

The stimulation of arginine transport by TNF α in human endothelial cells depends on NF- κ B activation

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Abstract

In human saphenous vein endothelial cells (HSVECs), tumor necrosis factor- α (TNF α) and bacterial lipopolysaccharide (LPS), but neither interferon γ (IFN γ) nor interleukin 1 β (IL-1 β), stimulate arginine transport. The effects of TNF α and LPS are due solely to the enhancement of system y⁺ activity, whereas system y^{+L} is substantially unaffected. TNF α causes an increased expression of SLC7A2/CAT-2B gene while SLC7A1/CAT-1 expression is not altered by the cytokine. The suppression of PKC-dependent transduction pathways, obtained with the inhibitor chelerythrine, the inhibitor peptide of PKC ζ isoform, or chronic exposure to phorbol esters, does not prevent TNF α effect on arginine transport. Likewise, ERK, JNK, and p38 MAP kinases are not involved in the cytokine effect, since arginine transport stimulation is unaffected by their specific inhibitors. On the contrary, inhibitors of NF- κ B pathway hinder the increase in CAT2B mRNA and the stimulation of arginine uptake. These results indicate that in human endothelial cells the activation of NF- κ B pathway mediates the TNF α effects on arginine transport.

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1. Introduction

L-Arginine, the precursor of nitric oxide (NO), enters mammalian cells through several membrane-bound transporters: system y⁺, b^{0,+}, B^{0,+}, and y^{+L} [1]. The widely expressed systems y⁺ and y^{+L} are referable, respectively, to the CAT family of monomeric transporters and to the

recently characterized group of heterodimeric amino acid transporters (HATs) [2–4]. Systems y⁺ and y^{+L} can be easily discriminated, since the former is sensitive to *N*-ethylmaleimide (NEM) while the latter can interact with neutral amino acids, such as leucine, although only in the presence of sodium [5]. Human umbilical vein endothelial cells (HUVECs) express CAT-1 and CAT-2B, for system y⁺, as well as y + LAT-1, y + LAT-2, and 4F2hc, for system y^{+L}, and transport arginine through the additive operations of the two systems [6].

In various cell models, pro-inflammatory mediators, such as tumor necrosis factor- α (TNF α), interleukin 1 β (IL-1 β), and interferon γ (IFN γ), enhance the expression of CAT transporters and the influx of arginine [7–9]. Also in HUVECs, as in other endothelial models [10,11], arginine transport is stimulated by TNF α through an increase in CAT-2B expression. On the contrary, the activity and the expression of system y^{+L} are not changed by the cytokine [6]. However, the signal transduction mechanisms associated with CAT induction and transport enhancement have been poorly characterized in endothelial cells.

Abbreviations: CAPE, caffeic acid phenethyl ester; CFPD, 2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1, 2-dihydropyrazol-3-one; DRB, 5,6-dichlorobenzimidazole riboside; EBSS, Earle's balanced salt solution; FBS, fetal bovine serum; HSVECs, human saphenous vein endothelial cells; HUVECs, human umbilical vein endothelial cells; IL-1 β , interleukin 1 β ; IFN γ , interferon γ ; LPS, lipopolysaccharide; M199, medium 199; MG132, peptide aldehyde Cbz-Leu-Leu-Leucinal; NEM, *N*-ethylmaleimide; NOS, nitric oxide synthase; PD98059, 2'-amino-3'-methoxyflavone; PDTIC, pyrrolidine dithiocarbamate; PPM-18, α -benzoylamino 1,4-naphthoquinone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, anthra [1,9]pyrazol-6[2*H*]-one; TNF α , tumor necrosis factor- α

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In this model, TNF α induces the expression of many genes through the activation of NF- κ B transcription factor [12]. NF- κ B is regulated primarily by phosphorylation of inhibitory proteins, the I κ Bs, which withhold the factor in the cytoplasm of non-stimulated cells. In response to TNF α , I κ B kinase (I κ BKK) phosphorylates the I κ Bs that are ubiquitinated and degraded, thus permitting the nuclear translocation of the NF- κ B [12]. In the nucleus, NF- κ B transcriptional activity can be modulated by several TNF α responsive protein kinases, such as the p38 subgroup of MAPKs, providing a cross-talk point with other signaling pathways [13].

This study was undertaken to identify the transduction pathways involved in TNF α stimulation of endothelial arginine transport through system y⁺. We demonstrate here that in a model of adult human endothelium, human saphenous vein endothelial cells (HSVECs), TNF α enhances the influx of cationic amino acid through the activation of NF- κ B.

2. Materials and methods

2.1. Cells and experimental treatment

Strains of HSVECs were obtained from three distinct patients undergoing coronary artery bypass grafting as already described [14]. Cells were routinely grown in collagen-coated 10-cm diameter dishes in medium 199 (M199), with glutamine concentration raised to 2 mM. The culture medium was supplemented with 20% fetal bovine serum (FBS), endothelial cell growth supplement (50 μ g/ml), and heparin (90 U/ml). The conditions of culture were as follows: pH 7.4, 5% CO₂ in air, and 37 °C. Cultures consisted of homogeneous endothelial populations, as determined by typical cobblestone morphology and positivity to von Willebrand's factor and CD31 antigens [14]. Three strains of HSVECs were isolated, characterized, and employed for the investigations presented here with qualitatively similar results. Culture medium was always renewed 24 h before the experiment.

For stimulation, all the cytokines and LPS (from *E. coli*, serotype O55:B5) were added from 100 \times stock solutions in water to complete growth medium for the times indicated for each experiment. As shown through routinely performed determinations of culture protein content or cell number, no significant cell loss was observed up to 48 h under the experimental conditions employed.

When inhibitors were required by the experimental protocols, they were added 1 h before TNF α treatment from 100 \times stock solutions in water or in growth medium.

2.2. Arginine influx

The experiments were carried out on HSVEC subcultures resulting from 3.5 \times 10⁴ cells seeded into 2-cm² wells of

disposable Falcon 24-well trays (Becton Dickinson Labware Europe, Le Pont De Claix, France) in 1 ml of growth medium. Cells were employed after 2–3 days when cultures were almost confluent (15 \pm 2 μ g of protein/cm²). All the experiments were performed using the cluster-tray method for the measurement of solute fluxes in adherent cells [15] with appropriate modifications. Cell monolayers were washed twice in Earle's balanced salt solution (EBSS), containing (in mM) 117 NaCl, 26 NaHCO₃, 5 KCl, 1.8 CaCl₂, 1 NaH₂PO₄, 0.8 MgSO₄, 5.5 glucose. L-Arginine uptake was determined from the initial influx of L-arginine, assayed with a 30-s incubation of the cells in the same solution containing L-[³H]arginine. The experiments were terminated by three rapid washes (<10 s) in ice-cold urea (300 mM). Cell monolayers were extracted in 0.2 ml of ethanol, and the radioactivity of extracts was determined with a Wallac Microbeta Trilux. Extracted cell monolayers 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 [15]. Preliminary characterization experiments (shown in Fig. 1) indicated that HSVEC, like HUVECs [6], transport arginine through two Na⁺-independent components: one, insensitive to N-ethylmaleimide (NEM) and suppressed by excess L-leucine in the presence of sodium, corresponds to system y⁺L; the other, inhibited by NEM and relatively insensitive to leucine, is identifiable with system y⁺. Therefore, when both systems

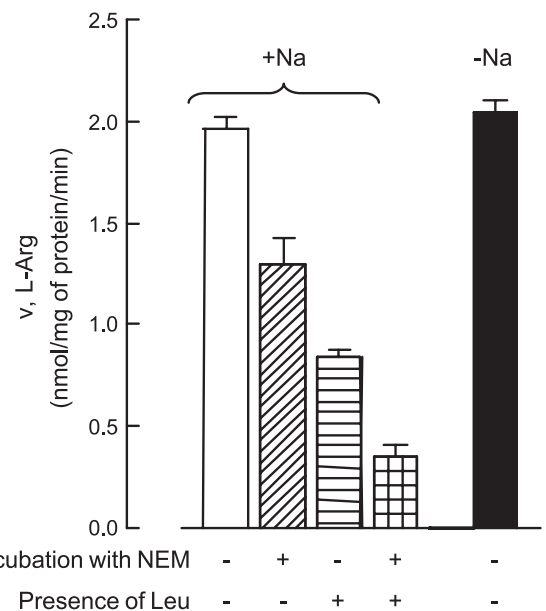


Fig. 1. Characterization of arginine influx in HSVECs. Cells were washed twice in EBSS or in Na⁺-free EBSS (as indicated) and L-arginine influx was assayed with 30-s incubations in the same solutions containing [³H]arginine (0.1 mM; 4 μ Ci/ml). The assay was performed in the absence or in the presence of 2 mM L-leucine. Where indicated, cells were pretreated for 5 min with 0.5 mM NEM. Points are means of four independent determinations within one representative experiment with S.D. indicated. The experiment was performed in three different cell strains with similar results.

had to be assessed, system y^+ was determined as the NEM-sensitive, leucine-resistant fraction of arginine transport, while NEM-insensitive, leucine inhibitable fraction represented the activity of system y^+L . When only the activity of system y^+ had to be determined, arginine uptake was measured in the presence of sodium and in the presence of leucine 2 mM, a treatment that completely abolished uptake through system y^+L . Arginine influx is expressed as nmol/mg protein/min⁻¹.

2.3. Reverse transcription

Total RNA from subconfluent cultures grown in 10-cm² wells was isolated with TRIZOL® (Life Technologies, Milan, Italy). RNA (2 µg), pretreated with RNase-free DNase, heated at 70 °C for 10 min, and placed on ice for 1 min, was incubated with a mixture containing 0.5 mM dNTPs mix, 25 ng/µl Oligo(dT)_{15–18} (Life Technologies), 10 mM dithiothreitol, 1 × first-strand buffer, 40 units of RNase inhibitor (Amersham Biotech, Milan, Italy), 200 units of SuperScript RT (Life Technologies), and water to a final volume of 20 µl for 1 h at 42 °C. The reaction was stopped by heating at 70 °C for 15 min. The duplex of RNA–DNA was treated with 2 units of RNase H (US Biochemicals, Cleveland, OH) at 37 °C for 20 min, and the amount of single-strand cDNA was evaluated using the fluorescent probe Oligreen (Molecular Probes, Eugene, OR) and phage M13+ as single-strand DNA standard with a Victor² 1420 Multilabel Counter, Wallac.

2.4. Polymerase chain reaction and “semi-quantitative PCR”

For PCR, 100 ng of single-strand cDNA from each sample was amplified in a total volume of 25 µl with 1 U of Platinum Taq DNA Polymerase (Invitrogen S.r.L., Milan, Italy), 1.6 × PCR buffer, 0.2 mM each dNTPs, 1.5 mM MgCl₂, along with both glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and proband sense and antisense primers employed at a concentration of 0.1 µM each. The primers were designed according to the sequences reported in GenBank with the help of Primer 3 program [16]. Primers for CAT-1 were 5' -ACT TGC TTC TAT GCC TTC GTG (sense) and 5' -TGT GGC GAT TAT TGG TGT TT (antisense) yielding an amplicon of 387 bp. The other primers were the same employed previously (see Table 1 of Ref. [6]). The sense primers for CAT-2B cDNA recognize the unique sequence resulting from the alternative splicing of the SLC7A2 transcript. A hot start PCR technique was utilized to reduce formation of nonspecific amplification products. An initial denaturation step at 92 °C for 2 min was followed by 34 cycles, with a 2-min denaturation step at 94 °C, the annealing at 59 °C for 30 s, and the extension step at 72 °C for 1 min. Another cycle was carried out, with the only difference being the final extension of 5 min. In preliminary experiments, these conditions were selected so

as to verify that the PCR products were in the exponential phase of amplification for all the proband and GAPDH transcripts. Amplified mixtures (10 µl) were electrophoresed through a 2% agarose gel (NuSieve 3:1-FMC), stained with ethidium bromide (0.5 µg/ml) in 0.5 × TBE buffer (0.1 M Tris, 90 mM boric acid, and 1 mM EDTA, pH 8.4), and visualized by ultraviolet light. Product size was established by co-migration with a 100-bp ladder marker (Bio-Rad Laboratories, Milan, Italy). Images of the electrophoresed cDNAs were recorded with a digital DC 120 Kodak camera and quantified by ID Image Analysis Software (Kodak Digital Science).

To confirm the identity with the reported human sequences, PCR products were ligated into pCRII-TOPO vector (TOPO TA cloning, Invitrogen BV, Groningen, The Netherlands) and used to transform CaCl₂ competent *E. coli*. Plasmid DNA was extracted with Jetquick plasmid miniprep spin kit (Genomed GmbH, Bad Oeynhausen, Germany); both sense and antisense strands of the cDNA were sequenced (MWG-Biotech AG, Ebersberg, Germany).

2.5. Statistical analysis

Statistical analysis of transport data was performed with analysis of variance, unless otherwise stated. The comparison between densitometry results of RT-PCR in control and treated cells was performed with the Wilcoxon test for nonparametric data.

2.6. Materials

FBS was purchased from Euroclone (Milan, Italy) and Medium 199 from Life Technology Italia (Milan, Italy). L-[2,3,4-³H]Arginine (45–70 Ci/mmol) was obtained from DuPont de Nemours (Bad-Homburg, Germany), TNF α , anthra [1,9-cd] pyrazol-6 [2H]-one (SP600125), and 2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1 (CFPD) from Alexis (San Diego, CA); other NF- κ B inhibitors, peptide aldehyde Cbz-Leu-Leu-Leucinal (MG132), 2'-amino-3'-methoxyflavone (PD98059), the PKC ζ pseudosubstrate inhibitor peptide, and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) from Calbiochem; Sigma (St. Louis, MO) was the source of *E. coli* (serotype O55:B5) lipopolysaccharide (LPS) as well as of all other chemicals.

3. Results

3.1. Effect of cytokines and LPS on arginine influx in HSVEC cells

In the experiment shown in Fig. 2, the effect of pro-inflammatory cytokines and LPS on the discriminated influx of arginine was studied in HSVECs. Treatments were prolonged for 48 h in complete growth medium and arginine

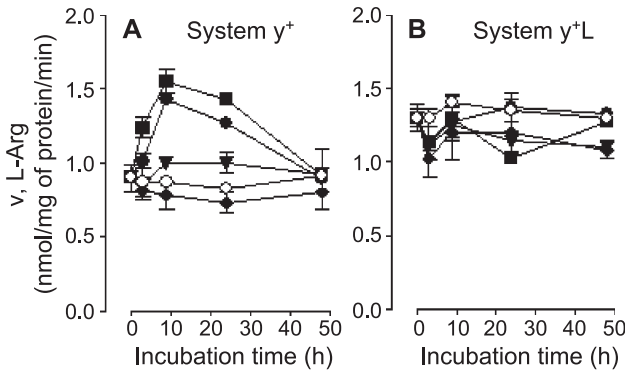


Fig. 2. Effect of pro-inflammatory cytokines and LPS on the discriminated influx of L-arginine. Cells were incubated in the absence (control, \circ) or in the presence of 1 $\mu\text{g/ml}$ LPS (\blacksquare), 10 ng/ml TNF α , (\bullet), 10 ng/ml IL-1 β (\blacklozenge) or 5 ng/ml IFN γ (\blacktriangledown). After the indicated times, cells were washed twice in EBSS and L-arginine influx was assayed with 30-s incubations in the same solution in the presence of [^3H]arginine (0.1mM; 4 $\mu\text{Ci/ml}$) both in cells pretreated for 5 min with 0.5 mM NEM (NEM-resistant influx) and in untreated cells (total influx). Panel A: System y^+ . Data represent the NEM-sensitive arginine influx calculated at any indicated time as the difference between total and NEM-resistant influx. Panel B: System y^+L . NEM-resistant arginine influx. Points are means of four independent determinations within one representative experiment with S.D. indicated. The experiment was repeated four times employing two different cell strains with similar results.

influx was determined at selected times in cells either exposed or unexposed to NEM. Both TNF α and LPS produced an increase of L-arginine transport through the sole NEM-sensitive component, i.e. system y^+ (Panel A). System y^+ stimulation required at least 6 h of treatment and was maximal after 9 h. At this time the V_{max} of the system was almost doubled in treated cells (1.62 ± 0.119 vs. 3.08 ± 0.126 nmol/mg protein/min in untreated and TNF α -treated cells, respectively). After 24 h, NEM-sensitive arginine influx was still significantly higher in treated cells than in control cells and returned to basal values only after 48 h of treatment. In contrast, IL-1 β and INF γ had no effect on arginine transport through system y^+ . No significant change was detected for NEM-resistant component, i.e. system y^+L , under any of the experimental conditions tested (Panel B). After 9 h of incubation the V_{max} of the system was 2.06 ± 0.182 vs. 1.97 ± 0.378 nmol/mg protein/min in untreated and TNF α -treated cells, respectively.

To ascertain if the increase of L-arginine transport induced by TNF α were dependent on macromolecular synthesis, we suppressed transcription with DRB. The inhibitor, added 1 h before treatment, substantially hindered TNF α -induced increase of system y^+ transport activity without altering the basal uptake of arginine (Fig. 3).

3.2. Effects of TNF α on the expression of genes for cationic amino acid transporters in HSVECs

A preliminary analysis of the expression of genes involved in high affinity system y^+ in HSVECs, performed with RT-PCR, indicated that this endothelial model

expresses SLC7A1, for CAT-1 transporter, and SLC7A2, for CAT-2B transporter (results not shown). Fig. 4 reports the RT-PCR of system y^+ -related genes (panels A and B), along with the densitometric analyses of the products (Panel C), obtained in HSVECs incubated in the presence of TNF α . The relative abundance of SLC7A1/CAT-1 RT-PCR product to GAPDH product remained constant throughout the treatment until 48 h. In contrast, the ratio of SLC7A2/CAT-2B to GAPDH increased after 4 h of incubation, reached a fourfold stimulation at 24 h, and decreased thereafter. The Wilcoxon test carried on with all the RT-PCR analyses performed on different cell strains revealed a highly significant increment ($P < 0.01$) of CAT-2B expression at all the times of treatment considered, except for the 48-h time point.

RT-PCR products of the y^+L -related genes SLC7A6 (coding for y^+LAT -2 protein), SLC7A7 (for y^+LAT -1), and SLC3A2 (for 4F2hc) did not show any significant variation with either TNF α or LPS (not shown).

3.3. Involvement of kinases in TNF α -dependent stimulation of system y^+ activity

Several inhibitors were used to assess the involvement of intracellular kinase cascades in TNF α -dependent stimulation of system y^+ transport activity. As shown in Fig. 5, Panel A, the stimulation of system y^+ by TNF α was unaffected by the MAPK inhibitors PD98059, a flavone compound which prevents ERK1/2 activation, SB 203580 and CFPD, both inhibitors of p38^{MAPK}, and by SP600125, a novel inhibitor

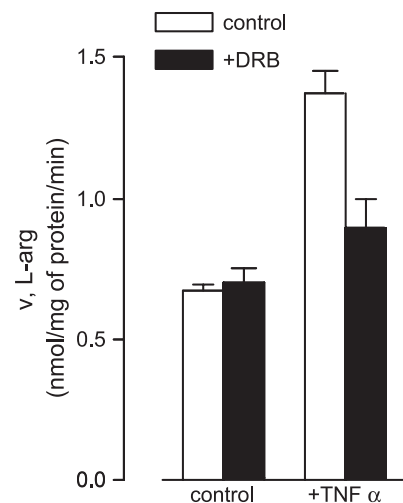


Fig. 3. Effect of DRB on TNF α -induced increase of system y^+ activity transport. HSVECs, grown to confluence in Medium 199, were incubated with TNF α (10 ng/ml) for 9 h. DRB (50 μM) was added 1 h before the treatment with the cytokine. Cells were washed twice in EBSS and the transport activity of system y^+ was assayed by 30-s incubations in the same solution containing [^3H]arginine (0.1 mM; 4 $\mu\text{Ci/ml}$) supplemented with L-leucine (2 mM). Data are means of three independent determinations, with S.D. indicated, obtained in a representative experiment repeated three times employing two different cell strains.

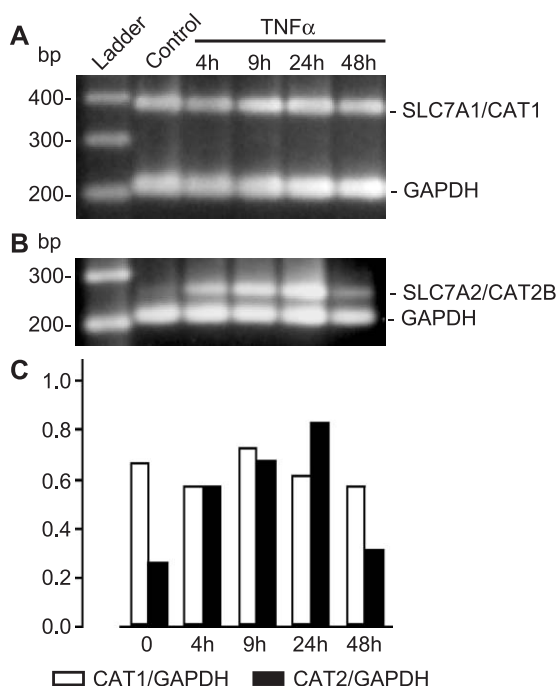


Fig. 4. Effect of TNF α on the expression of genes related to system y^+ in HSVECs. Cultures were incubated for the indicated times in the presence of 10 ng/ml of TNF α before RNA extraction. After reverse transcription, cDNA was used as template for PCR co-amplification reactions in which GAPDH primers were employed together with primers for SLC7A1/CAT-1 (Panel A) or SLC7A2/CAT-2B (Panel B). After the electrophoresis of RT-PCR products, gels were stained with ethidium bromide and photographed. Panel C reports the densitometric analysis in which the relative intensity of the SLC7A1 and SLC7A2/CAT-2B amplification products was normalized to those of the co-amplified GAPDH product. The analysis, repeated twice in three different cell strains, yielded comparable results.

of JNK [17]. Basal activity of system y^+ was not modified by any of the inhibitor tested.

Three distinct approaches were employed to evaluate the role of PKC in TNF α -induced stimulation of system y^+ transport activity. The first consisted in the chronic exposure to 100 nM PDBu, a treatment that down-regulates protein kinase C activity [18,19]. For the second approach, TNF α -treatment was performed in the presence of the specific inhibitor chelerythrine employed at 5 μ M, the maximal concentration found to be nontoxic in HSVECs (R. Visigalli, unpublished results). Third, TNF α treatment was performed in the presence of the inhibitor peptide for PKC ζ , an atypical PKC isozyme poorly sensitive to phorbol-induced PKC down-regulation. TNF α stimulation of system y^+ transport activity was still clearly detectable under all the three conditions adopted (Fig. 5, Panel B).

3.4. Effects of NF- κ B and proteasome inhibitors on system y^+ activity and CAT-2B mRNA expression

To investigate the involvement of NF- κ B in TNF α stimulation on system y^+ activity, we studied the effect of different inhibitors of the transcription factor. We have

selected inhibitors with different mechanisms of action, such as caffeic acid phenethyl ester (CAPE) and pyrrolidine dithiocarbamate (PDTC) that inhibit NF- κ B activation suppressing the interaction of NF- κ B proteins with the DNA [20]; PPM-18 that blocks the removal of I κ B α from NF- κ B/I κ B α complex [21]; and MG132 that inhibits the proteasome-dependent degradation of I κ B [22], thus preventing the nuclear translocation of the transcription factor.

As shown in Fig. 6, no inhibitor had significant effects on arginine transport in the absence of TNF α . Conversely, in the presence of TNF α , all the inhibitors markedly hindered the influx of the cationic amino acid. PDTC, CAPE, and MG132 completely suppressed the stimulatory effect of the

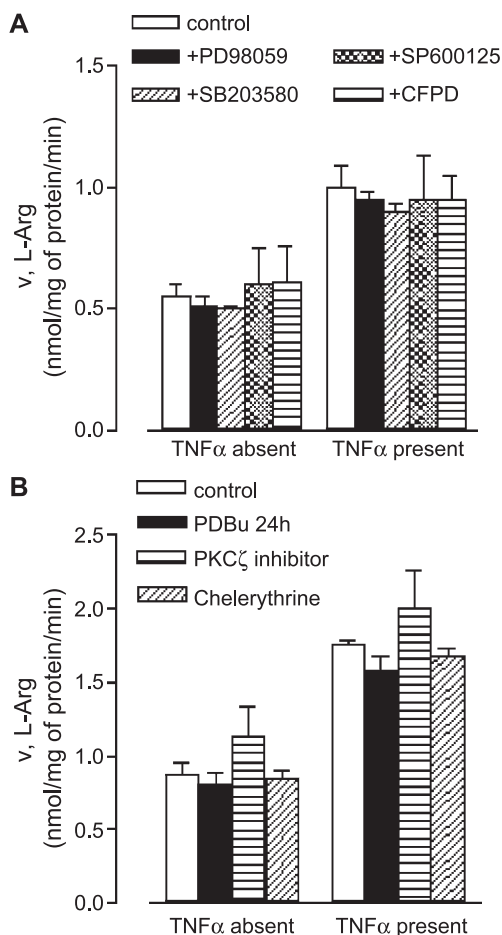


Fig. 5. Effect of kinase inhibitors on the TNF α -induced stimulation of system y^+ transport activity. After the preincubation in the presence of the various inhibitors and TNF α treatment, cells were washed twice in EBSS and the transport activity of system y^+ was assayed as described in Fig. 3. Data are means of three independent determinations, with S.D. shown, obtained in a representative experiment repeated three times employing two different cell strains. Panel A: Effects of MAPK inhibitors. Cells were incubated with PD98059 (50 μ M), SB 203580 (20 μ M), SP600125 (5 μ M), or CFPD (15 μ M), 1 h before the treatment with TNF α that was prolonged for 9 h in the presence of the same inhibitors. Panel B: Effects of PKC inhibitors. Cells were preincubated with chelerythrine (5 μ M) for 1 h, PKC ζ inhibitor peptide (10 μ M) for 1 h, or PDBu (100 nM) for 24 h. After the preincubation, cells were treated with TNF α for 9 h in the presence of the same inhibitors.

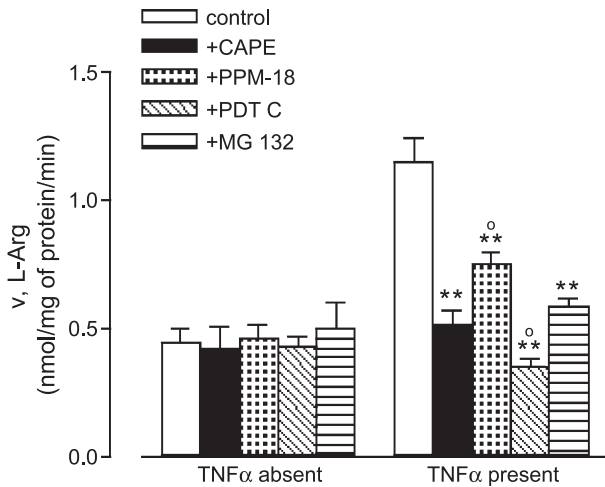


Fig. 6. TNF α -induced stimulation of system y^+ transport activity: sensitivity to NF- κ B inhibitors. HSVECs were pretreated for 1 h with the indicated NF- κ B inhibitors: CAPE (25 μ g/ml), PPM-18 (1 μ M), PDTC (100 μ M), MG132 (2 μ M) and then stimulated with 10 ng/ml TNF α for 9 h in the presence of the same inhibitors. The transport activity of system y^+ was determined as described in Fig. 3. Data are means of three independent determinations, with S.D. indicated, obtained in a representative experiment repeated three times employing two different cell strains. ** $P < 0.01$ (inhibitor present vs. inhibitor absent in TNF α -treated cells). $\circ P < 0.05$ (TNF α -present vs. TNF α -absent in inhibitor-treated cells).

cytokine that, in the presence of these compounds, did not modify significantly system y^+ transport activity. On the contrary, PPM-18 inhibited the effect of the cytokine only partially. Consistently, RT-PCR analysis indicated that PPM-18 markedly reduced, but did not abolish, the induction of CAT-2B caused by TNF α (Fig. 7). The Wilcoxon test carried on with all the RT-PCR analyses performed on different cell strains revealed a highly significant increment ($P < 0.01$) of CAT-2B expression for TNF α -treated cells and a significant increment ($P < 0.05$) for TNF α -treated cells incubated in the presence of the inhibitor.

4. Discussion

Although the intracellular concentration of arginine is well above the K_m of nitric oxide synthase (NOS) for the cationic amino acid, endothelial transport of arginine could represent a key factor for the appropriate fueling of the synthase under conditions of increased NO production [23]. Previous work performed with endothelial cells from human umbilical veins or from bovine aorta [6,10] has demonstrated the sensitivity of endothelial transport of arginine to pro-inflammatory cytokines. With endothelial cells obtained from saphenous veins (HSVECs), a model of adult human endothelium in vitro, we demonstrate that neither IFN γ nor IL-1 β have significant effects on arginine transport and point to TNF α and LPS as major factors for the up-regulation of arginine transport during the inflammatory response. As in HUVECs [6], also in HSVECs system y^+

is the sole target of TNF α . In the former model, no expression of CAT-2B is observed under control conditions and the gene is induced by the cytokine. In HSVECs, CAT-2B transcript is expressed at low levels even under control conditions and markedly increased by the cytokine. However, in both endothelial models, neither the expression of the genes related to system y^+L nor the activity of the transport system itself is sensitive to any pro-inflammatory compound. Interestingly, an increased abundance of CAT-2B mRNA, associated with a stimulated arginine transport through system y^+ , but not through system y^+L , has been also detected in human peripheral blood mononuclear cells obtained from patients with septic shock [24].

Previous work from this laboratory [6] has yielded a characterization of arginine transport in human endothelium in vitro and demonstrated that high extracellular concentrations of leucine can be employed as a convenient experimental condition to restrict the transport of the cationic amino acid to system y^+ . With this simple experimental device, we have measured the discriminated activity of system y^+ and demonstrated that the enhancement of arginine transport induced by the cytokine requires the activation of NF- κ B. Inhibitors of this transduction pathway hinder the transport stimulation, independently of their

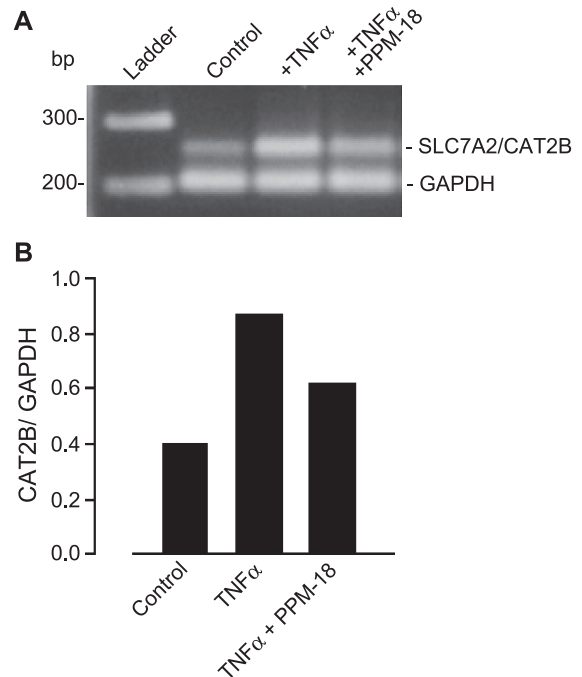


Fig. 7. Effect of PPM-18 on the expression of SLC7A2/CAT-2B induced by TNF α . Cultures were pretreated for 1 h with PPM-18 (1 μ M) and then stimulated with 10 ng/ml TNF α for 9 h in the presence of the same compound. RNA extraction, reverse transcription, and co-amplification were performed as detailed in Section 2. After electrophoresis of RT-PCR products, the gel was stained with ethidium bromide and photographed (Panel A). Panel B reports the densitometric analysis of SLC7A2/CAT-2B amplification products, normalized to those of the co-amplified GAPDH product. The analysis, repeated twice in two different cell strains, yielded comparable results.

mechanism of action. Some of these, such as the inhibitors of NF- κ B CAPE and PDTC and the proteasome inhibitor MG132, completely suppress arginine transport stimulation by TNF α . In contrast, another NF- κ B inhibitor, PPM-18, does not abolish the effect of the cytokine. In this case, also the increase in CAT-2B mRNA is only partially suppressed, indicating that a good correlation exists between CAT-2B mRNA levels and TNF α stimulation of arginine transport through system y^+ . The involvement of NF- κ B on the stimulation of arginine transport by inflammatory compounds, previously demonstrated only in non-endothelial models [25], adds CAT-2 gene to the enlarging list of targets of the transcription factor.

The study has been extended to ascertain if other transduction pathways are involved in the NF- κ B-dependent stimulation of arginine transport by TNF α . MAPK cascades could be good candidates for such a role since it is known that TNF α activates these transduction pathways in endothelial cells [26–28]. Moreover, short-term stimulation of arginine influx via system y^+ by adenosine [29] and D-glucose [30] is blocked by the inhibition of p44/42 MAPK in HUVECs. The stimulation of arginine transport by LPS in smooth muscle cells also appears to involve MAPK, and in particular p38 [31]. Nevertheless, inhibitors of the main MAPK groups, ERK1/2, p38, and JNK, do not interfere with TNF α stimulation of endothelial arginine transport, even if employed, as in the case of PD98059 and SB203580, at relatively high concentrations. These results, therefore, exclude an involvement of these transduction pathways in the stimulatory effect of the cytokine.

Also PKC isozymes may intervene in the transduction of TNF α signal that leads to the increased expression of CAT-2B transporter. The classical PKC α , the novel PKC ϵ , and the atypical PKC ζ have been demonstrated in HSVECs [18] and the two former isozymes, but not PKC ζ , are stimulated by TNF α [18]. In HUVECs PKC inhibition counteracts the effects of TNF α treatment on the expression of adhesion molecules [32]. Moreover, in one of the first reports concerning cytokine-mediated stimulation of arginine transport in HUVECs [33], the effect is blocked by chelerythrine, a PKC inhibitor endowed with poor isoform specificity. In contrast with that contribution [33], the results presented here indicate that chelerythrine does not hinder TNF α stimulation of arginine transport in HSVECs. Also the long-term exposure to phorbol esters, an experimental condition associated with the down-regulation of classical and novel PKC isozymes [19], does not affect the stimulation of system y^+ activity by TNF α . Moreover, the possibility that, as for other effects of TNF α [34], the cytokine activates system y^+ through PKC ζ , an atypical PKC insensitive to phorbols, has been further excluded through the employment of the specific inhibitor peptide. No PKC isozyme appears, therefore, to be requested for TNF α effect on arginine transport in HSVECs. Whether the discrepancy is due to difference in the endothelial model employed and/or to possible differences in the isozyme pattern is not clear. These

data do not imply that endothelial arginine transport is PKC-insensitive. Recent results indicate that the down-regulation of classical PKC isoform, obtained with an 18-h exposure to phorbols, indeed produces an increase in arginine transport [19]. In contrast, the results reported in this contribution indicate that down-regulation of PKC does not change appreciably arginine uptake. However, it should be stressed that although Krotova et al. [19] attribute changes in transport to modifications of system y^+ activity, no discrimination of arginine transport was reported in that paper. Therefore, PKC effects on arginine transport in endothelial deserve further investigations.

In conclusion, this report demonstrates that, in human endothelial cells, NF- κ B activation is required for TNF α -dependent stimulation of the expression of CAT-2B transporter while neither MAPK nor PKC appear to be involved in the regulatory mechanism.

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