Obesity Is Associated With Tissue-Specific Activation of Renal Angiotensin-Converting Enzyme In Vivo Evidence for a Regulatory Role of Endothelin

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Abstract—In the C57BL/6J mice model, we investigated whether obesity affects the function or expression of components of the tissue renin-angiotensin system and whether endothelin (ET)-1 contributes to these changes. ACE activity (nmol/L·L·His-Leu·mg protein⁻¹) was measured in lung, kidney, and liver in control (receiving standard chow) and obese animals treated for 30 weeks with a high-fat, low cholesterol diet alone or in combination with LU135252, an orally active ET₁ receptor antagonist. ACE mRNA expression was measured in the kidney, and the effects of LU135252 on purified human ACE were determined. Aortic and renal tissue ET-1 protein content was measured, and the vascular contractility to angiotensin II was assessed. Obesity was associated with a tissue-specific increase in ACE activity in the kidney (55±4 versus 33±3 nmol/L) but not in the lung (34±2 versus 32±2 nmol/L). Long-term LU135252 treatment completely prevented this activation (13.3±0.3 versus 55±4 nmol/L, P<0.05) independent of ACE mRNA expression, body weight, or renal ET-1 protein but did not affect pulmonary or hepatic ACE activity. Obesity potentiated contractions in response to angiotensin II in the aorta (from 6±2% to 33±5% KCl) but not in the carotid artery (4±1% to 3.6±1% KCl), an effect that was completely prevented with LU135252 treatment (6±0.4% versus 33±5% KCl). No effect of LU135252 on purified ACE was observed. Thus, obesity is associated with the activation of renal ACE in vivo independent of its mRNA expression and enhanced vascular contractility to angiotensin II. These effects are regulated by ET in an organ-specific manner, providing novel mechanisms by which ET antagonists may exert organ protection.

Key Words: risk factors ■ aorta ■ carotid arteries ■ vasoconstriction ■ diet ■ cholesterol ■ gene expression

Obesity is a major health problem in Western societies and an independent risk factor for cardiovascular disease. Body weight also appears to directly contribute to hypertension in that weight reduction significantly lowers blood pressure in hypertensive obese patients. Whether and via which mechanisms obesity affects regulation of the systems involved in vascular homeostasis and how obesity per se may affect vascular function are in large part unknown.

The renin-angiotensin system (RAS) is present in the vasculature and organs such as the heart and kidney, as well as in adipocytes, where it is functionally active and regulated by food intake. Angiotensin (Ang) II induces mRNA and protein expression of endothelin (ET)-1, a strong vasoconstrictor and mitogen, in vitro and in vivo and in the kidney and vasculature in vivo. Interestingly, ET-1 increases the formation of Ang II from Ang I, suggesting modulation of ACE activity. ET-1 is also involved in lipid metabolism and has been implicated in the pathogenesis of obesity-associated hypertension.

No data are available regarding whether obesity is associated with activation of the RAS or the ET system in nonadipose tissue such as the kidney or the vasculature. Also, it is unclear whether vascular responses to Ang II are affected and whether these effects are dependent on blood pressure. Using the C57BL/6J mouse model, which is particularly prone to diet-induced obesity, we investigated whether obesity in these animals affects tissue ACE activity in lung,
kidney, and renal and whether vascular structure and contractility to Ang II in the aorta and carotid artery are affected. To determine the potential regulatory role of ET on the RAS in vivo, we treated a subgroup of obese animals with an orally active ET₄ receptor antagonist.

Methods

Animal Treatments, Body Weight, and Tissue Preparation
Male C57BL/6J mice (4 to 5 weeks of age) obtained from The Jackson Laboratory were maintained at 24°C and kept at a 12-hour light/dark cycle with free access to water. Mice (n=10 per group) were randomly assigned to 30 weeks of treatment with either normal rodent chow or a high-fat, Western-type diet (Adjusted Calories Diet; Harlan Knoll TD 88137; 21% casein, high protein, 19.5% anhydrous milk fat, 0.15% cholesterol) with or without the nonpeptide ET₄ receptor antagonist LU135252 (50 mg/kg IP), and a blood sample was collected at the beginning and the end of treatment. On the day of the experiment, blood pressure was measured in conscious mice as described previously.31 animals were anesthetized with pentobarbital (50 mg/kg IP), and a blood sample was collected through puncture of the right ventricle. Both kidneys, as well as the thoracic aorta, mesenteric arteries, and both carotid arteries, were excised, and tissue samples from lung and liver were obtained. One half of the left kidney was preserved in 4% buffered formaldehyde for histological analysis, and the remaining kidney tissue and tissues from lung, liver, and aorta were snap-frozen in liquid nitrogen and stored at −70°C until further study. Study design and protocols were approved by the Institutional Animal Care Committee (Kommission für Tierversuche des Kantons, Zürich, Switzerland) and were in accordance with the American Heart Association guidelines for research animal use.

Plasma Lipid Measurements
EDTA blood samples were centrifuged at 5000 rpm for 10 minutes, frozen in 100-μL aliquots, and kept at −70°C until further determination. Plasma lipoproteins and triglycerides were determined enzymatically with a Cobas Mira Plus autoanalyzer (Roche Diagnostics).

Vascular Contractility to Ang II
Rings of aorta and carotid artery were placed into cold (4°C) Krebs-Ringer solution containing (in mmol/L) NaCl 118.6, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.1, EDTA calcium disodium 0.026, and glucose 11.1 and suspended in fine tungsten stirrups to avoid endothelial damage (diameter 100 m). 31 rings were repeatedly exposed to 100 mM KCl until optimal tension was reached. Each ring was then exposed to a single concentration of Ang II (3×10⁻⁷ mol/L) that had been found to cause maximal contraction as determined in pilot experiments. In some experiments, rings were pretreated for 30 minutes with either the cyclooxygenase inhibitor indomethacin (10⁻³ mol/L) or the thromboxane A₂ receptor antagonist SQ 30741 (10⁻⁷ mol/L; a gift from Bristol-Myers-Squibb, Princeton, NJ).

Tissue ACE Activity Measurements
Frozen tissue from whole kidney, lung, and liver was homogenized in 0.4 mol/L borate buffer, pH 7.2, containing 0.34 mol/L sucrose and 0.9 mol/L NaCl (1 mg tissue/mL). Homogenates were centrifuged (3000 rpm for 10 minutes), and supernatants were used for fluorimetric determination of ACE activity as described previously.32,33 Briefly, supernatants from homogenized tissues (20 μL) were incubated with 480 μL assay buffer containing 5 mmol/L Hip-His-Leu in 0.4 mol/L sodium borate buffer with 0.9 mol/L NaCl, pH 8.3; sodium borate buffer with 0.1% Triton X-100 with 0.9 mol/L NaCl, pH 8.0; or 0.1 mol/L phosphate buffer with 0.3 mol/L NaCl, pH 8.0, for 30 minutes at 37°C. The reaction was halted by the addition of 1.2 mL of 0.34 N NaOH. The product, His-Leu, was measured fluorimetrically at 365-nm excitation and 495-nm emission with a fluorocolorimeter (AMINCO) after the addition of 100 μL o-phthalaldehyde (20 mg/mL) in methanol. This was followed 10 minutes later by the addition of 200 μL of 3 N HCl and centrifugation at 3000 rpm for 10 minutes at room temperature. To correct for the intrinsic fluorescence of the tissues, blanks were made by adding the samples after NaOH had been added to the reaction. All experiments were performed in duplicate or triplicate. The samples were assayed within 10-second intervals. The specificity of the assay was determined on the basis of the inhibition of ACE activity with the compound enalaprilat MK-422 (3 pmol/L). ACE activity was related to tissue protein as measured with use of the Bradford method.34

Tissue ET Protein Content
The determination of tissue ET-1 protein content was made in a blinded fashion according to previously described protocols. Briefly, frozen renal or aortic vascular tissue was homogenized with the use of a polytron (model PT 1200; Kinematica AG) for 60 seconds in ice-cold chloroform/methanol (2:1) containing 1 mmol/L N-ethylmaleimide and 0.1% trifluoroacetic acid. Homogenates were left overnight at 4°C, and then 0.8 mL sterile distilled water was added. The mixture was vortexed and centrifuged at 4000 rpm for 15 minutes, and the supernatant was removed. Then, 1-μL aliquots of the extract were diluted with 9 μL of 4% acetic acid and then extracted. Eluates were dried in a Speed-Vac and reconstituted in working assay buffer for the radioimmunoassay, with isoform-specific rabbit antibodies against synthetic ET-1 (Peninsula Laboratories). Reversed phase HPLC was used for ET-1 identification, and renal ET-1 tissue content was related to tissue weight (in pg/g).19,31

RNA Isolation and RT-PCR
To determine the effects of LU135252 on expression of ACE, mRNA expression was quantified in the kidneys of obese and LU135252-treated obese mice. Total RNA was isolated through guanidinium thiocyanate/cesium chloride centrifugation.35 In RNA from kidneys, the ACE mRNA expression was determined with the use of RT-PCR. In RT-PCR experiments, equal amounts of total RNA (200 ng) were reverse transcribed into cDNA with the use of random hexamer primers and SuperScript II RNase H⁻ reverse transcriptase (Life Technologies) for 1 hour at 42°C. Then, 20% of each RT reaction were amplified in separate reactions with 20 pmol/μL ACE and GAPDH-specific sense and antisense primers by PCR. The PCRs were separated through standard agarose gel electrophoresis,36 stained with ethidium bromide, and documented with the use of photography with Polaroid 665 film. The optical density of standard and sample-specific PCR fragments was estimated with the use of a Personal Densitometer (Molecular Dynamics). The optical density of ACE PCR fragments was normalized versus GAPDH.

Histology
Kidneys, aorta, and carotid artery were examined with regard to renal morphology, glomerular abnormalities, and vascular hypertrophy by an experienced pathologist (C.C.H.), using standard histological methods. Briefly, paraffin-embedded sections (2 μm) of whole kidneys or vessels from all three treatment groups were stained with hematoxylin-eosin and were viewed with light microscopy at ×40.

ET Receptor Binding Studies
Frozen vascular rings of the thoracic aorta were sectioned at 10 μm. One section was stained with hematoxylin-eosin to determine the anatomic orientation of the vessel. The remaining sections were used
to determine total binding to radioactively labeled 125I-ET-1 (Amersham), given as amol ET-1/mm² vascular surface area. Specific ET₄ and ET₃ receptor binding capacity was assessed with the use of 125I-labeled ET receptor antagonists PD 151242 and BQ 3020 (Amersham), respectively, as described previously. 31,37

Vascular Cholesterol Content
Frozen aortic tissue was pulverized, homogenized in 500 μL of 12.5 mmol/L sodium cholate containing 0.4% Triton X-100 with the use of a polytron, and then left for 15 minutes at room temperature before sonication and centrifugation. After centrifugation, the supernatant was used for the determination of cholesterol with a microcolumn assay adapted for the Cobas Mira Plus autoanalyzer, based on the use of a polytron, and then left for 15 minutes at room temperature before sonication and centrifugation. After centrifugation, the supernatant was used for the determination of cholesterol with a microcolumn assay adapted for the Cobas Mira Plus autoanalyzer, based on the production of hydrogen peroxide from cholesterol and its esters through sequential action of cholesterol esterase and cholesterol oxidase. 38 In the presence of peroxidase, the hydrogen peroxide mediates the oxidative coupling of phenol and 4-aminoantipyrine to form a red quinoneimine derivative. The color intensity is directly related to the cholesterol concentration and is measured photometrically. Cholesterol calibrator (C7921; Sigma Chemical Co) was suitably diluted with sodium cholate/Triton X-100 to form standard curves in the appropriate range. Aortic cholesterol content was related to tissue protein content.

Purification of Human ACE and Activity Measurements
ACE protein was purified from fresh citrated human plasma according to the affinity chromatography method described by Bull et al. 39 Human blood was centrifuged at 12 500g for 60 minutes, and the supernatant was applied to a 300-μL lisinopril affinity column. Elution of the column with 0.1 mmol/L captopril resulted in a 110 000-fold enrichment of human ACE. The eluate was dialyzed and concentrated via a 30-kDa YM filter (Amicon). Enzymatic activity was measured (169 mU/mg) via a reversed phase HPLC assay with Ang I as substrate (5 × 10⁻⁵ mol/L) and incubation at 37°C for 60 minutes. After the incubation mixture was stopped with the addition of 0.5% trifluoroacetic acid and centrifugation, the formation of Ang II was monitored at 220 nm as a readout. In addition, enzymic specificity of the preparation for ACE was demonstrated through testing of a subset of 13 well characterized metalloprotease inhibitors for different proteases, including ACE, neutral endopeptidase 24.11, and ET-converting enzyme inhibitors (data not shown). Inhibition experiments with LU 135252 were performed following exactly the same procedure.

Calculations and Statistical Analysis
Data are given as mean±SEM (n=number of animals). Contractions of isolated vascular rings are given as percent contraction to potassium chloride (100 mmol/L). For multiple comparisons, results were analyzed by ANOVA, followed by Bonferroni’s correction. For comparison between two values, the unpaired Student’s t test or the nonparametric Mann-Whitney test was used when appropriate. A value of P<0.05 was considered significant.

Results

Body Weight, Plasma Lipids, and Blood Pressure
Body weight did not differ between the groups at the beginning of study. After 7 months of treatment, body weight had increased by 257% in the mice on the Western-type diet compared with 54% in the control mice (P<0.05). Concomitant treatment with LU135252 had no effect in obese mice (weight increase 263%, P=NS versus obese mice). In all animals, plasma lipid values were within the normal range. In obese mice, an increase in total cholesterol, LDL-cholesterol, and HDL-cholesterol levels was observed (P<0.05 versus control mice, Table 1). Concomitant treatment with LU135252 further increased total cholesterol levels, mainly due to increased HDL-cholesterol (P<0.05 versus Western-type diet and control). VLDL and triglyceride levels were not significantly affected by the different treatments (P=NS, Table 1), and the treatments had no effect on blood pressure (Table 1).

Tissue ACE Activity
In control animals, tissue ACE activity was comparable in whole kidneys and lung (33±3 versus 34±2 nmol His-Leu/mg protein, P=NS). In obese animals, ACE activity was increased in the kidney only (55±4 versus 33±3 nmol His-Leu/mg protein, P<0.05 versus control mice, Figure 1A). Long-term ET₄ receptor blockade completely prevented activation of ACE activity in the kidney and reduced activity to values below those seen in untreated obese animals (13±1, P<0.05 versus obese mice). Interestingly, long-term

![Table 1: Effects of Obesity Alone With or Without Chronic Endothelin ET₄ Receptor Blockade (LU135252) on Blood Pressure, Body Weight, and Plasma Lipids in C57BL/6J Mice](http://hyper.ahajournals.org/)
LU135252 treatment had no effect on ACE activity in the lung (Figure 1B) or liver (not shown).

**RT-PCR of ACE**

ACE mRNA expression in kidneys of obese mice chronically treated with LU135252 was not significantly different from that of obese control mice (3.48±0.33 versus 3.84±0.3 RU/μg RNA, n=6 per group, Figure 2).

**Tissue ET-1 Protein Content**

Obesity was associated with a small increase in tissue ET-1 protein content in the kidney (P<0.05); concomitant treatment with LU135252 had no effect on renal ET-1 content (Figure 3A). No change in vascular ET-1 content was observed in the aorta of obese mice compared with control animals, but long-term LU135252 treatment reduced tissue levels of ET-1 protein in the aorta (P<0.05 versus untreated mice, Figure 3B).

**Vascular Contractions in Response to Ang II**

The magnitude of contractions in response to Ang II was similar in the aorta and carotid artery of the control animals (6±2 versus 4±1% of KCl, Figure 4), and contractions were largely inhibited by indomethacin (Table 2) or the thromboxane receptor antagonist SQ30741 (Table 2). Although contractions to KCl in the aorta did not differ between groups (0.7±0.1g versus 0.6±0.1g, P=NS), obesity was associated with a 5.5-fold increase of the response to Ang II in the aorta (Figure 4A) but not in the carotid artery (Figure 4B). The enhanced contractile response to Ang II was completely prevented by long-term treatment with LU135252 in vivo (Figure 4A) or preincubation with indomethacin in vivo.

![Figure 1.](image1.png)

**Figure 1.** Tissue ACE activity in kidney and lung of C57BL/6J mice. Left, obesity was associated with a 67% increase in renal ACE activity compared with control C57BL/6J mice (P<0.05). Concomitant treatment with LU135252 for 30 weeks completely prevented the increase in ACE activity and lowered renal ACE activity below levels in control mice. Right, neither obesity nor treatment with LU135252 had any effect on pulmonary ACE activity. C indicates control mice; O, obese mice; O+LU, obese mice treated with LU135252. Data are mean±SEM. *P<0.05 vs control. †P<0.05 vs obese.

![Figure 2.](image2.png)

**Figure 2.** Expression of ACE and GAPDH mRNA in kidney. A, Renal ACE mRNA, as identified by RT-PCR yielding a 881-bp transcript, was unaffected by long-term LU135252 treatment. B, GAPDH mRNA expression (443-bp transcript).
vitro (Table 2) or SQ 30741, respectively (Table 2). Obesity in the absence or presence of LU135252 treatment had no effect on Ang II–induced contractions in the carotid artery.

**Vascular ET Binding and Cholesterol Content**
Neither obesity nor concomitant LU135252 treatment for 30 weeks had any significant effect on total ET-1 binding or binding to ETA receptors or ETB receptors in the aorta (Table 2). Similarly, cholesterol content in aortic rings was comparable in all treatment groups (Table 2).

**Histological Analysis**
Histological analysis showed normal glomeruli in control and obese mice and in obese mice treated with the ETA antagonist LU135252. With regard to other aspects of renal morphology, kidneys from untreated and LU135252-treated obese mice were not different from those of nonobese control animals. In mesenteric arteries and the thoracic aorta, completely normal vascular structure without signs of vascular hypertrophy was present in all treatment groups.

**Effects of LU135252 on Purified Human ACE**
Incubation of human ACE with LU135252 (10⁻⁶ to 10⁻⁴ mol/L) for 60 minutes had no effect on enzyme activity (data not shown).

**Discussion**
The present study presents evidence that obesity is associated with activation of tissue RAS in nonadipose tissue, resulting in the selective activation of ACE in the kidney independent of morphological changes and blood pressure. Although long-term ETA receptor blockade had no effect on body weight or renal ET-1 tissue levels, LU135252 treatment completely prevented ACE activation, suggesting a role for ETA receptors in ACE regulation. These effects likely require long-term actions on ACE, because LU135252 had no effect on activity of purified human ACE. In addition, in the aorta of C57BL/6J mice, contractions in response to Ang II, which are mediated by cyclooxygenase-dependent vasoconstrictors,

| Table 2. Effects of Obesity With or Without Chronic Endothelin ETα Receptor Blockade (LU135252) on Contractions to Ang II in Aorta and Carotid Arteries in the Presence of Indomethacin or Thromboxane A2 Receptor Antagonist SQ 30741, Vascular ET Receptor Binding, and Vascular Cholesterol Content |
|---------------------------------|-----------------|-----------------|-----------------|
| **Group**                      | Control         | Obese           | Obese + LU135252 |
| Vascular contractions          |                 |                 |                 |
| Aorta: Ang II + Indo, % KCl    | 0.7±0.5         | 0.3±0.2         | 0               |
| Aorta: Ang II + SQ 30741, % KCl| 0.5±0.2         | 0.2±0.1         | 0.1±0.1         |
| Carotid artery: Ang II + indomethacin, % KCl | 0.5±0.3         | 0.3±0.2         | 0.3±0.3         |
| Vascular [¹²⁵I]ET-1 binding     |                 |                 |                 |
| Aorta: total [¹²⁵I]ET-1 binding | ND              | 3.1±1.4         | 3.0±0.8         |
| Aorta: ETα receptor binding    | ND              | 0.47±0.4        | 0.38±0.25       |
| Aorta: ETβ receptor binding    | ND              | 0.63±0.4        | 0.5±0.1         |
| Vascular cholesterol content   |                 |                 |                 |
| Aorta: tissue cholesterol, ng/mg protein | 3±1            | 3±1             | 2±1             |

ND indicates not determined. Data are mean±SEM. *P<0.05 vs control. †P<0.05 vs obese.
were selectively enhanced in obese animals. Long-term ETₐ receptor blockade normalized the enhanced response to Ang II, indicating a role for ET-1 in the modulation of prostanoid-mediated contractions. The effects of obesity or LU135252 on vascular contractility were independent of ET receptor binding or vascular cholesterol content.

The exact mechanisms by which obesity increases cardiovascular risk are unknown, but they are likely multifactorial. Obesity is associated with changes in metabolic function, including alterations in glucose utilization and lipid abnormalities, and impairment of endothelium-dependent vasomotion and kidney function even in the absence of structural changes. Ang II–dependent hypertension in mice is associated with glomerulosclerosis and vascular hypertrophy. In the present study, renal ACE activity was increased despite in the absence of changes in renal or vascular structure, further underscoring a pressure-independent mode of action. Organ-specific activation of ACE may promote cell proliferation and vasoconstriction directly through increases in Ang II formation or indirectly through the activation of ET-1 (Figure 5), both of which contribute to renal disease.

A role for ET-1 in the pathogenesis of obesity-associated hypertension has been suggested. Impaired endothelium-dependent vasomotion is present in obese patients and may represent one of the manifestations of premature vascular disease. Components of the RAS, including ACE and AT₁ receptors, have recently been shown to contribute to diet-induced changes in endothelial function. Because anatomic heterogeneity of vascular dysfunction has been previously reported, we investigated two different vascular beds in mice to determining (1) the mechanisms by which vascular reactivity to Ang II is mediated and (2) whether obesity affects these parameters. Ang II induced contractions in both aorta and carotid artery that were in large part blocked by either indomethacin or a thromboxane A₂ receptor antagonist to a similar extent in control and obese mice, indicating that Ang II–induced contractions involve the release of vasoconstrictor prostanoids. Indeed, recent studies have suggested a role for mediators distinct from Ang II in mediation of the vasoconstrictor response to Ang II. Only in the aorta of obese mice were contractions to Ang II markedly enhanced, an effect completely prevented by concomitant ETₐ receptor blockade. Thus, obesity differentially affects contractility to Ang II, involving an ETₐ receptor–mediated component independent of tissue cholesterol or ET receptor binding capacity. Interestingly, aortic tissue ET-1 protein content was reduced in LU135252-treated mice. This raises the possibility that ET-1 tissue levels may contribute to increased prostanoid-mediated vascular contractility in vivo, as previously shown in the Dahl rat renal artery. In line with these observations, a role of ET-1 in mediation of the release of vasoconstrictor prostanoids in vitro has been demonstrated.

Total cholesterol and triglycerides levels were within the normal range in all animals. Obesity was associated with a 60-fold increase in LDL-cholesterol levels and a 3-fold increase in HDL-cholesterol levels. Changes in the lipoprotein profile have also been observed in obese humans, and low HDL-cholesterol/high LDL-cholesterol levels are associated with increased cardiovascular risk. In our study, ETₐ receptor blockade markedly increased plasma HDL cholesterol levels in obese mice independent of body weight, raising the possibility of a novel cardioprotective potential of these drugs in obesity-associated disorders. Clinical studies will
have to determine whether similar effects can be observed in humans.

In conclusion, obesity in mice is associated with tissue-specific activation of components of the renal and vascular tissue. As the level of enzyme activity and receptors, respectively. ETα receptor blockade had no effect on obesity but completely prevented the increase in renal ACE activity independent of its mRNA expression and normalized the enhanced vascular contractility to Ang II. Regulation of the tissue by ET may represent a new target for ET receptor antagonists to provide organ protection.

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