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A perchlorate sensitive iodide transporter in frogs

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Abstract

Nucleotide sequence comparisons have identified a gene product in the genome database of African clawed frogs (*Xenopus laevis*) as a probable member of the solute carrier family of membrane transporters. To confirm its identity as a putative iodide transporter, we examined the function of this sequence after heterologous expression in mammalian cells. A green monkey kidney cell line transfected with the *Xenopus* nucleotide sequence had significantly greater ^{125}I uptake than sham-transfected control cells. The uptake in carrier-transfected cells was significantly inhibited in the presence of perchlorate, a competitive inhibitor of mammalian Na^+/iodide symporter. Tissue distributions of the sequence were also consistent with a role in iodide uptake. The mRNA encoding the carrier was found to be expressed in the thyroid gland, stomach, and kidney of tadpoles from *X. laevis*, as well as the bullfrog *Rana catesbeiana*. The ovaries of adult *X. laevis* also were found to express the carrier. Phylogenetic analysis suggested that the putative *X. laevis* iodide transporter is orthologous to vertebrate Na^+-dependent iodide symporters. We conclude that the amphibian sequence encodes a protein that is indeed a functional Na^+/iodide symporter in *Xenopus laevis*, as well as *Rana catesbeiana.*

Keywords

Thyroid gland; endocrine disruption; amphibian; Na^+/iodide symporter

1. Introduction

Active absorption of iodide (I^-) from the bloodstream into thyroid follicle cells is a critical step in the synthesis of thyroid hormones. In mammals, this transport of I^- is accomplished by a Na^+-dependent I^- symporter (NIS) that is part of a large gene family of 12-14 transmembrane domain Na^+-dependent transporters (solute carrier family 5, member 5, SLC5A5) (Dai et al., 1996). The NIS is localized to the basolateral plasma membrane of thyrocytes (Castro et al., 1999), where it transports Na^+ and I^- into the cell at a ratio of $2Na^+$:1I. The transport of these two ions by NIS is driven by the electrochemical gradient for Na^+ across the basolateral cell membrane that is established via the actions of the Na^+/K^+-ATPase (Carr et al., 2006).

Ammonium perchlorate is a widespread contaminant of ground and surface waters in the United States. The only known mechanism of perchlorate toxicity is competitive inhibition of

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I^- binding to the NIS, resulting in disruption of thyroid hormone biosynthesis and subsequent elevations in plasma concentrations of thyroid stimulating hormone (TSH, thyrotropin) (Carr et al., 2006). The inhibitory effects of perchlorate are best observed in tadpoles, which require thyroid hormones for normal metamorphosis and development. Exposure to environmentally relevant concentrations of perchlorate during development can delay or prevent metamorphosis (Goleman et al., 2002a,b; Carr et al., 2003b; Sparling et al., 2003; Tietge et al., 2005; Goleman and Carr, 2006; Hu et al., 2006; Sparling et al., 2006; Theodorakis et al., 2006; Carr and Theodorakis, 2006). Observations of such developmental derangements are often the first indication of significant environmental contamination.

Despite the environmental impact of perchlorate contamination (Carr et al., 2003; Theodorakis et al., 2006; Carr and Theodorakis, 2006), there remains little known about the interaction between perchlorate and epithelial I^- transport in amphibian species. Identification of the amphibian version of NIS is a logical first step is increasing our knowledge of this interaction. From comparisons with mammalian gene products, a cloned sequence in the genome database of African clawed frogs (*Xenopus laevis*) was identified as a probable member of the solute carrier family of membrane transporters and seemed likely to represent the amphibian NIS. However, the recent characterization of another putative iodide transporter that proved to transport carboxylate, rather than iodide (Paroder et al., 2006), suggests that such identifications based on sequence similarity must be interpreted with caution. Accordingly, we examined explicitly the function of the putative *Xenopus* NIS (xNIS) after heterologous expression in mammalian cells. Moreover, we explored the tissue distribution of the carrier and analyzed its phylogenetic relationship to NIS proteins from other vertebrates. Portions of this work have been reported previously in abstract form (Carr et al., 2003a).

2. Materials and methods

2.1. Test materials

Sequences identified from GENBANK database searches were obtained from Stratagene. Radiolabeled iodide was purchased from Perkin Elmer. Remaining materials were reagent-grade or better and were obtained from various laboratory supply houses.

2.2. Experimental animals

Sexually mature male and female *X. laevis* were purchased from *Xenopus* Express (Homosassa, FL, USA). Adults were maintained in 160-L flow-through aquaria (Aquatic Habitats ™, Apopka, FL) containing dechlorinated water on a 12:12-h light:dark regime at 20 ± 2 °C. Frog brittle (Nasco, Ft. Atkinson, WI, USA) was provided 3 times weekly (4-6 brittle nuggets per frog). *X. laevis* tadpoles were purchased from Xenopus Express, maintained in dechlorinated tap water at a stocking density of 10 per 38 L, and fed daily with NASCO tadpole brittle. They were used for study at Nieuwkoop-Faber stage 58 of development (Nieuwkoop and Faber, 1994). Bullfrog (*Rana catesbeiana*) tadpoles were purchased from Charles D. Sullivan Co. Inc. and maintained in dechlorinated tap water in a large tank (178 cm L × 46 cm W × 51 cm D, 300 L) with filtration at a stocking density of 50/300 L. Tadpoles were fed boiled spinach three times per week. They were used for study at Taylor-Kollros stage XVII-XX of development (Taylor and Kollros, 1946). All animal use was approved by the TTU IACUC.

2.3 Tissue Collection

Tadpoles and frogs were anesthetized in MS-222 for isolation of specific tissues. These were removed by dissection and immediately flash frozen in liquid nitrogen for RNA extraction. Thyroid glands were removed as described by Wright et al. (1999).

2.4. Amplification and DNA sequencing

Tissue-specific expression was assessed at the mRNA level by amplification from isolated RNA via polymerase chain reaction (RT-PCR). Total RNA was extracted from tissues of *X. laevis* or *R. catesbeiana* tadpoles using the UltraSpec RNA Isolation System (Biotecx). cDNA was reverse transcribed from 2 μg total RNA using 1.0 μg oligo dT primer (Invitrogen), 40 mM dNTP mix (Roche) and 1 unit M-MLV reverse transcriptase (Promega). Amplification of the xNIS sequence by PCR was performed in 50 μl reactions using 50 μM dNTP mix, 1 unit Taq DNA polymerase (Roche) and 0.1 μM of each NIS-derived forward and reverse primer. The forward primer used for the putative xNIS was GGGTTGGACATCTGGGCTTC, and the downstream primer was CCTTCGAGGATCAGGATCAA. This was expected to amplify a fragment of 240 base pairs. PCR conditions consisted of 30 cycles at 58° C annealing and 68° C elongation in a MJ Research Minicycler. As a positive control for RNA integrity, we included primers for the ribosomal protein L8 (RPL8), which was expected to be expressed in all tissues. These primers consisted of forward primer GACATTATCCATGATCCAGG and reverse primer GGACACGTGGCCAGCAGTTT and were expected to amplify a fragment of 480 base pairs. Sequencing of PCR fragments was performed at the Texas Tech University Center for Biotechnology and Genomics using a Perkin Elmer Biosystems 310 Genetic Analyzer.

2.5. Transient Transfection

Exogenous expression of the putative xNIS was achieved by transfection of mammalian cells in culture. Green monkey kidney (COS-1) cells grown in 100-mm dishes were transfected at 80-90% confluency with 1μg/μl Green Fluorescent Protein (GFP)-tagged α1 sequence from the Na,K-ATPase (sham-transfected, vector a gift from Dr. Carlos Pedemonte) or xNIS - SLC5A5 (ATCC# 9336266), in pCMV Sport 6.1 plasmid (Invitrogen) with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The former was selected as a negative control because it is also a membrane protein with multiple spans, but is not capable of transporting iodide. Moreover, the GFP tag facilitated assessment of overall transfection efficiency. Incubations occurred at 37° C, 10% CO_2 in Dulbecco's modified Eagle's Medium (DMEM, Gibco). After 24 hr, the transfected cells were split into 13 33-mm plates with DMEM supplemented with 10% fetal bovine serum (Atlanta Biologicals) for subsequent analysis.

2.6. I^- uptake

Functional assessment of putative xNIS was accomplished using radiotracers as described previously (Pressley et al., 1995). In brief, assays for $^{125}I^-$ uptake were performed using sham- and xNIS-transfected cells 48 hr post transfection. Cells were typically at 95-100% confluency. DMEM/FBS was replaced by modified Hanks Balanced Salt Solution (HBSS) at pH 7.3 and 37° C. Cells were incubated in the presence and absence of 20 μM perchlorate for 5 min at 37° C in HBSS containing 20μM NaI and 1μCi ^{125}I (Weiss et al. 1984). Uptakes were terminated after 5 min by rinsing cell monolayers with ice-cold 100mM $MgCl_2$ and lysing the cells with 1 ml of 1.5 mM CsCl flame photometry standard. ^{125}I activity was measured in the lysates and medium on a gamma spectrophotometer and standardized by protein content for each plate. Sham- transfected COS-1 cells were analyzed by exactly the same method as NIS-transfected cells with the exception that successful transfection of the GFP--tagged α1 sequence was confirmed by the presence of fluorescence when the cells were examined under the confocal microscope, excitation at 488 nm and emission at 510 nm (Yokogawa Mod C-10 from Mc Bain Instruments). Protein content of cell lysates was measured using the Lowry method (Lowry et al., 1952).

2.7 Phylogenetic analysis

Thirty-one amino acid sequences homologous to the *X. laevis* NIS protein were obtained from the National Center for Biotechnology Information (NCBI), the *X. tropicalis* genome (JGI v. 3.0; Joint Genome Institute) database, and the Institute of Molecular and Cell Biology (IMCB, Ensembl). Sequences were aligned using ClustalW (Thompson et al., 1994) and phylogenetic analysis was performed using the neighbor-joining method (Saitou and Nei, 1987) in MEGA v 3.1 (http://www.megasoftware.net, Kumar et al., 2004). There were 686 positions used in the final data set with both pairwise deletion and complete deletion method options being used to generate separate trees. Nodal support was determined using 1000 bootstrap replications (Felsenstein, 1985). Topology of the two trees was identical with the exception of an inconsequential 1 point loss of bootstrap support in a few nodes using the pairwise deletion option.

NCBI accession numbers for translated proteins—XP_581578, bovine NIS; NP_777031, bovine SGLT1; XP_541946, canine NIS; NP_001007142, canine SGLT1; XP_429095, chicken NIS; XP_415247, chicken SGLT1; XP_524154, chimpanzee NIS; XP_522508, chimpanzee SMCTe; NP_000444, human NIS; AAI39840, human SGLT1; NP_666018, human SMCTe; NP_444478, mouse NIS; NP_999575, porcine NIS; NP_062784, mouse SGLT1; NP_663398, mouse SMCTe; NP_001009404, ovine SGLT1;; NP_443215, rat NIS; NP_037165, rat SGLT1; XP_576209, rat SMCTe; CAG00616, *Tetraodon nigroviridis* unidentified protein (putative NIS); AAH77614, *X. laevis* NIS; AAH60005, *X. laevis* SMCTe; NP_988910, *X. tropicalis* SMCTe; CAK04382, zebrafish NIS; AAH67621, zebrafish SGLT1; AAW55811, zebrafish SMCTe. Ensembl (The Institute of Molecular and Cell Biology, IMCB) IDs for translated proteins: SINFRUP00000132536, *Takifugu rubripes* NIS; ENSORLP00000008329, *Orzias latipes* (medaka) NIS.

2.8 Statistical analysis

Data on ^{125}I uptake in transfected and sham-transfected COS cells were analyzed by repeated measures one-way ANOVA followed by the Tukey-Kramer's multiple comparisons test using InStat software (GraphPad, San Diego, CA, USA). Data are reported as mean ± standard error of mean (SEM).

3. Results

To identify potential probes for use in characterizing the iodide uptake system of amphibians, we monitored submissions to various nucleotide sequence databases. Primers were initially generated against a partial cDNA sequence (Expressed Sequence Tag, GENBANK accession BF427625) with sequence homology to mammalian NIS (SLC5A5). Using RT-PCR we amplified a fragment of mobility consistent with the expected size of 230 bp using RNA prepared from whole *X. laevis* stage 60 tadpoles as template. Sequencing of the PCR product revealed that it was identical to the Genbank sequence. Moreover, using the same primers we amplified a fragment of comparable mobility from bullfrog tadpole thyroid (*R. catesbeiana).* The sequence of this fragment also proved to be identical to the *X. laevis* PCR fragment with the exception of a single G-T substitution (position 815). Additional amplifications revealed the presence of the putative NIS mRNA in thyroid tissues of *X. laevis* tadpoles, as well as several additional tissues in both species including kidney and gastrointestinal tract. An ovary sample from adult *Xenopus* also produced the NIS fragment (Fig. 1). Amplification of a housekeeping sequence, ribosomal protein L8, in all the tested samples confirmed the integrity of the RNA, even in those samples that failed to produce NIS-derived fragments.

While we were attempting to clone the full-length NIS cDNA from *X. laevis*, a full-length sequence identified as a probable member of the solute carrier family was submitted to

GENBANK (Genbank accession #BC077614). Indeed, the sequence contained the region targeted by the earlier PCR primers. This clone was obtained and subjected to functional analysis by heterologous expression. Mammalian COS-1 cells transfected with a vector containing the full length xNIS cDNA showed 2-4 fold greater ^{125}I uptake than sham-transfected cells. This uptake was significantly reduced in the presence of perchlorate. Perchlorate had no effect on ^{125}I uptake in sham-transfected cells. (Fig. 2). This strongly suggests that the putative sequence is indeed the NIS of amphibians.

Multiple sequence alignment analysis revealed that this perchlorate-sensitive *X. laevis* NIS shows 99% homology to the *X. tropicalis* NIS protein and shows 69 % homology to the human NIS protein at the amino acid level (p-distance). Interestingly, the *X. laevis* NIS shares far less similarity with other members of the solute carrier family. There is only 51% similarity with the *X. laevis* SMCTe and 49% similarity with human SMCTe (electrogenic sodium monocarboxylic acid transporter). Phylogenetic analysis using the neighbor-joining method provides high support for differentiation of the three families of sodium solute transporters (Fig. 3). The topology of the tree follows evolutionary lineages and does not show any incongruent relationships. This phylogenetic analysis offers additional support for the identity of the targeted sequence as the amphibian NIS.

4. Discussion

Despite numerous studies indicating that environmentally relevant concentrations of perchlorate inhibit thyroid hormone synthesis and disrupt metamorphosis (Goleman et al., 2002a, b; Carr et al., 2003; Sparling et al., 2003; Tietge et al., 2005; Goleman and Carr, 2006; Hu et al., 2006; Sparling et al., 2006; Theodorakis et al., 2006; Carr and Theodorakis, 2006), this is the first study to identify a functional perchlorate-sensitive iodide transport protein in an amphibian. This observation, along with the fact that this transporter shows sequence homology with mammalian NIS and other members of the sodium-dependent transport family, strongly suggests that this protein is indeed the NIS in these model species. Using an amplification strategy similar to ours, Optiz et al. (2006) has also shown expression of this transporter in *Xenopus* thyroid tissue. These investigators also observed a developmentally-distinct pattern of expression that correlates with thyroid hormone secretion during metamorphosis.

Our data indicate that the amphibian iodide transporter is expressed in a number of nonthyroid tissues in two frog species. The observation of xNIS expression in ovarian tissue is particularly interesting given that data from amphibians (Carr et al., 1984), birds (Newcomer, 1982; Newcomer et al., 1984) and mammals (Slebodziński, 2005) demonstrates the active transport of I^- in ovarian tissue. At present the physiological significance of ovarian I^- uptake is less well understood, but it may ensure that sufficient iodide is available in the ova for early embryonic development of the thyroid. Mammalian NIS is expressed by epithelial cells in a number of nonthyroid tissues, including salivary glands, mammary tissues, stomach, and kidney (Lacroix et al., 2001; Spitzweg et al., 2001; Wapnir et al., 2003, also see De La Vieja et al., 2000 for a review). Although the NIS is expressed in these other tissues, thyrocytes are much more efficient at concentrating I^- because of thyroid-specific transcription factors that promote NIS gene transcription (De La Vieja et al., 2000). In addition, the promoter region of the rat NIS gene has a TSH-responsive element that also up-regulates NIS expression (Ohmori et al., 1998). Recent work using TSH-receptor knockout mice indicates that NIS expression is virtually non-existent in thyrocytes lacking TSH receptors (Marians et al., 2002).

The xNIS shares sequence similarity with another member of the SLC solute transporter family, the so-called SMCTe protein. This transporter was initially described as an apical I^- transporter in human thyroid, based upon sequence homology with the NIS, its immunolocalization to the

apical plasma membrane of thyrocytes, and its ability to weakly transport I^- in an *in vitro* expression system (Rodriguez et al., 2002). Subsequent studies have shown that this protein is not a true I^- transporter (Coady et al., 2004), but instead functions to transport monocarboxylic acids such as pyruvate and lactate (Paroder et al., 2006). Whether this is a functional solute transporter in frog thyroid is an interesting area for future research.

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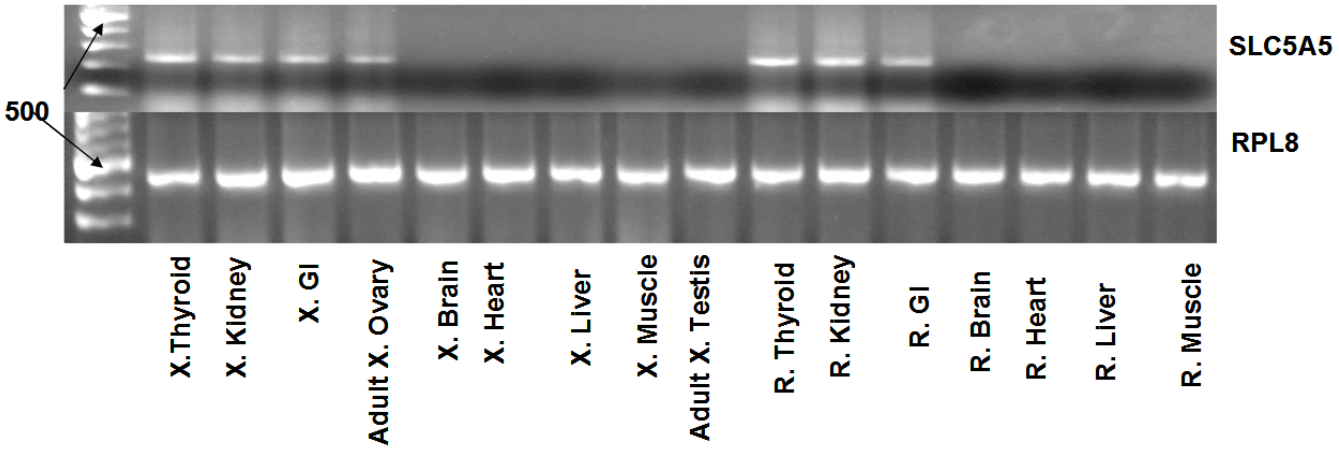

Figure 1.

RT-PCR analysis of the xNIS mRNA in different tissues of *X. laevis* (NF stage 58) and *R. catesbeiana* (Taylor-Kollros stages XVII-XX) tadpoles. Adult male and female *X. laevis* were used for analysis of testicular and ovarian tissue, respectively. Two micrograms of total RNA from thyroid, kidney, GI tract, skeletal muscle, brain, liver, or adult testes and ovaries were reversed transcribed before PCR amplification and separation on a 2% gel (1% agarose, 1% Nusieve, BioWhittaker Molecular Applications) for low molecular weight resolution. Relative expression of the ribosomal protein L8 (RPL8) is shown as a housekeeping control for the integrity of the total RNA.

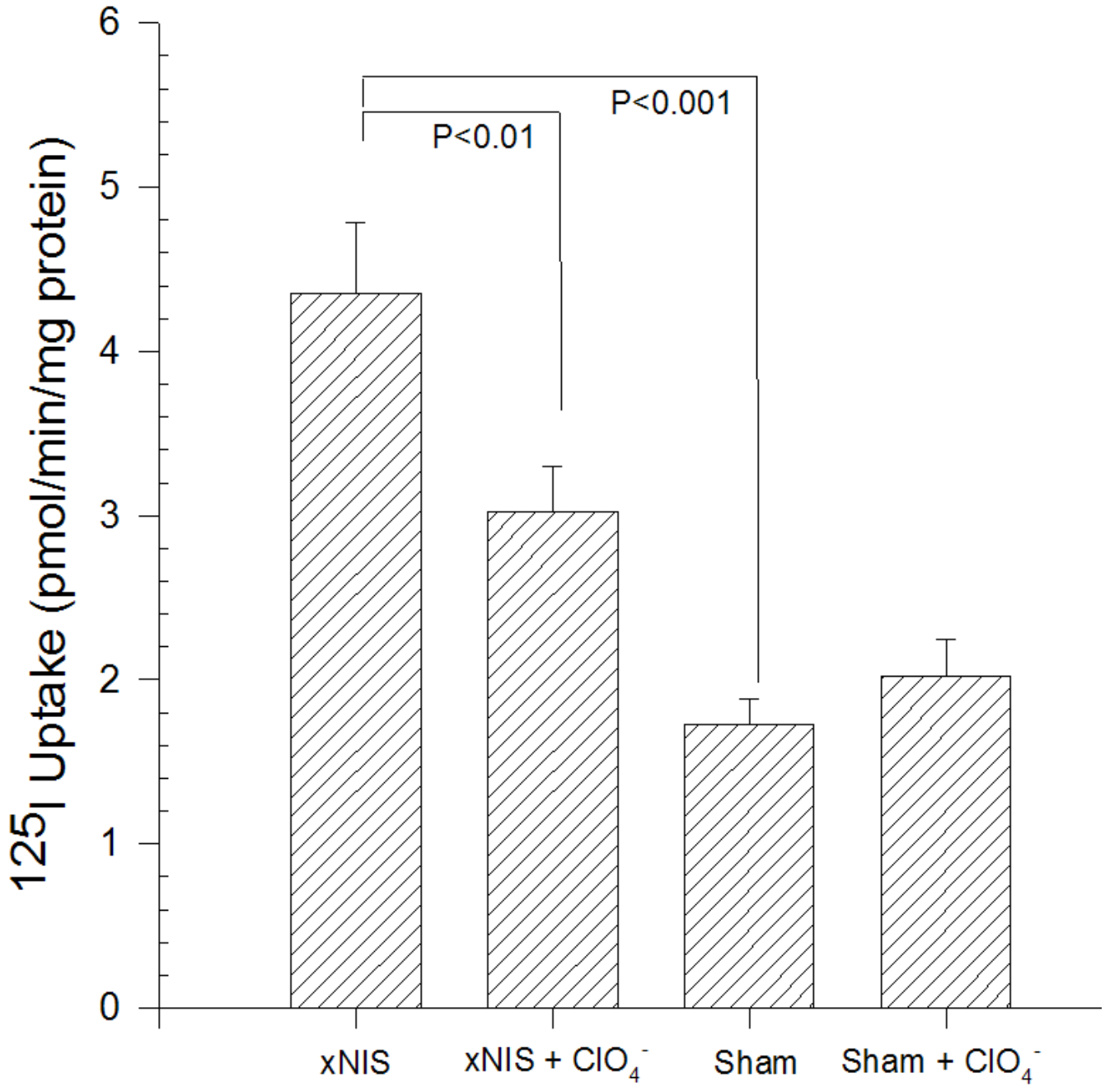

Figure 2.
In vitro ^{125}I uptake in the presence or absence of perchlorate (20μM) by COS cells transfected with xNIS or the negative control, GFP-tagged α subunit from the Na^+,K^+-ATPase (*Sham*). Bars represent the mean + SEM of 18 independent experiments.

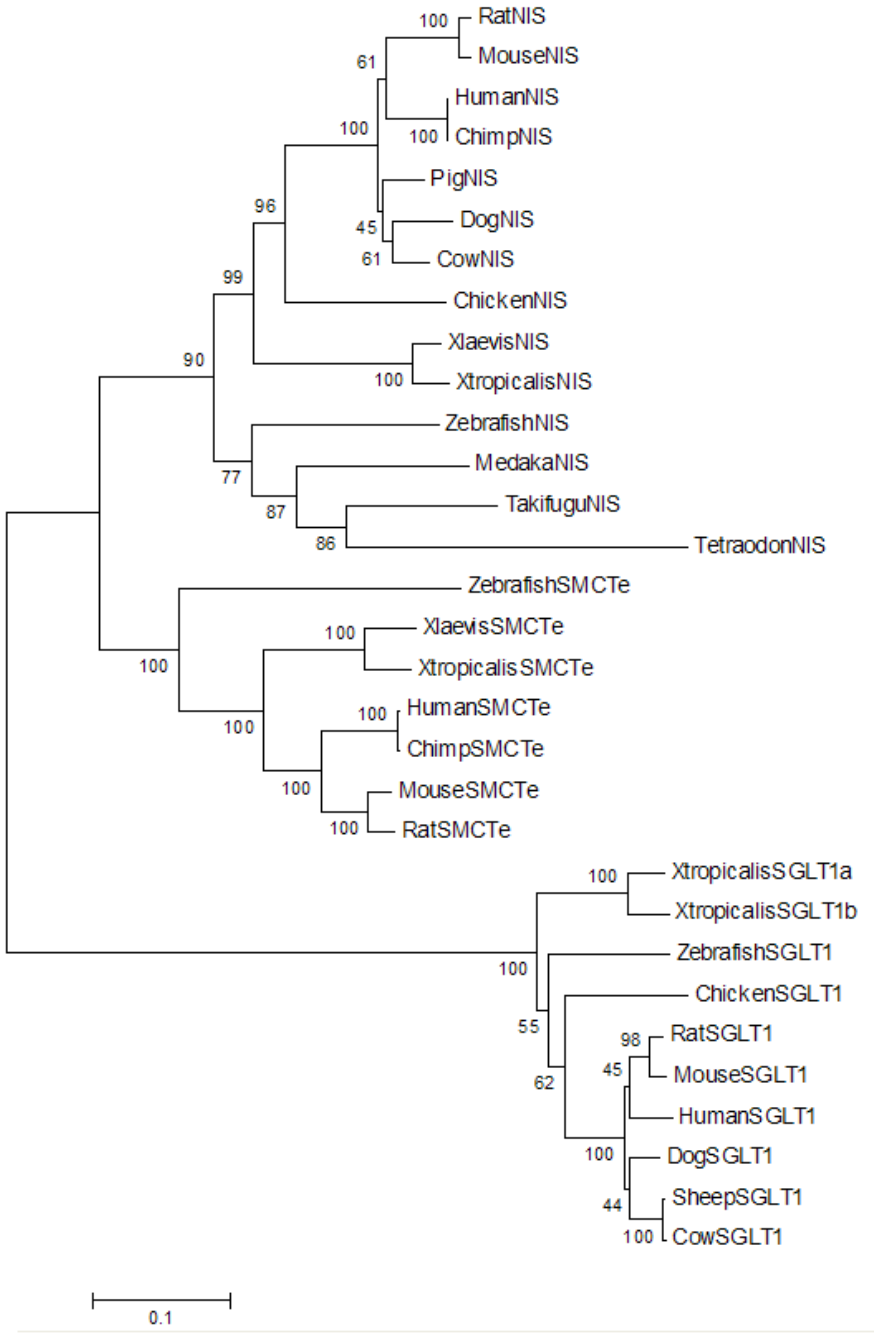

Figure 3.

Phylogenetic analysis of selected solute carrier proteins using the neighbor-joining method. Tree shown was derived from 686 positions using the complete deletion option and nodal support was determined using 1000 bootstrap replications. Scale bar indicates number of amino acid substitutions per site. Solute carrier proteins used in this analysis are NIS (Na^+-dependent I^- symporter); SGLT1 (Na(+)/glucose cotransporter); (SMCTe (electrogenic sodium monocarboxylic acid transporter).