Targeted Deletion of Murine CEACAM 1 Activates PI3K-Akt Signaling and Contributes to the Expression of (Pro)Renin Receptor via CREB Family and NF-κB Transcription Factors

Jiqian Huang, Kelly J. Ledford, William B. Pitkin, Lucia Russo, Sonia M. Najjar,* Helmy M. Siragy*

Abstract—The carcinoembryonic antigen–related cell adhesion molecule 1 regulates insulin sensitivity by promoting hepatic insulin clearance. Mice bearing a null mutation of Ceacam1 gene (Cc1−/−) develop impaired insulin clearance followed by hyperinsulinemia and insulin resistance, in addition to visceral obesity and increased plasma fatty acids. Because insulin resistance is associated with increased blood pressure, we investigated whether they develop higher blood pressure with activated renal renin-angiotensin system and whether this is mediated, in part, by the upregulation of renal (pro)renin receptor (PRR) expression. Compared with age-matched wild-type littermates, Cc1−/− mice exhibited increased blood pressure with increased activation of renal renin-angiotensin systems and renal PRR expression. Cytoplasmic and nuclear immunostaining of phospho-Pi3K p85α and phospho-Akt was enhanced in the kidney of Cc1−/− mice. In murine renal inner medullary collecting duct epithelial cells with lentiviral-mediated small hairpin RNA knockdown of carcinoembryonic antigen–related cell adhesion molecule 1, PRR expression was upregulated and phosphorylation of Pi3K (Tyr508), Akt (Ser473), NF-κB p65 (Ser276), cAMP response element–binding protein/activated transcription factor (ATF)-1 (Ser133), and ATF-2 (Thr71) was enhanced. Inhibiting Pi3K with LY294002 or Akt with Akt inhibitor VIII attenuated PRR expression. In conclusion, global null deletion of Ceacam1 caused an increase in blood pressure with increased renin-angiotensin system activation together with upregulation of PRR via Pi3K-Akt activation of cAMP response element–binding protein 1, ATF-1, ATF-2, and NF-κB p65 transcription factors. (Hypertension. 2013;62:317-323.) ● Online Data Supplement

Key Words: CEACAM1 ■ CREB protein ■ kidney ■ (pro)renin receptor ■ renin-angiotensin system

Insulin resistance is commonly associated with visceral obesity, hypertension, and hyperinsulinemia.1-5 It is also associated with increased activity of the renin-angiotensin system (RAS).6-7 Experimental studies and clinical trials demonstrated that blocking RAS components attenuates or improves certain aspects of the clinical manifestation of this syndrome.1-3-5

Several nutritionally and chemically induced in addition to genetically engineered mouse models of insulin resistance and obesity have been generated,2,11 but few have been fully characterized for increased blood pressure (BP). Mice bearing a null mutation of carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1, Cc1−/−) exhibit hyperinsulinemia resulting from impaired insulin clearance.4,12

Hyperinsulinemia in these mice causes insulin resistance in addition to increased hepatic lipid production and redistribution of the white adipose depot to cause visceral adiposity and a rise in plasma free fatty acids.14 Because insulin resistance is commonly associated with hypertension,4,5 this study was conducted to evaluate whether Cc1−/− mice develop an elevation in BP with activation of RAS and higher expression of (pro)renin receptor (PRR), a new component of RAS,16,17 which is associated with increased BP and diabetic nephropathy.18-22

Experimental Protocol

Cc1−/− null mice were generated and backcrossed for ≥12 times onto the C57BL/6 background.23 All procedures were approved by the University of Virginia Animal Care and Use Committee. Male mice at 6 months of age were studied. Body weight, blood glucose, plasma insulin and biochemistry, insulin tolerance, and systolic BP were monitored. Twenty-four–hour urine samples were collected to evaluate the urinary levels of albumin-to-creatinine ratio. Renal interstitial fluid was collected to determine angiotensin II level. Kidneys were harvested for protein and total RNA extraction, and parts were also fixed with Bouin fixative for immunohistochemical analysis of PRR,
phospho-PI3K p85α (Tyr 508), and phospho-Akt (Ser473). Renal PRR expression was also evaluated with semiquantitative real-time polymerase chain reaction and Western blotting. Procedures are detailed in the online-only Data Supplement.

Mouse renal inner medullary collecting duct (IMCD) epithelial cells were subjected to small hairpin RNA (shRNA) transduction with mouse CEACAM1 and scramble lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA) per manufacturer’s instruction. Stable shRNA expression was assessed using quantitative real-time polymerase chain reaction, Western blot analysis, and immunofluorescence staining (Figure S1 in the online-only Data Supplement). In some experiments, shRNA-transduced IMCD cells were treated with PI3K kinase inhibitor LY294002, Akt kinase inhibitor II (EMD Millipore, Billerica, MA), and CREB-binding protein (CBP)–cAMP response element (CRE)–binding protein (CREB) interaction inhibitor (CalBiochem, La Jolla, CA), as detailed in the online-only Data Supplement.

Data analysis was carried out using STATISTICA version 5.0 (StatSoft, Tulsa OK). Results are expressed as mean±SEM. Comparisons among different treatment groups were evaluated by ANOVA with repeated measures, and the Bonferroni correction method as a post hoc test. P<0.05 was statistically significant.

Results

Metabolic Phenotyping of Cc1−/− Mice

As previously shown,14 6-month-old Cc1−/− mice exhibited higher body weight, visceral obesity, plasma free fatty acids, fed blood glucose levels, and hyperinsulinemia compared with age-matched wild-type littermates (Cc1+/+; Table). Moreover, Cc1−/− exhibited reduced insulin tolerance relative to wild-type animals (Figure 1A), consistent with hyperinsulinemia-euglycemic clamp analysis showing the development of systemic insulin resistance in these mice.14,15 Although the mice are insulin resistant, they did not develop overt diabetes mellitus, as manifested by normal fasting glucose level (Table), consistent with no effect of Ceacam1 deletion on β-cell function.14

Elevation in BP and Upregulation of RAS Components in Murine Kidneys

Because insulin resistance is associated with changes in BP, we then evaluated this parameter. Cc1−/− mice exhibited...
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Activated PI3K-Akt Signaling-Enhanced PRR Expression

elevated systolic BP (Figure 1B) and urine albumin-to-creatinine ratio (Figure 1C). Moreover, their mRNA and protein levels of renal renin, angiotensin-converting enzyme, angiotensinogen, and angiotensin II type I receptor were also elevated (Figure 1E–1L). Cc1−/− mice had significantly higher levels of renal interstitial fluid angiotensin II than Cc1+/+ mice (Figure 1D).

**Elevated Renal PRR Expression in Cc1−/− Kidneys**

Because PRR is associated with a rise in BP, we examined its expression level in the kidney of these mice. Renal PRR mRNA (Figure 2A) and protein levels (Figure 2B) were elevated in Cc1−/− mice. Immunohistochemical staining revealed enhanced PRR expression in glomeruli and proximal tubules (PTs), distal tubules, and collecting ducts (Figure 2C). Of note, immunoglobulin G control was done for all immunohistochemical staining protocols and is shown in Figure S2.

**Increased Activation of PI3K/Akt Pathways in Cc1−/− Kidneys**

Immunohistochemical staining showed increased phosphorylation of PI3K p85α (Tyr 508) in the renal glomeruli, PTs, distal tubules, and collecting ducts in Cc1−/− mice (Figure 3A). Consistently, phosphorylation of Akt (Ser473) was also elevated in the cytosol and nuclei of renal glomeruli, PTs, distal tubules, and particularly, in collecting ducts of Cc1−/− mice (Figure 3B).

**Increased PRR Expression and Activation of the PI3K-Akt Pathway in CEACAM1 Knockdown IMCD Cells**

Next, we examined whether Ceacam1 deletion exerts a cell autonomous effect on PRR expression. Lentiviral-mediated shRNA knockdown of CEACAM1 in IMCD (Figure S1) caused a significant rise in PRR mRNA and protein expression (Figure 4A and 4B, KN versus scrambled [Sc] cells in the absence of inhibitors;
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Inhibiting Akt-Downstream CBP–CREB Interaction Prevented Elevation of PRR Expression in CEACAM1 Knockdown IMCD Cells

Akt blockade at 5, 15, 30, and 60 minutes significantly increased phosphorylation of CREB/activated transcription factor (ATF)-1 (Ser133), ATF-2 (Thr71), and NF-κB p65 (Ser276) in a time-dependent manner in IMCD cells with intact (scrambled [Sc]), but not reduced CEACAM1 expression (KN; Figure 5A). Moreover, the rise of PRR expression in CEACAM1 knockdown IMCD cells was significantly inhibited by 12-hour treatment with naphthamide compound, a CBP–CREB interaction inhibitor, in a dose-dependent manner at both mRNA and protein levels (Figure 5B). This suggests a role for CREB family in Akt-mediated CEACAM1 regulation of PRR in IMCD cells.

CREB Family Members Bind to CRE and AP-1 Regulatory Elements to Enhance PRR Expression

Computational analysis mapped 1 CRE and 5 AP-1 regulatory elements in murine PRR promoter (Figure 6A). Chromatin
immunoprecipitation analysis showed no significant difference in c-Jun–binding activity to any AP-1 site in PRR promoter between scramble and CEACAM1-shRNA–transducted IMCD cells (data not shown). In contrast, the binding activity of CREB-1 to CRE was about 6-fold higher in CEACAM1 knockdown cells (Figure 6B). Similarly, the binding activity of ATF-1 and ATF-2 to CRE was also elevated in these cells, albeit to a lower extent than CREB-1 (Figure 6C and 6D). In the absence of CEACAM1, CREB-1 and ATF-1 bound more significantly to AP-1.4 site, with a stronger effect by ATF-1 (Figure 6E–6G), and CREB-1 and ATF-2 bound more significantly (by ≈2-fold) to AP-1.5 site (Figure 6H–6J). The binding activity of these factors to the other 3 distal potential AP-1 sites was not examined.

**Discussion**

Mice with null deletion of *Ceacam1* (*Ccl^-/-*) develop systemic insulin resistance and visceral obesity resulting from impaired insulin clearance.14,15 The current studies demonstrate that these mice also develop albuminuria and increase in BP when fed a regular chow diet.

Elevated BP and albuminuria in *Ccl^-/-* mice were associated with activated renal RAS, as demonstrated by increased expression of renin, angiotensinogen, angiotensin-converting enzyme, angiotensin II, and angiotensin subtype 1 receptor in the kidney. Moreover, renal PRR level was also elevated, providing an in vivo demonstration of a role for this new member of RAS in insulin resistance associated with increased BP. Recently, several studies suggested that PRR contributes to the pathogenesis of diabetic nephropathy and conversely, reduction of its activity significantly improves renal inflammation and fibrosis in diabetes mellitus.18,21,24–29

The increase in PRR expression in *Ccl^-/-* kidney is mediated by activated PI3K-Akt pathway. This may seem paradoxical given the insulin resistance state in these mice. Dissociation between proximal insulin signaling and insulin resistance has been shown in humans30 and in several models of insulin resistance, including the well-characterized *Ob/Ob* mouse.31 We have detected a similar increase in basal signaling in the liver of the insulin-resistant L-SACC1 mouse with liver-specific inactivation of CEACAM1, which was attributed to activation of epidermal growth factor receptor, likely by white adipose tissue-derived plasma fatty acids and heparin-binding epidermal growth factor.32 Thus, it is possible that a similar mechanism involving epidermal growth factor receptor activation elevates basal Akt signaling in the kidney of the insulin-resistant *Ccl^-/-* mice with increased visceral obesity and elevation in fatty acids release. In addition, CEACAM1 exerts a cell autonomous regulatory effect on PI3K-Akt activity, as shown by elevated basal PI3K-Akt activation in CEACAM1 knockdown IMCD cells. The underlying mechanism remains to be determined, but it is of interest that siRNA-mediated
knockdown of CEACAM1 caused a similar effect on basal PI3K-Akt in human Daudi lymphoma B cells.\(^{29}\)

Moreover, increase in PRR expression seems to be regulated by CREB family binding to CRE of PRR promoter. Inhibiting Akt kinase attenuated the function of CREB-1, ATF-1, ATF-2, and NF-kB p65 transcription factors via reduction in their phosphorylation rather than changes in their expression levels. The role of NF-kB p65 activity in PRR regulation supports our previous reports showing that transcriptional regulation of PRR by NF-kB p65 altered binding to NF-kB regulatory elements in the presence of high glucose in rat mesangial cells\(^ {15,23}\) or in PT cells and IMCD cells exposed to low sodium levels.\(^ {33}\) In the presence of low salt, CREB-1 binding to CRE of PRR promoter increases in rat PT cells, resulting in transcriptional regulation of PRR by CREB/ATF family of transcription factors.\(^ {33}\) Similarly, this report showed that CEACAM1 knockdown increased binding of CREB-1, ATF-1, and ATF-2 to CRE of mouse PRR promoter in IMCD cells. Members of CREB/ATF, AP-1, and C/EBP bZIP subfamilies form homodimeric or heterodimeric complexes that bind specific DNA motifs. Several studies demonstrated that CREB/ATF influence AP-1 via protein interaction,\(^ {34}\) enhancing or suppressing expression of AP-1,\(^ {35}\) or occupying AP-1 DNA-binding elements.\(^ {36–38}\) Our findings demonstrated that in CEACAM1 knockdown IMCD cells, the binding of CREB-1, ATF-1, and ATF-2 to different AP-1 regulatory elements was significantly increased. These data suggest that the CREB family of transcription factors enhanced the transcriptional regulation of PRR via AP-1 DNA-binding sites in its promoter as well.

Collectively, null deletion of murine CEACAM1 activates PI3K-Akt signaling and contributes to the expression of PRR via transcription factors CREB family and NF-kB.

**Perspectives**

The present study demonstrates that null deletion of Ceacam1 significantly enhances renal expression of PRR via PI3K-Akt signaling pathways, CREB family, and NF-kB. These findings may help delineate new mechanisms underlying the regulation of the expression of this new RAS component in insulin resistance. Given that target deletion of Ceacam1 also causes insulin resistance, the elucidation of the mechanism underlying the effect of CEACAM1 on PRR expression promotes our understanding of the molecular link between insulin resistance, kidney disease, and hypertension, and identifies PRR as a potential therapeutic target.

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**Disclosures**

None.

**References**


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MATERIALS AND METHODS

Animal Husbandry, General Biological Characteristics and Renal Interstitial Fluid Collection

Cc1\(^{-}\) null mice were generated on a mixed C57BL/6x129sv background and backcrossed 12 times onto the C57BL/6 background (SJ). Animals were kept in a 12-h dark/light cycle and fed standard chow *ad libitum*. All protocols were approved by the Animal Care and Use Committees at the University of Virginia and the University of Toledo. Six months old male mice were studied. Before sacrifice, body weight (BW), blood glucose, plasma insulin and lipid, and systolic blood pressure (SBP) were monitored (S8). Additionally, insulin tolerance was assessed in awake mice following a 6 hour-fast starting by intra-peritoneal injection with Regular Human insulin (Novo Nordisk, 0.75U/kg BW) before drawing retro-orbital blood for glucose measurement (S8). 24 hour urine was collected as well for the evaluation of urinary level of albumin and creatinine. Renal interstitial fluid (RIF) was collected by microdialysis technique developed and successfully used in our laboratory (S2, S3) to determine angiotensin II (Ang II) level in the kidney. At the end of experiments, kidneys were harvested for protein and total RNA extraction, and parts of kidneys were also fixed with Bounin’s fixative.

Cell Culture, Small Hairpin RNA Interference and Cell Treatments.
Mouse renal inner medullary collecting duct epithelial cells (IMCD3) were obtained from the American Type Culture Collection (Manassas, VA) and cultured according to American Type Culture Collection recommended protocols. Cells were grown to confluence in DMEM/Nutrient Mixture F12 (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics. Serum free culture or serum starvation was conducted with Opti-MEM I (Life Technologies, Grand Island, NY) for 12 hours. IMCD cells were used for small hairpin RNA transduction. Target cells were plated in a 12-well plate 24 hours prior to viral infection. When the cells reached approximately 50% confluent, a mixture of complete medium with polybrene (Santa Cruz Biotechnology, Santa Cruz, CA) at a final concentration of 5µg/ml was prepared. Media from plate wells was removed and replaced with 1 ml of this polybrene / media mixture per well. Mouse CEACAM 1 (Santa Cruz Biotechnology, Santa Cruz, CA) and control shRNA lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA) were thawed at room temperature mixed gently before use and added to the culture to infect cells. After 24 hrs, culture medium was removed, replaced with 1 ml of complete medium and then the cells were incubated overnight. Stable clones expressing the shRNA were selected via puromycin dihydrochloride (Santa Cruz Biotechnology, Santa Cruz, CA) at a final concentration of 10 µg/ml. Puromycin-containing medium was refreshed every 3-4 days until resistant colonies could be identified. Several colonies were picked and expanded. Stable shRNA expression was assessed by CEACAM 1 expression using RT-PCR, Western blot assay and immunofluorescence staining. Identified cell colonies were expanded and employed in later experiments. For the experiments with different treatments, each drug was added to serum-free medium 30 minutes before the end of serum starvation. After 30 minutes of pretreatment, cells were refreshed with serum starvation medium with or without treatment, which included following reagents: PI3K kinase inhibitor LY294002, Akt kinase inhibitor II (EMD Millipore, Billerica, MA) and CBP-CREB interaction inhibitor (CalBiochem, La Jolla, CA).

Assessing Gene Expression and Protein Phosphorylation.
Determination of gene expression and protein phosphorylation were conducted with real-time PCR and Western blotting assay described in previous publication (S4-S7).

Validation of mRNA changes in the gene expression was achieved by quantitative real-time RT-PCR. Briefly RNA was extracted from kidney tissue and cultured cells with the RNeasy total RNA isolation kit (Qiagen, Valencia CA). The RNA integrity was accessed by 2% formaldehyde agarose gel electrophoresis. Expression level of PRR mRNA was measured by real-time RT–PCR iCycler according to the manufacturer’s instructions (Bio-Rad, Hercules CA). Single-stranded cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules CA). PCR was performed with iQTM SYBR green supermix (Bio-Rad, Hercules CA) according to the manufacturer’s instructions. Primers sequences are listed in Table S1. Reactions were performed in triplicate, and threshold cycle numbers were averaged. None-template control was used as negative control. Samples were calculated with normalization to 18S rRNA.

To analyze the expression and phosphorylation of proteins, whole cell lysates were extracted from kidney tissue or cultured cells with lysis buffer detailed in previous study (S4-S7). Fractionation of cytosolic, membranous and nuclear protein was conducted as previously reported (S5). Following primary antibodies against ATP6AP2 (PRR), TATA binding protein TBP (Abcam, Cambridge MA), phospho-PI3K p85α(Tyr508), PI3K p85α, phosphor-Akt (Ser473), Akt (Santa Cruz Biotechnology, Santa Cruz CA), phospho-CREB1/ATF1 (Ser133), CREB1, phosphor-ATF2(Thr71), ATF2, phosphor-p65(Ser276), NF-κBp65 (Cell Signaling Technology, Danvers MA) and β-actin antibody (Sigma, St. Louis MO) were employed. The bands densitometry was performed by Image Master TM Total Lab Version 2.0 (Amersham Pharmacia BioTech, Piscataway NJ). The band density of target protein was normalized to the corresponding density of β-actin. The arbitrary unit of band densities was represented as the expression level.

**Immunohistochemical and immunofluorescence staining**

Immunohistochemical staining was performed for localizing PRR, phospho-PI3K (Tyr508) and phosphor-Akt (Ser473) in the kidney. Briefly, 4-µm-thick sections were cut, deparaffined and rehydrated. Heat-induced antigen retrieval was conducted in 10 mM sodium citrate (pH 6.0). Endogenous peroxide activity was suppressed by 0.3% peroxide-methanol solution. VECTASTAIN®ABC KIT (Vector Laboratories, Burlingame, CA) was used for blocking and color reaction as recommended. The immunostaining was performed by incubating with polyclonal rabbit anti-(pro)renin receptor antibody (Abcam, Cambridge MA), phospho-PI3K p85α (Tyr508) and phosphor-Akt (Ser473) (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by 1 hr of incubation with secondary antibody conjugated with biotin at room temperature (Vector Laboratories, Burlingame, CA). Immunoreactive signal was detected with an avidin–biotin immunoperoxidase reaction (Vector Laboratories, Burlingame, CA) and visualized by exposure to diaminobenzidine (Sigma, St. Louis, MO). Non-specific binding was controlled by using the mouse immunoglobulin G isotype (Sigma, Sigma, St. Louis, MO) as a primary antibody.

IMCD cells were grown, treated and washed with PBS buffer and then fixed with 4% paraformaldehyde on coverslips. Cells were then blocked with 1% BSA in PBS. The immunostaining was performed by incubating with rabbit anti-CEACAM 1 antibody (S8), at 4°C overnight, followed by 1 hr of incubation with secondary antibody conjugated with Cynine (Cy3) at room temperature (Jackson ImmunoResearch, West Grove, PA). Coverslips were mounted in the slides with ant fade mounting medium with DAPI (Vector Laboratories, Burlingame, CA).
Measurement of Renal Ang II Production, Cell Viability and PI3K Activity

Angiotensin II levels of RIFs were measured by Angiotensin II EIA kit as recommended by manufacturer (Cayman Chemical, Ann Arbor, MI). Parallel experiments with the same design for measurement of PI3K activity in IMCDs were conducted simultaneously to determine the total cell number in each sample as previously described (S4). The total cell number is determined by quantitatively measuring the release of lactate dehydrogenase using CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison WI). The total cell number (1X 10^6 cells) was the average from triplicates and used for the normalization of PI3K activity of each sample.

PI3K activity of cell lysates from IMCDs was measured by PI3-Kinase ELISA kit as recommended by manufacturer (Echelon Inc., Salt Lake City, UT). Signal readout was normalized to total cell numbers of each sample and relative content (percentage) verse control was calculated. The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI (3,4,5) P3 produced by PI3K. We used readout of positive control to minute those of samples to get a positively proportional relative content verse control. Intra-assay and inter-assay variations were 2% and 5%.

Real-time Mapping of CRE and AP-1 Regulatory Elements in Mouse PRR promotor

The protocol for real time mapping of transcription factors cAMP response element binding protein 1(CREB-1) to cAMP response element (CRE) and activator protein 1 (AP-1) elements was detailed in previous publications (S5, S7).

Statistical analysis

The data analysis was carried out using STATISTICA version 5.0 (StatSoft, Tulsa OK). Results are expressed as mean ± SEM. Comparisons among different treatment groups were evaluated by analysis of variance (ANOVA) with repeated measures, and the Bonferroni correction method as a post-hoc test. A p-value of < 0.05 was defined as statistically significant.

SUPPLEMENT REFERENCES


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Figure S1. CEACAM 1 knockdown by small hairpin RNA in mouse innermedullary collecting duct cells. (A) RT-PCT; (B) Western blot; (C) Immunofluorescent staining of CEACAM 1. Cc1: CEACAM 1; shRNA: small hairpin RNA; Cc1 WT: Control cells with intact Cc1 expression and scramble shRNA; IgG control: immunofluorescence IgG control instead of Cc1 antibody.
Figure S2. IgG control for immunohistochemical staining. Scale bar 200 µm
Figure S3. Lentiviral-mediated shRNA knockdown of CEACAM1 in IMCD caused a significant rise in PRR mRNA and protein expression.