Amino Acids (1996) 10: 277–281

Amino
Acids

Correlation between nitric oxide synthase activity and reduced glutathione level in human and murine endothelial cells

D. Ghigo[1], D. Geromin[1], C. Franchino[1], R. Todde[1], C. Priotto[1], C. Costamagna[1], M. Arese[1], G. Garbarino[2], G. P. Pescarmona[1], and A. Bosia[1]

[1]Department of Genetics, Biology and Medical Chemistry and [2]Institute of Internal Medicine, University of Torino, Torino, Italy

Accepted October 1, 1995

Summary. The synthesis of nitric oxide (NO), detected as citrulline production, in human (HUVEC) and murine (tEnd.1) endothelial cells correlated with intracellular GSH. tEnd.1, which exhibited an intracellular GSH level 2.5-fold higher than HUVEC, showed a citrulline production (basally and after ionomycin stimulation) 5–8 times higher than human cells. Ionomycin-elicited citrulline synthesis in tEnd.1 cells increased 2.4-fold after loading with GSH, and decreased dose-dependently after GSH depletion. Cell loading with N-(2-mercaptopropionyl)-glycine neither significantly increased citrulline production nor relieved the effect of GSH depletion.

Keywords: Amino acids – Nitric oxide – Endothelial cells – Glutathione – Polyomavirus – N-(2-Mercaptopropionyl)-glycine – 1-Chloro-2,4-dinitrobenzene

Introduction

Nitric oxide (NO), a short-lived free radical gas produced by a wide variety of cell types (Schmidt and Walter, 1994), is generated by a class of NADPH-dependent NO synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline and NO with a 1:1 stoichiometry (Nathan and Xie, 1994). Reduced glutathione (GSH), present in high concentrations in mammalian cells, has been suggested to play a possible role in NO synthesis, as a reducing cofactor for NO production (Stamler et al., 1992) or, more likely, by preventing early inactivation of NOS by radical intermediates or NO itself (Griscavage et al., 1994). In a previuos work we have demonstrated that GSH is necessary to NO synthesis in human endothelial cells (Ghigo et al., 1993). Next we have found that tEnd.1, a murine endothelial cell line transformed by middle T antigen (mTa), exhibits a very high NOS activity when compared to

normal human and murine endothelial cells (Ghigo et al., 1995). Aim of the present work was to compare GSH levels between cells producing different amounts of NO, and to study NO synthesis in tEnd.1 cells after manipulations of GSH levels. GSH was depleted in a dose- and time-dependent way by treating cells with 1-chloro-2,4-dinitrobenzene (CDNB), which permeates the plasma membrane and forms a covalent GSH adduct via intracellular glutathione S-transferase activity: by this way GSH is irreversibly removed from the cell metabolism (Bosia et al., 1985). On the other hand, intracellular GSH was raised by incubating cells with exogenous GSH.

Since the direct measurement of NO, a free radical having an half-life of few sec, is difficult, several indirect techniques of detection have been developed. We used a simple and sensitive procedure (Bredt and Snyder, 1989), which monitors the NOS-mediated conversion of [^{3}H] arginine to [^{3}H] citrulline, which is produced stoichiometrically with NO.

Materials and methods

Endothelial cell cultures

Human endothelial cells from umbilical cord vein (HUVEC) were obtained, characterized and cultured as previously described (Ghigo et al., 1993). tEnd.1 (Williams et al., 1988) murine endothelial cell lines transformed by mTa of polyoma virus were grown in DMEM supplemented with 10% FCS (Ghigo et al., 1995). The viability of cells, assessed by trypan blue exclusion and by measuring the lactate dehydrogenase activity in the supernatant, ranged 93–97% after the incubation times under the different conditions described, including CDNB treatment.

Measurement of citrulline synthesis as a sensitive marker of NO production

Citrulline synthesis was measured as previously described (Ghigo et al., 1993): briefly, cells monolayers in 35 mm dishes, incubated at 37°C for 20 min with 5 μCi L-[^{3}H]arginine (L-[2,3,4,5-^{3}H]arginine monohydrochloride, 62 Ci/mmol, Amersham International, Bucks, UK) and 10 μM L-arginine, were stimulated for 15 min with 2 μM ionomycin or solvent alone; then cells were washed, and their [^{3}H]citrulline content was checked by cation exchange chromatography.

GSH depletion, GSH loading and intracellular GSH measurement

GSH depletion, loading and measurement were performed, as previously described (Ghigo et al., 1993), on cells monolayers in 35 mm dishes. GSH intracellular level was measured both by the 5,5′-dithio-bis-(2-nitrobenzoic acid) colorimetric method and the glutathione reductase-mediated recycling procedure: the former technique provides information about the cell content of soluble thiols, the latter is specific for GSH.

Statistics

All data in text and figures are provided as mean ± SEM.

Results

Both in HUVEC and tEnd.1 the calcium ionophore ionomycin increased citrulline synthesis (Fig. 1), due to the presence in these cells of a Ca^{++}-dependent NOS activity (Ghigo et al., 1995). Both basal and ionomycin-stimulated citrulline synthesis were totally inhibited by the NOS inhibitor

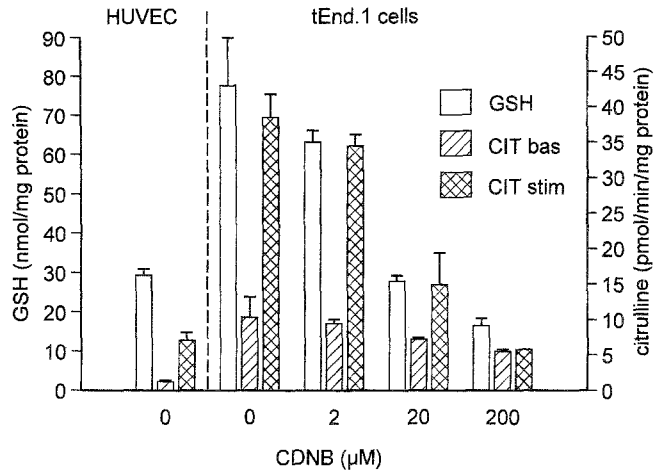

Fig. 1. Intracellular GSH level and citrulline synthesis in HUVEC and tEnd.1 cells. After 30 min incubation in buffer containing CDNB at the indicated concentrations, tEnd.1 cells were washed twice and tested for GSH content and citrulline production, in resting conditions (*CIT bas*) and after stimulation with 2μM ionomycin (*CIT stim*). Values are reported as means ± SEM (n = 3). GSH levels in HUVEC and in tEnd.1 incubated with 20 and 200μM CDNB were significantly different from control tEnd.1 ($p < 0.02$); basal citrulline: control tEnd.1 vs. 2–200μM CDNB = n.s., control tEnd.1 vs. HUVEC $p = 0.03$; citrulline after ionomycin: control tEnd.1 vs. 2μM CDNB = n.s., control tEnd.1 vs. 20–200μM CDNB $p \leq 0.01$, control tEnd.1 vs. HUVEC $p < 0.001$

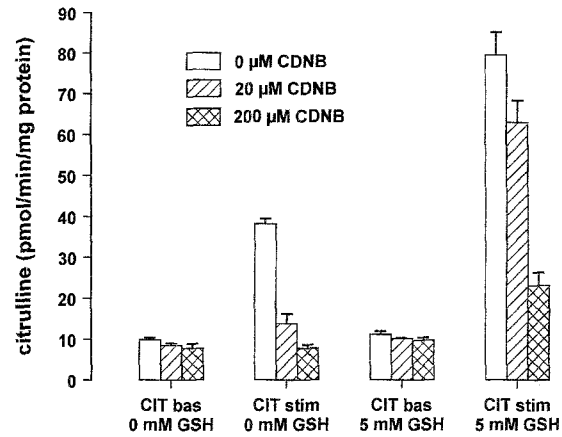

Fig. 2. Effect of tEnd.1 replenishments with GSH. After 3 h incubation in buffer alone (*0 mM GSH*), or buffer containing 5 mM GSH, cells were washed twice and tested for citrulline production, basally (*CIT bas*) or after 2μM ionomycin stimulation (*CIT stim*). Values are reported as means ± SEM (n = 3). CIT bas: 0 mM GSH vs. 5 mM GSH p n.s.; CIT stim: 0 mM GSH vs. 5 mM GSH $p \leq 0.002$

N^G-monomethyl-L-arginine (NMMA, 1 mM, 20 min preincubation, data not shown). tEnd.1, which exhibited an intracellular GSH level 2.65-fold higher than in HUVEC, showed a basal and ionomycin-elicited citrulline production 8 and 5 times higher than HUVEC, respectively. Preincubation of tEnd.1 with CDNB lowered the amount of intracellular GSH in a dose-dependent manner (Fig. 1): ionomycin-induced citrulline synthesis showed a clearcut dependence on GSH intracellular concentration; CDNB slightly decreased basal citrulline production. On the other hand (Fig. 2) incubation with exogenous GSH

doubled both intracellular GSH content and ionomycin-evoked citrulline production. Intracellular levels of GSH and citrulline showed a good correlation (GSH vs. basal citrulline $r = 0.977$, $p < 0.001$; GSH vs. ionomycin-stimulated citrulline $r = 0.938$, $p < 0.01$). Higher levels of intracellular GSH protected citrulline synthesis from the inhibitory effect of CDNB (Fig. 2), ruling out a direct effect of CDNB on NOS activity. On the other hand, when tEnd.1 cells were incubated for 30 min with 2 mM MPG, a sulfydrylic compound which permeates the plasma membrane (Mita, 1981), the amount of soluble thiols increased by 104 ± 35% ($n = 3$), basal and ionomycin-induced citrulline production increased only by 15.7 ± 4% and 14 ± 7%, respectively ($n = 3$).

Discussion

We have previously shown in HUVEC that NMMA-sensitive synthesis of citrulline is decreased by CDNB-elicited GSH depletion in a dose-dependent way and is increased by replenishing cells with GSH but not with the soluble thiol MPG, and only the former reverses the CDNB-induced inhibition of NO synthesis (Ghigo et al., 1993). Our data suggested that NO synthesis in HUVEC has a specific requirement for GSH, which can play a role by protecting NOS from inactivation by NO and other radical intermediates of the NOS reaction. In fact GSH protects NOS from inhibitory action of NO (Griscavage et al., 1994). In tEnd.1 cells we have previosly found a very high NOS activity (Ghigo et al., 1995): thus, it appeared interesting to check whether the correlation between GSH levels and NO production was maintained in cells overexpressing NOS activity. Our results in tEnd.1 confirm the GSH-dependence of NO synthesis already found in HUVEC: CDNB-elicited GSH depletion causes an inhibition of NO synthesis, which is prevented by loading cells with GSH. The cell-permeating thiol MPG is not able to replace GSH and does not increase citrulline production. Finally, mTa-transformed tEnd.1 cells, producing higher amounts of NO in comparison with normal murine and human endothelial cells, show a higher GSH content too. So far, GSH level in transformed endothelial cells may modulate their growth in vivo, as this depends on elevated NO production (Ghigo et al., 1995).

Acknowledgements

This study was supported by grants from CNR (CT14 92.02155 and CT14 93.01981, from MURST (grants 60% and 40%), from Programma Vigoni (Conferenza Permanente dei Rettori delle Università Italiane), from AIRC and from ISS (AIDS Project).

References

Bosia A, Spangenberg P, Ghigo D, Heller R, Lösche W, Pescarmona GP, Till U (1985) Effect of GSH depletion by 1-chloro-2,4-dinitrobenzene on human platelet aggregation, arachidonic acid oxidative metabolism and cytoskeletal proteins. Thromb Res 37: 423–434

Bredt DS, Snyder SH (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci USA 86: 9030–9033

Ghigo D, Alessio P, Foco A, Bussolino F, Costamagna C, Heller R, Garbarino G, Pescarmona GP, Bosia A (1993) Nitric oxide synthesis is impaired in glutathione-depleted human umbilical vein endothelial cells. Am J Physiol 265: C728–C732

Ghigo D, Arese M, Todde R, Vecchi A, Silvagno F, Costamagna C, Dong QG, Alessio M, Heller R, Soldi R, Trucco F, Garbarino G, Pescarmona GP, Mantovani A, Bussolino F, Bosia A (1995) Middle T antigen-transformed endothelial cells exhibit an increased activity of nitric oxide synthase. J Exp Med 181: 9–19

Griscavage JM, Fukuto JM, Komori Y, Ignarro LJ (1994) Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. J Biol Chem 269: 21644–21649

Mita I (1981) Chemistry, pharmacology and current clinical applications of tiopronin. In: Williams R, Gasbarrini G, Davis M (eds) Recent advances in 2-MPG treatment of liver diseases. Editrice Compositori, Bologna, pp 3–10

Nathan C, Xie Q-w (1994) Nitric oxide synthases: roles, tolls, and controls. Cell 78: 915–918

Schmidt HHHW, Walter U (1994) NO at work. Cell 78: 919–925

Stamler JS, Singel DJ, Loscalzo J (1992) Biochemistry of nitric oxide and its redox-activated forms. Science 258: 1898–1902

Williams RL, Courtneidge SA, Wagner EF (1988) Embryonic lethalities and endothelial tumors in chimeric mice expressing polyoma virus middle T oncogene. Cell 52: 121–131

Authors' address: Dr. D. Ghigo. Dipartimento di Genetica, Biologia e Chimica Medica – Sezione di Chimica Medica, Via Santena 5/bis, I-10126 Torino, Italy.

Received February 19, 1995