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Rate of transport of L-arginine is independent of the expression of inducible nitric oxide synthase in HEK 293 cells

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10 **Abstract**

11 Expression of inducible nitric oxide synthase (iNOS) is generally accompanied by a parallel upregulation in L-arginine transport
12 which is dependent, at least in part, on the synthesis of new carrier proteins. It is not clear however whether the induction of iNOS
13 and its subsequent utilisation of L-arginine for NO synthesis contribute to the enhancement in L-arginine transport rates observed
14 following induction of cells with pro-inflammatory mediators. To address this issue, we have transfected an iNOS construct in a
15 pEGFP-N1 vector into HEK-293 cells and investigated the effects this has on L-arginine transport. The expression of iNOS through
16 transfection resulted in the production of significant quantities of NO as detected by the standard Griess assay. Under these condi-
17 tions, the transport of L-arginine was found to be unaltered, with rate of uptake being comparable in both transfected and non-trans-
18 fected cells. Characterisation of the transporter(s) involved with uptake of L-arginine revealed features characteristic of the classical
19 cationic amino acid transport system y^+. Further analysis of the expression profile of the cationic amino acid transporter (CAT)
20 involved revealed the presence of transcripts for CAT-1 and CAT-2B. These data demonstrate that iNOS activity does not drive or
21 enhance L-arginine transport despite the fact that HEK-293 cells transport L-arginine via the CATs, including CAT-2B which is
22 thought to be critical for supply of substrate to iNOS.
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24 *Keywords:* L-Arginine transport; Cationic amino acid transporters; Nitric oxide; Inducible nitric oxide synthase; HEK293 cells

25 Expression of inducible nitric oxide synthase (iNOS)
26 in various cells and tissues is associated with the over-
27 production of nitric oxide (NO) which although critical
28 for immune defences also functions as an important
29 mediator of inflammatory responses with detrimental
30 consequences. For instance, iNOS produced NO is
31 believed to be responsible for the systemic hypotension
32 seen in septic shock [1–3] and has been implicated in the
33 pathogenesis of several other inflammatory diseases,
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including asthma [4] and inflammatory bowel disease [5]. 38
Once expressed, synthesis of NO by iNOS is critically 39
dependent on the availability and transport of L-arginine 40
[6–10]. The induction of iNOS by pro-inflammatory 41
mediators is accompanied by a parallel upregulation in 42
L-arginine transport [7,11–13] which is due at least in 43
part to enhanced expression of transcripts for cationic 44
amino acid transporters (CATs), including CAT-1, 2B, 45
and potentially 2A [14,15]. This parallel upregulation in 46
CAT expression and activity provides a mechanism for 47
sustaining substrate supply during enhanced synthesis of 48
NO by iNOS. It is not known, however, whether the 49
expression of iNOS and the subsequent enhanced utilisa- 50

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tion of L-arginine by this enzyme directly regulate CAT expression and function. One report in the literature has indicated that inhibition of iNOS activity using either 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), a potent and selective inhibitor of iNOS [16] or *S*-ethylisothiourea, a potent but non-selective NOS inhibitor [17], abolished LPS-induced L-arginine transport [18]. The same group has also demonstrated that expression of both iNOS and CAT-2B mRNA may be downregulated after prolonged (20 h) exposure of rat alveolar macrophages to dexamethasone. These effects were accompanied by corresponding decreases in both LPS-stimulated nitrite production and L-[^{3}H]arginine transport, suggesting a parallel regulation of the expression and function of iNOS and CAT-2B, at least in rat alveolar macrophages [19]. These finding are, however, at odds with previous studies in the murine macrophage J774 cell line [20] and in rat cultured aortic smooth muscle cells [13,21] in which dexamethasone selectively blocked iNOS expression whilst having no effect on induced L-arginine transport, suggesting that upregulation of transport may be independent of iNOS activity. These studies did not however take into account the fact that other pathways which utilise L-arginine may also be switched on in activated cells, thus masking any direct coupling between utilisation of L-arginine by iNOS and the subsequent regulation of transport that may occur as a consequence of the activity of this enzyme.

To determine unequivocally whether CAT function is influenced by the activity of iNOS we therefore deliberately chose to use an experimental system in which iNOS could be expressed and was fully active in cells in the absence of pro-inflammatory stimuli. Transient iNOS expression was therefore induced by transfection in human embryonic kidney-293 (HEK-293 cells). These cells can be easily transfected but do not express endogenous iNOS or show any upregulation in CAT activity following exposure to the standard cocktail of bacterial lipopolysaccharide (LPS) and cytokines generally used to induce these pathways in other cells (unpublished observations). In parallel studies, the characteristics of L-arginine transport have been fully investigated and the nature of the transporters involved with L-arginine uptake established. These studies are essential since very little is currently known about the mechanism of entry of L-arginine into HEK293 cells. Moreover, the transporters expressed may potentially determine the manner in which NO synthesis is regulated in these cells.

Experimental procedure

Materials

Tissue culture reagents were obtained from Invitrogen (Paisley, UK). Other reagents used were: SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Cowley, UK); Eukaryotic TOPO TA Cloning Kit and competent *Escherichia coli* (Invitrogen, Paisley, UK); PCR primers (MWG Biotech, Milton Keynes, UK); Restriction enzymes and T4 DNA ligase (Promega, Southampton, UK); *Taq* DNA Polymerase, M-MLV reverse Transcriptase, and dNTP (Invitrogen, Paisley, UK); Plasmid miniprep and maxiprep kits (Qiagen, Crawley, UK); anti-iNOS and anti-GFP monoclonal antibodies (BD Biosciences Clontech, Cowley, UK). L-[2,3-^{3}H]arginine (36.1 Ci/mmol) was obtained from New England Nuclear (Dreieich, Germany). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Perbio Science UK, Cheshire, UK). GW274150 was a gift from GlaxoSmithKline (Stevenage, UK). All other chemicals were obtained from Sigma or BDH and were of the highest analytical grade obtainable.

Cell culture

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and 10% foetal bovine serum. Cells were harvested by trypsinisation and passaged every 5 days by dilution of a suspension of the cells 1:4 in fresh medium.

RNA isolation

Total cellular RNA was isolated from the cultured cells using RNA STAT-60 (Biogenesis, Poole, UK) according to the manufacturer's protocol. The concentration of RNA solutions was determined spectrophotometrically and the quality verified by visualisation of 28S and 18S rRNA bands after electrophoresis through a 1% ethidium bromide stained agarose gel.

Construction of iNOS-pEGFP-N1 Plasmid

Total RNA was extracted from rat (Wistar) cultured aortic smooth muscle cells activated with bacterial lipopolysaccharide (LPS; 100 μg ml^{-1}) and interferon-γ (IFN-γ; 50 U ml^{-1}) for 24 h. The first strand cDNA was synthesised from 1 μg total RNA using the Clontech SMART system (BD Biosciences Clontech, Cowley, UK) and an iNOS gene-specific reverse primer (5′-GAG TCT TGT GCC TTT GGG CT-3′) based on a published rat iNOS cDNA sequence (Accession No.: X76881). The cDNA products were subsequently amplified by PCR using the SMART RACE cDNA Amplification Kit together with the above reverse primer, lacking the nucleotide sequence for the iNOS stop codon. To facilitate translation initiation and enhance iNOS-GFP expression levels, a modified iNOS forward primer was designed with a Kozak consensus sequence

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introduced around the initiation codon of iNOS (5′-CAC CGC CAT GGC TTG CCC CTG GAA-3′). A 3.449 kb cDNA fragment amplified was ligated into pcDNA3.1 using the Eukaryotic TOPO TA Cloning Kit. After transformation into *E. coli*, a mini-preparation of the plasmid was made and the purified cDNA sequenced using a Beckman Coulter CEQ2000XL Sequencer. The iNOS-GFP expression vector was subsequently constructed by digesting pcDNA 3.1 harbouring the iNOS cDNA with *Hin*dIII and *Sac*II, and then subcloning the restricted iNOS cDNA from pcDNA3.1 into the *Hin*dIII and *Sac*II sites of the pEGFP-N1 vector. The iNOS sequence was fused at its C terminal to GFP and was driven directly by the CMV promoter of the plasmid.

Transfection of HEK293 cells with iNOS-pEGFP-N1

Confluent monolayers of HEK-293 cells in T75 tissue culture flasks were trypsinised and plated into 24-well plates at a seeding density of 2×10^4 cells per well. Cells were allowed to grow to 60% confluency prior to transfecting with iNOS-pEGFP-N1 using an α5β1 integrin-binding polycationic peptide ($[K]_{16}$GACRRETAWA CG; Peptide 6) [22]. For optimum transfection efficiency, conditions previously described [22] were adhered to. Briefly, culture medium was removed from wells and the adherent cells washed twice with PBS. 0.5 ml of transfection complex in Opti-MEM I medium containing lipofectin (0.75 µl), plasmid (1 µg), and peptide 6 (4 µg) was then added to each well. Cells were incubated for a further 3 h at 37 °C, after which the medium was removed, replace with normal culture medium containing 10% FCS and incubated in a tissue culture incubator at 37 °C for 6–48 h before determining accumulated nitrite levels in the culture medium as described below. In parallel experiments cells were also incubated with the potent and highly selective iNOS inhibitor GW274150 (S-[2-[(1-iminoethyl)amino]ethyl]-L-homocysteine) to inhibit NO production [23]. In control experiments cells were transfected with either empty vector or pEGF-N1 vector alone. Transfected cells were routinely visualised using a Nikon EFD 3 Labophot-2 fluorescence microscope and the transfection efficiency determined from the proportion of green fluorescing cells. The estimated transfection efficiency generally obtained with the above protocol was between 60 and 70%. Batches of cells showing <50% transfection were discarded. There was no detectable fluorescence in cells transfected with the empty non-GFP vector alone.

Western blot analysis of iNOS expression

Western blotting was carried out as described previously [24]. Cell lysates (20 µg protein per lane) were separated by SDS–PAGE electrophoresis, transferred onto PVDF membrane (Sigma–Aldrich, Poole, UK) and blocked for 2 h in 100 mM NaCl, 10 mM Tris, 0.1% (v/v) Tween 20, pH 7.4 (STT), containing 5% (w/v) non-fat milk. Membranes were then incubated overnight with either a monoclonal anti-iNOS (1:2500 dilution) or anti-GFP (1:1000 dilution) antibody in STT containing 5% (w/v) non-fat milk). Blots were washed with STT (6×5 min) and incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody for 1 h. Following further washing (6×5 min) in STT, immunoreactive bands were visualised using ECL detection System (Amersham–Pharmacia, Buckinghamshire, UK).

Measurement of nitrite formation

Measurement of nitrite production as an assay of NO release was carried out as described previously [24] using the Griess reaction with sodium nitrite as standard [25].

Measurement of L-arginine transport

Unidirectional transport of L-arginine was measured in cell monolayers as described previously [20]. Cells were rinsed twice with a modified Hepes-buffered Krebs solution (composition (mM): NaCl, 131; KCl, 5.5; $MgCl_2$, 1; $CaCl_2$, 2.5; $NaHCO_3$, 25; NaH_2PO_4, 1; D-glucose, 5.5; Hepes, 20; pH 7.4) at 37 °C. Uptake was initiated by adding 50 µl of Krebs containing either 100 µM or increasing concentrations (10–1000 µM) of L-[^{3}H]arginine (2 µCi ml^{-1}) to each well and influx measured over 1 min. The effects of pH on transport were examined in Krebs solution with pH values ranging between pH 6 and 7.4. In parallel experiments, the sodium-dependency of transport was examined in modified buffer in which NaCl, $NaHCO_3$, and NaH_2PO_4 were replaced with choline chloride, choline bicarbonate, and KH_2PO_4, respectively. Uptake of L-[^{3}H]arginine was terminated by placing plates on ice and rinsing cells twice with 200 µl ice-cold Krebs containing 10 mM unlabelled L-arginine. Cell protein was determined using bicinchoninic acid (BCA) protein assay reagent following the manufacturer's protocol. Radioactivity (dpm) in cell lysates was measured by liquid scintillation counting. Transport was expressed in pmol µg protein^{-1} min^{-1}.

Profile of CAT expression in HEK-293 cells

The identification of the CATs expressed in HEK-293 cells was determined by RT-PCR. The first-strand cDNA was synthesised from 2 µg total RNA using 0.5 µl (250 ng) random hexamer primers, 1 µl M-MLV reverse transcriptase (200 U/µl), 1 µl dNTP (10 mM), 4 µl of 5× first-strand buffer, 2 µl dithiothreitol (0.1 M), 1 µl RNase-OUT (40 U/µl), and 9 µl sterile distilled water. The reaction was allowed to proceed according to the supplier's recommendation (Invitrogen, Paisley, UK) for M-MLV

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reverse transcreptase. The cDNA product was amplified by PCR using CAT-specific primers designed from Genbank sequences for human CAT-1 (Accession No.: NM_003045), CAT-2 A (Accession No.: U76368), and CAT-2B (Accession No.: D29990). The following primer pairs were used: hCAT-1 (656 bp), 5′-TCG GCC ATG GTC AAC AAA AT-3′ and 5′-TCA AAG AGG AAG GCC ATC ACA-3′; hCAT-2A (599 bp), 5′-GAG TCT GCT TGG GTG AAT AAA-3′ and 5′-GAC TGC CTC TTA CTC ACT CT-3′; and hCAT-2B (642 bp), 5′-GAG TCT GCT TGG GTG AAT AAA-3′ and 5′-AGC TGC CAC TGC ACC CGA TGA-3′. Amplification was carried out in a Techne touchgene programmable thermal cycler (Techne, Cambridge, UK) using *Taq* DNA polymerase (0.5 U). The reaction mixture (0.5 μl of a 10 μM stock of each primer, 0.5 μl of dNTP (10 mM), 2.5 μl of 10× PCR buffer, 0.75 μl of 50 mM $MgCl_2$, and 19.75 μl sterile distilled water) was initially heated to 94 °C for 2 min, followed by 33 cycles under the following conditions: denaturing at 94 °C for 30 s, annealing at 55 °C for 40 s, extension at 72 °C for 40 s, and a final extension step at 72 °C for 5 min. Ten microliters of the PCR product was used for DNA electrophoresis on a 1% agarose gel, stained with ethidium bromide, and documented using a Bio-Rad imager system.

Statistics

All values are means ± SE of measurements in at least three different cell cultures with two replicates per experiment. ANOVA was used to determine statistical significance between paired values with the overall confidence levels set at 95% (0.05).

Results

Cloning, sequence, and analysis of iNOS cDNA

A 3.449 kb cDNA fragment was amplified by PCR as described in methods using iNOS gene specific primers and the sequence obtained submitted to GenBank (Accession No.: AY211532). The cDNA produced encodes the full-length iNOS protein when inserted into the multiple cloning site of the pEGP-N1 plasmid and driven by the CMV promoter of the plasmid. The first six bases at the 5′ end of the cDNA produced were not part of the iNOS sequence. Instead, these six bases constituted the Kozak consensus translation initiation site for iNOS. The remaining sequence represents that for iNOS. Sequence comparison revealed our cloned iNOS cDNA to be highly homologous (>99.5%) with two other published rat iNOS sequences, showing 17 single-base mismatches to that published by Nunokawa et al. [26, Accession No.: D14051], and 13 single-base mismatches to the sequence published by Geng et al. [27, Accession No.: X76881]. These small differences occur as sporadically placed amino acid substitutions (Fig. 1) with no insertion or deletion mutations being apparent between the clones. Our cDNA also shows a high level of homology at the cDNA level to the inducible enzyme from human [28] and mouse [29]. It contains a single open reading frame beginning with an AUG methionine at codon 1 and encodes a protein of 1147 residues with a calculated molecular mass of 130.67 kDa. It also contains the predicted consensus recognition sites for the various cofactors including FMN (at amino acids: 620–639), FAD-PPi (at amino acids: 764–777), FAD-ISO (at amino acids: 900–910), NADPH–Ribose (at amino acids: 973–992), and NADPH-Ade (at amino acids: 1074–1088) in the C-terminal and for calmoduline (at amino acids 502–527) in the mid portion. Following sequencing and analysis, the fragment produced was subsequently subcloned into the *Hin*dIII and *Sac*II sites of the pEGFP-N1 vector and used in expression studies.

Efficiency of transfection of iNOS using peptide 6

Transfection of HEK-293 cells with iNOS-pEGFP-N1 using peptide 6 under optimised conditions reported previously [22] resulted in detectable expression of GFP in 60–70% of cells. As shown in Fig. 2, visualisation of transfected cells by fluorescence microscopy confirms significant expression of GFP at 6 h (Fig. 2A) with levels increasing over time, reaching a peak at 24–48 h (Fig. 2D) but declining by 72 h due to marked decreases in cell viability (data not shown).

Western blot analysis of lysates carried out in parallel reflects a time-dependent expression of GFP which as shown in Fig. 3A, was detectable after 6 h, peaking at 24 h. Expression of iNOS was evident after 6 h and was maximal at 12 h remaining at this level over the 48 h incubation period (Fig. 3B).

Amino acid: Position	72	348	349	380	591	679	680	714	721	722	740	844	974	999	1084	1133	1138
Clone 1:	H	A	V	L	G	E	T	P	L	S	L	A	R	R	M	V	A
Clone 2:	Y	P	V	F	V	V	P	S	L	R	L	G	C	H	M	A	T
Clone 3:	H	A	A	F	V	E	T	S	P	R	P	A	C	H	I	A	T

Fig. 1. Sequence analysis of rat iNOS highlighting random amino acid substitutions between our clone (clone 1, Accession No.: AY211532) and that published by Nunokawa et al. (clone 2; Accession No.: D14051), and by Geng et al. (clone 3; Accession No.: X76881).

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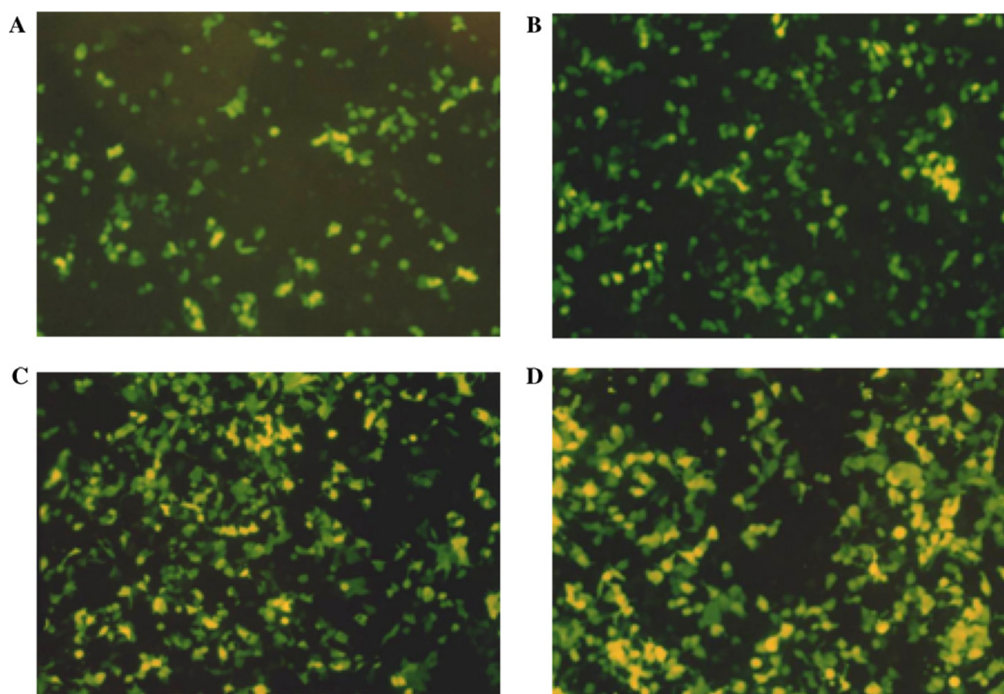

Fig. 2. Expression of iNOS-EGFP in HEK293 cells. Cells were transfected with pEGFP-N1 harbouring the iNOS cDNA construct as described in Experimental procedure. Cells were visualised at 6 h (A), 12 h (B), 24 h (C), and 48 h (D) after transfection using a Nikon EFD3, LABOPHOT-2 fluorescence microscope. These pictures are representative of observations made in at least three different experiments.

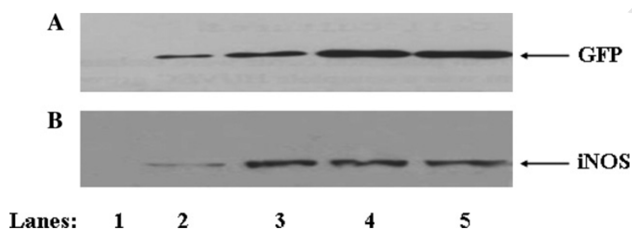

Fig. 3. Time course of expression of GFP (A) and iNOS (B) in transfected HEK-293 cells. Lysates were obtained from control cells (lane 1) and at 6 h (lane 2), 12 h (lane 3), 24 h (lane 4), and 48 h (lane 5) after transfection with iNOS-pEGFP-N1. Proteins were separated by SDS–PAGE electrophoresis and probed with either a monoclonal anti-GFP or anti-iNOS antibody as described in Experimental procedure. These blots are representative of three separate experiments using three different batches of cells.

Effects of iNOS expression on nitrite production

Transfection of cells with iNOS-pEGFP-N1, but not with empty or pEGFP-N1 vector, resulted in the expression of iNOS (Fig. 3B) and a marked increase in NO production which was reflected in a time-dependent increase in accumulated nitrite levels in the culture medium (Fig. 4A). Moreover the maximum amount of NO produced (56 ± 0.03 pmol μg protein^{-1} nitrite at 48 h) was well within the range detected following induction of iNOS in various cells systems using inflammatory mediators such as bacterial lipopolysaccharide and/or cytokines. To confirm that the detected nitrite is generated by iNOS, additional experiments were carried out in which transfected cells were co-incubated with the highly selective iNOS inhibitor GW274150. As shown in Fig. 5A, accumulated nitrite production was abolished by 10 μM GW274150, confirming iNOS as the source of the NO produced.

Effects of iNOS expression on L-arginine transport

HEK-293 cells transported L-[^{3}H]arginine in a time-dependent manner which was linear over 5 min in both transfected and non-transfected cells (data not shown). As a result all subsequent uptake studies were carried out over 1 min. The rate of uptake in 48 h controls was 1.91 ± 0.03 pmol μg protein^{-1} min^{-1} and there was no significant change in transporter activity in NO-generating transfected cells expressing iNOS (Fig. 4B). Inhibition of NO synthesis with GW274150 did not cause any significant change in transport (Fig. 5B). Transfection of cells with empty (data not shown) or pEGFP-N1 vector alone did not alter L-arginine transport (Fig. 4B).

Profile of CAT expression and activity in HEK-293 cells

Total RNA isolated from HEK-293 cells was probed by RT-PCR for CAT-1, CAT-2A, and CAT2B using isoform specific primers. The data obtained show that HEK-293 cells express the high affinity CAT 1 and CAT 2B but not the low affinity CAT 2 A (Fig. 6).

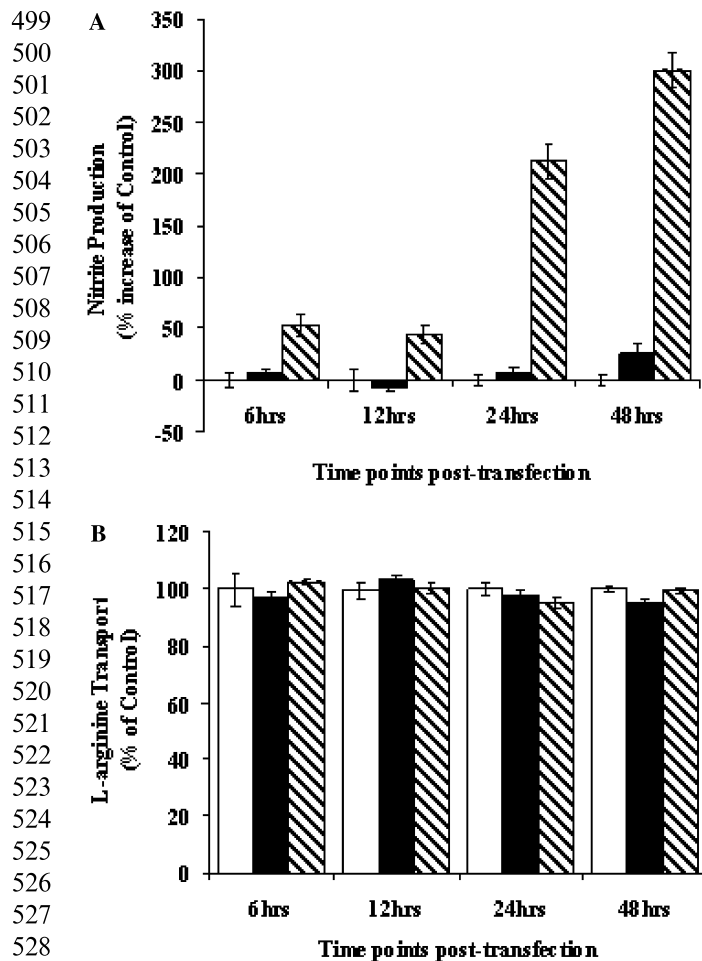

Fig. 4. Effects of iNOS-pEGFP-N1 transfection on NO production (A) and L-arginine transport (B) in HEK-293 cells. Nitrite and transport were measured at 6–48 h after transfection. Open bars represent control non-transfected cells, solid black bars represent cells transfected with a GFP only vector and hatched bars represent cells transfected with iNOS-pEGFP-N1. Results represent means ± SEM of four separate experiments with six replicates in each.

In kinetic studies, entry of L-arginine was monitored over the concentration range 5–1000 μM. Transport was found to be saturable (Fig. 7) with a K_m of 0.21 mM and V_{max} of 5.3 nmol μg protein^{-1} min^{-1} and occurred via a single carrier system as evident from the Eadie–Hofstee plot in Fig. 7 (inset).

In competition experiments the substrate specificity of L-arginine transport was examined by screening the inhibitory effects of a series of 1 mM unlabelled amino acids on uptake of 0.1 mM L-[^{3}H]arginine. As shown in Table 1, transport of L-arginine was unaffected by selective substrates for system A (preferred substrate: 2-methylaminoisobutyric acid (MeAIB; [30]), ACS (preferred substrate: L-serine, L-alanine [31]), L (preferred substrate: 2-aminobicyclo-(2,2,1)heptane-2-carboxylic acid (BCH, [32]), and N (preferred substrate: 6-diazo-5-oxo-L-norleucine (DON, [33]) but significantly inhibited by other cationic amino acids, L-lysine and L-ornithine (Table 1). In addition, transport was found to be unaffected by changes in extracellular Na^+ or pH ranging from 6 to 7.4 (Table 2).

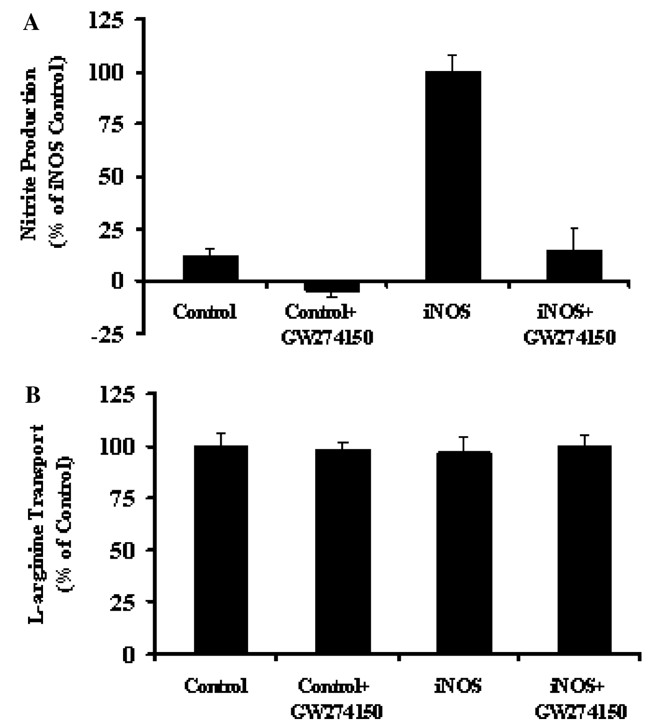

Fig. 5. Effect of GW274150 on NO production (A) and L-arginine transport (B) in control and iNOS-pEGFP-N1 transfected HEK-293 cells. Nitrite and transport were measured 48 h after transfection. Results represent means ± SEM of three separate experiments with two replicates in each.

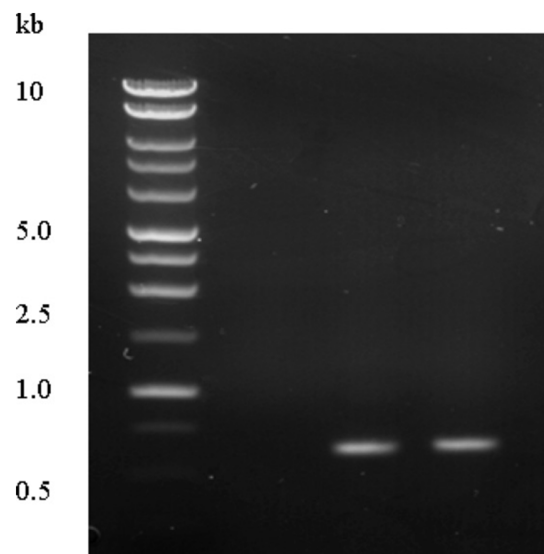

Fig. 6. CAT expression in HEK-293 cells. Total RNA was isolated from control, non-activated cells and RT-PCR performed (33 cycles) using primer pairs specific for CAT-1, CAT-2A, and CAT-2B as outlined in Experimental procedure. Lanes represent: 1 = ladder, 2 = CAT-2A, 3 = CAT-2B, and 4 = CAT-1.

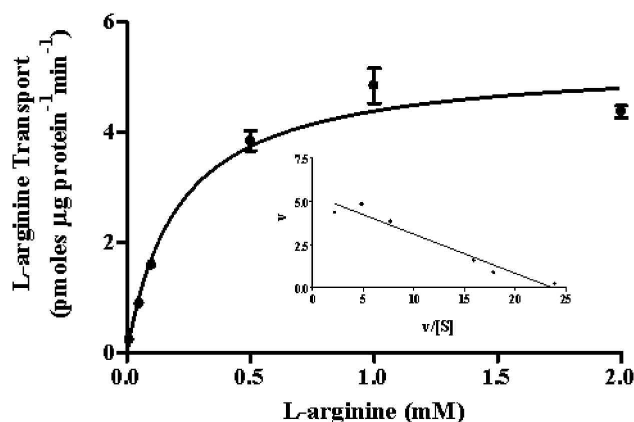

Fig. 7. Kinetics of L-arginine transport in HEK-293 cells. Rates of L-arginine transport (0.005–2 mM) were measured in control cells. The inset shows an Eadie–Hofstee plot of saturable transport, where V is the initial velocity (pmol µg protein^{-1} min^{-1}) and S is the substrate concentration (mM). Values are means ± SEM of three different experiments with six replicates in each.

Table 1
Selectivity of L-arginine transport in control HEK-293 cells

Condition	Transport rate (pmol µg protein^{-1} min^{-1})
Control	2.09 ± 0.10
System A/ASC substrates	
MeAIB	2.56 ± 0.14
Alanine	2.57 ± 0.12
Serine	2.33 ± 0.11
Proline	2.03 ± 0.09
System N substrates	
DON	1.74 ± 0.08
L-Histidine	2.06 ± 0.06
System L substrates	
BCH	1.96 ± 0.13
L-Phenylalanine	2.36 ± 0.10
L-Leucine	1.85 ± 0.13
System y^+ substrates	
L-Arginine	0.61 ± 0.05*
L-Lysine	0.91 ± 0.05*
L-Ornithine	0.97 ± 0.09*

Transport of 100 µM L-arginine was measured over 1 min in the absence or presence of a 10-fold excess (1 mM) of a given inhibitor. Data are expressed as a percentage of the respective control influx in controls. Values are means ± SE of three different experiments with six replicates in each.

* $P < 0.01$ relative to control.

Discussion

Production of NO by iNOS in several cell systems depends critically on the availability and transport of L-arginine. More importantly, the expression of iNOS under these conditions is accompanied by a parallel upregulation in L-arginine transporter activity. This widely reported phenomenon [7,12,13,34–36] provides a mechanism for sustaining substrate supply during enhanced synthesis of NO, and is thus a key target for regulating NO synthesis by iNOS. Previous work has, however, not determined whether the increase in transporter activity is a direct consequence of increased iNOS activity resulting in enhanced substrate uptake into the cells.

To resolve this question we have examined whether transiently expressing iNOS in non-induced cells can regulate the rate of L-arginine transport as a consequence of the increased utilisation of the amino acid for NO synthesis. These studies were carried out in HEK-293 cells transfected with an iNOS construct in pEGFP-N1 vector using a synthetic peptide comprising of an integrin-targeting ($\alpha_5\beta_1$ integrin selective) arginine-glycine-aspartic acid tripeptide motif and a DNA-binding moiety of 16 lysine residues [22]. This novel transfection strategy resulted in consistently high transfection efficiency where ⩾50% of cells appeared transfected. More importantly, the levels of iNOS protein expressed and the amounts of NO produced were comparable to those detected in various cell systems following induction with pro-inflammatory mediators [7,10,13,35,36]. This finding validates the use of peptide 6 as an effective nonviral system for iNOS gene transfer in HEK-293 cells.

However, despite the prominent expression of iNOS and marked production of NO achieved, parallel transport studies failed to show any modification of L-arginine transport: the transport rate in transfected cells was not significantly different when compared to rates determined in control non-transfected cells. This observation demonstrates that the expression of iNOS alone is not sufficient to upregulate L-arginine uptake into cells. In this regard, we and others have demonstrated that increases in L-arginine transport are sensitive to inhibition by cycloheximide and associated with a marked increase in the expression of transcripts for CATs, indicating a critical requirement for protein synthesis of the L-arginine transporter itself [7,14,15,20,21].

Table 2
Effects of Na^+ and pH on L-arginine transport in HEK-293 cells

Condition	Transport rate (pmol µg protein^{-1} min^{-1})
Na^+-dependency	
Control	
+Na^+	1.8 ± 0.11
−Na^+	1.6 ± 0.10
pH-dependency	
7.4 (control)	1.85 ± 0.08
7.0	1.89 ± 0.11
6.5	1.86 ± 0.06
6.0	1.82 ± 0.12

Transport of 100 µM L-arginine was measured over 1 min in the presence (normal Krebs solution) or absence of Na^+ and at varying pH values ranging from pH 7.4 to pH 6). Data are expressed as a percentage of the respective control influx in controls. Values are means ± SE of three different experiments with six replicates in each.

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Our results therefore provide the first direct evidence that expression of active iNOS is by itself insufficient to upregulate CAT function and hence arginine transport. This conclusion is apparently in conflict with a recent report by Hammermann et al. [18], which suggests that direct inhibition of iNOS activity in rat alveolar macrophages is sufficient to abolish LPS-activated L-arginine uptake. The inhibitor of iNOS primarily used in those experiments (AMT) did not inhibit basal arginine influx in control cells but inhibited enhanced arginine uptake in LPS-pretreated cells even with brief pre-incubations. The authors therefore concluded that this was an indirect effect due to reduced arginine utilisation by iNOS. This association is however questionable since L-arginine transport: (i) occurs by facilitated diffusion driven by the membrane potential, (ii) accumulates L-arginine against a concentration gradient, and (iii) is sensitive to trans-stimulation when substrate concentrations are high on the opposite side of the cell membrane [37,38]. Thus, if inhibition of L-arginine utilisation by iNOS results in elevated intracellular levels of this amino acid, entry of radiolabelled L-arginine would be enhanced via trans-stimulation causing an increase in L-[^{3}H]arginine uptake. As we observed no significant changes in accumulated L-[^{3}H]arginine in cells transfected with our iNOS construct, we conclude that activation of iNOS itself does not influence rates of L-arginine transport. It could be argued that in the system used by Hammermann et al. [18], activation of cells may result in the co-induction of linker proteins which may facilitate compartmentalised regulation of L-arginine transport and NO synthesis. This, however, still remains to be established and the coupling of L-arginine transport to NO synthesis may not be a widespread phenomenon in all activated cell types [13,20,21].

Differences in the profile of expression of L-arginine transporter systems may explain the discrepancy between our study and those of Hammermann et al. [18]. This latter point is important since there are suggestions in the literature that CATs may be linked to different NOS enzymes, thereby providing a direct supply of substrate for that isoform. In this regard, CAT-1 is thought to supply substrate to endothelial NOS by virtue of its co-localisation with this enzyme [39]. CAT-2B on the other hand may regulate supply of L-arginine to iNOS as these proteins are co-induced in cells where neither is constitutively expressed. Moreover, in such systems, the induction of CAT-2B can be accompanied by a parallel downregulation in CAT-1 mRNA [14], thereby making CAT-2B the critical transporter in these cells.

Since the nature of the transporters associated with L-arginine uptake had not previously been characterised in HEK-293 cells, we have therefore examined the characteristics of L-arginine transport and the profile of expression of CATs in these cells. Kinetic studies and Eadie–Hofstee analysis show that entry of L-arginine occurs predominantly via a single saturable carrier that is of relatively low capacity but of high affinity for L-arginine. Transport was selectively inhibited by the cationic amino acids L-lysine, L-ornithine, and by L-arginine itself. Substrates for amino acid transport systems A/ASC, N, and L were without significant effect. This inhibition profile together with the Na^+ and pH insensitivity strongly implicate the CATs as the predominant carrier of L-arginine in HEK-293 cells and excludes other systems such as y^+L, $b^{o,+}$, and $B^{o,+}$ which are broad scope in nature, transporting both cationic and neutral amino acids [38,40].

To identify the CAT or CATs expressed in HEK-293 cells we carried out RT-PCR of total RNA isolated from these cells using CAT-1, CAT-2A, and CAT-2B-specific primers respectively. CAT-3 was not investigated since this transporter is thought to be expressed predominantly in brain [41,42] and thymus tissue [43] and also interacts with neutral amino acids [41]. The data obtained show that HEK-293 cells express transcripts for CAT-1 and CAT-2B but not for CAT-2A. This expression profile agrees with the functional studies in that the kinetics of uptake reflects a high affinity system or systems with indistinguishable characteristics at the functional level. Although, CAT-2A and CAT-2B are spliced variants with high percentage structural homology they are functionally distinguishable by virtue of the fact that CAT-2A is a high capacity but low affinity carrier while CAT-2B exhibits similar functional characteristics to those of CAT-1 [44–46]. Both these carriers have a high affinity for L-arginine, transporting the latter with a Michaelis constant (K_m) of between 0.14 and 0.38 mM. The K_m of 0.21 mM determined in our study therefore supports CAT-1 and CAT-2B as being the predominant carriers of L-arginine into HEK-293 cells.

At present we cannot distinguish between CAT-1 and CAT-2B in terms of their relative contribution to total L-arginine uptake nor can we specify which CAT directly supplies L-arginine to transfected iNOS in our cell system. However, the detection of CAT-2B transcripts, generally believed to be expressed under inflammatory conditions in parallel with iNOS, suggests that this protein can exist endogenously in control conditions and may not require induction. More importantly, its detection excludes the possibility that iNOS was not able to alter transport in our studies because of lack of expression of the critical CAT (CAT-2B) associated with uptake of L-arginine for utilisation by this enzyme.

In conclusion, our current findings provide the first direct evidence that CATs function independently of iNOS and that the rate of transport of L-arginine into cells is not modulated by the activity of iNOS. Thus, the enhancement in L-arginine transport observed in systems where expression of iNOS and the subsequent generation of large quantities of NO are induced must occur via mechanisms not directly related to the rate of con-

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835 sumption of L-arginine or generation of NO by iNOS.
836 These mechanisms presumably involve activation by
837 external stimuli of common upstream signalling path-
838 ways leading to upregulation of iNOS and CAT expres-
839 sion [15].

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843 **References**

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