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SHORT COMMUNICATION

Human cationic amino acid transporters are not affected by direct nitros(yl)ation

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Abstract

A direct inhibiting effect of NO on the function of CAT-1 and -2A has been postulated to occur via nitrosylation of cysteine residues in the transporters. Neither the NO donor SNAP nor a mixture of SIN-1 and Spermine NONOate, that generates the strong nitrosating agent N_2O_3, reduced CAT-mediated L-arginine transport. Direct nitros(yl)ation does either not occur in CATs or does not affect their transport function. A regulatory effect of NO or nitrosating agents on CAT-mediated transport under physiological conditions seems, therefore, unlikely.

Keywords Nitric oxide donors · SNAP · SIN-1 · SPENO · Arginine · *Xenopus laevis* oocytes · Nitrosylation · Nitrosation

Abbreviations

CAT	Cationic amino acid transporter (SLC7A1-A3, prefix: human)
CAA	Cationic Amino Acids
cRNA	RNA transcribed from cDNA
GSH	Reduced glutathione
GSNO	S-Nitrosoglutathione
HPLC	High-performance liquid chromatography
IUGR	Intrauterine growth restriction
NEM	*N*-Ethylmaleimide
PKC	Protein kinase C
SLC7	Solute carrier family 7
SIN-1	5-Amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride
SNAP	S-Nitroso-*N*-acetylpenicillamine
SNO	S-Nitrosothiols
SPENO	Spermine NONOate; (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino] diazen-1-ium-1,2-diolate

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Introduction

Cationic amino acid transporters (CATs, SLC7A1-3) mediate specifically the transport of cationic amino acids (CAA) such as L-arginine. In most cells, they are the main CAA import route and are thus essential for all metabolic pathways involving these amino acids (Closs et al. 2006), e. g. the synthesis of proteins, urea, NO and creatine (Lu et al. 2009). Previous studies have shown that CATs are inhibited by *N*-ethylmaleimide (NEM) that acts on two defined cysteine residues conserved in all mouse and human CAT proteins corresponding to Cys33 and 273 in human CAT-2A (Beyer et al. 2013; Devés et al. 1993). These cysteine residues are thus essential for transporter function and consequently a probable target by endogenous mediators to regulate transporter activity.

In rat cardiac myocytes, inhibition of both low- and high-affinity arginine transport through nitric oxide has been observed and it has been suggested that this inhibition was mediated by S-nitros(yl)ation of cysteine residues in CAT-1 and CAT-2A (Zhou et al. 2010). As transport inhibition by exogenous NO has also been observed in myocyte-derived membrane vesicles lacking all intracellular components such as CAA, or soluble enzymes, a direct interaction between

NO and the transporters has been proposed. S-nitros(yl)ation is the redox-based formation of S-nitrosothiols (SNO) in cysteine residues in proteins (or smaller molecules such as glutathione, GSH) that may lead to pronounced structural and functional changes (Foster et al. 2003). SNO formation can be achieved by reaction of either NO with cysteine thiyl radicals (known to be present in a number of proteins) or of nitrosating agents with cysteine thiol (Heinrich et al. 2013). Alternatively, SNO may be generated by a process called trans-nitrosation from nitros(yl)ated small molecules (e. g. S-nitrosoglutathione, GSNO) or other proteins (Heinrich et al. 2013). A powerful nitrosating agent is dinitrogen trioxide (N_2O_3) that can be formed from NO and O_2^-. The physiological concentrations of the two reactants are considered to be too low for the reaction to occur efficiently in the aqueous environment of the cytoplasm. However, both compounds accumulate in the hydrophobic milieu of the plasma membrane thus accelerating N_2O_3 formation and subsequent nitrosation (Liu et al. 1998). As CATs are integral membrane proteins, S-nitros(yl)ation seems a plausible mechanism of transporter regulation. The aim of our current study was, therefore, to identify the cysteine residues responsible for transporter inhibition by NO/nitros(yl)ation.

Materials and methods

L-[^{3}H]arginine uptake

Isolation of *Xenopus laevis* oocytes (approved by the Rhineland-Palatinate Investigative Office, A 12-1-002) and injection with cRNA coding for hCAT-1 or hCAT-2A (or with water only for baseline correction) were performed as previously described (Beyer et al. 2013). The injected oocytes were incubated in modified Leibovitz medium for 2 days at 18 °C to enable protein expression. Oocytes were then rinsed three times with ice-cold uptake solution ND96 (96 mM NaCl, 1.8 mM $CaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.3) and subsequently incubated for 10 min in ND96 in the absence or presence of 100 µM SNAP (Sigma Aldrich, Deisenhofen, Germany) or 1 mM each, Sin-1 and SPENO (both Cayman Chemical, Ann Arbor, USA), as indicated. With SNAP being light sensitive, incubation tubes were wrapped with aluminum foil during the experiments.

hCAT activity was determined by measuring either 100 µM (hCAT-1) or 1 mM (hCAT-2A) L-[^{3}H]arginine (10 µCi/ml, ICN Biomedicals GmbH, Eschwege, Germany) uptake in the continuous presence or absence of the respective NO/O_2^- donor. The time points within the linear range of initial uptake were determined by performing a time-course. Incubation times were 15 min for experiments performed at 20 °C, and 1 min (hCAT-1) or 3 min (hCAT-2A) for experiments performed at 37 °C. The uptake was stopped by transferring the incubation tubes on ice followed by immediate washing three times with ice-cold ND96. Oocytes were then solubilized in 2% SDS and radioactivity was determined by scintillation counting.

Quantification of GSH and GSNO

3 oocytes each were lysed in 500 µl borate buffer and GSH to GSNO concentrations were determined in 50 µl lysate, each, using an HPLC-based method as previously described (Tsikas et al. 1999), except that NEM was supplemented at a final concentration of 1 mM where suitable. GSNO was determined as GSH peak after treatment of oocyte lysates with NEM (reacts with free GSH) and subsequent 2-mercaptoethanol (transforms GSNO, but also oxidized glutathione to GSH). GSNO generated by exposure to Sin-1/SPENO was quantified by subtracting the GSH peak obtained after NEM and 2-mercaptoethanol treatment of lysates of untreated oocytes from the corresponding peak of Sin-1/SPENO-exposed oocytes thereby canceling the endogenous background of oxidized glutathione and GSNO (Supplemental Fig. 1).

Statistical analyses

For statistical analyses, the mean amount of L-[^{3}H]arginine taken up by the respective batch of oocytes without exogenous transporter expression was subtracted from the value obtained for each transporter-expressing oocyte of the same batch. Values for each individual oocyte were then calculated as percentage of the mean of the respective untreated oocytes. *t* tests between untreated and treated oocytes from all oocyte batches were then performed.

Results and discussion

To examine the effect of nitric oxide on CAT function, we expressed human CAT-1 or -2A in *X. leavis* oocytes where the activity of each individual transporter can be monitored against a very low background of endogenous transport. To get maximal exposure of the transporters to NO, we followed the protocol of Zhou et al. (2010) and incubated oocytes in buffer containing 100 µM of the NO donor SNAP, 10 min prior and during the transport measurement. In our initial experiments carried out at 20 °C (the temperature routinely used for oocytes), we did not see any difference in transport activity between SNAP-exposed and control oocytes, either expressing hCAT-1 (Fig. 1a, $p = 0.684$, $n = 13–17$) or hCAT-2A (Fig. 1b, $p = 0.0827$, $n = 12–16$). Sufficient NO release by SNAP was verified on norepinephrine-precontracted mouse aortic rings: as expected (Leone and Boyle 2006)

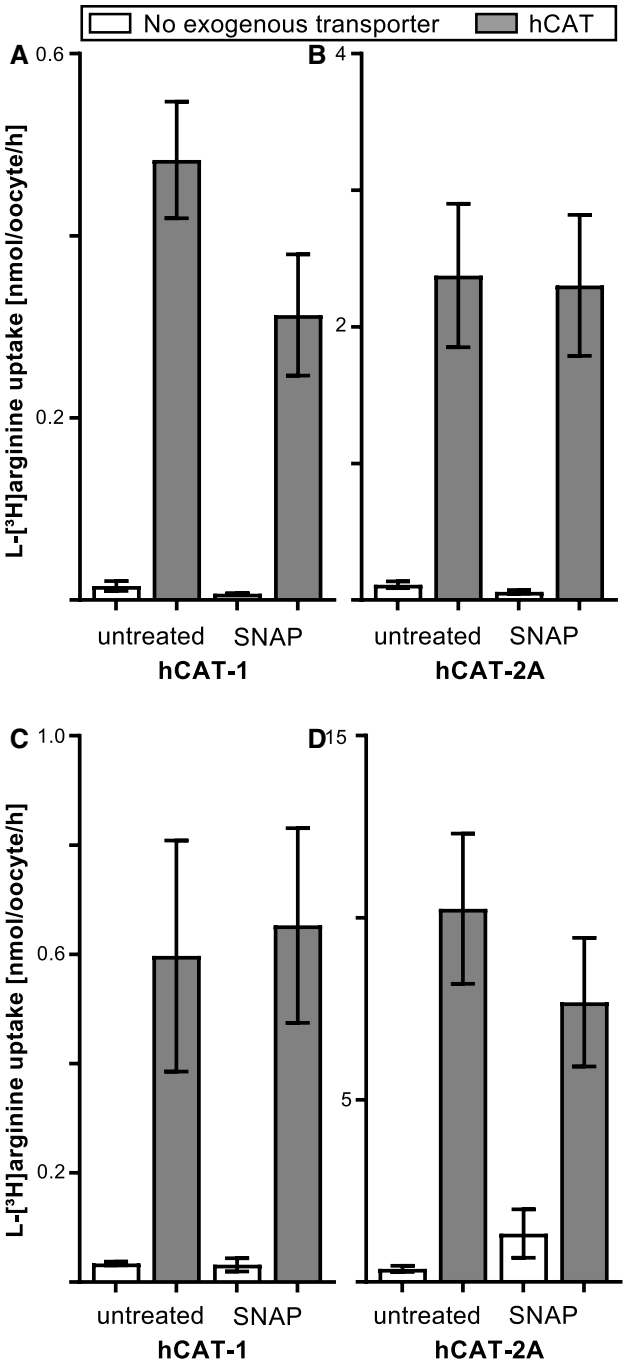

Fig. 1 hCAT-mediated transport is not altered by exposure to NO. *X. leavis* oocytes expressing hCAT-1 or hCAT-2A as indicated (dark columns) or no exogenous transporter (open columns) were exposed to 100 µM *S*-Nitroso-*N*-acetylpenicillamine (SNAP) for 10 min in ND96 buffer or left untreated, followed by incubation in the same solution, respectively, but containing L-[^{3}H]arginine (100 µM for hCAT-1 and 1 mM for hCAT-2A) for 15 min at an incubation temperature of 20 °C or for 1 or 3 min, respectively, at an incubation temperature of 37 °C. Incubation temperature was 20 °C (**a**, **b**) and 37 °C (**c**, **d**), respectively, for both, the 10 min preincubtion and the time of L-[^{3}H]arginine uptake. After intensive washing, the radioactivity per each oocyte was determined and the amount of absorbed L-[^{3}H]arginine was calculated in nmol per hour. Columns represent means ± SD (n = 4–6 from one representative oocyte batch)

half maximal relaxation was achieved at a range of 60 nM SNAP (data not shown).

Because CATs are mammalian proteins operating at 37 °C under physiologic conditions, we repeated our oocyte experiments at an incubation temperature of 37 °C. However, even under this elevated temperature, we did not observe an effect of 100 µM SNAP on either hCAT-1 (Fig. 1c, $p = 0.853$, $n = 13–16$) or hCAT-2A (Fig. 1d, $p = 0.079$, $n = 13–16$).

From these experiments, we conclude that NO has no direct effect on hCAT-mediated transport. Zhou et al. (2010) observed inhibition of L-arginine transport by NO generated endogenously in cardiomyocytes, indicating that NO concentrations lower than 1 µM (Nakamura and Lipton 2016) are sufficient for transport inhibition. The amount of NO initially released by 100 µM SNAP is about 4 µM (Zhou et al. 2010). The lack of hCAT inhibition under exposure to 100 µM SNAP in the *X. leavis* oocytes, thus indicates that CATs do not possess thiyl radicals that can be directly nitrosylated by NO.

Alternatively, SNO formation can occur via nitrosation, e. g. reaction of thiol with NO^+ or rather NO^+ donors (Heinrich et al. 2013) that may have been built from NO in rat cardiomyocytes, but not in *X. leavis* oocytes. To test directly if CATs are inhibited by reaction with NO^+ donors, we adapted the protocol developed by Daiber and co-authors (Daiber et al. 2009) who observed maximal SNO formation under a flux of 3NO and $1O_2^-$. Such a stoichiometric release of 3NO and $1O_2^-$ can be achieved by equimolar amounts of Sin-1 (that releases NO and O_2^- at equal rates) and SPENO (that generates two molecules of NO at the same rate) and leads to SNO formation independent of any cellular components. Exposure of *X. leavis* oocytes expressing either hCAT-1 or hCAT-2A, to 1 mM each, Sin-1 and SPENO, did not alter their transport activity (Fig. 2, $p = 0.6271$ for hCAT-1 and $p = 0.6951$ for hCAT-2A).

Under 1 mM Sin-1/SPENO, about 10% of the GSH was transformed into GSNO (Supplemental Fig. 1), demonstrating that significant nitrosation had occurred. In addition, this GSNO concentration should be sufficient for trans-nitrosating susceptible proteins (Paige et al. 2008). These experiments demonstrate that CATs are either not directly nitrosated (or trans-nitrosated by GSNO) or SNO formation in CATs does not affect their function.

There are three alternative explanations for the discrepancy between the NO effect on rat and human CATs in cardiomyocytes and *X. leavis* oocytes, respectively: (i) Rat and human CAT-1 and CAT-2A may differ in their sensibility to NO/NO^+. A comparison of the amino acid sequences of rat and human CAT-1 and -2 proteins shows indeed one exclusive cysteine residue for rCAT-1 (position 202) and four exclusive cysteine residues for rCAT-2 (positions 438, 454, 620, 657). If they were responsible for the observed difference, this would imply that a

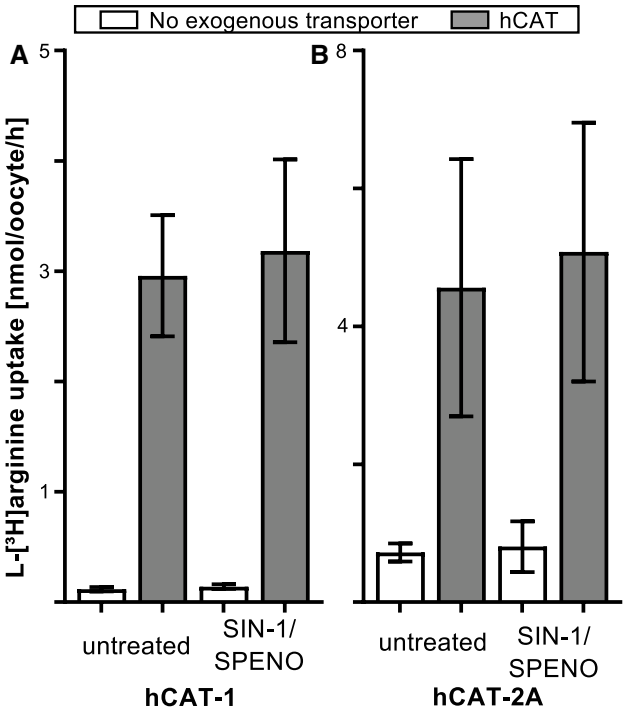

Fig. 2 hCAT-mediated transport is not altered by exposure to N_2O_3. *X. leavis* oocytes expressing hCAT-1 (A) or hCAT-2A (B) were exposed to 5-Amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride (Sin-1) and spermine-NONOate (SPENO), 1 mM each, for 10 min in ND96 buffer or left untreated, followed by incubation in the same solution, respectively, but containing L-[^{3}H]arginine (100 µM for hCAT-1 and 1 mM for hCAT-2A) for 1 or 3 min, respectively. Incubation temperature was 37 °C throughout. After intensive washing, the radioactivity per each oocyte was determined and the amount of absorbed L-[^{3}H]arginine was calculated in nmol per hour. Columns represent means ± SD ($n = 6$ from one representative oocyte batch).

modulation of the transport activity would be mediated by nitros(yl)ation of completely different cysteine residues in rCAT-1 and -2. This seems rather unlikely. (ii) NO may not act directly on CATs, but rather on interacting proteins. An indirect inhibitory effect on hCAT-mediated transport has also been demonstrated for protein kinase C (PKC) (Rotmann et al. 2004). However, PKC inhibits hCATs in a variety of mammalian cells (Rotmann et al. 2007) as well as in *X. leavis* oocytes (Graf et al. 2001), indicating that the interacting protein responsible for PKC inhibition is rather ubiquitous. This may be different for an interacting component responsible for NO/NO^+-mediated CAT inhibition. (iii) Nitros(yl)ation of CATs may require a multiplex enzymatic machinery as recently reported in *E. coli*, where the presence of the hybrid cluster protein HCP is necessary for efficient SNO formation in a variety of proteins (Seth et al. 2018). In either case, our study implies that a membrane component (most likely an interacting protein) responsible for NO/NO^+-mediated CAT inhibition would be present in membrane vesicles derived from cardiomyocytes, but not in *X. leavis* oocytes. Further studies are required to identify such an interacting protein.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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