Functional regulation of Na^+-dependent neutral amino acid transporter ASCT2 by *S*-nitrosothiols and nitric oxide in Caco-2 cells

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Abstract **We describe the regulation mechanisms of the Na^+-dependent neutral amino acid transporter ASCT2 via nitric oxide (NO) in the human intestinal cell line, Caco-2. Exposure of Caco-2 cells to *S*-nitrosothiol, such as *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and *S*-nitrosoglutathione, and the NO-donor, NOC12, concentration- and time-dependently increased Na^+-dependent alanine uptake. Kinetic analyses indicated that SNAP increases the maximal velocity (V_{max}) of Na^+-dependent alanine uptake in Caco-2 cells without affecting the Michaelis–Menten constant (K_t). The stimulatory effect was partially eliminated by actinomycin D and cycloheximide. Increased Na^+-dependent alanine uptake by SNAP was partially abolished by the NO scavengers, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide sodium salt (carboxy-PTIO) and *N*-(dithiocarboxy)sarcosine disodium salts (DTCS), as well as the NADPH oxidase inhibitor, diphenyleneiodonium. RT-PCR revealed that Caco-2 cells expressed the Na^+-dependent neutral amino acid transporter ASCT2, but not the other Na^+-dependent neutral amino acid transporters $ATB^{0,+}$ and B^0AT1. These results suggested that functional up-regulation of ASCT2 by SNAP might be partially associated with an increase in the density of transporter protein via de novo synthesis.**

Keywords: Amino acid transport; System ASC; Nitric oxide; *S*-Nitrosothiol; Transport regulation; Caco-2 cells

1. Introduction

Amino acid transporters provide amino acid nutrients to cells, re-uptake neurotransmitters in the central nervous system, (re)absorb materials in the intestine and kidney, and modulate intracellular redox reactions. The Na^+-dependent transport of neutral amino acids by intestinal cells is catalyzed by various systems [1]. Among them, system B^0 is preferentially responsible for transporting Na^+-dependent neutral amino acids from the luminal membrane into enterocytes [2,3] and renal epithelial cells [2]. The Na^+-dependent neutral amino acid transporter ATB^0 has recently been cloned from human placental (JAR) [2] and intestinal (Caco-2) [4] cell lines, the functional characteristics of which are similar to those of the amino acid transport system B^0 [5]. Although, ATB^0 mediates the transport of several neutral amino acids in a Na^+-dependent manner and acts as a Na^+-dependent exchanger of neutral amino acids [6], system B^0 was originally reported to mediate the net uptake of neutral amino acids. In addition, the amino acid sequences of human ATB^0 are highly homologous to those of rat and mouse ASCT2 [7,8], and in fact ATB^0 is the human ortholog of mouse and rat ASCT2. Recently, Bröer et al. [9] have cloned from the mouse kidney the Na^+-dependent neutral amino acid transporter B^0AT1, which belongs to the Na^+-dependent neurotransmitter transporter family (SLC6A) and is responsible for the "real" amino acid transport system B^0. Therefore, we used "ASCT2" instead of ATB^0 in this study.

Nitric oxide (NO) plays important roles in a multitude of physiological processes. It is implicated in a wide range of disease processes, exerting both detrimental and beneficial effects at the cellular level [10] and it also participates in many of the physiological and pathophysiological processes in the gastrointestinal tract [11]. Inducible nitric oxide synthase (iNOS) is inactive under normal conditions, but some inflammatory agents such as endotoxin, interleukin-1β, and tumor necrosis factor-α induce the expression iNOS [12,13]. Although, NO provides antimicroviral properties during infection [14], overproduction of NO by iNOS causes tissue injury under conditions of inflammation or infection [15,16].

Compounds that can donate NO and its redox active forms are widely used to mimic the effects of NO synthase [17]. In particular, the *S*-nitrosothiol class of NO donors can modulate protein function by transnitrosylation as well as NO release [18,19]. Bridges et al. [20,21] have shown that the cystine/glutamate transporter and the taurine transporter are up-regulated by long-term exposure to NO donors such as *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and SIN-1, in retinal pigment epithelial cell. In addition, Smith et al. [22] reported that a NO donor decreased the activity of reduced folate transporter (RFT) in a cyclic GMP-independent manner. Furthermore, Kaye et al. [23] also reported that NO or its congeners (NO_x) generated from SNAP inhibit desipramine-sensitive norepinephrine transport in a concentration-dependent and cyclic GMP-independent manner. These reports suggest that NO is an important regulatory factor in the expression and function of transporters.

The Na^+-dependent neutral amino acid transport system ASCT2 is a preferential neutral amino acid transport systems in enterocytes. To provide insight into the NO/NO_x-mediated regulation of the Na^+-dependent neutral amino acid transport system under inflammatory conditions or during enterocyte differentiation, we examined the effects of *S*-nitrosothiol and

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NO on this system in the human intestinal cell line Caco-2. This line is a useful model of intestinal epithelial cells because it undergoes spontaneous enterocytic differentiation in vitro [24,25]. In addition, the features of the Caco-2 transport for various organic ions and nutrients closely resemble those of the intestinal epithelium [4,26–28]. Here, we investigated the effects and regulatory mechanisms of *S*-nitrosothiol type or NONOate type NO-donors on Na^+-dependent alanine uptake mediated by the amino acid transporter ASCT2 in Caco-2 cells.

2. Materials and methods

2.1. Materials

We purchased L-[2,3-^{3}H]alanine (47 Ci/mmol) and L-[2,3,4,5-^{3}H]-arginine (61 Ci/mmol) from Amersham Biosciences Co. (Piscataway, NJ, USA) and [^{3}H]glycylsarcosine (40 Ci/mmol) and [^{3}H]carnitine (80 Ci/mmol) from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). All unlabeled amino acids, diphenyleneiodonium chloride (DPI), actinomycin D, and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SNAP, *S*-nitrosoglutathione (GS-NO), 1-hydroxy-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC12), 3-(4-morpholinyl)sydnonimine hydrochloride (SIN-1), 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide sodium salt (carboxy-PTIO) and *N*-(dithiocarboxy)sarcosine disodium salts (DTCS) were purchased from Dojindo Laboratory (Kumamoto, Japan). Cell culture media and reagents were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest commercially available purity.

2.2. Cell culture

The human intestinal cell line Caco-2 (passages 88–95) and normal human intestinal epithelial cells were obtained from Dainippon Pharmaceuticals (Osaka, Japan). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% non-essential amino acids [29]. Normal human intestinal epithelial cells were cultured using the CS-2.0 serum-free medium kit (Applied Cell Biology Research Institute, Kirkland, WA, USA) according to the manufacturer's instructions. Cells passaged by dissociation in 0.05% trypsin and 0.02% EDTA were seeded in 24-well culture plates (BD Biosciences) at a density of 1×10^4 cells/well and reached confluence 7 days later. On the following day, the cells were processed and uptake was measured.

2.3. Uptake measurements

Confluent cells were rinsed three times with uptake buffer consisting of 25 mM HEPES/Tris (pH 7.4), 140 mM NaCl (or choline chloride), 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose [29]. Uptake was initiated by adding 250 μl of uptake buffer containing [^{3}H]alanine and measured after a 15-min incubation at 37 °C. We investigated the Na^+-dependence of the transport process using uptake buffer in which NaCl was isoosmotically substituted with choline chloride. Uptake was terminated by aspiration followed by two washes with ice-cold uptake buffer. The cells were then solubilized in 1% SDS in 0.2 M NaOH and placed in scintillation vials to measure radioactivity.

2.4. NO donor effects

To determine the effects of NO/NO_x on the Na^+-dependent alanine uptake in Caco-2 cells, confluent cultures were incubated with serum-free medium in the presence or absence of the *S*-nitrosothiol type NO donor SNAP or the NONOate type NO donor NOC12 (half-life of NO release: ~100 min) for the designated period (usually 16 h). To test the specificity of SNAP in stimulating Na^+-dependent alanine uptake, Caco-2 cells were incubated with 1 mM SNAP for 16 h at 37 °C with [^{3}H]alanine and then levels of [^{3}H]arginine, [^{3}H]glycylsarcosine, and [^{3}H]carnitine uptake were measured for 15 min. The capacity of antioxidant and NO scavengers to inhibit the SNAP-induced stimulation of Na^+-dependent alanine uptake was measured by incubating Caco-2 cells in serum-free culture medium containing SNAP in the presence of cysteine (1 mM), carboxy-PTIO (300 μM) and DTCS (500 μM). The cells were also incubated for 16 h with dibutylyl cyclic GMP (0.1 and 1 mM), a cell permeable cyclic GMP analog, and then Na^+-dependent [^{3}H]alanine uptake was measured.

2.5. RT-PCR

Total RNA was isolated from Caco-2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription proceeded with 5 μg of total RNA using Superscript II (Invitrogen). The primer sets for ASCT2 were 5′-CCG CTG ATG ATG AAG TGC-3′ (forward primer) and 5′-CCC CCG ATA GTG TTT GAG-3′ (reverse primer), which correspond to nucleotides 1692–2197 of cDNA [Accession No. U53347]. The primer sets for $ATB^{0,+}$ were 5′-AAA TGC CCG AGT TTC TTC-3′ and 5′-TCT CAT TCC GGT TTT CTG-3′ (reverse primer), which correspond to nucleotides 101–2002 of cDNA [Accession No. AF151978]. The primer sets for B^0AT1 were 5′-CAA TGG CAT CGT CTT CCT CT-3′ (forward primer) and 5′-TTG AAC CTG TCC ACA CCG TA-3′ (reverse primer), which correspond to nucleotides 744–1544 of cDNA [Accession No. NM_001003841]. The primer sets for GAPDH were 5′-CCA TCA CCA TCT TCC AGG AG-3′ (forward primer) and 5′-CCT GCT TCA CCA CCT TCT TG-3′ (reverse primer), which correspond to nucleotides 278–853 of cDNA [Accession No. M33197]. The PCR procedure using these primer sets consisted of denaturation for 2 min at 94 °C, and 30 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 90 s. Final extension proceeded for 7 min at 72 °C. The RT-PCR products were separated on 1% agarose gels and stained with ethidium bromide.

2.6. Data analysis

Each experiment proceeded using duplicate or triplicate monolayers. Results are expressed as means ± S.E.M. Statistical significance was determined by Student's *t* test for unpaired samples assuming equal variance and $P < 0.05$ was considered significant. Statistical differences among multiple groups were determined by the one-way analysis of variance and $P < 0.05$ was considered significant by Dunnett's test using commercial software (Instat, GraphPad, San Diego, CA, USA).

3. Results

3.1. Functional identification of Na^+-dependent neutral amino acid transport system in Caco-2 cells

The transport systems B^0, B^{0+} and ASC are currently thought to be responsible for Na^+-dependent alanine uptake from the brush-border membrane in the intestine. We initially investigated the features of Na^+-dependent alanine uptake in Caco-2 cells (Table 1). Unlabeled alanine, serine and cysteine totally inhibited Na^+-dependent [^{3}H]alanine (48 nM) uptake. Phenylalanine, an aromatic neutral amino acid, also inhibited Na^+-dependent [^{3}H]alanine uptake moderately but significantly. On the contrary, arginine (cationic amino acid), the selective system A substrate, α-methyl-aminoisobutyric acid (MeAIB), and the selective system L substrate, 2-amino-2-norbornane-carboxylic acid (BCH) did not inhibit Na^+-dependent [^{3}H]alanine uptake (Table 1). These results agreed with those of previous reports regarding the characteristics of Na^+-dependent alanine uptake in Caco-2 cells [30]. In addition, these results indicated that the functional Na^+-dependent neutral/basic amino acid transport system $B^{0,+}$ is not expressed in Caco-2 cells. Because the Na^+-dependent neutral amino acid transport system B^0 accepts phenylalanine as a substrate, we studied the effect of Na^+ on [^{3}H]phenylalanine (40 nM) uptake in Caco-2 cells. The presence or absence of Na^+ in the buffer did not