# Cationic amino acid transporter gene expression in cultured vascular smooth muscle cells and in rats

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**Hattori, Yoshiyuki, Kikuo Kasai, and Steven S. Gross.** Cationic amino acid transporter gene expression in cultured vascular smooth muscle cells and in rats. *Am. J. Physiol.* 276 (*Heart Circ. Physiol.* 45): H2020–H2028, 1999.—Immunostimulants trigger vascular smooth muscle cells (VSMC) to express the inducible isoform of NO synthase (iNOS) and increased arginine transport activity. Although arginine transport in VSMC is considered to be mediated via the $y^+$ system, we show here that rat VSMC in culture express the *cat-1* gene transcript as well as an alternatively spliced transcript of the *cat-2* gene. An RT-PCR cloning sequence strategy was used to identify a 141-base nucleotide sequence encoding the low-affinity domain of alternatively spliced *CAT-2A* and a 138-base nucleotide sequence encoding the high-affinity domain of *CAT-2B* in VSMC activated with lipopolysaccharide (LPS) in combination with interferon-γ (IFN). With this sequence as a probe, Northern analyses showed that *CAT-1* mRNA and *CAT-2B* mRNA are constitutively present in VSMC, and the expression of both mRNAs was rapidly stimulated by treatment with LPS-IFN, peaked within 4 h, and decayed to basal levels within 6 h after LPS-IFN. *CAT-2A* mRNA was not detectable in unstimulated or stimulated VSMC. Arginine transporter activity significantly increased 4–10 h after LPS-IFN. iNOS activity was reduced to almost zero in the absence of extracellular arginine uptake via system $y^+$. Induction of arginine transport seems to be a prerequisite to the enhanced synthesis of NO in VSMC. Moreover, this work demonstrates tissue expression of *CAT* mRNAs with use of a model of LPS injection in rats. RT-PCR shows that the expression of *CAT-1* and *CAT-2B* mRNA in the lung, heart, and kidney is increased by LPS administration to rats, whereas *CAT-2A* mRNA is abundantly expressed in the liver independent of LPS treatment. These findings suggest that together CAT-1 and CAT-2B play an important role in providing substrate for high-output NO synthesis in vitro as well as in vivo and implicate a coordinated regulation of intracellular iNOS enzyme activity with membrane arginine transport.

nitric oxide synthase; arginine; inducible isoform of nitric oxide synthase

IMMUNOSTIMULANTS TRIGGER vascular smooth muscle cells (VSMC) to express the inducible isoform of nitric oxide (NO) synthase (iNOS) (3, 5, 15). Induction of iNOS and overproduction of NO in VSMC have been implicated in the genesis of septic and cytokine-induced circulatory shock (23, 36). Elucidation of the factors that control iNOS activity should provide information for the design of therapeutics that effectively limit pathophysiological NO overproduction. The activity of iNOS appears to be regulated mainly at the transcriptional level (41). However, regulation of arginine availability can also determine the cellular rate of NO production, since arginine is the only physiological substrate for the NO synthase reaction. Recent reports indicate that iNOS activity is strictly dependent on the presence of extracellular L-arginine. The cytokine-stimulated production of NO by VSMC and the NO-mediated vascular hyporeactivity after endotoxin exposure can be reversed by removing L-arginine from the extracellular environment (3, 34). These findings indicate that the transport of L-arginine into VSMC may be an important regulatory mechanism for determining the rate of VSMC NO production.

The transport of cationic amino acids by VSMC appears to be mediated by the system $y^+$ carrier (14, 26, 27). Recently, genes encoding the proteins responsible for the activity of the murine system $y^+$ carrier have been cloned and designated *CAT-1, CAT-2A*, and *CAT-2B* (1, 8–10, 22, 29, 30). *CAT-1* was initially identified as an ectropic retrovirus receptor in murine fibroblasts (1) and was subsequently shown to be a basic amino acid transporter in *Xenopus* oocytes (24, 37). *CAT-2B* was first detected in activated thymocytes (29) and has recently been cloned from lipopolysaccharide (LPS)-treated macrophages (10). CAT-1 and CAT-2B are low-capacity transporters that have a high affinity for cationic amino acids. In contrast, *CAT-2A* is an alternate splice variant of *CAT-2B* that was cloned from murine liver and possesses low affinity but high transport capacity (9, 22).

In the present study, to elucidate the molecular mechanism of L-arginine transport in immunostimulated VSMC, we characterized the expression of *CAT* mRNAs in these cells. Rat VSMC express mRNAs for *CAT-1* as well as *CAT-2B*, both of which are upregulated by LPS in combination with interferon-γ (IFN). Here we report the partial cloning of a cDNA for *CAT-2A* and *CAT-2B* from rat VSMC. These isoforms result from mutually exclusive alternative splicing of the transcript in a tissue-specific manner. Using a model of LPS injection in rats, we also investigated the tissue distribution of *CAT* mRNAs and alteration of their expression during endotoxic shock. We show that *CAT-1* and *CAT-2B* mRNAs are increased by endotoxin treatment of cardiovascular tissues such as lung, heart, and kidney, whereas *CAT-2A* mRNA, which is highly expressed in the liver, is unaffected by LPS administration. These data suggest that an immunostimulant-elicited increase in arginine transport activity plays a key role in NO formation by VSMC and that arginine transport is stimulated by endotoxin in rat tissues during sepsis.

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## METHODS

*Cell culture, animal treatment, and extraction of RNA.* VSMC were isolated by elastase and collagenase digestion of thoracic aortas from male Wistar rats (6). Cultures were fed twice weekly with DMEM containing 10% fetal bovine serum and antibiotics (100 µg/ml piperacillin and 100 µg/ml streptocymin). Cells in *passages 10–15* were seeded and grown to confluence in 96-well plates for nitrite assay and in 75-cm$^2$ culture flasks for preparation of cell lysate and RNA. The cells exhibited a classical VSMC phenotype, with hill-and-valley morphology, and stained positively for smooth muscle α-actin with a monoclonal antibody (DAKO, Via Real, CA). Male Wistar rats (250–300 g) were injected intravenously with LPS (*Escherichia coli* serotype 0111:B4, 10 mg/kg; Sigma, St. Louis, MO) or saline (1 ml/kg) in the endotoxin and control groups, respectively. The animals were killed 3 h later by exsanguination, and various organs were removed, immediately frozen in liquid nitrogen, and stored at −70°C until RNA extraction. The guanidinium isothiocyanate-acid phenol method (7) was used to extract total RNA from the VSMC and rat tissues.

*Nitrite measurement.* Nitrite production, an indicator of NO synthesis, was measured in the supernatant of VSMC, as previously described (18). Nitrite was measured by adding 100 µl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) to 100-µl samples of cell culture medium. Absorbance at 550 nm was determined with a microplate reader. Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium.

*Arginine transport measurement.* After incubation in the presence of LPS-IFN, the cells were washed with HEPES-buffered saline (HBS) and further incubated in HBS at 37°C for 10 min. The reaction was started by changing the medium to HBS containing L-[$^3$H]arginine (2.15 TBq/mmol). The concentration of L-[$^3$H]arginine was 100 nM, and the reaction time was 2 min. The reaction was stopped by washing the cells three times with ice-cold HBS. The cells were then lysed in 0.32 N NaOH with 1% SDS, and the radioactivity incorporated was determined by a liquid scintillation counter. The protein concentration of the cell lysate was measured using the Lowry method, with BSA as a standard (28).

*Determination of arginine concentration in cells and medium.* After 12- and 24-h incubation periods, the medium was collected, centrifuged at 10,000 *g* for 5 min, and stored at −80°C for subsequent analysis by HPLC. Cells were rinsed with PBS ($Ca^{2+}$ and $Mg^{2+}$ free) and lysed with methanol, and cell lysates were stored at −80°C for HPLC analysis. The deproteinized samples were injected into a Wakosil 5C18 column (Wako, Tokyo, Japan) attached to an HPLC system of Waters chromatography (Millipore, Milford, MA), and the concentrations of arginine and other amino acids in the samples were determined as previously described (21). Intracellular amino acid concentrations are expressed in millimolar, where the volume is the intracellular water space (0.5 ± 0.05 pl, $n = 3$) measured as described previously (25).

*CAT mRNA expression.* RT-PCR was performed using a standard method (25). cDNA was synthesized from total RNA from LPS-IFN-activated VSMC by avian myeloblastosis virus RT with random 9 mers as primers and then amplified by PCR with primers derived from the published sequences of rat *CAT-1* and murine *CAT-2A*. The forward 5′-GCCATCGTCATCTCCTTCCTG-3′ [corresponding to sense bp 272–292 of *CAT-1* (23)] and reverse 5′-CCCTCCCTCACCGTATTTCAC-3′ [corresponding to sense bp 782–802 of *CAT-1* (23)] primers were used to detect the presence of a 531-bp *CAT-1* transcript, whereas the forward 5′-AACGTGCTTTTATGCCTTTGT-3′ [corresponding to sense bp 795–815 of *CAT-2A* (13)] and reverse 5′-GGTGACCTGGGACTCGCTCTT-3′ [corresponding to sense bp 1387–1407 of *CAT-2A* (13)] primers common to *CAT-2A* and *CAT-2B* were used to detect *CAT-2A* and/or *CAT-2B* transcript(s). The PCR products were subcloned into pCR2.1 plasmids (Invitrogen, San Diego, CA) and sequenced to ensure that they corresponded to the expected 613-bp *CAT-2A* and 616-bp *CAT-2B* transcripts. From this sequencing result of rat partial cDNA for *CAT-2A* and *CAT-2B*, second primers that distinguish *CAT-2A* from *CAT-2B* were made to detect the presence of 115-bp *CAT-2A* and 121-bp *CAT-2B* transcripts; the primers are 5′-CCTTACCCCGCATTCTGTTTG-3′ [forward ($P_{2A}$-F)] and 5′-AAATGACCCCTGCAGTCATCG-3′ [reverse ($P_{2A}$-R)] for 115-bp *CAT-2A* and 5′-CCCAATGCCTCGTGTAATCTA-3′ [forward ($P_{2B}$-F)] and 5′-TGCCACTGCACCCGATGACAA-3′ [reverse ($P_{2B}$-R)] for 121-bp *CAT-2B*. The PCR products for *CAT-1*, 115-bp *CAT-2A*, and 121-bp *CAT-2B* were labeled with d-[α-$^{32}$P]CTP by random priming and used as probes for Northern blot analysis of VSMC mRNA. Blotting procedures were performed as described elsewhere (19). The blots were quantitated for radioactivity with a BAS2000 image analyzer (Fuji Photo Film, Tokyo, Japan). In addition, expression of the *CAT* mRNAs in rat tissues was evaluated by RT-PCR with use of primers specific for *CAT-1* and *CAT-2*. The identity of the PCR products was confirmed by direct sequencing.

iNOS mRNA was also analyzed by Northern blot analysis with a rat iNOS cDNA as a probe for VSMC (31) and by an RT-PCR method with iNOS-specific primers for rat tissues (19).

Sequencing was accomplished by using the *Taq* DyeDeoxy Terminater and DyePrimer Cycle Sequencing protocols developed by Applied BioSystems (Foster City, CA) with use of fluorescent-labeled dideoxynucleotides and primers. The labeled extension products were analyzed on a DNA sequencer (model 373A, Applied Biosystems).

*Statistical analysis.* Values are means ± SD. Multiple comparisons were evaluated by ANOVA followed by Fisher's protected least significant difference test. Student's unpaired *t*-test was used for comparisons between two experiments. $P < 0.05$ was considered significant.

## RESULTS

Exposure of rat VSMC to LPS in culture triggers the production of nitrite, an accumulating oxidation product of NO. Nitrite accumulation is not observed in untreated cells but appears 6–8 h after addition of LPS to the culture medium. Although IFN alone does not elicit nitrite production, it has a potent synergistic effect on LPS-induced nitrite accumulation. Figure 1 shows the time course of nitrite production in VSMC treated with a combination of LPS (30 µg/ml) and IFN (100 U/ml) (LPS-IFN). Nitrite accumulation 24 h after stimulation was 960 ± 24 nmol/mg cell protein. On Northern blot analysis of iNOS mRNA, a dominant transcript of ∼4.6 kb is recognized in LPS-IFN-stimulated VSMC by means of a probe for iNOS. The transcript is barely detectable at 2 h, becomes evident at 4 h, peaks at 8 h, and is sustained at slightly lower levels at 24 h after stimulation (Fig. 1, *inset*).

Nitrite formation was examined in the culture media containing various concentrations of L-arginine in the VSMC activated with LPS-IFN for 24 h. The rate of nitrite formation was saturable to arginine concentra-

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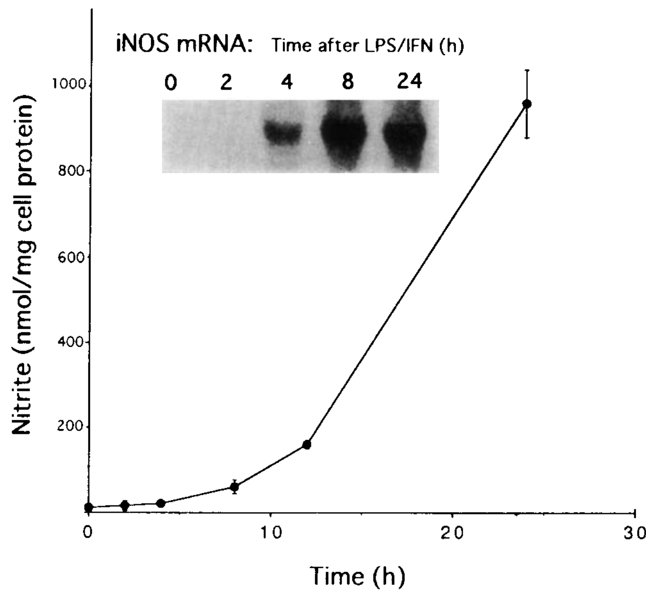


Fig. 1. Time course of nitrite production and induction of inducible isoform of nitric oxide synthase (iNOS) mRNA in rat vascular smooth muscle cells (VSMC) stimulated with lipopolysaccharide (LPS) and interferon-γ (IFN). Nitrite accumulation in culture medium was measured at times indicated. *Inset*: Northern blot of VSMC total RNA probed with a rat iNOS cDNA probe. Values are means ± SD of 6 determinations. When not shown, error bars are contained within symbols.

tion within an apparent Michaelis-Menten constant ($K_m$) of 40 μM and almost saturated at 100 μM arginine (Fig. 2*A*). The transport of L-arginine into immunostimulant-activated VSMC has been shown to be mediated by the cationic amino acid transport system $y^+$ (12–15, 26, 27). The dependency of NO formation on L-arginine transport was studied by incubating VSMC for 24 h with LPS-IFN with dialyzed serum, amino acid-depleted medium containing L-arginine (0.1 mM), and increasing concentrations of L-lysine (0–10 mM), which shares this transport system and competitively inhibits the transport of arginine (38–40). Under these conditions, the production of nitrite was inhibited in a concentration-dependent manner by lysine (Fig. 2*B*) but not by serine, which does not share the $y^+$ system (data not shown). This inhibition by lysine was bypassed by the presence of higher concentrations of arginine (1 mM; Fig. 2*B*).

Changes in initial arginine uptake were investigated in VSMC cultured with LPS-IFN. As shown in Fig. 3*A*, the rate of uptake was significantly augmented in VSMC at 4–10 h after LPS-IFN, and the rate declined thereafter. Treatment of VSMC with cycloheximide (5 μg/ml) abolished the increase in arginine uptake without reducing uptake below control levels (data not shown). We measured changes in the intracellular concentration of arginine when VSMC were activated with LPS-IFN. At 12 and 24 h after LPS-IFN, the intracellular and extracellular arginine concentrations were determined. The intracellular arginine concentration was higher in LPS-IFN-stimulated cells, and the intracellular-to-extracellular distribution ratio of arginine was much higher in these stimulated cells (Fig. 3*B*). The intracellular concentration of arginine decreased when lysine was present in the medium (data not shown). We also measured intracellular concentrations of cationic amino acids other than arginine in VSMC (Table 1). After 12 h in culture the total concentration of cationic amino acids (arginine + lysine + ornithine) was 0.54 mM in unstimulated cells and 1.53 mM in LPS-IFN-treated cells. After 24 h the concentration of cationic amino acids in the LPS-IFN-treated cells was further increased to 2.19 mM.

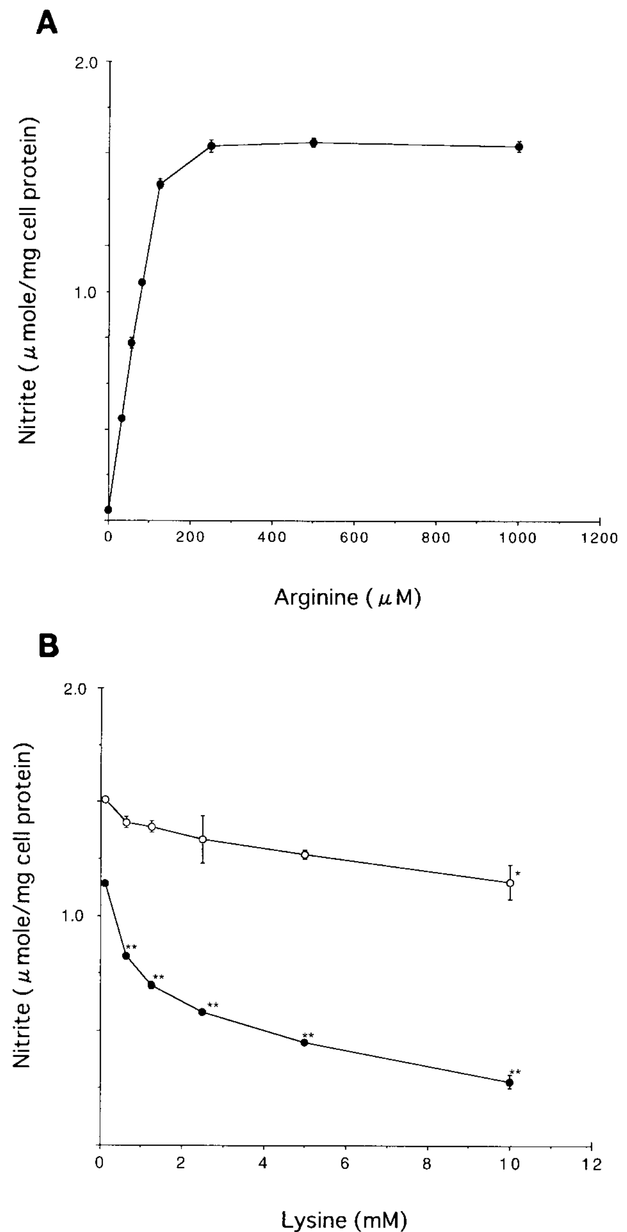


Fig. 2. Effect of changes in arginine and lysine concentrations in culture medium on nitrite production in rat VSMC. Cells were cultured in routine culture medium (DMEM with 10% serum) and then stimulated with LPS-IFN in similar DMEM with dialyzed serum containing various concentrations of L-arginine (*A*) or 0.1 mM (●) or 1 mM (○) arginine supplemented with lysine (*B*). After 24 h, nitrite accumulation in culture medium was measured. Values are means ± SD of 6 determinations from 2 independent experiments. When not shown, error bars are contained within symbols. * $P < 0.05$; ** $P < 0.01$ vs. control (no lysine).

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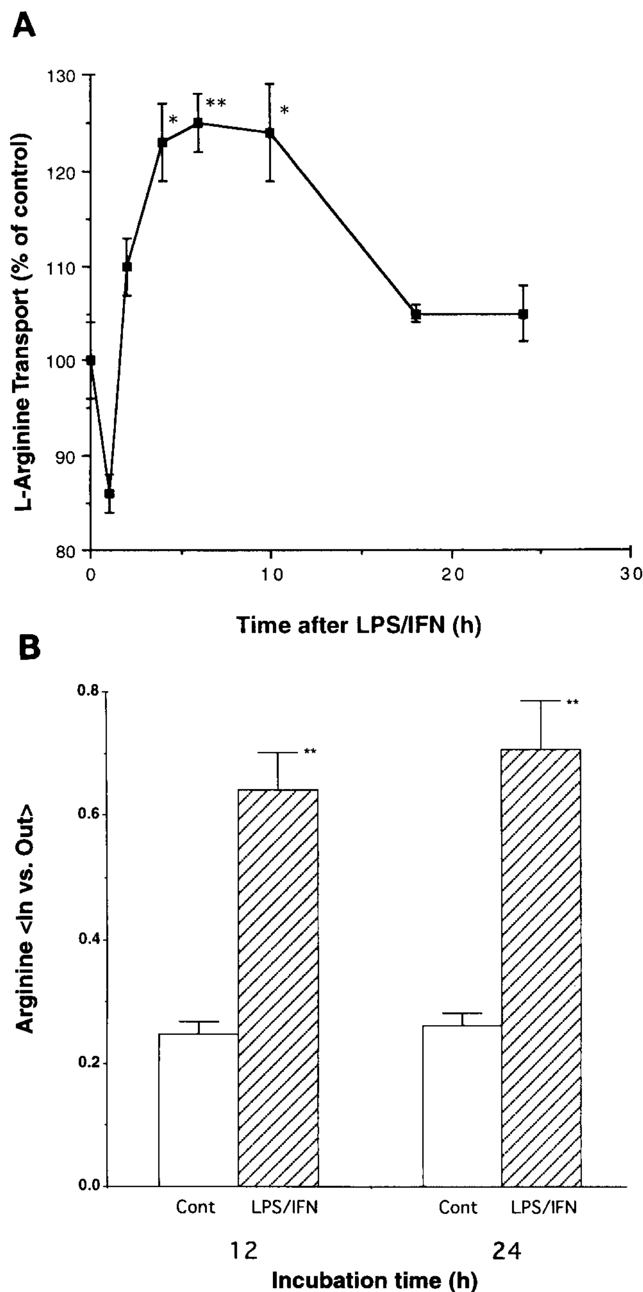


Fig. 3. Changes in rate of arginine uptake and ratio of intracellular to extracellular arginine concentrations in rat VSMC. Cells were stimulated with LPS-IFN in routine culture medium (DMEM with 10% serum). *A*: rate of uptake of [$^3$H]arginine. Radioactivity incorporated was normalized for protein recovery from each well. *B*: ratio of intracellular to extracellular arginine concentrations at 12 and 24 h. Values are means ± SD of 6 determinations from 2 independent experiments. * $P < 0.05$; ** $P < 0.01$ vs. control (no LPS-IFN).

cDNA prepared by RT from LPS-IFN-activated rat VSMC RNA served as a template for PCR. The products obtained were for a 531-bp *CAT-1* and the mutually exclusive alternatively spliced isoforms of *CAT-2* (high-affinity isoform *CAT-2B* or low-affinity isoform *CAT-2A*). As shown in Fig. 4, a specific DNA sequence (138 or 141 bp) was nested within a 613- or 616-nt sequence obtained by RT-PCR by using the primers that flanked the region of alternative splicing. One of these sequences encodes the L-arginine high affinity of the *CAT-2* isoform polypeptide (CAT-2B), which, expressed in *Xenopus oocytes,* acts as cationic amino acid transport system y$^+$, and the other encodes CAT-2A, which is the low-affinity liver isoform of this cationic amino acid transport activity (13–15). The deduced amino acid sequences, based on the rat nucleotide sequences, of the domain that differs between the two isoforms (46 and 47 amino acids) displays 100% identity with that of mouse CAT-2A (8) and differs by only one residue from that of mouse CAT-2B (29) (Fig. 4). On the basis of the nucleotide sequence differences in this domain between *CAT-2A* and *CAT-2B*, we made new primer sets for PCR amplification of cDNA prepared from rat VSMC and tissue RNAs to discriminate *CAT-2A* mRNA and *CAT-2B* mRNA. PCR primer sets for amplification of 115-bp *CAT-2A* cDNA are $P_{2A}$-F and $P_{2A}$-R, and for 121-bp *CAT-2B* they are $P_{2B}$-F and $P_{2B}$-R, respectively (Fig. 4). The identity of the PCR products 115-bp *CAT-2A* and 121-bp *CAT-2B*, as those corresponding to rat *CAT-2A* and *CAT-2B*, was confirmed by direct sequencing of the PCR products.

The time course of *CAT* mRNAs was investigated by Northern analysis of total RNA from LPS-IFN-activated VSMC with rat *CAT* cDNAs (531-bp *CAT-1* cDNA, 115-bp *CAT-2A* cDNA, and 121-bp *CAT-2B* cDNA) as probes. As shown in Fig. 5, two mRNAs for *CAT-1* (7.9 and 3.4 kb) and for *CAT-2B* (8.5 and 4.5 kb) were identified in vascular smooth muscle. *CAT-1* mRNA, which was detectable in untreated VSMC, substantially increased after the stimulation with LPS-IFN, peaked at 2 h and then decreased below basal levels by 8 h. The *CAT-2A* signal was absent or negligible in the untreated and stimulated VSMC throughout the time course (data not shown). *CAT-2B* mRNA, which was detectable in untreated VSMC, increased within 2 h after the stimulation, peaked by 4 h, and subsequently decreased up to 24 h.

Figure 6 shows the effects of dexamethasone, cycloheximide, and actinomycin D on the expression of *CAT* mRNAs in VSMC. The steady-state level of *CAT-1* mRNA in VSMC stimulated with LPS-IFN was decreased by 6 h, but it remained increased by treatment of the cells with cycloheximide. The steady-state level

Table 1. *Intracellular concentrations of cationic amino acids*

| | Arginine | Lysine | Ornithine |
|---|---|---|---|
| | *12-h incubation* | | |
| Control | 0.16 ± 0.02 | 0.37 ± 0.04 | 0.01 ± 0.01 |
| LPS-IFN | 0.40 ± 0.03† | 1.09 ± 0.08† | 0.04 ± 0.01* |
| | *24-h incubation* | | |
| Control | 0.19 ± 0.02 | 0.50 ± 0.05 | 0.02 ± 0.01 |
| LPS-IFN | 0.37 ± 0.04† | 1.78 ± 0.03† | 0.04 ± 0.01 |

Values are means ± SD of 3 determinations, expressed in mM. Vascular smooth muscle cells were stimulated with lipopolysaccharide (LPS) and interferon-γ (IFN) in routine culture medium (DMEM with 10% serum). Intracellular concentrations of cationic amino acids (arginine, lysine, and ornithine) were determined after 12- and 24-h incubations. * $P < 0.05$; † $P < 0.01$ vs. control (no LPS-IFN).

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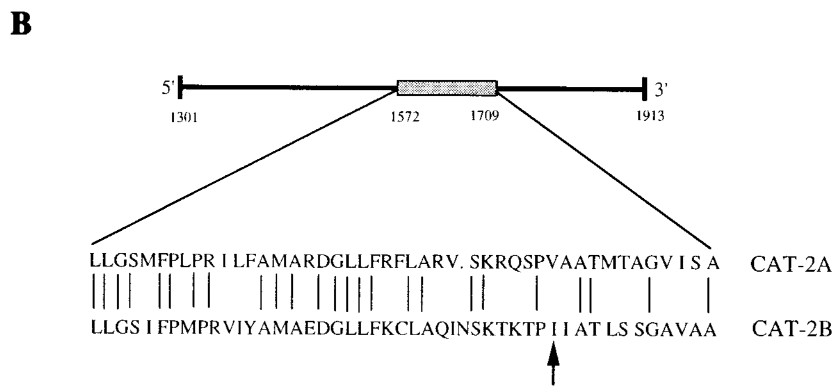


Fig. 4. DNA sequence and predicted amino acid sequence of alternatively spliced region of rat VSMC *CAT-2*. *A*: nucleotide sequence of entire RT-PCR product. Nucleotide number is shown with respect to cDNA sequence of mouse liver *CAT-2A*. Top 21-bp and tail 21-bp sequences are those of PCR primers derived from mouse *CAT-2A* cDNA sequence. *B*: nested 46- and 47-amino acid sequences predicted for *CAT-2A* and *CAT-2B*. This coding region for *CAT-2B* confers high-affinity binding of L-arginine to system $y^+$ in mouse lymphocytes and that for *CAT-2A* confers low-affinity binding in mouse hepatocytes. Position of this region (bases 1572–1709) is shown with respect to nucleotide number of mouse liver *CAT-2A*. *Nucleotide differences for rat sequences compared with mouse lymphocyte or liver sequences. Arrow, single amino acid difference for rat vs. mouse lymphocytes. PCR primer sequences for 121-bp *CAT-2A* ($P_{2A}$-F and $P_{2A}$-R) and for 121-bp *CAT-2B* ($P_{2B}$-F and $P_{2B}$-R) are underlined.

of *CAT-2B* mRNA in VSMC after LPS-IFN, which returned to near basal level by 6 h, was abolished by actinomycin D at 6 h. In contrast, *CAT-2B* mRNA in VSMC treated with dexamethasone or cycloheximide was increased compared with control (LPS-IFN only) at 6 h.

We next used a model of LPS injection in rats to investigate the tissue distribution of *CAT* mRNAs and alteration of their expression during endotoxic shock. As has been shown previously, iNOS mRNA is markedly increased in the lung, heart, liver, and kidney from LPS-treated rats but is absent from all tissues studied in control rats (20). The *CAT-1* signal was absent or negligible in the control lung, heart, and liver, whereas a signal was detected in the control kidney. After LPS the *CAT-1* signal became detectable in the lung and

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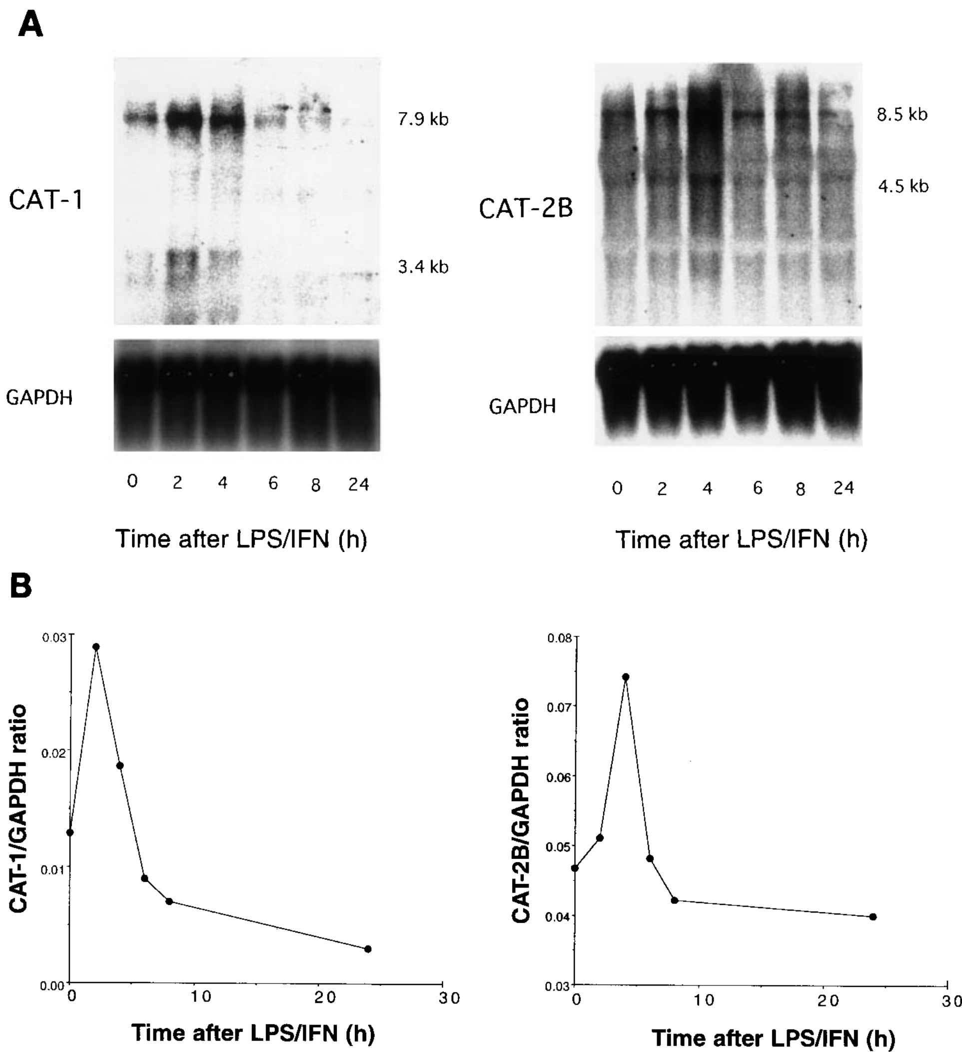


Fig. 5. Time course of steady-state *CAT-1* and *CAT-2B* mRNAs in rat VSMC activated by LPS-IFN. *A*: Northern blot of VSMC RNA probed with a *CAT-1* 531-bp cDNA probe or with a 121-bp *CAT-2B* cDNA clone (alternative spliced region shown in Fig. 4). *B*: time course of relative intensity of 7.9-kb *CAT-1* and 8.5-kb *CAT-2B* transcripts on Northern blot. Quantitative data were obtained by normalizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript signals in each lane.

heart and was substantially increased in the kidney; however, it remained absent in the liver (Fig. 7). Although the *CAT-2* signal was very low in the control lung and heart, a large signal was observed in the control liver and a weak signal in the control kidney. After LPS the *CAT-2* signal remained elevated in the liver and was further increased in the kidney (Fig. 7). By separate PCR amplification with use of primers $P_{2A}$-F and $P_{2A}$-R for *CAT-2A* and $P_{2B}$-F and $P_{2B}$-R for *CAT-2B*, a large *CAT-2A* signal in the liver was detected independently of LPS treatment, and a modest induction by LPS of *CAT-2A* mRNA was observed in the kidney. The *CAT-2B* mRNA was clearly induced in the lung, heart, and kidney by LPS treatment and also was detectable in the liver before and after LPS (Fig. 7). Control PCR experiments demonstrated equivalent expression of the glyceraldehyde 3-phosphate dehydrogenase gene in all samples.

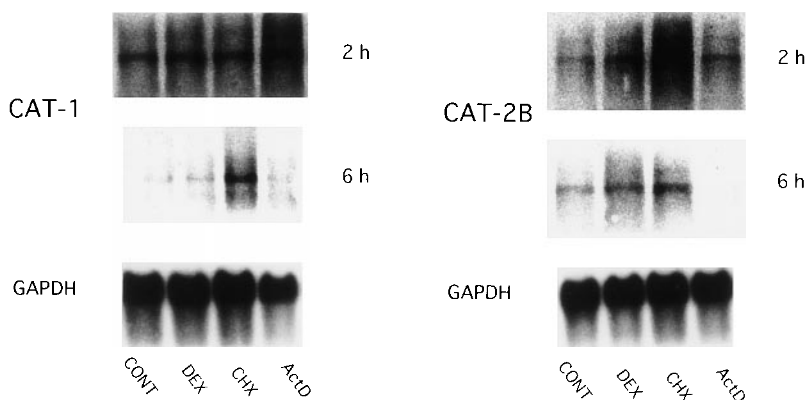


Fig. 6. Effect of dexamethasone (Dex), cycloheximide (Chx), and actinomycin D (ActD) on LPS-IFN-induced changes in mRNA levels for *CAT-1* and *CAT-2B*. VSMC were treated with LPS-IFN in absence or presence of dexamethasone (1 µM), cycloheximide (1 µg/ml), or actinomycin D (1 µg/ml). After 2 and 6 h, cells were harvested and total RNA was prepared and subjected to Northern blot analysis with use of *CAT-1* and *CAT-2B* probes and, subsequently, a GAPDH-specific probe. Cont, control.

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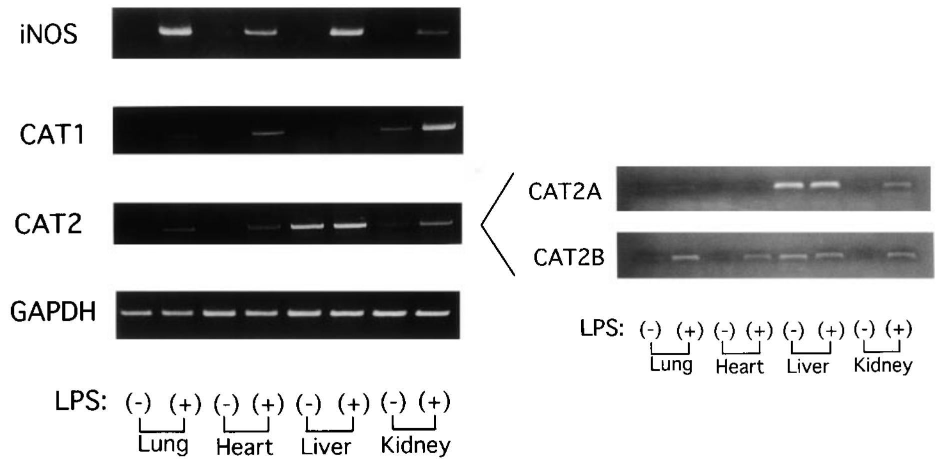


Fig. 7. Effects of LPS treatment in vivo on *CAT-1, CAT-2*, and iNOS mRNA expression in lung, heart, liver, and kidney. Total RNA was prepared from tissues of rats 3 h after intravenous administration of saline [control, LPS(−)] or LPS [10 mg/kg, LPS(+)] and assayed by RT-PCR by use of gene-specific primers. For *CAT-2*, RT-PCR was performed using primer set common to *CAT-2A* and *CAT-2B* and, subsequently, other sets of primers each specific for *CAT-2A* or *CAT-2B*.

## DISCUSSION

Several lines of evidence argue that L-arginine transport by rat aortic VSMC is mediated by the system $y^+$ carrier and that LPS or cytokines stimulate the rate of L-arginine transport by upregulation of the system $y^+$ family of cationic amino acid transporters (12–14, 16, 26, 27). This study shows clearly that immunostimulants can stimulate the system $y^+$ activity of VSMC and the mRNA concentrations of *CAT-1* and *CAT-2B*. Moreover, this work defines the profile of tissue expression of *CAT* mRNAs in LPS-treated rat: expression of *CAT-1* and *CAT-2B* mRNA in lung, heart, and kidney is increased by LPS administration, whereas *CAT-2A* mRNA is abundantly expressed in liver in the presence or absence of LPS treatment.

The cationic amino acid transport system $y^+$ has been well studied in mammalian cells (8, 30, 38–40). Physiological transport system $y^+$ activity is encoded by the cationic amino acid transporter genes (*cat-1* and *cat-2*), with cDNA clones denoted as *CAT-1, CAT-2A*, and *CAT-2B* (1, 7–9, 22, 29, 30). Three different polypeptides encoded by these genes each catalyze sodium-independent cationic amino acid uptake. CAT-2A and CAT-2B are distinct proteins encoded by two mature mRNAs originating from one primary transcript of the *cat-2* gene (10, 22). These isoforms result from the mutually exclusive alternative splicing of the transcript in a tissue-specific manner. In this study we used PCR primers for rat *CAT-2* that flanked the region of alternative splicing and found that the rat and mouse sequences differ by only one base within this alternatively spliced region of *CAT-2A* or *CAT-2B*. This results in a single amino acid conservative substitution of "I" in the rat for "V" in the mouse predicted the 47-amino acid polypeptide sequence of CAT-2B, whereas the rat and mouse predicted sequences are 100% identical within this 46-amino acid region of CAT-2A (10, 22). The optimal alignment between the alternatively spliced regions of rat CAT-2 amino acid sequence shows only 50% identity.

In mouse lymphocytes, expression of CAT-2B results in high-affinity ($K_m$ ~ 40–100 μM) system $y^+$ uptake. The CAT-2A transporter isoform has a much lower substrate affinity ($K_m$ ~ 5 mM) and is expressed predominantly in liver. In the rat VSMC we could detect mRNAs for *CAT-1* and *CAT-2B*, but not for *CAT-2A*, by Northern blot analysis. Using RT-PCR, we were able to detect the presence of *CAT-2A* mRNA in rat VSMC, the product of which was not derived from genomic DNA but was confirmed by sequencing to be the product derived from *CAT-2A* mRNA. Indeed, the *CAT-2A* signal was detectable at lower levels even by Northern blot analysis when VSMC were treated with LPS-IFN in the presence of cycloheximide. Nonetheless, CAT-1 and CAT-2B are the isoforms expected to take up arginine from the medium (DMEM), in which the arginine concentration routinely used is 400 μM. Because rat plasma L-arginine concentrations are ~100 μM or lower under septic shock conditions (11, 33), CAT-1 and CAT-2B are most likely to be the physiologically relevant isoforms in most tissues, including lung, heart, and kidney, with a negligible contribution by CAT-2A, except in liver (9, 10, 22).

We reported earlier that, along with iNOS, immunostimulants synergistically induce VSMC to express argininosuccinate synthase (AS) mRNA and activity (17). With constitutively expressed argininosuccinate lyase (AL), AS confers on cells an "arginine-citrulline cycle" (17, 32), which can sustain NO production via continuous regeneration of the iNOS substrate L-arginine from the iNOS coproduct L-citrulline. This allows for NO synthesis from citrulline even in the absence of extracellular arginine. We also showed the coinduction of AS/AL and iNOS in vivo in tissues of LPS-treated rats most clearly in the kidney, where arginine is regenerated from citrulline by use of AS and AL (20). We found the apparent $K_m$ to be 40 μM L-arginine for NO biosynthesis from extracellular substrate L-arginine and that nitrite production was a hyperbolic function of extracellular L-arginine concentration, such that upregulated nitrite production was reduced to almost zero when extracellular L-arginine was 0 mM (Fig. 2). Thus extracellular L-arginine concentration is a rate-limiting factor for NO synthase activity in VSMC exposed to immunostimulants under normal conditions where intracellular citrulline concentration is not high enough to support arginine supply via the arginine-citrulline cycle (17, 32). This dependency on extracellu-

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lar L-arginine concentration could be determined by the arginine transport $y^+$ system activity. Indeed, increasing concentrations of L-lysine dose dependently inhibited NO synthesis. It has been shown that the activity of iNOS was not directly inhibited by lysine in a murine macrophage cell line and in rat peritoneal macrophages (4, 35). The induction of *CAT-1* and *CAT-2B* mRNAs and arginine transport activity in LPS-IFN-treated VSMC preceded iNOS mRNA induction and NO biosynthesis. Thus upregulated arginine transport activity may play a more important role in supporting high-output NO synthesis by VSMC, especially in the earlier stages before the recycling system from citrulline to arginine by AS and AL becomes effective. It is likely that arginine must be concentrated from the extracellular fluid into the cells for continuous production of NO. Under these conditions, intracellular concentrations of other cationic amino acids (lysine and ornithine) in VSMC also are increased. Transport of arginine via system $y^+$ is subject to transstimulation; i.e., the influx of arginine is significantly increased by the presence of competing substrates in the cells (38). Therefore, the increase in the arginine transport activity in LPS-IFN-treated cells might be due, in part, to transstimulation.

It is now well established that high-output NO synthesis by immunostimulant-activated cells coincides with the induction of arginine transport activity in cultured cells. Our study shows that expression of mRNAs for *CAT-1* and *CAT-2B* resulting in high-affinity system $y^+$ uptake is induced in response to immunostimulants, providing a molecular basis for this in VSMC. We also show the concomitant induction of *CAT-1* and *CAT-2B* mRNA and iNOS mRNA in various tissues of LPS-treated rats. These findings suggest that together CAT-1 and CAT-2B play a special function in providing substrate for high-output NO synthesis in vitro as well as in vivo. Several important questions await answers: *1*) iNOS of VSMC has been well characterized, and the reported value of $K_m$ for arginine is 5–10 μM. The intracellular concentration of arginine shown here was much higher than this, even in VSMC incubated with lysine, yet under these conditions NO synthesis was considerably inhibited. How can this discrepancy be explained? *2*) What is the advantage for a cell to express CAT-1 and CAT-2B, both coding functionally active transporters in the VSMC and the other tissues? *3*) What are the relative contributions of activation of transporter(s) and the recycling pathway of citrulline to arginine to sustaining NO synthesis?

Inhibiting arginine availability affords a potential therapeutic opportunity for limiting NO production in pathophysiological conditions arising from NO excess. The challenge will be to develop specific modulators of arginine availability to the cells that can be targeted to specific cell types.

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