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Angiotensin-Converting Enzyme (ACE) Inhibitors Modulate Cellular Retinol-Binding Protein 1 and Adiponectin Expression in Adipocytes via the ACE-Dependent Signaling Cascade

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ABSTRACT

Inhibitors of the angiotensin-converting enzyme (ACE) decrease angiotensin II production and activate an intracellular signaling cascade that affects gene expression in endothelial cells. Because ACE inhibitors have been reported to delay the onset of type 2 diabetes, we determined ACE signaling-modulated gene expression in endothelial cells and adipocytes. Using differential gene expression analysis, several genes were identified that were 3-fold up- or down-regulated by ramiprilat in cells expressing wild-type ACE versus cells expressing a signaling-dead ACE mutant. One up-regulated gene was the cellular retinol-binding protein 1 (CRBP1). In adipocytes, the overexpression of CRBP1 enhanced (4- to 5-fold) the activity of promoters containing response elements for retinol-dependent nuclear receptors [retinoic acid receptor (RAR) and retinoid X receptor (RXR)] or peroxisome proliferator-activated receptors (PPAR). CRBP1 overexpression also enhanced the promoter activity (by 470 ± 40%) and expression/release of the anti-inflammatory and antiatherogenic adipokine adiponectin (cellular adiponectin by 196 ± 24%, soluble adiponectin by 228 ± 74%). Significantly increased adiponectin secretion was also observed after ACE inhibitor treatment of human preadipocytes, an effect prevented by small interfering RNA against CRBP1. Furthermore, in *ob*/*ob* mice, ramipril markedly potentiated both the basal (approximately 2-fold) and rosiglitazone-stimulated circulating levels of adiponectin. In patients with coronary artery disease or type 2 diabetes, ACE inhibition also significantly increased plasma adiponectin levels (1.6- or 2.1-fold, respectively). In summary, ACE inhibitors affect adipocyte homeostasis via CRBP1 through the activation of RAR/RXR-PPAR signaling and up-regulation of adiponectin. The latter may contribute to the beneficial effects of ACE inhibitors on the development of type 2 diabetes in patients with an activated renin-angiotensin system.

Several clinical studies have assessed the effects of angiotensin-converting enzyme (ACE) inhibitor therapy in patients with a high risk of experiencing cardiovascular events and revealed an apparent link between ACE inhibition and improved insulin sensitivity (Lithell et al., 1990), as well as increased regression to normoglycemia (Bosch et al., 2006). Indeed, ACE inhibition was associated with a lower rate of new-onset type 2 diabetes (self-reported) in high-risk patients with hypertension, coronary artery disease, or heart failure (Oksa et al., 1994; Yusuf et al., 2000; Opie and Schall, 2004; Gillespie et al., 2005; Dagenais et al., 2006). It has been suggested that these beneficial effects may be related to the modulation of the anti-inflammatory and antiatherosclerotic adipokine adiponectin (Furuhashi et al., 2003; Hermann et al., 2006).

Of the pharmaceutical agents currently prescribed to patients with cardiovascular disease, a similar action has been attributed to telmisartan, a specific inhibitor of the AT-1 angiotensin II receptor that is known to directly activate peroxisome proliferator-activated receptor-γ (PPARγ) (Benson et al., 2004; Clasen et al., 2005). The molecular mechanism(s) by which ACE inhibitors affect plasma adiponectin levels is unclear, but their actions are not necessarily related to changes in the local concentrations of either angiotensin II

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ABBREVIATIONS: ACE, angiotensin-converting enzyme; Adn, adiponectin; atRA, all-*trans*-retinoic acid; CRBP1, cellular retinol-binding protein 1; 9cisRA, 9-*cis*-retinoic acid; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; RAR, retinoic acid receptor; siRNA, small interfering RNA; GFP, green fluorescent protein.

or bradykinin. Indeed, several of the apparently beneficial effects of ACE inhibitor therapy cannot be directly related to changes in enzyme activity and changes in angiotensin II or bradykinin levels, and we have reported previously that the binding of an ACE inhibitor to ACE results in the phosphorylation of the cytoplasmic tail of the enzyme (on serine 1270) and activation of a specific signaling cascade that can affect the expression of endothelial genes (Fleming, 2006). Because adipose tissue expresses all of the components required to constitute a fully active renin-angiotensin system (Engeli et al., 2000) and obesity is positively correlated with the development of type 2 diabetes (Ferchak and Meneghini, 2004), we analyzed the mechanisms by which ACE inhibition affects adiponectin levels, concentrating on the potential role of the recently described ACE signaling cascade.

Materials and Methods

Materials

Rosiglitazone was from Alexis Biochemicals (ALEXIS Corporation, Lausen, Switzerland). All other substances were obtained from Sigma-Aldrich (Deisenhofen, Germany).

Cell Culture

Endothelial Cells. Human umbilical vein endothelial cells were isolated and cultured as described previously (Busse and Lamontagne, 1991). In some experiments, human umbilical vein endothelial cells were infected with adenoviruses encoding human somatic full-length wild-type ACE (wtACE) or the nonphosphorylatable signaling-dead ACE mutant (ACE S1270A).

Adipocytes. Primary human visceral preadipocytes (Lonza Verviers SPRL, Verviers, Belgium) were cultured as recommended by the supplier in Preadipocyte Basal Medium 2, containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. After reaching confluence, cells were incubated in for a further 14 days with or without ramiprilat (100 nM), rosiglitazone (100 nM), or a combination of both. As a positive control, some cells were differentiated to adipocytes using commercially available differentiation medium (Lonza Verviers SPRL). 3T3-L1 preadipocytes (Sigma-Aldrich, St. Louis, MO) were cultured and differentiated as described previously (Schupp et al., 2004). Because ACE expression was not detectable in 3T3 L1 cells, experiments with ACE inhibitors were performed in 3T3 L1 cells stably transfected with human somatic wild-type ACE, as described for endothelial cells previously (Kohlstedt et al., 2002).

Transfection and Luciferase Reporter Gene Assay

The human adipocyte apM-1 promoter luciferase construct was kindly provided by A. Schäffler (Regensburg, Germany), the luciferase reporter gene constructs carrying promoters with DR1 (RXR-RXR) or DR5 (RAR-RXR) response elements were from S. J. Collins (Seattle, WA), and the PPRE reporter gene construct was from N. Marx (Ulm, Germany). In some experiments, cells were also transfected with a pQE30-plasmid carrying the His-tagged cellular retinol binding protein 1 (CRBP1) sequence (provided by Dr. K. Palczewski, Cleveland, OH) or green fluorescent protein (GFP). 3T3-L1 preadipocytes were transiently transfected using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA), and luciferase activity was assessed using a commercially available kit (Promega, Mannheim, Germany).

Down-Regulation of CRBP1 Using Small Interfering RNAs

To down-regulate CRBP1 in adipocytes, small interfering RNAs (siRNAs) were obtained from Invitrogen (Karlsruhe, Germany). An unrelated siRNA ("sense" soluble epoxide hydrolase 2: 5′-AAC CTC AGA TCT ACA AGT TTC CCT GTC TC-3′) was used as control siRNA. Transfection was performed using the Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol.

Microarray Analysis

DNA microarray analysis was performed using Affymetrix microarray oligonucleotide chips (HG-U133A; Affymetrix Inc., Santa Clara, CA). Human umbilical endothelial cells (second passage, and no longer expressing endogenous ACE) were transduced with either wtACE or the S1270A ACE mutant adenoviruses. Twenty-four hours after infection, the cells were treated with either solvent (phosphate-buffered saline) or ramiprilat (100 nM) for up to 48 h. Total RNA was isolated using the Absolutely RNA Miniprep Kit (Stratagene, Cambridge, UK), and RNA quality was assessed using the Bioanalyzer 2100 system (Agilent, Waldbronn, Germany). Data analysis was performed with the GeneSpring software version 4.2 (Silicon Genetics, San Carlos, CA) and Microarray Analysis Suites 5.0 (MAS 5.0; Affymetrix). Genes, which were determined as "present" and which consistently (i.e., in experiments using three different endothelial cell batches) exhibited a more than 3-fold increase or decrease in ramiprilat-treated versus solvent-treated wtACE-infected cells but that did not change in cells expressing the S1270A ACE mutant, were considered to be "ACE signaling-regulated genes."

Immunoblotting

Cells were either boiled in SDS sample buffer or lysed in Nonidet lysis buffer [20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 25 mM β-glycerophosphate, 10% (v/v) glycerol, 1 mM $Na_4P_2O_7$, 10 nM okadaic acid, 2 mM Na_3VO_4, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 10 μg/ml trypsin inhibitor, 44 μg/ml phenylmethylsulfonyl fluoride, and 1% (v/v) Nonidet P-40], left on ice for 10 min, and centrifuged at 10,000*g* for 10 min. Plasma or cell supernatants were heated in SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis, as described previously (Kohlstedt et al., 2002). Proteins were detected using their respective antibodies and were visualized by enhanced chemiluminescence using a commercially available kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The adiponectin antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), the antibody against CRBP1 was from Affinity BioReagents Inc. (Golden, CO), and the anti-His antibody was from QIAGEN GmbH (Hilden, Germany).

Reverse-Transcriptase Polymerase Chain Reaction

Equal amounts of RNA were reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen) and specific CRBP-1 primers (forward: 5′-AGATGAGAGTGGAAGGTGTG-3′, and reverse: 5′-GACAAAGCCAAGAGACAGAC-3′) as described previously (Lepreux et al., 2004). Polymerase chain reaction products were separated on a 2% Tris-acetate-EDTA agarose gel and visualized by staining with ethidium bromide. The same samples were used for amplification of the housekeeping gene, β-actin.

Plasma Adiponectin Levels

Mice. Obese mice (*Lep*^{ob}; referred to as *ob* or *ob/ob* mice, 10 weeks old) were obtained from Charles River (Sulzfeld, Germany) and treated with ramipril (5 mg/kg/day orally), rosiglitazone (3 mg/kg/day orally), or a combination of both drugs for 10 weeks. Thereafter, the animals were anesthetized (isofuran 1.5%) and euthanized by a transverse cut through the large abdominal vessel. Plasma adiponectin levels were determined by enzyme-linked immunosorbent assay (Phoenix Pharmaceuticals, Inc., Belmont, CA) according to the manufacturer's instructions.

Patients. Circulating levels of adiponectin were measured by Sandwich ELISA (Millipore Bioscience Research Reagents, Temecula, CA) in plasma obtained from patients visiting the outpatients department with either coronary artery disease or type 2 diabetes some of whom were already undergoing treatment with an ACE inhibitor (ramipril 5 mg/day). Concurrent medication did not

differ between the patients. In the case of type 2 diabetes, we also analyzed the plasma of patients receiving rosiglitazone (4 mg/day). Plasma samples were obtained with informed consent of the patient.

Statistical Analysis

Data are expressed as the means ± S.E.M., and statistical evaluation was performed using Student's t test for unpaired data or one-way analysis of variance followed by a Bonferroni t test where appropriate. Values of $p < 0.05$ were considered statistically significant.

Results

Identification of Genes Influenced by ACE Inhibitor-Activated ACE Signaling. To identify genes regulated by the ACE signaling cascade, we performed a differential gene expression analysis (Affymetrix microarray HG-U133) using human umbilical vein endothelial cells (passage 2). These cells, which no longer endogenously expressed ACE protein, were infected with either human somatic ACE (wtACE) or the nonphosphorylatable, signaling-dead ACE (S1270A) mutant 24 h before incubation with either solvent or ramiprilat for an additional 48 h. Gene expression profiles were then determined and compared between wtACE and S1270A ACE. Genes were considered to be ACE signaling-regulated when more than 3-fold differences in the RNA levels were detected in response to ramiprilat stimulation in wtACE-expressing cells but not in cells expressing the S1270A mutant. Following the stepwise data analysis strategy described, 12 genes (Table 1) were found to be significantly more highly expressed 72 h after infection with wtACE compared with the control cells (non–ACE inhibitor-treated and infected with

TABLE 1

Genes identified by microarray analysis to be at least 3-fold more highly expressed in human umbilical vein endothelial cells 72 hours after infection with wtACE compared with the control cells (non–ACE-inhibitor-treated and infected with ACE-S1270A)

Exactvalues of changes are indicated in the table (x-fold). CRBP1 is in boldface type.

HG-U133A	Gene	Gene Bank	Description	Map	x-Fold Changes
213665_at	SOX4	AI989477	SOX4 protein	6p22.3	4
211074_at		AF000381	EST	N.A.	3.6
217317_s_at	MN7	AB002391	D15F37 (pseudogene)	15	3.5
211478_s_at	CD26	M74777	Dipeptidylpeptidase IV	2q24.3	4.6
216894_x_at	KIP2	D64137	Cyclin-dependent kinase inhibitor p57^{KIP2}	11p15.5	3.6
203372_s_at	SSI-2	AB004903	STAT induced STAT inhibitor-2	12q	3.3
203423_at	**CRBP1**	**NM_002899**	**Retinol-binding protein 1, cellular**	**3q23**	**3.2**
220755_s_at	G8	NM_016947	G8 protein	6p21.3	3.1
204340_at		NM_003492	Chromosome X open reading frame 12	Xq28	3.1
213156_at		AL049423	EST	3	3.1
205392_s_at	SY14	NM_004166	Small-inducible cytokine subfamily A, member 14	17q11.2	3.1
213593_s_at		AW978896	EST	7	3.1

EST, expressed sequence tag; N.A., not available.

TABLE 2

Genes identified by microarray analysis to be at least 3-fold lower expressed in human umbilical vein endothelial cells 72 hours after infection with wtACE compared with the control cells (non–ACE-inhibitor-treated and infected with ACE-S1270A)

HG-U133A	Gene	Gene Bank	Description	Map
202859_x_at	IL8	NM_000584	Interleukin 8	4q13-q21
212909_at	GPR39	AL567376		2q21-q22
216532_x_at		AL138831	EST	10
214011_s_at	HSPC111	BE314601	EST	5
201155_s_at	CPRP1	NM_014874	Mitofusin 2	1p36.21
204826_at	FBX1	NM_001761	Cyclin F	16p13.3
218264_at	BCCIP	NM_016567	BRCA2 and CDKN1A-interacting protein	10
206055_s_at	SNRPA1	NM_003090	Small nuclear ribonucleoprotein polypeptide A′	22q
218244_at	FLJ20736	NM_017948	Hypothetical protein FLJ20736	9
206461_x_at	MT1	NM_005951	Metallothionein 1H	16q13
209408_at	MCAK	U63743	Mitotic centromere-associated kinesin	1
208152_s_at	GURDB	NM_004728	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide	10q21
202094_at	BIRC5	AA648913		17q25
204146_at	PIR51	BE966146		12p13.2-p13.1
204531_s_at	PSCP	NM_007295	Breast cancer 1	17q21
219306_at	HKLP2, hklp2	NM_020242	Kinesin-like 7	3p21.32
202095_s_at	Survivin	NM_001168	Survivin	17q25
202580_x_at	MPP2	NM_021953	Forkhead box M1	12p13
204768_s_at	RAD2	NM_004111		11q12
215218_s_at		AC004144	EST	19q13.1
204318_s_at	B99, GTSE-1	NM_016426	G_2 and S phase-expressed 1	22q13.2-q13.3
218782_s_at	PRO2000	NM_014109	PRO2000 protein	8
222077_s_at	RACGAP1	AU153848		12p13.2-p13.1
211814_s_at	CYCE2	AF112857	Cyclin E2	8q22.2
218542_at	FLJ10540	NM_018131	Hypothetical protein FLJ10540	10
218039_at	ANKT	NM_016359	Nucleolar protein ANKT	15
217010_s_at	CDC25C	AF277724	Cell division cycle 25C	5q31
216870_x_at	LEU2	AF264787	Deleted in lymphocytic leukemia, 2	13q14.3
220060_s_at	FLJ20641	NM_017915	Hypothetical protein FLJ20641	12q23.3

EST, expressed sequence tag.

ACE-S1270A). On the other hand, 29 genes (Table 2) were significantly down-regulated in cells infected with wtACE compared with the appropriate control cells.

One of the genes up-regulated by ramiprilat was CRBP1, a ubiquitous cytoplasmic protein of 134 amino acids (15.7 kDa) belonging to the family of fatty-acid binding proteins. To confirm the microarray results, we determined the effect of ramiprilat (100 nM) on CRBP1 mRNA (Fig. 1a) and protein (Fig. 1b). Indeed, in human umbilical vein endothelial cells, ramiprilat up-regulated CRBP1 levels to approximately the same extent as all-*trans*-retinoic acid (atRA, 1 μM) and 9-*cis*-retinoic acid (9cisRA, 1 μM), which have been reported previously to increase CRBP1 expression (Harada et al., 1995). Ramiprilat was without effect on CRBP1 levels in ACE-deficient 3T3 L1 cells (data not shown), demonstrating that the ACE inhibitor requires the presence of ACE to affect CRBP1 gene expression.

Role of CRBP1 in Adipocyte Gene Expression. CRBP1 mediates the intracellular transport of retinol and might consequently activate retinol-dependent nuclear receptors, the retinoid X receptor (RXR) and the retinoic acid receptor (RAR) (Ghyselinck et al., 1999), both of which are reported to transactivate PPARs via heterodimerization (Michalik and Wahli, 2007). We therefore determined whether or not CRBP1 overexpression affected the activity of promoters carrying response elements for PPAR (PPRE), RXR (RXRE) or RAR (RARE). These experiments were performed in 3T3 L1 cells, which express higher PPAR levels than the endothelial cells initially screened. All of the ACE inhibitors tested up-regulated CRBP1 expression in these cells (Fig. 2a) and in differentiated human adipocytes (Fig. 2b), implying that the ability to modulate adipocyte CRBP1 expression is shared by the ACE inhibitor class of compounds.

The overexpression of CRBP1 in 3T3 L1 cells significantly increased the activity of all of the promoters studied compared with cells overexpressing GFP (Fig. 3a). Ramiprilat had no direct effect on PPARγ activity because the activity of the PPRE-carrying promoter was not modulated in ACE-transfected 3T3 L1 cells or endothelial cells after short-term stimulation with ramiprilat (100 nM, 24 h, data not shown). Therefore, in contrast to some AT-1 receptor antagonists (Clasen et al., 2005), the ACE inhibitor ramiprilat had no direct effect on PPARγ activity.

Because adiponectin is classified as a PPARγ-regulated gene, we next assessed the consequences of CRBP1 overexpression on the activity of a luciferase-coupled adiponectin reporter construct. CRBP1 overexpression led to the activation (4.7 $\pm$ 0.4-fold, $n = 10$) of the human adiponectin promoter expressed in 3T3 L1 cells (Fig. 3b).

Effect of ACE Inhibitors on Adiponectin Expression in Vitro and in Vivo. To verify that the increase in adiponectin promoter activity reflected an increase in the production of adiponectin protein, 3T3 L1 cells were transfected with either GFP or CRBP1, and the levels of cellular adiponectin and the soluble adiponectin secreted into the cell culture medium were determined. Cellular adiponectin and soluble adiponectin levels were significantly enhanced in response to CRBP1 overexpression (Fig. 4a).

To determine whether ACE inhibitors affect adiponectin gene expression, ACE-overexpressing 3T3 L1 preadipocytes were incubated with imidaprilat, enalaprilat, or ramiprilat (each 100 nM for 48 h), and soluble adiponectin levels in the cell supernatant were determined (Fig. 4b). All three ACE inhibitors significantly increased soluble adiponectin production. Adiponectin release was not affected by ACE inhibitors in ACE-deficient 3T3 L1 cells (data not shown). In addition, to confirm the effect of ACE inhibitors on adiponectin gene expression, cultured human preadipocytes were treated for 48 h (data not shown) or 2 weeks with ramiprilat or enala-

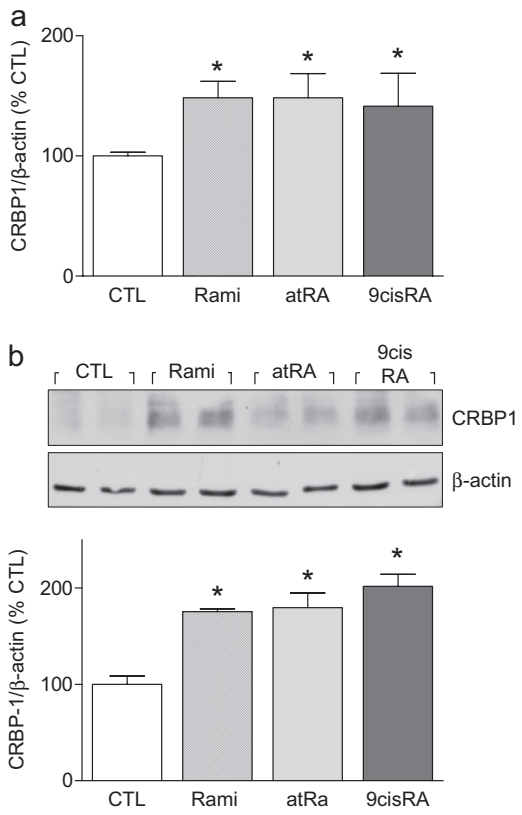

Fig. 1. Effect of ACE inhibitors on the expression of CRBP1. Effect of ramiprilat (Rami, 100 nM), atRA (1 μM), and 9cisRA (1 μM) on CRBP1 mRNA (a) and protein (b) expression in human endothelial cells. The bar graphs summarize data obtained in three different experiments. $*$, $p <$ 0.05 versus control (CTL).

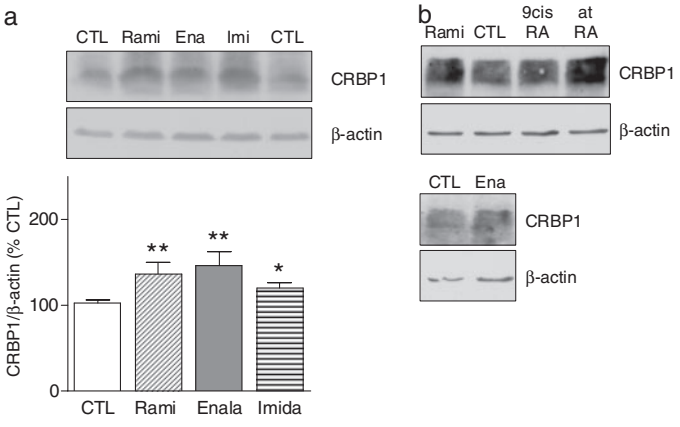

Fig. 2. CRBP1 expression after ACE inhibition in adipocytes. a, effect of solvent (CTL), ramiprilat (Rami), enalaprilat (Ena), and imidaprilat (Imi), each 100 nM, for 48 h on CRBP1 protein expression in 3T3 L1 cells. b, representative Western blots showing the effect of solvent, ramiprilat, enalaprilat, atRA (1 μM), or 9cisRA (1 μM) on CRBP1 expression in human visceral adipocytes 48 h after stimulation. Similar results were obtained in three additional experiments.

prilat (each 100 nM, Fig. 4c). Cell culture medium was changed once within the stimulation and collected over the last 48 h to recover soluble adiponectin from the cell supernatant. Compared with solvent-treated cells, both of the ACE inhibitors significantly increased the release of adiponectin from preadipocytes. Moreover, the increase in adiponectin expression was comparable with that detected in cells treated with the rosiglitazone (100 nM) or a commercially available differentiation medium.

To verify that the ACE inhibitor-induced up-regulation of adiponectin secretion is dependent on CRBP1 in human preadipocytes, we performed experiments using an siRNA approach. In human embryonic kidney 293 cells transiently overexpressing histidine-tagged CRBP1, the siRNA treatment significantly decreased CRBP1 levels (Fig. 5a). In primary human preadipocytes, the siRNA directed against CRBP1 had little effect on basal CRBP1 levels but effectively blocked both the ramiprilat (100 nM, 48 h)-induced increase in CRBP1 expression and adiponectin release (Fig. 5, b and c). Treatment with the unrelated siRNA was without effect on either CRBP1 or adiponectin levels.

We next compared the effects of ramipril (5 mg/kg/day orally), rosiglitazone (3 mg/kg/day orally), or the combination of both (10 weeks) on circulating adiponectin levels in *ob/ob* mice. There were no significant differences in body weight, blood glucose levels, circulating free fatty acids, or triglycerides associated with the respective therapies (data not shown). Although ramipril treatment was associated with a significant increase in circulating adiponectin levels in the animals studied (Fig. 6a), the response was not as pronounced as the increase detected in rosiglitazone-treated animals or animals receiving both compounds. However, treatment with ramiprilat markedly potentiated the effect of rosiglitazone on adiponectin levels.

Plasma adiponectin was also determined in patients with coronary artery disease who were either not receiving an ACE inhibitor or receiving ramipril (5 mg/day) and from patients with type 2 diabetes, some of whom were being treated with an ACE inhibitor or with rosiglitazone. Even though the cohort of patients studied was small, the results obtained clearly showed that ACE inhibition increased circulating levels of soluble adiponectin (Fig. 6b) and thereby confirm findings from earlier studies (Furuhashi et al., 2003; Hermann et al., 2006). Circulating levels of leptin did not differ between the samples studied (data not shown).

Discussion

In the present study, we observed that treatment with an ACE inhibitor enhances circulating adiponectin levels in vitro and in vivo and potentiates the adiponectin-enhancing

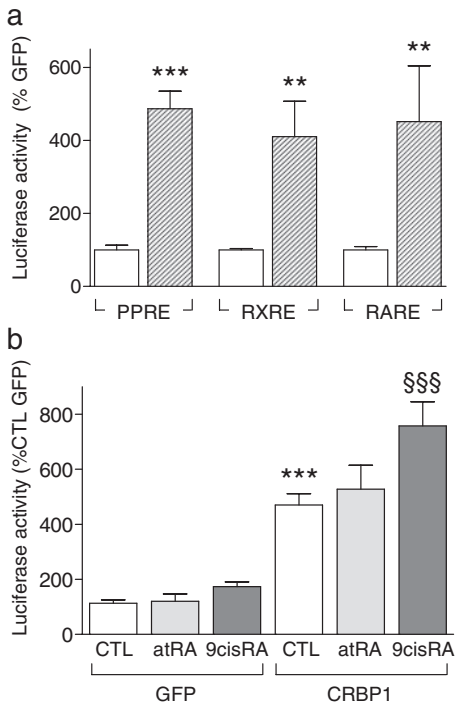

Fig. 3. Effect of CRBP1 on PPRE, RXRE, RARE and adiponectin promoter activity. a, effect of CRBP1 overexpression (shaded bars) on the activity of luciferase-coupled promoters carrying PPREs, an RXR response element (RXRE), or RAR response element (RARE) in 3T3 L1 cells. Control cells were transfected with GFP (□). b, activity of the adiponectin promoter construct in 3T3 L1 cells cotransfected with either GFP or CRBP1 and treated with solvent (CTL), atRA (1 μM) or 9cisRA (1 μM) for 36 h. The bar graphs summarize data obtained in 3 to 12 separate experiments; **, $p < 0.01$, and ***, $p < 0.001$ versus CTL or CTL/GFP; §§§, $p < 0.001$ versus control CRBP1 transfection (CTL CRBP1).

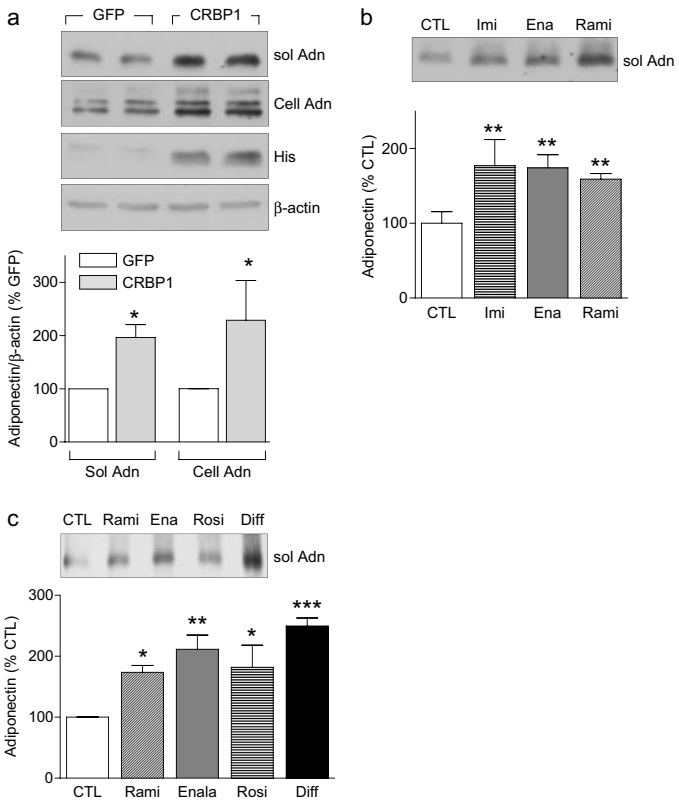

Fig. 4. Effect of CRBP1 overexpression and ACE inhibition on adiponectin levels in vitro. a, effect of GFP and CRBP1 overexpression on soluble adiponectin (sol Adn) and cellular adiponectin (Cell Adn) levels in 3T3 L1 cells. To ensure transfection and equal protein loading, membranes were reprobed with an anti-histidine antibody and for β-actin. b, representative Western blot showing the effect of solvent (CTL), imidaprilat (Imi, 100 nM), enalaprilat (Ena, 100 nM), and ramiprilat (Rami, 100 nM) on the amount of soluble adiponectin (sol Adn) recovered from the media of differentiated 3T3 L1 cells. c, effect of solvent, ramiprilat, enalaprilat, rosiglitazone (Rosi, 100 nM), and differentiation medium (Diff) on the release of soluble adiponectin from human visceral adipocytes. Cells were stimulated for 12 days before collecting culture medium over the last 48 h to recover soluble adiponectin. Bar graphs summarize data obtained in four to eight separate experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus GFP or control (CTL).

effect of the PPARγ agonist rosiglitazone in *ob/ob* mice. The molecular mechanisms underlying this effect were linked to the expression of CRBP1, which was identified as a gene regulated by the ACE signaling cascade. Increases in the CRBP1 protein led to the up-regulation of adiponectin most probably via the activation of retinol-dependent nuclear receptor proteins (RAR/RXR) and cross-activation of PPARs that are activated via heterodimerization with RAR/RXR.

The beneficial effects of adiponectin on glucose and lipid metabolism have been well described, and direct administration of adiponectin has been shown to be beneficial in animal models of diabetes, obesity, and atherosclerosis (Oh et al., 2007). A decrease in adiponectin levels, as observed in cases of coronary artery disease (Nakamura et al., 2004) or type 2 diabetes (Hotta et al., 2000), has been associated with disease progression. Thus, adiponectin has been identified as a new therapeutic target, and increased adiponectin levels improve insulin sensitivity and cardiovascular function. There is certainly circumstantial evidence linking the activation of the renin-angiotensin system with adiponectin levels, and both AT-1 receptors and ACE inhibitors can elicit the same effect in patients with essential hypertension (Furuhashi et al., 2003). However, although AT-1 receptor blockers increase adiponectin levels by directly activating PPARγ (Clasen et al., 2005), the mechanism by which ACE inhibitors affects adiponectin levels is unknown. However, we observed that ramiprilat has no direct effect on PPARγ activity (K. Kohlstedt, unpublished observations) and that adiponectin up-regulation also occurs in vitro, suggesting that the ACE inhibitor-induced increase in adiponectin is not simply related to a reduction in blood pressure. Interestingly, ramipril also potentiated the adiponectin-enhancing effect of the PPARγ agonist rosiglitazone. The latter effect cannot be explained by changes in body weight, triglyceride, free fatty acid, or blood glucose levels, parameters that have been shown previously to affect adiponectin expression (Esposito et al., 2003).

Many ACE inhibitor-mediated effects cannot be explained by a change in blood pressure and have been attributed to pleiotropic actions that are not necessarily related to the activity of the enzyme per se. One such effect is the activation of the ACE signaling cascade, which involves the dimerization of the enzyme in response to ACE inhibitor binding (Kohlstedt et al., 2006), the activation of the kinase CK2, which phosphorylates the cytoplasmic tail of the enzyme, and c-Jun NH_2-terminal kinase/activator protein-1 signaling (Kohlstedt et al., 2002, 2004). Although ACE and cyclooxygenase 2 were identified previously as ACE signaling-regulated genes, the microarray used identified CRBP1 as being regulated by a signal transduction pathway that requires the phosphorylation of the cytoplasmic tail of ACE, although at this stage, it is unclear whether or not the molecular events resulting in its up-regulation are the same. CRBP is a small (15 kDa) cytosolic protein that is widely expressed and highly conserved among mammals, it is essential for retinol homeostasis (Ghyselinck et al., 1999) and acts as a chaperone mediating the intracellular transport of retinol to its metabolizing enzymes (Napoli, 1999). Because the retinoids regulate a myriad of physiological processes, including hematopoiesis and differentiation, changes in CRBP1 expression can markedly affect cellular homeostasis; indeed, the loss of CRBP1 has been linked with cancer progression (Esteller et al., 2002). The results of the present investigation indicate that ACE inhibitors increase the expression of CRBP1. However, because this protein would be expected to have greater effects on adipocytes, which in addition to being involved in retinoid storage and metabolism express high levels of ACE (Wang et al., 2006), the majority of our study investigated its effects either in adipocytes or in vivo. Studies in rats indicate that 15 to 20% of the total retinoid content in the body can be stored in adipose tissue (Tsutsumi et al., 1992), and CRBP1 expression in adipocytes is regulated by retinoic acid, dexamethasone, and tri-iodothyronine (Okuno et al., 1995). More-

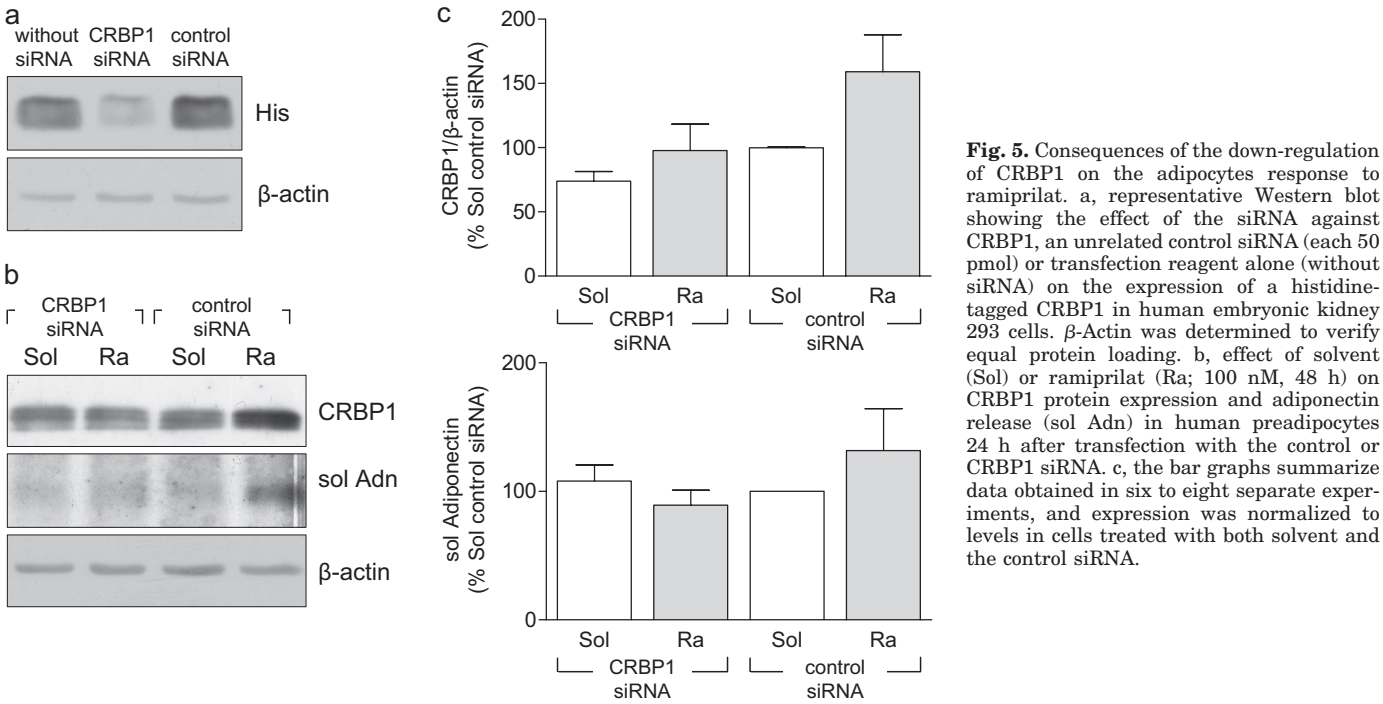

Fig. 5. Consequences of the down-regulation of CRBP1 on the adipocytes response to ramiprilat. a, representative Western blot showing the effect of the siRNA against CRBP1, an unrelated control siRNA (each 50 pmol) or transfection reagent alone (without siRNA) on the expression of a histidine-tagged CRBP1 in human embryonic kidney 293 cells. β-Actin was determined to verify equal protein loading. b, effect of solvent (Sol) or ramiprilat (Ra; 100 nM, 48 h) on CRBP1 protein expression and adiponectin release (sol Adn) in human preadipocytes 24 h after transfection with the control or CRBP1 siRNA. c, the bar graphs summarize data obtained in six to eight separate experiments, and expression was normalized to levels in cells treated with both solvent and the control siRNA.

over, the overexpression of CRBP1 is reported to increase retinol metabolism (Lissoos et al., 1995) and has been linked to the activity of vitamin A nuclear receptors such as RAR (Farias et al., 2005b).

Two conserved families of nuclear receptors encode retinoic acid receptors; RARs, which bind both the natural isoform, all-*trans* retinoic acid, and the 9-*cis* retinoic acid isomer, and RXRs, which bind only 9-*cis* retinoic acid (Farias et al., 2005a). Our data indicate that the overexpression of CRBP1 in 3T3 L1 adipocytes not only leads to the activation of promoters carrying response elements for RAR and RXR but also to those carrying the PPRE. The transactivation of PPAR via RXR or RAR has indeed been well described previously (Chawla et al., 2001; Michalik and Wahli, 2007) and probably represents the molecular mechanism by which ACE inhibitors affect the expression of adipocyte-derived genes, such as adiponectin. The findings that the overexpression of CRBP1 was sufficient to increase adiponectin promoter activity, as well as protein synthesis and secretion, and that the down-regulation of CRBP1 in adipocytes prevented the ramiprilat-induced increase in adiponectin release support this conclusion.

Taken together, the results of the present investigation indicate that ACE inhibitors modulate adipocyte gene expression via the induction of CRBP1. The subsequent up-regulation and release of adiponectin potentially affects many tissues in addition to the cardiovascular system that might contribute to the effects of ACE inhibitors on the progression of atherosclerosis and/or insulin insensitivity.

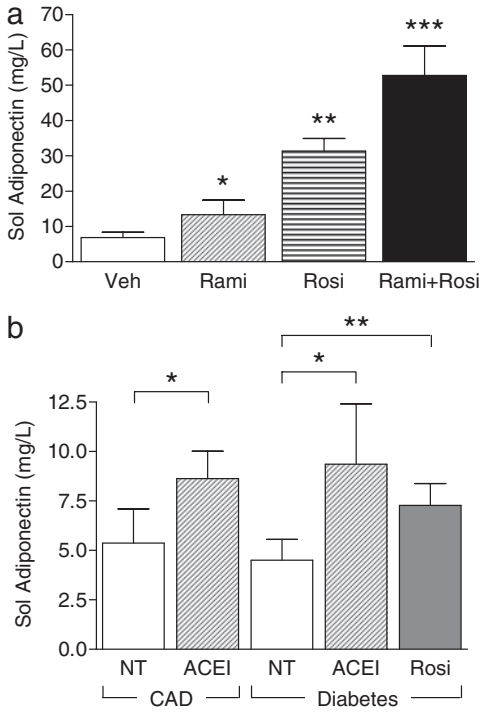

Fig. 6. Effect of ACE inhibition on adiponectin levels in vivo. a, effect of ramiprilat and/or rosiglitazone on adiponectin levels in *ob/ob* mice treated with either vehicle (Veh, 0,25% carboxymethylcellulose), ramipril (Rami, 5 mg/kg/day orally), rosiglitazone (Rosi, 3 mg/kg/day orally), or a combination of both drugs for 10 weeks. b, adiponectin levels in plasma from patients with either coronary artery disease (CAD) or type 2 diabetes (Diabetes) who were either untreated (NT) or treated with ramipril (Rami, 5 mg/day) or rosiglitazone (Rosi, 4 mg/day). The bar graphs summarize data obtained in 4 to 12 independent mice/patients; *, $p < 0.05$, **, $p < 0.01$, effect of drug treatment versus CTL or NT.

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