Endothelin-converting enzyme is a plausible target gene for hypoxia-inducible factor

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Renal endothelin-converting enzyme (ECE)-1 is induced in experimental diabetes and following radiocontrast administration, conditions characterized by renal hypoxia, hypoxia-inducible factor (HIF) stabilization, and enhanced endothelin synthesis. Here we tested whether ECE-1 might be a HIF-target gene *in vitro* and *in vivo*. ECE-1 transcription and expression increased in cultured vascular endothelial and proximal tubular cell lines, subject to hypoxia, to mimosine or cobalt chloride. These interventions are known to stabilize HIF signaling by inhibition of HIF-prolyl hydroxylases. In rats, HIF-prolyl-hydroxylase inhibition by mimosine or FG-4497 increased HIF-1α immunostaining in renal tubules, principally in distal nephron segments. This was associated with markedly enhanced ECE-1 protein expression, predominantly in the renal medulla. A progressive and dramatic increase in ECE-1 immunostaining over time, in parallel with enhanced HIF expression, was also noted in conditional von Hippel-Lindau knockout mice. Since HIF and STAT3 are cross-stimulated, we triggered HIF expression by STAT3 activation in mice, transfected by or injected with a chimeric IL-6/IL-6-receptor protein, and found a similar pattern of enhanced ECE-1 expression. Chromatin immunoprecipitation sequence (ChIP-seq) and PCR analysis in hypoxic endothelial cells identified HIF binding at the ECE-1 promoter and intron regions. Thus, our findings suggest that ECE-1 may be a novel HIF-target gene.

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Endothelins (ETs) have profound physiological effects in the kidney, affecting its oxygen supply and expenditure through the regulation of renal microcirculation and tubular transport activity, respectively.[1–3] ET-1, the biologically most active ET isoform, induces intense renal cortical vasoconstriction but exerts medullary vasodilation, mediated by specific ET_A and ET_B receptors, respectively,[4] and inhibits tubular sodium transport through nitric oxide and the activation of ET_B receptors.[2,3]

ETs are formed by sequential proteolysis of their precursors, pre-pro-ETs. The intermediate peptides, big ETs, undergo a final proteolysis by ubiquitous ET-converting enzymes (ECE), principally the ECE-1 isoform, with the formation of bioactive ETs.[5] Although most studies of ET synthesis have focused on the induction of the pro-hormone, the potential role of ECE-1 in the regulation of ET-1 synthesis has been largely overlooked.

Hypoxia has long been recognized to stimulate ET expression,[6–8] and more recently hypoxia-inducible transcription factors (HIFs) have been defined as potent stimuli for induction of the pro-hormone.[6,9–12] However, we have recently reported that renal ECE-1 expression is markedly enhanced in diabetic rat kidneys and following the administration of iodinated contrast agents,[13] conditions characterized by elevated plasma and renal ET[14,15] and intensified renal parenchymal hypoxia and HIF expression.[16,17] This provides the basis for the hypothesis that ECE-1 induction, related to hypoxia and HIFs, might contribute to enhanced renal ET-1 generation.

HIFs are key regulators of gene response during hypoxia, which consist of a constitutive β-subunit and one of at least two alternative α-subunits.[18,19] HIF αβ heterodimers are formed in the cytoplasm, undergo nuclear translocation, and bind to numerous hypoxia-responsive elements. This leads to epigenetic DNA changes that generate cellular hypoxia response, with quiescence of some genes and the induction of over 200 genes

involved in cell metabolism and survival.[18,19] The regulation of HIF signal is governed by HIF-α proteasomal degradation, initiated by oxygen-sensitive HIF-prolyl hydroxylases (PHDs), and subsequent polyubiqidation and binding with von Hippel–Lindau (VHL) tumor suppressor. Thus, in well oxygenated cells, PHDs initiate HIFα degradation and prevent the generation of the HIF signal. By contrast, PHDs become inactive during hypoxia, permeating HIFα accumulation and dimerization with β subunits, initiating HIF response. Indeed, PHDs can be considered as cellular oxygen sensors, as their activity varies in the range of physiological/pathological oxygen tensions.[18,19]

Signal Transducer and Activator of Transcription (STAT) 3 is another master regulator of cellular gene response to hypoxia and inflammation.[20] We have recently reported a plausible renal HIF–STAT3 cross-talk *in vivo*, with reciprocal co-stimulation.[21] Furthermore, HIF αβ heterodimers and activated (phosphorylated) p-STAT3 dimers were found to form a transcription complex together with p300, inducing shared target genes such as vascular endothelial growth factor in malignant cell lines.[22–24]

In this perspective, we explored the possibility that ECE-1 might be a HIF-target gene. *In vitro*, ECE-1 expression was studied in cultured vascular endothelial cells and in proximal tubular cell lines subjected to hypoxia or hypoxia-mimetic conditions. Furthermore, in line with our previous experimental strategies,[21] we studied ECE-1 expression following HIF induction *in vivo*. This has been achieved by specific HIF-PHD inhibitors and in VHL conditional knockout mice.[21,25] To further explore a possible STAT3-induced HIF stimulation with subsequent ECE-1 induction, tubular STAT3 phosphorylation was induced by an interleukin (IL-6)/soluble IL-6 receptor fusion protein ('hyper IL-6'), which can trigger IL-6 signaling on cells that express gp130, also in the absence of membrane-bound IL-6R expression, as previously shown for kidney parenchymal cells.[26] Complementary ChIP-seq analysis was carried out in cultured endothelial cells to evaluate potential HIF-binding sites within the ECE-1 promoter and intron regions.

RESULTS

ECE-1 induction *in vitro*

As illustrated in Figure 1a, cultured human umbilical vascular endothelial cells (HUVEC), incubated for 24 h under hypoxia (pO_2 5%), displayed a 83% rise in ECE-1 transcription, assessed by real-time PCR. ECE-1 transcription also increased 67 and 80% following incubation with the hypoxia-mimetic cobalt chloride or with the PHD-inhibitor mimosine, respectively ($P<0.05$). A parallel transcriptional enhancement was noted as well under these conditions with the ET-1 pre-prohormone and with Glut-1, a confirmed HIF-dependent gene. In addition, ECE-1 transcription increased 2.3-, 2.2-, and 2-fold in HK-2 proximal tubular cell culture following hypoxia, cobalt chloride, and mimosine, respectively, with a parallel enhanced transcription of ET-1 and Glut-1 (Figure 1b). ET-1 synthesis substantially increased by these HIF-stimulating strategies in the two cell lines, as illustrated in the corresponding Figures 1c and d.

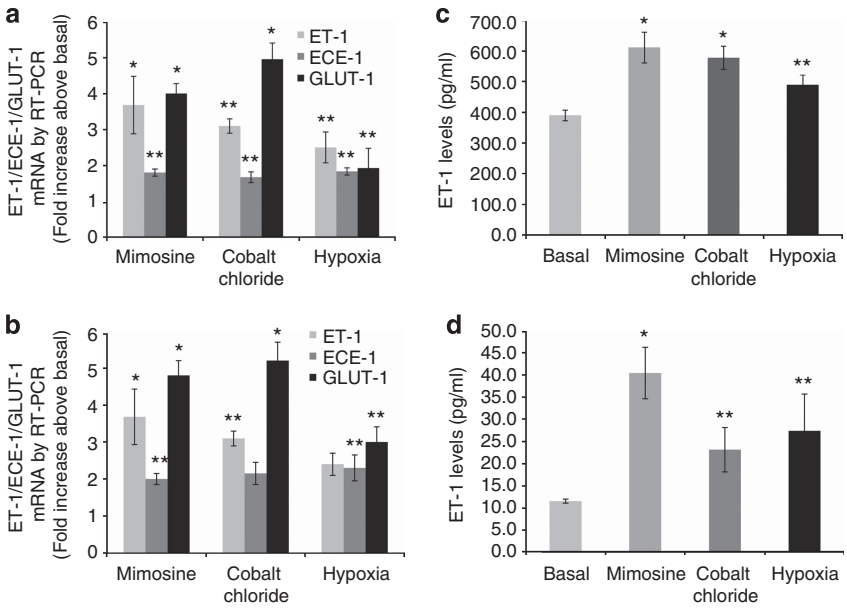

Figure 1 | Endothelin-converting enzyme (ECE)-1 and endothelin (ET)-1 induction in human umbilical vein endothelial cells (HUVECs) (a and c) and in HK-2 proximal tubular cells (b and d) following hypoxia-inducible factor (HIF) stimulation. Cells were incubated for 24 h under hypoxia (pO_2 5%) or normoxia (pO_2 20%) with or without the addition of the hypoxia-mimetic prolyl hydroxylases (PHD) inhibitor $CoCl_2$ (200 μM) or mimosine (800 μM). ECE-1 pre-pro ET-1 and Glut-1 transcription were assessed using RT-PCR with 36B4 serving as a control housekeeping gene. In HUVEC (**a**), ECE-1 transcription rose 1.8-, 1.7-, and 1.8-fold following exposure to hypoxia, cobalt chloride, and mimosine, respectively. In (**b**), corresponding 2.3-, 2.2-, and 2-fold ECE-1 transcriptional increments were noted in HK-2 cells as well. The transcription of pre-pro ET-1 and of Glut-1, confirmed HIF-target genes, rose in parallel. Corresponding ET-1 content in the supernatants substantially increased in both HUVEC (**c**) and HK-2 cell lines (**d**). ($n=6$ per group, $^*P<0.01$; $^{**}P<0.05$ for the various modes of HIF stimulation vs. normoxia.)