

Original Paper

Regulation of Expression of Renal Organic Anion Transporters OAT1 and OAT3 in a Model of Ischemia/Reperfusion Injury

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OK cells • HEK cells • Ischemic acute kidney injury model • Regulation of expression • OAT1 • OAT3 • Transport experiments • Reporter gen assay • Cloning of putative human promotor sequence • Translation

Abstract:

Background: Recently, we gained evidence that impairment of rOat1 and rOat3 expression induced by ischemic acute kidney injury (AKI) is mediated by COX metabolites and this suppression might be critically involved in renal damage. **Methods:** (i) Basolateral organic anion uptake into proximal tubular cells after model ischemia and reperfusion (I/R) was investigated by fluorescein uptake. The putative promoter sequences from hOAT1 (SLC22A6) and hOAT3 (SCL22A8) were cloned into a reporter plasmid, transfected into HEK cells and (ii) transcriptional activity was determined after model ischemia and reperfusion as a SEAP reporter gen assay. Inhibitors or antagonists were applied with the beginning of reperfusion. **Results:** By using inhibitors of PKA (H89) and PLC (U73122), antagonists of E prostanoid receptor type 2 (AH6809) and type 4 (L161,982), we gained evidence that I/R induced down regulation of organic anion transport is mediated by COX1 metabolites via E prostanoid receptor type 4. The latter signaling was confirmed by application of butaprost (EP2 agonist) or TCS2510 (EP4 agonist) to control cells. In brief, the latter signaling was verified for the transcriptional activity in the reporter gen assay established. Therein, selective inhibitors for COX1 (SC58125) and COX2 (SC560) were also applied. **Conclusion:** Our data show (a) that COX1 metabolites are involved in the regulation of renal organic anion transport(ers) after I/R via the EP4 receptor and (b) that this is due to transcriptional regulation of the respective transporters. As the promoter sequences cloned were of human origin and expressed in a human renal epithelial cell line we (c) hypothesize that the regulatory mechanisms described after I/R is meaningful for humans as well.

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Introduction

The organic anion transport system of the renal proximal tubule plays a crucial role in the excretion of a variety of potentially toxic compounds [1, 2]. This system consists of organic anion exchangers located at the basolateral membrane and mediates the rate determining step of elimination and the efflux step through the apical membrane [3, 4]. The classical basolateral organic anion exchanger is the terminal step in the tertiary active transport system, which is dependent on an inward-directed Na^+ gradient that drives the uptake of α -ketoglutarate, which is then exchanged for organic anions [5, 6]. Oat1 and Oat3 are components of the basolateral polyspecific transporter for organic anions [7, 8], which was functionally described since substantial time [5]. In summary, the classical renal basolateral polyspecific uptake transporter for organic anions is represented by Oat1 and Oat3 [9, 10].

In human renal allografts, a condition of ischemic acute kidney injury (iAKI), the clearance of the prototypical organic anion para-aminohippurate (PAH) was reduced for at least 7 days after transplantation [11]. Based on the latter observation, we demonstrated down regulation of both Oat1 and Oat3 following iAKI in a rodent model [12]. This was subsequently confirmed by independent groups [13, 14]. We also demonstrated that prostaglandin E2 (PGE2) leads to down regulation of the expression of both Oat1 and Oat3 in a proximal tubular cell line after long-term exposure (up to 72 h) [15]. In general, PGE2 levels are increased in the kidney cortex after AKI [16, 17], as well as during chronic renal ischemia [18]. In a rat model of iAKI, we described that low-dose (1mg/kg) indomethacin, an inhibitor of prostaglandin E2 synthesis, abolishes ischemia/reperfusion (I/R)-induced down regulation of both Oat1 and Oat3. This correlated with an abrogated down regulation of either organic anion secretory transport (PAH) and renal clearance of endogenous organic anions (PGE2) [19]. With respect to Oat1 and Oat3, the same was demonstrated in a model system of renal I/R [20] using proximal tubular cells from rat [21].

Most notably, low-dose (1mg/kg) indomethacin did not only rescue organic anion transport but also had a significantly beneficial effect on general renal outcome (as determined by GFR) without negatively affecting renal perfusion (RPF) [19]. This effect was even more pronounced when selective COX1 inhibition was applied [22]. In the respective study, we gained serious evidence that I/R induced impairment of rOat1 and rOat3 expression is mediated by COX1 metabolites, whereas COX2 metabolites are most likely not involved. From the data obtained from the above mentioned studies and a recent study [23] we concluded that impaired expression of Oat1 and Oat3 may be mechanistically involved in renal damage after I/R [23].

Consequently, we aimed for a) additional insight into the regulation of the latter transporters after I/R. Therefore, we investigated organic anion transport in a proximal tubular epithelial transport assay (OK cells) after applying of model I/R [20, 21]. In order to (b) test whether the data obtained in the physiological readout are due to regulation of expression, we cloned the putative promoter sequences and set up a reporter gen assay. The cloned promoter sequences were of human origin thus (c) gaining evidence whether the data obtained might also be transferable to men.

Materials and Methods

Cell culture

HEK-293 cells were from ATCC (Rockville, MD, USA) grown in DMEM/HAM F-12 medium with 10% fetal calf serum. Opossum kidney (OK) cells were obtained from Dr. Biber, Department of Physiology, University of Zürich and were cultured as described [24]. For transport assay OK cells were cultured on permeable supports (Falcon, Becton Dickinson Labware, Franklin Lakes, USA). The cells were maintained at pH 7.4 and 37°C, and gassed with 95% air / 5% CO_2 . The seeding density was $0.4 \cdot 10^6 \text{ cm}^{-2}$. The medium was changed every third day and the cells were used for experiments at day 10 after seeding. All experiments

were performed with cells that were serum starved for 24 hours before the experiments. E prostanoid specific agonists (TCS2510, butaprost; both from Tocris Bioscience, Bristol, UK) were applied thereafter in serum free growth medium for 6h (reporter gen assay), or 48h (transport assay).

In vitro ischemia reperfusion model

Model ischemia and reperfusion was performed as published and described in detail by Sauvant et al. [20, 21]. In brief, cells were grown as described above. Control cells were exposed to bicarbonate-HEPES buffered Ringer solution adjusted to pH 7.4 (NaHCO₃ 24.0 mM, Na₂HPO₄ 0.8 mM, NaH₂PO₄ 0.2 mM, NaCl 86.5 mM, KCl 5.4 mM, CaCl₂ 1.2 mM, MgCl₂ 0.8 mM, HEPES 20 mM; pH adjustment with 1N NaOH) containing 5 mM glucose and kept at 37°C and ambient pO₂ partial pressure. Model ischemia was applied by exposing the cells to a bicarbonate-MES buffered Ringer at pH 6.6 (NaHCO₃ 4.5 mM, Na₂HPO₄ 0.8 mM, NaH₂PO₄ 0.2 mM, NaCl 106.0 mM, KCl 5.4 mM, CaCl₂ 1.2 mM, MgCl₂ 0.8 mM, MES (morpholinoethanesulfonic acid) 20 mM; pH adjustment to 6.6 with 1N NaOH) without glucose and at an O₂ fraction below 1%. Cells were exposed for 2h to either control or ischemic (pH 6.6, aglycemia, O₂ fraction below 1%) conditions and then cultivated under standard cell culture conditions representing the reperfusion period for 6h (reporter gen assay), or 48h (transport assay). Hypoxia was applied to the cells using a hermetic chamber filled with gas consisting of 95% N₂ and 5% CO₂ (see acidosis). During filling, the fraction of O₂ was measured and filling was continued until the O₂ fraction in the chamber fell below 1%. Stability of pH was assured by using a bicarbonate-MES buffered Ringer in a 5% CO₂ atmosphere. Correct pH was moreover assured by measurement before and after the incubation period. Aglycemia mimics the absence of glucose due to its consumption without redelivery in ischemia and was applied by using a glucose-free buffer (as mentioned above). Application of inhibitors (U-73122, H89; SC560 and SC58125 from Tocris Bioscience, Bristol, UK) or antagonists (AH6809, L161,982; both from Tocris Bioscience, Bristol, UK) occurred directly after model ischemia with the beginning of reperfusion.

Fluorescein uptake

Fluorescein transport assays were done as described before [21]. In brief, OK cells were seeded on 24well filters (Falcon, Becton Dickinson Labware, Franklin Lakes, USA) and grown until confluency. The volumes of the apical and basolateral compartments were 0.2 ml and 0.5 ml, respectively, in order to avoid hydrostatic pressure differences. Ischemia was induced as described above, using the latter volumes. Fluorescein uptake was determined as described [15]. In brief, the cells were washed three times with phosphate buffered Ringer (PBS; 138 mmol/l NaCl, 1 mmol/l NaH₂PO₄, 4 mmol/l Na₂HPO₄, 4 mmol/l KCl, 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 5 mmol/l glucose, pH 7.4). Transport measurements were performed in phosphate buffered ringer at pH 7.4 and 37°C. Fluorescein uptake transport was determined by measuring the uptake of 10 • 10⁻⁶ M fluorescein after 2 min. After that, cells were washed 4 times with ice-cold PBS until no fluorescein was detectable in the washing solution. The cells were then lysed in 1 ml 0.1 % Triton - X100 in 20 mM MOPS (3-(N-morpholino)propanedulfonic acid) buffered at pH 7.4 and fluorescence was counted in a multiwell - plate-reader (Victor², Wallac Instruments, Finland). Counts were corrected for extracellular binding and unspecific adhesion to the cells by subtraction of fluorescein counts on cells at 4°C. Fluorescein counts were normalized to protein content in the lysate measured by BCA protein assay (Pierce, Rockford, USA).

Cloning of putative hOAT1/3 promoter sequences. hOAT1

Based on the human genomic sequence (accession no. NT_033903.7), the 1999-base pair flanking region upstream of the transcription start site was cloned by PCR using the primers listed in Table 1 and human genomic DNA (Clontech). Primers were chosen in similar to previously described [25].

hOAT3

Based on the human genomic sequence (accession no. NT_033903.7), the 2083-base pair flanking region upstream of the transcription start site was cloned by PCR using the primers listed in Table 1 and human genomic DNA (Clontech). Primers were chosen in similar to previously described [26].

The respective PCR product was isolated by electrophoresis and subcloned into the secretory alkaline phosphatase reporter vector pSEAP2-Basic (Promega; accession no. U89937), at NheI and XhoI sites. The sequences were verified by sequencing of the plasmids gained (Eurofins/MWG Operon; Martinsried,

Table 1. Primers used for cloning the putative promotor sequences of hOAT1 and hOAT3

	hOAT1-Prom	hOAT3-Prom
gene	hOAT1 / SLC22A6	hOAT3 / SLC22A8
accession no.	NC_000011.9	NC_000011.9
location	62754161 to 62752162	62784509 bis 62782427
product size	1999	2082
upstream primer (Nhe I)	GGGCTAGCAGTGAGGATGTGGTCCAAG	GGGCTAGCGTGATAGATCCCCAATAAGG
downstream primer (Xho I)	GGCTCGAGTGGGCCAGGCCAGCCAGT	GGCTCGAGGCAGCTCAGTCTAACAAGC

Germany). This full-length reporter plasmids are hereafter referred to as hOAT1-Prom or hOAT3-Prom, respectively.

Reporter-Gen-Assay (SEAP)

Expression status was assessed by the Mercury™ Pathway Profiling reporter gene assay system from Clontech Inc. using secretory alkaline phosphatase (SEAP) as reporter, essentially as described earlier [27]. In brief, the HEK cells were transfected (FuGene® transfection kit from Promega) with the respective SEAP plasmid (hOAT1, hOAT3) or empty vectors (SEAP-Basic) and cultivated for 24h. Growth medium was collected for determination of SEAP activity and cells were subjected to model ischemia and reperfusion as described above. Then again, growth medium was collected and SEAP activity was assayed. The SEAP signal after model ischemia and reperfusion (6h) from each well was normalized to the respective signal before the start of ischemia in the same well in order to obtain an internal normalization. Subsequently, the respective SEAP signals from controls were set as 100%. SEAP-activity in the media was determined with the AttoPhos® System (Promega, Mannheim, Germany).

R(verse)T(ranscriptase)-PCR

RNA from HEK cells was extracted using Qiagen RNA Isolation Kit (Qiagen, Hilden, Germany). RNA concentration was determined and cDNA was synthesized using iScript cDNA synthesis kit (Biorad, CA, USA) according to the manufacturers instructions. In brief, RT-PCR was performed according to iQ SYBR-Green Supermix RT-PCR system protocol (Biorad, CA, USA). Initial denaturation was performed at 95°C for 3 min. For human E prostanoid receptor type 2 (hEP2), the primers were 5'- AGA GGG GAA AGG GTG TCC AT -3' (sense) and 5'- CCA AAG GCC AAG TAC CAT GC -3' (antisense), resulting in an 562 bp RT-PCR-product. Primers were spanning a 11829 bp intron (accession no. NT_026437.12). For human E prostanoid receptor type 4 (hEP4), the primers were 5'- TGC TTC ATC GAC TGG ACC AC -3' (sense) and 5'- TTC TCG CTC CAA ACT TGG CT -3' (antisense), resulting in an 408 bp RT-PCR-product. Primers were spanning a 9918 bp intron (accession no. NT_006576.16). The RT-PCR products were tested for correct size by agarose gel electrophoresis and melting point analysis. PCR products were eluted and sequenced by Eurofins/MWG Operon (Martinsried, Germany).

Data analysis

Data are presented as means ± SEM respectively. The *n* value is given in the text or in the figures and represents the number of supports used for the respective experiments (wells, filters, culture dishes). Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls test. Differences were considered statistically significant when *P* < 0.05.

Materials

If not stated otherwise, chemicals used were from Sigma or Tocris. - Concentrations of H89 and butaprost were chosen according to our former study [28]. The same is true for SC560 and SC58125 [22]. U-73122 was applied according to the supplier (Tocris) giving an IC₅₀ below 5 μM. Concentrations of TCS2510 were chosen according to [29] indicating a biological effect at around 10 μM. Concentrations and effects of AH6809 and L161,982 are discussed in detail in the discussion section.

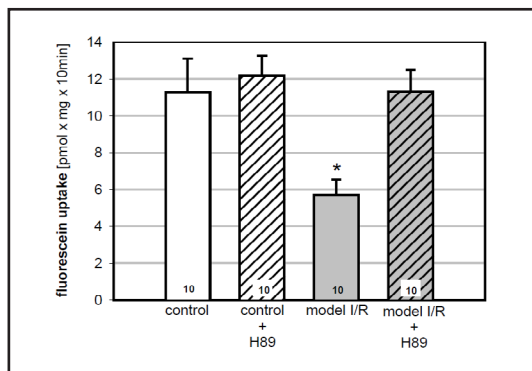


Fig. 1. Basolateral uptake of organic anions into OK cells after model ischemia and reperfusion: Effect of PKA inhibition. Fluorescein uptake was determined as described in the methods section. Fluorescein uptake is expressed as pmol per mg cell protein and uptake time. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for 48h with or without application of 5 μ M H89. n is given in the respective bars. Statistical significance is indicated by * (indicating difference to untreated control).

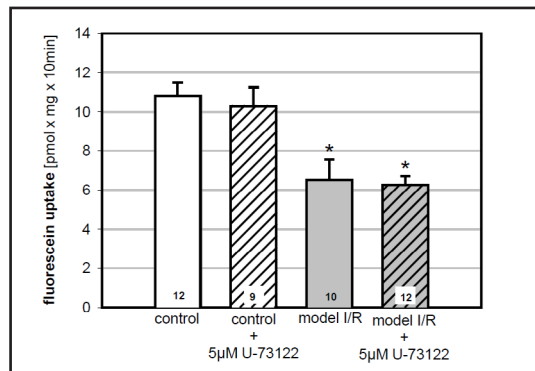


Fig. 2. Basolateral uptake of organic anions into OK cells after model ischemia and reperfusion: Effect of PLC inhibition. Fluorescein uptake was determined as described in the methods section. Fluorescein uptake is expressed as pmol per mg cell protein and uptake time. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for 48h with or without application of 5 μ M U-73122. n is given in the respective bars. Statistical significance is indicated by * (indicating difference to untreated control).

Results

Fluorescein transport into proximal tubular cell line (OK)

Recently, we demonstrated I/R induced down regulation of (a) the transport of the prototypic organic anion fluorescein in OK cells, (b) the renal transport of organic anions and (c) the rOat1 and rOat3 regulation is most likely mediated by COX1 metabolites [15, 21, 22]. In kidney tissue, the expression of the G protein coupled E prostanoid receptors type 1 to 4 is well characterized. Among these, activation of EP2 and EP4 leads to cAMP mediated stimulation of the PKA, whereas EP3 inhibits the mentioned cAMP signaling pathway. EP4 additionally generates PLC mediated activation of PKC [30, 31]. Consequently, we tested the effect of PKA inhibition by 5 μ M H89 on fluorescein uptake into proximal tubular OK cells. As shown in Fig. 1, uptake of fluorescein is impaired after model I/R, which is abrogated when PKA is inhibited by H89. H89 itself has no effect on PKA. Inhibition of PLC however had no effect on ischemia induced impairment of fluorescein uptake transport (Fig. 2). Therefore, in this particular model system I/R induced impairment of fluorescein uptake is most probably mediated via activation of EP2 and/or EP4.

Subsequently, we investigated the effect of antagonizing EP2 (by 35 μ M AH6809) or EP4 (by 5 μ M L161,982) receptor on fluorescein uptake after I/R. As shown in Fig. 3, fluorescein uptake is still impaired despite application of AH6809, whereas L161,982 abolishes I/R induced down regulation of fluorescein transport. Both substances have no effect in control cells. In order to validate these data, we incubated OK cells with the respective agonists and detected fluorescein uptake as described. As depicted in Fig. 4A, TCS2510 (EP4 agonist) dose dependently down regulates fluorescein uptake into proximal tubular OK cells, whereas butaprost (EP2 agonist) failed. Consequently, we conclude that ischemia and reperfusion induced impairment of fluorescein uptake into proximal tubular OK cell is mediated by COX1 products dominantly via activation of E prostanoid receptor type 4. Of note however, only EP4 receptor and not the EP2 receptor was detected in OK cells [28], which may be due to the fact that RNA sequence data of the opossum are not available and the primers in the respective study were generated from the rat sequence. Therefore we cannot decide whether the absence of effects for the EP2 antagonist AH6809 or the EP2 agonist butaprost is due to

Fig. 3. Basolateral uptake of organic anions into OK cells after model ischemia and reperfusion: Effect of EP2 or EP4 antagonists. Fluorescein uptake was determined as described in the methods section. Fluorescein uptake is expressed as pmol per mg cell protein and uptake time. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for 48h with or without application of 35 μ M AH6809 (EP2 antagonist) or 5 μ M L161,982 (EP4 antagonist). n is given in the respective bars. Statistical significance is indicated by * (indicating difference to untreated control) and # (indicating difference of model I/R + L161,982 to model I/R without treatment).

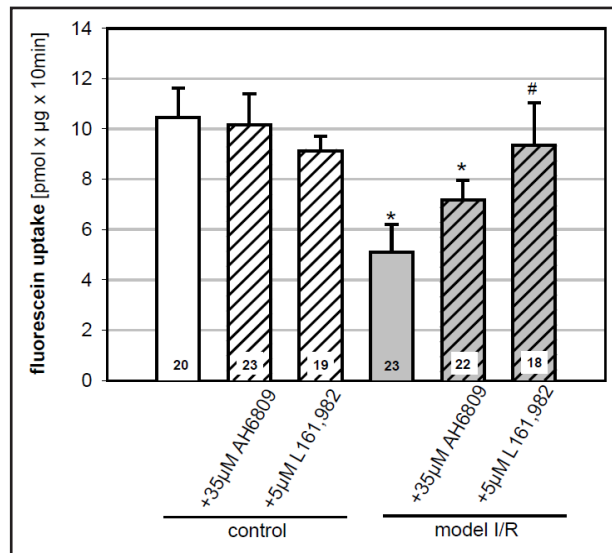
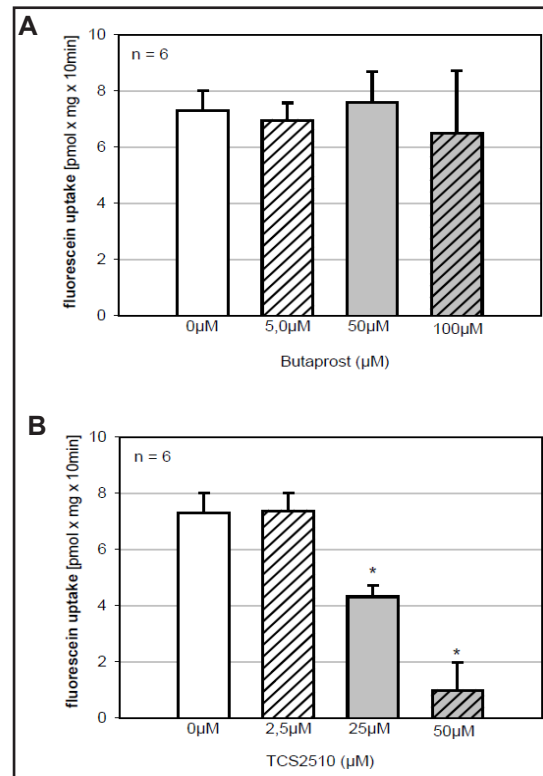


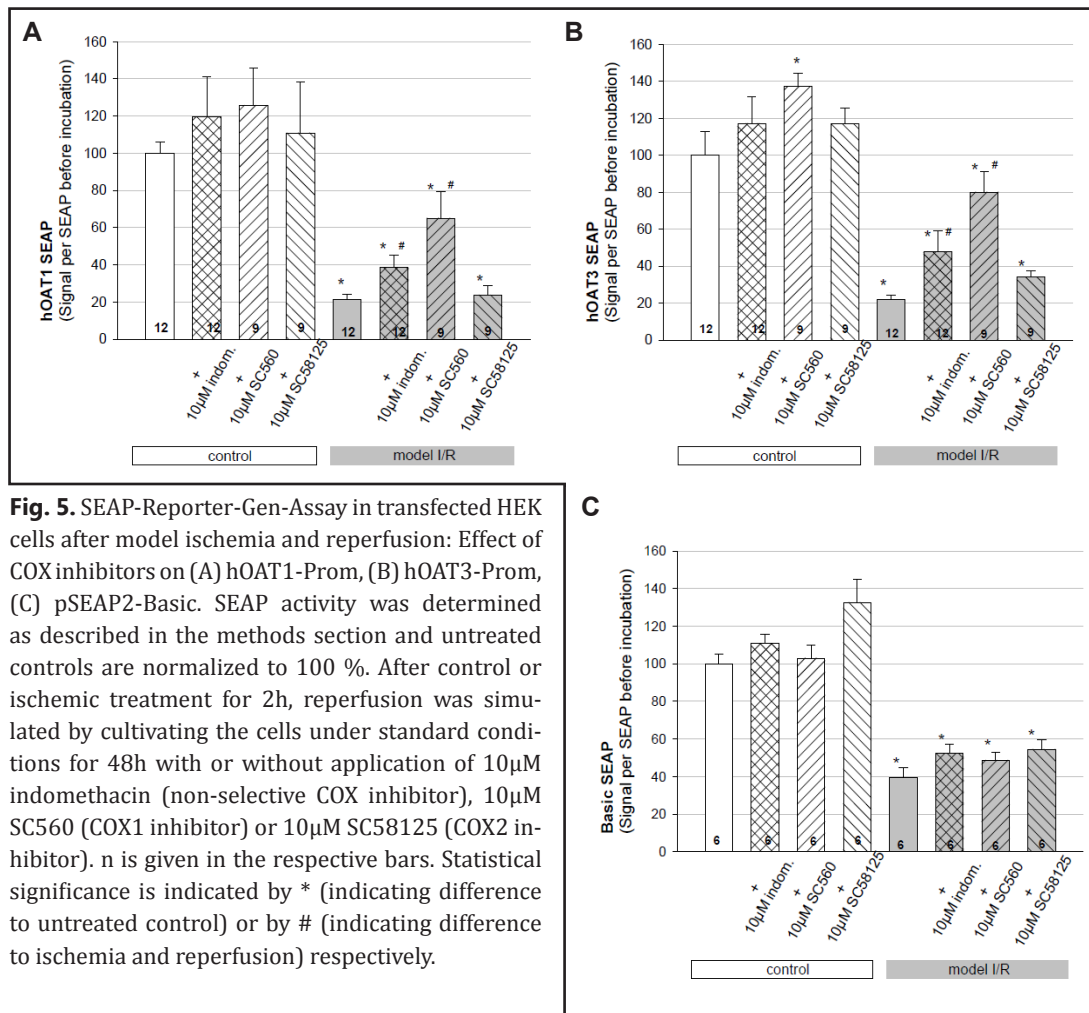
Fig. 4. Basolateral uptake of organic anions into OK cells under control conditions: Effect of EP2 (A) or EP4 (B) agonists. Fluorescein uptake was determined as described in the methods section. Fluorescein uptake is expressed as pmol per mg cell protein and uptake time. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for 48h with or without application of up to 100 μ M butaprost (EP2 agonist) or 50 μ M TCS2510 (EP4 agonist). n is given in the respective bars. Statistical significance is indicated by * (indicating difference to untreated control).



the absence of the respective EP2 receptor in OK cells or due to the fact that this receptor is not involved in regulation of fluorescein transport in OK cells in general.

Reporter Gen Assays

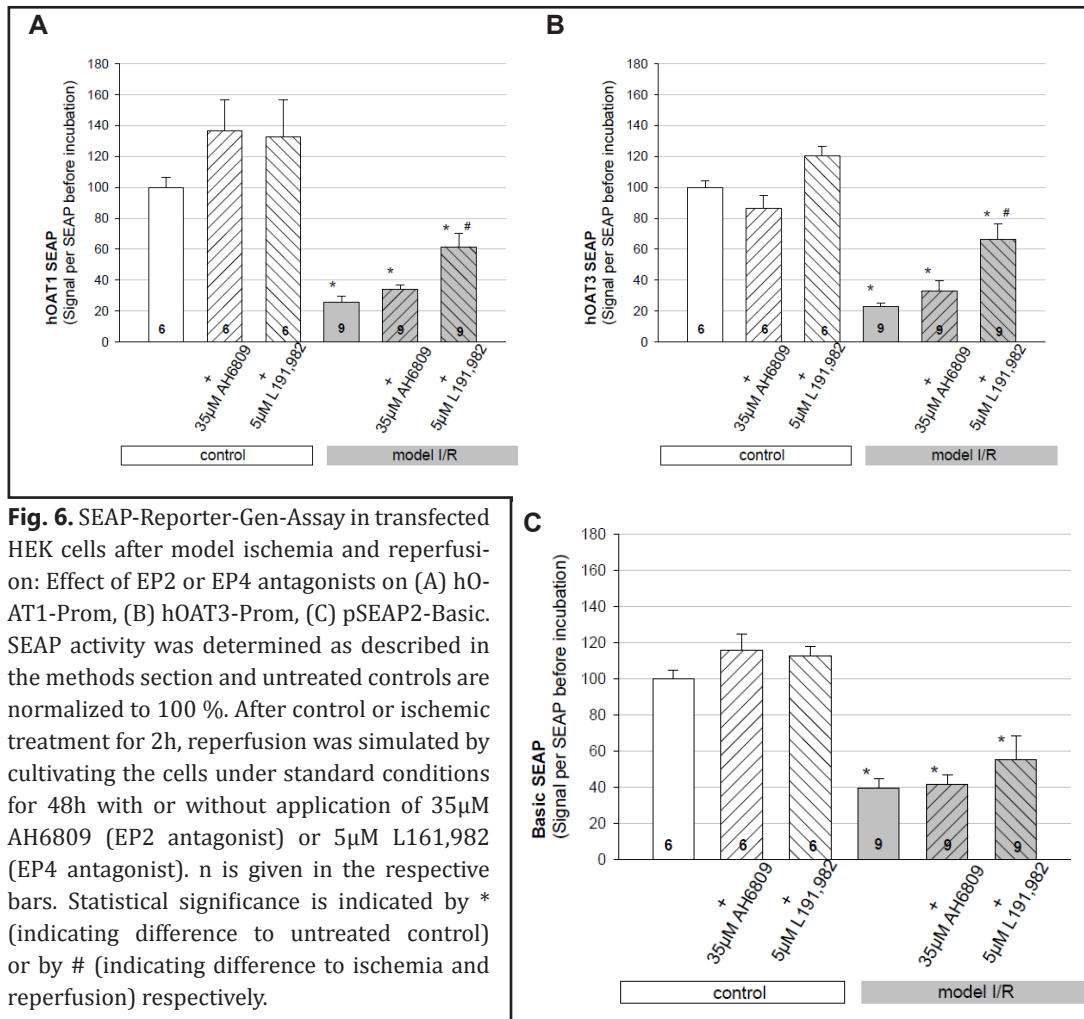
The putative promoter sequences of hOAT1 or hOAT3 were cloned into pSEAP2-Basic and the respective vector plasmids were transfected into human embryonic kidney (HEK-293) cells (see methods section). Model I/R was applied as described. In accordance to what we have previously shown for fluorescein uptake in proximal tubular cells [21] and for expression of rOat1 and rOat3 in rat [19], indomethacin partly abolished I/R induced impairment of SEAP-activity for both the hOAT1-Prom and the hOAT3-Prom (Fig. 5 A/B). Specific inhibition of COX1 (by SC560) even acted more pronounced (Fig. 5 A/B), whereas



SC58125 (selective COX2 inhibitor) had no effect. This improvement is in accordance with fluorescein uptake data from proximal tubular cells and with expression of rOat1 and rOat3 in rat [21, 21]. SC560 alone led to a slight increase in SEAP activity of the hOAT3-Prom, whereas otherwise no effects in controls were detected. In HEK cells transfected with the pSEAP2-Basic Vector, model I/R impaired SEAP activity in similar to what was shown for hOAT1-Prom and hOAT3-Prom (Fig. 5C). In contrast however, I/R induced down regulation by indomethacin or SC560 is not abolished (Fig. 5C). Thus, impairment of transcriptional regulation after I/R seems to be a rather unspecific effect, whereas inhibition of COX1 (after ischemia) seems to specifically counteract this phenomenon if putative promoter sequences for hOAT1 and hOAT3 are present in the vector (hOAT1-Prom, hOAT3-Prom).

In accordance to what we demonstrated above for fluorescein uptake in proximal tubular cells, antagonizing EP4 receptor (by 5µM L161,982) partly abolished I/R induced impairment of SEAP-activity for both, the hOAT1-Prom and the hOAT3-Prom (Fig. 6 A/B), whereas 35µM AH6809 (EP2 antagonist) had no effect. In HEK cells transfected with the pSEAP2-Basic vector, there is no abrogation of I/R induced down regulation by AH6809 or L161,982 (Fig. 6C). Thus again, impairment of transcriptional regulation after I/R seems to be a rather unspecific effect, whereas antagonizing EP4 (after ischemia) seems to specifically counteract this phenomenon if putative promoter sequences for hOAT1 and hOAT3 are present in the vector (hOAT1-Prom, hOAT3-Prom).

As shown in Fig. 7A/B, TCS2510 (EP4 agonist) dose dependently down regulates SEAP-activity for both the hOAT1-Prom and the hOAT3-Prom, whereas butaprost (EP2 agonist) has no such effect (Fig. 8 A/B). In HEK cells transfected with the pSEAP2-Basic Vector



both, TCS2510 or butaprost, had no effect on the respective SEAP signal (data not shown). Consequently, we conclude that after I/R COX1 product(s) induced activation of E prostanoid receptor type 4 is involved in increased expression of hOAT1-Prom and hOAT3-Prom.

Both EP4 and EP4 were detected by RT-PCR in HEK cells (Fig. 9). Thus, the absence of effects for the EP2 antagonist AH6809 or the EP2 agonist butaprost are obviously not due to the absence of the respective EP2 receptor in HEK-293 cells.

Discussion

From the data presented in our study, we conclude that the impaired expression of Oat1 and Oat3 observed in the rat model of iAKI [12, 19, 32] is most likely also due to a down regulation of gene transcription. Interestingly, the signal of the empty control vector pSEAP2-Basic was decreased to a similar extent after model I/R, indicating an inhibitory effect of I/R on gene transcription in general. In opposite to what was found for hOAT1-Prom and hOAT3-Prom, this particular pSEAP-Basic signal was not increased due to inhibition of COX1. Thus, we conclude that the particular putative human promoter sequences are responsible for the induction of OAT expression after inhibition of COX1. Moreover, as the inhibitors were only applied after ischemia with the very beginning of reperfusion, we conclude that during reperfusion COX1 metabolites are responsible for further impairing expression of both OAT1

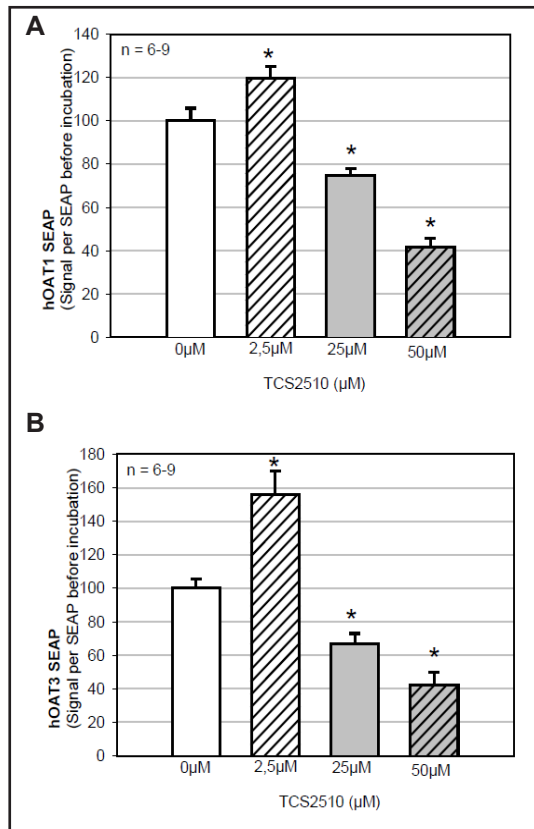


Fig. 7. SEAP-Reporter-Gen-Assay in transfected HEK cells under control conditions: Effect of TCS2510 on (A) hOAT1-Prom or (B) hOAT3-Prom. SEAP activity was determined as described in the methods section and untreated controls are normalized to 100 %. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for 48h with or without application of up to 50μM TCS2510 (EP4 agonist). n is given in the respective figure. Statistical significance is indicated by * (indicating difference to untreated control).

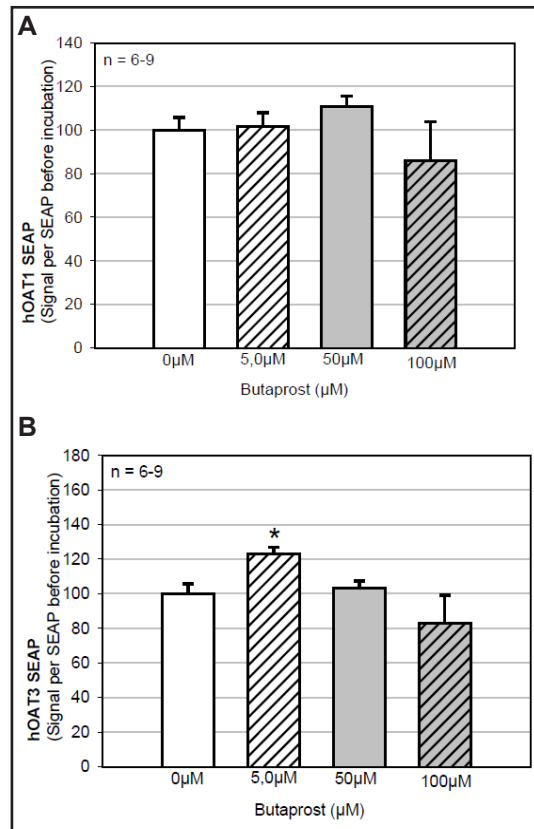
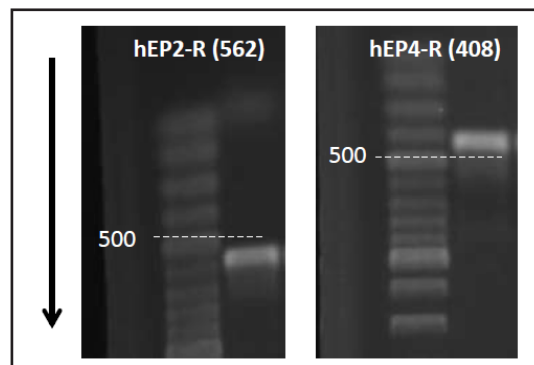


Fig. 8. SEAP-Reporter-Gen-Assay in transfected HEK cells under control conditions: Effect of butaprost on (A) hOAT1-Prom or (B) hOAT3-Prom. SEAP activity was determined as described in the methods section and untreated controls are normalized to 100 %. After control or ischemic treatment for 2h, reperfusion was simulated by cultivating the cells under standard conditions for 48h with or without application of up to 100μM butaprost (EP2 agonist). n is given in the respective figure. Statistical significance is indicated by * (indicating difference to untreated control).

Fig. 9. Detection of EP2 or EP4 mRNA in HEK cells. RT-PCR was performed as described in the methods section. Primers were intron spanning. Products of correct size were generated for E prostanoid receptor type 2 (hEP2-R; left panel; size 562 bp) and E prostanoid receptor type 4 (hEP4-R; right panel; size 408 bp). Products were eluted and sequenced. Both products were of correct sequence indicating correct detection of the respective receptors.



and OAT3. In opposite, down regulation of translation itself seems to be due to an effect which is not specific for OAT1 and OAT3.

Subsequently, we investigated how the COX1 metabolites are impairing expression (and therefore function) of the renal basolateral organic anion transporters. In our functional readout (fluorescein transport), we found that I/R induced impairment of organic anion uptake is dependent on PKA activity, whereas it is independent from PLC. In accordance to what is known about E prostanoïd receptor signaling [30, 31] and to what was already mentioned in the results section, this was evidence that activation of E prostanoïd (EP) receptor type 2 or 4 is responsible for I/R induced impairment of organic anion uptake into proximal tubular cells. Application of selective EP receptor antagonist directly after model ischemia at the beginning of reperfusion gave evidence that EP receptor type 4 rather than type 2 is part of the signaling cascade. However, AH6809 (applied at 35 μ M) is not only antagonizing EP2 (K_i = 0.35 μ M) but also EP1 (with a much lower affinity). In similar, L161,982 (applied at 5 μ M) is not only antagonizing EP4 (K_i = 0.024 μ M) but also EP1, EP2 and EP3 (K_i is 19 μ M, 23 μ M and 2 μ M, respectively). Therefore we applied specific agonists for the EP2 (butaprost) or the EP4 receptor (TCS2510) in increasing concentrations in order to generate a dose response for this particular substances and organic anion transport. Together, the data indicate that organic anion uptake into renal proximal tubular OK cells after I/R is impaired by COX1 metabolites which most probably act via the EP4 receptor.

Again, we tested whether the data obtained in the physiological readout are due to regulation of expression in a reporter gene assay consisting of the putative promoter sequences from human OAT1 or OAT3 in order to gain evidence whether the data obtained will possibly also hold true for the human system. Likewise, we found the predicted pattern to be true for both vectors (hOAT1-Prom or hOAT3-Prom), showing an increased transcription after model I/R by application of 5 μ M L161,982 (EP4 antagonist). In accordance to the transport assay, TCS2510 (EP4 agonist) alone abolished expression of both hOAT1-Prom and hOAT3-Prom (dose response; Fig. 8 A/B), whereas butaprost (EP2 agonist) had no effect. Taken together, the data indicate that transcription of hOAT1 and hOAT3 after I/R is impaired by COX1 metabolites which most probably act via the EP4 receptor.

Unfortunately, any data on regulation of both Oat1 (Slc22a6) or Oat3 (Slc22a8) other than those regarding renal ischemia from our group are very sparsely. There are some data published on sex dependent regulation in mice [33] and rat [34]. And some addressed the effect of hyperuricemia [35], ureteral obstruction [36] and the effect of cytostatics [37]. The same is true when it comes to molecular data on transcriptional regulation, except from [26] where a conserved cAMP response element in the promoter sequence of hOAT3 (SLC22A8) was demonstrated and data indicating constitutive and inducible transcriptional activation via CRE, which is well in accordance with the particular signaling described in our study. Moreover, the data available for hOAT3 (SLC22A8) are in accordance with a report [38] pointing to down regulation of ocular Slc22a8 in rat and calf in case of hypoxia. Exactly this phenomenon of hypoxia also takes place in renal tissue in iAKI and is part in the model ischemia applied [20]. Unfortunately, data on molecular regulation of hOAT1 (SLC22A6) are very rare and constricted to a study demonstrating regulation by the transcription factor HNF4 α [25]. Own in silico analysis of the cloned promoter sequence of hOAT1 (SLC22A6) indicates the presence of possible CREB binding sites (data not shown), however the importance of these sites for expression still has to be verified. However, due to genomic clustering of hOAT1 (SLC22A6) and hOAT3 (SLC22A8) described by Bhatnagar et al. [39] it at least seems highly reasonable that both transporters are regulated in a similar manner, as we found in our study.

Taken altogether, we can answer the questions posed in the introduction as follows: We (a) gained additional insight in the regulation of renal organic anion transport(ers) after I/R showing that COX1 metabolites are involved by acting via the EP4 receptor. We (b) obtained serious evidence that the data already published and from physiological readout are due to transcriptional regulation of the respective transporters. As the promoter sequences cloned were of human origin and expressed in a human renal epithelial cell line (HEK-293) we speculate that this is increased (c) evidence that the regulatory mechanisms described after

renal I/R will possibly be transferable to humans. At least, this model represents a suitable tool for research on expression of hOAT1 and hOAT3.

Consequently, we finally hypothesize that the beneficial effect of low dose indomethacin [19] or COX1-inhibitor [22] on expression and function of rOat1 (Slc22a6) and rOat3 (Slc22a8) detected in rats after renal I/R might also take place in humans. Even more important, the beneficial effect on renal outcome might also take place in humans. Just recently, we gained serious evidence that down regulation of rOat1 (Slc22a6) and rOat3 (Slc22a8) in rats is mechanistically involved in renal damage due to I/R [23]. Thus, we hypothesize that an OAT dependent mechanism may also be of clinical importance in humans. If this hypothesis proves true, it will represent a new mechanistic model of the induction of renal damage after I/R injury.

Abbreviations

hOAT1-Prom (putative hOAT1 promoter sequence cloned into pSEAP2-Basic vector according to methods section); hOAT3-Prom (putative hOAT3 promoter sequence cloned into pSEAP2-Basic vector according to methods section); MES (2- (N-morpholino)ethanesulfonic acid); BCA (bicinchoninic acid).

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Disclosure Statement

None of the authors has competing interests with respect to any issue of the respective study.

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