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Biochimica et Biophysica Acta 1768 (2007) 1479–1487

Biochimica et Biophysica Acta BBA

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Rapamycin stimulates arginine influx through CAT2 transporters in human endothelial cells

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Received 2 November 2006; received in revised form 25 January 2007; accepted 15 February 2007
Available online 24 February 2007

Abstract

In endothelial cells Tumor Necrosis Factor-α (TNFα) stimulates arginine transport through the increased expression of SLC7A2/CAT2 transcripts. Here we show that also rapamycin, an inhibitor of mTOR kinase, stimulates system y^+-mediated arginine uptake in human endothelial cells derived from either saphenous (HSVECs) or umbilical veins (HUVECs). When used together with TNFα, rapamycin produces an additive stimulation of arginine transport in both cell models. These effects are observed also upon incubation with AICAR, a stimulator of Adenosine-Monophosphate-dependent-Protein Kinase (AMPK) that produces a rapamycin-independent inhibition of the mTOR pathway. Rapamycin increases the V_{max} of high affinity arginine transport and causes the appearance of a low affinity component that is particularly evident if the treatment is carried out in the presence of TNFα. RT-qPCR studies have demonstrated that these kinetic changes correspond to the induction of both the high affinity transporter CAT2B and the low affinity isoform CAT2A. Western blot and immunocytochemical analyses indicate that, consistently, the expression of CAT2 proteins is also stimulated under the same conditions. These changes are associated with an increase of the intracellular arginine concentration but with a decrease of NO production. Thus, our data suggest that mTOR activity is associated with the repression of CAT2 expression at mRNA and protein level.

Keywords: mTOR; CAT transporter; SLC7A gene; Arginine; System y^+

In human endothelial cells L-arginine transport is mediated by the leucine-resistant, NEM sensitive system y^+ and by the leucine-inhibitable system y^+L [1]. System y^+ is due to the activity of CAT transporters, encoded by SLC7A genes: the ubiquitous CAT1, encoded by SLC7A1, and the two transporters, CAT2A and CAT2B, derived from alternative splicing of SLC7A2 transcripts, are the best characterized members of the family [2,3]. Conversely, system y^+L is a member of the group of heterodimeric amino acid transporters (see [4] for review), formed by a light and a heavy subunit. In the case of system y^+L, the heavy subunit is 4F2hc/CD98, the product of SLC3A2 gene, while two alternative light chains have thus far been characterized, y^+LAT1 (encoded by SLC7A7) and y^+LAT2 (encoded by SLC7A6).

While a regulatory mechanism for system y^+L expression has been described only for human monocytes [5], the regulation of CAT expression and transport activity has been extensively studied under a variety of hormonal and inflammatory stimuli. In particular, proinflammatory cytokines modulate cationic amino acid transport activity in several endothelial cell

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranosyl 3′-5′-cyclic-monophosphate; AU, Arbitrary Unit; CAA, Cationic Amino Acid; CLSM, Confocal Laser Scanning Microscopy; DAN, 2,3-diaminonaphtalene; DTT, Dithiothreitol; EBSS, Earle's Balanced Salt Solution; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HSVECs, Human Saphenous Vein Endothelial Cells; HUVECs, Human Umbilical Vein Endothelial Cells; M199, Medium 199; NEM, N-ethylmaleimide; TNFα, Tumor Necrosis Factor-α

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doi:10.1016/j.bbamem.2007.02.016

models [6,7]. Studies performed with human endothelial cells have demonstrated that TNFα enhances system y^+ activity through an increase of CAT2B expression, while the activity and the expression of system y^+L are not changed by the cytokine [1]. The transduction pathway employed by TNFα to induce the increase of arginine transport has been partially characterized in Human Saphenous Vein Endothelial Cells (HSVECs) [8]. Those results demonstrated that the activation of NF-κB is required for the TNFα effect on CAA transport, while the involvement of other transduction pathways, such as PKC and MAPK, was excluded [8]. However, given the complexity of TNFα-dependent signalling pathways, it is apparent that other transduction routes may be involved in the cytokine-dependent stimulation of arginine transport.

Among the possible candidates, the PI3K/Akt/mTOR pathway appears of great interest since it is known that TNFα activates PI3K and Akt in human endothelial cells [9] and, hence, is expected to raise the activity of mTOR (mammalian Target Of Rapamycin). Consistently, TNFα-dependent stimulation of PI3K effectors, including Akt, activates mTOR in non-endothelial models [10,11]. mTOR, known also as FRAP or RAFT, is a serine/treonine kinase that plays an important role in the regulation of cell growth and proliferation, nutrient transport, protein and RNA stability, acting as a fundamental controller of protein translation [12–14]. Studies about mTOR functions have been greatly favored by the availability of a specific inhibitor, rapamycin (sirolimus), a macrolide antibiotic, isolated from *Streptomyces hygroscopicus*, that specifically inhibits mTOR activity after binding to the protein FKBP12 [15].

In this study, we demonstrate that rapamycin stimulates SLC7A2 gene expression, thus leading to the enhanced expression of CAT2 transporters and to the stimulation of arginine transport. Moreover, we provide evidence that the rapamycin and TNFα effects on arginine transport are additive.

1. Materials and methods

1.1. Cells and experimental treatments

HSVECs were obtained from vessel remnants of patients undergoing coronary artery bypass grafting and cultured as already described [8]. Five strains of HSVECs from different patients were employed with qualitatively similar results. HUVECs were obtained from three distinct cords according to the method of Jaffe, with minor modifications as described previously [16]. 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 (ECGS, 37.5 μg/ml), and heparin (75 U/ml). Cells were kept at 37 °C, pH 7.4, in an atmosphere of 5% CO_2. Cultures consisted of homogeneous endothelial populations, as demonstrated by the typical cobblestone morphology and the positivity to von Willebrand's factor and CD31/PECAM-1 antigens. Culture medium was always renewed 24 h before the experiment.

Cells were employed 2–3 days after seeding, when cultures were almost confluent (12±3 g of protein/cm^2). Unless otherwise stated, TNFα was used at 10 ng/ml in complete growth medium from a 100× stock in sterile water for the times indicated for each experiment. Rapamycin (100 nM) and 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranosyl 3′-5′-cyclic-monophosphate (AICAR, 2 mM) were added 1 h before the cytokine treatment from 100× stocks in growth medium.

1.2. Arginine influx

For transport studies, cells were seeded on 2-cm^2 wells of disposable Falcon 24-well trays (Becton Dickinson Labware Europe, Le Pont De Claix, France) in 1 ml of growth medium. All the experiments were performed using the cluster-tray method for the measurement of solute fluxes in adherent cells [17] with appropriate modifications. Cell monolayers were washed twice in Earle's balanced salt solution (EBSS), containing (in mM) 117 NaCl, 26 $NaHCO_3$, 5 KCl, 1.8 $CaCl_2$, 1 NaH_2PO_4, 0.8 $MgSO_4$, 5.5 glucose. L-Arginine influx was assayed with a 30 s incubation of the cells in the same solution containing L-[^{3}H] arginine. Preliminary experiments indicated that, in this interval, arginine influx approached linearity under all the experimental conditions adopted (results not shown). To restrict arginine uptake to CAT transporters (system y^+), the activity of system y^+L was inhibited adding leucine to the uptake solution [1]. The experiments were terminated by two rapid washes (<10 s) in ice cold 0.3 M urea. Cell monolayers were extracted in 0.2 ml ethanol and the radioactivity of extracts was determined with a Wallac Microbeta Trilux (Perkin Elmer, Wellesley, Ma, USA). Extracted cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 M NaOH and protein content was determined directly in each well using a modified Lowry procedure as previously described [17].

Amino acid influx is expressed as nmoles·mg of $protein^{-1}\cdot min^{-1}$. Kinetic parameters of arginine influx were determined by non-linear regression analysis using a GraphPad Prism3™ software. The equations used for fitting the experimental data were:

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

for a single saturable system, or

$$V = \frac{V_{max1}\cdot[S]}{K_{m1} + [S]} + \frac{V_{max2}\cdot[S]}{K_{m2} + [S]} \quad (2)$$

for two saturable systems, where v is the initial influx, V_{max} the maximal influx and K_m the Michaelis constant.

1.3. RT-qPolymerase chain reaction

1 μg of total RNA, isolated with RNeasy Mini Kit® (Qiagen S.p.a., Milano, Italy), was reverse transcribed as described previously [1]. For real time PCR (40 cycles), cDNA was amplified with 2X Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen s.r.l., Milano, Italy), along with the following forward and reverse primers (5 pmol each): 5′ CTT CAT CAC CGG CTG GAA CT 3′ and 5′ GGG TCT GCC TAT CAG CTC GT 3′ for SLC7A1/CAT1; 5′ TTC TCT CTG CGC CTT GTC AA 3′ and 5′ TCT AAA CAG TAA GCC ATC CCG G 3′ for SLC7A2/CAT2A; 5′ TTC TCT CTGCGC CTT GTC AA 3′ and 5′ CCA TCC TCC GCC ATA GCA TA 3′ for SLC7A2/CAT2B; 5′ AGC CTC AAG ATC ATC AGC AAT G 3′ and 5′ CAC GAT ACC AAA GTT GTC ATG GA 3′ for GAPDH. The primer set was designed according to the known sequences reported in GenBank with the help of Primer 3 program [18]. Quantitative PCR was performed in a 36 well Rotor Gene 3000 (Corbett Research, Rotor-Gene™ 3000, version 5.0.60, Mortlake, Australia). Each cycle consisted of a denaturation step at 95 °C for 15 s, followed by separate annealing (30 s, 57 °C) and extension (30 s, 72 °C) steps. Fluorescence was monitored at the end of each extension step. A no-template, no-reverse transcriptase control was included in each experiment. At the end of the amplification cycles a melting curve analysis was added. The analysis of the data was made according to the Relative Standard Curve Method [19].

1.4. Western analysis

Cells, grown in 10-cm^2 dishes, were washed twice with ice-cold phosphate-buffered saline, scraped in the same solution and collected by low speed centrifugation. The pellet was suspended in 0.3 ml of Laemmli buffer (62.5 M Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.2 M DTT). For the analysis of CAT2, DNA was sheared by passing repeatedly the sample through 22G and 27G needles until the viscosity dropped [20]. After total protein quantification by a modified micro Lowry protein assay, 30 μg of protein samples were separated on 8%

acrylamide gels by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Bio Rad, Milano, Italy). Membranes were blocked at 4 °C overnight with an incubation in Tris-buffer saline (TBS; 50 mM Tris–HCl pH 7.5, 150 mM NaCl), containing 3% BSA, 1% Casein, 0.33% gelatin and 10% goat serum and exposed for 2 h at room temperature to a filtered anti-hCAT2 polyclonal antiserum (diluted 1:2000 in blocking solution). This antiserum was generated by Neosystem Groupe SNPE (Strasbourg, France) against the C-terminus peptide Y-17-F (Y-R-N-L-S-S-P-F-I-F-H-E-K-T-S-E-F) of human CAT2 proteins. The blot was then exposed to horseradish peroxidase-conjugated anti-rabbit IgG (1:3000) for 1 h at room temperature. The membranes were washed and the immunoreactivity visualized with enhanced chemiluminescence (Bio Rad, Milano, Italy). The expression of p70S6 kinase and of phospho-p70S6 kinase was determined as described by PhosphoPlus® p70S6 kinase (Thr389, Thr421/Ser424) Antibody kit from Cell Signaling (Celbio, Milano, Italy). β-Tubulin and GAPDH, detected with a monoclonal antiserum (1:50 and 1:500, respectively), were employed for standardization.

1.5. Immunocytochemistry

Immunostaining for CAT2 was performed on cell monolayers grown on Falcon two-well chamber slides (Becton Dickinson Labware Europe, Le Pont De Claix, France). Cells, after a 24 h incubation in the indicated media, were washed twice with PBS and fixed with 3.7% paraformaldehyde in PBS (pH 7.4). After additional washing, non-specific absorption of antibodies was blocked by incubation for 1 h in PBS containing 3% bovine serum albumin after which the cells were incubated for 1 h at 37 °C with anti-CAT2 polyclonal antiserum (see Western analysis) diluted 1:500 in blocking solution. After three washing, cells were incubated for 1 h at room temperature with Alexa Fluor 488–conjugated secondary antibodies (Molecular Probes, Invitrogen, Milano, Italy) (1:400 dilution in blocking solution). The cells were finally washed, and the slides were mounted with Pro-Long® antifade kit (Molecular Probes, Invitrogen, Milano, Italy), and examined with a confocal microscope ZEISS, LSM 510 META equipped with inverted microscope Axiovert 200 M Carl Zeiss Meditec GmbH, Munchen-Hallbergmoos, Germany. For each condition, a series of confocal images of representative fields were taken and the section yielding the maximal signal was selected.

1.6. Determination of amino acid content and NO production

The intracellular content of lysine and arginine was determined on cell extracts in a 5% solution of acetic acid in ethanol by HPLC analysis with a Biochrom 20 Amino Acid Analyzer (Biochrom, Cambrige, UK) employing a High Resolution Column Bio 20 Peek Lithium and the Physiological Fluid Chemical Kit (Biochrom, Cambrige, UK) for elution. The column effluent was mixed with ninhydrin reagent, passed through the high temperature reaction coil, and read by the photometer unit. The intracellular concentration of amino acids was calculated from the amino acid contents (expressed in nmol/mg of protein) and cell volumes determined in parallel cultures under the same experimental conditions by [^{14}C] urea distribution, as described previously [21].

The accumulation of nitrite, stable derivative of NO, in the culture media of HUVECs was assessed through a fluorimetric approach, based upon the production of the fluorescent molecule 1-(H)-naphtotriazole from DAN in the presence of nitrite in an acid environment [22] as described previously [1]. Fluorescence was determined with Wallac Victor^2 1420 Multilabel Counter (Perkin Elmer, Wellesley, Ma, USA). Nitrite production was expressed in nmoles per ml of extracellular medium (μM).

1.7. Statistical analysis

Unless otherwise specified, statistical analysis of data was performed with a one-way ANOVA with the Bonferroni post hoc test. P values >0.05 were considered not significant.

1.8. Materials

Endotoxin-free FBS and Medium 199 (Euroclone) were purchased from Celbio (Pero, MI, Italy). L-[2,3,4-^{3}H]Arginine (45–70 Ci/mmol) was obtained from Perkin Elmer (Monza, Italy), TNFα (Alexis) from Vinci-Biochem (Firenze, Italy). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-βTubulin antibodies (Santa Cruz Biotechnology) were purchased from DBA Italia (Milano, Italy), while GAPDH (Chemicon) was obtained from Prodotti Gianni (Milano, Italy). Sigma (Milano, Italy) was the source of rapamycin and AICAR, as well as of all other chemicals.

2. Results

2.1. Rapamycin and TNFα additively stimulate arginine transport

The effect of rapamycin on arginine transport through system y^+ is shown in Fig. 1. The experiments were performed in HUVECs and in HSVECs in the absence or in the presence of TNFα that, as expected [1,8], significantly stimulated system y^+ activity in both cell types. In the absence of TNFα, rapamycin (100 nM) significantly stimulated arginine transport in HUVECs after either 8 or 24 h of treatment. In HSVECs the rapamycin-dependent stimulation of arginine transport was somewhat dependent on the cell strain, ranging from +10% to +50%. However, when simultaneously treated with rapamycin and TNFα, both HUVECs and HSVECs consistently exhibited values of arginine transport markedly higher than those obtained with either compound alone. Indeed, in cells incubated for 8 h or 24 h in the presence of both TNFα and rapamycin, system y^+-mediated arginine transport increased by 2- or 3-fold compared with untreated, control cells.

Fig. 2 shows the transport activity of system y^+ in HUVECs treated with different concentrations of TNFα in the absence or in the presence of rapamycin. TNFα-dependent stimulation of arginine transport was significant at 1 ng/ml and maximal at 10 ng/ml, with no further increases at higher cytokine concentrations. In all the range of cytokine concentrations tested (from 0.1 to 100 ng/ml), cells treated with both rapamycin

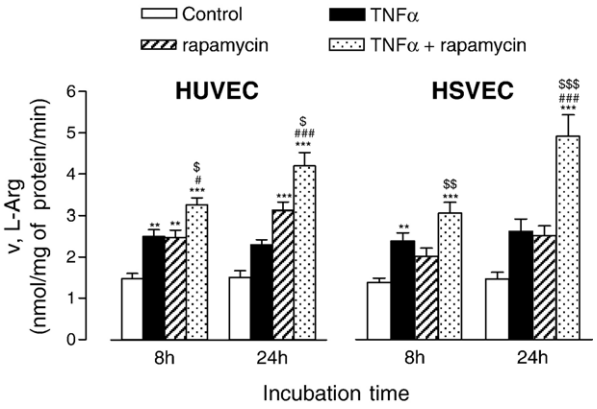

Fig. 1. Effect of rapamycin and TNFα on arginine transport in HUVECs and HSVECs. Cells were incubated in the absence or in the presence of 10 ng/ml TNFα, 100 nM rapamycin, or both, as indicated. After 8 h or 24 h cells were washed in EBSS and L-arginine influx was assayed with 30-s incubations in the same solution supplemented with [^{3}H]arginine (100 μM; 4 μCi/ml) in the presence of 2 mM L-leucine to restrict arginine influx to system y^+. Bars are means±SEM of eight experiments on three different HUVEC strains and seven experiments on 5 distinct HSVEC strains. $^{**}p<0.01$, $^{***}p<0.001$ vs. control, untreated cells; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. cells treated with TNFα; $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. cells treated with rapamycin.

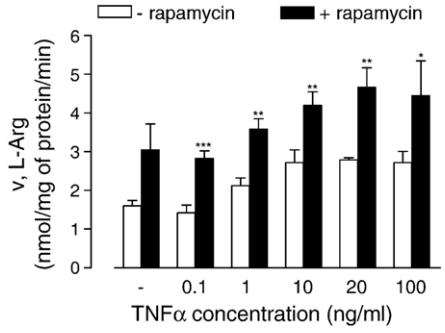

Fig. 2. Dose–response curve of TNFα effects on arginine transport in the absence and in the presence of rapamycin. HUVECs were incubated for 20 h with the indicated concentrations of TNFα in the absence (empty bars) or in the presence (solid bars) of 100 nM rapamycin. After this period, cells were washed in EBSS and system y^+ transport activity was measured as described in the legend of Fig. 1. Points are means of three independent determinations within one representative experiment with SD indicated. The experiment was repeated twice with similar results. Statistical analysis was performed with two-tail *t*-test for unpaired data. ***$p<0.001$, **$p<0.01$, *$p<0.05$ vs. cells incubated with the same TNFα concentration in the absence of rapamycin.

and TNFα had an higher arginine transport activity than cells treated with the cytokine alone.

A kinetic analysis of arginine transport, employing a wide range of amino acid concentrations, was performed with untreated HSVECs or with cells treated for 24 h in the presence of TNFα and rapamycin. (Fig. 3). Uptake was measured in the presence of excess extracellular leucine (see the legend) to inhibit system y^+L. The diffusive component (K_d of 0.95 ± 0.02 min^{-1}), derived from the linear regression of influx values at high arginine concentrations (from 2 to 10 mM) in control cells, was subtracted to influx data to yield the saturable arginine influx. The results are presented both in v vs. [S] representation (Panel A) and in the Eadie–Hofstee graphical transformation (Panel B) so as to allow a better discrimination between the transport components. Saturable arginine transport in untreated cells was satisfactorily fitted with a single system ($K_m=0.08\pm0.027$ mM, V_{max} of 2.51 ± 0.18 nmol/mg of protein/min, $R^2=0.88$, see the insets of Panels A and B). On the contrary, in cells treated with TNFα and rapamycin, transport data were best fitted ($R^2=0.99$) by the operation of two saturable transport systems with very divergent affinities (see Panel B): the high affinity component whose V_{max} was markedly increased (10.4 ± 1.85 nmol/mg of protein/min) while the K_m (0.12 ± 0.036 mM) was not significantly modified and a low affinity component, not detectable in control cells, with a V_{max} of 27.34 ± 2.7 nmol/mg of protein/min and a K_m of 5 ± 1.56 mM. While the K_m of the high affinity component is in the range attributed to the high affinity system y^+-related CAT isoforms (CAT1 and CAT2B), the K_m of the low affinity component is compatible with the operation of CAT2A transporter [2]. Similar analyses indicated that incubation with TNFα or rapamycin alone raised the V_{max} of the high affinity component and caused the appearance of the low affinity component, although at a lesser extent than the two compounds together (results not shown).

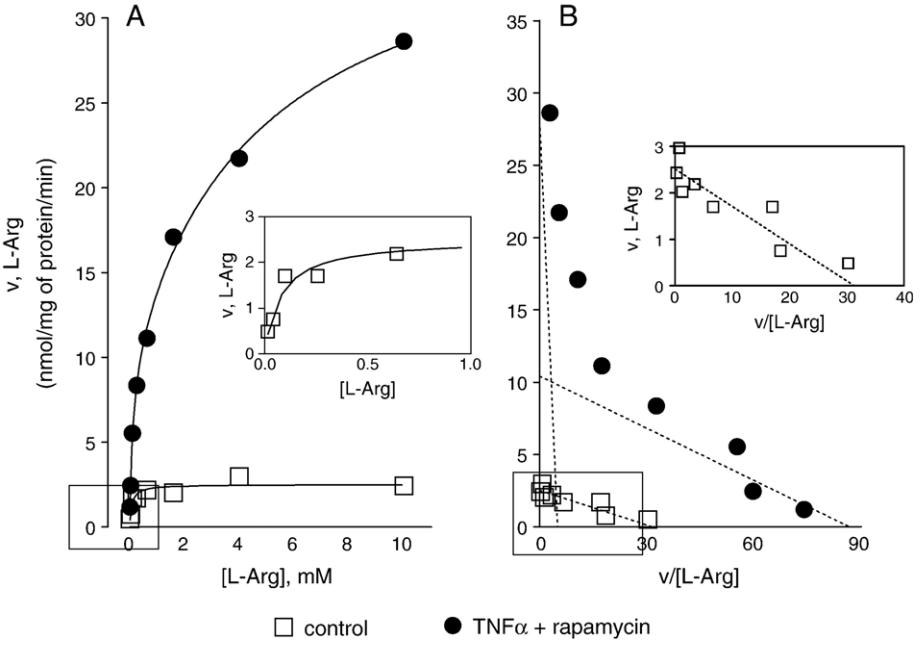

Fig. 3. Kinetic analysis of L-arginine transport in HSVECs: effect of the combined treatment with rapamycin and TNFα. Cells were incubated for 24 h in the absence (control) or in the presence of TNFα + rapamycin. After this period, the 30 s influx of increasing concentrations of L-[^{3}H]Arg (0.016 − 10 mM) was measured in the presence of 2.5 mM (for [Arg] from 0.016 to 0.256 mM) or 25 mM L-leucine (for [Arg] from 0.64 to 10 mM). Influx data were corrected for K_d (see text). Points are means of three independent determinations within one representative experiment repeated twice with comparable results. Panel A, Curves represent the best fit of the data to Eq. (1) for control cells or to Eq. (2) for TNFα + rapamycin-treated cells (see Materials and methods). In the inset, data of arginine influx obtained with arginine concentrations up to 1 mM (control, untreated cells) are represented in an amplified scale. Panel B, Data reported in Panel A are shown in the Eadie–Hofstee graphical representation. In the inset, data of arginine influx obtained in control, untreated cells are represented in an amplified scale. Lines are drawn from the kinetic parameters obtained from the non-linear regression analysis shown in Panel A.

2.2. Rapamycin-induced stimulation of arginine transport is due to the induction of CAT2 mRNAs and proteins

To investigate the mechanism underlying the changes of arginine transport in HUVECs treated with TNFα, rapamycin, or both compounds, we performed a real time PCR analysis of the expression of SLC7A1, coding for the constitutive CAT1 transporter, and SLC7A2, that encodes for two distinct isoforms of CAT2 transporter, CAT2A and CAT2B [23–25]. The expression of CAT1 (Fig. 4, Panel A) was comparable under all the experimental conditions adopted. On the contrary, either TNFα or rapamycin induced an increase in both CAT2A (Panel B) and CAT2B expression (Panel C), with rapamycin producing the largest effect. If rapamycin treatment was performed in the presence of TNFα, a massive (>5–10 fold) increase in both CAT2A and CAT2B mRNA levels was consistently observed after 8h and, even more clearly, after 24 h of incubation.

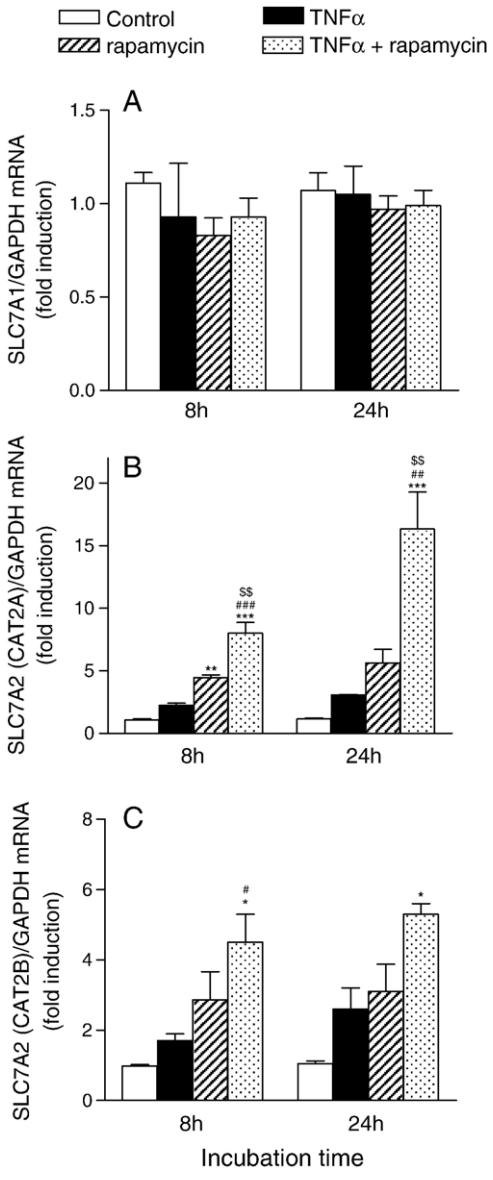

Fig. 4. Effect of rapamycin and TNFα on the expression of genes related to system y^+ in HUVECs. Cells were incubated for 8 h or 24 h in the absence or in the presence of 10 ng/ml TNFα, 100 nM rapamycin, or TNFα + rapamycin, as indicated. After RNA extraction and reverse transcription, cDNA was employed as template for qPCR with CAT1, CAT2A, CAT2B, or GAPDH primers (see Materials and methods). The intensity of the SLC7A1/CAT1, (Panel A) SLC7A2/CAT2A (Panel B) and SLC7A2/CAT2B (Panel C) amplification products was normalized to that of the GAPDH product amplified from the same cDNA sample and referred to the corresponding value obtained in control, untreated cells (=1). Data are means±SEM of three separate experiments on 3 distinct HUVEC strains. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control, untreated cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. cells treated with TNFα; $^{\$\$}p<0.01$ vs. cells treated with rapamycin.

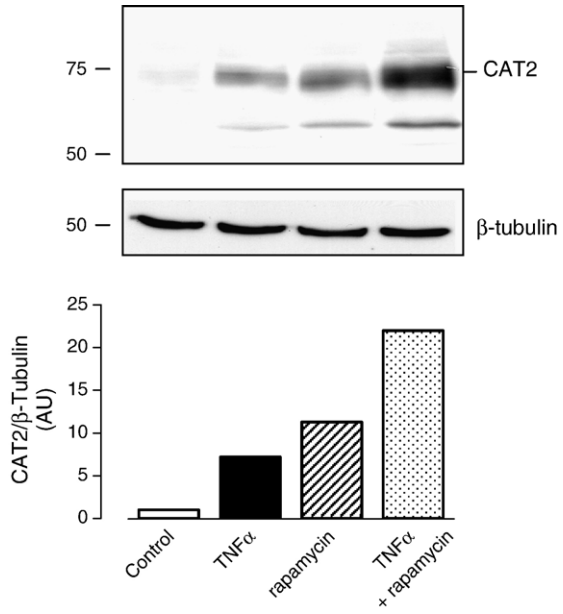

Fig. 5. Effect of rapamycin and TNFα on CAT2 protein in HUVECs. Cells were incubated for 24 h in the absence or in the presence of 10 ng/ml TNFα, 100 nM rapamycin, or both, as indicated. After protein extraction, Western Blot analysis was performed using anti-CAT2 (1:2000) and anti-β tubulin antisera (1:50, see Materials and methods). Lower panel presents the results of the densitometric analysis of CAT2 expression, normalized to β-tubulin expression. A representative Western blot is shown; the experiment was repeated three times with similar results.

To assess whether the observed changes of transport activity and gene expression were associated with changes in protein levels, the cell content of CAT2 proteins was determined in HUVECs incubated with TNFα, rapamycin, or both. It should be noted that the polyclonal antiserum used for these studies does not discriminate between CAT2A and CAT2B isoforms.

The results of the Western Blot analysis (Fig. 5) indicate that CAT2 proteins are clearly detectable by the antiserum used. Marked differences in CAT2 expression among the experimental conditions adopted are evident. While carrier proteins were almost undetectable in control, untreated HUVECs, a 8-fold increase in CAT2 expression was detected in response to a 24-h treatment with TNFα; under the same conditions, also rapamycin caused a similar, significant increase on the expression of CAT2 protein but the largest induction (20-fold) was detectable in cells treated with both compounds.

The immunocytochemical analysis of CAT2 expression in HUVECs, presented in Fig. 6, confirmed these results. While

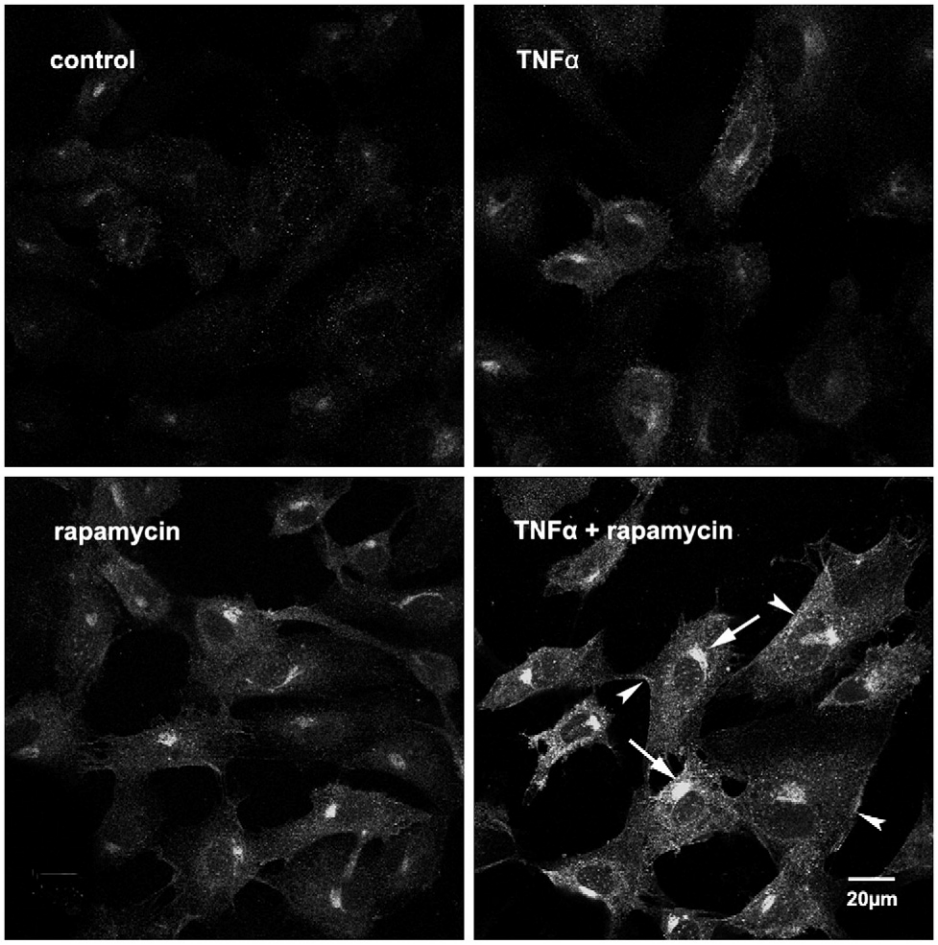

Fig. 6. Expression of CAT2 proteins in HUVECs. Cells were incubated for 24 h in the absence or in the presence of 10 ng/ml TNFα, 100 nM rapamycin, or both, as indicated. At this time immunocytochemical analysis was performed with anti-CAT2 antiserum and the slides observed in CLSM. Single confocal sections of representative fields are shown (see Materials and methods). Arrows, perinuclear areas of high CAT2 expression. Arrowheads, membrane expression of CAT2 proteins.

the carrier proteins are barely expressed in control cells, they became evident in cells treated for 24 h with TNFα or rapamycin. Interestingly, the confocal sections of treated cells demonstrate that the carrier proteins localize both at the plasma membrane and inside the cells, with a particular abundance in the perinuclear area. Cells treated with both rapamycin and TNFα uniformly presented a much higher CAT2 signal, both in the cytoplasm and at the plasma membrane (see arrows).

2.3. The stimulation of arginine transport by rapamycin and TNFα causes an increase in cell content of cationic amino acids without a stimulation of NO production

The cell contents of arginine and lysine were determined with HPLC and corrected for the values of cell volume measured in parallel to yield the intracellular concentration of cationic amino acids. The results (Fig. 7, Panel A) indicate that rapamycin, either alone or together with TNFα, caused a significant increase in the intracellular concentration of both lysine and arginine. However, the production of NO, as assessed with the determination of nitrites in the extracellular medium, was not increased under any experimental condition but, rather,

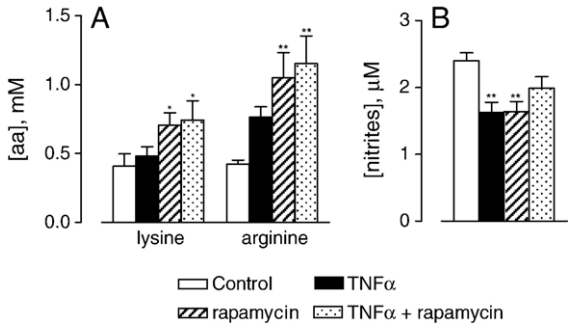

Fig. 7. Effect of rapamycin and TNFα on the intracellular concentration of cationic amino acids (Panel A) and NO production (Panel B) in HUVECs. Cells were incubated in the absence or in the presence of 10 ng/ml TNFα, 100 nM rapamycin, or both, as indicated. (Panel A) After 24 h cells were washed twice in PBS and extracted as described under Materials and methods. The amino acid content of the extracts was determined with HPLC and corrected with the cell volume, measured in parallel, to yield the intracellular concentrations (see Materials and methods). Bars represent means of three independent determinations with SEM indicated. (Panel B) After 48 h, the concentration of nitrites in the incubation medium was determined in parallel cultures as described under Materials and methods. Bars represent means of six independent determinations in three experiments with SEM indicated. *$p<0.05$, **$p<0.01$ vs. control, untreated cells.

was significantly lower in cells treated with either TNFα or rapamycin (Fig. 7, Panel B).

2.4. AICAR stimulates arginine transport

To verify if the effect of rapamycin was detectable also in other conditions associated with the inhibition of mTOR, we used AICAR, which activates AMPK (Adenosine-Monophosphate-dependent-Protein Kinase), a rapamycin-independent inhibitor of mTOR. Fig. 8, Panel A, shows the effects of a 20-h treatment with AICAR on arginine influx in the absence or presence of TNFα. In the absence of TNFα, AICAR produced a modest, although significant, stimulation of arginine uptake. In cells incubated with the AMPK stimulator in the presence of TNFα, the transport activity was higher than in cells incubated with TNFα alone. However, the effect of rapamycin was larger than that of AICAR, both in the absence or in the presence of TNFα.

To verify if the stimulatory effect of AICAR was associated with the inhibition of mTOR, we assessed the phosphorylation status of p70S6 kinase, a well known substrate of mTOR kinase. The results, presented in Fig. 8, Panel B, indicated that AICAR produced a clear cut inhibition of p70S6 kinase phosphorylation, although at a lesser degree than rapamycin.

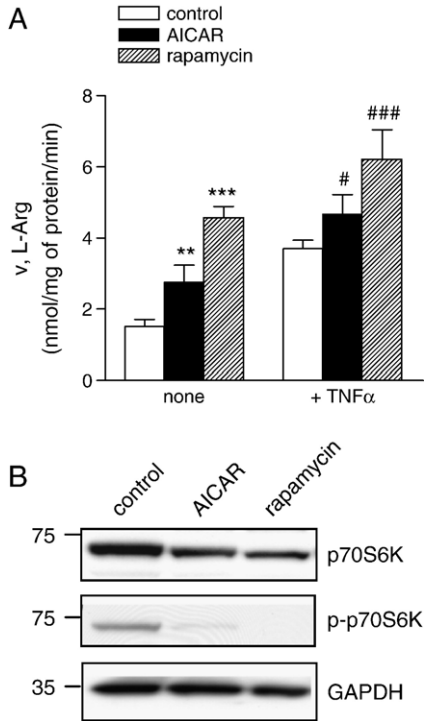

Fig. 8. Effect of rapamycin and AICAR on system y^+ transport activity and p70S6K phosphorylation in HUVECs. Cells were incubated for 20 h in the absence or in the presence of rapamycin (100 nM) or AICAR (2 mM), as indicated. (Panel A) The treatments were performed in the absence (left) or in the presence (right) of TNFα (10 ng/ml). After this period, cells were washed in EBSS and system y^+ transport activity was measured as described in the legend of Fig. 1. Points are means of three independent determinations within one representative experiment with SD indicated. The experiment was repeated twice with two distinct HUVEC strains with similar results. $^{**}p<0.01$, $^{***}p<0.001$ vs. control, untreated cells (left); $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. cells treated with TNFα (right). (Panel B) After protein extraction, Western blot analysis was performed using anti-p70S6 kinase, anti-phospho-p70S6 kinase (Thr389) both at a concentration of 1:1000, and anti-GAPDH antisera (1:500, see Materials and methods). A representative Western blot is shown. The experiment was repeated twice with similar results.

3. Discussion

We have recently demostrated that the stimulation of arginine transport and CAT2B expression, elicited by TNFα in human endothelial cells, is associated with the activation of NF-κB pathway [8]. Since other effects of TNFα have been attributed to the activation of the PI3-kinase/Akt/mTOR transduction pathway [26,27], we undertook this study to establish the role of mTOR in TNFα-dependent stimulation of system y^+. Surprisingly, rapamycin did not inhibit the stimulation of arginine transport and CAT2 expression by TNFα but, rather, it significantly enhanced the effect of the cytokine. Consistent with recently published evidences [28,29], our results suggest that, in spite of the antagonism described for rapamycin and TNFα in vascular smooth muscle cells [30], the effects of the two compounds may actually synergize in endothelial cells.

Interestingly, rapamycin alone was also able to stimulate arginine uptake through an increased expression of SLC7A2, suggesting that the inhibitor relieves a repressive effect of the mTOR transduction pathway on the gene.

While the molecular mechanisms underlying rapamycin effect deserve future investigations, they should not be ascribed to the induction of TNFα production by endothelial cells. Indeed, although it is known that human endothelial cells are able to produce this cytokine [31], rapamycin had no significant effect on its expression in HUVECs (Barilli, A., results not shown). Moreover, the dose–response curve for TNFα-dependent stimulation of arginine transport, shown in Fig. 2, indicates that at 10 ng/ml the effect of the cytokine is maximal but still further enhanced by rapamycin.

It should be stressed that SLC7A2 induction seems due to mTOR inhibition rather than to a specific effect of rapamycin. Indeed, a condition that leads to an indirect, rapamycin-independent inhibition of the kinase, such as treatment with AICAR, also causes the stimulation of arginine uptake either in the presence or in the absence of TNFα (see Fig. 8).

The expression of CAT2 transporters was studied here also at the protein level using a polyclonal anti-CAT2 antibody. This antiserum is apparently CAT2-specific and does not seem to interact with CAT1 since with either Western blot or immunocytochemical analysis a signal is detectable only in cells treated with TNFα, rapamycin, or both, that is, under conditions in which only the expression of SLC7A2 is stimulated. With this antiserum we have demonstrated that rapamycin-dependent stimulation of arginine transport in endothelial cells seems quite a straight forward process, with increased SLC7A2 transcription followed by increased abundance of CAT2 proteins and transport stimulation. Interestingly, the immunocytochemical analysis indicates that in cells stimulated with both TNFα and rapamycin, most transporters have an intracellular distribution, although a clear cut positivity

of plasma membrane is also detected, a finding thus far unreported for CAT2 transporters.

Under basal conditions the expression of CAT2 proteins is very low and, hence, their contribution to arginine transport should be negligible. However, given that mTOR inhibition does not change the expression of CAT1, the 2–3 fold stimulation of arginine transport detected in cells treated with both compounds is only ascribable to the induction of CAT2 transporters that, under these conditions, account for a substantial portion of arginine influx. Both CAT2 isoforms, the high affinity CAT2B and the low affinity CAT2A (K_m=5 mM), are induced by rapamycin and, more markedly, by the combined treatment with the mTOR inhibitor and TNFα. However, the significance of CAT2A induction remains unclear since, even at the supraphysiological arginine concentration of the culture medium (0.3 mM vs. normal plasma concentrations ranging around 0.1 mM), the kinetic parameters (see Fig. 3) indicate that no more than 20% of arginine influx in cells treated with both rapamycin and TNFα occurs through CAT2A, with the remaining 80% attributable to CAT2B and CAT1 activity.

Nevertheless, the induction of the low affinity CAT2A isoform appears of particular interest. In control, untreated endothelial cells SLC7A2/CAT2A mRNA was undetectable [8], suggesting that the expression of the low affinity isoform is, at best, marginal under basal conditions. High constitutive expression of CAT2A has been described only in hepatocytes, while the induction of the transporter by cytokines has been reported in other animal cell models, such as rat cardiac myocytes [32] or rat vascular smooth muscle cells [33]. Moreover, CAT2A expression is induced also in rat skeletal muscle under stress conditions, such as surgical trauma or food deprivation [34]. It has been proposed that CAT2A could act as an export route for arginine derived from the breakdown of proteins [35]. This hypothesis appears of great interest since rapamycin is known to induce autophagy in cultured cells [36,37]. Thus, the increase in intracellular arginine, observed in cells stimulated with rapamycin (see Fig. 7), could be the result of both the increase of arginine influx, mostly due to CAT2B expression, and the stimulation of cell proteolysis. If this is the case, CAT2A would provide a device to prevent the excessive accumulation of cationic amino acids in the intracellular compartment.

More comprehensive investigations are required in order to define the functional consequences of these changes in CAT expression and arginine transport. Since the stimulation of mTOR by growth factors and nutrients promotes protein synthesis and cell growth [12–14], the inhibition of this pathway should cause a decrease in nutrient transport. Nevertheless, literature reports examples of inhibitory [38,39] as well as of stimulatory [40,41] effects of rapamycin on nutrient transporters. As far as genes for arginine transporters are concerned, this contribution represents the first investigation on the effects of mTOR inhibition and clearly indicates that only CAT2 expression is modified by rapamycin, while CAT1, previously linked to nutritional stimuli [42], appears unaffected, at least at mRNA level. In non-endothelial models CAT2 induction has been repeatedly associated with an increase in NO synthesis mediated by NOS2 [43]. In contrast, the results presented here suggest that, in human endothelium, CAT2 induction can be uncoupled from the stimulation of NO synthesis. Indeed, rapamycin-treated cells express CAT2 and increase the intracellular arginine concentration but synthesize less NO than untreated controls (see Fig. 7). Unpublished results from our laboratory indicate that this effect is due to the inhibition of NOS3 expression by rapamycin, while NOS2 expression is not detectable under the same conditions (Dall'Asta, V. et al., manuscript in preparation). Given the important protective role of NOS3-derived NO in the vascular endothelium, these results point to the relevance of mTOR activity for the preservation of endothelial function and viability.

Acknowledgments

This study was partially supported by MIUR, Ministero dell'Istruzione, dell'Università e della Ricerca Rome, Italy, (PRIN "In vitro models of endothelial damage of ischemia–reperfusion: role of arginine and nitric oxide"), by I.R.C.C.S. Centro Cardiologico Fondazione Monzino, Milan, Italy, and by Grants Cl 100/4-1 and the Collaborative Research Center SFB 553 (Project B4) from the Deutsche Forschungsgemeinschaft, Bonn, Germany.

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