The Isoquinolone Derived Prolyl Hydroxylase Inhibitor ICA Is a Potent Substrate of the Organic Anion Transporters 1 and 3

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Key Words

Hypoxia · Renal proximal tubule · Drugs

Abstract

Objective: Many cellular responses to hypoxia are mediated by the transcription factor complex hypoxia-inducible factor (HIF). HIF stability is governed by a family of dioxygenases called HIF prolyl hydroxylases (PHDs). Isoquinolone-derived PHD inhibitors, like 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetate (ICA), which stabilize the intracellular HIF- α have been suggested as a potentially beneficial therapeutic strategy for the treatment of disorders associated with ischemia. To stabilize HIF- α , ICA has to be taken up into proximal tubule cells (PCTs) across the basolateral membrane by one of the organic anion transporters 1, 2 or 3 (OAT1, OAT2 or OAT3). The release into the urine across the luminal membrane may be mediated by OAT4. **Method:** To demonstrate interaction of ICA with human OAT1, OAT2, OAT3 and OAT4, ICA was tested on these transporters stably transfected in HEK293 cells by using p-aminohippurate (PAH), cGMP and estrone-3-sulfate (ES) as reference substrates, respectively. **Results:** Uptakes of PAH and ES in OAT1- and OAT3-transfected HEK293 cells were inhibited by ICA with half-maximal inhibition values of 0.29 ± 0.05 and $2.58 \pm 0.16 \mu\text{M}$, respectively. OAT2 was less sensitive to ICA. Efflux experiments identified ICA as an OAT1 and OAT3 sub-

strate. Preloading OAT4-transfected HEK293 cells with ICA stimulated ES uptake by $18.3 \pm 3.8\%$. **Conclusion:** The uptake of ICA across the basolateral membrane of PCTs occurs mainly by OAT1 and the efflux into the tubular lumen by OAT4.

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Introduction

Renal ischemia is known to play an important role in the pathophysiology of acute kidney injury as well as in chronic kidney disease. The balance between hypoxia and normoxia is regulated by transcription factors, commonly named hypoxia-inducible factors (HIFs). Under normoxic conditions, constitutively expressed HIF- α subunits are hydroxylated by prolyl hydroxylases (PHDs) which leads to recognition and ubiquitination by the E3 ubiquitin ligase von Hippel-Lindau protein and subsequent degradation by the proteasome. Under hypoxia, hydroxylation does not occur due to the lack of molecular oxygen and HIF can accumulate in the cell. In the nucleus, HIF- α dimerizes with the HIF- β /aryl hydrocarbon receptor nuclear receptor subunit and induces the transcription of its target genes [1, 2]. HIF target genes are numerous, including enzymes of the glycolytic pathway, vascular endothelial growth factor and PHDs. PHDs are increasingly considered as promising therapeutic targets

Fig. 1. Chemical structures of NOG and ICA. Structures were constructed by the free version of ChemSketch. Note that another nomenclature for ICA is 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetic acid. ICA was originally synthesized by HOECHST as S950086 and later by FibroGen as FG-2216.

to improve kidney function in acute or chronic hypoxia [3, 4]. Recently, we showed that the 2-oxoglutarate (2-OG) analogues, N-oxalylglycine (NOG) and pyridine-2,4-dicarboxylic acid, were transported by the organic anion transporter 1 (OAT1) and thus enter proximal tubule cells (PCTs) and stabilize HIF-1 α [5]. Two-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetic acid (ICA, FG-2216) is another promising drug in establishing renal function after acute kidney injury in animal models [6, 7] and has also been shown to improve erythropoietin production in healthy volunteers and patients suffering from end-stage renal disease [8]. Since ICA is structurally related to NOG (fig. 1), we investigated whether this drug is a substrate for one of the OATs. OATs are potent drug transporters occurring preferentially in PCTs and transport a wide variety of structurally unrelated compounds such as antivirals, anti-neoplastics, non-steroidal anti-inflammatory drugs, β -lactam antibiotics, diuretics, sartanes as well as metabolite intermediates and endogenous compounds such as flavonoids or coumarins [9–12].

Concise Methods

Reagents and Chemicals

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (Taufkirchen, Germany) or AppliChem (Darmstadt, Germany). The [^3H]-labeled compounds, p-aminohippurate (PAH), cGMP and estrone-3-sulfate (ES), were from Perkin Elmer (Rodgau, Germany). ICA was synthesized as described in Schley et al. [6].

Cell Culture and Transport Experiments

OAT1-, OAT2-, OAT3-, OAT4- and vector-transfected HEK293 cells are used routinely in our laboratory and were prepared and treated as described previously [5, 13]. For transport measurements, cells were incubated at room temperature in Mammalian Ringer (MRi) that contained the [^3H]nuclide at the concentration indicated in the respective figure legend and various experimental conditions. ICA was dissolved in DMSO. Uptake was terminated at 5 min by removal of the radio-labeled medium and immediately washing 3 times with ice-cold PBS. Cells were dissolved in 0.5 ml 1 N NaOH by gently shaking for 120 min, and the

[^3H]-content was determined by liquid scintillation counting (Tri-carb 2810, Perkin Elmer). Protein quantification was performed by the Bradford assay [14].

Statistics and Calculations

Data are provided as means $\pm$ SD. Unpaired 1-tailed Student's t tests were used to show statistical significant differences for the inhibition of the uptake of PAH, cGMP or ES by ICA or by trans-stimulation of ICA on OAT1-, OAT3-, OAT4- and vector-transfected HEK293 cells. Statistical significance was set to *** at $p < 0.001$. The constants for half-maximal inhibition (IC_{50}) of PAH, cGMP and ES uptake by ICA were calculated using SigmaPlot software (Systat Software, San Jose, Calif., USA).

Results

In OAT1-, OAT2- and OAT3-expressing HEK293 cells, ICA at a concentration of 100 μM reduced the uptake of the reference substrate PAH for OAT1, cGMP for OAT2 and ES for OAT3 from 2.31 ± 0.46 , 0.232 ± 0.052 and $0.034 \pm 0.052 \text{ pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ to 0.104 ± 0.038 , 0.126 ± 0.030 and $0.006 \pm 0.001 \text{ pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, respectively. Inhibitors of OAT1 (probenecid), OAT2 (indomethacin) and OAT3 (probenecid), all at concentrations of 100 μM , inhibited uptake of PAH by 94.8, of cGMP by 96.6 and of ES by 78.5%, respectively (fig. 2a). A detailed analysis revealed IC_{50} values for the inhibition of uptake of PAH, cGMP and ES by ICA of 0.29 ± 0.05 , 158.4 ± 22.2 and $2.58 \pm 0.16 \mu\text{M}$, respectively, identifying ICA as a potent OAT1 and OAT3 inhibitor (fig. 2b). Competitive inhibition of PAH uptake by ICA in OAT1-transfected HEK293 cells was shown by a Dixon plot with a K_i value of $1.17 \pm 0.28 \mu\text{M}$ (fig. 2c). The competitive inhibition suggested that ICA was a substrate of OAT1. Therefore, OAT1- and vector-transfected HEK293 cells were incubated for 20 min in 0.5 μM [^3H] PAH to achieve an equilibrium between intra- and extra-cellular PAH. By replacing 0.5 μM [^3H]PAH by 0.05 μM [^3H]PAH and by 0.05 μM [^3H]PAH plus 10 μM ICA, this equilibrium was disturbed and changes in the cellular [^3H] PAH content were followed as a function of time (fig. 2d). Whereas, in vector-transfected HEK293 cells only a mar-

Fig. 2. Effect of ICA on OAT1-, OAT2- and OAT3-transfected HEK293 cells. **a** Inhibition of transporter-mediated substrate uptake by ICA. The 5-minute uptake of $0.5 \mu\text{M}$ [^3H]PAH, of 100 nM [^3H] cGMP plus $9.9 \mu\text{M}$ un-labeled cGMP and of 10 nM [^3H]ES in OAT1-, OAT2- and OAT3-transfected HEK293 cells (dark grey columns) was inhibited by $100 \mu\text{M}$ ICA and by the transporter-specific inhibitors: probenecid ($100 \mu\text{M}$) for OAT1 and OAT3 and indomethacin ($100 \mu\text{M}$) for OAT2. The uptake obtained in vector-transfected HEK293 cells was subtracted from the uptake in transporter-transfected cells to obtain the transporter-mediated uptake. For a better comparison of the data, the uptake of the respective reference substrate was set to 100%. **b** Determination of IC_{50} for the inhibition of substrate-mediated uptake by ICA. Plots were obtained by using $0.5 \mu\text{M}$ [^3H]PAH (●), 100 nM [^3H]cGMP plus $9.9 \mu\text{M}$ un-labeled cGMP (▼) and 10 nM [^3H]ES (○) and increasing concentrations of ICA. For better comparison, the uptake in the absence of ICA was set to

100% (hatched bar). **c** Dixon plot to determine competitive inhibition of PAH uptake by ICA in OAT1-transfected HEK293 cells. Each line represents a linear regression analysis using 2 PAH concentrations (●: $10 \mu\text{M}$; ○: $100 \mu\text{M}$). From the intercept, a K_i value of $1.17 \pm 0.28 \mu\text{M}$ was obtained. **d** Efflux of PAH in OAT1- and vector-transfected HEK293 cells in the absence and presence of ICA as a function of time. Cells were incubated for 20 min at 37°C with $0.5 \mu\text{M}$ [^3H] PAH. After washing, cells were incubated for the time indicated in MRI containing $0.05 \mu\text{M}$ [^3H]PAH in the absence (OAT1: ●; vector: ▼) or presence of $10 \mu\text{M}$ ICA (OAT1: ○; vector: △). After the indicated time, the cellular content of [^3H]PAH was determined. Efflux of [^3H]PAH in OAT1-transfected HEK293 cells was larger in the presence of ICA (○) than in its absence (●) and was independent of ICA (▼, △) in vector-transfected cells. All data presented in (a–d) were obtained in triplicate on each of 3 different cell passages and statistical significance in (a) was set at *** $p < 0.001$.

ginal ICA-independent decrease of the intracellular [^3H] PAH content was noticed, OAT1-transfected HEK293 cells responded with a more rapid decrease of cellular PAH in the presence of ICA than in its absence, indicating uptake of ICA in exchange against [^3H]PAH by OAT1. Simi-

lar experiments with OAT3-transfected HEK293 cells were unsuccessful, because ES uptake did not reach equilibrium, even after 2 h of incubation. Therefore, OAT3- and vector-transfected HEK293 cells were preloaded for 120 min at 37°C with MRI in the absence and presence of $100 \mu\text{M}$ ICA.

Fig. 3. Interaction of ICA with OAT4. **a** Inhibition of OAT4-mediated ES uptake by ICA. The 5-minute uptake of 10 nM [^3H]ES in OAT4-transfected HEK293 cells (dark grey columns) was inhibited by 100 μM ICA (grey columns) and by probenecid (100 μM , black columns). **b** Preloading of OAT4- and vector-transfected HEK293 cells with ICA. Cells were preloaded for 120 min at 37°C with either 220 μl 0.1% DMSO in DMEM or 220 μl 100 μM ICA, 0.1% DMSO in DMEM. After extensive washings in PBS, the

5-minute uptake of 10 nM [^3H]ES in OAT4- and vector-transfected HEK293 cells was measured. Thereafter the cells were treated as described above for the uptake of the radio-labeled substrate. The uptake in vector-transfected cells was low and independent of the preloading treatment (data not shown). Columns represent mean values of experiments obtained in triplicate on each of 3 different cell passages; *** $p < 0.001$.

Preloading resulted in an uptake of ES enhanced by $26.7 \pm 9.3\%$ by ICA (measurements in triplicate on 3 subsequent cell preparations, $p < 0.001$). The uptake in vector-transfected cells was not influenced by preloading the cells with ICA ($0.004 \text{ pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ in both preparations), excluding unspecific effects of ICA, which may induce additional substrate uptake (data not shown).

Whereas OAT1, OAT2 and OAT3 are localized in the basolateral membrane, OAT4 is found in the luminal membrane of PCTs. The uptake of ES by OAT4 was only moderately inhibited by ICA (100 μM ; fig. 3a); ES uptake decreased from 0.050 ± 0.002 to $0.034 \pm 0.004 \text{ pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ in the presence of 100 μM ICA. To test whether ICA interacts from the intracellular side with OAT4, OAT4- and vector-transfected HEK293 cells were preloaded for 120 min at 37°C with 100 μM ICA and subsequently the uptake of ES was measured for 5 min. This experimental design stimulated ES uptake in OAT4-transfected cells by $18.3 \pm 3.8\%$ from 0.088 ± 0.004 to $0.106 \pm 0.010 \text{ pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ (fig. 3b).

Discussion

Stabilization of HIF-1 α by inhibition of PHDs is considered a potential therapy for anemia and renal ischemia. There exist 3 isoforms of the HIF PHD enzyme:

PHD1, PHD2 and PHD3 [10]. PHDs are members of the dioxygenase family that require oxygen, iron and the tri-carboxylic acid intermediate 2-OG for their catalytic activity. Among the PHDs, PHD2 is considered the most important one, because PHD2 knock-out mice are not viable and die between embryonic day 12.5 and 14.5 [3]. PHD2 is present mainly in the cytoplasm and has a substrate preference for HIF-1 α [15, 16]. PHD inhibitors derived from 2-OG bind, similar to 2-OG, at the catalytic site of the enzyme as a substrate mimic [3]. A potent PHD2 inhibitor is ICA (FG-2216). Its inhibitory activity against the PHD2 enzyme was determined to be 0.3 μM [17] or 3.9 μM [18], depending on the assay used.

The first step in renal secretion of organic anions is their uptake across the basolateral membrane of PCTs by one of the OATs (OAT1, OAT2 or OAT3). Since we recently identified NOG [5] as an OAT1 substrate, we speculated that ICA, due to its structural similarity to NOG, is also an OAT1 substrate. ICA turned out to be a very potent inhibitor of PAH uptake in OAT1-transfected HEK293 cells, exhibiting an IC_{50} of $0.29 \pm 0.05 \mu\text{M}$. In the former study, the IC_{50} for NOG was determined to be $79.6 \pm 10.2 \mu\text{M}$ [5], that is, the IC_{50} for ICA is approximately 275 times smaller than the IC_{50} for NOG in a similar experimental setup. The IC_{50} of ICA for the inhibition of the uptake of ES in OAT3-transfected HEK293 cells was $2.58 \pm 0.16 \mu\text{M}$. Both IC_{50} values are in the range

of the plasma concentration of ICA (approximately 3–5 μM), which can be calculated from the maximal plasma concentration (C_{max} 179 $\pm$ 31 $\mu\text{g/ml}$) given by Bernhardt et al. [8]. Efflux- or trans-stimulation experiments identified ICA as an OAT1 and an OAT3 substrate. However, due to the low stimulation of ES uptake by preloading OAT3-transfected cells with ICA, the contribution of OAT3 to ICA uptake may be low.

Whereas ICA is taken up primarily by OAT1, its release into the final urine is likely to be mediated by OAT4. In addition, ICA filtered by the glomerulus is able to block ES uptake by OAT4. The cooperation between inhibition by and transport of ICA by OAT4 may modulate intracellular ICA concentrations, thereby contributing to the beneficial effect of ICA on stabilizing HIF-1 α . Beneficial effects of ICA were already demonstrated in mouse models of renal ischemia reperfusion injury, where application of ICA improved kidney structure and preserved renal function [6]. In a phase 1 study, ICA was applied orally to 12 hemodialysis patients, 6 of them anephric and 6 healthy volunteers [8]. The area under the curve was 3,660 $\pm$ 813 in healthy

volunteers and increased to 5,730 $\pm$ 2,420 and 6,572 $\pm$ 2,349 h/ μg per ml in nephric and anephric patients. Similarly, the $T_{1/2}$ changed from 15.7 $\pm$ 1.1 to 29.8 $\pm$ 11.4 and 33.3 $\pm$ 8.4 h in the same cohorts [8], and an increase in erythropoietin production in all subjects was observed. The increase in the area under the curve and in $T_{1/2}$ in anephric patients highlights the role of renal secretion of ICA, a process in which OATs are likely to be involved.

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Disclosure Statement

No conflict of interest exists. Y. Hagos is a Professor of Physiology at the Institute of Systemic Physiology and Pathophysiology at the University Medical Center Göttingen and also CEO and shareholder of PortaCellTec Biosciences GmbH.

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