Original Paper

Downregulation of Peptide Transporters PEPT1 and PEPT2 by Oxidative Stress Responsive Kinase OSR1

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Key Words

Peptide transport • Cell volume • OSR1 • Voltage clamp • *Xenopus* oocytes

Abstract

Background/Aims: OSR1 (oxidative-stress-responsive kinase 1) participates in the regulation of renal tubular ion transport, cell volume and blood pressure. Whether OSR1 contributes to the regulation of organic solute transport remained; however, elusive. The present study thus explored the OSR1 sensitivity of the peptide transporters PEPT1 and PEPT2. **Methods:** cRNA encoding PEPT1 or PEPT2 were injected into *Xenopus* oocytes without or with additional injection of cRNA encoding wild-type OSR1, WNK1 insensitive inactive ^{T185A}OSR1, constitutively active ^{T185E}OSR1, and catalytically inactive ^{D164A}OSR1. Electrogenic peptide (glycine-glycine) transport was determined by dual electrode voltage clamp, the abundance of hemagglutinin-tagged PEPT2 (PEPT2-HA) by chemiluminescence. **Results:** In *Xenopus* oocytes injected with cRNA encoding PEPT1 or PEPT2, but not in oocytes injected with water, the dipeptide gly-gly (2 mM) generated an appreciable inward current ($I_{\text{gly-gly}}$). Coexpression of OSR1 significantly decreased $I_{\text{gly-gly}}$ in both PEPT1 and PEPT2 expressing oocytes. The effect of OSR1 coexpression on $I_{\text{gly-gly}}$ in PEPT1 expressing oocytes was mimicked by coexpression of ^{T185E}OSR1, but not of ^{D164A}OSR1 or ^{T185A}OSR1. Kinetic analysis revealed that coexpression of OSR1 decreased maximal $I_{\text{gly-gly}}$. OSR1 further decreased the PEPT2-HA protein abundance in the cell membrane. **Conclusion:** OSR1 has the capacity to downregulate the peptide transporters PEPT1 and PEPT2 by decreasing the carrier protein abundance in the cell membrane.

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Introduction

The oxidative-stress-responsive kinase 1 (OSR1) is engaged in the regulation of epithelial transport [1-4]. OSR1 and the related SPAK (STE20-related proline/alanine-rich

kinase) upregulate the NaCl (NCC) and Na⁺,K⁺,2Cl⁻ (NKCC2) cotransporters and thus impact on blood pressure [2-17]. By stimulating cellular ion uptake SPAK and OSR1 contribute to cell volume regulation [9, 18]. OSR1 is under the control of with-no-K(Lys) (WNK) kinases [5-7], which are similarly involved in the regulation of renal tubular ion transport and blood pressure [7, 19-22]. Mutations of genes encoding WNK kinases may lead to Gordon's syndrome, a monogenic disease characterized by hypertension and hyperkalaemia [23-26].

OSR1 and/or SPAK participate in the regulation of further epithelial transport processes, such as Na⁺ coupled phosphate transport [27, 28], and Na⁺/H⁺ exchanger [29].

The present study explored whether OSR1 modifies the peptide transporters 1 (PEPT1) and/or 2 (PEPT2), carriers accomplishing electrogenic cellular uptake of di- and tripeptides [30-32] including peptide-like drugs [30, 31]. Regulators of peptide transporters include glucocorticoids [33], leptin [34] and growth hormone [35]. Signaling participating in the regulation of peptide transporters include phosphoinositide (PI) 3 kinase [36], phosphoinositide dependent kinase PDK1 [36], serum & glucocorticoid inducible kinase SGK1 [37] and AMP activated kinase [38].

In order to possibly disclose an effect of OSR1 on the peptide transporters, cRNA encoding PEPT1 and PEPT2 were injected into *Xenopus* oocytes with or without cRNA encoding OSR1. In those oocytes peptide transport was estimated from peptide induced current.

Materials and Methods

Constructs

Constructs encoding PEPT1 or PEPT2 or PEPH2HA [39], wild-type OSR1, WNK1 insensitive inactive^{T185A}OSR1, constitutively active ^{T185E}OSR1, and catalytically inactive ^{D164A}OSR1 [5], were used for generation of cRNA as described previously [27, 28, 40, 41].

Voltage clamp in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [42, 43]. Where not indicated otherwise, 10 ng cRNA encoding PEPT1 or 20 ng cRNA encoding PEPT2 was injected on the first day and 10 ng cRNA encoding wild type OSR1, constitutively active ^{T185E}OSR1, catalytically inactive ^{D164A}OSR1 or WNK1 insensitive inactive^{T185A}OSR1 were injected on the same day after preparation of the oocytes [44]. The oocytes were maintained at 17°C in ND96 solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 2.5 NaOH, 5 HEPES (pH 7.4), 5 sodium pyruvate (C₃H₃NaO₃), Gentamycin (100 mg/l), Tetracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), Theophylline (90 mg/l). The voltage clamp experiments were performed at room temperature 3 days after injection of cRNA encoding PEPT1 or 4 days after injection of cRNA encoding PEPT2. Concentration of cRNA and time for expression have been chosen according to previous experiences and preliminary experiments utilizing 10 or 20 ng cRNA and 3 or 4 days of expression. Comparisons were always made between oocytes injected with the same amount of cRNA and after identical expression times. Two-electrode voltage-clamp recordings were performed at a holding potential of -70 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter (1322 Axon Instruments) and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) [45]. The control superfusate (ND96) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 2.5 NaOH and 5 HEPES, pH 7.4. [46]. Glycine-glycine was added to the solutions at a concentration of 2 mM, unless otherwise stated [41]. The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [40, 47].

Chemiluminescence

For detection of PEPT2-HA cell surface expression, the oocytes were first incubated with primary mouse monoclonal anti-HA antibody conjugated to horseradish peroxidase (1:1000, Miltenyi Biotec Inc, CA, USA). Individual oocytes were placed in 96 well plates with 20 µl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer) by integrating the signal over a period of 1 sec [44]. Results display normalized relative light units. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol [46].

Fig. 1. Coexpression of OSR1 decreases electrogenic peptide transport in PEPT1-expressing *Xenopus* oocytes. A: Representative original tracings showing glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus* oocytes injected with water (a) or expressing PEPT1 without (b) or with additional coexpression of wild type OSR1 (c). B: Arithmetic means $\pm$ SEM ($n = 20$) of glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus* oocytes injected with water (dotted bar), or expressing PEPT1 without (white bar) or with (black bar) wild type PEPT1. **($p < 0.01$) indicates statistically significant difference from the absence of OSR1.

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of oocytes investigated. All voltage clamp experiments were repeated with at least 2-3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA (Tukey test or Kruskal-Wallis test) or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether OSR1 contributes to the regulation of the peptide transporters PEPT1 and PEPT2. To this end, cRNA encoding PEPT1 or PEPT2 was injected into *Xenopus* oocytes with or without additional injection of cRNA encoding OSR1. Peptide transport was estimated from the inward current observed following addition of the dipeptide glycine-glycine (2 mM) to the bath solution ($I_{\text{gly-gly}}$).

No $I_{\text{gly-gly}}$ was detectable in water-injected *Xenopus* oocytes (Fig. 1Aa) indicating that *Xenopus* oocytes did not express sizable endogenous electrogenic glycine-glycine transport. In *Xenopus* oocytes injected with cRNA encoding PEPT1, however, addition of glycine-glycine to the bath was followed by the appearance of $I_{\text{gly-gly}}$. The additional injection of cRNA encoding wild type OSR1 resulted in a significant decrease of $I_{\text{gly-gly}}$ in PEPT1 expressing *Xenopus* oocytes (Fig. 1).

Further experiments explored whether OSR1 was effective by modifying maximal transport rate and/or affinity of PEPT1. To this end, the oocytes were exposed to glycine-glycine concentrations ranging from 0.01 to 5 mM. As illustrated in Fig. 2 the increase of peptide concentration was followed by an increase of $I_{\text{gly-gly}}$ in both, *Xenopus* oocytes expressing PEPT1 alone and *Xenopus* oocytes expressing PEPT1 and OSR1. The increase of $I_{\text{gly-gly}}$ was, however, larger in *Xenopus* oocytes expressing PEPT1 alone than in *Xenopus* oocytes expressing PEPT1 with OSR1. Calculation of maximal currents yielded values, which were significantly ($p < 0.05$) higher in *Xenopus* oocytes expressing PEPT1 alone (78 ± 11 nA, $n = 10-12$) than in *Xenopus* oocytes expressing both, PEPT1 and OSR1 (48 ± 5 nA, $n = 10-12$). The glycine-glycine concentrations required for halfmaximal current (K_M) were not

Fig. 2. Coexpression of OSR1 decreases maximal electrogenic peptide transport in PEPT1-expressing *Xenopus* oocytes. Arithmetic means $\pm$ SEM (n = 10-12) of glycine-glycine-induced current ($I_{\text{gly-gly}}$) as a function of glycine-glycine concentration in *Xenopus* oocytes expressing PEPT1 without (black squares), or with (black circles) additional coexpression of wild type OSR1.

Fig. 3. The effect of OSR1 is mimicked by active T^{185E} OSR1 but not by inactive mutants D^{164A} OSR1 or T^{185A} OSR1. A: Representative original tracings showing glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus laevis* oocytes injected with water (a), expressing PEPT1 alone (b) or with constitutively active T^{185E} OSR1 (c), with catalytically inactive D^{164A} OSR1 (d), or with WNK1 insensitive inactive T^{185A} OSR1 (e). B: Arithmetic means $\pm$ SEM (n = 8-10) of glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus laevis* oocytes injected with water (dotted bar) or expressing PEPT1 without (white bar) or with constitutively active T^{185E} OSR1 (black bar), with catalytically inactive D^{164A} OSR1 (light grey bar) or with WNK1 insensitive inactive T^{185A} OSR1 (dark grey bar). ***($p < 0.001$) indicates statistically significant difference from the absence of T^{185E} OSR1.

significantly different between *Xenopus* oocytes expressing PEPT1 alone ($795 \pm 95 \mu\text{M}$, n = 10-12) and in *Xenopus* oocytes expressing PEPT1 together with OSR1 ($684 \pm 63 \mu\text{M}$, n = 10-12).

Further experiments explored whether the effect of OSR1 was dependent on kinase activity. As illustrated in Fig. 3, similar to wild type OSR1, the constitutively active T^{185E} OSR1 decreased $I_{\text{gly-gly}}$. In contrast, neither the catalytically inactive D^{164A} OSR1 nor the WNK1 insensitive inactive T^{185A} OSR1 significantly affected $I_{\text{gly-gly}}$ in PEPT1 expressing *Xenopus* oocytes.

A further series of experiments tested whether OSR1 similarly influences the peptide transporter isoform PEPT2. Similar to what has been observed in *Xenopus* oocytes expressing PEPT1, in *Xenopus* oocytes injected with cRNA encoding PEPT2 addition of glycine-glycine

Fig. 4. Coexpression of OSR1 decreases electrogenic peptide transport in PEPT2-expressing *Xenopus* oocytes. A: Representative original tracings showing glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus* oocytes injected with water (a) or expressing PEPT2 without (b) or with additional coexpression of wild type OSR1 (c). B: Arithmetic means $\pm$ SEM ($n = 16-18$) of glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus* oocytes injected with water (dotted bar), or expressing PEPT2 without (white bar) or with (black bar) wild type OSR1. **($p < 0.01$) indicates statistically significant difference from the absence of OSR1.

Fig. 5. OSR1 coexpression decreases PEPT2 protein abundance in the *Xenopus laevis* oocyte cell membrane. Arithmetic means $\pm$ SEM ($n = 72-73$) of PEPT2 protein abundance determined by chemiluminescence in *Xenopus laevis* oocytes injected with water (dotted bar) or expressing PEPT2-HA alone (white bar), or expressing PEPT2-HA together with wild-type OSR1 (black bar). **($p < 0.01$) indicates statistically significant difference from *Xenopus laevis* oocytes expressing PEPT2 alone (Kruskal-Wallis test).

to the bath was followed by the appearance of $I_{\text{gly-gly}}$, which was significantly decreased following additional injection of cRNA encoding wild type OSR1 (Fig. 4). The down-regulation of $I_{\text{gly-gly}}$ in PEPT1 or PEPT2-expressing *Xenopus laevis* oocytes following coexpression of OSR1 could have resulted

from a decline of protein abundance in the cell membrane. Thus, chemiluminescence was employed to quantify the carrier protein abundance in the cell membrane. As illustrated in Fig. 5, coexpression of wild-type OSR1 resulted in a significant decrease of the PEPT2 protein abundance in the cell membrane of PEPT2 expressing *Xenopus laevis* oocytes.

Discussion

The present study discloses a novel function of the oxidative-stress-responsive kinase 1 (OSR1), i.e. the down-regulation of the peptide transporter isoforms PEPT1 and PEPT2. Electrogenic dipeptide transport in PEPT1 expressing *Xenopus* oocytes was significantly decreased by coexpression of OSR1, of constitutively active $T185E$ OSR1, but not of kinase dead $D164A$ OSR1 or WNK1 insensitive inactive $T185A$ OSR1. The inefficacy of the mutants indicates that the effect of OSR1 may require kinase activity. According to chemiluminescence the effect is at least in part due to a decrease of (PEPT2) carrier protein abundance in the cell membrane. Along those lines, OSR1 decreases the maximal transport rate without appreciably influencing the affinity of the (PEPT1) carrier.

The present observations reveal the ability of OSR1 to modify PEPT1 and PEPT2 peptide transporter activities. To which extent those effects are relevant for the regulation of peptide transport *in vivo*, remains, however, to be shown. It must be kept in mind that the expression level of the kinase or the transporters as well as signaling molecules regulating OSR1 and/or the peptide transporters may be different in the oocyte expression system and given cells in mammalian organisms.

The peptide transporters accomplish cellular uptake of peptides into renal [48] and intestinal [31, 49] epithelial cells. Moreover, the peptide transporters are expressed in pancreatic cancer cells [50] and prostate cancer cells [51]. Substrates of peptide transporters include several drugs, such as beta-lactam antibiotics, angiotensin-converting enzyme inhibitors, antiviral drugs, and anti-cancer agents [31, 49, 52-55]. The peptide transporters thus participate in cellular drug uptake [30, 38, 56, 57] and the carriers may be potential targets for tumor therapy [51].

Regulators of OSR1 include dietary salt [58], vasopressin [59, 60] and insulin [61, 62]. Whether or not regulation of OSR1 by dietary salt, vasopressin or insulin contributes to the regulation of peptide transporters remains to be shown.

Conclusion

OSR1 has the potency to downregulate the peptide transporters PEPT1 and PEPT2. Further experiments are needed to define the *in vivo* significance of OSR1 sensitive peptide transport.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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