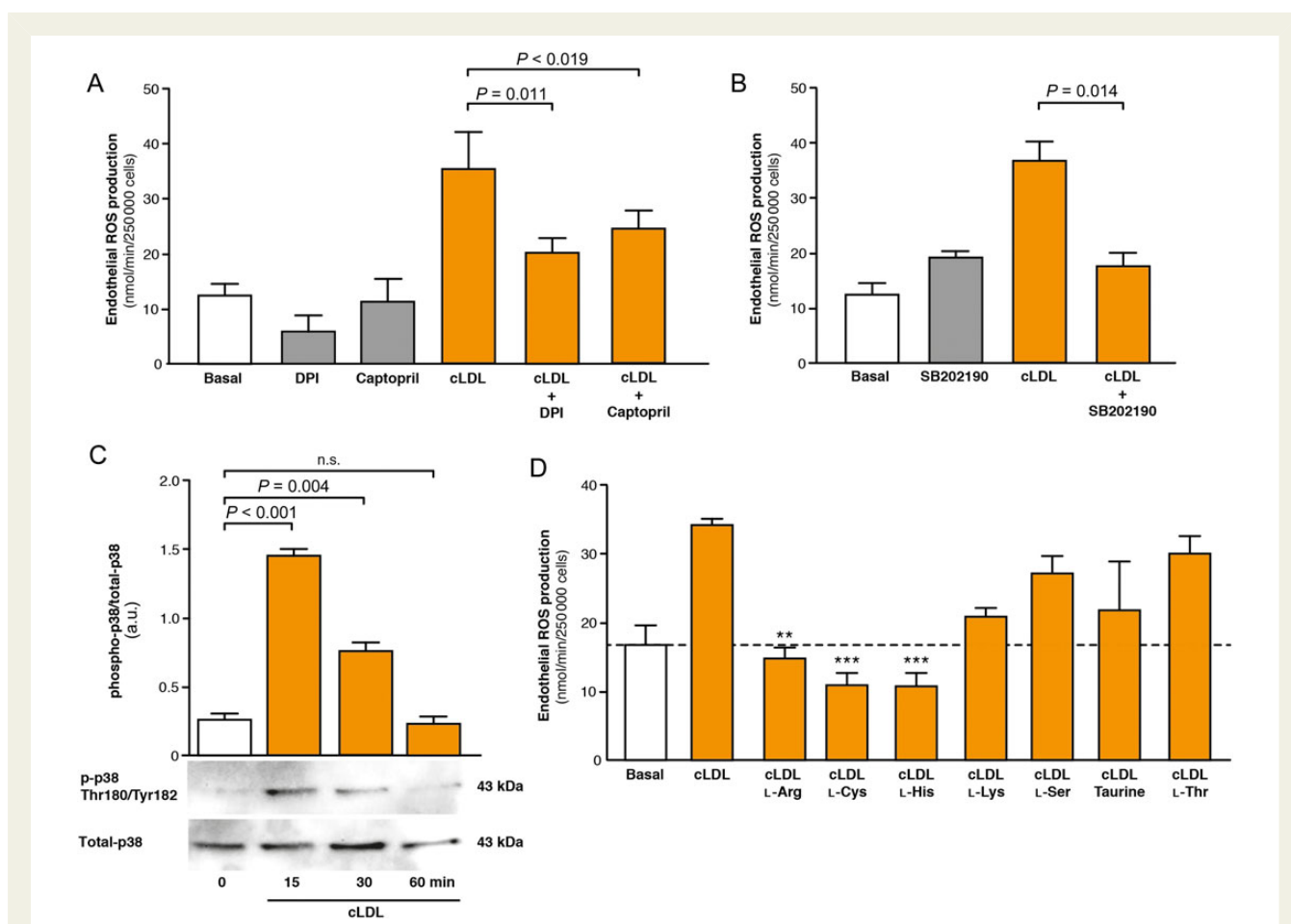
Figure 6 (*A*) Reactive oxygen species production in human aortic endothelial cells treated with carbamylated low-density lipoprotein (100 μg/mL, 1 h) after preincubation with the NADPH-oxidase inhibitor diphenylene iodinium (5 μM, 30 min) or captopril (10 μM; $n = 3–5$ per group). (*B*) Reactive oxygen species production in human aortic endothelial cells treated with carbamylated low-density lipoprotein (100 μg/mL, 1 h) after preincubation with the p38 mitogen-activated protein kinase inhibitor SB202190 (5 μM, 30 min; $n = 3–5$ per group). (*C*) Time course of phosphorylation of p38 mitogen-activated protein kinase at Thr180/Tyr182 in human aortic endothelial cells after treatment with carbamylated low-density lipoprotein (100 μg/mL, $n = 4$). (*D*) Reactive oxygen species production in human aortic endothelial cells treated with carbamylated low-density lipoprotein (100 μg/mL, 1 h) carbamylated in the presence of amino acids (each 10 mM, $n = 4$ per group) as indicated. **$P < 0.01$ and ***$P < 0.001$ for comparison with carbamylated low-density lipoprotein alone. Results are presented as means ± SE and P-values were adjusted for multiple comparisons where appropriate.

Lipoproteins are important regulators of endothelial function. Whereas high-density lipoprotein (HDL) mainly exerts protective effects,[32] LDL promotes endothelial dysfunction and in turn atherosclerosis once it has been modified by ROS induced oxidation.[33] Recently, novel forms of lipoprotein modification have been described. Urea- or myeloperoxide-driven carbamylation of lipoprotein lysine residues indeed impairs the functional integrity of HDL and LDL particles.[16,19] Of note, serum levels of carbamylated proteins and in particular lipoproteins are elevated in patients with coronary artery disease and particularly in those with CKD.[16,17,19]

In the present study, LDL isolated from healthy donors was carbamylated *ex vivo* using potassium cyanate in order to generate well-defined experimental conditions. Notably, the number of carbamylation sites in LDL from CKD patients was even higher when compared with the *ex vivo* cLDL used for the experiments. This

underscores the clinical relevance of the present findings and we assume an enzymatic carbamylation process *in vivo*. The detrimental effects of cLDL observed in this study may well occur in such patients as well as in those with a high myeloperoxidase activity.

In organ chamber experiments using aortic rings from C57Bl/6J mice, we observed that cLDL impaired acetylcholine- and calcium-ionophore induced endothelium-dependent relaxation, while nLDL did not affect vascular reactivity. Thus, the effect is specific for the carbamylated form of the lipoprotein and must involve a mechanism other than the muscarinic receptor and its signal transduction, since the response to the calcium-ionophore was equally affected. Because the relaxation to the NO donor sodium nitroprusside was not affected by cLDL, the latter specifically impairs the function of the endothelium, but not the vasodilating capacity of vascular smooth muscle cells. The effect of cLDL on vascular relaxation was
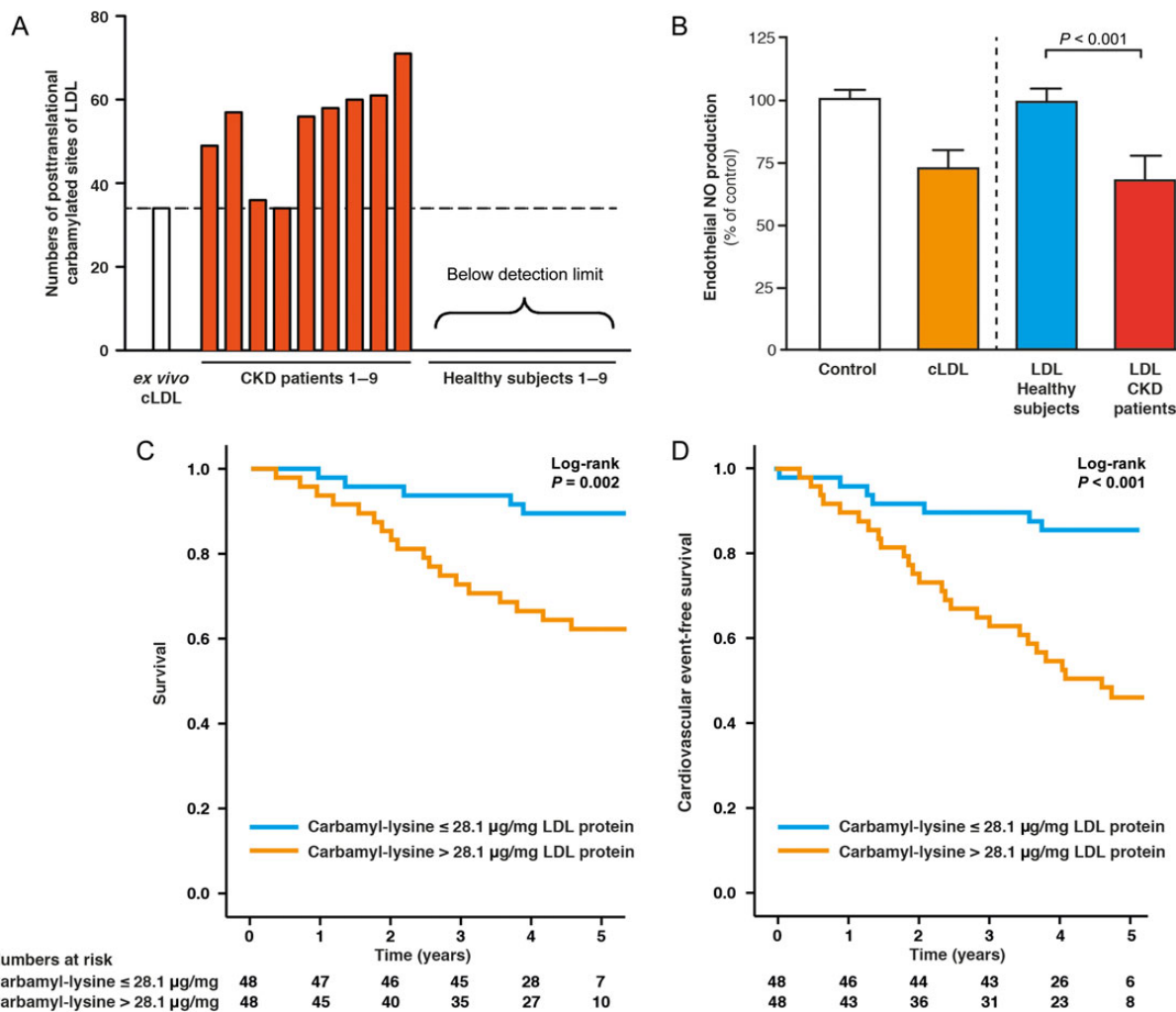
Figure 7 (*A*) Quantification of carbamylated sites in low-density lipoprotein isolated from chronic kidney disease patients and healthy subjects (*n* = 9 per group). The left bar represents the number of carbamylation sites in low-density lipoprotein carbamylated *ex vivo* by incubation with potassium cyanate for 4 h as a positive control. Each bar represents an individual subject. (*B*) Effect of low-density lipoprotein (100 µg/mL, 1 h) isolated from the same patients as in (*A*) on endothelial nitric oxide production in human aortic endothelial cells (*n* = 9 per group). (*C*) Kaplan–Meier curve for all-cause mortality and (*D*) cardiovascular events of patients with chronic kidney disease (*n* = 96) divided into two categories according to median low-density lipoprotein-carbamyl-lysine levels. Results are presented as means ± SE and *P*-values were adjusted for multiple comparisons where appropriate.

comparable with that of oxLDL, indicating that different modifications of LDL can exert a similar effect on vascular function possibly via similar pathways.[12] Moreover, the findings that LDL from healthy subjects did not altered endothelium-dependent vasodilation as well as endothelial NO and ROS production suggests that the effect of LDL on endothelial function is mainly determined by modifications of the LDL particle such as carbamylation or oxidation.[34,35]

A recent study revealed that LOX-1 may be the receptor predominantly mediating cellular effects of cLDL at least in endothelial cells.[26] Thus, the effect of cLDL on endothelium-dependent relaxation to acetylcholine was determined in aortic rings from transgenic mice exhibiting endothelium-specific overexpression of LOX-1. Endothelial dysfunction in response to cLDL was indeed enhanced in LOX-1/Tg, but not in WT mice. In contrast, nLDL did not affect the relaxation

of aortic rings from LOX-1/Tg or WT mice. The involvement of the LOX-1 receptor was confirmed using genetic silencing in HAEC, since down-regulation of LOX-1 by siRNA prevented the cLDL-induced inhibition of NO production in HAEC. Notably, *in vivo* experiments clearly confirmed the role of LOX-1 in mediating the effect of cLDL on ED. This is of relevance since LOX-1 is crucially involved in atherosclerosis and highly expressed in human atherosclerotic lesions.[36] Furthermore, activation of LOX-1 with oxLDL has been documented to enhance endothelial oxidative stress and inflammatory activation.[27] Therefore, the detrimental effects of cLDL may not only be important for endothelial dysfunction, but also for the development of atherosclerotic lesions.

The specific activation pattern of the endothelial LOX-1 receptor by cLDL together with the absence of any effect of the cLDL on
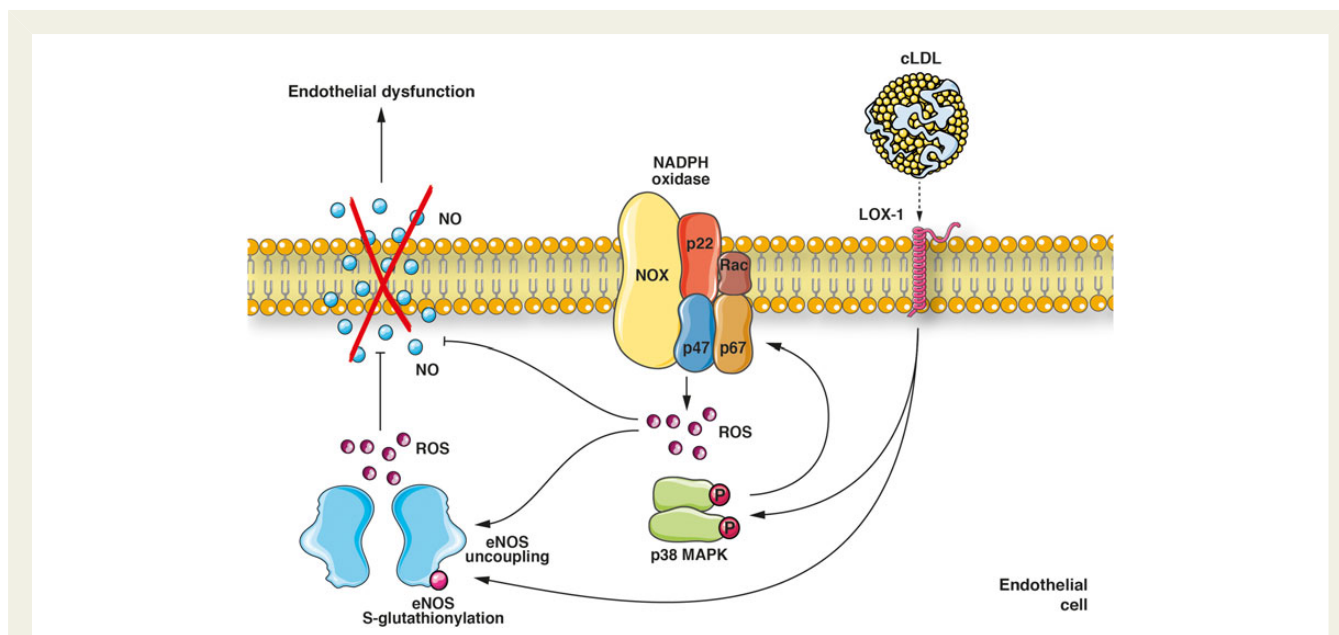
Figure 8 Schematic representation of molecular mechanisms of carbamylated low-density lipoprotein-induced endothelial dysfunction.

vascular smooth muscle cells suggest that either NO production or NO bioavailability or, alternatively, vasoconstrictor prostaglandins must be involved in cLDL-induced endothelial dysfunction. However, unselective inhibition of cyclooxygenase using indomethacin did not alter endothelium-dependent relaxations to acetylcholine in response to cLDL.

Contrarily, scavenging of ROS using PEG-SOD /PEG-catalase prevented cLDL-induced endothelial dysfunction. Therefore, cLDL must stimulate the endothelial production of ROS. Consistent with this interpretation, cLDL induced ROS production both in aortic rings and in cultured HAEC. These findings are in line with a recent study demonstrating that cLDL induces oxidative stress in endothelial progenitor cells.[37]

Reactive oxygen species production in endothelial cells may originate from NADPH oxidases, mitochondria, or eNOS itself if it is uncoupled. The data generated with the NADPH-oxidase inhibitor DPI indicate that activation of NADPH-oxidase occurring downstream of the MAPK p38 may contribute to ROS formation in response to cLDL.

Several agents are known to induce eNOS uncoupling.[22,24] In marked contrast to dimeric eNOS which oxidizes L-arginine resulting in the formation of L-citrulline and NO, uncoupled eNOS loses its ability to produce NO and instead generates ROS. We observed that cLDL leads to eNOS uncoupling as indicated by a higher ratio of monomeric to total eNOS. Recently, S-glutathionylation of eNOS has been shown to induce eNOS uncoupling.[25] Here, we found that cLDL up-regulated eNOS S-glutathionylation, which can at least partially explain the molecular mechanisms leading to eNOS uncoupling in response to cLDL.

Several amino acids such as cysteine, histidine, and arginine are known to inhibit carbamylation of albumin.[29] Here, we observed that cysteine, histidine, and arginine present during *ex vivo* carbamylation of LDL prevented cLDL-induced endothelial ROS production.

This indicates that dietary supplementation of these amino acids may provide a potential treatment strategy to preclude cLDL-induced endothelial dysfunction in patients with increased protein carbamylation such as CKD.

Finally, in the clinical part of the study, we documented a significant association between LDL-carbamyl-lysine levels and all-cause mortality and cardiovascular events in patients with CKD. These findings point to an important role of cLDL in the development of cardiovascular disease in CKD patients and highlight cLDL as a novel cardiovascular risk factor in these patients.

In summary, our data indicate that cLDL induces ED via activation of the endothelial LOX-1 receptor leading to p38-dependent NADPH-oxidase activation, ROS production, S-glutathionylation-dependent eNOS uncoupling, and reduced NO bioavailability. In addition, cLDL inhibits eNOS activation in a direct manner by affecting eNOS phosphorylation (*Figure 8*). These findings represent a novel pathomechanism of endothelial dysfunction potentially important for initiation of atherosclerotic vascular disease in patients with CKD and in smokers, notably patient groups with a very high-cardiovascular risk.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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