ELSEVIER

European Journal of Pharmacology 339 (1997) 289–293

ejp

Short communication

Cationic amino acid transporter-2 mRNA induction by tumor necrosis factor-α in vascular endothelial cells

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Received 10 September 1997; revised 26 September 1997; accepted 30 September 1997

Abstract

Nitric oxide (NO) synthesis may be coupled to the activity of the cellular L-arginine transporter, namely the cationic amino acid transporter. The present study examined tumor necrosis factor (TNF)-α-induced alterations in the gene expression of the cationic amino acid transporter (CAT) and NO production in human umbilical vein endothelial cells. In quiescent endothelial cells, CAT-1 mRNA expression, determined by reverse transcription-polymerase chain reaction, was dominant to that of CAT-2. TNF-α (10 ng/ml for 1–24 h) induced a time-dependent increase in CAT-2 but not CAT-1 expression. Moreover, TNF-α (1–30 ng/ml) treatment for 6 h induced a concentration-dependent increase in CAT-2 mRNA expression. The upregulation of CAT-2 expression by TNF-α was associated with enhanced nitrite accumulation in the culture medium (70% increase compared with vehicle-treated cells at 24 h). Thus, induction of the cationic amino acid transporter may constitute one mechanism for the TNF-α-induced NO production in human umbilical vein endothelial cells. © 1997 Elsevier Science B.V.

Keywords: Cationic amino acid transporter; Gene expression; TNF-α (tumor necrosis factor-α); Nitric oxide (NO); Umbilical vein endothelial cell; (Human)

1. Introduction

Nitric oxide (NO) in vascular cells contributes to a decrease in vascular tone under both physiological and pathophysiological conditions (Moncada et al., 1991). The availability of L-arginine, the substrate for NO synthase, provides a site for the regulation of NO production (Moncada et al., 1991). L-Arginine is endogenously synthesized from L-citrulline by argininosuccinate synthetase and argininosuccinate lyase, and is also taken up into the cell from the circulation by membrane-bound transporters (system y^+, $b^{0,+}$, $B^{0,+}$ and y^+L) (Kakuda and MacLeod, 1994). The genes for the cationic amino acid transporter, which encode system y^+ transporters, were originally cloned in mouse and named CAT-1 and CAT-2 (for review, see MacLeod et al., 1994). CAT-1 is expressed constitutively, while CAT-2, which has been subcloned as CAT-2A and -2B by alternative splicing, is an inducible gene (MacLeod et al., 1994). CAT expression in humans has not been characterized, except as an ecotropic retroviral receptor, the original name for CAT-1 (Yoshimoto et al., 1992).

Recent studies have shown that inflammatory mediators, such as interleukin-1β, tumor necrosis factor (TNF)-α and lipopolysaccharide, enhance the gene expression of CAT-2 but not CAT-1 in rat vascular smooth muscle cells and astroglial cells (Gill et al., 1996; Stevens et al., 1996). In rat cardiac myocytes and microvascular endothelial cells, however, the expression of CAT-1, -2A and -2B mRNA is enhanced by cytokine stimulation (Simmons et al., 1996a,b). Thus, there are some discrepancies in CAT gene expression among cell types. TNF-α, a potent cytokine produced by activated leukocytes, plays a crucial role in endotoxin shock, partly through an overproduction of NO by vascular endothelial and smooth muscle cells (Petros et al., 1991). In human umbilical vein endothelial cells, TNF-α has been shown to increase L-arginine uptake (Pan et al., 1995). In the present study, human

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umbilical vein endothelial cells subjected to TNF-α treatment were analyzed for the expression of CAT mRNA and for metabolites of NO.

2. Materials and methods

2.1. Materials

Medium 199, Trypsin (1:250), Oligo $(dT)_{12-18}$ and SuperScript™ II reverse transcriptase were purchased from Gibco BRL (Gaithersburg, MD), fetal bovine serum and recombinant human TNF-α were from Dainippon Pharmaceutical (Osaka), endothelial cell growth supplement was from Upstate Biotechnology (Lake Placid, NY), ISOGEN was from Nippongene (Tokyo), AmpliTaq® DNA polymerase was from Perkin Elmer (Foster City, CA), and pT7Blue T-vector was from Novagen (Madison, WI).

2.2. Cell culture and TNF-α treatment

Human umbilical vein endothelial cells were isolated from fresh human umbilical veins by 0.125% trypsin digestion and cultured on collagen-coated plates in Medium 199 supplemented with 20% fetal bovine serum, 30 μg/ml endothelial cell growth supplement, 50 μg/ml heparin, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells in passages 3–5 were seeded at a density of 2.1×10^4 cells/cm^2 into culture plates (6 cm in diameter) and subconfluent monolayer cells were used for experiments. Medium was replaced 2 h before experiments. Cells were treated with 1–30 ng/ml of TNF-α (2.6×10^6 JRU/mg protein) for 1–24 h.

2.3. Extraction of RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from endothelial cells, using ISOGEN. The first strand cDNA was synthesized from 0.5 μg of total RNA, using Oligo $(dT)_{12-18}$ primer and SuperScript™ II reverse transcriptase under the conditions recommended by the supplier, and the reversely transcribed product was used for subsequent amplification by PCR. For monitoring genomic DNA contamination in RNA samples, a reaction mixture without the reverse transcriptase was also prepared. Oligonucleotide primers were constructed based on the published sequences for human CAT-1 (Yoshimoto et al., 1991), CAT-2 (GenBank accession number d29990), and β-actin (Ponte et al., 1984). Amplification of the cDNA with the primers for β-actin served as a positive control for the quantity of cDNAs. Primer pairs for CAT-1 were 5′-CCAACGTCAATGATAGGACC-3′ (nucleotides 1274–1293) for the sense primer and 5′-CTGGTCCAGCTGCATCATGA-3′ (nucleotides 1896–1877) for the antisense primer; CAT-2 5′-AGCCTGGCTTATCTTACGAC-3′ (nucleotides 1304–1323) for the sense and 5′-AATCTGACCCAAGTGTCTGC-3′ (nucleotides 1760–1741) for the antisense; β-actin 5′-CCCAGATCATGTTTGAGACC-3′ (nucleotides 400–419) for the sense and 5′-TAGCTCTTCTCCAGGGAGGA-3′ (nucleotides 760–741) for the antisense. PCR amplification was performed by using AmpliTaq® DNA polymerase and specific primers in a Programmable Temp Control System PC-700 (ASTEC, Fukuoka) with initial heating for 5 min at 94°C, followed by 25 cycles (for CAT-1 and CAT-2) or 21 cycles (for β-actin) of 1 min each of denaturation at 94°C, annealing at 54°C, and extension at 72°C and a final extension for 10 min at 72°C. PCR products were analyzed by electrophoresis on 2% agarose gels with an ATTO densitograph (ATTO, Tokyo). To confirm the sequence of PCR products, they were subcloned into pT7Blue T-vectors and sequenced by the dideoxynucleotide chain termination method, using a HITACHI SQ-5500 sequencer (Hitachi, Tokyo).

2.4. Measurement of nitrite formation

Nitrite accumulation in the culture medium was used as an index of cellular NO synthesis. Endothelial cells cultured in 6-well plates were treated with TNF-α (10 ng/ml) for 6 or 24 h. After treatment, the medium was mixed with 2-volumes of cold ethanol and placed for 30 min at 4°C for precipitation of serum protein. Nitrite in the supernatant was determined by chemiluminescence analysis (Palmer et al., 1987), using a SIEVERS NO analyzer model 270B (Sievers, Boulder, CO).

3. Results

Amplification of cDNA from human umbilical vein endothelial cells with the CAT-1, -2 or β-actin primers generated various amounts of PCR products in both control and TNF-α-treated cells (Fig. 1A). The size of each PCR product corresponded to the predicted size of CAT-1 (623 bp), CAT-2 (457 bp) or β-actin (361 bp) (Fig. 1A). RNA samples that were processed without reverse transcriptase gave no detectable PCR products. Although not shown, the sequence of each product was identical to that of the nucleotides reported as human CAT-1, -2 and β-actin cDNAs. There was a significant enhancement in RT-PCR product of CAT-2 in endothelial cells treated with TNF-α (10 ng/ml, 1–24 h), while the expression of CAT-1 and β-actin was unaffected by TNF-α (Fig. 1A). Densitometric analysis showed that the relative density of CAT-1 to β-actin remained constant throughout 1–24 h treatment with TNF-α (Fig. 1B). In contrast, the relative density of CAT-2 to β-actin increased progressively for 1–4 h of treatment and remained elevated up to 24 h (Fig. 1C).

Fig. 2 shows that the stimulatory effect of TNF-α on CAT-2 mRNA level was concentration-dependent. Thus,

while CAT-1 and β-actin expression was not changed by increasing concentrations of TNF-α, there was a concentration-dependent increase in PCR products for CAT-2 (Fig. 2A). The densitometric analysis determined that there was a significant difference, by one-way analysis of variance, in CAT-2/β-actin ratio with different concentrations (Fig. 2C).

In a separate study, nitrite concentrations in the medium of vehicle- and TNF-α-treated cells were compared. The nitrite concentration was significantly higher in the TNF-α (10 ng/ml)-treated cells (1477 ± 115 pmol/ml, mean $\pm$ S.E.M.) than in the vehicle-treated cells (894 ± 218 pmol/ml) at 24 h ($P < 0.05$ by Student's t-test). Thus, an enhanced production of NO by TNF-α treatment was demonstrated.

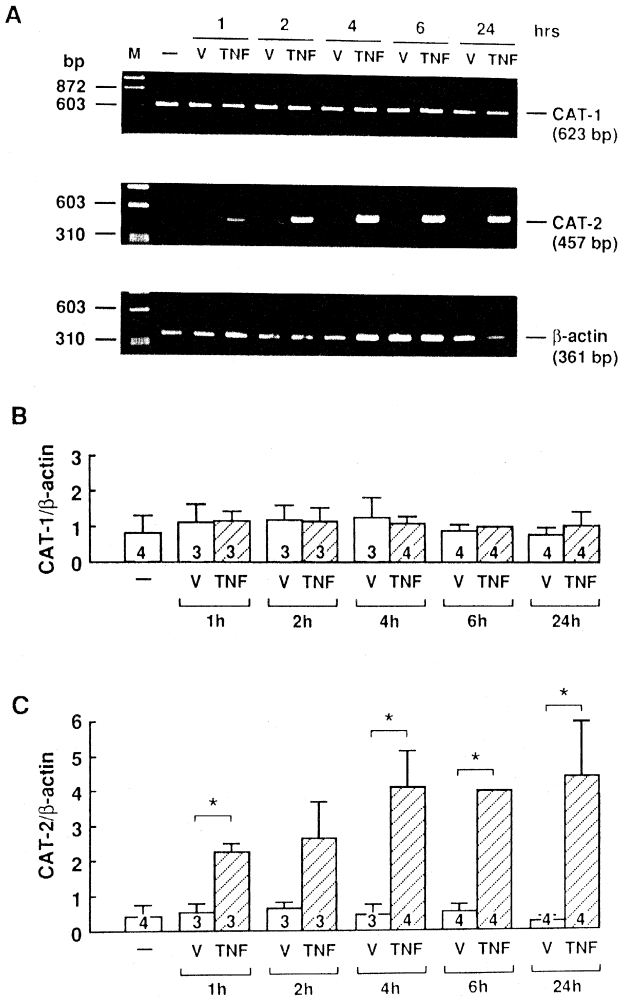

Fig. 1. Expression of CAT-1 and CAT-2 mRNAs in human umbilical vein endothelial cells treated with TNF-α (10 ng/mg). (A) Typical agarose gel electrophoresis of RT-PCR products. M; DNA size markers (ϕX174-*Hae* III digest). V; vehicle (Medium 199) for TNF-α. (B) and (C) Densitometric analysis of relative expression of CAT-1 (B) and CAT-2 (C). PCR products normalized to those of β-actin. In each experiment, the value of CAT-1/β-actin from cells treated with TNF-α for 6 h was estimated as 1 for an overall reference value (in B), and CAT-2/β-actin as 4 (in C). Values are means $\pm$ S.E.M ($n = 3$–4). $^{*}P <$ 0.05 by Student's t-test.

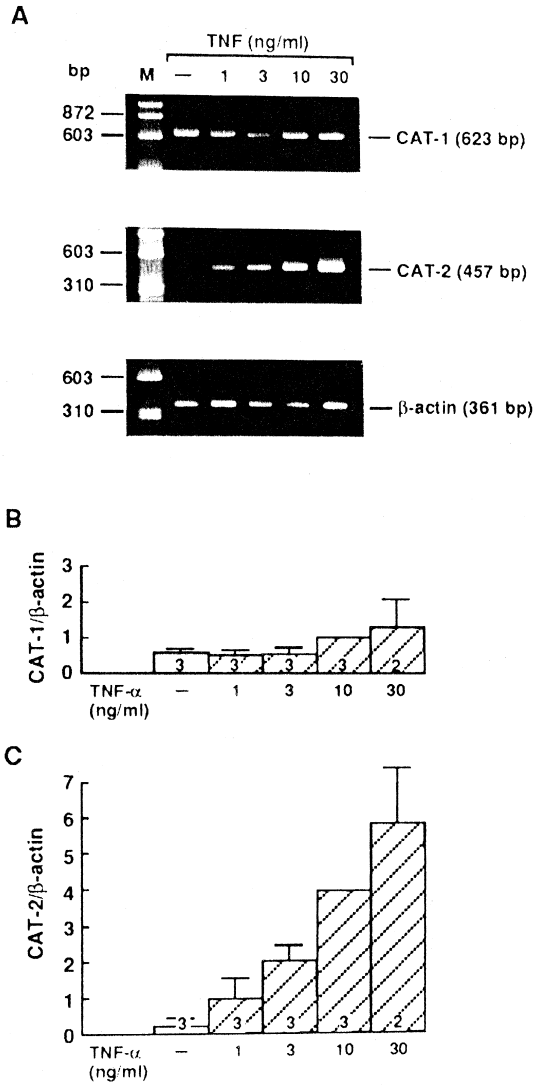

Fig. 2. Effects of increasing concentrations (1–30 ng/ml) of TNF-α for 6 h on CAT-1 and CAT-2 mRNA expression in human umbilical vein endothelial cells. (A) Typical agarose gel electrophoresis of RT-PCR products. M; DNA size markers (ϕX174-*Hae* III digest). (B) and (C) Densitometric analysis of relative intensity of CAT-1 and CAT-2 PCR products normalized to those of β-actin. In each experiment, the value of CAT-1/β-actin from cells treated with TNF-α 10 ng/ml was estimated as 1 for an overall reference value (in B), and CAT-2/β-actin as 4 (in C). Values are means $\pm$ S.E.M. of range ($n = 2$–3). Differences among various concentrations were significant by one-way analysis of variance ($P < 0.05$).

4. Discussions

The expression of genes for cationic amino acid transporter-1 and -2 was investigated in human vascular endothelial cells stimulated by TNF-α. In quiescent endothelial cells, CAT-1 expression was dominant to that of

CAT-2, but CAT-2 expression was markedly upregulated in TNF-α-stimulated endothelial cells without significant changes in CAT-1 expression. This result is consistent with the original classification that CAT-1 is a housekeeping gene and that CAT-2 is an inducible type (MacLeod et al., 1994). In rat cardiac myocytes and microvascular endothelial cells, however, interleukin-1β in combination with interferon-γ stimulates the levels of CAT-1, -2A and -2B mRNA (Simmons et al., 1996a,b). Since Simmons and his coworkers used serum-starved cells as quiescent cells in their studies, the stimulatory effects of cytokines on CATs gene expression were probably expressed more strongly in these cells than in cells maintained in 20% serum-containing medium as in our study.

Since L-arginine is the substrate for NO synthase, the cellular transport of L-arginine, as well as its de novo synthesis from L-citrulline, may determine NO production by NO synthase, especially when a large amount of NO is produced during inflammation. The results of the present study suggest that NO production by human umbilical vein endothelial cells treated with TNF-α was upregulated in association with enhanced CAT-2 expression. Several previous studies found similar results in other cell types. Lipopolysaccharide in combination with cytokines has been shown to stimulate both L-arginine transport and NO production in vascular smooth muscle cells (Wileman et al., 1995). In other studies, CAT-2 but not CAT-1 mRNA was induced by interleukin-1β in combination with TNF-α in vascular smooth muscle cells and also by lipopolysaccharide in combination with interferon-γ in astroglial cells (Gill et al., 1996; Stevens et al., 1996). The upregulation of CAT-2 was associated with L-arginine transport, gene expression for inducible NO synthase or NO production in these studies (Gill et al., 1996; Stevens et al., 1996).

Lipopolysaccharide and cytokines are also reported to induce argininosuccinate synthetase, the enzyme that produces L-arginine from L-citrulline, in rat cardiac myocytes and microvascular endothelial cells (Simmons et al., 1996a,b). Therefore, de novo synthesis as well as uptake of L-arginine may control the intracellular concentration of L-arginine. However, lipopolysaccharide has been shown to induce arginase, which catalyzes the conversion of L-arginine to L-ornithine and urea, in rat aortic endothelial cells (Buga et al., 1996). The induction of arginase by cytokines may lower L-arginine availability, which may explain the relatively small increase (70%) in NO production in spite of the great enhancement in CAT-2 mRNA expression by TNF-α seen in this study. Several other possibilities can be postulated for the cytokine-induced upregulation of NO synthesis by endothelial cells. First, induction of inducible NO synthase mRNA by cytokines has been reported in vascular endothelial cells (Moncada et al., 1991; Suschek et al., 1993). Second, cytokines may increase NO production in human umbilical vein endothelial cells by inducing guanosine triphosphate (GTP)-cyclohydrolase I (Rosenkranz-Weiss et al., 1994), which is the rate-limiting enzyme for the production of tetrahydrobiopterin, a cofactor for both endothelial and inducible isoforms of NO synthase.

An overproduction of NO by endothelial cells may cause vascular hyporeactivity to vasoconstrictor agents in endotoxemia (Schaller et al., 1985; Szabó et al., 1993). In clinical endotoxemia, the induction of cationic amino acid transporter-2, argininosuccinate synthetase, inducible NO synthase and GTP cyclohydrolase I may contribute in concert to an increase in NO production by vascular endothelial cells, leading to hypotension and vascular hyporeactivity. In this paper, we demonstrated the induction of CAT-2 mRNA by TNF-α in relation to enhanced NO production. Thus, the cationic amino acid transporter may constitute another regulator of NO production under pathophysiological conditions in which the substrate for NO synthase is increased.

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