ANNALS OF SURGERY
Vol. 221, No. 5, 590–601

TNF-Stimulated Arginine Transport by Human Vascular Endothelium Requires Activation of Protein Kinase C

Ming Pan, M.D., Ph.D., Masafumi Wasa, M.D., D. Scott Lind, M.D., Jonathan Gertler, M.D., William Abbott, M.D., and Wiley W. Souba, M.D., Sc.D.

From the Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts; and the Department of Surgery, University of Florida College of Medicine, Gainesville, Florida

Objective

The authors determined the endothelial arginine transport mechanism and the potential role of a tumor necrosis factor (TNF)-α-mediated signal transduction pathway involving protein kinase C (PKC) in regulating this transport in cultured endothelial cells.

Summary Background Data

The vascular endothelium metabolizes arginine to generate nitric oxide (NO), and an increase in NO production can be stimulated by several cytokines. The mechanism(s) responsible for the accelerated arginine transport are poorly understood.

Methods

Arginine transport was assayed in confluent human umbilical vein endothelial cells in the presence of TNF ± the PKC inhibitor chelerythrine chloride.

Results

Carrier-mediated arginine transport was accomplished by two Na^+-independent transporters, System y^+ (80% of total transport) and System $b^{0,+}$ (20% of transport). Tumor necrosis factor (0.1–2 ng/mL) increased System y^+-mediated arginine transport in a time- and dose-dependent manner by augmenting System y^+ transport maximal capacity (control V_{max} = 1325 ± 60 pmol/mg protein/minute *vs.* TNF V_{max} = 3015 ± 110 pmol/mg protein/minute, $p < 0.01$) without affecting transporter affinity (control Km = 30 ± 1.4 μM *vs.* 34 ± 1.3 μM arginine, p = NS). Stimulation was maximal at the 8-hour time point and was inhibited by both actinomycin D and cycloheximide. In addition, inhibition of PKC with chelerythrine abrogated the TNF-augmented arginine transport. Similarly, incubation of cells with the direct PKC activator TPA (phorbol ester 12-myristate 13-acetate) stimulated System y^+-mediated arginine transport nearly fivefold, secondary to an increase in transporter V_{max} (TPA V_{max} = 5349 ± 310 pmol/mg protein/minute, $p < 0.001$ *vs.* control), with no change in Km. This TPA-induced stimulation of arginine transport also was blocked by chelerythrine Cl, actinomycin D, and cycloheximide. Incubation of TNF-stimulated cells with two NO synthase inhibitors did not reduce transport activity, suggesting that the arginine transporter and the NO synthase enzyme may, in part, be independently regulated.

Tumor necrosis factor and TPA only slightly increased System $b^{0,+}$-mediated arginine transport. Treatment of cells with dcAMP (dibutyryl cyclic adenosine monophosphate, a protein kinase A activator) did not alter arginine transport activity, indicating that PKA activation was not required for the response to occur.

Conclusions

These data indicate that TNF stimulates arginine transport in human umbilical vein endothelial cells via a process that requires activation of the intracellular messenger PKC, which in turn signals *de novo* protein synthesis, possibly of the y^+ arginine transporter protein itself.

L-arginine is the exclusive precursor for the biosynthesis of nitric oxide (NO), a short-lived molecule that plays a crucial role in maintaining vascular tone.[1-4] It has been suggested that enhanced NO production by the endothelium may be dependent on accelerated uptake of circulating arginine under certain circumstances.[1,2,5] Although endothelial cells were the first cell type identified to produce NO, little is known about the factors that regulate arginine transport across the endothelial cell membrane. Although it is known that endotoxin[6,7] and certain cytokines[8] can stimulate endothelial cell expression of the NO synthase gene, less information regarding cytokine control of arginine transport is available. Lind and associates[9] demonstrated that both tumor necrosis factor (TNF) and interleukin-1 stimulated arginine transport by the porcine pulmonary artery endothelium, but these events have not been studied in human endothelial cells. Moreover, the specific signal transduction pathways involved in cytokine-mediated increases in endothelial arginine transport require further elucidation.

For complex organisms to develop and differentiate, cells must respond to extracellular signals with a specific set of mechanisms that regulate gene expression. Between the extracellular signal (hormone, cytokine, growth factor, etc.) and the gene (the protein the gene encodes is the final product), a system of diverse cellular components has been assembled that guarantee specific pathways of signal transduction (Fig. 1). Within the cell are specific proteins (targets) that play a central role in controlling this response. Among these targets is a family of closely related enzymes collectively called protein kinase C (PKC), which plays a crucial role in regulating cellular proliferation, differentiation, and function.[10,11] There are at least nine PKC iso-enzymes that exist in different organs and determine tissue biological responses when PKC is activated.[12] Naturally occurring PKC activators that are found in all cells include diacylglycerol, calcium, and certain phospholipid derivatives. Synthetic derivatives known as phorbol esters (e.g., phorbol ester 12-myristate 13-acetate [TPA]) directly activate PKC by acting at the diacylglycerol binding site[13] and have been used experimentally to study the regulation of PKC.

Because of the potential link between arginine transport and NO production and because recent studies have demonstrated that PKC activation can modulate amino acid transport, the current experiments were undertaken. In the current studies, the endothelial arginine transport mechanism and the potential role of a TNF-mediated signal transduction pathway involving PKC in regulating this transport were investigated in cultured human umbilical vein endothelial cells. Our data indicate that TNF stimulates arginine transport in human umbilical vein endothelium via a process that requires activation of the intracellular messenger PKC, which in turn signals *de novo* protein synthesis, possibly of the y^+ arginine transporter protein itself.

MATERIALS AND METHODS

Reagents

Human umbilical venous endothelial cells (HUVECs), trypsin neutralizing solution, HEPES-buffered saline solution, trypsin/ethylenediaminetetraacetic acid, and endothelial cell growth medium were purchased from Clonetics Corporation, San Diego, California. Tumor necrosis factor-α was obtained from Quality Controlled Biochemicals. Inc., Hopkinton, Massachusetts. Chelerythrine chloride was obtained from LC Laboratories, Woburn, Massachusetts. Phorbol ester 12-myristate 13-acetate (TPA), cycloheximide, actinomycin D, dibutyryl cyclic adenosine monophosphate (dcAMP), N ω-nitro-L-arginine (L-NA), N ω-nitro-L-arginine methyl ester (L-NAME), and all other reagents were obtained from Sigma Chemical Inc., St. Louis, Missouri. Cell culture flasks were purchased from Corning Co., Corning, New York. Twenty-four-well cell culture dishes were ob-

Presented at the 106th Annual Session of the Southern Surgical Association, December 4–7, 1994, Palm Beach, Florida.

Supported by NIH Grant R01 HL44986 (Dr. Souba).

Address reprint requests to Wiley W. Souba, M.D., Sc.D., Chief, Division of Surgical Oncology, Massachusetts General Hospital, Cox 626, 100 Blossom Street, Boston, MA 02114.

Accepted for publication January 13, 1995.

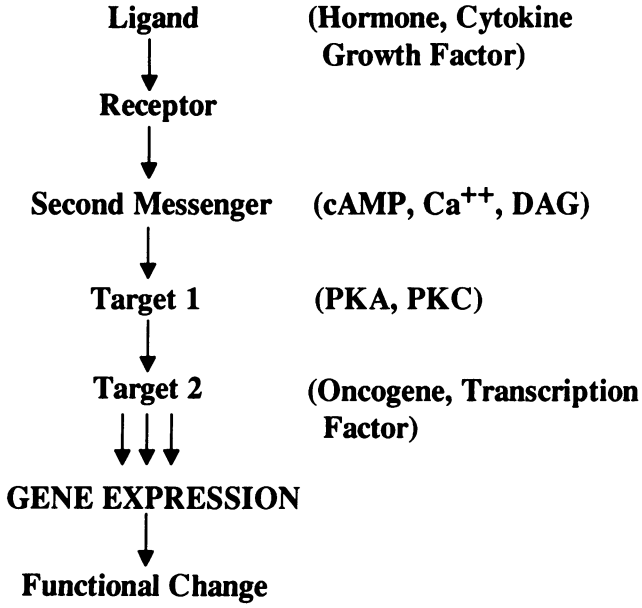

Figure 1. Simplified schema of the signal transduction pathway. Extracellular ligands bind to their receptors to stimulate the production of second messengers, which activate a target cascade leading to gene transcription. In many cases, PKC is the first target. Gene transcription leads to a functional change, generally from the enhanced expression of protein(s).

tained from Becton and Dickinson Co, Lincoln Park, New Jersey.

Preparation of Human Umbilical Vein Cell Cultures

Cryopreserved primary (1 C) HUVECs were thawed at 37 C in a water bath. The cells were immediately transferred into growth medium which had been equilibrated in a 5% CO_2/95% air humidified incubator for 30 minutes. Human umbilical venous endothelial cells were seeded on 75 cm^2-culture flasks at a density of 2500 cells/cm^2 and grown under similar conditions. The culture medium was changed the next day, then every other day thereafter. When cell confluence exceeded 60%, medium was increased from 1 mL/5 cm^2 to 2 mL/5 cm^2.

Passage of Cells

Culture medium was aseptically removed from HUVEC monolayers (approximately 80% confluent) and 10 mL of trypsin/ethylenediaminetetraacetic acid solution was then added to cover the cells. Trypsinization lasted for 3 to 4 minutes, until approximately 50% of the cells were detached. Gentle shaking facilitated the release of cells from culture surface. Cells were transferred to a sterile centrifuge tube containing 10 mL of trypsin-neutralizing solution and were centrifuged at 220 × g for 5 minutes to form a pellet. The supernatant was discarded and the cell pellet was resuspended in 10 mL of growth medium. Human umbilical venous endothelial cells (2nd–5th passage) were plated in 24-well dishes and were allowed to grow to confluence (~7 days) before transport experiments.

Cell Treatments

Twenty-four hours after reaching confluence, cells were treated with TNF-α (0–2 ng/mL), TPA (0–1 μM), or dcAMP (1 μM) for various periods of time (0–24 hours). Cells also were incubated with cycloheximide (20 μM), actinomycin D (1 μM), chelerythrine chloride (6.5 μM), L-NA (100 μM), or L-NAME (100 μM) before transport experiments were undertaken.

^{3}H-Arginine Transport Assay

^{3}H-arginine transport uptake measurements were done as described previously.[14] Transport activity was measured in either choline chloride Krebs-Ringer Buffer (KRP) or NaCl-KRP (choline Cl or NaCl 119 mM, KCl 5.9 mM, $MgSO_4$-$7H_2O$ 1.2 mM, $KHCO_3$ 1.2 mM, glucose 5.6 mM, $CaCl_2$-$2H_2O$ 0.5 mM, and choline phosphate 25 mM). Transport was initiated by adding 0.25 mL of 37 C choline-KRP or Na-KRP containing ^{3}H-arginine at various concentrations (1 μM–10 mM according to experiments) to HUVEC monolayers and incubating for 30 seconds in the 37 C water bath. Transport was terminated by discarding the uptake buffer and rinsing the cells with ice-cold buffer three times. The cells were allowed to dry and were solubilized in 200 μl 0.2 N NaOH/0.2% sodium dodecyl sulfate solution.

Liquid scintillation spectrometry was used to measure the radioactivity in cell suspensions and uptake mixes. Protein content was determined by bicinchoninic acid protein method.[15] Na^+-independent transport was determined by subtracting the nonsaturable portion of the arginine uptake in choline-KRP from total uptake. System y^+ arginine transport was defined as the Na^+-independent transport of arginine in the presence of 10 mM L-leucine in the uptake buffer. The leucine-inhibitable portion of carrier-mediated Na^+-independent uptake was defined as System $b^{0,+}$.[9] Transport velocities were expressed in picomoles per milligram of protein per minute. Data (mean ± SD) were analyzed with Student's t test or one way analysis of variance. A p level < 0.05 was considered statistically significant.

RESULTS

Arginine Transport Characteristics

Arginine uptake by HUVECs, measured in both choline-KRP and Na-KRP buffers, was linear for at least 1

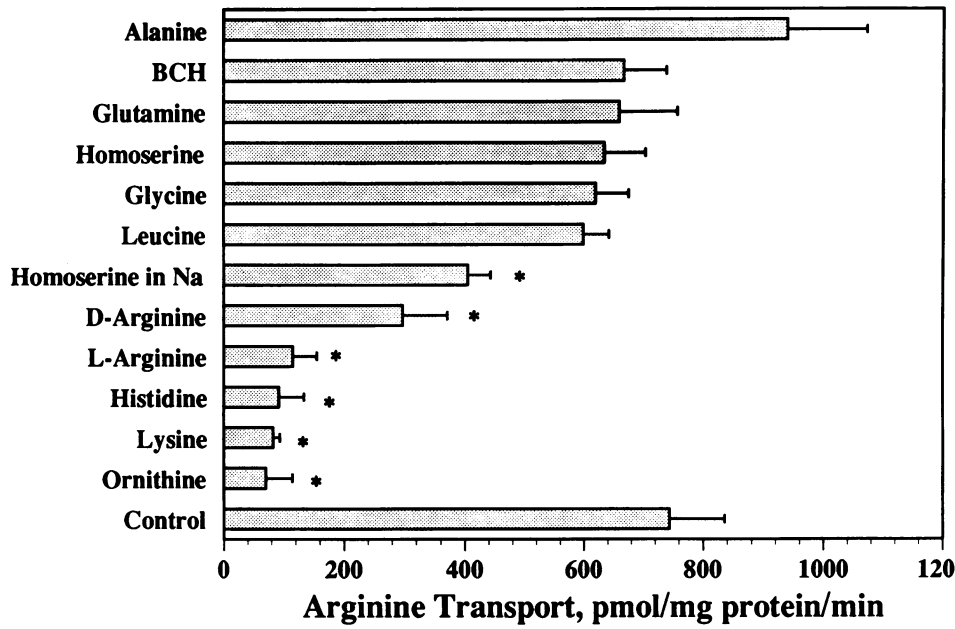

Figure 2. Amino acid inhibition profile of Na^+-independent arginine transport. Arginine (50 μM) uptake was measured in confluent HUVECs with 10-mM individual amino acids in choline-KRP uptake buffer. Transport velocity units are pmol/mg protein/minute. Transport values are means ± SD (n = 6). *p < 0.01 *vs.* control.

minute. Therefore, a 30-second uptake time was chosen for subsequent experiments to ensure uptake linearity. At [arginine] = 50 μM, Na^+-independent arginine uptake accounted for 75% of total arginine uptake (the Na^+-dependent component was only 25% of total uptake). The addition of leucine (10 mM) to the uptake buffer blocked 15% to 20% of total Na^+-independent arginine uptake, indicating a minor contribution of System $b^{0,+}$.[9,16] The majority of arginine uptake, not leucine-inhibitable, had an amino acid inhibition profile consistent with the well-known cationic amino acid transport System y^+ (Fig. 2).[17,18] Given these initial observations, all subsequent transport studies were done in the presence of excess leucine to block any contribution of System $b^{0,+}$ and in the absence of sodium ion to focus on System y^+.

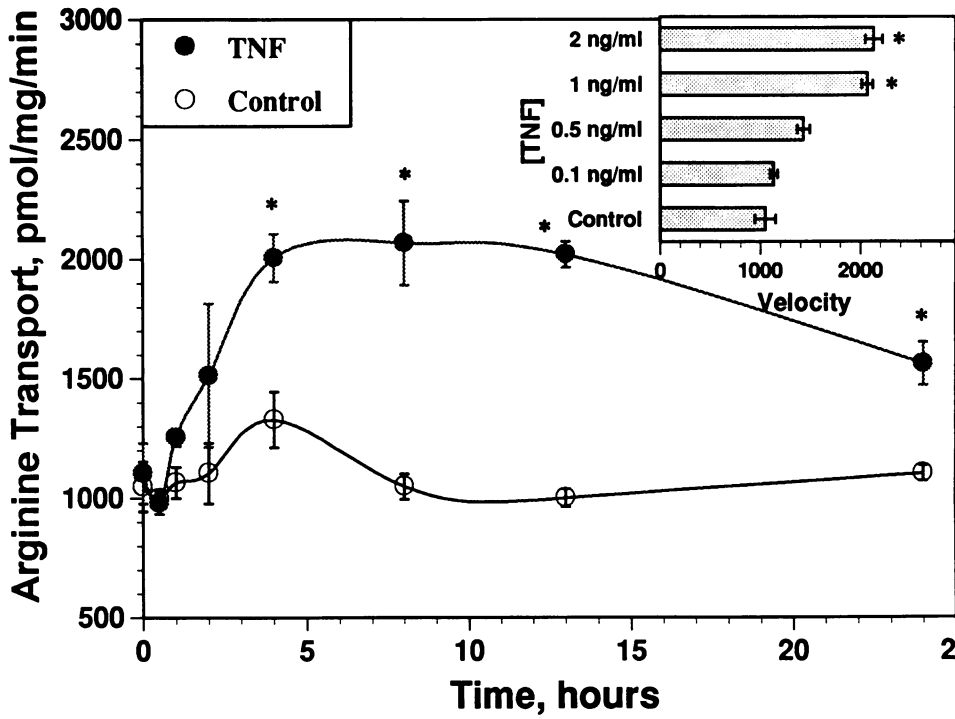

Figure 3. Effect of TNF on System y^+-mediated arginine transport. Arginine transport was measured after cells had been incubated with TNF (1 ng/mL) for various periods of time (0–24 hours). Inset: dose response of TNF stimulation of arginine transport. Cells were incubated with TNF (0–2 ng/mL) for 8 hours before measurements were made. Transport values are means ± SD (n = 6). *p < 0.01 *vs.* control.

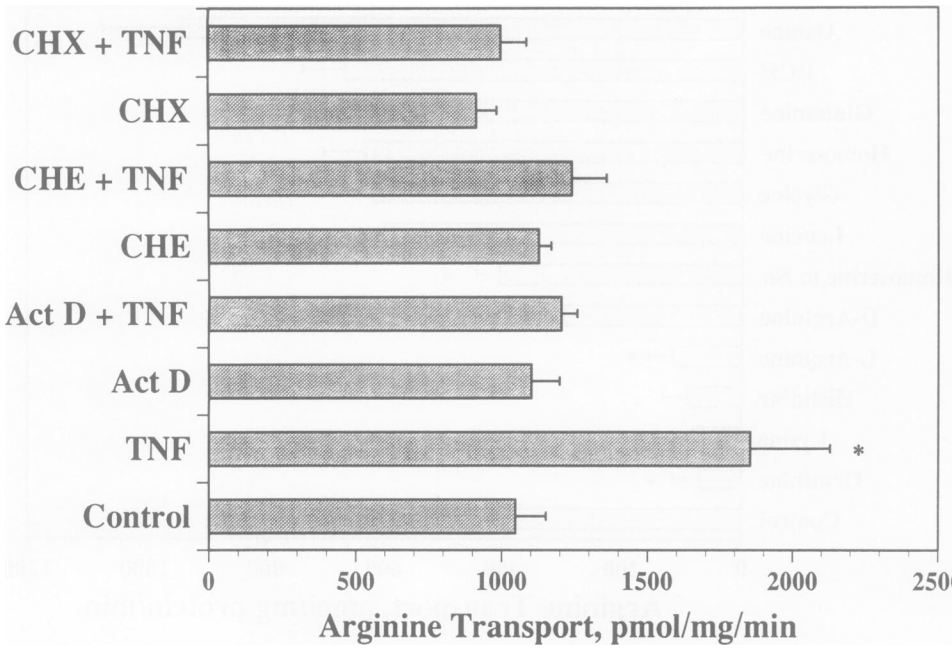

Figure 4. Effects of actinomycin D (Act D), cycloheximide (CHX), and chelerythrine chloride (CHE) on TNF stimulation of System y^+-mediated arginine transport. HUVECs were incubated with TNF (1 ng/ml) ± Act D (1 μM), CHX (20 μM), or CHE (6.5 μM) for 8 hours before measurements were made. Transport values are means ± SD (n = 6). *$p < 0.01$ *vs.* control.

Effect of TNF on Arginine Transport

We next investigated the effect of TNF (1 ng/mL) on arginine transport. Over a 5-minute to 24-hour incubation period, TNF stimulated System y^+ arginine uptake in a dose- and time-dependent manner (Fig. 3). The response was first observed at 2 hours, and lasted for 24 hours. A slightly greater than twofold increase in y^+-mediated arginine uptake was observed in TNF-treated cells at the 8-hour time point. Higher TNF concentration (1–2 ng/mL, Fig. 3 inset) resulted in an enhanced stimulatory effect.

The TNF-induced increase in arginine transport activity was abrogated by both actinomycin D and cycloheximide (Fig. 4), indicating a requirement for DNA tran-

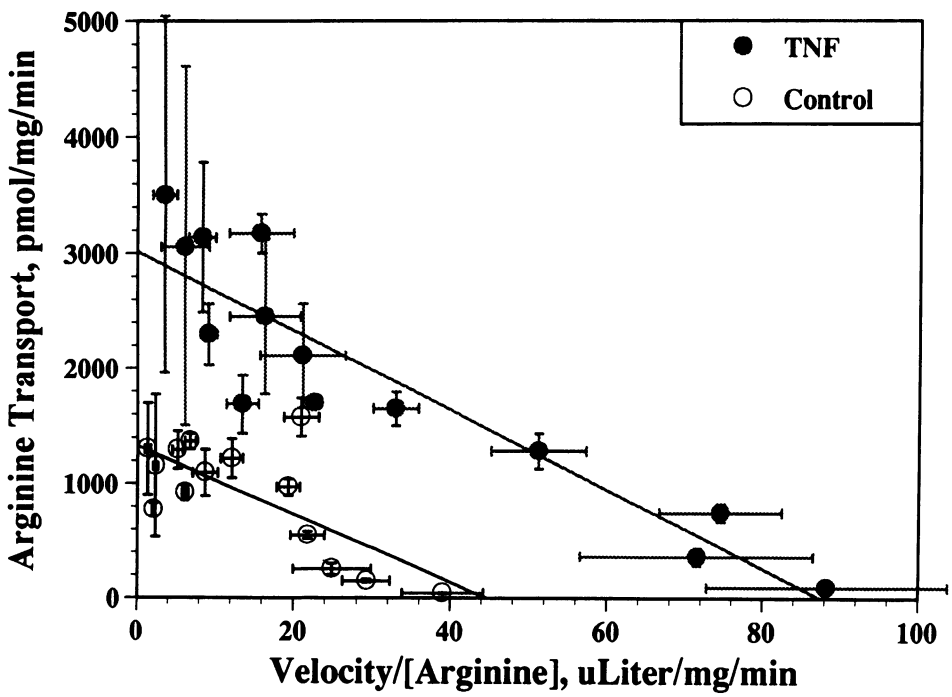

Figure 5. Effect of TNF on System y^+ arginine transport kinetics. Eadie-Hofstee linear transformation of the transport data (n = 6) is accomplished when arginine transport velocity is determined as a function of velocity/arginine concentration. TNF increased arginine transporter maximal capacity Vmax (y-intercept) without affecting the apparent transporter affinity Km (negative slope of each line). See text for actual kinetic values.

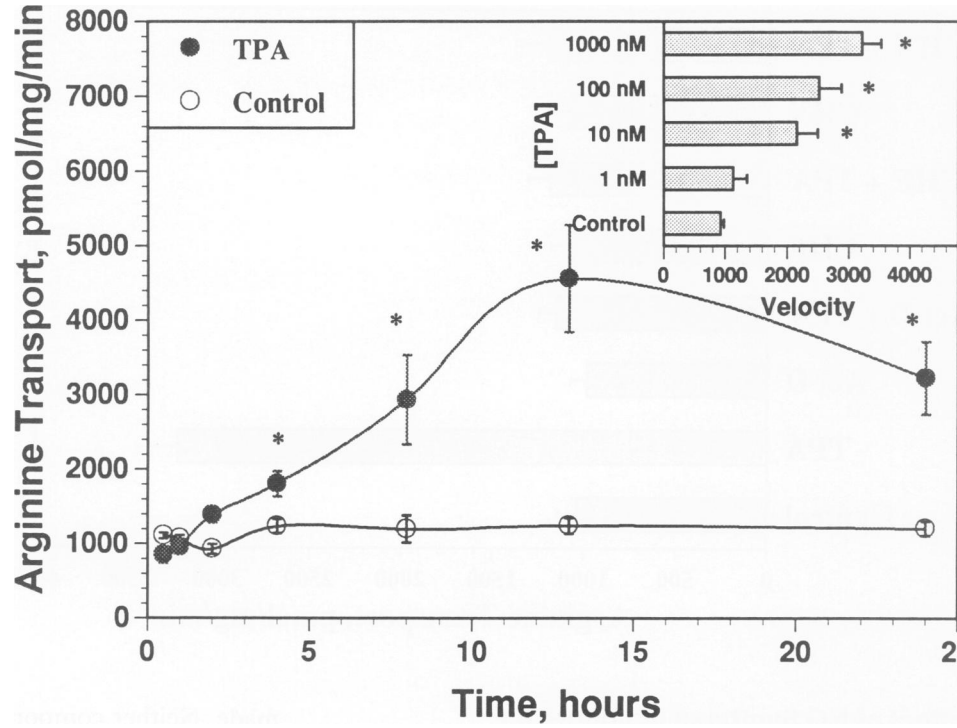

Figure 6. Effect of phorbol 12-myristate 13-acetate (TPA) on arginine transport. System y^+ arginine transport was measured after HUVECs had been incubated with TPA (0.5 μM) for 0–24 hours. Inset: dose response of TPA stimulation of arginine transport. Cells were incubated with TPA (0–1 μM) for 8 hours before measurements were made. Transport values are means ± SD (n = 6). *p < 0.01 *vs.* control.

scription and *de novo* protein synthesis. Arginine (1 μM–1 mM) transport kinetics (V_{max}, maximal transport velocity and Km, transporter affinity) were then determined in control and TNF-treated cells (Fig. 5). Tumor necrosis factor-augmented transporter maximal capacity (control V_{max} = 1325 ± 60 pmol/mg protein/minute *vs.* Tumor necrosis factor V_{max} = 3015 ± 110 pmol/mg protein/minute, $p < 0.01$) without affecting the transporter affinity (control Km = 30 ± 1.4 *vs.* TNF Km = 34 ± 1.3 μM arginine, p = NS). These kinetic characteristics are consistent with an increase of functional transporter units rather than modification of existing transporter affinity for its substrate arginine. In light of these kinetic data, the newly synthesized protein may be the System y^+ carrier itself. The specific PKC inhibitor chelerythrine chloride also blocked the TNF-mediated increase in arginine transport (Fig. 4) without affecting baseline transport activity, indicating that PKC was involved in this TNF-induced response.

Effect of TPA on Arginine Transport

To further test the hypothesis that PKC activation was required for TNF to augment System y^+ activity, we investigated effects of the direct PKC activator TPA on arginine transport in HUVECs. Like TNF, TPA stimulated arginine transport in a dose- and time-dependent fashion (Fig. 6). TPA required 2 to 4 hours to increase System y^+ activity and reached peak stimulation at 13 hours, at which time a threefold increase in transport activity was noted. This TPA stimulation also was abrogated by both actinomycin D and cycloheximide (Fig. 7). Kinetically, TPA increased the System y^+ transporter maximal capacity (Fig. 8) fourfold (control V_{max} = 1325 ± 60 pmol/mg protein/minute *vs.* TPA V_{max} = 5349 ± 310 pmol/mg protein/minute, $p < 0.001$) without affecting transporter affinity (control Km = 32 ± 1.4 μM *vs.* TPA Km = 32 ± 1.5 μM arginine, p = NS). As aforementioned, these kinetic characteristics are consistent with an increase in functional transporter units rather than a modification of the existing transporter affinity. Chelerythrine chloride also blocked the TPA-induced increase in arginine transport (Fig. 7) without affecting baseline transport activity. These data further indicate that PKC activation was involved in this response.

Effect of Protein Kinase A Activation on Arginine Transport Activity

To test for a role for the intracellular messenger protein kinase A (PKA) in regulating the TNF-mediated enhancement of System y^+ activity, we incubated cells with dcAMP for various periods of time (0–24 hours). dcAMP did not alter arginine transport, indicating that PKA activation was not part of the signal transduction pathway that resulted in a functional change in arginine transport (Fig. 9).

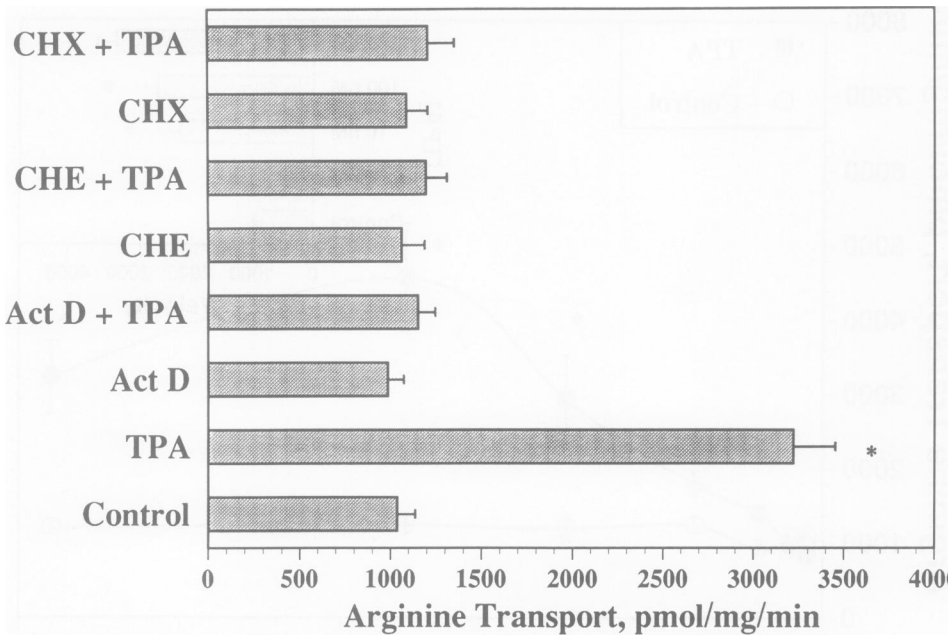

Figure 7. Effects of actinomycin D (Act D), cycloheximide (CHX), and chelerythrine chloride (CHE) on TPA stimulation of System y^+-mediated arginine transport. Human umbilical venous endothelial cells were incubated with TPA (0.5 μM) ± Act D (1 μM), CHX (20 μM), or CHE (6.5 μM) for 8 hours before measurements were made. Transport values are means ± SD (n = 6). *$p < 0.01$ *vs.* control.

Effect of NO Synthase Inhibitors on Arginine Transport

The effects of the NOS inhibitors L-NA and L-NAME on arginine transport activity were also investigated (Fig. 10). Human umbilical vein endothelial cells were incubated with TNF or TPA, with or without L-NA or L-NAME, for 8 hours before uptake measurements were made. Neither compound significantly altered arginine transport in control and treated cells.

DISCUSSION

In this study, we characterized arginine transport by human umbilical vein endothelial cells, investigated the

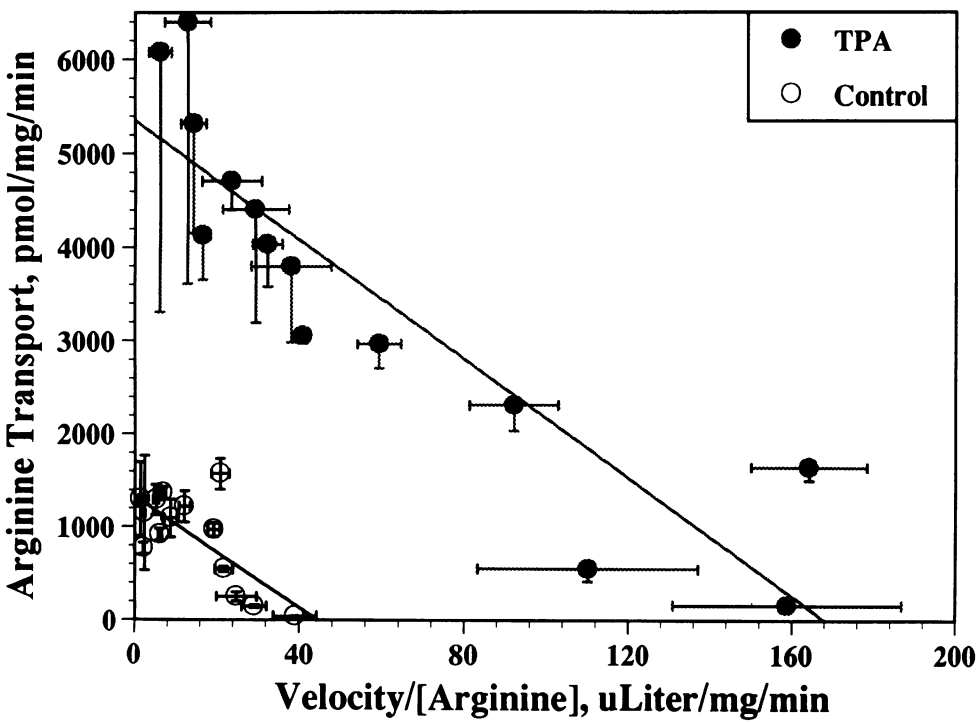

Figure 8. Effect of TPA on System y^+ arginine transport kinetics. Eadie-Hofstee linear transformation of the transport data is shown (n = 6). TPA increased arginine transporter maximal capacity V_{max} (y-intercept) without affecting the apparent transporter affinity Km (negative slope of each line). See text for actual kinetic values.

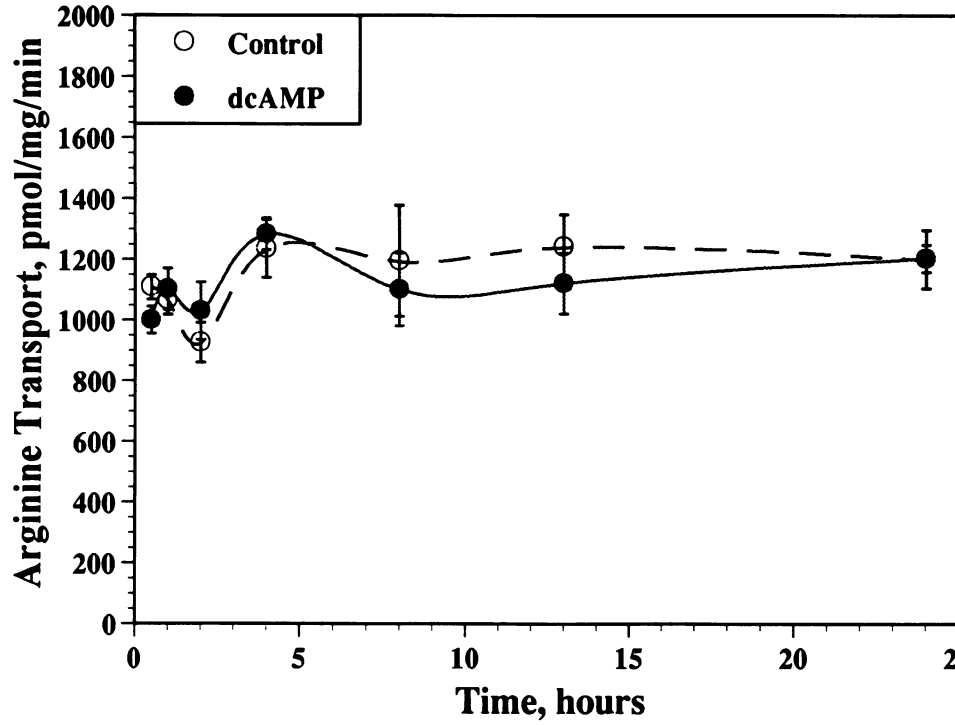

Figure 9. Effect of dibutyryl cyclic adenosine monophosphate (dcAMP, 1 μM) on System y^+-mediated arginine transport. Transport values are means ± SD (n = 6).

effects of TNF on this transport activity, and studied the potential requirement of PKC activation in the TNF-mediated signal transduction pathway that led to an increase in arginine transport activity. Our impetus for studying these responses was stimulated by the central role that arginine occupies in endothelial cell nitrogen metabolism and by our interest in the mechanism by which cytokines modulate transport in different tissues. Clearly, the control of the signal transduction pathway initiated by TNF binding to its extracellular receptor, which results in accelerated arginine transport, is a complex one, and the current studies have attempted to address only one of the mechanisms involved.

The vascular endothelium plays a key role in regulating vascular tone, and recent studies have demonstrated that this is accomplished partially through the elabora-

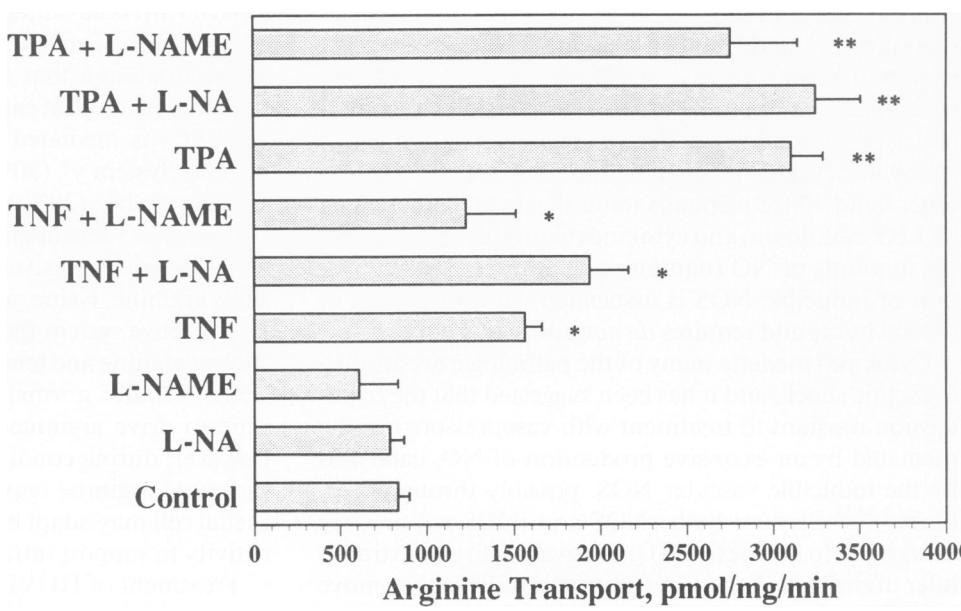

Figure 10. Effects of NOS inhibitors L-NA and L-NAME on System y^+-mediated arginine transport. Human umbilical venous endothelial cells were incubated with TPA (0.5 μM) or TNF (1 ng/mL) ± L-NA (100 μM) or L-NAME (100 μM) for 8 hours before measurements were made. Transport values are means ± SD (n = 6). *p < 0.05, **p < 0.01 *vs.* control.

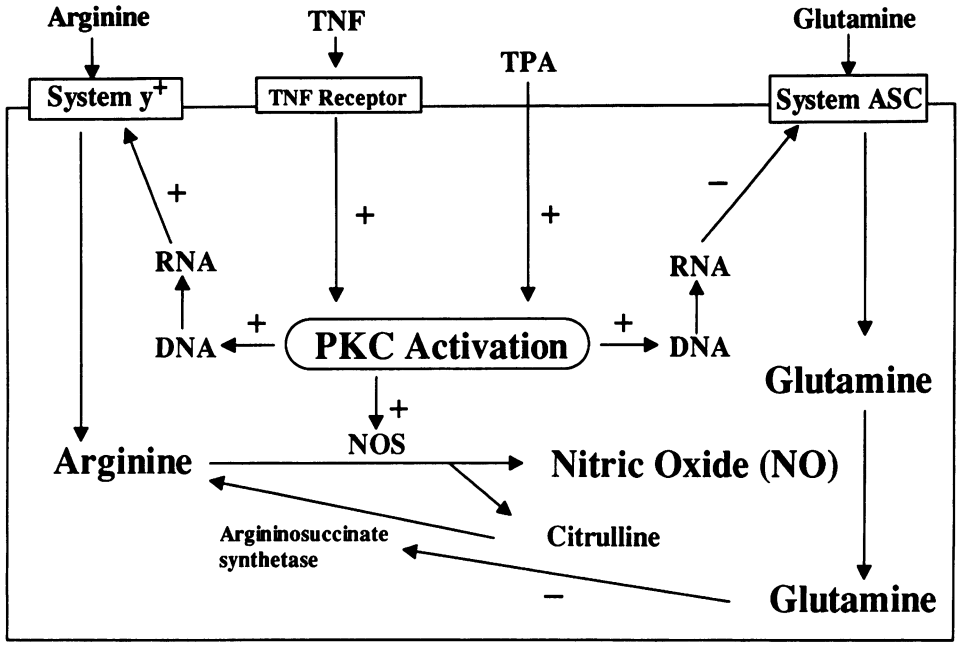

Figure 11. Putative regulation of arginine and glutamine transport in HUVECs. Activation of PKC iso-enzymes by TNF or TPA stimulates transmembrane arginine transport by enhancing *de novo* synthesis of the System y^+ arginine transporter protein. Simultaneously, glutamine transport (System ASC) is inhibited secondary to the synthesis of an inhibitory protein that diminishes ASC activity. A portion of the transported arginine is metabolized to NO and citrulline by nitric oxide synthetase (NOS), a process that is stimulated by TNF. Intracellular citrulline can be converted back to arginine by argininosuccinate synthetase, an enzyme inhibited by intracellular glutamine. The combination of increased arginine uptake concurrent with a decreased glutamine transport, mediated by PKC activation, may be one mechanism by which the endothelial cell controls NO production.

tion of the short-lived bioregulatory molecule NO.[1,2] In physiologic states, NO acts as endothelium-derived relaxing factor, regulating cardiovascular tone and blood flow in response to receptor stimulation. In pathologic states, NO mediates tumoricidal and microbicidal cytotoxicity and has been shown to be the mediator of hypotension in septic shock.[2,19–21] Nitric oxide is generated from the terminal guanidino nitrogen atom(s) of L-arginine, a process catalyzed by nitric oxide synthase (NOS).[22] The other product of this reaction is citrulline, which can be reconverted to arginine in the cytoplasm.[23] Two forms of NOS have been described, a constitutive NOS (cNOS), which is a calmodulin/Ca^{2+}-dependent enzyme, and an inducible NOS (iNOS), which is a calmodulin/Ca^{2+}-independent enzyme. cNOS is a rapidly responding enzyme that synthesizes short-lasting NO (picomole amounts) in response to physiologic stimuli and acts as endothelium-derived relaxing factor through guanylate cyclase to regulate vascular tone.[1,2] On the other hand, iNOS responds more slowly and is activated by LPS, endotoxin, and cytokines to producing long-lasting amounts of NO (nanomole quantities). The activation of inducible NOS is associated with a lag time of several hours and requires *de novo* protein synthesis.[1]

Cytokines mediate many of the pathologic events during septic shock, and it has been suggested that the hypotension resistant to treatment with vasopressors may be mediated by an excessive production of NO, catalyzed by the inducible vascular NOS, possibly through cytokines.[2,24,25] Because both cNOS and iNOS require free L-arginine to synthesize NO, the availability of extracellular arginine and the transport mechanisms that move extracellular arginine into cells may be key pathways in regulating NO production.

Plasma membrane amino acid transport is a complex process that involves specific membrane-bound transport proteins. In general, each transport system relates to a group of homogeneous transporter proteins that reside in and span the cell membrane and serve to translocate a group of structurally related amino acids into the cytoplasm. Transport can be broadly classified into Na^+-dependent and Na^+-independent processes. In the case of Na^+-independent transport processes, sodium is not an essential cotransporter ion. The cluster tray method of measuring amino acid transport in cultured cells *in vitro* is advantageous because it avoids the *in vivo* complexities of cell-cell interactions and changes in the microcirculation that may alter transport. The current studies revealed that carrier-mediated arginine uptake by HUVECs is mediated by two Na^+-independent transporters, System y^+ (80% of Na^+-independent transport) and System $b^{0,+}$ (20% of Na^+-independent uptake). Only a minor Na^+-dependent component was identified. System y^+ has an exclusive preference for the cationic amino acids arginine, lysine, and ornithine.[17,18] System $b^{0,+}$ is a less selective system that transports dipolar amino acids, such as alanine and leucine, as well as the cationic amino acids.[22] Under normal conditions, there is ample arginine to drive arginine-dependent metabolic pathways; however, during conditions of arginine deprivation and increased arginine requirements (i.e., sepsis), the endothelial cell may adapt by increasing membrane transport activity to support intracellular arginine metabolism.

Treatment of HUVECs with TNF stimulated System

y^+-mediated arginine transport activity in a time- and dose-dependent manner. In contrast, only minimal enhancement of System $b^{0,+}$-mediated arginine transport was observed (data not shown). This latter transporter is a low affinity carrier that contributes little to total uptake at physiologic arginine concentrations. At least 2 hours were required for TNF to exert its stimulatory effect, which is consistent with the time lag required for TNF to activate iNOS. The augmented System y^+ arginine transport activity required DNA transcription and *de novo* protein synthesis and was the result of an increase in transporter V_{max}. These results are consistent with *de novo* synthesis of the carrier protein itself. Incubation of stimulated cells with chelerythrine chloride abrogated the TNF effect, indicating the involvement of PKC activation in the TNF-induced response.

Protein kinase C is a family of intracellular enzymes that control diverse biological functions.[10–12] It mediates signaling pathways through which several inflammatory mediators exert several of their biological properties.[10–12] Consistent with our observations in TNF-treated endothelial cells, direct PKC activation by the phorbol ester 12-myristate 13-acetate also stimulated arginine transport. The TPA-stimulated arginine transport required DNA and protein synthesis and was due to a V_{max} effect. The timing and magnitude of this effect strongly suggest that TNF and TPA act similarly to up-regulate arginine transport in HUVECs. In contrast, when cells were incubated with the PKA activator dcAMP for various periods of time (0–24 hours), arginine transport was not affected, indicating that PKA was not involved in the TNF-induced augmentation of arginine transport.

L-arginine analogues such as N ω-monomethyl-L-arginine (L-NMMA), N ω-L-arginine (L-NA), or N ω-L-arginine methyl ester (L-NAME) compete with L-arginine for NOS and are specific NOS inhibitors. Baseline, LPS-, and cytokine-induced NO production are blocked by these inhibitors.[7,8] In the current study, we investigated effects of L-NA and L-NAME on arginine transport. Unlike NOS, the TNF or TPA-induced increase in endothelial arginine transport was not blocked by L-NA or L-NAME. These data suggest that TNF activation of iNOS and arginine transport may be partially independent events. Arginine is required for other intracellular reactions, it has been shown to play a central role in polyamine biosynthesis, and it has both immunomodulatory and secretory functions.[9]

Activation of PKC in the endothelial cells we studied does not result in a generalized increase in amino acid transport. In contrast to arginine, the transport of glutamine is diminished by PKC activation.[27] This divergent regulation of two distinctly different carrier proteins (System y^+ and System ASC) may allow the endothelium to regulate its production of NO (Fig. 11). Sessa and colleagues have shown that intracellular glutamine inhibits the regeneration of arginine from citrulline by blocking argininosuccinate synthetase.[28] Conceivably, to enhance NO biosynthesis, PKC activation simultaneously activates iNOS and stimulates arginine transport, facilitating extracellular arginine transport across cellular membrane into cells to provide the essential substrate for NO synthesis; at the same time, intracellular accumulation of glutamine is inhibited, thereby allowing the regeneration of arginine to occur. The combination of increased arginine uptake concurrent with a decreased glutamine transport, mediated by PKC activation, may be one mechanism by which the endothelial cell controls NO production.

This study is consistent with TNF-activation of its membrane receptor and subsequent stimulation of second messengers (diacylglycerol, phospholipids, or Ca^{++}), which activate PKC. In response to TNF, an increase in System y^+ transport activity was observed, presumably because of activation of the y^+ gene with enhanced synthesis of the transport protein. Alternatively, a comodulator protein could have been synthesized that increased the functional activity of existing membrane bound y^+ transporters. In light of the relatively recent cloning of the arginine transporter,[29] molecular studies will confirm whether there was an increase in transcription of the y^+ gene.

References

1. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43: 109–142.
2. Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992; 6:3051–3064.
3. Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. Ann Rev Pharmacol Toxicol 1990; 30:535–560.
4. Ignarro LJ. Signal transduction mechanisms involving nitric oxide. Biochem Pharmacol 1991; 41:484–490.
5. Hibbs JB, Taintor RR, Vavrin Z, et al. Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. *In* Moncada S, Higgs EA, eds. Nitric Oxide from L-Arginine: A Bioregulatory System. Amsterdam: Elsevier Science Publishers B V, 1990, pp 189–223.
6. Thiemermann C, Vane J. Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat *in vivo*. Eur J Pharmacol 1990; 182:591–595.
7. Kilbourn RG, Jubran A, Gross SS, et al. Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide symthesis. Biochem Biophys Res Commun 1991; 172:1132–1138.
8. Kilbourn RG, Gross SS, Jubran A, et al. N^G-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implication for the involvement of nitric oxide. Proc Natl Acad Sci U S A 1990; 87:3629–3632.
9. Cendan JC, Souba WW, Copeland EM, Lind DS. Cytokines regulate endotoxin stimulation of endothelial cell arginine transport. Surgery 1995; 117:213–219.

10. Hug H, Sarre TF. Protein kinase C isoenzymess: divergence in signal transduction? Biochem J 1993; 291:329–343.
11. Clemens MJ, Trayner I, Menaya J. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. J Cell Sci 1992; 103:881–887.
12. O'Brian CA, Ward NE. Biology of the protein kinase C family. Cancer Metastasis Rev 1989; 8:199–214.
13. Castanga M, Takai Y, Kaibuchi K, et al. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 1982; 257:7847–7853.
14. Gazzola GC, DAll'Asta V, Franchi-Gazzola R, et al. The cluster tray method for rapid measurement of solute fluxes in adherent cultured cells. Anal Biochem 1981; 115:368.
15. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985; 150:76.
16. Van Winkle LJ, Campione AL, Gorman JM. Na^+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between these substrates. J Biol Chem 1988; 263:3150–3163.
17. Christensen HN, Liang M. Transport of diamino acids into the Enrlich cells. J Biol Chem 1966; 241:5542–5551.
18. Greene B, Pacitti AJ, Souba WW. Characterization of L-arginine by pulmonary artery endothelial cells. Am J Physiol 1991; 260: L241–L246.
19. Drapier JC, Hibbs JB. Differentiation of Murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. J Immunol 1988; 140:2829–2838.
20. Ochoa JB, Udekwu AO, Billiar TR, et al. Nitrogen oxide levels in patients after trauma and sepsis. Ann Surg 1991; 214:621–626.
21. Westenberger U, Thanner S, Ruf HH, et al. Formation of free radicals and nitric oxide derivative of hemoglobin in rats during shock syndrome. Free Rad Res Commun 1990; 11:167–178.
22. Stuehr DJ, Griffith OW. Mammalian nitric oxide synthetase. Adv Enzymol 1992; 65:287–346.
23. Hecker M, Sessa WC, Harris HJ, et al. The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. Proc Natl Acad Sci U S A 1990; 87:8612–8616.
24. Nussler AK, di Silvio M, Billiar TR, et al. Stimulation of the nitric oxide synthase pathway in human hepatocytes by cytokines and endotoxin. J Exp Med 1992; 176:261–266.
25. Hibbs JB, Westenfelder C, Taintor R, et al. Evidence of cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. J Clin Invest 1992; 89:867–877.
26. Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 1988; 333:664–666.
27. Pan M, Wasa M, Souba WW. Glutamine transport in human endothelium is inhibited by protein kinase C activation. Surg Forum 1994; XLV:340–343.
28. Sessa WC, Hecker M, Mitchell JA, et al. The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: L-Glutamine inhibits the generation of L-arginine by cultured endothelial cells. Proc Natl Acad Sci USA 1990; 87:8607–8611.
29. Closs EI, Lyons CR, Kelly C, et al. Characterization of the third member of the MCAT family of cationic amino acid transporters. J Biol Chem 1993; 268:20796–20800.

Discussion

DR. MARK A. WILSON (Louisville, Kentucky): President McDonald, Secretary Copeland, Fellows, and Guests. As many of us are aware, nitric oxide has been recognized as a central mediator of many physiologic and pathophysiologic processes as evidenced by the myriad publications about this molecule. However, there is little data and only a handful of studies that have examined intracellular availability of arginine and its transport across cell membranes.

Dr. Souba and his coauthors are to be congratulated for this current elegant study which, I believe, adds significantly to our understanding of endothelial cell biology as related to the second messenger and mechanisms of arginine transport. I have three questions for the authors.

First, can you tell us whether these responses of the arginine transporter also occur in other cell types or with other mediators and, if so, whether the mechanisms are similar? Secondly, the importance of extracellular arginine in the function of nitric oxide synthase is controversial, particularly in endothelial cells and particularly in the case of the constitutive nitric oxide synthase. Several studies by Bogle have demonstrated that hormones such as bradykinin increase the y+ transporter affinity for arginine and increase the rapidity of arginine within just seconds after stimulation.

Yet the significance of this finding has been questioned because intracellular levels of arginine in endothelial cells are several-fold higher than those levels that are necessary for optimal function of the constitutive enzyme. In light of these studies, would you please elaborate on the presumed importance of extracellular arginine for function of both the constitutive and inducible nitric oxide synthases, both in endothelial cells and other cell types.

Next, much of your previous research has been focused on the decrease that occurs in glutamine transport under similar conditions. Because glutamine can serve as an intracellular source of arginine, please speculate about the interrelationships of these two systems.

Finally, are there any prospects for selective antagonists of arginine transport which might be useful in differentiating the potentially beneficial effects of constitutive nitric oxide synthase from the potentially detrimental effects of larger amounts of nitric oxide produced by the inducible system?

I very much enjoyed the opportunity to review the manuscript and thank the Association for the privilege of the floor.

DR. R. NEAL GARRISON (Louisville, Kentucky): President McDonald, Secretary Copeland. Dr. Souba asked me to discuss this paper, and I feel honored to do so, but find it difficult to follow Dr. Wilson's in-depth discussion.

One aspect that I would like to focus on relates to the endothelial cell and its overall function. The endothelial cell is very complex. At times, it is an immunologically reactive type of cell, and the transplant surgeons worry about it. At other times, it is an antithrombogenic type of cell, which brings comfort to the hearts of vascular surgeons. And, thirdly, it redistributes blood flow or regulates—autoregulates blood flow, a process the trauma surgeons worry about quite often.

These studies utilize umbilical vein endothelial cells; yet most, if not all, inflammation occurs under a different set of circumstances. In our *in vivo* microscopy studies, the most reactive vessels are the small precapillary microvessels where au-