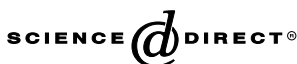


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# Rate of transport of L-arginine is independent of the expression of inducible nitric oxide synthase in HEK 293 cells

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## Abstract

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

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**Keywords:** L-Arginine transport; Cationic amino acid transporters; Nitric oxide; Inducible nitric oxide synthase; HEK293 cells

Expression of inducible nitric oxide synthase (iNOS) in various cells and tissues is associated with the over-production of nitric oxide (NO) which although critical for immune defences also functions as an important mediator of inflammatory responses with detrimental consequences. For instance, iNOS produced NO is believed to be responsible for the systemic hypotension seen in septic shock [1–3] and has been implicated in the pathogenesis of several other inflammatory diseases,

including asthma [4] and inflammatory bowel disease [5]. Once expressed, synthesis of NO by iNOS is critically dependent on the availability and transport of L-arginine [6–10]. The induction of iNOS by pro-inflammatory mediators is accompanied by a parallel upregulation in L-arginine transport [7,11–13] which is due at least in part to enhanced expression of transcripts for cationic amino acid transporters (CATs), including CAT-1, 2B, and potentially 2A [14,15]. This parallel upregulation in CAT expression and activity provides a mechanism for sustaining substrate supply during enhanced synthesis of NO by iNOS. It is not known, however, whether the expression of iNOS and the subsequent enhanced utilisation

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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-[<sup>3</sup>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 findings 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.

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## 101 Experimental procedure

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### 103 Materials

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105 Tissue culture reagents were obtained from Invitro-  
106 gen (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-<sup>3</sup>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.

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HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U ml<sup>-1</sup>), streptomycin (100 µg ml<sup>-1</sup>), 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.

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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.

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Total RNA was extracted from rat (Wistar) cultured aortic smooth muscle cells activated with bacterial lipopolysaccharide (LPS; 100 µg ml<sup>-1</sup>) and interferon-γ (IFN-γ; 50 U ml<sup>-1</sup>) 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 *Hind*III and *Sac*II, and then subcloning the restricted iNOS cDNA from pcDNA3.1 into the *Hind*III 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  $2 \times 10^4$  cells per well. Cells were allowed to grow to 60% confluency prior to transfecting with iNOS-pEGFP-N1 using an  $\alpha 5\beta 1$  integrin-binding polycationic peptide ([K]<sub>16</sub>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  $\mu$ l), plasmid (1  $\mu$ g), and peptide 6 (4  $\mu$ g) 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 pEGFP-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  $\mu$ g 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  $\times$  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  $\times$  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<sub>2</sub>, 1; CaCl<sub>2</sub>, 2.5; NaHCO<sub>3</sub>, 25; NaH<sub>2</sub>PO<sub>4</sub>, 1; D-glucose, 5.5; Hepes, 20; pH 7.4) at 37 °C. Uptake was initiated by adding 50  $\mu$ l of Krebs containing either 100  $\mu$ M or increasing concentrations (10–1000  $\mu$ M) of L-[<sup>3</sup>H]arginine (2  $\mu$ Ci ml<sup>-1</sup>) 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<sub>3</sub>, and NaH<sub>2</sub>PO<sub>4</sub> were replaced with choline chloride, choline bicarbonate, and KH<sub>2</sub>PO<sub>4</sub>, respectively. Uptake of L-[<sup>3</sup>H]arginine was terminated by placing plates on ice and rinsing cells twice with 200  $\mu$ l 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  $\mu$ g protein<sup>-1</sup> min<sup>-1</sup>.

#### 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  $\mu$ g total RNA using 0.5  $\mu$ l (250 ng) random hexamer primers, 1  $\mu$ l M-MLV reverse transcriptase (200 U/ $\mu$ l), 1  $\mu$ l dNTP (10 mM), 4  $\mu$ l of 5 $\times$  first-strand buffer, 2  $\mu$ l dithiothreitol (0.1 M), 1  $\mu$ l RNase-OUT (40 U/ $\mu$ l), and 9  $\mu$ l sterile distilled water. The reaction was allowed to proceed according to the supplier's recommendation (Invitrogen, Paisley, UK) for M-MLV

reverse transcriptase. The cDNA product was amplified by PCR using CAT-specific primers designed from Genbank sequences for human CAT-1 (Accession No.: NM\_003045), CAT-2A (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<sub>2</sub>, 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.

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301 *Statistics*

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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).

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310 **Results**

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312 *Cloning, sequence, and analysis of iNOS cDNA*

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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

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Amino acid:	72	348	349	380	591	679	680	714	721	722	740	844	974	999	1084	1133	1138
Position																	
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).

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 calmodulin (at amino acids 502–527) in the mid portion. Following sequencing and analysis, the fragment produced was subsequently subcloned into the *Hind*III 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).

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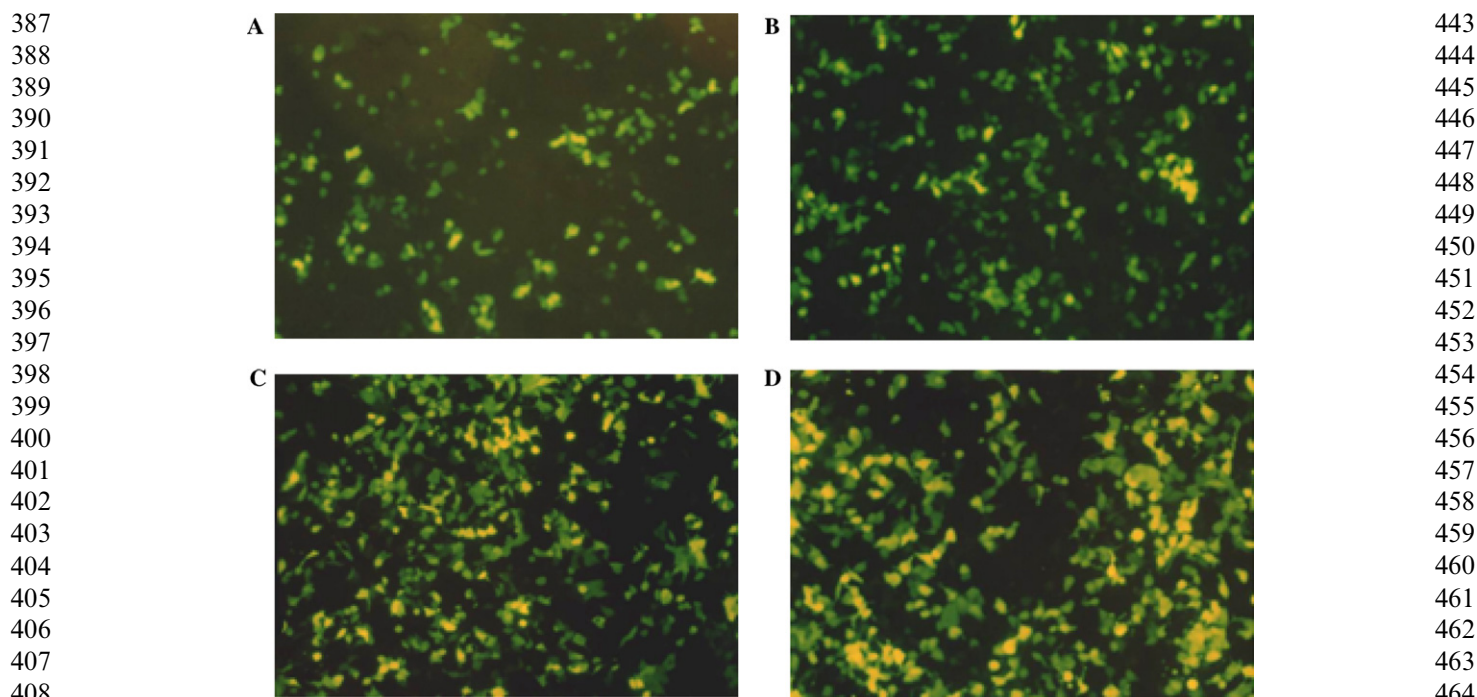


Fig. 2. Expression of iNOS-EGFP in HEK293 cells. Cells were transfected with pEGFP-N1 harbouring the iNOS cDNA construct as described in Experimental procedure. Cells were visualised at 6 h (A), 12 h (B), 24 h (C), and 48 h (D) after transfection using a Nikon EFD3, LABOPHOT-2 fluorescence microscope.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

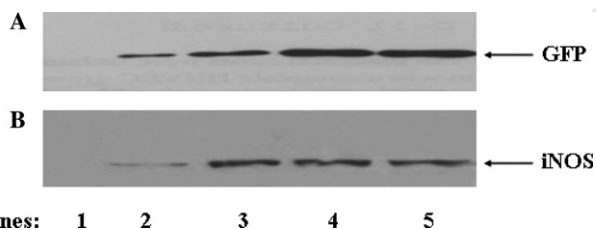


Fig. 3. Time course of expression of GFP (A) and iNOS (B) in transfected HEK-293 cells. Lysates were obtained from control cells (lane 1) and at 6 h (lane 2), 12 h (lane 3), 24 h (lane 4), and 48 h (lane 5) after transfection with iNOS-pEGFP-N1. Proteins were separated by SDS-PAGE electrophoresis and 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 ( $56 \pm 0.03 \text{ pmol } \mu\text{g protein}^{-1}$  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 gener-

ated 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  $10 \mu\text{M}$  GW274150, confirming iNOS as the source of the NO produced.

#### Effects of iNOS expression on L-arginine transport

HEK-293 cells transported L-[ $^3\text{H}$ ]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  $1.91 \pm 0.03 \text{ pmol } \mu\text{g protein}^{-1} \text{ min}^{-1}$  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).

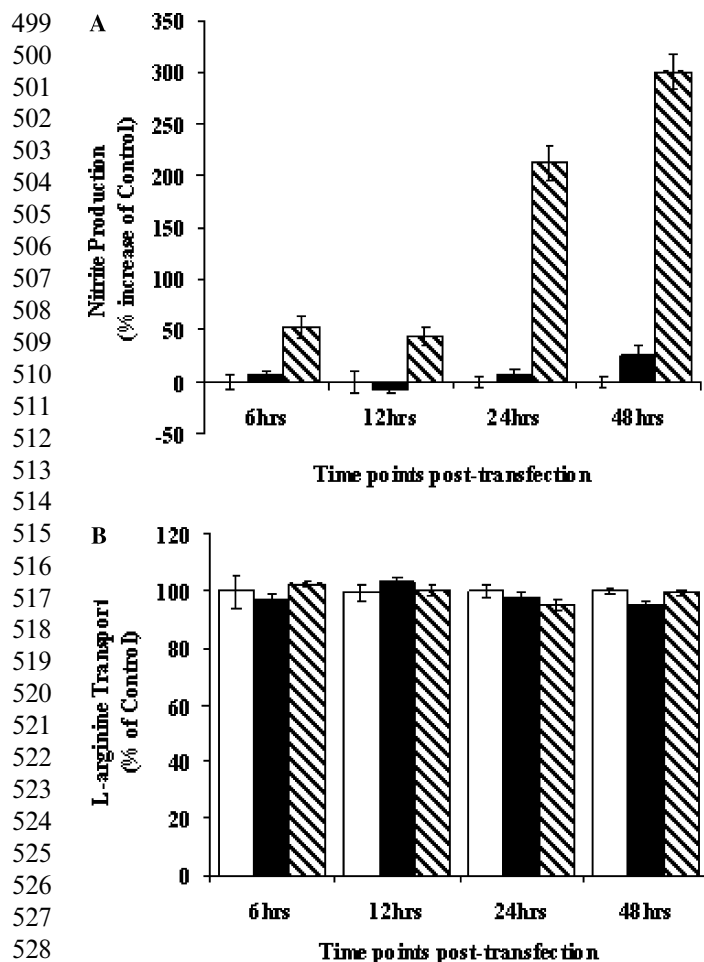


Fig. 4. Effects of iNOS-pEGFP-N1 transfection on NO production (A) and L-arginine transport (B) in HEK-293 cells. Nitrite and transport were measured at 6–48 h after transfection. Open bars represent control non-transfected cells, solid black bars represent cells transfected with a GFP only vector and hatched bars represent cells transfected with iNOS-pEGFP-N1. Results represent means  $\pm$  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  $\mu$ M. Transport was found to be saturable (Fig. 7) with a  $K_m$  of 0.21 mM and  $V_{max}$  of 5.3 nmol  $\mu$ g protein<sup>-1</sup> min<sup>-1</sup> 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-[<sup>3</sup>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

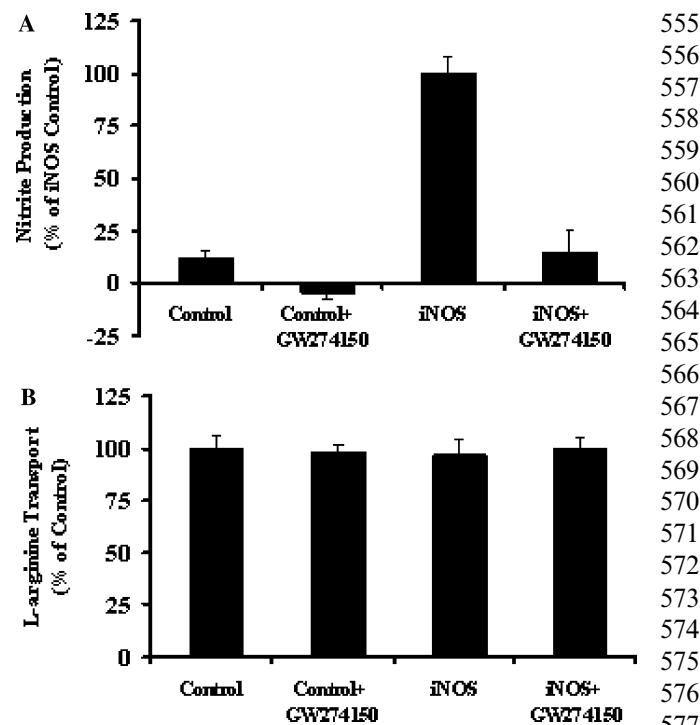


Fig. 5. Effect of GW274150 on NO production (A) and L-arginine transport (B) in control and iNOS-pEGFP-N1 transfected HEK-293 cells. Nitrite and transport were measured 48 h after transfection. Results represent means  $\pm$  SEM of three separate experiments with two replicates in each.

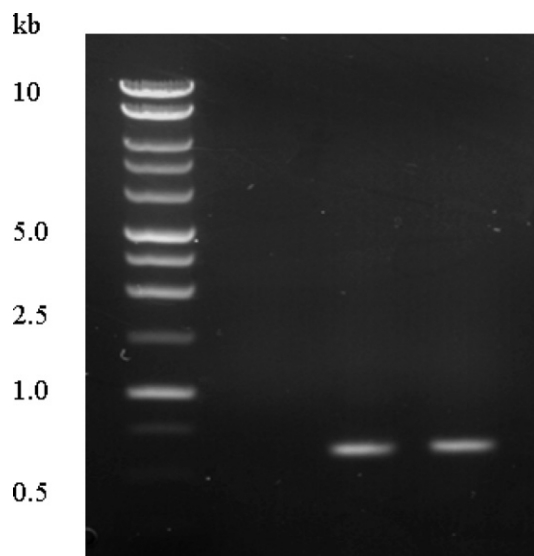


Fig. 6. CAT expression in HEK-293 cells. Total RNA was isolated from control, non-activated cells and RT-PCR performed (33 cycles) using primer pairs specific for CAT-1, CAT-2A, and CAT-2B as outlined in Experimental procedure. Lanes represent: 1=ladder, 2 = CAT-2A, 3 = CAT-2B, and 4 = CAT-1.

(Table 1). In addition, transport was found to be unaffected by changes in extracellular Na<sup>+</sup> or pH ranging from 6 to 7.4 (Table 2).

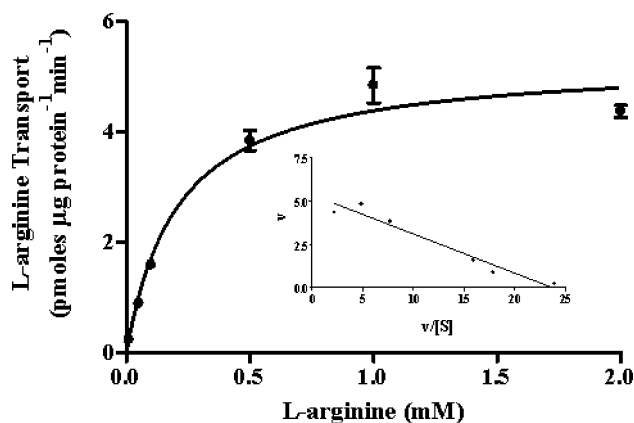


Fig. 7. Kinetics of L-arginine transport in HEK-293 cells. Rates of L-arginine transport (0.005–2 mM) were measured in control cells. The inset shows an Eadie–Hofstee plot of saturable transport, where  $V$  is the initial velocity ( $\text{pmol } \mu\text{g protein}^{-1} \text{ min}^{-1}$ ) and  $S$  is the substrate concentration (mM). Values are means  $\pm$  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 ( $\text{pmol } \mu\text{g protein}^{-1} \text{ min}^{-1}$ )
Control	$2.09 \pm 0.10$
<i>System A/ASC substrates</i>	
MeAIB	$2.56 \pm 0.14$
Alanine	$2.57 \pm 0.12$
Serine	$2.33 \pm 0.11$
Proline	$2.03 \pm 0.09$
<i>System N substrates</i>	
DON	$1.74 \pm 0.08$
L-Histidine	$2.06 \pm 0.06$
<i>System L substrates</i>	
BCH	$1.96 \pm 0.13$
L-Phenylalanine	$2.36 \pm 0.10$
L-Leucine	$1.85 \pm 0.13$
<i>System <math>\gamma^+</math> substrates</i>	
L-Arginine	$0.61 \pm 0.05^*$
L-Lysine	$0.91 \pm 0.05^*$
L-Ornithine	$0.97 \pm 0.09^*$

Transport of  $100 \mu\text{M}$  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  $\pm$  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

Condition	Transport rate ( $\text{pmol } \mu\text{g protein}^{-1} \text{ min}^{-1}$ )
<i>Na<sup>+</sup>-dependency</i>	
Control	
+Na <sup>+</sup>	$1.8 \pm 0.11$
-Na <sup>+</sup>	$1.6 \pm 0.10$
<i>pH-dependency</i>	
7.4 (control)	$1.85 \pm 0.08$
7.0	$1.89 \pm 0.11$
6.5	$1.86 \pm 0.06$
6.0	$1.82 \pm 0.12$

Transport of  $100 \mu\text{M}$  L-arginine was measured over 1 min in the presence (normal Krebs solution) or absence of Na<sup>+</sup> 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  $\pm$  SE of three different experiments with six replicates in each.

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  $\geq 50\%$  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].

723 Our results therefore provide the first direct evidence  
 724 that expression of active iNOS is by itself insufficient to  
 725 upregulate CAT function and hence arginine transport.  
 726 This conclusion is apparently in conflict with a recent  
 727 report by Hammermann et al. [18], which suggests that  
 728 direct inhibition of iNOS activity in rat alveolar macro-  
 729 phages is sufficient to abolish LPS-activated L-arginine  
 730 uptake. The inhibitor of iNOS primarily used in those  
 731 experiments (AMT) did not inhibit basal arginine influx  
 732 in control cells but inhibited enhanced arginine uptake  
 733 in LPS-pretreated cells even with brief pre-incubations.  
 734 The authors therefore concluded that this was an indi-  
 735 rect effect due to reduced arginine utilisation by iNOS.  
 736 This association is however questionable since L-arginine  
 737 transport: (i) occurs by facilitated diffusion driven by the  
 738 membrane potential, (ii) accumulates L-arginine against  
 739 a concentration gradient, and (iii) is sensitive to trans-  
 740 stimulation when substrate concentrations are high on  
 741 the opposite side of the cell membrane [37,38]. Thus, if  
 742 inhibition of L-arginine utilisation by iNOS results in ele-  
 743 vated intracellular levels of this amino acid, entry of  
 744 radiolabelled L-arginine would be enhanced via trans-  
 745 stimulation causing an increase in L-[<sup>3</sup>H]arginine uptake.  
 746 As we observed no significant changes in accumulated L-  
 747 [<sup>3</sup>H]arginine in cells transfected with our iNOS con-  
 748 struct, we conclude that activation of iNOS itself does  
 749 not influence rates of L-arginine transport. It could be  
 750 argued that in the system used by Hammermann et al.  
 751 [18], activation of cells may result in the co-induction of  
 752 linker proteins which may facilitate compartmentalised  
 753 regulation of L-arginine transport and NO synthesis.  
 754 This, however, still remains to be established and the  
 755 coupling of L-arginine transport to NO synthesis may  
 756 not be a widespread phenomenon in all activated cell  
 757 types [13,20,21].

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

773 Since the nature of the transporters associated with L-  
 774 arginine uptake had not previously been characterised in  
 775 HEK-293 cells, we have therefore examined the charac-  
 776 teristics of L-arginine transport and the profile of expres-  
 777 sion of CATs in these cells. Kinetic studies and Eadie-  
 778 Hofstee analysis show that entry of L-arginine occurs

779 predominantly via a single saturable carrier that is of rel-  
 780 atively low capacity but of high affinity for L-arginine.  
 781 Transport was selectively inhibited by the cationic  
 782 amino acids L-lysine, L-ornithine, and by L-arginine itself.  
 783 Substrates for amino acid transport systems A/ASC, N,  
 784 and L were without significant effect. This inhibition  
 785 profile together with the Na<sup>+</sup> and pH insensitivity  
 786 strongly implicate the CATs as the predominant carrier  
 787 of L-arginine in HEK-293 cells and excludes other sys-  
 788 tems such as y<sup>+</sup>L, b<sup>0,+</sup>, and B<sup>0,+</sup> which are broad scope in  
 789 nature, transporting both cationic and neutral amino  
 790 acids [38,40].

791 To identify the CAT or CATs expressed in HEK-293  
 792 cells we carried out RT-PCR of total RNA isolated from  
 793 these cells using CAT-1, CAT-2A, and CAT-2B-specific  
 794 primers respectively. CAT-3 was not investigated since  
 795 this transporter is thought to be expressed predomi-  
 796 nantly in brain [41,42] and thymus tissue [43] and also  
 797 interacts with neutral amino acids [41]. The data  
 798 obtained show that HEK-293 cells express transcripts  
 799 for CAT-1 and CAT-2B but not for CAT-2A. This  
 800 expression profile agrees with the functional studies in  
 801 that the kinetics of uptake reflects a high affinity system  
 802 or systems with indistinguishable characteristics at the  
 803 functional level. Although, CAT-2A and CAT-2B are  
 804 spliced variants with high percentage structural homol-  
 805 ogy they are functionally distinguishable by virtue of the  
 806 fact that CAT-2A is a high capacity but low affinity car-  
 807 rier while CAT-2B exhibits similar functional character-  
 808 istics to those of CAT-1 [44–46]. Both these carriers have  
 809 a high affinity for L-arginine, transporting the latter with  
 810 a Michaelis constant (*K<sub>m</sub>*) of between 0.14 and 0.38 mM.  
 811 The *K<sub>m</sub>* of 0.21 mM determined in our study therefore  
 812 supports CAT-1 and CAT-2B as being the predominant  
 813 carriers of L-arginine into HEK-293 cells.

814 At present we cannot distinguish between CAT-1 and  
 815 CAT-2B in terms of their relative contribution to total L-  
 816 arginine uptake nor can we specify which CAT directly  
 817 supplies L-arginine to transfected iNOS in our cell sys-  
 818 tem. However, the detection of CAT-2B transcripts, gen-  
 819 erally believed to be expressed under inflammatory  
 820 conditions in parallel with iNOS, suggests that this pro-  
 821 tein can exist endogenously in control conditions and  
 822 may not require induction. More importantly, its detec-  
 823 tion excludes the possibility that iNOS was not able to  
 824 alter transport in our studies because of lack of expres-  
 825 sion of the critical CAT (CAT-2B) associated with  
 826 uptake of L-arginine for utilisation by this enzyme.

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



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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