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Downregulation of duodenal SLC transporters and activation of proinflammatory signaling constitute the early response to high altitude in humans

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Wojtal KA, Cee A, Lang S, Götze O, Frühauf H, Geier A, Pastor-Anglada M, Torres-Torronteras J, Martí R, Fried M, Lutz TA, Maggiorini M, Gassmann M, Rogler G, Vavricka SR. Downregulation of duodenal SLC transporters and activation of proinflammatory signaling constitute the early response to high altitude in humans. *Am J Physiol Gastrointest Liver Physiol* 307: G673–G688, 2014. First published June 26, 2014; doi:10.1152/ajpgi.00353.2013.—Solute carrier (SLC) transporters mediate the uptake of biologically active compounds in the intestine. Reduced oxygenation (hypoxia) is an important factor influencing intestinal homeostasis. The aim of this study was to investigate the pathophysiological consequences of hypoxia on the expression and function of SLCs in human intestine. Hypoxia was induced in human intestinal epithelial cells (IECs) in vitro (0.2; 1% O_2 or $CoCl_2$). For human in vivo studies, duodenal biopsies and serum samples were obtained from individuals ($n = 16$) acutely exposed to 4,554 meters above sea levels. Expression of relevant targets was analyzed by quantitative PCR, Western blotting, or immunofluorescence. Serum levels of inflammatory mediators and nucleosides were determined by ELISA and LC/MS-MS, respectively. In the duodenum of volunteers exposed to high altitude we observed decreased mRNA levels of apical sodium-dependent bile acid transporter (ASBT), concentrative nucleoside transporters 1/2 (CNT1/2), organic anion transporting polypeptide 2B1 (OATP2B1), organic cation transporter 2 (OCTN2), peptide transporter 1 (PEPT1), serotonin transporter (SERT), and higher levels of IFN-γ, IL-6, and IL-17A. Serum levels of IL-10, IFN-γ, matrix metalloproteinase-2 (MMP-2), and serotonin were elevated, whereas the levels of uridine decreased upon exposure to hypoxia. Hypoxic IECs showed reduced levels of equilibrative nucleoside transporter 2 (ENT2), OCTN2, and SERT mRNAs in vitro, which was confirmed on the protein level and was accompanied by activation of ERK1/2, increase of hypoxia-inducible factor (HIF) proteins, and production of IL-8 mRNA. Costimulation with IFN-γ and IL-6 during hypoxia further decreased the expression of SERT, ENT2, and CNT2 in vitro. Reduced oxygen supply affects the expression pattern of duodenal SLCs that is accompanied by changes in serum levels of proinflammatory cytokines and biologically active compounds demonstrating that intestinal transport is affected during systemic exposure to hypoxia in humans.

solute carrier; intestine; hypoxia; inflammation

PHYSIOLOGICAL RESPONSE TO hypoxia is one of the most evolutionary conserved adaptive mechanisms in humans (48). It is initiated by the drop of oxygen concentration and recognized by well-characterized hypoxia-inducible factors (HIFs) present in every cell. Once stabilized and heterodimerized, these transcription factors bind to hypoxia-responsive elements (HRE) followed by either induction or repression of gene expression (17, 24). Hypoxia-induced responses are initiated at a cellular level and propagated onto tissue and eventually to systemic levels. Numerous targets associated with response to hypoxia have been identified and validated. They are typically associated with generation of ATP, oxidative stress, iron absorption, and immune responses (3, 4, 19, 32, 37, 39, 42, 50).

Not surprisingly, hypoxia plays an important role also in intestinal (patho)physiology. In a steady state, the intestinal wall is exposed to a steep oxygen gradient reaching from highly oxygenated submucosa to the almost anoxic intestinal lumen; because of this phenomenon, intestinal epithelial cells (IECs) are to some extent permanently facing "physiological hypoxia" (9). In concert with this notion, it has been reported that both intestinal absorption and permeability are affected by oxygen concentration (30, 31). It is known that electrical currents decrease rapidly in hypoxic conditions, specifically when applied from serosal side of intestinal tissue (7). This implies that the ion transport across gut epithelium depends on the local oxygen concentration. Interestingly, in some pathological conditions such as colitis, the inflammatory microenvironment induces hypoxic signaling, a phenomenon generally described as "inflammatory hypoxia" (9, 14). The activation of proinflammatory signaling in the hypoxic environment occurs via mutual interplay between HIF-1α and NF-κB transcription factors (5) that subsequently leads to the release of proinflam-

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matory mediators as described in both in vitro (46) and in vivo studies (27). The presence of these mediators is an important feature of intestinal inflammation as demonstrated earlier (49).

Solute carrier (SLC) transporters mediate the uptake of a large variety of nutrients, ions, and biologically active compounds across plasma membranes and organelles. The biological significance of these proteins is high since they participate in important processes such as differentiation, absorption, stress, inflammation, neurotransmission, and metabolism (29). In the intestine, solute carriers are predominantly expressed in the IECs, but some of them also in other cell types, including immune cells (35). It has been demonstrated that oxygen deprivation affects the intestinal iron absorption via HIF-2-dependent mechanisms (23, 32, 54). In addition, the expression levels of equilibrative nucleoside transporters in IECs are suppressed by hypoxia and this phenomenon may have a direct effect on the attenuation of inflammation in the gut (13, 36). It has been shown earlier that low oxygen levels can decrease the levels of carnitine transporter organic cation transporter (OCTN) 2 (8, 40) as well as cystic fibrosis transmembrane conductance regulator (CFTR) (56). Despite the fact that these studies were performed in specific pathological settings, they can provide some indications about the control of other immunologically and physiologically relevant intestinal SLC transporters during hypoxia in humans.

Previously, we reported that the mRNA expression levels of SLCs are altered in patients with inflammatory bowel diseases (IBD) (52). In the present study we investigated the impact of hypoxia on expression and function of specific subset of physiologically relevant intestinal SLC transporters and proinflammatory responses in the gut. We observed that both hypoxia-driven changes in the intestinal SLCs and proinflammatory signaling are coexisting events and can provide insights into the novel, immunologically relevant adaptive mechanisms to oxygen deprivation in the intestine.

MATERIALS AND METHODS

Human subjects and exposure to high-altitude hypoxia. Description of human subjects and the study design for high-altitude hypoxia was given in detail elsewhere (2, 44); the study was approved by the Ethics Committee of the Canton of Zurich (EK-1677). Briefly, after blood and biopsy sampling at the University Hospital Zurich (USZ), healthy individuals ($n = 16$) ascended to the Capanna Regina Margherita (CM; 4,559 m above sea level) where both duodenal biopsies and peripheral blood were taken at *days 2* and *4* after ascent to high altitude. Acute mountain sickness (AMS) was diagnosed in some subjects using the Lake Louise Score (LLS >5) accompanied by medical examination. These individuals ($n = 8$) were given dexamethasone (9-fluor-16a-methylprednisolone, dexamethasone; Galepharm, Kuesnacht, Switzerland) at 2 × 8 mg/day as soon as the AMS symptoms appeared (after all samples were collected on *day 2*) and distinguished retrospectively from the control group ($n = 8$) during analysis of duodenal and serum targets. All biological specimens were stored and transported in liquid nitrogen until the analysis in the laboratory of Gastroenterology and Hepatology of USZ.

Antibodies, chemicals, Taqman assays, and recombinant cytokines. Rabbit anti-phospho Akt (no. 4058), rabbit anti-Akt (no. 9272), rabbit anti-phospho p44/42 (no. 9101), rabbit anti-p44/42 (no. 4695), rabbit anti-p38 (no. 9212), rabbit anti-STAT1 (no. 9172), and rabbit anti-β-actin (no. 4970) antibodies were purchased from Cell signaling (Danvers, MA). Rabbit anti-ENT2/SLC29A2 (ARP43822) and rabbit anti-OCTN2/SLC22A5 (ARP63404) antibodies were obtained from Aviva Systems Biology (San Diego, CA). Rabbit anti-human SERT/SLC6A4 (BS-1893R) was purchased from Bioss (Woburn, MA). Rabbit anti-HIF-1α antibody (NB100–479) was obtained from Novus Biologicals (Littleton, CO). Mouse anti-HIF-2α was purchased from Abcam (Cambridge, UK). Human recombinant IFN-γ (IFG40012) and IL-6 (I1395), and $CoCl_2$ (15862) were purchased from Sigma-Aldrich (Buchs, Switzerland). FM19G11 (sc-364490) and FR180204 (sc-203945) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). β-actin (4310881E), GLUT-1 (Hs00892681_m1), IFN-γ (Hs00989291_m1), and IL-6 (Hs00174131_m1) Taqman assays were purchased from Applied Biosystems (Foster City, CA). All Taqman assays used for detection of duodenal SLC transporters and villin-1 by RT-PCR were identical with those used in our previous studies (34, 52).

Quantification of chemokines, cytokines, and other metabolites in human serum. The analysis of the cytokine content in serum samples of at least six human subjects was completed at Rules-Based Medicine (Austin, TX) using Human Cytokine MAP A 1.0 array. Serotonin concentration in serum samples of at least six human subjects was determined using Serotonin Research ELISA purchased from Rocky Mountains Diagnostics (BA E-5900; Colorado Springs, CO) according to the manufacturer's protocol. The levels of total bile acids were quantified by colorimetric assay (BQ 092A-EALD; BQKits Diagnostics, San Diego, CA).

Cultivation of IECs. Human epithelial intestinal cell line T84 were cultivated at 37°C in a 5% CO_2 incubator in high-glucose DMEM medium (Sigma-Aldrich) containing L-glutamine, sodium bicarbonate, and 10% FCS. T84 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA).

RNA isolation, RT-PCR, and analysis of data. Frozen tissues were lysed in RLT buffer containing DTT and processed by QIACUBE (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Reverse transcription was performed using RT kit (Life Sciences, Zug, Switzerland) according to the manufacturer's protocol. RT-PCR analysis of target genes was performed relatively to the expression of either villin-1 (SLC transporters) or β-actin (other targets) as housekeeping genes. The expression levels of targets for human mRNA expression levels were assessed by setting the expression levels of ground level (Zurich, 400 m above sea level) to 1. The expression of housekeeping genes was measured in triplicates and the levels of other targets in duplicates for each duodenal specimen. For experiments with cell lines, the measurements were performed in duplicates for every target.

Induction of hypoxia and stimulation with cytokines. To induce hypoxia in T84 cells, we used hypoxic chambers. Briefly, cells were plated on Polyester (PET) Membrane Transwell-Clear Inserts (3470; Costar; Sigma-Aldrich) for 4 wk. Afterwards cells were incubated in either 0.2 or 1% of O_2 (5% CO_2) hypoxic chambers (InVivo400, Ruskinn, Bridgend, UK) in standard cultivation medium (Sigma-Aldrich) for the indicated time points. For costimulation with proinflammatory cytokines, both IL-6 and IFN-γ were added to the basal chamber at the concentration of 50 ng/ml 24 h before harvesting. Alternatively, to induce hypoxia chemically, T84 cells were stimulated with different concentrations of $CoCl_2$ (Sigma-Aldrich) in standard cell culture incubator for 48 h as described elsewhere (51).

Cell lysis and protein determination. Cells were scraped from the Transwells in the presence of ice-cold M-PER buffer (Pierce, Wohlen, Switzerland) containing 1 mM DTT and cocktail of protease inhibitors (Roche, Basel, Switzerland). Protein concentrations in the cell lysates were determined using the BCA Protein Assay Reagent (Pierce). Cell lysates containing 10 μg of total protein were suspended in NuPAGE LDS Sample Buffer (Life Sciences) containing 50 mM DTT.

SDS electrophoresis and Western blotting. Proteins were separated in 10% polyacrylamide gel (Tris/glycine) and transferred onto nitrocellulose membranes. Before incubation with primary antibodies, membranes were blocked with 5% solution of nonfat dried milk buffer in TBS-T (TBS + 0.1% Tween) for 1 h at room temperature (RT) with gentle shaking. Primary antibodies were diluted in TBS-T containing 5% BSA and incubated with the membranes overnight at 4°C.

Corresponding secondary antibodies were diluted in TBS-T buffer containing 5% blocking milk for 1 h at RT. Bands were detected by using ECL detection kit (GE Healthcare, Glattbrugg, Switzerland).

Immunofluorescence. T84 cells were grown on Transwells until full confluence and incubated in hypoxic chambers for indicated time points. Monolayers were then fixed with 4% PFA for 30 min, permeablized with 0.1% Triton X-100 for 15 min, and blocked with 5% BSA for 1 h at RT. Primary antibodies were applied overnight at 4°C followed by respective secondary ABs 1 h at RT. Monolayers were mounted on glass slides using Dako mounting medium.

Cell viablility, proliferation, and cytotoxicity assays. T84 cells were grown on 96-well plates to reach ~80% confluency. LDH release assay (11644793001) purchased at Roche as well as cell proliferation (G3582) and cell viability assay (G9241) purchased at Promega (Dubendorf, Switzerland) were performed in according to the manufacturers' protocols.

Quantification of uridine and hypoxanthine in serum. Serum uridine and hypoxanthine were measured by liquid chromatography coupled to tandem mass spectrometry using an Acquity UPLC-Xevo TQ Mass Spectrometer (Waters, Milford, MA). Two-hundred microliters of serum samples were deproteinized by addition of 9 μl of perchloric acid 11.6 M (final concentration 0.5 M) and centrifuged at 20,000 *g* and 4°C for 10 min. Then, 5 μl of supernatant were injected into the UPLC-MS/MS apparatus. The stationary phase was an Acquity UPLC BEH C18 column (100 × 2.1 mm, 130-Å pore, 1.7-μm particle; Waters), and the mobile phase was set at 0.5 ml/min through a binary gradient elution using a saline buffer (20 mM ammonium acetate, pH 5.6) and acetonitrile, as follows: *time 0* to 1.1 min, isocratic 100% saline buffer; 1.1 to 5 min, gradient from 0 to 13.6% acetonitrile; 5 to 5.1 min, gradient from 13.6 to 100% acetonitrile; 5.1 to 6.1 min, isocratic 100% acetonitrile; and 6.1 to 7.2 min, isocratic 100% saline buffer. Detection of the eluate components was performed using multiple reaction monitoring, with positive electrospray mode with the following *m/z* transitions: 244.8 > 113.0 for uridine (12-V cone voltage, 10-eV collision energy) and 136.8 > 110.1 for hypoxanthine (32-V cone voltage, 18-eV collision energy). Identification of the compounds was based on retention time and ion transitions. Calibration curves made with aqueous standards were processed in parallel, and concentrations were obtained from interpolation of the peak areas using Target Lynx software (Waters). The levels of uridine and hypoxanthine were analyzed in serum of nine human subjects at both ground level and high altitude.

Statistical analysis. Two-tailed *P* values of duodenal targets were calculated by either ANOVA or Mann-Whitney test. Two-tailed *P* values between groups of serum analytes were assessed by paired *t*-test, using Graph Pad Prism 5 software (La Jolla, CA). To test for correlations between serum (and/or tissue) analytes and the expression levels of SLC in the duodenum, the data were logarithmically transformed and measured by a linear regression analysis using IBM SPSS Statistics Version 21. Graphs were plotted using GraphPad Prism version 5.00 for Windows (GraphPad Software).

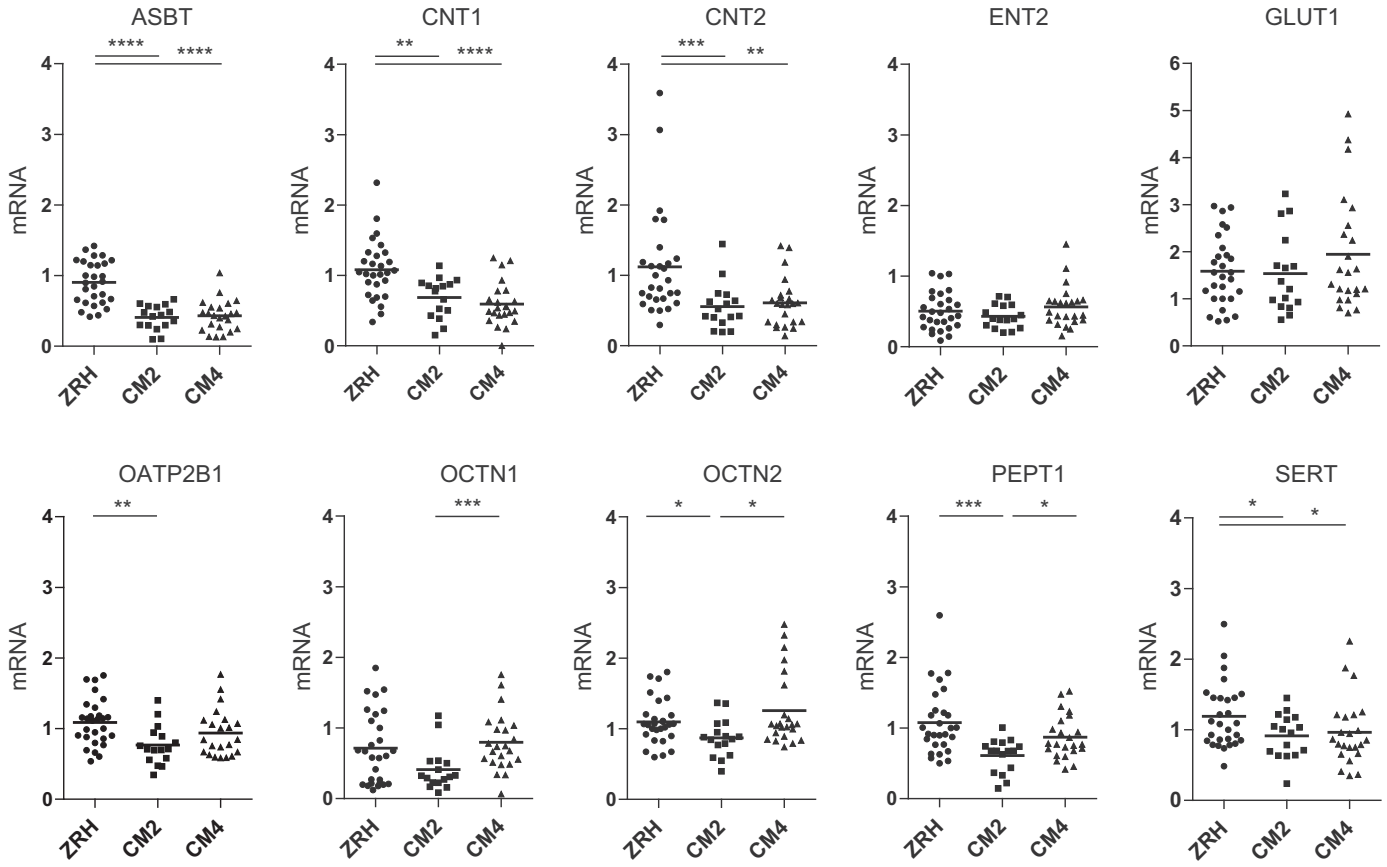

Fig. 1. mRNA expression levels of duodenal Solute carrier (SLC) transporters in healthy human subjects exposed to high-altitude hypoxia. All available duodenal specimens from ground level of Zurich (ZRH; $n = 27$), 4,555 m above sea level at *day 2* (CM2; $n = 18$) and at *day 4* (CM4; $n = 21$) were analyzed by RT-PCR. The mRNA expression levels of implicated SLC transporters were assessed vs. villin-1, each specimen measured in duplicates. ASBT, apical sodium-dependent bile acid transporter; CNT, concentrative nucleoside transporters; ENT equilibrative nucleoside transporter; OATP, organic anion transporting polypeptide; OCTN, organic cation transporter; PEPT, peptide transporter; SERT, serotonin transporter. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Table 1. *Impact of high-altitude hypoxia on the expression of SLC transporters and inflammatory markers in human duodenum*

	High-Altitude Hypoxia				
	Healthy (n = 7)		AMS (n = 9)		
Duodenum	(−) *Day 2*	(−) *Day 4*	(−) *Day 2*	Dex *Day 4*	*P* Value
SLC transporters					
ASBT	0.40 (±0.14)†	0.68 (±0.43)*	0.46 (±0.16)‡	0.37 (±0.15)‡	*0.0100*
CNT 1	0.62 (±0.35)*	0.60 (±0.48)*	0.68 (±0.20)†	0.51 (±0.25)*	0.1281
CNT 2	0.44 (±0.21)*	0.60 (±0.44)*	0.57 (±0.21)*	0.48 (±0.19)*	0.3679
ENT 2	0.70 (±0.33)	1.03 (±0.93)	1.32 (±0.86)	1.63 (±1.51)	0.5359
GLUT 1	0.85 (±0.45)	1.01 (±0.55)	1.14 (±0.54)	1.56 (±1.15)	0.4279
OATP2B1	0.62 (±0.17)‡	0.82 (±0.26)*	0.71 (±0.26)*	0.76 (±0.24)*	0.7953
OCTN 1	1.12 (±0.83)	3.47 (±2.82)*	0.77 (±0.44)	1.50 (±1.31)	0.2561
OCTN 2	0.82 (±0.41)	0.86 (±0.13)*	0.86 (±0.31)	1.17 (±0.45)	*0.0398*
PEPT 1	0.53 (±0.39)*	0.90 (±0.53)	0.65 (±0.22)*	0.80 (±0.34)	0.2120
SERT	0.87 (±0.48)	0.98 (±0.57)	0.86 (±0.27)	0.75 (±0.36)	0.5808
Cytokines					
IFN-γ	0.78 (±0.38)	1.02 (±1.01)	2.52 (±2.20)*	0.54 (±0.41)†	*0.0189*
IL-6	0.83 (±0.89)	0.40 (±0.34)*	2.52 (±1.85)*	1.43 (±1.42)	*0.0361*
IL-10	1.16 (±0.23)	0.45 (±0.21)*	1.56 (±0.62)*	0.75 (±0.45)	*0.0143*
T cells					
IL-17A	2.41 (±0.71)*	1.26 (±1.48)	2.22 (±0.75)†	0.66 (±0.50)	*0.0447*
ROR-γ	1.52 (±1.33)	0.42 (±0.18)*	0.66 (±0.24)*	0.52 (±0.26)*	0.1124
Foxp3	0.96 (±0.51)	1.10 (±0.78)	0.70 (±0.40)*	0.32 (±0.15)*	*0.0334*

Numbers in parentheses represent ± SD. Duodenal biopsies from healthy subjects were analyzed at the beginning of the study [Zurich (ZRH)] as well as 2 and 4 days after ascent to high altitude. The mRNA expression levels of solute carrier (SLC) transporters were determined vs. villin-1 and relevant proinflammatory cytokines relative to mRNA expression levels of β-actin. The basal expression levels in both control (n = 7) and acute mountain sickness (AMS)/dexamethasone (Dex)-treated (n = 9) groups were set to 1 for each individual at the ground level of Zurich and displayed as a fold difference as compared with the basal levels. The effect of Dex in AMS group was assessed by comparing the mRNA levels before (*day 2*; CM2) and after (*day 4*; CM4) administration of the drug (last column; *P* values). ASBT, apical sodium-dependent bile acid transporter; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; OCTN, organic cation transporter; OATP, organic anion transporting polypeptide; PEPT, peptide transporter; SERT, serotonin transporter. Statistical analysis was performed using Friedman test with Dunn's multiple comparison test: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ vs. ZRH levels.

RESULTS

High-altitude hypoxia changes the expression levels of SLC transporters in human duodenum. To investigate the impact of hypoxia on SLC expression in vivo, we quantified the mRNA expression levels of implicated transporters in human duodenal specimens obtained from healthy subjects after ascent to high altitude. Interestingly, the mRNA expression levels of tested duodenal SLCs were changed at high altitude compared with the near sea levels of Zurich. Specifically, the mRNA levels of apical sodium-dependent bile acid transporter (ASBT), OATP2B1, OCTN2, concentrative nucleoside transporters 1 and 2 (CNT1 and CNT2), OCTN1, PEPT1, and SERT were significantly decreased upon exposure to high-altitude hypoxia (Fig. 1) The levels of ENT2 and GLUT1 were not changed significantly; however, in the case of latter we observed a trend towards increased expression over time (Fig. 1). Because some individuals suffered from AMS, the entire cohort was subdivided according to the use of dexamethasone (44) (also see *Human subjects and exposure to high-altitude hypoxia*). We observed that changes in the expression of SLCs were generally independent of dexamethasone treatment (Table 1). Notably, mRNA levels of OCTN1 increased significantly compared with basal levels but only in the control group (Table 1).

High-altitude exposure changes the expression levels of pro- and anti-inflammatory cytokines in human duodenum. To investigate the potential link of hypoxia-induced changes in SLC levels and intestinal inflammation, we also screened for the expression levels of proinflammatory cytokines along with important targets of innate immunity. Hypoxia significantly increased duodenal mRNA levels of proinflammatory cytokines IFN-γ and IL-6 (both ~2.5-fold) detected 2 days after ascend to high altitude but only in subjects suffering from AMS (Table 1). This effect was attenuated on *day 4*, i.e., after the administration of dexamethasone that decreased the expression levels of all other proinflammatory targets, even below the basal levels (Table 1). Interestingly, high-altitude hypoxia also affected the mRNA expression levels of regulatory T-cell markers, such as IL-17, ROR-γ, and Foxp3. Hypoxia-mediated induction of IL-17 was independent of AMS; however, the mRNA levels of Foxp3 were markedly different between these two subgroups (Table 1).

High-altitude exposure modulates the serum levels of pro-inflammatory and anti-inflammatory mediators. To investigate if the observed changes in duodenum are accompanied by the differences in the release of immunologically relevant mediators including substrates of SLC transporters, we next analyzed serum samples before and after exposure to high altitude. We detected higher levels of proinflammatory mediators, namely IFN-γ, IL-8, macrophage inflammatory protein-1β (MIP-1β), and matrix metalloproteinase-2 (MMP-2), in serum of all subjects upon elevation to high altitude (Fig. 2). In addition, we also detected higher levels of anti-inflammatory IL-10, which increased in the time of exposure to hypoxia. Conversely, the levels of CCL-2 were significantly lower but only at *day 4* (Fig. 2), which was in concert with the inhibitory effect of hypoxia on the production of this chemokine in alveolar macrophages (47). Next, to investigate the functional consequences of changes in the expression of intestinal transporters, we mea-

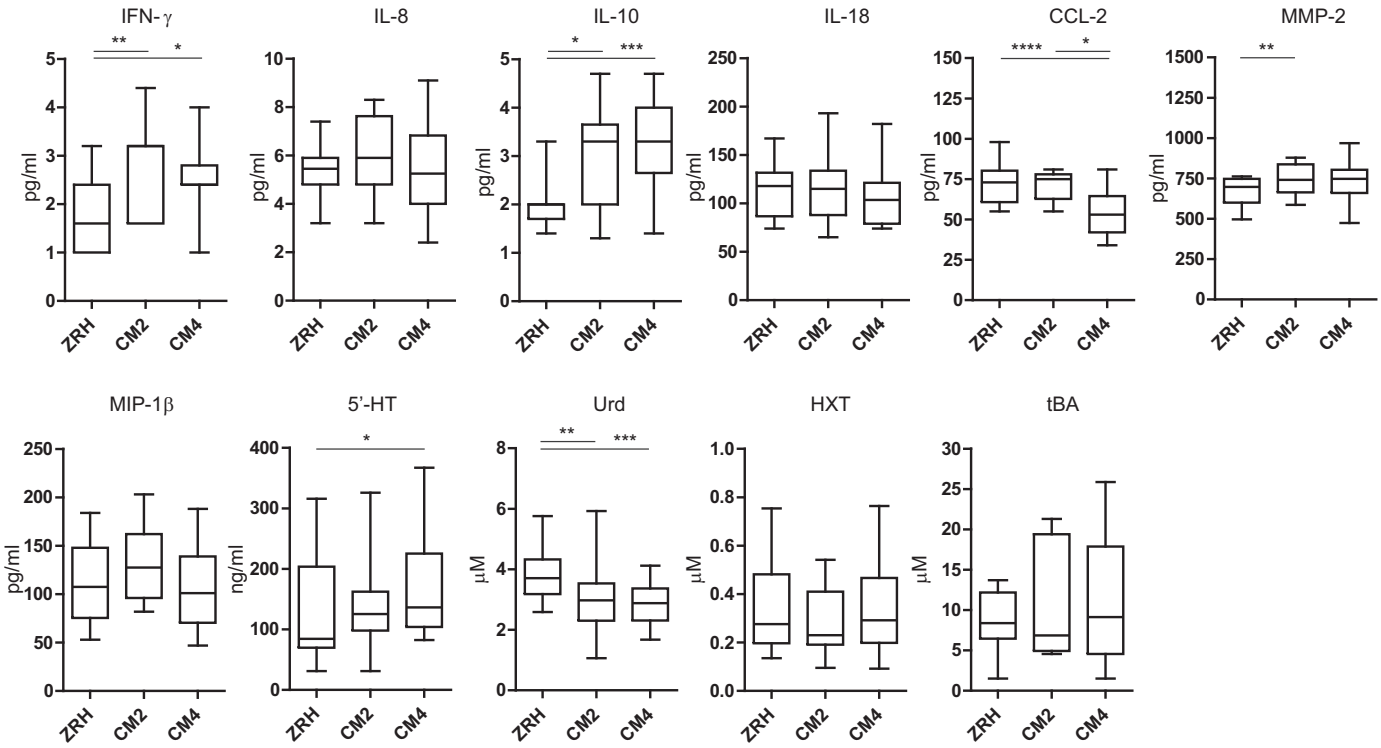

Fig. 2. Effect of high-altitude hypoxia on serum levels of inflammatory mediators and biologically active compounds in humans. Serum samples from healthy subjects were analyzed at the beginning of the study at the level of Zurich (ZRH) as well as on *day 2* (CM2) and *day 4* (CM4) after assent to high altitude (Capanna Margheritta; 4,555 m above sea level; $n = 16$). MMP-2, matrix metalloproteinase-2; MIP-1β, macrophage inflammatory protein-1β; 5′-HT, 5′-hydroxytryptamine; Urd, uridine; HXT, hypoxanthine; tBA, total bile acids. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison test: $*P < 0.05$; $**P < 0.01$.

sured the levels of SLC substrates in serum of the same human subjects exposed to high-altitude hypoxia. Interestingly, the amounts of serotonin [5′-hydroxytryptamine (5′-HT)], a substrate of SERT, significantly increased at *day 4*. A different trend was observed for uridine, which is a substrate of nucleoside transporter CNTs and ENTs (Fig. 2). The serum levels of hypoxanthine, which is a substrate of ENT2, did not change upon elevation to high altitudes, and this observation was consistent with unchanged mRNA levels of this transporter in the duodenal tissue (Fig. 1 and Table 1). The serum levels of

Table 2. *Effect of high-altitude hypoxia on serum levels of inflammatory mediators and biologically active SLC substrates in humans*

	Healthy ($n = 7$)			AMS ($n = 9$)			
	ZRH	High Altitude		ZRH	High Altitude		
Serum	(−) *Day 0*	(−) *Day 2*	(−) *Day 4*	(−) *Day 0*	(−) *Day 2*	Dex *Day 4*	*P* Value
Cytokines and Chemokines							
IFN-γ	1.9 (±0.53)	2.8 (±1.08)	2.6 (±0.82)	1.6 (±0.77)	2.6 (±0.66)*	2.2 (±0.74)	0.1946
IL-8	5.9 (±0.93)	5.0 (±1.22)	6.5 (±1.68)	4.9 (±1.03)	6.9 (±1.37)†	4.9 (±2.03)	0.0710
IL-10	1.9 (±0.50)	2.9 (±0.88)	3.6 (±1.13)*	2.1 (±0.53)	2.9 (±1.29)	3.1 (±0.99)†	0.7869
IL-18	134 (±18.7)	119 (±28.0)	117 (±20.5)*	99 (±25.1)	112 (±38.9)	100 (±36.2)	*0.0076*
CCL-2	80.3 (±12.3)	68.5 (±10.0)*	67.3 (±7.2)*	67.4 (±11.4)	72.7 (±8.3)	44.4 (±7.0)†	*0.0001*
MIP-1β	128 (±37.6)	128 (±37.6)	139 (±42.4)	100 (±44.1)	137 (±45.9)*	88.9 (±36.6)	*0.0040*
MMP-2	718 (±50.0)	758 (±104.4)	795 (±92.6)	634 (±97.2)	736 (±91.8)†	685 (±120.0)	0.3544
Other							
5′-HT	126 (±97)	162 (±102)	203 (±97)*	126 (±78)	112 (±40)	136 (±50)	0.2755
Urd	3.8 (±1.0)	3.4 (±1.1)	3.2 (±0.6)	3.9 (±0.8)	2.6 (±0.9)‡	2.5 (±0.6)‡	0.5502
HPX	0.35 (±0.2)	0.33 (±0.15)	0.35 (±0.22)	0.33 (±0.19)	0.23 (±0.12)	0.32 (±0.17)	0.0969
tBA	10.3 (±4.1)	15.2 (±6.0)	7.9 (±6.4)	7.8 (±3.4)	7.6 (±6.1)	13.9 (±8.7)	0.1439

Numbers in parentheses represent ± SD. Serum samples from healthy subjects were analyzed at the beginning of the study at the level of Zurich (ZRH) as well as on *day 2* (CM2) and *day 4* (CM4) after assent to high altitude (Capanna Margheritta; 4,555 m above sea level). Effect of Dex (*P* values) in the AMS group was assessed by comparing the values before (CM2) and after (CM4) the administration of Dex. The levels of cytokines and chemokines in serum are displayed in pg/ml The levels of 5′-hydroxytryptamine (5′-HT) are displayed in ng/ml. Total bile acids (tBA), uridine (Urd), and hypoxanthine (HXT) levels are expressed in μM. MIP-1β, macrophage inflammatory protein-1β; MMP-2, matrix metalloproteinase-2. Statistical analysis was performed using Friedman test with Dunn's multiple comparison test: $*P < 0.05$; $†P < 0.01$; $‡P < 0.001$ vs. ZRH levels.

bile acids (substrates of ASBT) were not altered upon exposure to high-altitude hypoxia. To evaluate the influence of AMS and concomitant dexamethasone treatment on the serum levels of these mediators, we subdivided our cohort in according to the presence or absence of AMS symptoms (Table 2). Similar to the anti-inflammatory effect observed in duodenal tissues, dexamethasone effectively decreased the levels of all proinflammatory targets measured, which was correlated with the remission of AMS symptoms in this subgroup of subjects. Conversely, despite some increasing trends in healthy group, we did not observe any significant changes in proinflammatory targets. This observation was consistent with the absence of high-altitude sickness symptoms among these subjects. Moreover, in the control group we observed a significant decrease in the levels of the proinflammatory cytokine IL-18 and chemokine CCL-2, suggesting that these subjects developed an anti-inflammatory response when exposed to high altitude, which could also be supported by increased levels of anti-inflammatory IL-10 (Table 2). Increased levels of serotonin, known to inflict a broad range of physiological effects in the gut (20), were significantly higher only in control group. Interestingly, serum levels of uridine, known to exert an anti-inflammatory effect in animal models of lung inflammation (16), were significantly lower only in AMS/dexamethasone group (Table 2). The levels of bile acids did not change significantly in any of the tested groups.

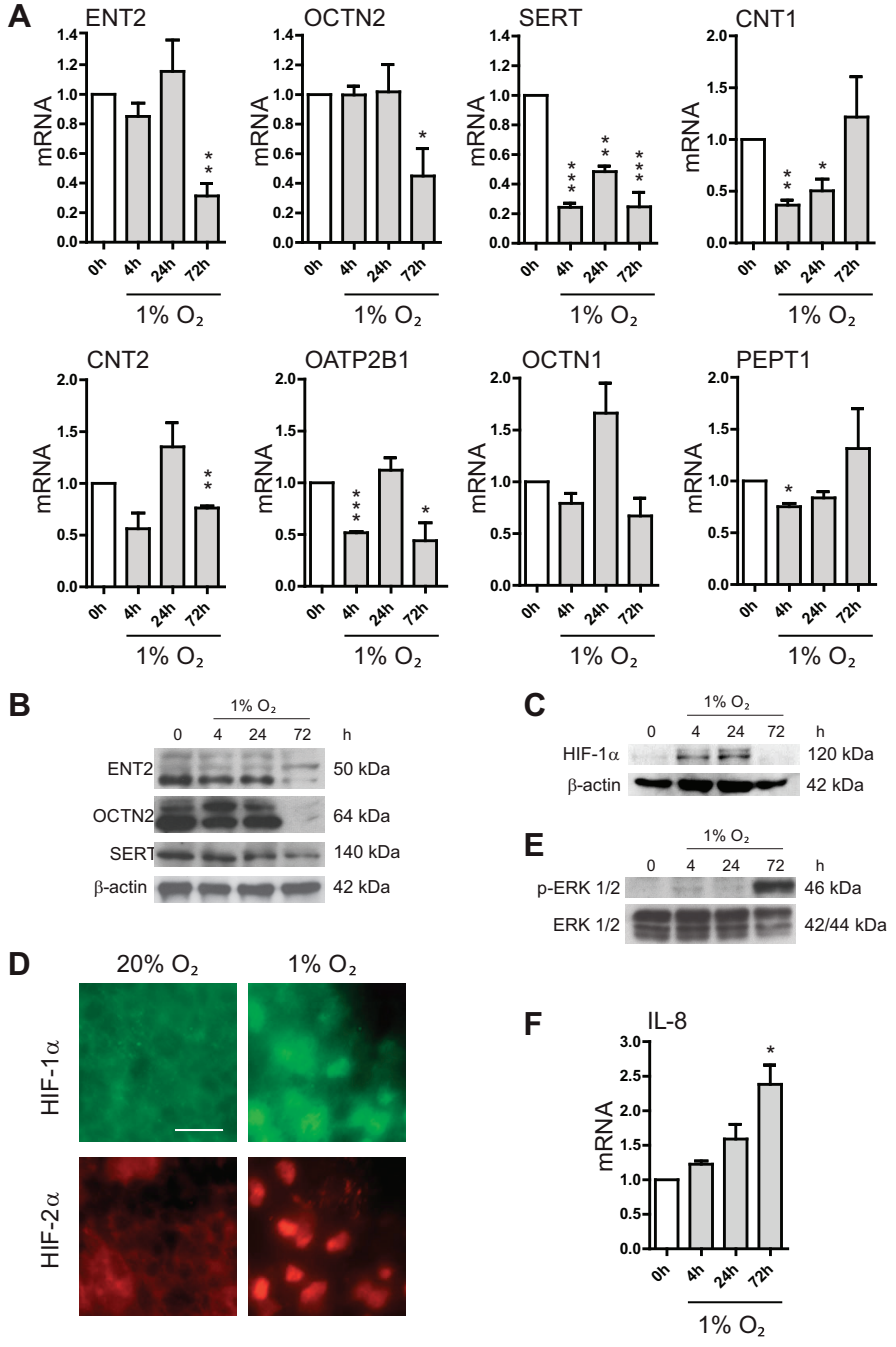

Fig. 3. Effect of hypoxia on the mRNA levels of SLC transporters and proinflammatory signaling in human intestinal cells. *A*: mRNA levels of ENT2, OCTN 2, and SERT are decreasing during the oxygen deprivation in vitro. T84 cells grown on Transwells were incubated in 1% O_2 for different time points. The results represent mean values of 3 independent experiments each measured in triplicates relatively to the basal levels of a target vs. β-actin and set to 1 for each experiment. Error bars indicate SE. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. normoxic cells. *B*: protein levels of OCTN2, ENT2, and SERT are decreased in response to hypoxia in vitro. The protein expression levels of SLCs were assessed by Western blotting. β-Actin was used as a loading control. *C*: Western blotting analysis of hypoxia-inducible factor-1α (HIF-1α) protein in hypoxic T84 cells. *D*: immunofluorescence analysis of HIF-1α and HIF-2α proteins in T84 cells. Size bar = 20 μm. *E*: hypoxia triggers proinflammatory signaling in intestinal epithelial cells (IECs) in vitro. The activation of ERK was assessed by Western blotting. *F*: hypoxia induces production of IL-8 mRNA. The results represent mean values of 3 independent experiments each measured in triplicates relatively to the basal levels of a target vs. β-actin and set to 1 for each experiment. Error bars indicate SE. Statistical analysis was performed using repeated-measures ANOVA with Tukey's multiple comparison test: $*P < 0.05$ vs. normoxic cells.

Hypoxia decreases the expression levels of SERT, ENT2, and OCTN2 transporters and activates proinflammatory signaling in human IECs in vitro. To confirm an observed effect of hypoxia on the expression levels of SLC transporters observed at high altitudes in human subjects, we incubated human intestinal epithelial T84 cells in humidified atmosphere containing 1% O_2 over a period of 3 days and subsequently we monitored the mRNA expression levels of specific subset of SLCs involved in the transport of nutrients, hormones, and biologically active compounds in the intestine that were reported to be altered upon intestinal inflammation in humans (52). We observed that hypoxia decreased the levels of carnitine transporter (OCTN2) and SERT mRNAs (Fig. 3*A*). The expression levels of ENT2 were also decreased. Concerning other SLCs of potential importance to inflammatory responses in the gut, we observed only transient trends in mRNA levels of CNT1 and CNT2, PEPT1, OATP2B1, and OCTN1. Consistent with mRNA data for ENT2, OCTN2, and SERT, we observed decreased protein levels of these three SLCs 72 h after induction of oxygen deprivation in hypoxic chambers (Fig. 3*B*). Western blotting analysis confirmed an increased expression of HIF-1α protein as early as 4 h after the induction

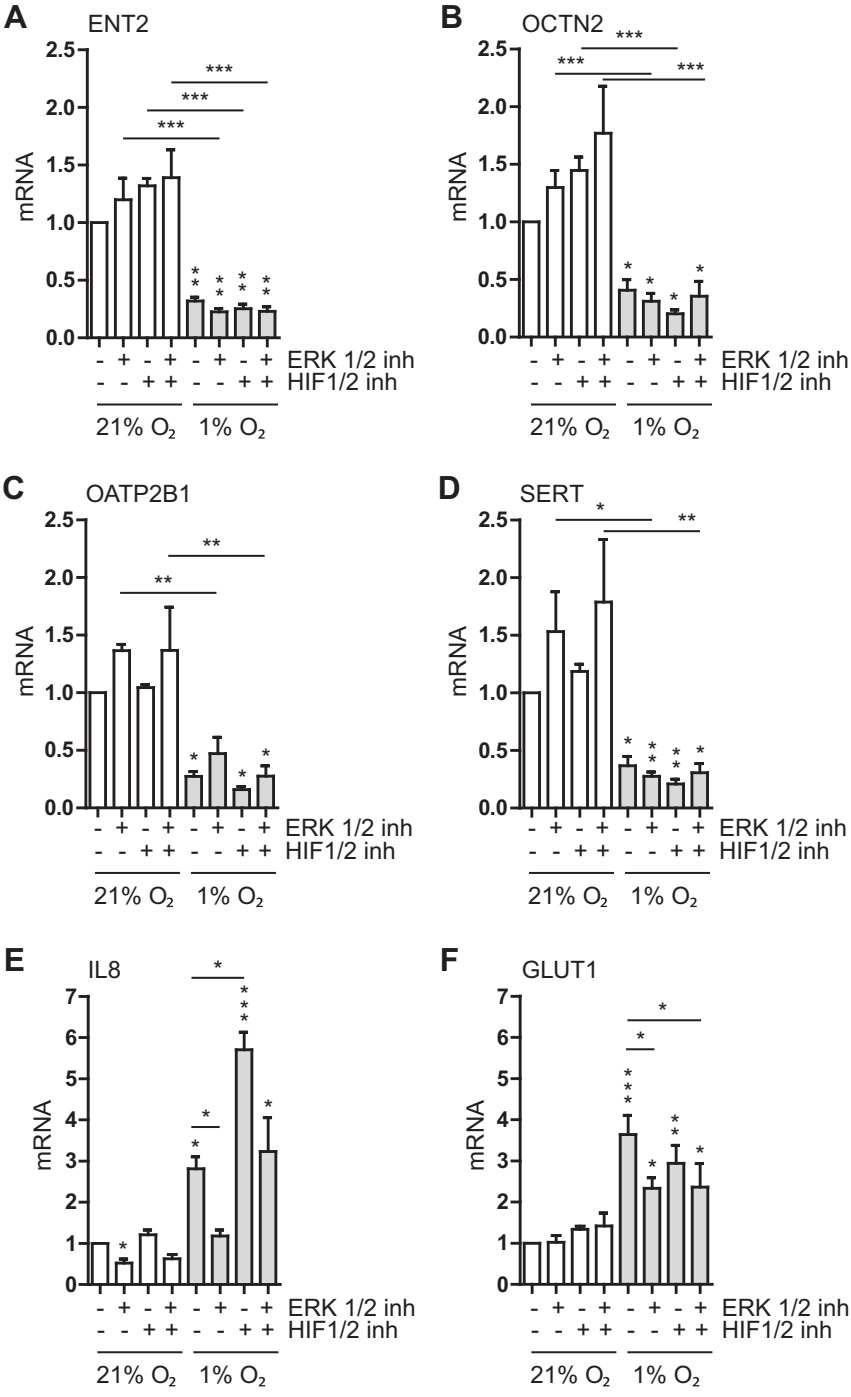

Fig. 4. Effect of HIF1/2 and ERK1/2 inhibitors on hypoxia-induced changes in SLCs mRNA levels. T84 cells grown on Transwells were incubated in either in 20 or in 1% of O_2 for 72 h in the presence or absence of inhibitors of ERK (FR180204) and HIFs (FM19G11). The results represent mean values of 3 independent experiments each measured in triplicate. *A*: ENT2. *B*: OCTN2. *C*: OATP2B1. *D*: SERT. *E*: IL8. *F*: GLUT1. Statistical analysis was performed using repeated-measures ANOVA with Tukey's multiple comparison test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. untreated cells.

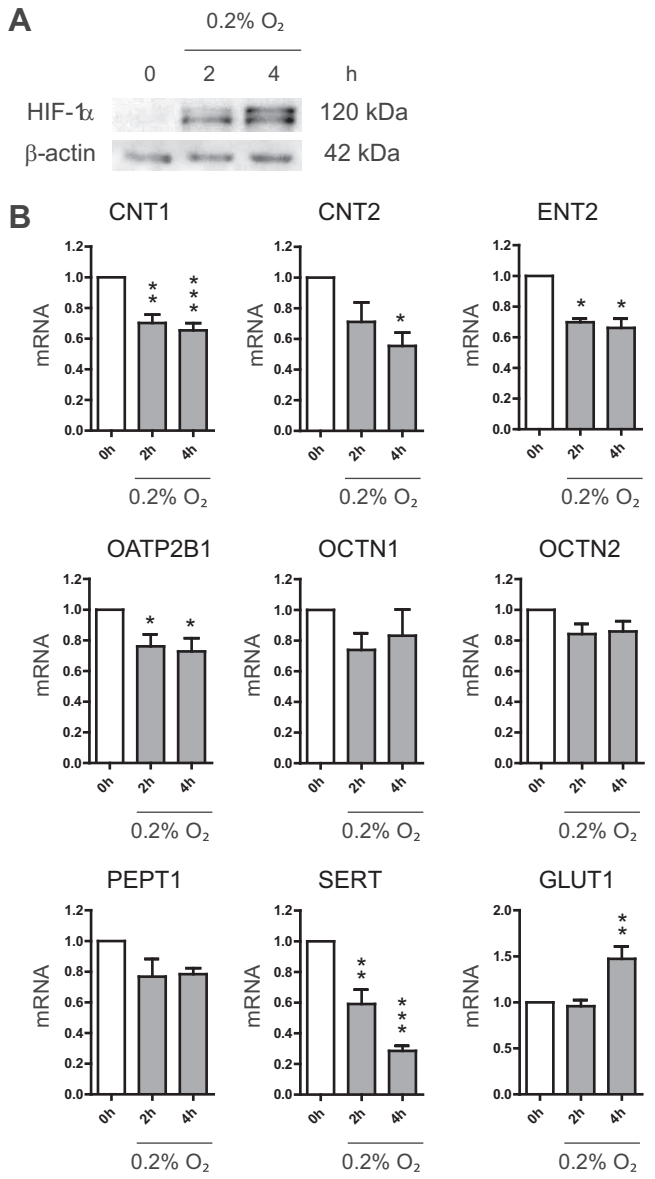

Fig. 5. Effect of hypoxia on SLCs mRNA levels in early time points. T84 cells grown on Transwells were incubated in 0.2% of O_2 for 2 and 4 h. *A*: Western blotting analysis of HIF-1α protein. *B*: relative mRNA expression levels of SLC transporters. The results represent mean values of 3 independent experiments each measured in triplicate. Statistical analysis was performed using repeated-measures ANOVA with Tukey's multiple comparison test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. normoxic cells.

of hypoxia (Fig. 3*C*). This was confirmed by immunofluorescence, which revealed an increase of HIF-1α and -2α proteins 48 h after induction of hypoxia (Fig. 3*D*), targets expected to be upregulated by hypoxia. Because of the known cross talk between hypoxia and proinflammatory signaling (9, 14, 22), we next tested if the observed hypoxia-induced changes in SLC levels are accompanied by changes in proinflammatory signaling pathways in human IECs. Interestingly, hypoxia activated ERK signaling at 72 h (Fig. 3*E*) and stimulated production of IL-8 mRNA (Fig. 3*F*).

Hypoxia-induced late effects on the expression of SLCs are independent on HIF and ERK signaling in vitro. To investigate the role of HIF and ERK signaling in the regulation of the expression of ENT2, OATP2B1, OCTN2, and SERT, next we induced hypoxia in IECs in the presence of pharmacological inhibitors of these pathways. Interestingly, we did not observe any significant effects on the expression levels of these SLCs 72 h after induction of hypoxia (Fig. 4, *A-D*), although both ERK and HIF classical targets, namely IL-8 and GLUT-1, were clearly affected (Fig. 4, *E* and *F*, respectively).

Hypoxia decreases the expression of SLCs during early time points in vitro. Until now we did not observe any changes in the expression of SLCs early (0–4 h) when we incubated cells in 1% O_2 atmosphere but mostly in the late time points (24–72 h). Based on the absence of the early response, it was difficult to exclude that the observed effects of hypoxia on the expression of SLCs were only secondary and/or unspecific. To investigate if oxygen deprivation indeed triggers specific changes in SLC expression, we incubated T84 cells in a more steep oxygen gradient (O_2%) allowing therefore a more rapid hypoxic signaling to occur and monitored the changes over the first 4 h. Western blotting analysis showed that the increase of protein levels of HIF-1α was achieved already after 2 h (Fig. 5*A*). Consistent with the previous observations (Fig. 3 and 4), we detected a decrease in expression levels of all SLC tested (Fig. 5*B*). The downregulation was statistically significant for CNT1, CNT2, ENT2, OATP2B1, and SERT. The expression

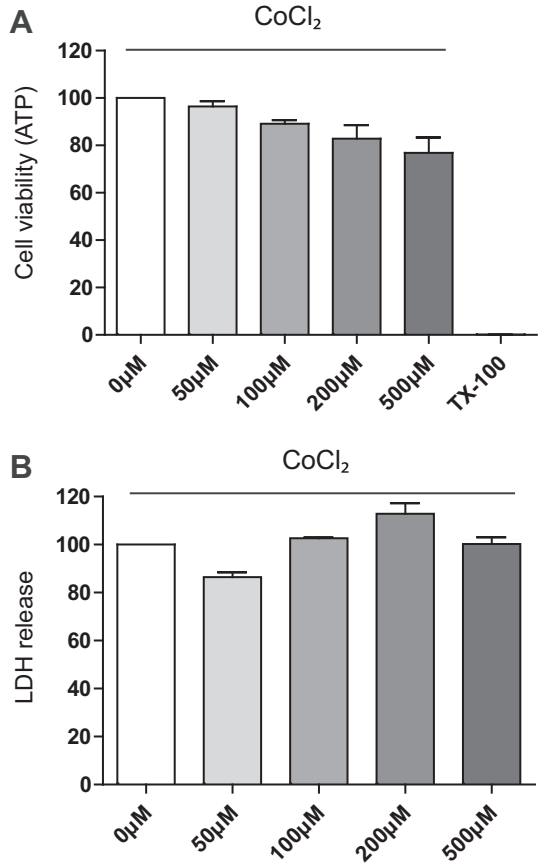

Fig. 6. The impact of $CoCl_2$ on the cell viability of human IECs T84. *A*: cell viability was determined by measurement of ATP content in growing T84 cells. *B*: cell toxicity was determined by LDH release. Results show mean values of 2 independent experiments measured in triplicate. Triton X-100 (0.1%) was used as a negative control of cell viability.

levels of GLUT-1, a target gene of HIF-1α, were increased, confirming the specificity of hypoxic signaling.

Chemically induced hypoxia triggers downregulation of intestinal SLC genes. Because of hypoxia-induced trends in SLC expression levels were only transient for some SLCs, we next induced hypoxia chemically by incubating T84 cells with cobalt chloride ($CoCl_2$), a compound that prevents PHD-mediated degradation of HIFs (55). Because cobalt chloride could potentially affect cell viability, we have tested this compound on T84 cells (Fig. 6). Increase concentrations of $CoCl_2$ had only minimal effect on cells viability as measured by ATP content in cell monolayers (Fig. 6*A*). When LDH release was assessed, a standard toxicity assay, only minor fluctuations were detected (Fig. 6*B*), confirming an absence of overall harmful effect. With regard to the mRNA expression levels of SLCs, $CoCl_2$ induced a time- and concentration-dependent significant decrease of CNT1, PEPT1, OCTN2, OATP2B1, and SERT (Fig. 7) expression in T84 cells. The mRNA expression levels of CNT2, ENT2, and OCTN1 were also significantly decreased but mostly at early time points and at high concentrations of $CoCl_2$. Simultaneously, chemically induced hypoxia increased the levels of GLUT1 mRNA, confirming the HIF-1-specific signaling (Fig. 7*I*). To further investigate these findings, $CoCl_2$ was next added to Caco2 cells, another human IEC line, in a time- and a concentration-dependent manner, similar to the previous experiments with T84 cells. Interestingly, the mRNA levels of SLCs expressed in Caco2 were also decreased mostly with a minimum between 8 and 24 h after treatment, but it was not the case for all the tested SLCs (Fig. 8). Notably, the mRNA levels of ASBT, exclusively expressed in this particular cell line, were also decreased in time (Fig. 8*A*), which was in a strong concert with in vivo data (Fig. 1 and Table 1). Similar to T84, mRNA levels of GLUT-1 expressed in Caco2 were increased in both a time-dependent and concentration-dependent manner (Fig. 8*I*)

IL-6 and IFN-γ potentiates hypoxia-induced decrease of nucleoside and serotonin transporters in IECs. To assess the potential impact of proinflammatory mediators on mRNA levels of SLC genes during hypoxia, we incubated T84 cells in hypoxic chambers and stimulated them subsequently with IL-6 and IFN-γ, two cytokines that were elevated in both serum and tissue of individuals suffering from AMS (23). Before this we tested if the addition of proinflammatory cytokines, especially during hypoxia, would affect cell viability. As anticipated,

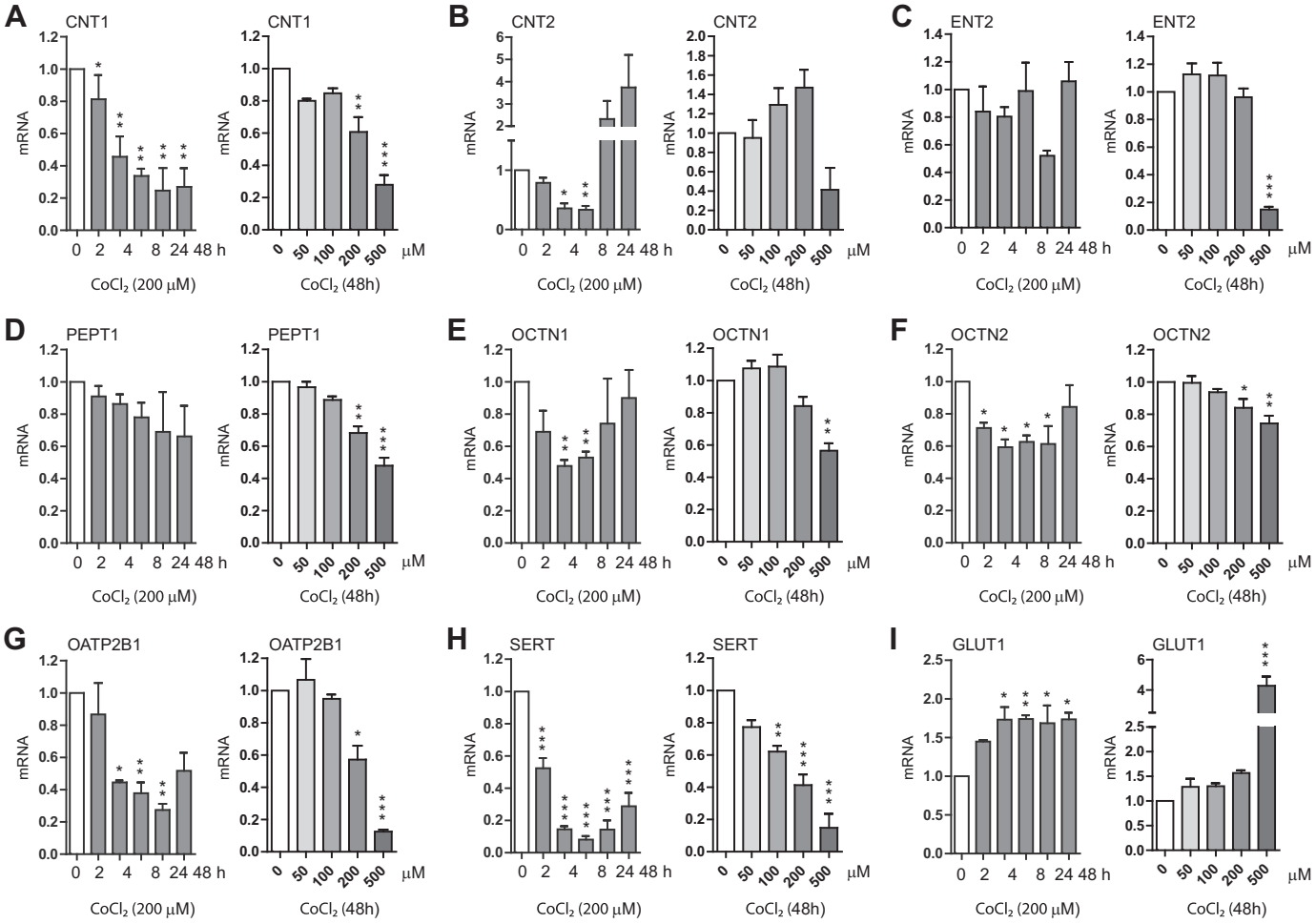

Fig. 7. Effect of chemically induced hypoxia on mRNA expression levels of SLC transporters in human IEC line T84. T84 cells were stimulated with different concentrations of $CoCl_2$ and harvested at different time points. The mRNA expression levels of implicated SLC transporters were assessed by RT-PCR in 4 independent experiments each measured in triplicates relatively to the basal levels of a target vs. β-actin and set to 1 for each experiment. *A*: CNT1. *B*: CNT2. *C*: ENT2. *D*: PEPT1. *E*: OCTN1. *F*: OCTN2. *G*: OATP2B1. *H*: SERT. *I*: GLUT1. Error bars indicate SE. Statistical analysis was performed using repeated-measures ANOVA with Tukey's multiple comparison test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. normoxic cells.

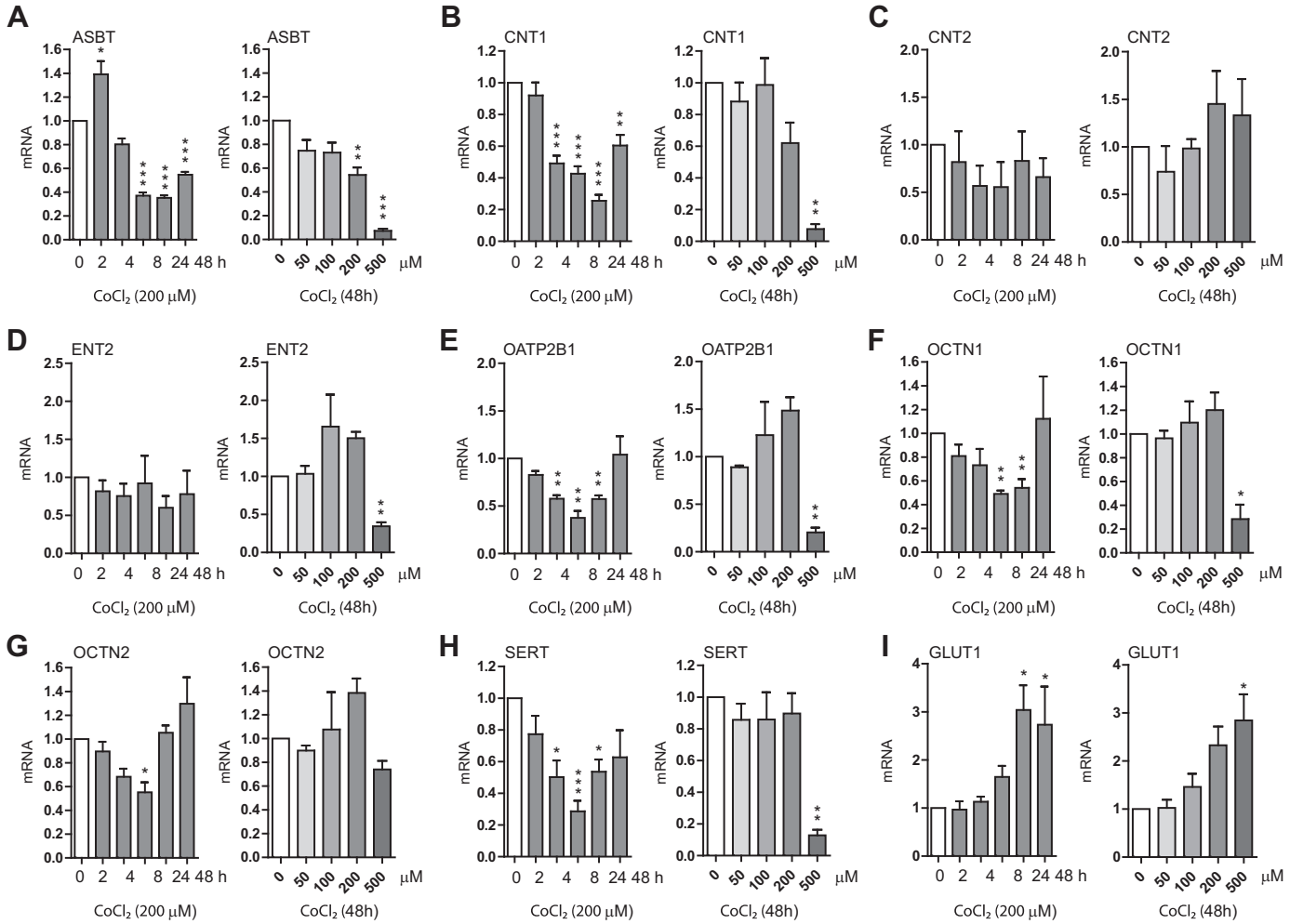

Fig. 8. Effect of chemically induced hypoxia on mRNA expression levels of SLC transporters in human IEC line Caco2. Caco2 cells were stimulated with different concentrations of $CoCl_2$ and harvested at different time points. The mRNA expression levels of implicated SLC transporters were assessed by RT-PCR in four independent experiments each measured in triplicates relatively to the basal levels of a target vs. β-actin and set to 1 for each experiment. *A*: ASBT. *B*: CNT2. *C*: CNT2. *D*: ENT2. *E*: OATP2B1. *F*: OCTN1. *G*: OCTN2. *H*: SERT. *I*: GLUT1. Error bars indicate SE. Statistical analysis was performed using repeated-measures ANOVA with Tukey's multiple comparison test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. normoxic cells.

hypoxia alone decreased viability of T84 cells for ~20% as measured by ATP content (Fig. 9*A*). Interestingly, the presence of IFN-γ, but not IL-6, decreased cell viability further of ~10%. To investigate if these effects were due to changes in cell division rate, we performed cell proliferation assays using the same conditions. Surprisingly, we detected a marked decrease of proliferating cells during hypoxia compared with normoxia (Fig. 9*B*). These changes were independent on the addition of IL-6 and/or IFN-γ but only during oxygen deprivation. During normoxia, implicated proinflammatory cytokines inhibited cell proliferation only when added together and the effect on cell proliferation was comparable to hypoxia alone (Fig. 9*B*). The assessment of RNA content in the treated samples revealed that hypoxia indeed decreases cell proliferation rate (Fig. 9*C*), but this effect was rather independent on the addition of cytokines. Conversely, the assessment of cytotoxicity by LDH assay confirmed that hypoxia has some toxic effect on cells, but this was not changed upon addition of cytokines (Fig. 9*D*). Stimulation with either IL-6 and/or IFN-γ during hypoxia had overall the same effect on the expression levels of most of the tested SLCs compared with normoxia but, in the case of some targets, to a greater extent (Fig. 10). Specifically, this was the case for CNT2, OATP2B1, and SERT. Stimulation with IL-6 and IFN-γ had a markedly different outcome on the expression levels of OCTN1 and OCTN2 compared with normoxia. Little or no effect was observed in case of ENT2 and GLUT-1, which were differently affected by hypoxia alone. Last, IFN-γ had a dramatically stronger effect on the production of ICAM-1 mRNA during hypoxia compared with normoxia (Fig. 10). Since colonic T84 cells were not an ideal system to confirm in vitro effects of hypoxia in human duodenum, we next tested if hypoxia changes mRNA levels of implicated SLCs in the intestinal cells from duodenum. To test this, we stimulated human duodenal cell line Hutu80 in a similar fashion as in our previous experiments. Surprisingly, this cell line expressed detectable amounts of only two transporters from our panel, namely ENT2 and OCTN2; however, incubation in hypoxic environment with or without addition of proinflammatory cytokines resulted in similar effects on the mRNA levels of these transporters as in colonic T84 cells (Fig. 11*A*) An increased expression of HIF-1α-specific target COX-2 confirmed that hypoxic

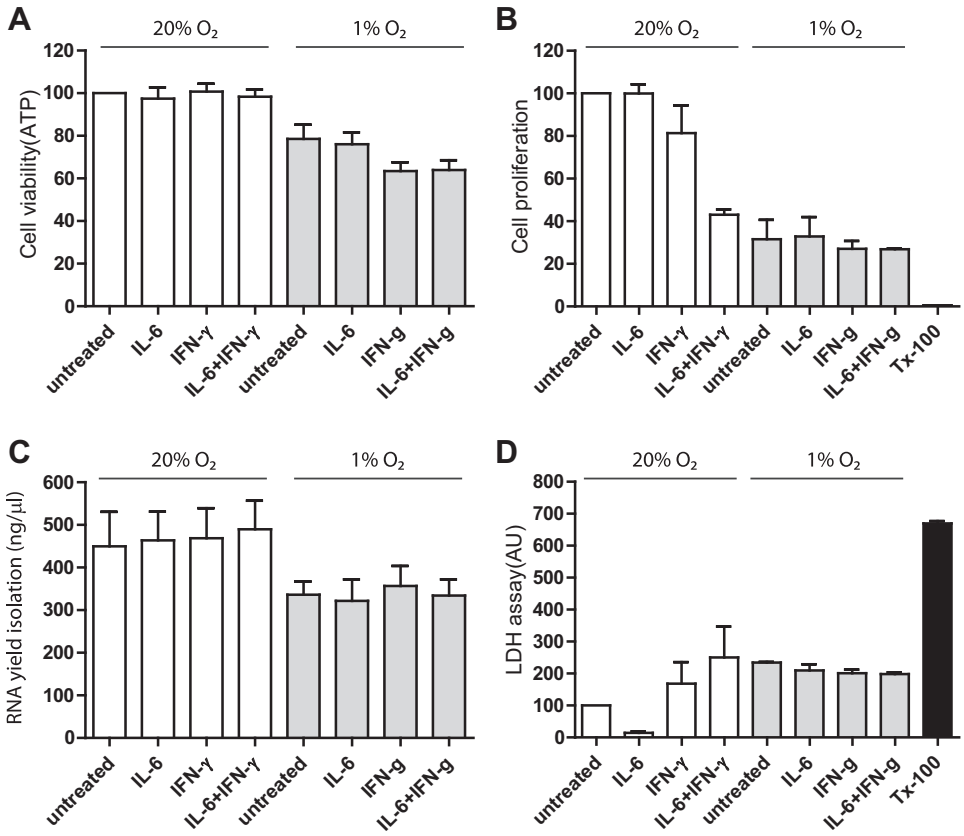

Fig. 9. The effect of IFN-γ and IL-6 on the cell viability and proliferation during normoxia and hypoxia. *A*: cell viability was determined by measurement of ATP content in growing T84 cells. *B*: cell proliferation is decreased during hypoxia, but the addition proinflammatory cytokines does not influence this effect. *C*: yield of RNA isolation is lower in hypoxic cells compared with normoxic cells. *D*: hypoxia alone, but not together with IFN-γ and IL-6, induces cell toxicity of T84 cells. Triton X-100 (0.1%) was used as a negative control of cell viability. AU, arbitrary units.

signaling was effectively induced. In concert with these observations, $CoCl_2$-induced hypoxia decreased the mRNAs of both ENT2 and OCTN2 in a time- and a concentration-dependent manner (Fig. 11*B*), essentially confirming the effects observed in colonic cell lines (Figs. 7 and 8) at least for these two SLCs.

Serum levels of inflammatory mediators correlate with the expression of specific SLCs in human duodenum. To test for potential correlation between implicated serum analytes and duodenal mRNA levels of SLCs, we performed regression analysis of all human samples obtained from ground level and at high altitude. As partially expected, we found a significant correlation between the expression of CNT1 and serum levels of uridine, which is a substrate of this transporter (Fig. 12*A*). Interestingly, we found inverse correlations between serum levels of IFN-γ and mRNA expression levels of ASBT and ENT2 (Fig. 12, *B* and *C*, respectively). In addition, we also observed inverse, statistically significant correlations between serum levels of IL-10 and mRNA expression levels of ASBT and CNT1 (Fig. 12, *D* and *E*, respectively).

DISCUSSION

Oxygen sensing is one of the most important mechanisms controlling the body metabolism in states of health and disease. In the present study, we have identified a novel pleiotropic effect of hypoxia on the intestinal physiology, involving a set of SLC genes. Low oxygen levels and/or increase of HIF proteins were correlated with a decrease in mRNA levels of several intestinal SLC in vitro. Most importantly, our study demonstrates the existence of this physiological phenomenon in human duodenum upon exposure to high-altitude hypoxia. Furthermore, decreased expression levels of duodenal SLCs are accompanied by changes of systemic levels of biologically active compounds, such as serotonin and uridine. Last, but not least, we show that the levels of circulating IFN-γ, MMP-2, and IL-10 are elevated upon ascent to high altitude in humans, an observation not reported earlier.

Previously, we reported that mRNA expression levels of selected SLC transporters in IBD intestine were upregulated upon inflammation (52). Here, we report that hypoxia decreased mRNA levels of SERT in both human duodenum in vivo (Fig. 1 and Table 1) and in human intestinal cells in vitro (Figs. 3, 4, and 5). These effects were correlated with elevated systemic levels of serotonin (5′-HT) (Table 2), which is mainly produced by enterochromaffin cells acting as oxygen sensors in the gut (28). Higher levels of 5′-HT are typically associated with pulmonary hypertension and hypoxia (1, 12), but it is not clear at this point if this effect elicits an overall harmful or beneficial effects in human duodenum, since next to its rather proinflammatory role during colitis, serotonin can also promote mucosal growth and intestinal motility (20). Consistently, stimulation with proinflammatory cytokines, which were upregulated both locally and systemically during high-altitude hypoxia (Tables 1 and 2 and Fig. 2), resulted in a profound decrease in the expression levels of SERT, even in normoxic conditions (Fig. 10). Based on these results we postulate that hypoxia-mediated downregulation of SERT in duodenum, which represents serotonin recycling system of serotonin, could contribute to the observed rise in serum concentration of this hormone.

We report that the expression levels of carnitine transporter OCTN2 were repressed during hypoxia both in human duode-

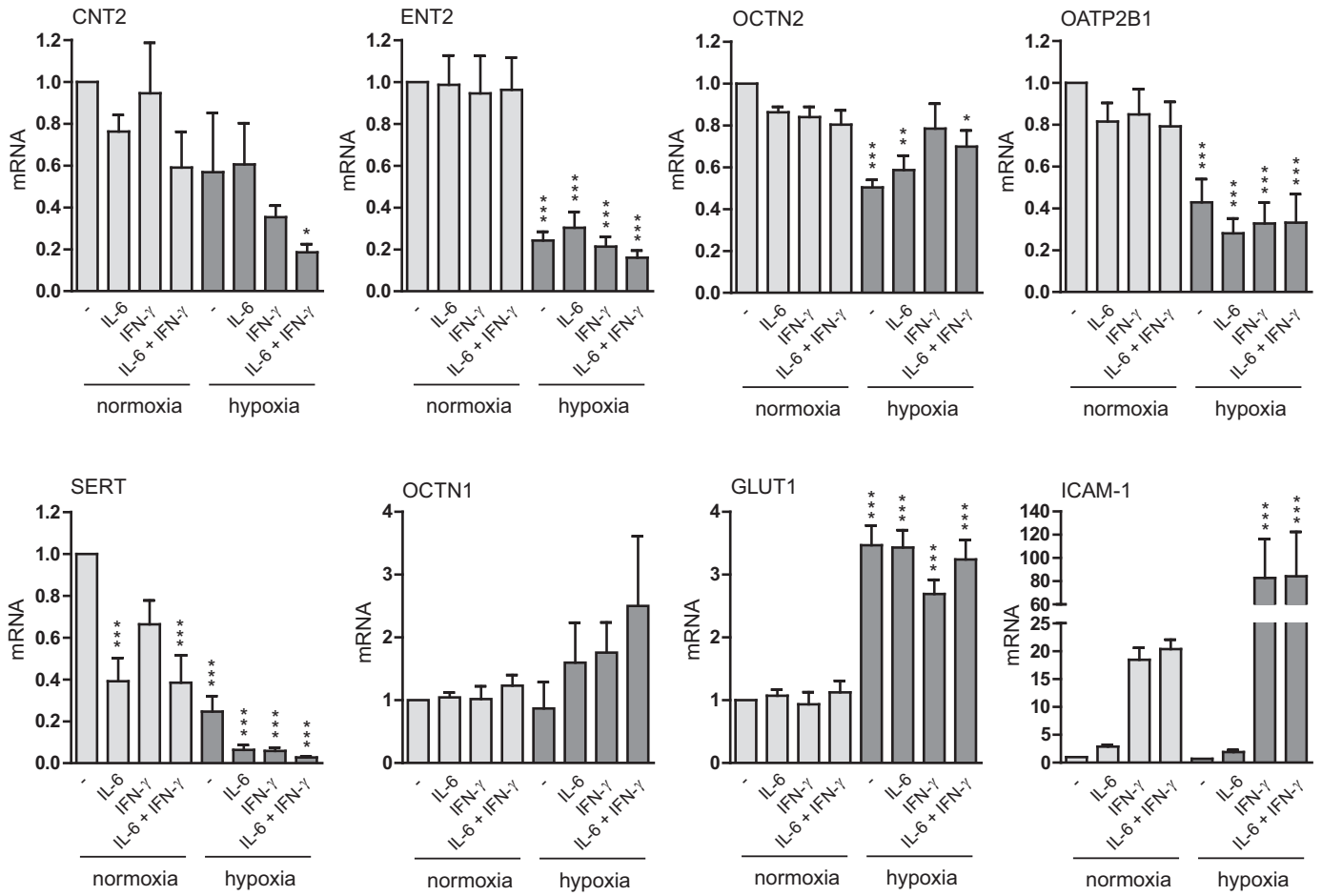

Fig. 10. Effect of IFN-γ and IL-6 on mRNA levels of SLCs in the IECs during hypoxia. T84 cells grown on Transwells were incubated in either 1 or 20% O_2 for 72h. Both cytokines were added to the basolateral chambers 24 h before harvesting. To confirm the specificity of IFN-γ -mediated signaling, ICAM1 mRNA levels were assessed. The results represent mean values of 3 independent experiments, each measured in triplicates relatively to basal mRNA expression levels set to 1 for untreated normoxic cells in each experiment. Error bars indicate SE. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. normoxic cells.

num and in the IECs in vitro (Fig. 1 and Table 1; Figs. 3–7, respectively). This observation is supported by earlier studies conducted in placenta both in vitro and in vivo (8, 36). Because L-carnitine was reported to have an anti-inflammatory effect during colitis (18, 45), it can be anticipated that a decrease in the expression of OCTN2 could have important, long-term consequences for gut physiology as demonstrated in knockout animal models (43) as well as IBD patients (38, 52). Of note, therapeutic administration of dexamethasone increased the expression levels of OCTN2, which was on one hand correlated with attenuation of AMS symptoms at high altitude and on the other strongly supported by positive influence of steroids on the expression of this transporter accompanied by their overall therapeutic effect in IBD patients (52).

In addition, we observed that hypoxia-induced changes in nucleoside transporters markedly differ between in vitro and in vivo analyses. Whereas both the mRNA and protein expression levels of ENT2 were significantly reduced in in vitro experiments, there was only a small decrease observed in vivo (Figs. 1 and 3). Concerning the expression levels of CNTs, we observed a strong decreasing effect of hypoxia in duodenum, which could be supported by our in vitro data (Figs. 3, 5, 7, and 8). The hypoxia-mediated impact on CNTs became visible, when we detected decreasing amounts of uridine in serum of all human subjects exposed to high-altitude hypoxia (Figs. 1, 2, and 12 and Table 2). This was in a strong concert with observations that hypoxia not only inhibits CNT2-mediated uptake of this nucleoside in neuronal cells but also reduces the expression levels of CNT2 in the ischemic brain (33). Based on the fact, that uridine is known to have anti-inflammatory effects in animal models of lung inflammation, as well as protective function in hypoxia/ischemia-induced damage on vascular and central nervous systems (6, 10, 16), it can be concluded that human subjects exposed to high-altitude hypoxia, especially those suffering from AMS, may indeed be affected by the drop in physiological levels of this nucleoside. It would be tempting to speculate that hypoxia-induced downregulation of CNTs could either act as a protective mechanism to prevent from scavenging uridine back to the intestinal tissue or it would be an effect directly linked to limited intestinal absorption of nucleosides. Of note, the levels of hypoxanthine, an endogenous substrate of ENTs, were not changed significantly (Fig. 2 and Table 2), which coincided with unchanged expression levels of ENT2 in duodenum (Fig. 1 and Table 1). It is tempting to speculate if other nucleosides, which have been reported to play an important role in mediating responses during intestinal inflammatory hypoxia, such as adenosine (15, 53), are affected upon exposure to high altitude as reported

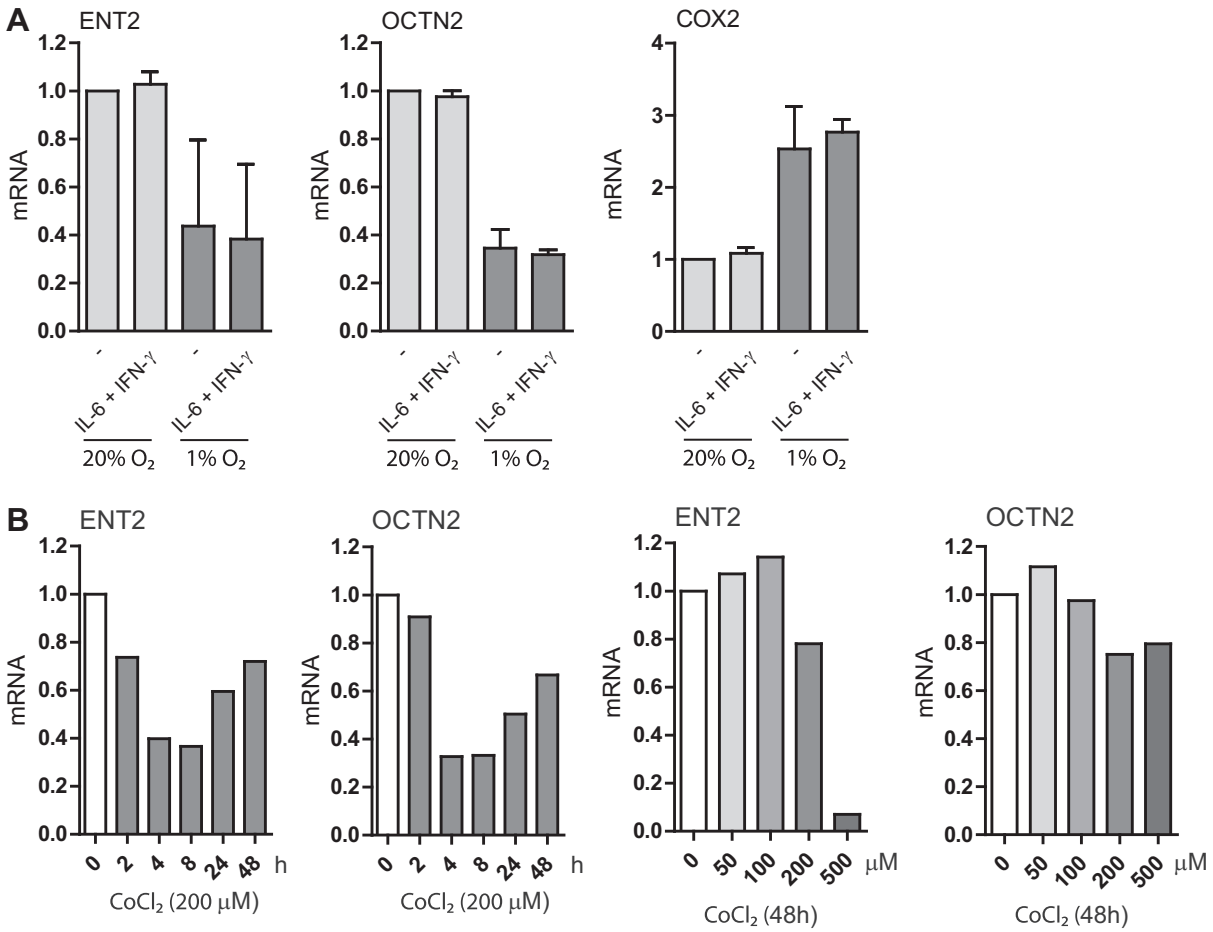

Fig. 11. Effect of hypoxia on mRNA expression levels of ENT2 and OCTN2 in human duodenal Hutu80 cell line. *A*: Hutu80 cells were incubated in either normoxic (20% O_2) or hypoxic (1% O_2) environment for 4 days with or without human recombinant IFN-γ. The mRNA expression levels of SLCs were determined by RT-PCR. Graphs show the results from 2 independent experiments, each measured in duplicate. Error bars indicate SE. *B*: chemically induced hypoxia decreases the mRNA expression levels of OCTN2 and ENT2 in Hutu80. The graphs show the results of a single experiment measured in duplicate.

earlier (41). Further studies are required to investigate this hypothesis.

It is still unknown if any of HIFs are directly responsible for the observed decrease in mRNA levels of implicated transporters by repressing transcription of SLC genes. The results obtained using selective inhibitor of HIFs do not favor this hypothesis (Fig. 4), but stimulation with $CoCl_2$ showed that HIF-1α could play a role in this repression (Figs. 6, 7, and 11). To provide more insight into possible roles of HIFs in this phenomenon, one should attempt to employ more complex and sophisticated systems of studying transcriptional control mediated by HIFs, such as knockdowns and promoter binding assays in combination with more potent and selective inhibitors. The potential scope of the investigation would aim at the involvement of also other components of hypoxic signaling upstream of HIF-1α, such as PHDs. Nevertheless, it could not be excluded that observed decrease in SLC mRNA levels is a result of a hypoxia-induced depletion and/or limited availability/activity of pivotal transcription factors responsible for maintenance of basal levels of these particular transcripts. For OATP2B1 and OCTN2 this could be CREB-binding protein or any member of p300 family of transcription factors, which on one hand can bind to the promoter sites of their target genes and on the other mediate HIF-specific effects. Another mechanism of action could involve a cross talk with proinflammatory signaling at the level of transcriptional control, since many of implicated SLC genes bind transcription factors linked to the implicated pathways, such as STAT1, NF-κB, and AP-1. However, a highly probable mechanism of repression of SLC mRNA levels could involve a hypoxia-mediated inhibition of transcription preinitiation complex by negative cofactor 2 (NC2), a phenomenon related to the control of energy demand on a cellular level, which was reported earlier by Denko et al. (11). These issues will need to be addressed in the near future.

The direct consequences of the observed changes in intestinal SLCs at the systemic level are still largely unknown. It has been reported previously that animals transferred to high altitude increase the mass of both heart and lungs, while the mass of the intestines significantly decreases (25). In support of this, our findings indicate that intestinal absorption may be affected, since the expression levels of most of tested SLCs were decreased upon exposure to hypoxia. Interestingly, Aeberli et al. (2) observed that among the same individuals elevation to high altitude triggered a significant weight loss with a simultaneous decrease in energy intake, which was not related to the changes in the release of gastrointestinal hormones controlling appetite. The results presented within this article suggest that observed weight loss could be at least partially attributed to

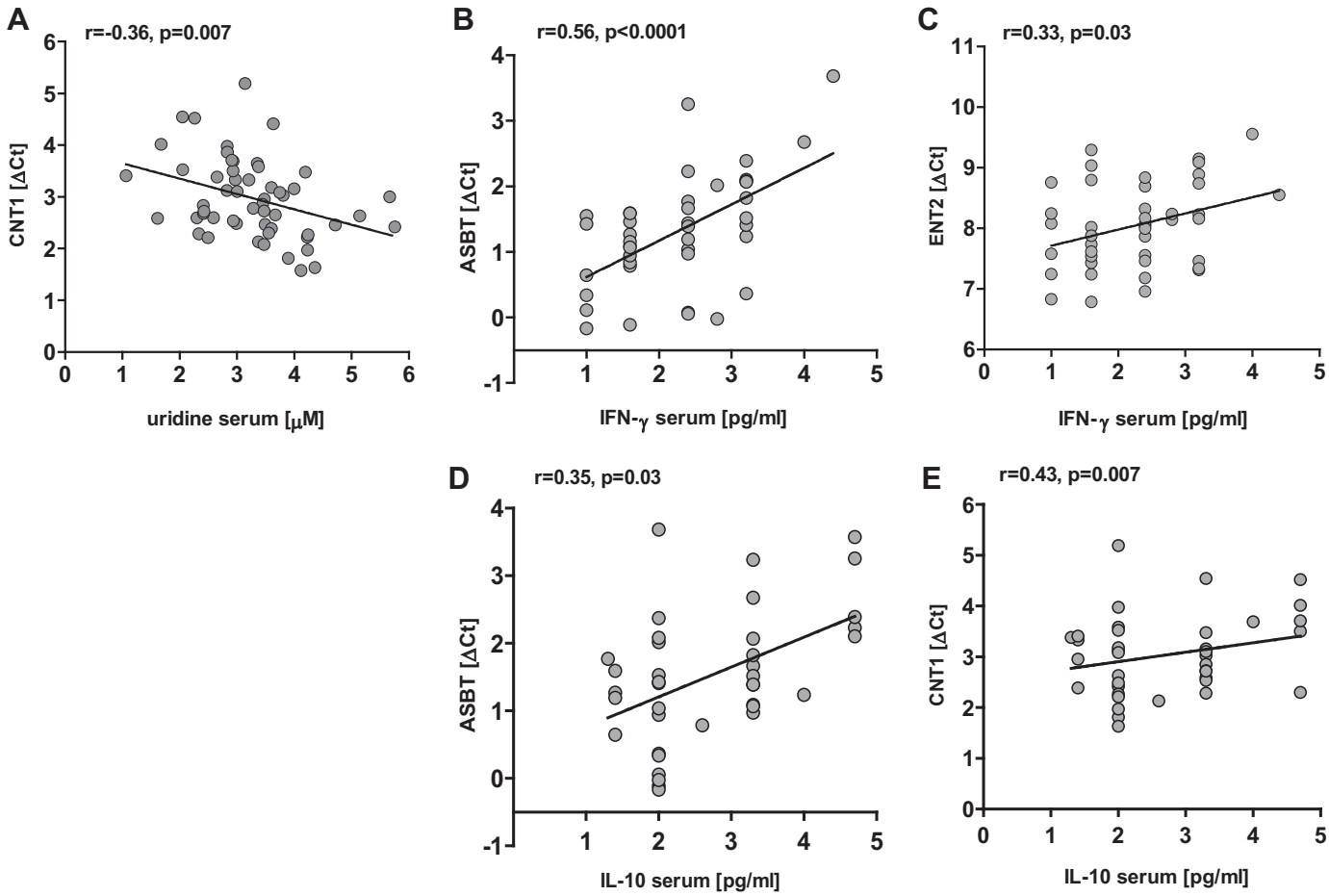

Fig. 12. Correlations among serum levels of metabolites, cytokines, and selected SLCs mRNAs expressed in human duodenum during high-altitude ascent. *A*: mRNA expression levels of CNT1 are inversely proportional to the serum levels of its substrate uridine. Serum levels of IFN-γ are inversely correlated with duodenal mRNA levels of ASBT (*B*) and ENT2 (*C*). Serum levels of IL-10 were significantly correlated with mRNA levels of ASBT (*D*) and CNT1 (*E*). Statistical significance was determined by linear regression analysis. ΔC_t, change in critcal threshold.

decreased intestinal absorption of nutrients, drugs, as well as biologically active mediators of systemic homeostasis, such as L-carnitine, nucleosides, serotonin, and possibly many others. This effect would be a direct consequence of hypoxia-mediated active repression of genes, which can be dispensable for cells under oxygen deprivation (11).

Altogether, our results strongly suggest that both local and systemic oxygen levels should be considered not only important for intestinal absorption but also as pivotal for immune responses in healthy intestine. This has been supported by studies of intestinal pathological conditions, such as IBD (21, 22, 26). Last, but not least, we reported that high altitude induces production of proinflammatory cytokines in human duodenum and confirmed that hypoxia triggers proinflammatory signaling in the IECs in vitro, which were supported by findings of Taylor et al. (46). Changes in both pro- and anti-inflammatory cytokines were coinciding with changes in the levels of regulatory T-cell markers, such as Foxp3, IL-17A, and ROR-γ (Table 1), which in turn may hint at the misbalance in immunoregulation occurring in hypoxic duodenum. Interestingly, the link between systemic levels of pro- and anti-inflammatory mediators and the expression levels of duodenal SLCs became evident when significant correlations between the members of these two groups were found (Fig. 12). It is still a question of a debate what the exact role of nucleoside and bile acid transporters in mediating inflammatory signals in hypoxic duodenum is; however, our earlier study in IBD patients had already implied such function (52). The observations of current study could also provide clues for the increased incidence of flares in IBD patients traveling to high altitude, as reported recently by our group (49). Our study has also limitations, e.g., relatively small number of human subjects, lack of dexamethasone randomization, and lack of functional data on the effect of hypoxia on transporter activity as well as the absence of in-depth investigation on mechanistic and molecular levels. These issues need to be addressed during the future studies, where we will aim at identification of therapeutically relevant molecular mechanisms, which operate in IBD patients during exposure to hypoxic environment.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

K.A.W., O.G., H.F., T.A.L., M.M., M.G., G.R., and S.R.V. conception and design of research; K.A.W., A.C., S.L., J.T.-T., and R.M. performed experiments; K.A.W., S.L., O.G., J.T.-T., and R.M. analyzed data; K.A.W., G.R., and S.R.V. interpreted results of experiments; K.A.W. and O.G. prepared figures; K.A.W. drafted manuscript; K.A.W., O.G., H.F., A.G., M.P.-A., J.T.-T., R.M., M.F., T.A.L., M.G., G.R., and S.R.V. edited and revised manuscript; K.A.W., A.C., S.L., O.G., H.F., A.G., M.P.-A., J.T.-T., R.M., M.F., T.A.L., M.M., M.G., G.R., and S.R.V. approved final version of manuscript.

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