Vol. 217, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 5, 1995 Pages 363-369

STIMULATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE mRNA LEVELS BY ENDOGENOUS NITRIC OXIDE IN CYTOKINE-ACTIVATED ENDOTHELIUM

Joanna Bereta* and Michal Bereta

Department of Biochemistry, Jagiellonian University, Cracow, Poland

Received October 27, 1995

Previous studies have shown that endogenous nitric oxide (NO) potentiates glycolysis in the cytokine-activated murine microvascular endothelial cells (MME). In the present study we investigate the influence of NO on the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme of the glycolytic pathway. Activation of MME with TNF-α and IFN-γ resulted in a strong elevation of GAPDH mRNA levels. This effect was impaired in the presence of L-NMMA, the inhibitor of NO synthesis. We discuss the possibility that NO-mediated elevation of GAPDH mRNA levels may compensate for NO-mediated inhibition of GAPDH enzymatic activity, representing another adaptive mechanism which protects cells producing large amounts of NO against its cytotoxic effects.

Cytokine-inducible nitric oxide synthase (iNOS) is responsible for the production of abundant amounts of NO during inflammation. Under these conditions NO released by cytokine-activated cells acts as a potent cytotoxic agent against some bacteria, viruses, protozoa or tumor cells (1). Cytotoxicity of NO results in part from its interactions with the centers of iron sulfur clusters in the mitochondrial electron transport chain (2, 3). As a consequence, the production of energy which is required for vital cellular processes is strongly impaired and to survive cells might increase utilization of glycolysis to meet their energy demands. However, GAPDH, one of the glycolytic pathway enzymes, is also inhibited by NO or its derivatives (4 - 6). The inhibition of both energy sources: respiratory chain and glycolysis, is probably responsible for the cytotoxic effects of NO against pathogens (3). However, the inhibition of energy production by NO may

*To whom correspondence should be addressed at Institute of Molecular Biology, Jagiellonian University, Al. Mickiewicza 3, 31-120 Cracow, Poland.
Fax: 48-12-33-69-07. E-mail: Joannab@mol.uj.edu.pl.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MME, murine brain microvascular endothelial cells; iNOS, inducible nitric oxide synthase; L-NMMA, *N*-monomethyl-L-arginine; FCS, fetal calf serum; VCAM-1, vascular cell adhesion molecule-1.

0006-291X/95 $12.00

also contribute to self-destruction of NO-generating cells, as it was shown in the case of pancreatic β-cells damaged during inflammatory states (7).

It has been suggested that cytokines which stimulate cells to produce large amounts of NO simultaneously activate mechanisms of defence against its cytotoxicity (3). Among the best described mechanisms of self-protection is elevated expression of superoxide dismutase which decomposes superoxide suppressing the formation of highly toxic peroxynitrite (8). However, the mechanisms which might be utilized by cells to counteract the NO-mediated impairment of energy production are largely unknown.

The consequences of the inhibition of mitochondrial respiration might resemble those of hypoxic stress. Among mammalian cells which are generally hypoxia-sensitive, endothelial cells are relatively tolerant to decreases in oxygen concentration although the mechanisms by which they adapt their metabolic functions are unclear (9). Recently Graven et al. have shown that the unique response of endothelial cells to hypoxia is characterized by up-regulation of mRNA for a set of proteins including GAPDH (10). We examined whether a similar process takes place in cytokine-stimulated MME, which are resistant to endogenously produced NO.

MATERIALS AND METHODS

Reagents. Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN) and recombinant murine IFN-γ from Life Technologies Ltd.(Paisley, UK). DMEM, RPMI 1640, RPMI 1640 Select Amine Kit, fetal calf serum (FCS) were purchased from Life Technologies Ltd. (Paisley, UK). α-^{32}P-dCTP was obtained from Amersham Life Science, (Little Chalfont, UK). Unless indicated otherwise all remaining reagents were purchased from Sigma Chemicals Co., (St. Louis, MO).

Maintenance of cells. Murine brain microvascular endothelial cells (MME) were a gift from Dr. R. Auerbach (Madison, WI). They were grown in medium consisting of DMEM, 20% FCS, endothelial cell growth supplement (30 μg/mL), 2 mM glutamine and antibiotics.

Nitrite assay. MME were cultured in 6-well plates in 1 mL of culture medium. For experiments the medium was replaced with RPMI 1640 containing 5% FCS, 10 mM HEPES, antibiotics and factors indicated for each experiment. Nitrite concentration in the medium was determined after 24 h by a microplate assay as described previously (11). Briefly, 80 μL aliquots of the culture medium were incubated with equal volumes of Griess reagent at room temperature for 10 minutes and then the absorbance at 540 nm was measured using ELISA reader. Nitrite concentration was determined using dilutions of sodium nitrite in water as a standard.

Counting of cells. Cells were released from plates with trypsin, washed once with RPMI 1640, centrifuged, resuspended in 0.5 mL of PBS, and counted in heamocytometer in the presence of Trypan Blue.

RNA preparation and Northern blot analysis. MME were cultured in 94-mm tissue culture plates. After the cells reached confluence, medium was replaced with RPMI 1640 containing 2% FCS, 10 mM HEPES and antibiotics. The monolayers were incubated with cytokines for a period of time indicated for each experiment. Total RNA was prepared using phenol extraction method (12). RNA samples (5 μL) were separated electrophoretically in 1% agarose gel under denaturing conditions (13). RNA was then transferred to Hybond-N membranes (Amersham, UK) according to the manufacturer's instruction. The blots were baked in 80°C for 2 h, prehybridized overnight and hybridized to cDNA probes specific for the human GAPDH (ATCC, GenBank/EMBL: M17851), murine macrophage iNOS (a gift from Drs. Q.-W. Xie and C. Nathan, New York,

NY), murine VCAM-1 (a gift from Dr. L. Osborn, Cambridge, MA) and for 18S rRNA (kindly provided by N. Bhownick, Athens, GA). The probes were labeled by the Random Primers DNA labeling System (Amersham, UK). Hybridizations were performed at 68°C for 24 h. Non-specifically bound radioactivity was removed by washing the blots in 2 x standard saline solution (SSC) at room temperature, followed by two subsequent washes in 2 x SSC/0.1% SDS at 68°C for 30 min. each. The blots were then subjected to autoradiography at -70°C using intensifying screens.

RESULTS AND DISCUSSION

We have previously demonstrated that the inflammatory cytokines, TNF-α and IFN-γ induce the expression of iNOS in MME (11). The iNOS-dependent NO synthesis can be monitored by measuring the accumulation of nitrite in the culture medium. MME were able to synthesize large quantities of NO for at least 7 days in the continuous presence of cytokines (Fig. 1). During that time endogenous NO did not affect growth and viability of MME. This observation indicated presence of protective mechanisms against NO-mediated cytotoxicity in MME cells. The accumulation of nitrite in MME culture medium was accompanied by increased synthesis of lactate, the final product of anaerobic glycolysis (14). These processes did not occur when MME were stimulated with the cytokines in the presence of L-NMMA or in the absence of arginine, a substrate for NO synthesis. This suggested that substantial amounts of endogenous NO inhibited mitochondrial respiration in MME and the cells utilized predominantly glycolytic pathway to meet their basic energy requirements. NO-dependent inhibition of mitochondrial

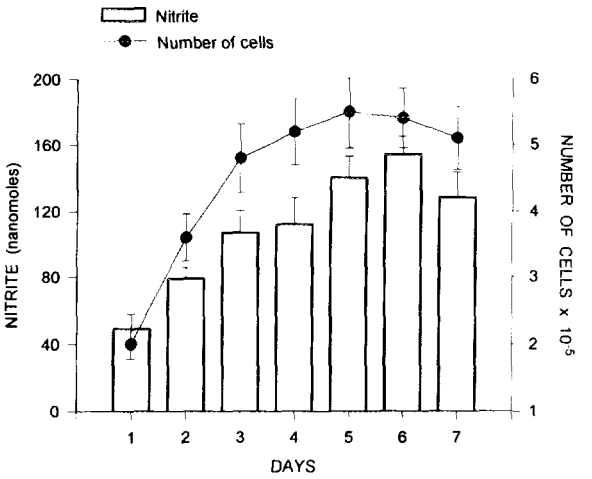

Figure 1. Nitrite accumulation and cell number in a long-term culture of MME. Cells were seeded on 6-well plates at a density of 1 x 10^5/well. 24 h later medium was replaced with RPMI 1640 containing 5% FCS, TNF-α (2 ng/mL) and IFN-γ (200 U/mL). Culture medium containing the cytokines was replaced daily. Every 24 h media were assayed for nitrite content and cells were released from plates and counted. Data represent means ± SD of 3 experiments and each determination was done in duplicates.

respiration and a switch to glycolytic pathway was also demonstrated in smooth muscle cells upon their treatment with TNF-α and IFN-γ (15). However, it has been shown that NO or its derivatives such as peroxynitrite ($ONOO^-$) inhibits enzymatic activity of GAPDH (6, 16) while it enhances utilization of glucose through glycolysis in some cells (15, 17). These apparently conflicting data could be explained by the existence of regulatory mechanisms, including increased synthesis of GAPDH, compensating for partial inhibition of its enzymatic activity. The increased GAPDH levels could be achieved by the stimulation of its gene transcription and/or increased stability of GAPDH mRNA.

Although GAPDH gene was for a long time classified as a housekeeping gene, a number of recent studies demonstrate that its expression is under control of several regulatory mechanisms (18-20). We found that stimulation of MME with TNF-α and IFN-γ led to a strong increase in GAPDH mRNA levels (Fig. 2). The presence of L-NMMA in the culture medium during incubation of MME with the cytokines strongly limited the increase indicating that NO was involved in the process (Fig 2). Similar results were obtained when NO synthesis in cytokine-activated MME was inhibited by a lack of L-arginine in the culture medium (data not shown). The increase in iNOS mRNA levels in cytokine-activated MME was also diminished by L-NMMA. To exclude a possibility that the observed effect of L-NMMA resulted from suppression of the entire transcription process in MME, we utilized VCAM-1 mRNA levels as an additional control. Unlike GAPDH- and iNOS-mRNAs, L-NMMA increased the level of mRNA specific for VCAM-1. This

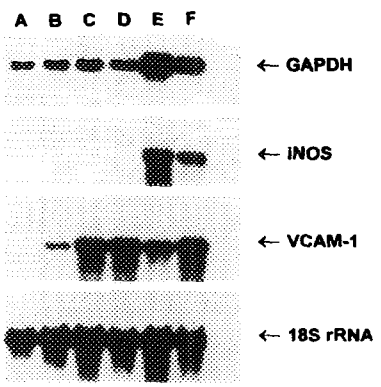

Figure 2. Northern blot analysis of GAPDH, iNOS and VCAM-1 mRNAs in MME cells. Cells were incubated for 18 h with medium alone (lane A), with IFN-γ (200 U/mL) (lane B), TNF-α (2 ng/mL) (lane C), TNF-α in the presence of L-NMMA (1 mM) (lane D), TNF-α and IFN-γ (lane E), TNF-α and IFN-γ in the presence of L-NMMA (lane F). Equal loading of wells with RNA was verified by hybridization with 18S rRNA-specific cDNA probe.

result could be expected since it has been shown that NO inhibits VCAM-1 expression in endothelial cells (21).

Concomitant analyses of the time-courses of nitrite accumulation and changes in GAPDH mRNA levels in the cytokine-activated MME cells and the influence of L-NMMA on both processes confirmed the involvement of NO in the regulation of GAPDH mRNA levels (Fig 3). After 3 h of MME incubation with TNF-α and IFN-γ a moderate stimulation of GAPDH mRNA levels was observed and this process was not inhibited by L-NMMA. Significant increase in GAPDH mRNA levels observed after 6 h of stimulation of the cells with cytokine was only slightly inhibited by the presence of L-NMMA, suggesting that the initial increase resulted directly from the action of TNF-α and/or IFN-γ. Strong elevation of GAPDH mRNA levels observed after 12 h was significantly diminished by L-NMMA. After 24 h, the amount of transcript was still very high but this increase was almost completely abolished by L-NMMA. The time course of changes in GAPDH mRNA levels and its susceptibility to inhibition by L-NMMA correlated well with the levels of nitrite accumulation in MME culture medium (Fig. 3B). These results show that the initial increase in GAPDH mRNA levels caused by cytokines was further potentiated and prolonged due to NO activity. The possible mechanism of NO-mediated increase in GAPDH mRNA levels may involve either costimulation of transcription or stabilization of GAPDH mRNA.

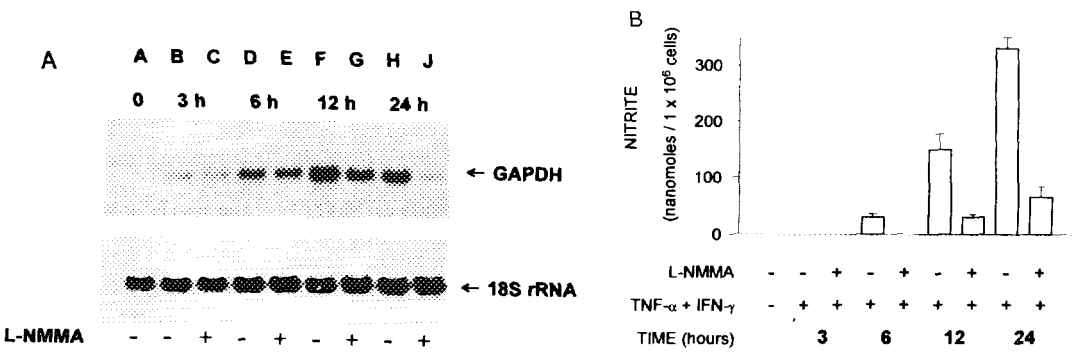

Figure 3. Comparison of time-dependence of GAPDH mRNA levels and nitrite accumulation in MME. (A) Northern blot analysis of GAPDH mRNA. Cells were incubated for indicated periods of time with medium alone (lane A), with TNF-α (2 ng/mL) and IFN-γ (200 U/mL) (lanes B, D, F, H), TNF-α and IFN-γ in the presence of L-NMMA (lanes C, E, G, J). Equal loading of wells with RNA was verified by hybridization with 18S rRNA-specific cDNA probe. Only the relevant portions of autoradiogram are shown. (B) Accumulation of nitrite in MME culture medium. Cells were incubated for indicated periods of time with medium alone, with TNF-α (2 ng/mL) and IFN-γ (200 U/mL), or with TNF-α and IFN-γ in the presence of L-NMMA (1 mM). The amount of nitrite was determined as described in Materials and Methods. Bars represent means ± SD of 5 experiments and each determination was done in triplicate.

The possible role of NO in the regulation of transcription is not well understood. Nitric oxide, through its interactions with thiols and transition metals, might modulate the activity of transcription factors. It has been postulated that NO influences the activity of NF-κB and AP-1, two transcription factors which regulate expression of large number of genes involved in the response to infection and stress. However, the results of distinct studies are often contradictory. For example Tabuchi et al. (22) showed that sodium nitroprusside, a donor of NO, inhibited AP-1 activity in vitro in the presence of dithiothreitol (DTT) while Peunova and Enikolopov demonstrated a synergistic effect of NO donors on calcium-mediated activation of AP-1 in neuronal cells (23). Conflicting reports were also presented in respect to the regulation of NF-κB activity by NO (21, 24, 25). Since regulatory elements of GAPDH gene are not well characterized, it is difficult to speculate on a possible mechanism of stimulation of GAPDH gene expression by NO.

Another possible explanation of the stimulatory effect of NO on GAPDH mRNA levels is its influence on GAPDH mRNA stability. Recently, Quail and Yeoh demonstrated that GAPDH mRNA levels might be regulated by post-transcriptional mechanisms dependent on iron status. Iron deficiency evokes more than 2-fold increase in GAPDH mRNA levels in rat liver (26). It has been well documented that NO plays a direct role in post-transcriptional gene regulation mediated by iron regulatory protein (IRP). NO activates the RNA binding activity of IRP and mimics the consequences of iron starvation influencing translation rate or stability of mRNA encoding proteins involved in iron uptake, storage and utilization (27, 28).

NO inhibits enzymatic activity of GAPDH but at the same time its action leads to increase in GAPDH mRNA levels making possible increased synthesis of this protein. The overall activity of GAPDH in cells may therefore remain stable or even increased, thus allowing for the effective utilization of glycolytic pathway and to maintain sufficient levels of ATP. This mechanism likely represents another adaptation of cells producing large amounts of NO to become resistant to its cytotoxic effects.

ACKNOWLEDGMENT

This work was supported by Committee of Scientific Research grant 6-P203-033-05 (KBN, Warsaw, Poland).

REFERENCES

1. Nathan, C., Xie, Q-W. (1994) Cell 78, 915-918
2. Cleeter, M.W.J., Cooper, J.M., Darley-Usmar, V.M., Moncada, S. and Schapira, A.H.V. (1994) FEBS Lett. 345, 50-54

3. Stamler, J.S. (1994) Cell 78, 931-936
4. Mohr, S., Stamler, J.S., Brüne, B. (1994) FEBS Lett. 348, 223-227
5. Ravichandran, V., Seres, T., Moriguchi, T., Thomas, J.A., Johnston, Jr., R.B. (1994) J. Biol. Chem. 269, 25010-25015
6. Dimmeler, S., Ankarcrona, M., Nicotera, P., Brüne, B. (1993) J. Immunol. 150, 2964-2971
7. Corbett, J.A., Sweetland, M.A., Lancaster, Jr., J.R., McDaniel, M.L. (1993) FASEB J.7, 369-374
8. Lewis-Molock, Y., Suzuki, K., Tanigushi, N., Nguyen, D.H., Mason, R.H., White, C.W. (1994) Am. J. Respir. Cell. Mol. Biol. 10, 133-141
9. Ogawa, S., Clauss, M., Kuwabara, K., Shreeniwas, R., Butura, C., Koga, S., Stern, D. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 9897-9901
10. Graven, K.K., Troxler, R.F., Kornfeld, H., Panchenko, M.V., Farber, H.W. (1994) J. Biol. Chem. 269, 24446-24453
11. Bereta, M., Bereta, J., Cohen, S., Cohen, M. (1992) Biochem. Biophys. Res. Commun. 186, 315-320
12. Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY
13. Rose-John, S., Dietrich, A., Marks, F. (1988) Gene 74, 465-471
14. Bereta, M., Bereta, J., Georgoff, I., Coffman, F.D., Cohen, S., Cohen, M. (1994) Exp. Cell. Res. 212, 230-242
15. Geng, Y-J., Hansson, G.K., Holme, E. (1992) Circ. Res.71, 1268-1276
16. Molina y Vedia, L., McDonald, B., Reep, B., Brüne, B., Di Silvio, M., Billiar, T.R. (1993) J. Biol. Chem. 268, 3016
17. Mateo, R.B., Reichner, J.S., Mastrofrancesco, B., Kraft-Stolar, D., Albina, J.E. (1995) Am. J. Physiol. 268, C669-C675
18. Alexander-Bridges, M., Dugast, I., Ercolani, L., Kong, X.F., Giere, L., Nasrin, N. (1992) Adv. Enzyme Regul. 32, 149-159
19. Mansur, N.R., Meyer-Siegler, K., Würzer, J.C., Sirover, M.A. (1993) Nucleic Acids Res. 21, 993-998
20. Rolland, V., Dugail, I., Le Liepvre, X., Lavau, M. (1995) J. Biol. Chem. 270, 1102-1106
21. De Caterina, R., Libby, P., Peng, H-B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone, Jr., M.A., Shin, W.S., Liao, J.K. (1995) J. Clin. Invest. 96, 60-68
22. Tabuchi, A., Sano, K., Oh, E., Tsuchiya, T., Tsuda, M. (1994) FEBS Lett. 351, 123-127
23. Peunova, N., Enikolopov, G. (1993) Nature 364, 450-453
24. Peng, H-B., Libby, P., Liao, J.K. (1995) J. Biol. Chem. 270, 14214-14219
25. Lander, H., Schajpal, P. K., Novogrodsyk, A. (1993) Immunology 151, 7182-7187
26. Quail, E.A., Yeoh, G.C.T. (1995) FEBS Lett. 359, 126-128
27. Drapier, J-C., Hirling, H., Wietzerbin, J., Kaldy, P., Kühn, L.C. (1993) EMBO J. 12, 3643-3649
28. Pantopoulos, K., Weiss, G., Hentze, M.W. (1994) Trends Cell Biol. 4, 82-86