

# Endothelin-converting enzyme-1 increases in atherosclerotic mice: potential role of oxidized low density lipoproteins

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**Abstract** The aim of our study was to analyze the relationships between atherosclerosis and endothelin-converting enzyme-1 (ECE-1). Four-week-old C57BL/6J [wild-type (WT)] and apolipoprotein E-deficient (apoE) mice were fed with a standard or Western-type fat diet for 8 wks. ApoE showed atherosclerotic lesions in the aorta, higher blood pressure and vascular lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) protein content than WT. ApoE showed a significant increase in ECE-1 protein content and mRNA expression in aorta, lung, and kidney, without changes in heart. When an ECE-1 inhibitor, FR-901533, was administered to them, blood pressure decreased in apoE on fat diet versus apoE on normal diet and WT. ECE-1 and LOX-1 protein content were elevated in peripheral blood mononuclear cells (PBMC) from hypercholesterolemic patients. In order to study the mechanism involved in this ECE-1 up-regulation, bovine aortic endothelial cells (BAEC) were treated with oxidized-low density lipoproteins (oxLDL). OxLDL, but not LDL, increased ECE-1 protein content, mRNA expression and promoter activity. Our results demonstrate that ECE-1 increases in different atherosclerosis situations. **■** Up-regulation of ECE-1 could contribute, at least partially, to the development of hypertension seen in apoE mice, because FR-901533 avoided it. Probably, atherosclerotic situations course with an increase of oxLDL, which is able to induce ECE-1 expression with the subsequent potential pathological effects.—Martínez-Miguel, P., V. Raoch, C. Zaragoza, J. M. Valdivielso, M. Rodríguez-Puyol, D. Rodríguez-Puyol, and S. López-Ongil. **Endothelin-converting enzyme-1 increases in atherosclerotic mice: potential role of oxidized low density lipoproteins.** *J. Lipid Res.* 2009. 50: 364–375.

**Supplementary key words** hypercholesterolemia • endothelial function • hypertension

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Atherosclerosis is a slowly evolutive age-linked disease of large arteries, characterized by local lipid deposition associated with chronic inflammatory response, leading potentially to acute plaque rupture, thrombosis, and ischemic diseases (1, 2). Atherogenesis includes a complex sequence of events, such as over expression of adhesion molecules, recruitment of mononuclear cells to the endothelium, local activation of leukocytes and inflammation, lipid accumulation, and foam cell formation (2–4).

The mechanisms involved in atherogenesis have been extensively studied but incompletely defined. Oxidized low density lipoproteins (oxLDL), generated by the local oxidation of LDL (5), seem to play a role in the development of atherosclerosis, at least in part through the interaction with lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) (6), the activation of the transcription factor NF- $\kappa$ B (7), and the subsequent up-regulation of proinflammatory gene expression (8). Endothelial dysfunction seems to be also an early event in atherosclerosis, and it predisposes to the development of the structural vascular changes (9–11).

Different mediators have been proposed to be involved in the development of the atherosclerosis-linked endothelial dysfunction. Endothelin (ET) has been one of these mediators (12). In fact, increased circulating levels of ET-1 have been detected in patients with hypercholesterolemia (13, 14). Moreover, in apoE mice, an experimental model of hypercholesterolemia and atherosclerosis,

Abbreviations: apoE, apolipoprotein E-deficient; BAEC, bovine aortic endothelial cells; ET, endothelin; ECE-1, endothelin-converting enzyme-1; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; oxLDL, oxidized low density lipoproteins; PBMC, peripheral blood mononuclear cells; TTBS, Tween Tris buffered saline; WT, wild-type.

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chronic ET-A receptor blockade normalized endothelial dysfunction and reduced atheroma formation (15, 16).

This increased synthesis of ET-1 may be related to the overexpression of prepro-ET-1 mRNA, as increased steady-state levels of this messenger have been demonstrated in the aorta from hypercholesterolemic mice (15, 16). However, changes in prepro-ET-1 intracellular levels are not the sole mechanism involved in the regulation of vascular ET-1 synthesis. Endothelin-converting enzyme (ECE-1), the enzyme that regulates the conversion from big ET-1 to ET-1, seems to be increased in atherosclerotic plaques (17), and its activity may be enhanced in patients with atherosclerosis (18). However, other studies propose that ECE-1 content and activity are decreased in hypercholesterolemic patients (19).

LDLs have been suggested to play a role in the modulation of the ET-1 synthesis. The oxidized form of these proteins increased the prepro-ET-1 mRNA expression in different cell types, with the subsequent increased ET-1 synthesis (20, 21). Moreover, native and oxLDL seem to stimulate ECE-1 expression in cultured endothelial cells (22), although indirect evidence from human studies suggests an inverse relationship between LDL levels and ECE-1 vascular activity (19).

Considering this information, we planned to analyze the possible role of ECE-1 in the pathogenesis of atherosclerosis, as well as the relationship between oxLDL and ECE-1, in order to clarify the previously published scarce and contradictory information. By a combined *in vivo* and *in vitro* approach, we tried to obtain consistent data and perform an analysis of the mechanisms responsible for the observed changes.

## MATERIALS AND METHODS

### Materials

Culture plates, SuperSignal detection system and secondary horseradish peroxidase-conjugated goat anti-mouse IgG were from CultiK (Pierce, Rockford, IL). Paragon electrophoresis system was from Beckman Coulter Inc. (Fullerton, CA). Dual Luciferase Reporter Assay System, pGL3 vector, pRL-SV40 vector and T4 polynucleotide kinase were from Innogenetics (Walkersville, MD). Lipofectamine reagent and OptiMEM I media were from GIBCO-Invitrogen (Barcelona, Spain). Acrylamide-bisacrylamide was from Hispanlab-Pronadisa (Madrid, Spain). MXB films were from Kodak (Rochester, NY). Protein markers, BioRad protein assay kit, plates and electrophoresis equipment were from Bio-Rad Laboratories (Richmond, CA, USA). Protease inhibitor cocktail tablets were from Roche Diagnostics (Madrid, Spain). The ET-1 ELISA system,  $\alpha$ - $^{32}$ P]dCTP and  $\gamma$ - $^{32}$ P]ATP were from GE Healthcare Bio-Sciences (Buckinghamshire, UK). Advantage Genomic PCR Kit was from Clontech Lab (Palo Alto, CA). Unless otherwise indicated, the rest of the drugs, culture media, antibodies, and reagents were from Sigma-Aldrich-Fluka Chemical Co. (St. Louis, MO).

### In vivo studies

Male homozygous apoE and C57BL/6J control [wild-type (WT)] mice from 4 wks old were obtained from The Jackson

Laboratory (Charles River España, Barcelona, Spain). WT and apoE were fed with a normal or Western type diet (TD88137, Harlan Teklad) to induce atherosclerosis for 8 wks. Animals had free access to water, were maintained at 24°C, and kept at a 12 h light/dark cycle. One week before the sacrifice, arterial blood pressure was measured in conscious animals by means of a tail-cuff sphygmomanometer (LE 5001 Pressure Meter, Letica Scientific Instruments, Hospitalet, Spain). Animals were trained for 3 days before starting the measurement to prevent stress and were prewarmed at 30°C with a heater (LE5660/6, Letica Scientific Instruments). Blood pressure was recorded in 2 consecutive days, with at least 20 determinations by day. In a subgroup of animals, blood pressure was also recorded at 7 wks, before and after the intraperitoneal administration of 1 mg/kg weight of FR-901533, a rather selective ECE antagonist (kindly provided by Dr. Yuriyo Yamamoto, Fujisawa Pharmaceutical Co.). After the 8 wks, animals were anesthetized with pentobarbital (50 mg/kg *i.p.*), and a blood sample was collected through puncture of the right ventricle. Plasma was separated (3,500 rpm, 10 min) and stored until biochemical determination (Hitachi 917). Plasma lipoproteins (LDL and HDL cholesterol), total cholesterol and triglycerides were determined using a colorimetric-based assay on a Cobas Mira Plus autoanalyzer (Roche Diagnostics, Basel, Switzerland) as described (15). Aorta, lungs, kidneys, and heart were removed via a thoracic-abdominal incision and stored until analysis. Aorta, lung, kidney, and heart portions were collected in 4% paraformaldehyde for histological studies. Because of the scarce tissue it was impossible to assay ECE-1 mRNA expression in aortas. The investigation was conducted in conformity with the Public Health Service policy on the Humane Care and Use of Laboratory Animals incorporated in the Institute for Laboratory Animal Research (ILAR), Guide for the Care and Use of Laboratory Animals published by the US National Academies. All the studies were approved by our Institutional Committee of Alcalá University.

Preparations of aorta, lung, kidney, and heart tissues were subjected to immunostaining (23) with anti-ECE-1 antibody (mAb AEC32-236, generous gift from Dr. Kohei Shimada) (24). Antibody-protein complexes were detected with anti-HRP-horseradish secondary antibodies using diaminobenzidine reagent following manufacturer's instructions (Dako Cytometrix, Fort Collins, CO). At least four sections per animal were analyzed for each immunostaining.

Blood samples were taken from six male patients, with ages between 52 and 70 years and hypercholesterolemia (range: 250–320 mg/dl), as well as from six normocholesterolemic males (range: 172–215 mg/dl) between 49 and 71 years of age. Everyone gave their informed consent to make the protocol approved by Institutional Committee from our hospital, in accordance with the Principles outlined in the Declaration of Helsinki (*Cardiovascular Research* 1997; 35: 2–4). Peripheral blood mononuclear cells (PBMC) were isolated from blood samples with Ficoll solution (Commercial Rafer, Madrid, Spain), in order to extract total proteins, as described below.

### Cell culture

Bovine aortic endothelial cells (BAEC) were isolated from bovine thoracic aortas using previously described methods (25). Characterization was based on their typical cobblestone appearance and uniform uptake of fluorescent acetylated LDL. Cells were maintained in RPMI 1,640 supplemented with 15% calf serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in an atmosphere of 95% air and 5% CO<sub>2</sub>. Experiments were routinely performed on confluent monolayers at passages 2–5, made quiescent by serum deprivation. Cellular toxicity was evaluated in

all experimental conditions by the trypan blue dye exclusion method and by measurement of lactic dehydrogenase (LDH) activity in the incubation media. No significant toxicity was detected.

Human endothelial cell line, EA.hy926 (EA) were from Dr. Cora-Jean S. Edgell (Yale University School of Medicine, New Haven, CT), and they were grown in DMEM with high glucose supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in an atmosphere of 95% air and 5% CO<sub>2</sub>. Mouse aortic endothelial cells (MAEC) were isolated from WT animals by Dr. Carlos Zaragoza (CNIC, Madrid, Spain), and they were grown in DMEM-F12 supplemented with 20% fetal calf serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin and endothelial growth factor.

### Oxidation of LDL

Human LDL ( $d = 1.019\text{--}1.063$ ) was isolated from fresh plasma of healthy humans by sequential ultracentrifugation at 4°C as described (26). Oxidative modification of LDL was performed by incubation with 25  $\mu$ M CuSO<sub>4</sub> in PBS for 24 h at room temperature (27). Protein concentrations of lipoprotein preparations were determined using the BioRad protein assay kit. OxLDL was assessed by electrophoretic mobility under nondenaturing conditions using a Paragon electrophoresis system, showing a single band with a 2-fold faster migration rate than native LDL. A final concentration between 50–200  $\mu$ g protein/ml of native LDL or oxLDL was used in cells.

### Western blot assays

Proteins were obtained from tissue, human PBMC, and BAEC, by using the Lysis Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, 10 mM sodium pyrophosphate) containing a protease inhibitor cocktail. Protein concentration was determined with BioRad protein assay kit. Proteins were separated on SDS-PAGE 6% (30  $\mu$ g protein/lane), and transferred onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) nonfat dry milk in Tween Tris buffered saline (TTBS) (20 mM Tris-HCl pH 7.5, 0.9% NaCl, 0.05% Tween 20) at room temperature, and then incubated for 90 min with 10  $\mu$ g/ml of the monoclonal anti-ECE-1 antibody (mAb AEC32-236) or with 1:1,000 dilution of the monoclonal anti-LOX-1 antibody (mAb anti-LOX-1 #5-2 from Dr. Tatsuya Sawamura). After washing in TTBS, blots were incubated with 200-fold-diluted horseradish peroxidase-conjugated goat anti-mouse IgG. The immunoreactive bands were visualized with the SuperSignal detection system after 30 s of exposure to MXB film. Then blots were reblotted with a monoclonal anti- $\beta$ -tubulin antibody for samples from mice tissues and BAEC or with a rabbit anti-actin antibody for human PBMC in order to normalize ECE-1 and LOX-1 levels.

### Northern blot analysis

Total cellular RNA was isolated from tissues or BAEC with the guanidinium thiocyanate-phenol-chloroform method (28). For Northern analysis, blots of RNA were hybridized with  $\alpha$ -<sup>32</sup>P]dCTP labeled specific ECE-1 mice or bovine probes (29, 30) in hybridization solution (50% Formamide, 5 $\times$  Denhardt's solution, 5 $\times$  SSPE, 0.5% SDS, and 100  $\mu$ g herring sperm DNA) at 42°C. The filters were stripped by boiling in 0.1% SDS solution and reprobed with a <sup>32</sup>P-labeled 18 S cDNA (5.8 kb fragment digested by *Eco*RI). The densitometric analysis of the films was performed with an image scanner using the public domain software package National Institutes of Health Image 1.55 (Bethesda,

MD). Levels of ECE-1 were normalized by using 18 S expressions within the same lane, and expressed in relative densitometric units with respect to control values.

### Measurement of ET-1 levels and ECE-1 activity

Supernatants and membrane proteins from BAEC treated with LDL or oxLDL were collected as described (31, 32) in order to measure ET-1 production and ECE-1 activity, respectively, using an enzyme-linked immunosorbent assay (ELISA). The membrane proteins from BAEC treated for 24 h with 100  $\mu$ g/ml OxLDL or LDL were isolated, and then 30  $\mu$ g were incubated with a fixed amount of big ET-1 (100 ng) for 4 h at 37°C in the presence or not of 100  $\mu$ M phosphoramidon. In order to generate a standard curve for ET-1 serial dilutions of ET-1 ranging from 1–16 fmol per well were used. A cubic-spline curve was fit to the standards and unknown values interpolated from the standard curves automatically. The cross-reactivity of the ELISA ET-1 antibody, as determined by the concentration giving 50% B/B<sub>max</sub>, was: ET-1 (100), ET-2 (100), ET-3 (<0.001), big-ET-1 human (<0.07), and atrial natriuretic peptide (<0.0006).

### Transient transfection experiments

To determine whether the effect of oxLDL on ECE-1 gene expression was mediated by the 5'-flanking region of the gene, a human ECE-1 promoter/luciferase reporter gene plasmid was constructed (pGL3-ECE-1) (33). We used the PCR of HeLa cell genomic DNA to create serial deletion fragments of the human ECE-1 gene promoter with the 5' ends at nucleotides -650 (AP-1), -596 (NF- $\kappa$ B), -542 (Acute phase), -483 (CAAT box), -444 (Shear stress), -328 (STAT), and -216 (Glucocorticoid receptor element), and the 3' end nucleotide +1 using the Advantage Genomic PCR Kit. Fragments were subcloned in the Xho I-Hind III site of pGL3 vector, upstream from a luciferase reporter gene.

BAEC were grown at 60–80% of confluence in 12-well plates and transfected with promoter/luciferase constructs, by mixing 0.1  $\mu$ g/ $\mu$ L of pGL3-ECE-1 with 1 ng/ $\mu$ L of plasmid control from *Renilla* luciferase (pRL-SV40 vector) and 4  $\mu$ g/ml of Lipofectamine into OptiMEM I media. After 24 h of transfection, cells were refed with complete RPMI 1,640 for at least 16 h, and then native LDL or oxLDL was added at different doses and times using RPMI without serum. Luciferase activity was assessed using a Dual Luciferase Reporter Assay System, and expressed as relative light units of pGL3-ECE-1/*Renilla*/mg protein of each well.

### Electrophoretic mobility shift assays

BAEC were incubated with oxLDL at different times and electrophoretic mobility shift assays was displayed to check on the activation of NF- $\kappa$ B, as previously described (34). Oligonucleotide sequences were based on the putative NF- $\kappa$ B binding element in the ECE-1 promoter (from nucleotides -617 to -591) as follow: *sense* 5'-GGC TGG AGG GAT TTT TCC TCC TTT CA-3' and *antisense* 5'-TGA AAG GAG GAA AAATCC CTC CAG CC-3' (35). Oligonucleotides were labeled with  $\gamma$ -<sup>32</sup>P]ATP at the 5' end with T4 polynucleotide kinase and then incubated with nuclear extracts. Protein-DNA complexes were separated in a 6% nondenaturing polyacrylamide gel in 0.25  $\times$  Tris buffer EDTA. The gels were dried under vacuum and exposed to X-ray film. For competition experiments, 125-fold molar excess of competitor DNA (AP-1 oligonucleotide, NF- $\kappa$ B oligonucleotide) was coincubated with the labeled oligonucleotide probe (NF- $\kappa$ B). Sequences of the oligonucleotides for AP-1 were: *sense* 5'-CAT GGC TGT GTC ACC CTT GTC CC-3' and *antisense* 5'-GGG ACA AGG GTG ACA CAG CCA TG-3'.

## Statistical analysis

Data are expressed as means  $\pm$  SEM. Animal studies were analyzed by ANOVA, followed by the Scheffe multiple comparison test after confirming the normality of the distribution of data. Human studies were analyzed with the Mann-Whitney test. The in vitro studies include at least three separate experiments and are usually expressed as a percentage of the control values. Because the number of data in these experiments was never over 10, nonparametric statistics, particularly the Wilcoxon (two groups) or Friedman (more than two groups) tests, were selected to compare the paired results from the different experimental groups. The level of statistical significance was defined as  $P < 0.05$ .

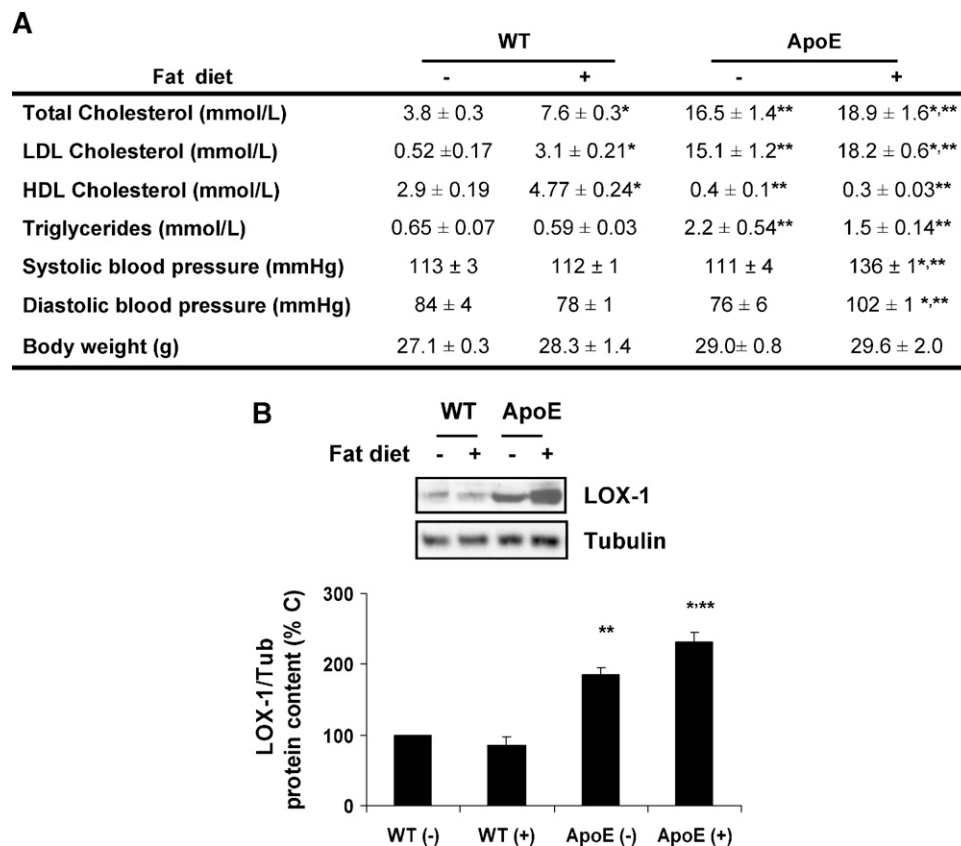
## RESULTS

### ApoE mice and hypercholesterolemic patients show an increased expression of ECE-1

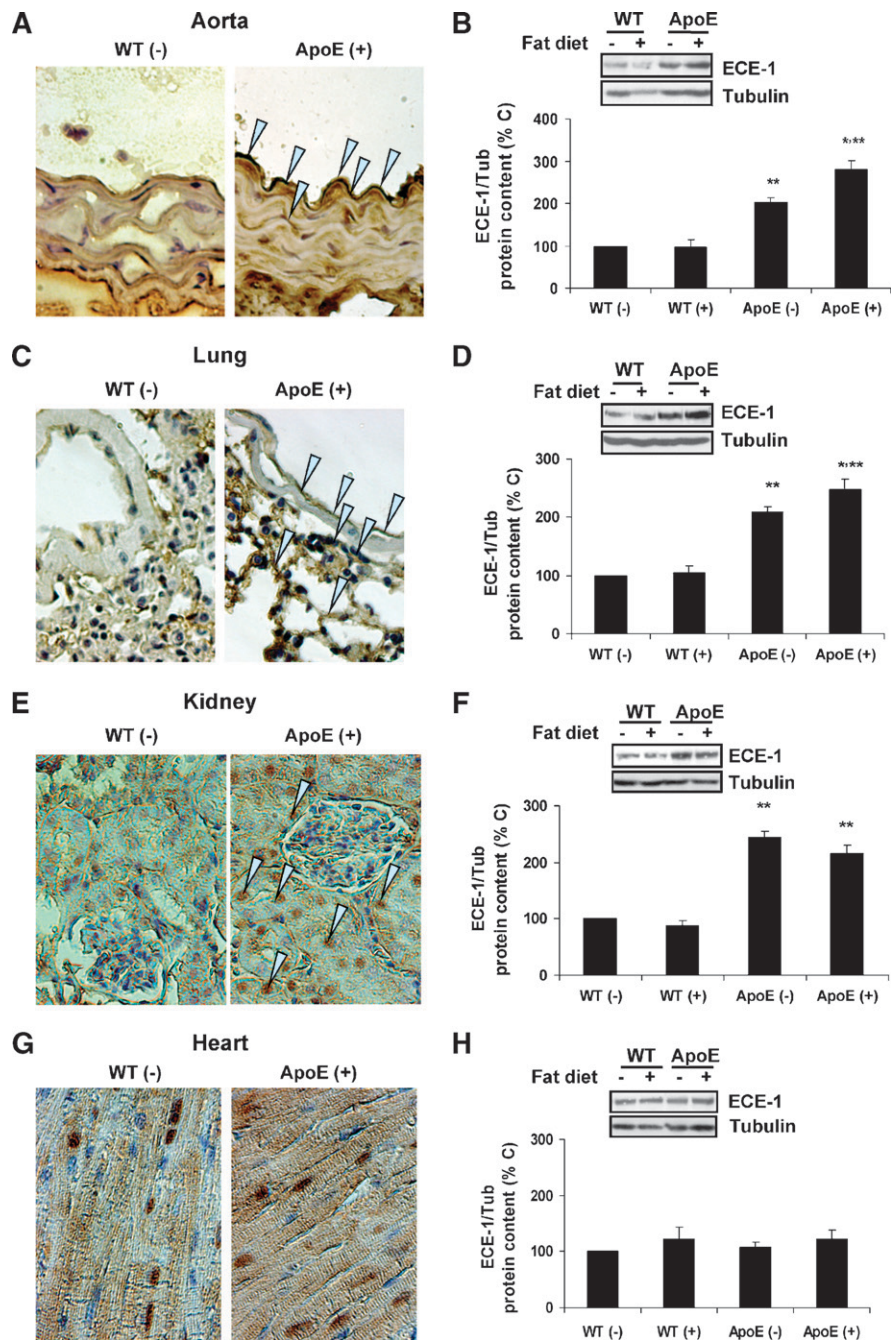
We used apoE mice, an animal model that resembles human atherosclerosis, to investigate the relationships between ECE-1 and vascular disease. ApoE mice developed atherosclerotic lesions in their aortas and had higher levels

of cholesterol and lipids than WT animals (Fig. 1A). ApoE mice fed with the fat diet showed higher values of blood pressure than WT and apoE animals on the standard diet (Fig. 1A). LOX-1 protein content was increased in the aorta of apoE mice, a change that was also magnified by the fat diet (Fig. 1B).

In apoE mice, we found an increased ECE-1 protein content in aorta, lungs, and kidneys, with respect to their wild-type counterparts, as detected by immunohistochemistry (Fig. 2, left part) and immunoblot (Fig. 2, right part). However, no significant differences were found in the heart (Fig. 2). The fat diet induced a slight but significant increase of ECE-1 protein content in aorta and lungs from apoE animals (Fig. 2, right part). The analysis of ECE-1 mRNA in these animals revealed an increased expression in the same organs as above, without significant differences in heart tissue (Fig. 3). When FR-901533, an ECE-1 inhibitor (36), was administered to mice via intraperitoneal at 1 mg/kg, blood pressure was reduced significantly in apoE mice on the fat diet versus WT mice and apoE on the normal diet (Fig. 4A).



**Fig. 1.** Characteristics of wild-type (WT) and apolipoprotein E-deficient mice (apoE), on a normal (–) or fat diet (+). A: Body weight, lipids levels, and blood pressure in 12 animals per group. B: Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) content, assessed by Western blot, in the aorta. The upper part shows a representative experiment, whereas the lower part shows the mean  $\pm$  SEM of the densitometric analysis of 12 animals per group (values are expressed as the percentage of WT animals on a normal diet). \*  $P < 0.05$  vs. animals of the same strain with a normal diet, \*\*  $P < 0.05$  vs. WT mice on the same diet.

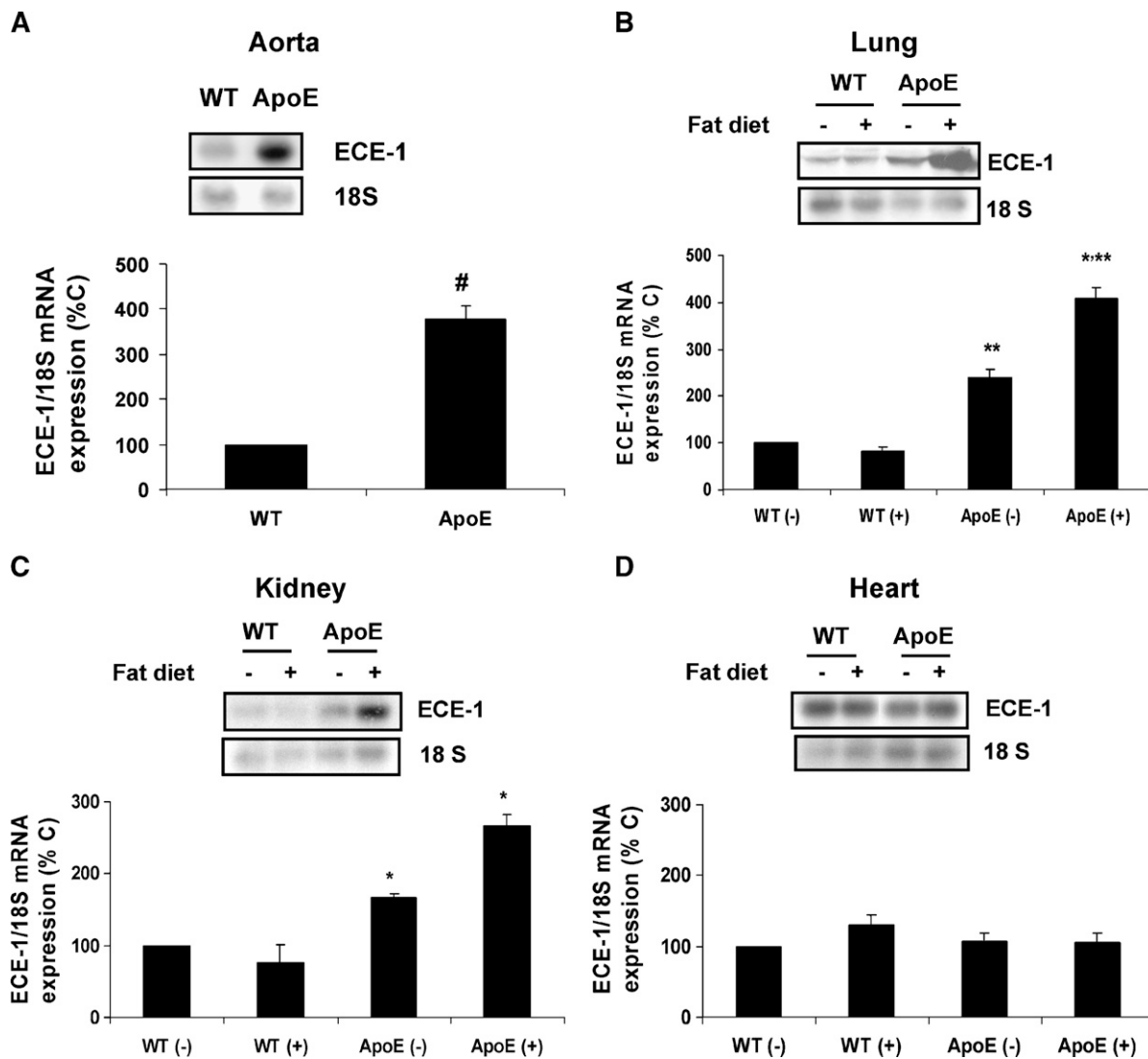


**Fig. 2.** Changes in endothelin-converting enzyme-1 (ECE-1) protein content in the aorta (A and B), lung (C and D), kidney cortex (E and F), and heart (G and H) from WT and apoE-deficient mice, on a normal (-) or fat diet (+). On the left part of the figure, photomicrographs of the immunostaining for ECE-1 in the different tissues (arrows point to positive ECE-1 immunostaining) are shown. On the right, their corresponding immunoblots are shown. At the top of each one, a representative Western blot is shown and at the bottom the mean  $\pm$  SEM of the densitometric analysis of 12 animals per group (values are expressed as the percentage of WT animals on a normal diet) is provided. \*  $P < 0.05$  vs. animals of the same strain with a normal diet, \*\*  $P < 0.05$  vs. WT mice on the same diet.

To evaluate these animal findings in human beings, we isolated PBMC from clinically diagnosed hypercholesterolemic patients and healthy people, and then, ECE-1 and LOX-1 protein content was measured. Both proteins significantly increased in hypercholesterolemic patients (Fig. 4B).

#### OxLDL up-regulates ECE-1 in cultured BAEC

To analyze the potential mechanisms involved in the up-regulation of ECE-1 found in hypercholesterolemic animals and patients, we used BAEC treated with LDL or oxLDL. ECE-1 protein content increased in BAEC incubated with oxLDL at different doses and times (Fig. 5).



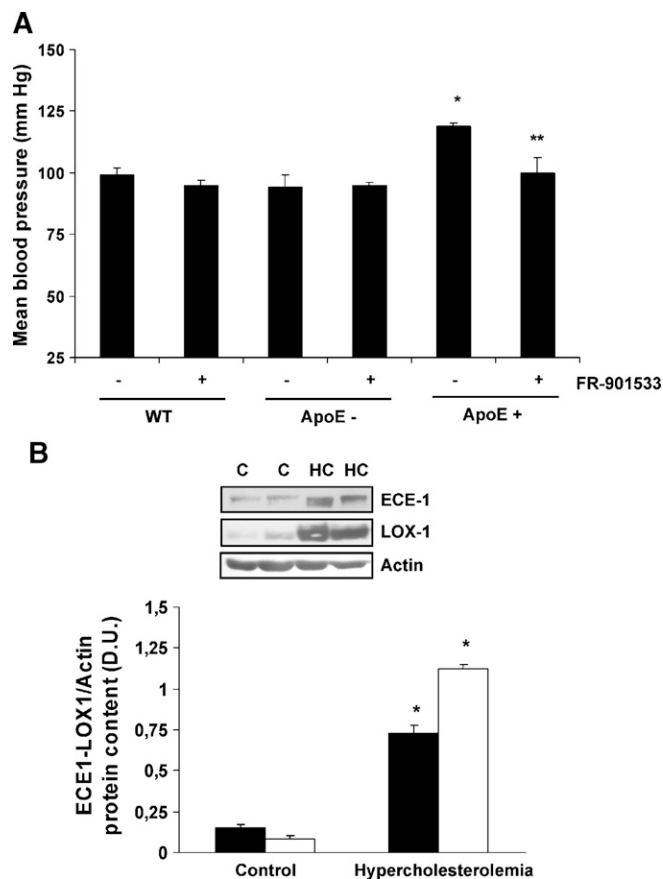
**Fig. 3.** Changes in ECE-1 mRNA expression in aorta (A), lung (B), kidney cortex (C), and heart (D) from wild-type (WT) and apoE-deficient mice, on a normal (-) or fat diet (+). The upper part of each panel shows a representative Northern blot experiment, whereas the lower part shows the mean  $\pm$  SEM of the densitometric analysis of 12 animals per group (values are expressed as the percentage of WT animals on a normal diet). #  $P < 0.05$  vs. WT mice, \*  $P < 0.05$  vs. animals of the same strain with a normal diet, \*\*  $P < 0.05$  vs. WT mice on the same diet.

The stimulatory effect started after 6 h of incubation and remained for 24 h (Fig. 5A). It was maximal at 100  $\mu\text{g}/\text{ml}$  oxLDL (Fig. 5B). This same concentration of oxLDL significantly increased ECE-1 activity and ET-1 synthesis in cells (Fig. 5C). No significant changes were found with LDL (Fig. 5).

#### OxLDL regulates ECE-1 expression in endothelial cells through activation of NF- $\kappa$ B

To clarify the mechanisms involved in the oxLDL-dependent ECE-1 up-regulation, we tested the ability of oxLDL to modulate ECE-1 transcription. A significant increase of ECE-1 mRNA steady-state levels was detected in cells incubated for 8 h with 100  $\mu\text{g}/\text{ml}$  oxLDL, an effect that was not observed with LDL (Fig. 6A). ECE-1 promoter activity was stimulated by oxLDL in a time- (Fig. 6B) and

dose-dependent manner (Fig. 6C), reaching its maximum after 6 h of incubation with 100  $\mu\text{g}/\text{ml}$  oxLDL. LDL incubation, by contrast, did not modify the promoter activity. On the other hand, incubation of transfected cells with the antioxidants catalase and N-acetyl cysteine prevented the stimulatory effect of oxLDL on ECE-1 promoter activity (Fig. 6D). To relate our *in vitro* results in BAEC to those obtained in animals and human beings, we transfected ECE-1 promoter also in human endothelial cells (EA) and in mouse aortic endothelial cells (MAEC) from WT animals. We found a significant increase in promoter activity after 6 h of incubation with 75  $\mu\text{g}/\text{ml}$  oxLDL in both types of cells (Fig. 6E); in contrast LDL did not induce any change. To investigate the signaling that leads to ECE-1 promoter stimulation, serial deletions of the ECE-1 regulatory region were evaluated in a luciferase assay of



**Fig. 4.** A: Changes in the mean blood pressure of mice treated with the ECE-1 inhibitor FR-901533. The mean blood pressure was registered in three groups of animals, wild-type fed with a normal diet (WT), apoE-deficient fed with a normal diet (apoE<sup>-</sup>), and apoE fed with a fat diet (apoE<sup>+</sup>), in basal conditions (-) and after the intraperitoneal administration of 1 mg/kg FR-901533 (+). \*  $P < 0.05$  vs. other groups, \*\*  $P < 0.05$  vs. basal apoE<sup>+</sup>. B: Changes in ECE-1 and LOX-1 protein content in peripheral blood mononuclear cells (PBMC) from normo- and hypercholesterolemic patients. The upper panel of the figure shows the representative Western blots, whereas the lower panel shows the densitometric analysis of six patients per group. Closed bars: ECE-1 values; Open bars: LOX-1 values. \*  $P < 0.05$  vs. control (normocholesterolemic people). Error bars represent SEM.

transiently-transfected BAEC. When the NF- $\kappa$ B responsive element was deleted (Fig. 7A, upper panel), the stimulatory effect of oxLDL fell down near control levels (Fig. 7A, lower panel). The involvement of NF- $\kappa$ B on ECE-1 transcriptional regulation was further confirmed by electrophoretic mobility shift assays, showing an increase of nuclear NF- $\kappa$ B binding after 30 min of incubation with oxLDL (Fig. 7B).

## DISCUSSION

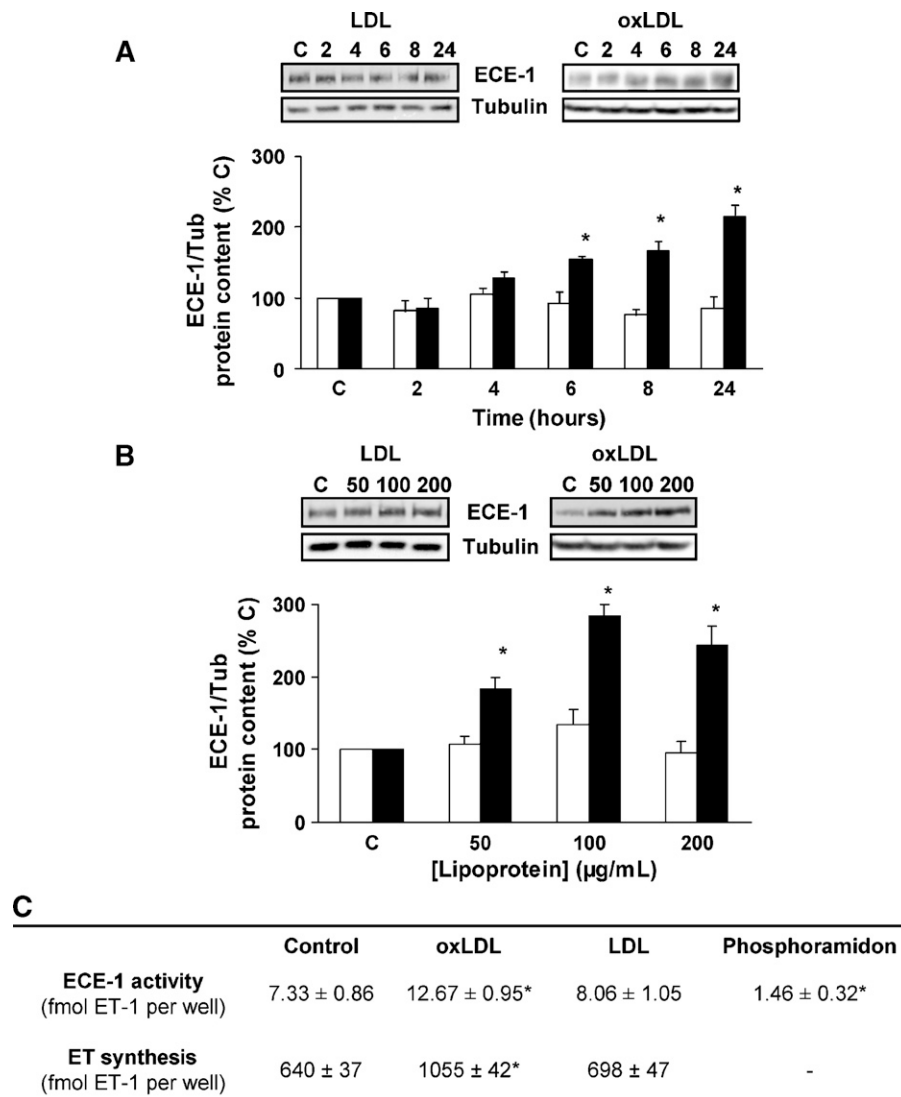
Present results demonstrate that apoE-deficient mice exhibit an increased content of ECE-1 in aorta, lungs, and kidneys, without changes in heart tissue. The changes

observed seem to be the consequence of an increased gene expression, as steady-state ECE-1 mRNA levels in these tissues paralleled the protein content. These results confirm previous descriptions in human beings (17–19), suggesting a role for ECE-1 overproduction in the genesis of the endothelial dysfunction in atherosclerosis. Mitani et al. (37) also reported that rabbits with high levels of cholesterol showed higher levels of ECE-1. Additionally, our results provide the first demonstration of the tissue-specificity of the changes in ECE-1 over expression, where the most striking finding was the lack of changes in ECE-1 expression on heart tissue.

The mechanisms involved in the ECE-1 up-regulation in these animals have been indirectly explored in the present work. The relevance of hypercholesterolemia in this experimental model, the fact that the fat diet increased ECE-1 content in some tissues even more, and the general acceptance of the pathogenic role of LDL, particularly in its oxidized form, in atherosclerosis (38, 39), point to oxLDL as the possible intrinsic factor that stimulate ECE-1 expression. In spite of the fact that we did not measure plasma oxLDL levels in our mice, we found significant changes in LOX-1 protein content in the aorta of apoE mice. As LOX-1 expression can be stimulated by oxLDL (39–42), this finding could suggest that hypercholesterolemic animals could have increased levels of oxLDL.

Oxidized LDL induced an increased expression of the ECE-1 mRNA in cultured endothelial cells, with the subsequent increase in protein content. Protein changes were paralleled by an increase in the enzyme activity and the ET-1 synthesis. Previous work from Niemann et al. (22) reported a similar oxLDL-dependent stimulation of ECE-1 synthesis, but in that work LDL also increased the cellular content of ECE-1. Moreover, Ruschitzka et al. (19) proposed an inverse relationship between serum LDL levels and vascular ECE-1 activity; however, in our hands, LDL did not modify the synthesis of ECE-1 by cultured cells. OxLDL up-regulated the cellular content of ECE-1 in a concentration range between 50–200  $\mu$ g/ml. These concentrations are similar to those previously measured in the plasma of hypercholesterolemic patients (43).

The mechanism responsible for the oxLDL-dependent ECE-1 up-regulation has been also partially explored in the present work in BAEC. The changes in the ECE-1 mRNA steady-state levels observed in endothelial cells seem to be the consequence, at least partially, of an increased activity of the ECE-1 promoter. Regulation of ECE-1 promoter by oxLDL was also confirmed in human and mouse endothelial cells, suggesting that our results are not specific of BAEC. These results demonstrate the relevance of oxLDL in the ECE-1 up-regulation not only in different species of cells but also in hypercholesterolemic mice. The analysis of the promoter activity after serial deletions and gel-shift assays support the relevance of NF- $\kappa$ B in this ECE-1 promoter-increased activity. The fact that antioxidants prevented the oxLDL-dependent changes in promoter activity also supports a role for reactive oxygen species in the genesis of the observed effects. Reactive oxygen species and NF- $\kappa$ B seem also to play a role



**Fig 5.** Effect of native (LDL) and oxidized (oxLDL) low density lipoproteins on ECE-1 protein content, ECE-1 activity, and endothelin (ET) synthesis. A, B: Bovine aortic endothelial cells (BAEC) were incubated with 100  $\mu\text{g}/\text{ml}$  LDL (open bars) or oxLDL (closed bars) at different times (A) or at different concentrations for 24 h (B), and ECE-1 protein content was measured by Western blot. In the upper part of each panel a representative Western-blot is shown, whereas in the lower part the densitometric analysis of five independent experiments is given (mean  $\pm$  SEM). Values are expressed as the percentage of control cells (C). \*  $P < 0.05$  vs. C and LDL. C: ECE-1 activity in membrane proteins and ET concentration in supernatants from BAEC treated with 100  $\mu\text{g}/\text{ml}$  LDL or oxLDL for 24 h were measured by ELISA. Values are expressed as fmol of ET-1 per well, and are the mean  $\pm$  SEM of four independent experiments in triplicate. \*  $P < 0.05$  vs. other groups. In the ECE-1 activity assay, phosphoramidon (100  $\mu\text{M}$ ) was used as negative control (ECE-1 activity inhibition: 80  $\pm$  7%).

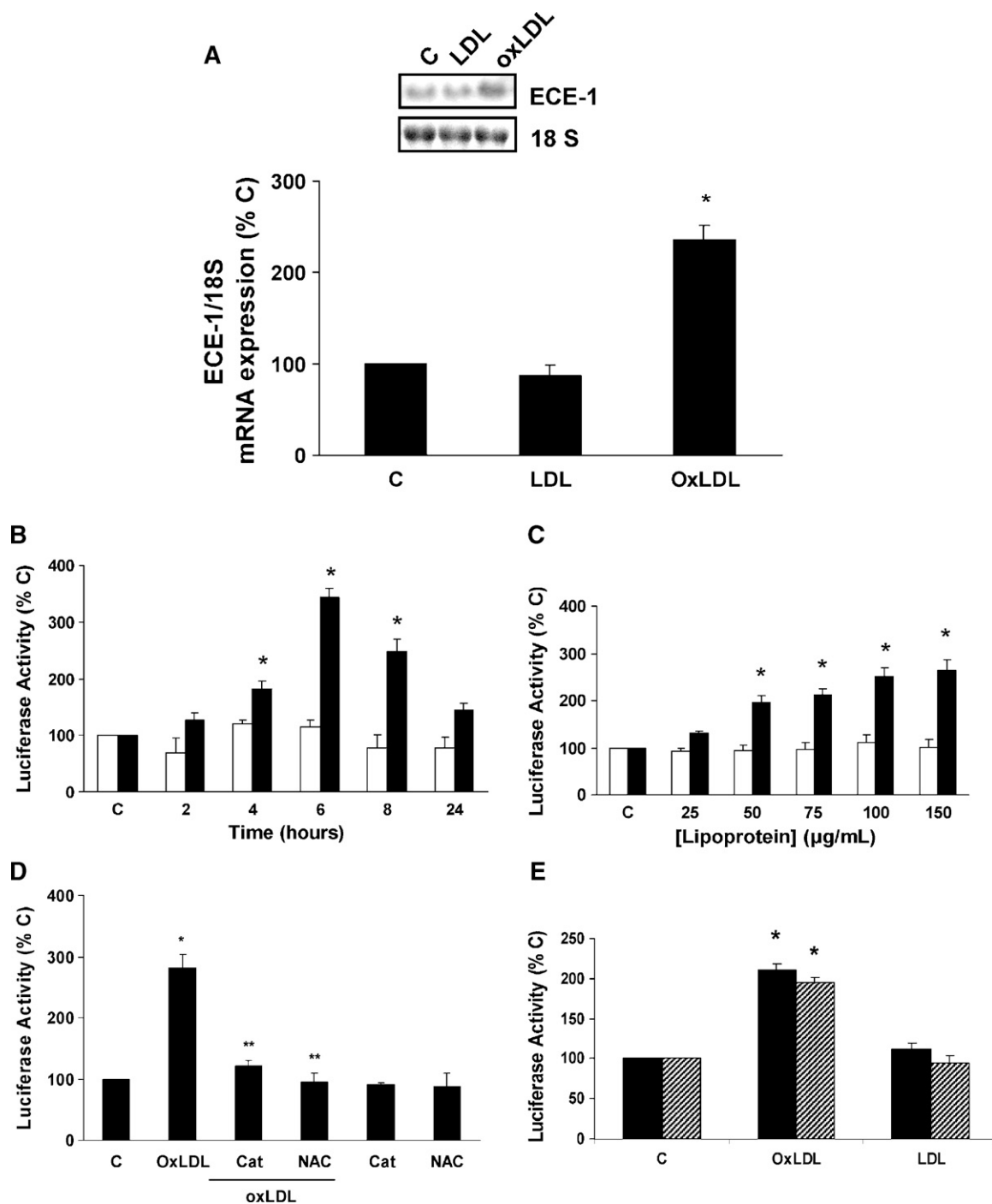
in the regulation of cell function by oxLDL in other pathophysiological conditions (7, 44).

The relevance of the changes detected in ECE-1 tissue content in the development of the alterations detected in apoE mice must also be discussed. If ECE-1 overexpression were involved in the genesis of the morphological and functional changes observed in these mice, the blockade of the enzyme would improve these pathological changes. Previous experimental work demonstrated that the chronic blockade of the ETA receptor prevented the vascular dam-

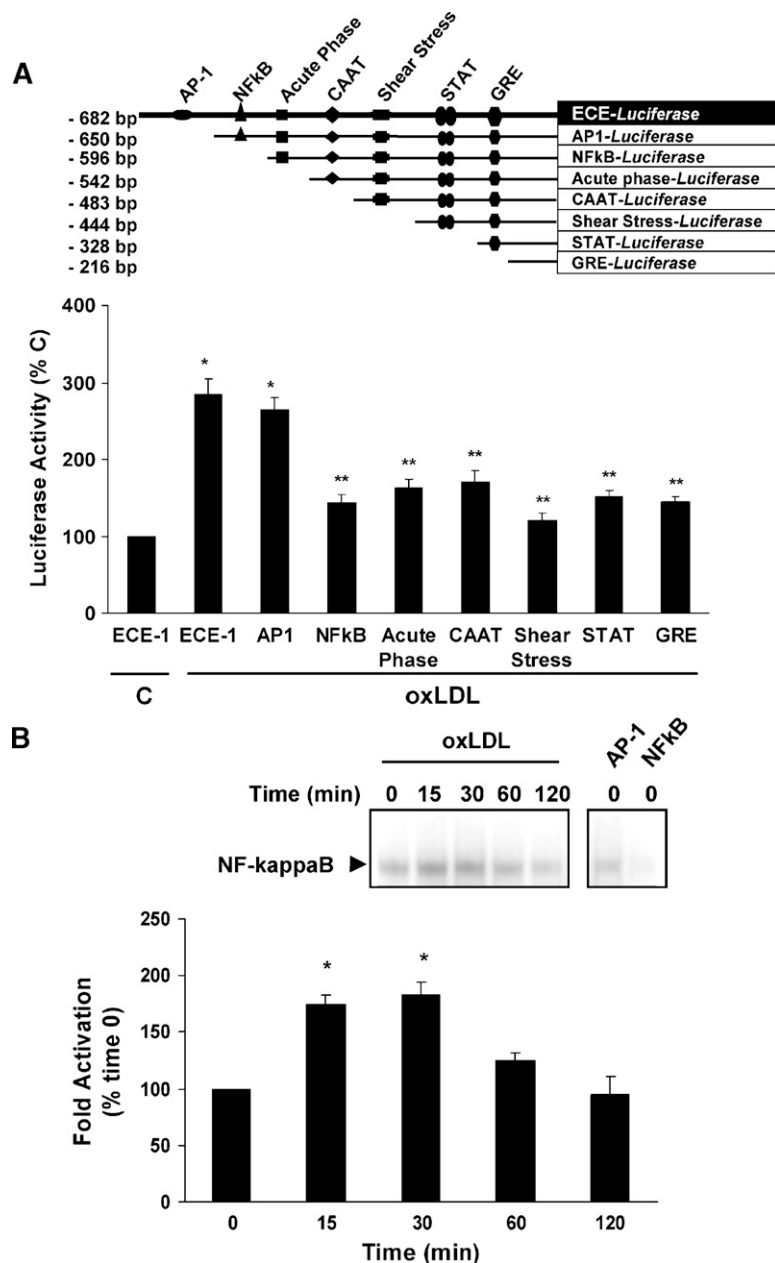
age that characterizes this experimental model (15, 16). In our hands, acute blockade of ECE-1 with FR-901533 decreased blood pressure in apoE-deficient mice fed with a fat diet, but did not modify blood pressure in WT and apoE animals on a normal diet, suggesting that ECE-1 overexpression is only one of the factors involved in the high blood pressure maintenance in apoE mice.

Studies in experimental models and cultured cells must be considered critically when analyzing pathophysiological mechanisms in human beings. To perform a preliminary





**Fig. 6.** Effect of native (LDL) and oxidized (oxLDL) low density lipoproteins on ECE-1 mRNA steady-state levels, and activity of ECE-1 promoter. **A:** BAEC were incubated with 100 µg/ml LDL or oxLDL for 8 h, and ECE-1 mRNA was measured by Northern blot. In the upper part of the panel, a representative Northern-blot is shown, whereas in the lower part the densitometric analysis of three independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C), \*  $P < 0.05$  vs. C and LDL. **B, C:** BAEC were transfected with an ECE-1 promoter/luciferase reporter gene plasmid. Transfected BAEC were then incubated with 100 µg/ml LDL (open bars) or oxLDL (closed bars) at different times (B) or concentrations for 6 h (C), and the ECE-1 promoter activity was measured. Values are expressed as the percentage of control cells (C), and are the mean ± SEM of three independent experiments in triplicate. \*  $P < 0.05$  vs. C and LDL. **D:** BAEC were transfected as above and then incubated with 100 µg/ml oxLDL for 6 h, in the presence or not of 80 U/ml catalase (Cat) or 100 µM N-acetyl cysteine (NAC), and then assayed for luciferase activity. Results are the mean ± SEM of four independent experiments in triplicate, and are expressed as the percentage of control cells (C). \*  $P < 0.05$  vs. the other groups, \*\*  $P < 0.05$  vs. oxLDL. **E:** EA (closed bars) and MAEC (striped bars) were transfected with an ECE-1 promoter/luciferase reporter gene plasmid, incubated with 75 µg/ml oxLDL or LDL for 6 h and then assayed for luciferase activity. Results are the mean ± SEM of three independent experiments in triplicate, and are expressed as the percentage of control cells (C). \*  $P < 0.05$  vs. C and LDL. In the transfection experiments, phorbol myristate acetate ( $3 \times 10^{-7}$  M, 6 h) was used as positive control (stimulation:  $283 \pm 18\%$ ).



**Fig. 7.** Effect of oxLDL on transactivation of ECE-1 promoter deletions and on the binding of NFkB to nuclear extracts. **A:** BAEC were transfected with plasmids containing serial deletion fragments of ECE-1 promoter linked to luciferase, treated with 100  $\mu$ g/ml oxLDL for 6 h, and assayed for luciferase activity. Top: Schematic of ECE-1 5' flanking region deletions, showing some of the putative response elements in these regions: -650 (AP-1), -596 (NF- $\kappa$ B), -542 (Acute phase), -483 (CAAT box), -444 (Shear stress), -328 (STAT), and -216 [glucocorticoid receptor element (GRE)]. Bottom: The quantitative results are the mean  $\pm$  SEM of four different experiments in triplicate, and are expressed as the percentage of induction with respect to control cells (C). \*  $P < 0.05$  vs. control (100%), \*\*  $P < 0.05$  vs. ECE-1 and AP-1. Phorbol myristate acetate ( $3 \times 10^{-7}$  M, 6 h) was used as positive control (stimulation:  $258 \pm 13\%$ ). **B:** Nuclear extracts were harvested from BAEC previously incubated with 100  $\mu$ g/ml oxLDL for different times, and then incubated with the labeled oligonucleotide containing the NFkB consensus sequence. Top left: Effect of oxLDL on NFkB binding activity of BAEC nuclear extracts. Top right: Competition assay using unlabeled AP-1 and NFkB oligonucleotides. Bottom: Densitometric analysis of gel-shift experiments (mean  $\pm$  SEM of four different experiments and are expressed as the percentage of time 0, with \*  $P < 0.05$  vs. time 0).

approach to the analysis of the relevance of the association between hypercholesterolemia and increased ECE-1 activity in humans, we hypothesized that ECE-1 could be expressed in PBMC, and we found a link between hypercholesterolemia and the increased content of ECE-1 in these cells. LOX-1 protein content also increased in PBMC of hypercholesterolemic patients, as it did in aortas of apoE-deficient mice. These findings point to the association between hypercholesterolemia and increased ECE-1 activity, suggesting that PBMC could be used as a valuable system to explore the changes in the enzyme in pathological conditions.

In summary, present results demonstrated a direct relationship between LDL and ECE-1 content, in contrast with Ruschitzka's results, and provide some information about

the mechanisms involved in this relationship. ECE-1 expression increased in aorta, lungs, and kidneys of apoE mice, suggesting that ECE-1 could play a role in the hypertension found in some of these animals. Increased oxLDL levels may be proposed as one of the factors responsible for the ECE-1 up-regulation, throughout an increased reactive oxygen species synthesis, and NF- $\kappa$ B activation, with the subsequent stimulation of ECE-1 promoter activity and increased mRNA steady-state levels of ECE-1. Thus, ECE-1 could be proposed as a therapeutic target in atherosclerosis prevention. **Fig. 7**

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