INFγ stimulates arginine transport through system y^+L in human monocytes

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Abstract Freshly isolated human monocytes transport L-arginine mostly through a sodium independent, NEM insensitive pathway inhibited by L-leucine in the presence, but not in the absence of sodium. Interferon-γ (IFNγ) stimulates this pathway, identifiable with system y^+L, and markedly enhances the expression of SLC7A7, the gene that encodes for system y^+L subunit y+LAT1, but not of SLC7A6, that codes for the alternative subunit y+LAT2. System y^+ plays a minor role in arginine uptake by monocytes and the expression of system y^+-related genes, SLC7A1 and SLC7A2, is not changed by IFNγ. These results demonstrate that system y^+L is sensitive to IFNγ.

Keywords: Monocyte; Interferon-γ; Arginine transport; Lysinuric protein intolerance; System y^+L

1. Introduction

Four distinct mechanisms, named systems y^+, y^+L, $b^{0,+}$, and $B^{0,+}$, account for the transport of cationic amino acids (CAA) in mammalian cells (see [1] for review). Among these, system y^+L is an exchange route that recognizes CAA in the absence of sodium but requires the cation to interact with neutral amino acids such as leucine [2]. Its activity can be easily discriminated from system y^+ due to the sensitivity of the latter to NEM inhibition [3]. System y^+L is a glycoprotein-associated amino acid heterodimeric transporter, whose heavy chain is the glycoprotein CD98/4F2hc and the alternative light chains, y+LAT1 or y+LAT2, are responsible for the recognition and operational features of the transport process [4]. While the system has been implied in the *trans*-epithelial absorption of CAA [4], its functional role in non-epithelial tissues is still unclear and definite examples of regulatory changes of its activity have not yet been reported.

In several cell models of monocyte/macrophage lineage, pro-inflammatory compounds, such as LPS and IFNγ, stimulate the transport of CAA. This effect has been usually referred to the induction of the system-y^+-related CAT-2B transporter [5–9], while no information is available on possible changes in system y^+L activity under the same conditions. However, most of those studies were performed on murine models and recent evidence suggests that the regulation of CAA transport in murine and human monocytes/macrophages may be different [10]. On the other hand, although the expression of system y^+L in human leukocytes has been reported [11,12], it has not been neither referred to a particular cell type nor studied in cytokine-treated cells.

Here, we demonstrate that system y^+L is the main transport mechanism for arginine in human monocytes and that it is specifically upregulated by IFNγ through the induction of SLC7A7/y+LAT1.

2. Materials and methods

2.1. Monocyte isolation and culture conditions

Mononuclear cells were separated from buffy coats, obtained from six normal healthy volunteers, supplied by the Unità di Immunoematologia e trasfusione of Azienda Ospedaliera di Parma (Parma). The buffy coats, diluted 1:4 with PBS, were layered on 10 ml of Fycoll Hypaque and centrifuged at $750\times g$ for 30 min at 20 °C. Peripheral blood mononuclear cells (PBMC) at the interface were removed, washed three times in PBS, and centrifuged at $150\times g$ for 10 min at 20 °C. After the final wash, PMBC were suspended in RPMI containing 2% endotoxin-free fetal bovine serum (FBS) and seeded on plastic-ware appropriate for the various determinations. After a 30-min incubation at 37 °C in an atmosphere at 5% CO_2, non-adherent cells were removed with three vigorous washes with prewarmed sterile Earle's Balanced Salt Solution (EBSS). Adherent monocytes were employed immediately (for characterization of arginine transport and expression of CAA transporters in freshly isolated cells) or collected, resuspended in RPMI supplemented with 10% FBS in the absence or in the presence of IFNγ (10 ng/ml), and maintained at 37 °C under gentle agitation for the indicated times. After the incubation, monocytes were let to sediment for 1 h before the experimental determinations. The experiments shown are typical experiments with variability among transport values not exceeding 30%. To assess the purity of the preparation, cells were stained with monocyte-specific anti-CD14 mAb. More than 75% of the isolated cells express CD14 (not shown).

2.2. L-Arginine influx

For transport studies, cells were seeded on 96-well dishes. Monocytes, washed once with a modified bicarbonate-free EBSS buffered at pH 7.4 with 20 mM Tris/HCl, were incubated for 1 min in 50 μl of the same solution containing [^{3}H]-arginine (4 μCi/ml, 100 μM). In this interval of time, arginine uptake approached linearity (results not

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Abbreviations: CAA, cationic amino acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFNγ, Interferon-γ; LPI, lysinuric protein intolerance; NEM, *N*-ethyl-maleimide

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shown). The experiment was terminated by two rapid washes (<10 s) in cold PBS and cell monolayers were extracted in 50 μl ethanol. The radioactivity in cell extracts was determined with Microbeta Trilux (Wallac). Extracted cells were then dissolved with 0.5% sodium deoxycholate in 1 M NaOH and protein content was determined directly in the well using a modified Lowry procedure as previously described [13]. In the experiments in which Na^+-independent transport was to be measured, a modified Na^+-free EBSS (NMG-EBSS) was employed. In this solution NaCl and NaH_2PO_4 were replaced, respectively, by *N*-methyl-D-glucamine and choline salts.

2.3. Expression of influx data

Amino acid influx is expressed as nmoles per mg of protein per minute. Kinetic parameters of arginine influx were determined by non-linear regression analysis using the equation:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} + K_D \cdot [S] \quad (1)$$

for a transport process resulting from the additive operations of a saturable system and a non-saturable component

$$v = v_0 - \frac{I_{max} \cdot [I]}{I_{0.5} + [I]} \quad (2)$$

for a competitively inhibited system, where v_0 is the influx in the absence of the inhibitor, I_{max} is the maximal inhibition and $I_{0.5}$ is the inhibitor concentration required for half maximal inhibition.

2.4. Reverse transcription and semi-quantitative PCR

For expression studies, monocytes were seeded on 6-well trays. Isolation of total RNA and reverse transcription were performed as described previously for endothelial cells [13]. Briefly, 100 ng of single-stranded cDNA from each sample was amplified with 1.0× PCR buffer, 0.2 mM each dNTPs, 2.5 mM $MgCl_2$, along with proband primers and 1.25 U of Hot Master *Taq* DNA polymerase. Primer sequences were reported previously [13]. Number of cycles is detailed for each experiment. Images of the electrophoresed cDNAs were recorded with a digital DC 120 Kodak camera and quantified by ID Image Analysis Software (Kodak Digital Science). In semi-quantitative experiments, GAPDH primers were also added in the amplification mixture and the results were expressed as the densitometric ratio of proband vs. GAPDH product.

2.5. Materials

Endotoxin-free FBS was purchased from Celbio and culture medium (RPMI 1640) from Cambrex Bio Science. Human recombinant INFγ and CD14 mAb were from Vinci Biochem. [L-2,3,4-^{3}H]Arginine 45–70 Ci/mmol) was obtained from Perkin–Elmer Italia, Hot Master *Taq* polymerase from Eppendorf SrL, and Fycoll Hypaque from Amersham Pharmacia Biotech. Sigma was the source of all the other chemicals.

3. Results

3.1. Characterization of L-arginine transport in human monocytes

To discriminate the different components of CAA uptake in freshly isolated human monocytes, maintained in control medium (FBS-supplemented RPMI), L-arginine uptake was inhibited, in the presence or in the absence of sodium, with increasing concentrations of L-leucine (Fig. 1, upper panel). The assay was performed with cells either treated or untreated with NEM, a well known inhibitor of system y^+ but not of system y^+L [3]. In the absence of leucine, the substitution of extracellular sodium did not inhibit L-arginine uptake, thus excluding a significant contribution of sodium dependent transport systems, such as $B^{0,+}$. Leucine inhibited arginine transport only in the presence of sodium, a result compatible with system y^+L activity, but excluding a significant contribution of system $b^{0,+}$. The kinetic analysis of inhibition by leucine indicated that, at an arginine concentration of 100 μM, the influx of the CAA was leucine-inhibitable by more than 65% with an $I_{0.5}$ of 0.21 mM. However, if L-leucine was present at the maximal concentration employed (2 mM), the addition of 2 mM lysine did not produce any further decrease of arginine transport, thus indicating the complete inhibition of high affinity arginine influx by leucine. In the absence of sodium, leucine actually exerted a significant *cis*-stimulation of arginine influx. Under all conditions, the pre-incubation of monocytes with NEM (0.4 mM for 2 min) did not inhibit L-arginine uptake, confirming that system y^+ contribution was, at best,

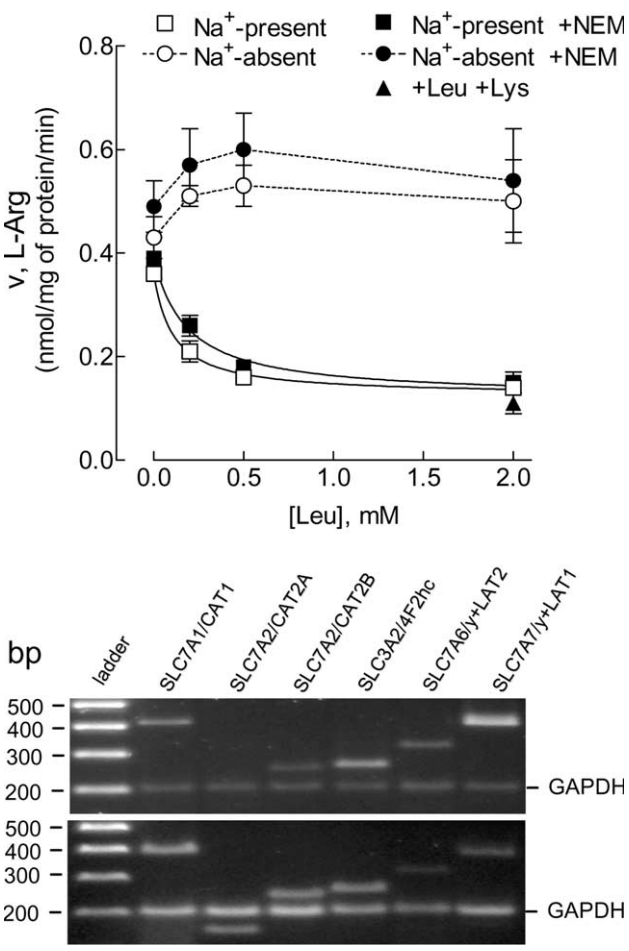

Fig. 1. Characterization of L-arginine influx in human monocytes. Upper panel: Freshly isolated monocytes, seeded for 1 h in RPMI supplemented with 10% FBS, were washed in EBSS or Na^+-free NMG-EBSS, as indicated. Arginine uptake was then assayed with 1-min incubations in the same solution employed for washing, supplemented with 100 μM L-[^{3}H]arginine in the presence of the indicated concentrations of L-leucine. (▲) Arginine uptake in the presence of 2 mM L-leucine + 2 mM L-lysine. Where indicated, before the uptake assay, cells were pre-treated for 2 min with NEM (0.4 mM). Points are means of four independent determinations with S.D. indicated when greater than point size. Lines represent the best fit of experimental data to Eq. (2) (see Section 2). Middle panel: RT-PCR products (35 cycles of amplification) obtained with the primers of the indicated genes (see Section 2). The experiment was repeated in cells isolated from three different subjects with comparable results. Lower panel: RT-PCR products obtained with the same primers employing RNA isolated from Calu-3 human lung adenocarcinoma cells as positive control. 35 cycles of amplification were employed.

marginal. The kinetic analysis of total arginine transport (see Eq. (1)) was compatible with the operation of a single saturable system, endowed with a K_m of 51.8 ± 9.6 μM and a V_{max} of 1.028 ± 0.17 nmol/mg of protein/min (results not shown).

Fig. 1, middle panel, shows the RT-PCR analysis of CAA transporters expressed in human monocytes. The expression of SLC7A1, coding for system y^+ transporter CAT1, was clearly detectable, whereas SLC7A2 expression was less evident and detected only for the CAT2B transcript. Transcripts encoded by all the known genes for system y^+L were detectable in human monocytes: SLC3A2, that codes for system y^+L heavy chain CD98/4F2hc, SLC7A7, that codes for the light chain y+LAT1, and SLC7A6, for the alternative y+LAT2 light chain.

3.2. Effect of IFNγ on L-arginine uptake in human monocytes

To study the effect of IFNγ on the discriminated influx of arginine in human monocytes, cells were maintained in a complete growth medium in the presence of 10 ng/ml IFNγ for 24 or 48 h (Fig. 2). L-arginine influx was determined either in the absence of inhibitors or in the presence of 2 mM leucine, to inhibit system y^+L transport activity, or of 2 mM leucine + 2 mM lysine, to inhibit the whole saturable component of arginine transport. Therefore, the portion of arginine transport inhibited by leucine was ascribed to system y^+L activity, while the transport further inhibited by lysine was considered the contribution of system y^+. After a 24 h-treatment (panel A), IFNγ produced a modest, although significant, increase of L-arginine transport, that was fully attributable to an increase of system y^+L activity. After 48 h of treatment (panel B), the stimulation of arginine influx by IFNγ was much more evident and, again, fully accounted for by a marked increase of system y^+L transport activity (+290% compared with control values). Values of arginine transport in control, untreated cells remained substantially unchanged throughout the 48-h incubation.

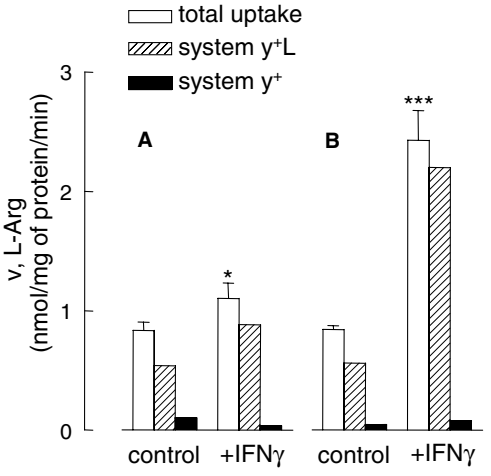

Fig. 2. Effect of incubation with IFNγ on the discriminated transport of L-arginine in human monocytes. Monocytes were incubated in RPMI containing 10% FBS in the absence or in the presence of IFNγ (see Section 2) for 24 h (Panel A) or 48 h (Panel B). After this time, L-arginine uptake assay was performed as described in the legend of Fig. 1. System y^+L: Leucine-inhibitable component of arginine influx, calculated as the difference between total influx and the influx measured in the presence of 2 mM leucine. System y^+: the component of arginine influx, calculated as the difference between the influx measured in the presence of 2 mM leucine and the influx measured in the presence of both 2mM leucine and 2 mM lysine. Bars are means of four independent determinations with S.D. indicated. The experiment was repeated in cells isolated from five different donors with comparable results. $*P < 0.05$; $***P < 0.001$.

3.3. Effects of IFNγ on the expression of CAA transporter genes in human monocytes

Fig. 3 reports the results of a semi-quantitative RT-PCR analysis of the expression of genes for CAA transporters in monocytes treated with IFNγ. With the number of cycles adopted for each proband cDNA, all the proband transcripts were clearly detectable and their signal was not saturated. The only gene significantly induced by the cytokine was SLC7A7 (top left panel). The densitometric analysis (top right panel) revealed that, in IFNγ-treated monocytes, the relative abundance of SLC7A7 expression, corrected for GAPDH, was increased more than twofold at 24 h and more than threefold at 48 h compared with control, untreated cells. Under the same conditions, the expression of SLC3A2, the gene for system y^+L heavy chain CD98/4F2hc, and of SLC7A6, the gene for the alternative light chain y+LAT2, exhibited no significant change (middle panel, left) as demonstrated by the densitometric analysis (middle panel, right). Also the RT-PCR products of system y^+-related genes SLC7A1 and SLC7A2 (CAT2B transcript) were not changed by the treatment with the cytokine (bottom panels).

4. Discussion

Arginine transport in monocytes/macrophages has been extensively investigated, but most of these studies have employed rodent cell lines [5–9]. In those models, the contribution of system y^+ to CAA transport has been emphasized due to the regulatory links that exist between CAT transporters and nitric oxide production [8]. Much less data are available for human models. In a preparation of human PBMC (85% lymphocytes/15% monocytes), comparable contributions of systems y^+ and y^+L to CAA transport have been reported [14]. Moreover, with a similar cell preparation, Reade et al. [15] attribute only a minor role to system y^+ in non-stimulated PBMC, although its activity is increased in septic shock patients. The present study demonstrates that, while the contribution of system y^+ appears marginal, system y^+L is the major transport system for arginine under basal conditions and its activity is markedly stimulated by IFNγ. Since monocytes are the typical targets of this cytokine, the stimulation of system y^+L may be involved in the activation process induced by IFNγ in these cells. It should be stressed that circulating monocytes are quiescent cells that, once migrated into peripheral tissues, undergo a complex maturation process to reach full differentiation and functional competence. Indeed, the production of nitrites by these cells is very low and uncorrelated to the stimulation of arginine transport through system y^+L by IFNγ (B.M. Rotoli, unpublished results).

The main characteristics shown by system y^+L in human monocytes are similar to those found in human mesenchymal models [13,16]. At variance with those models, however, human monocytes exhibit a significant stimulation of arginine transport by extracellular leucine under sodium-free conditions

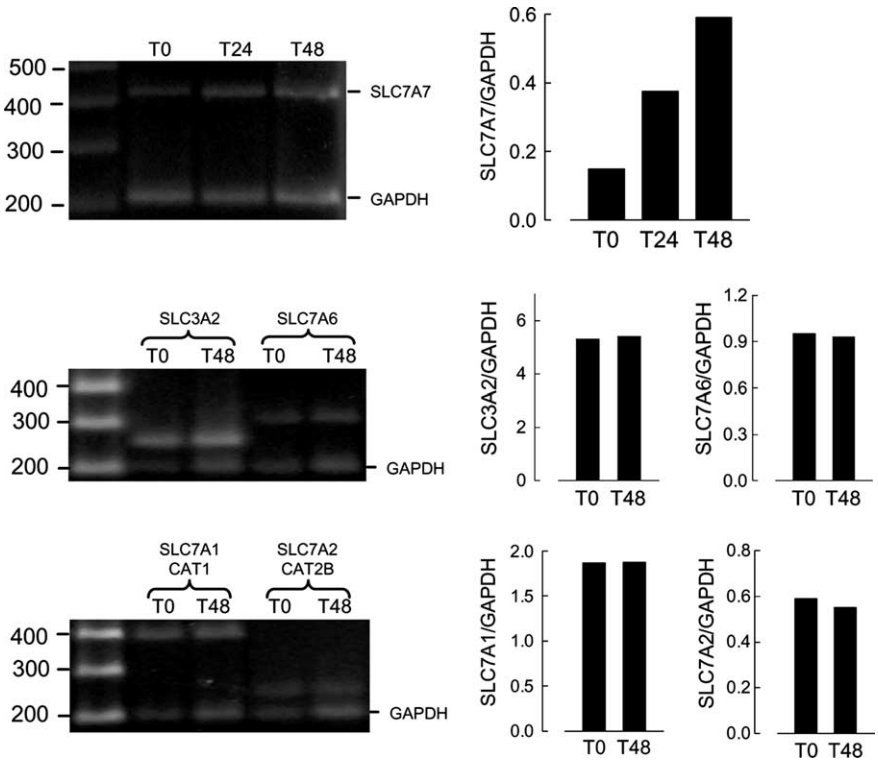

Fig. 3. Effect of IFNγ on the expression of genes related to transporters for CAA in human monocytes. Cells were incubated for the indicated times in the presence of 10 ng/ml of IFNγ before RNA extraction. For the analysis of SLC7A1, SLC7A2/CAT2B, SLC3A2, and SLC7A6, after reverse transcription the cDNA solution was divided into two aliquots. One, supplemented with GAPDH primers, underwent 25 cycles of amplification to detect GAPDH transcript. The other, supplemented with the proband primers, underwent 35 cycles of amplification. The amplification products of GAPDH and the proband transcript, mixed together, were then put on the same gel. For the analysis of SLC7A7, proband and GAPDH primers were put in the same amplification mixture and underwent 32 cycles of co-amplification. Left: Images of RT-PCR products. SLC7A7 (upper panel), SLC3A2 and SLC7A6 (middle panel), SLC7A1 and SLC7A2/CAT2B (lower panel) at the indicated times of incubation in the presence of IFNγ. Right: Densitometric analyses of the gels shown at left. In each panel, the relative intensity of the amplification product of the proband cDNA was normalized to that of the GAPDH product obtained from the amplification of the same cDNA mixture. The analysis, repeated in three different monocytes preparations, yielded comparable results.

(Fig. 1). This effect may be explained by a *trans*-stimulation of arginine influx by leucine entered into the cells through parallel pathways other than system y^+L during the transport assay. This explanation would require conditions, such as an outwardly directed sodium gradient and a low intracellular amino acid pool, that may actually occur in the experimental setting adopted.

It is generally accepted that the y^+L light chain y+LAT1 is the subunit expressed in absorbing epithelia, while the alternative isoform y+LAT2 would be preferentially expressed in non-epithelial tissues [4]. However, it is known that y+LAT1 also is expressed in non-epithelial cells, such as human fibroblasts [16], human endothelial cells [13], and leukocytes [11,12,15]. This contribution not only demonstrates that SLC7A7/y+LAT1 is clearly expressed by human monocytes but also shows the stimulation of its expression by IFNγ, a thus far unknown effect of the cytokine. Although no previous report had indicated SLC7A7 as a target of IFNγ, Mykkanen et al. [17] have identified in the 5′ regulatory region of the gene several putative transcription factor binding sequences, such as NFAT, that could be involved in IFNγ signal transduction. Mutations of *SLC7A7* gene cause the autosomal recessive condition lysinuric protein intolerance, LPI [18]. LPI is characterized by selective CAA loss in the urine associated with several extra-renal alterations, such as a severe alveolar proteinosis, suggestive for the involvement of cells of monocyte–macrophage lineage. The clear-cut expression of SLC7A7/y+LAT1 in human monocytes and its induction by IFNγ strengthen the possibility that these cells are a phenotypic target of LPI-associated mutations and prompt further investigations of this issue.

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