CAT2-mediated L-arginine transport and nitric oxide production in activated macrophages

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Activated macrophages require L-arginine uptake to sustain NO synthesis. Several transport systems could mediate this L-arginine influx. Using competition analysis and gene-expression studies, amino acid transport system y^+ was identified as the major carrier responsible for this activity. To identify which of the four known y^+ transport-system genes is involved in macrophage-induced L-arginine uptake, we used a hybrid-depletion study in *Xenopus* oocytes. Cationic amino acid transporter (CAT) 2 antisense oligodeoxyribonucleotides abolished the activated-macrophage-mRNA-induced L-arginine transport. Together with expression studies documenting that CAT2 mRNA and protein levels are elevated with increased L-arginine uptake, our data demonstrate that CAT2 mediates the L-arginine transport that is required for the raised NO production in activated J774 macrophages.

Key words: amino acid, antisense inhibition, iNOS, NO, *Xenopus* oocyte.

INTRODUCTION

Macrophages primed with interferon-γ (IFN-γ) and activated with bacterial lipopolysaccharide (LPS) transcriptionally induce the inducible isoform II of nitric oxide synthase (iNOS or NOS II), leading to NO production [1–5]. LPS has been shown to induce L-arginine transport and NO synthesis in mouse peritoneal macrophages [4] and in the mouse macrophage cell line J774 [6,7], with this activity being dependent on *de novo* synthesis of arginine-transport proteins [2]. Further treatment of J774 cells with IFN-γ leads to additional increases in arginine utilization and NO synthesis [2,6]. To sustain the high levels of NO produced by macrophages after this activation, external arginine uptake is required [6,8]. This requirement for additional L-arginine uptake was postulated to occur via system-y^+ transport in J774 cells [9], but this study did not exclude the possible involvement of other L-arginine-transport systems, such as y^+L or b^{0+}. Several amino acid transport systems mediate mammalian L-arginine transport, namely via systems y^+, y^+L and b^{0+} [10]. In fact, we and others have cloned several cDNAs that encode transport proteins with properties corresponding to systems y^+, y^+L and b^{0+} [11–16]. Although the cationic amino acid transporter (CAT) 2a transporter was cloned from activated macrophages [17], the reported kinetic properties encoded by this splice variant of CAT2 are distinct from the high-affinity transporter reported previously [6]. Thus to date there has been no study that has yet identified the protein(s) that mediate the induced L-arginine transport in activated murine macrophages. The present study has identified CAT2 as the protein that mediates the L-arginine system-y^+ transport in cytokine- and endotoxin-activated J774-cell macrophages.

MATERIALS AND METHODS

Arginine uptake

The mouse macrophage-like cell line J774 was maintained as described previously [5]. J774 cells were plated in 24-well plates (2.5×10^5 cells/well) in 1 ml of supplemented RPMI 1640 media. Following a 2-h attachment period, cells were primed with mouse IFN-γ (20 units/ml; Sigma) for 2 h, then treated with bacterial LPS from *Escherichia coli* 055:B5 (100 ng/ml; Sigma) for 17 h. Arginine uptake (2 min at 37 °C) occurred as described previously [5] with cells incubated in 0.5 ml of uptake solution [137 mM NaCl/5.4 mM KCl/2.8 mM $CaCl_2$/1.2 mM $MgSO_4$/10 mM Hepes/Tris (pH 7.4)] supplemented with 0.1 mM L-arginine containing 1.0 μCi/ml L-[^{3}H]arginine. For uptakes using an Na^+-free solution, NaCl was substituted with equimolar amounts of *N*-methyl-D-glucamine. Uptake was stopped with ice-cold stop solution [137 mM NaCl/14 mM Tris/HCl (pH 7.4)]. Specific transport was measured by adding 5 mM of the indicated L-amino acid or N^G-monomethyl-L-arginine and N^G-nitro-L-arginine to the uptake solution. Cells were lysed in 0.5 ml of 0.5 % Triton X-100. Two 150-μl samples were removed per well and radioactivity determined by liquid scintillation. Uptake velocities per dish (2.5×10^5 cells) were calculated as pmol of L-arginine/min.

Northern-blot analysis

Total RNA was isolated from control J774 cells or those treated with IFN-γ (20 units/ml) for 2 h and/or LPS (100 ng/ml) for 6 h using 1.0 ml of RNA-isolation solution (Advanced Biotechnologies Ltd.) per 10-cm Petri dish. Northern blots contained

Abbreviations used: iNOS, inducible isoform II of nitric oxide synthase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; ODN, oligodeoxyribonucleotide; RT-PCR, reverse transcriptase PCR; GST, glutathione S-transferase; CAT, cationic amino acid transporter.

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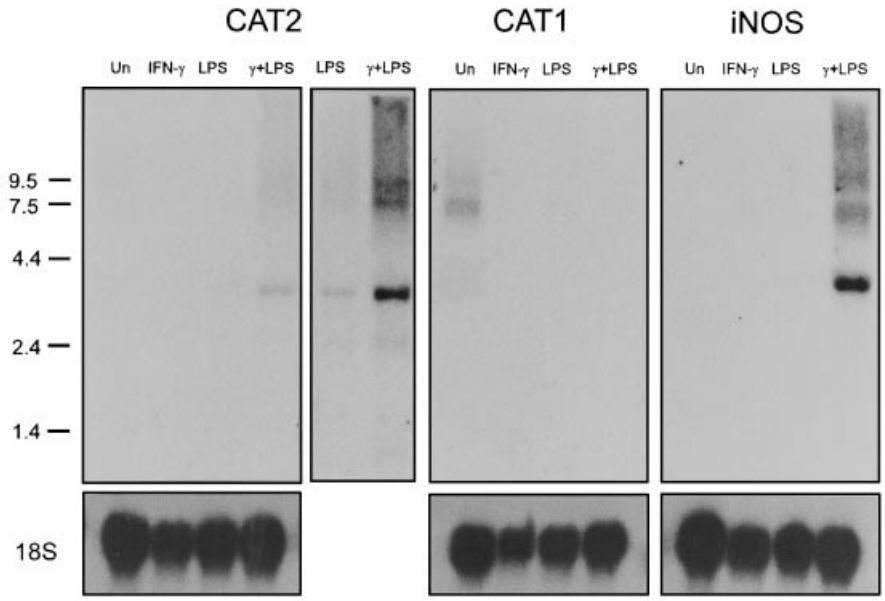

Figure 1 CAT and iNOS mRNA expression in J774 cells

Northern blot of total RNA (30 μg/lane) isolated from J774 cells cultured under the following conditions. Cells were either untreated (Un), treated with IFN-γ (20 units/ml) for 8 h (IFN-γ), with LPS (100 ng/ml) for 6 h (LPS) or with a combination of 2-h IFN-γ followed by 6-h LPS (γ + LPS). Triplicate blots were hybridized with full-length CAT2 (3.9 kb), CAT1 (2.3 kb) or iNOS (4.1 kb) cDNA probes; the loading control was an 18 S rRNA oligonucleotide probe. For signal clarity, the CAT2-probed blot shows an additional longer exposure for LPS alone and for IFN-γ and LPS.

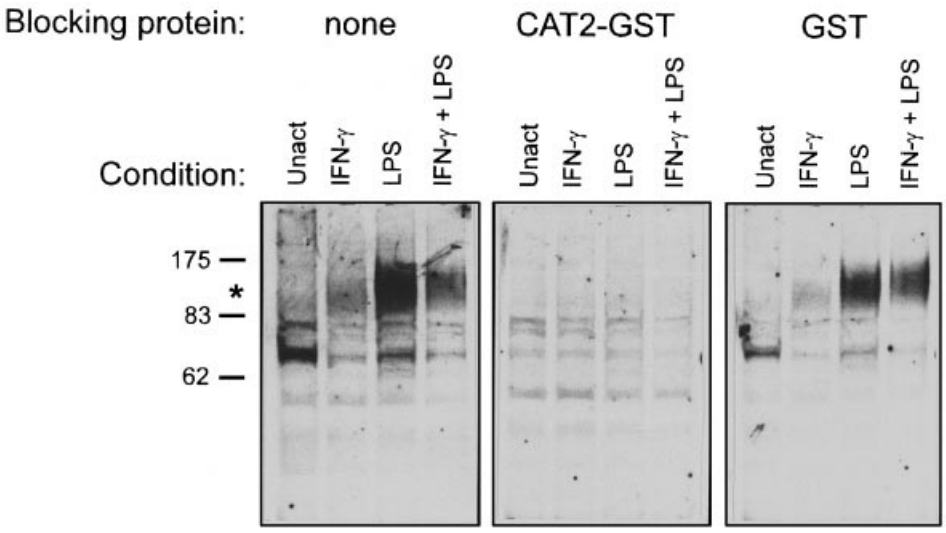

Figure 2 CAT2 protein expression in J774 cells

Protein lysates were isolated from J774 cells that were either untreated (Unact), treated with IFN-γ (20 units/ml) for 19 h (IFN-γ), with LPS (100 ng/ml) for 17 h (LPS) or with a combination of 2-h IFN-γ followed by 17-h LPS (IFN-γ + LPS) and were separated (60 μg/lane) by SDS/PAGE (10% gel). Western blots were probed with anti-CAT2 antiserum (1 μg/ml). Antibody specificity was tested using CAT2–GST fusion protein (2.5 μg/ml), GST alone (2.5 μg/ml) or no blocking protein (none). * denotes the CAT2 protein signal.

J774 total RNA (30 μg/lane) probed with full-length CAT1, CAT2 [14,18] and iNOS [19] cDNAs random primed and ^{32}P-dCTP labelled, as described previously [15]. Loading was standardized using an 18S rRNA oligo-DNA probe (5′-CATG-GTAGGCACGGCGACTACCAT-3′). Blots were washed with high stringency [0.1 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS, 42 °C] and autoradiographs quantified by scanning densitometry (Bio-Rad).

Western blotting

Anti-CAT2 antiserum was generated in rabbits by injecting CAT2–glutathione S-transferase (GST)-fusion proteins encoding the C-terminal 70 amino acids. The antiserum was IgG-enriched on a Protein-A column and by ammonium sulphate precipitation. J774 total cell lysates were prepared by adding 1 ml of RIPA buffer [1 % deoxycholate/1 % Triton X-100/0.1 % SDS/150 mM

NaCl/2 mM $MgCl_2$/10 mM Tris/HCl (pH 7.2)/2 μg/ml aprotinin/1 μg/ml pepstatin A/0.5 μg/ml leupeptin] per 10 cm dish. The lysate was centrifuged for 3 min at 12000 ***g*** and the pellet discarded. Protein content was determined by either Lowry assay (DC protein assay, Bio-Rad) or UV spectrophotometric readings at A_{280} (both methods showed similar values). Total cell lysates (60 μg/lane) were denatured with 6 M urea and separated by SDS/PAGE (10 % gel) [17]. Proteins were then transferred electrophoretically on to supported nitrocellulose (Trans-blot, BioRad). Membranes were treated at room temperature for 2 h with blocking buffer (5 % non-fat dried milk/PBS/0.1 % Tween-20/0.5 M NaCl), then with 1.0 μg/ml anti-CAT2 antiserum for 1 h, with or without GST or CAT2-C-terminus–GST-fusion proteins (pGEX-3X, Pharmacia) (2.5 μg/ml in 4 ml), followed by secondary goat anti-rabbit IgG (1:5000, BioRad) for 1 h (antiserum incubations were in blocking buffer). Proteins were detected by chemiluminescence (Renaissance, NEN Life Science Products) and autoradiographs were quantified by scanning densitometry (Bio-Rad).

Xenopus oocyte studies

Xenopus laevis oocytes were isolated and prepared as described previously [15,20]. Poly(A^+) RNA was purified from J774 cells [15,20]. Stage-VI oocytes were injected with water (control) or with 10 ng of poly(A^+) RNA (0.2 μg/μl) from unactivated or activated macrophages. Amino acid uptake was performed as described previously [15,20]. Hybrid-depletion experiments were performed using CAT2 oligodeoxyribonucleotides (ODNs): sense (5′-TATCCAAGACTTCTTTGCCGTGTGC-3′, 489–513 bp) and antisense (5′-GTAGGCTGAAACCCTGTCCTT-GC-3′, 1406–1429 bp), as described previously [20].

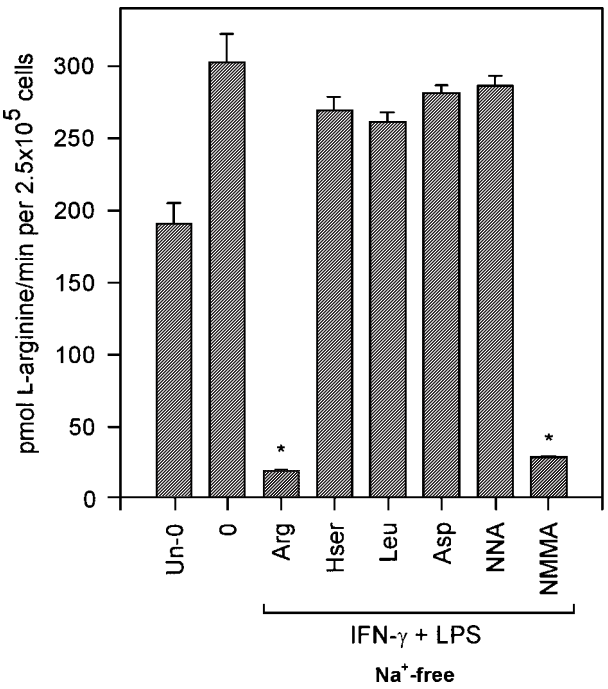

Figure 3 Characterization of L-arginine transport in J774 cells

[^{3}H]L-Arginine uptake was performed using unactivated (Un) or activated (INF-γ + LPS) J774 cells treated with INF-γ (20 units/ml, 2 h) followed by LPS (100 ng/ml, 17 h) in the absence of sodium. *N*-Methyl-D-glucamine (Na^+-free) was used, with no inhibitor (0) or in the presence of L-arginine (Arg), L-homoserine (Hser), L-leucine (Leu), L-aspartic acid (Asp), L-N^G-nitro-arginine (NNA) or L-N^G-methyl-arginine (NMMA), each at 5.0 mM. These data represent the means ± S.E.M. of three independent experiments (*$P < 0.001$).

RESULTS

As demonstrated previously [5,6,8,9,21,22], our uptake studies in J774 macrophage cells confirmed that there is an increased L-arginine transport following activation with IFN-γ and LPS (results not shown). To identify the transporter(s) responsible for the induced L-arginine uptake, the expression of CAT1, CAT2, CAT3, rBAT and 4F2hc mRNAs (where rBAT and 4F2hc encode amino acid transporters of systems b^{0+} and y^+L) were measured and compared with iNOS mRNA levels in J774 cells. Northern-blot analysis revealed that CAT2 mRNA (3.9 and 8.5 kb) was nearly undetectable in unactivated and IFN-γ-treated J774 cells, but was induced more than 5-fold by LPS alone, and by IFN-γ and LPS (Figure 1). In contrast, CAT1 mRNA (7.4 kb) levels decreased following all three treatments (Figure 1). The induction of iNOS mRNA (4.1 kb) paralleled CAT2 mRNA levels, with a 25-fold increase upon exposure to both IFN-γ and LPS (Figure 1). CAT2a (a splice variant of CAT2) transcripts [17] were only faintly detectable by reverse transcriptase PCR (RT-PCR) and the abundance of this message did not change following macrophage activation (results not shown). CAT3 mRNA was detected in neither J774 cells nor peritoneal macrophages when assessed by RT-PCR and Northern-blot analysis (B. Nicholson and C. L. Macleod, unpublished work). Neither rBAT [15] nor 4F2hc [16] mRNAs were detected by Northern-blot analysis, although 4F2hc (but not rBAT) was amplified measurably by RT-PCR in both resting and activated J774 cells (results not shown).

CAT2 protein expression was assessed in unstimulated and activated J774 cells by Western-blot analysis (Figure 2). CAT2 protein levels increased slightly following the addition of IFN-γ and to a greater extent following treatment with LPS alone or with IFN-γ and LPS (Figure 2). CAT2 antiserum detected a $\approx$ 90-kDa glycoprotein. The signal was competed away by the immunogen CAT2–GST, but not by GST alone (Figure 2). Transport analysis of activated J774 cells was performed to confirm that CAT2-mediated system-y^+ transport activity was induced following activation and to assess whether systems y^+L (encoded in part by 4F2hc) or b^{0+} (encoded by rBAT) may mediate a portion of the L-arginine transport in these cells. The failure of L-leucine (a competitive inhibitor of systems y^+L and b^{0+}) to inhibit L-arginine uptake in the absence of Na^+ eliminates the involvement of these systems in activated J774 cells (Figure 3). Furthermore, the fact that L-leucine transport was not induced in activated J774 cells (data not shown), excludes the involvement of systems y^+L or b^{0+}. Consistent with previous studies [7], activated J774-cell-mediated NO synthesis was decreased 78 % in the absence of exogenous L-arginine (results not shown). Thus we show that in the J774 macrophage cell line (Figure 3) and in bone-marrow-derived macrophages [5], system y^+ was indeed induced, which is consistent with CAT2 expression and argues against the involvement of other transporters such as CAT1, CAT2a, CAT3, 4F2hc or rBAT.

Although CAT2 was identified as a candidate carrier responsible for increased L-arginine influx in activated J774 cells, it could be possible that other, as yet unidentified, carriers may contribute to the increased L-arginine transport. We therefore used the *Xenopus* oocyte expression system to establish the quantitative importance of CAT2 as the induced L-arginine transporter in activated J774 cells. System-y^+ transport activity was induced in *Xenopus* oocytes injected with mRNA from IFN-γ- and LPS-activated J774 cells (Figure 4A), paralleling the

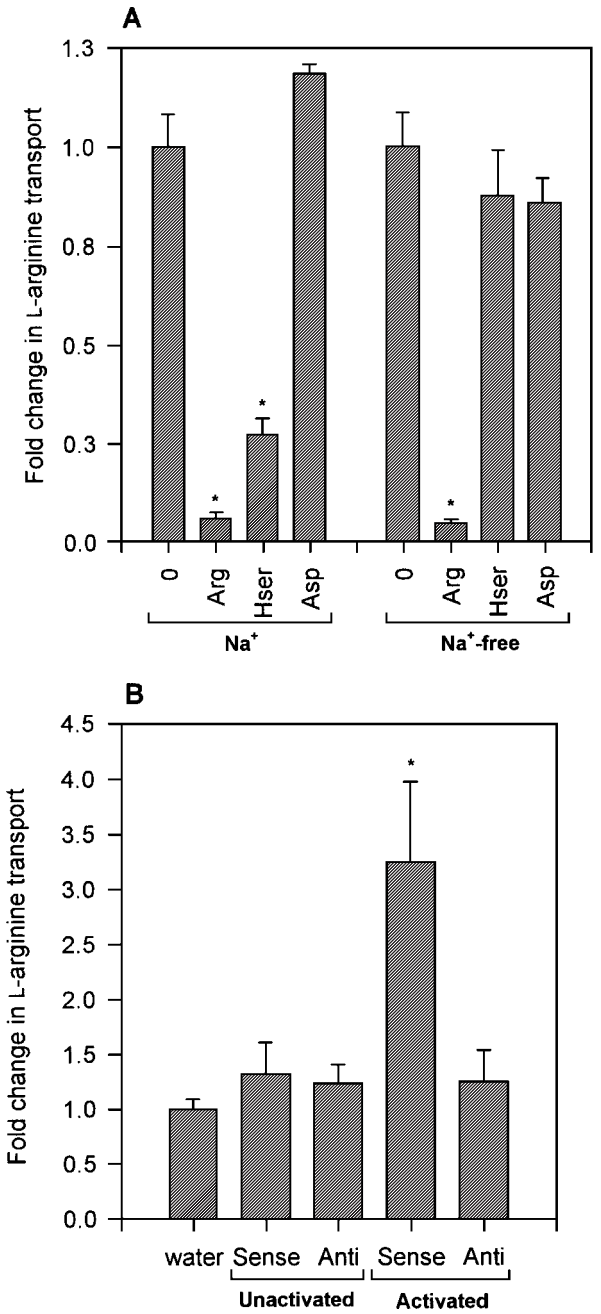

Figure 4 Specificity of L-arginine transport (A) and CAT2-specific antisense inhibition of J774 mRNA (B) in *Xenopus* oocytes

(**A**) Individual oocytes were injected with 600 ng of mRNA derived from J774 cells activated with IFN-γ (20 units/ml, 2 h) and LPS (100 ng/ml, 6 h). [^{3}H]L-Arginine uptake was performed in oocytes 3 days post injection, in the presence (Na^+) or absence (Na^+-free; choline chloride) of sodium, with no inhibitor (0) or in the presence of L-arginine (Arg), L-homoserine (Hser) or L-aspartic acid (Asp) each at 5.0 mM. (**B**) Oocytes were injected with either water or 600 ng of mRNA from unactivated or activated J774 cells, as described for (**A**). Prior to injection, mRNA was subjected to CAT2 sense (Sense) or antisense (Anti) ODN hybrid depletion. Data are shown as the means $\pm$ S.E.M. of four (**A**) or three (**B**) independent experiments (*$P < 0.001$).

induced L-arginine transport observed in activated J774 cells (Figure 3). In addition, L-homoserine inhibition of L-arginine transport was only observed in the presence of Na^+ ($P < 0.001$), with no inhibition by L-aspartic acid (Figure 4A), suggesting that system y^+ was expressed in J774-mRNA-injected oocytes. To finally provide conclusive evidence for CAT2 involvement in the induced L-arginine transport in activated J774 cells, hybrid-depletion experiments in *Xenopus* oocytes were performed. CAT2 antisense ODNs annealed to mRNA from activated J774 cells showed complete inhibition of the induced L-arginine transport to levels observed in water-injected (control) oocytes ($P < 0.05$, Figure 4B), whereas CAT2 sense ODNs had no effect. Similar inhibitory effects with CAT2 antisense ODNs were observed on the induced NO synthesis in *Xenopus* oocytes injected with mRNA from activated J774 cells (results not shown).

DISCUSSION

LPS and IFN-γ combine to induce iNOS mRNA and protein levels in macrophages, leading to the release of the cytotoxic mediator NO [1–4]. Arginine is essential for macrophage cytotoxic killing of target cells [3] and NO synthesis requires the uptake of external arginine into macrophages, cardiac myocytes, astrocytes and vascular smooth-muscle cells [7,19,21,23]. Since several distinct transport proteins could mediate macrophage L-arginine uptake [10–16,24], we investigated the presence of known transporter mRNAs in J774 cells. The multiple genes that encode L-arginine transporters CAT1, CAT2, CAT3, rBAT and 4F2hc have distinct expression patterns [10,12,15,16] and their expression changes in response to external signals. Within the CAT family of transporters, there are differences in expression and promoter usage depending on the tissue and conditions [12,26–29]; even the alternatively spliced CAT2 and CAT2a isoforms have a distinct tissue distribution and inducibility in response to different conditions and stresses [29]. Studies of induced L-arginine transport and CAT2 expression have been reported recently [22]. Our studies show that CAT1, CAT2 and 4F2hc mRNAs, but not rBAT or CAT2a mRNAs, were detected by either Northern-blot analysis or RT-PCR, suggesting that several transporters could be involved in L-arginine uptake; however, some may only have a minor contribution. Our analysis of gene-expression patterns following activation with LPS and IFN-γ revealed that 4F2hc mRNA levels remained unchanged (results not shown) and that CAT1 mRNA decreased (Figure 1). CAT2 mRNA did not increase discernibly in cells treated with IFN-γ alone, but required both IFN-γ and LPS for maximal induction (Figure 1), suggesting that IFN-γ and LPS may work synergistically on CAT2 mRNA synthesis or stability. The decrease in CAT1 mRNA may suggest that it makes a minimal contribution to total L-arginine transport in activated cells, whereas the increases in CAT2 mRNA (Figure 1) and protein (Figure 2) indicate that CAT2 may provide a greater role in the induced L-arginine transport in activated J774 cells. The increase in CAT2 mRNA was concordant with increases in iNOS mRNA (Figure 1), suggesting co-ordinate mRNA expression.

LPS alone was sufficient to induce maximal CAT2 protein expression (Figure 2), further correlating CAT2 involvement in L-arginine transport in J774 cells. However, CAT2 protein levels were reduced in IFN-γ- and LPS-treated cells (Figure 2), suggesting that IFN-γ may block the stimulatory effect of LPS on CAT2 translation or reduce protein stability, without affecting its synergistic effects on increases in CAT2 (and iNOS) mRNA levels. Our experiments (results not shown) and those of others [2,5–7,22] have demonstrated that the combination of IFN-γ with LPS stimulates NO production to a greater extent than IFN-γ or LPS alone. The raised NO synthesis may possibly down-regulate CAT2 protein at the level of translation and/or protein degradation. This hypothesis is consistent with the CAT2 transporter playing a pivotal role in NO production; however, further work is required to clarify this issue.

Although CAT1 and 4F2hc mRNAs were detected in untreated J774 cells, neither transporter is likely to contribute significantly towards L-arginine transport in IFN-γ- and LPS-activated J774 cells, since CAT1 mRNA levels in fact declined following activation (Figure 1) and there was no increase in L-leucine transport (system y^+L, encoded in part by 4F2hc) following activation (results not shown). rBAT mRNA was not detected by Northern blotting or RT-PCR in untreated or activated J774 cells (results not shown), supporting our transport data (Figures 3 and 4A) that system b^{0+} is not expressed in these cells. Furthermore, our *Xenopus* oocyte hybrid-depletion experiments documented clearly the fact that the induced L-arginine transport in activated J774 cells was due to CAT2 (Figure 4B). CAT2 antisense ODNs not only blocked the induced L-arginine transport (Figure 4B), but also reduced the elevated NO synthesis in oocytes injected with mRNA from activated J774 cells (results not shown), suggesting that CAT2 protein expression may be essential for maintaining NO synthesis in activated J774-cell macrophages.

In summary, the CAT2 transporter was identified as the specific protein mediating increased L-arginine transport in activated J774 macrophage cells. Transport analysis revealed that system y^+, and not systems y^+L or b^{0+}, was induced in J774 cells by IFN-γ and LPS. The increase in both CAT2 mRNA and protein in IFN-γ- and LPS-activated cells parallels the induction of iNOS mRNA and NO synthesis. By hybrid depletion in *Xenopus* oocytes, direct evidence was presented that CAT2 is essential for the stimulated L-arginine transport. Taken together, the data support a central role for CAT2 in providing L-arginine for the synthesis of NO. Ultimate confirmation of the importance of this transporter comes from examination of peritoneal macrophages from mice lacking the CAT2 gene (*Cat2*$^{-/-}$ mice), which indicate an essential role of CAT2-mediated L-arginine transport for NO production (C. L. MacLeod, unpublished work). Defining the transport systems and identifying the regulatory mechanisms of specific proteins involved in L-arginine transport in resting and activated macrophages may provide new targets and specificity for the clinical management of NO synthesis.

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