FEBS Letters 392 (1996) 242–244 FEBS 17424

Decreased L-arginine-nitric oxide pathway in cultured myoblasts from spontaneously hypertensive versus normotensive Wistar-Kyoto rats

G. Dubois

Pharmacologie des régulations cardiovasculaires, CNRS URA 1482, Faculté de Médecine Necker, 156, rue de Vaugirard, 75730 Paris Cedex 15, France

Received 10 June 1996; revised version received 9 July 1996

Abstract The hypertrophy of the arterial media exhibited by spontaneously hypertensive rats is related to the hyperproliferation of their smooth muscle cells in culture. Using two methods to assay nitric oxide synthase activity, we showed that this abnormal proliferation results from a decreased activity of the inducible NOS II and less modulation of this enzymatic activity by these cells compared to control Wistar Kyoto cells.

Key words: Spontaneously hypertensive rat; Nitric oxide synthase; Smooth muscle cell

1. Introduction

Spontaneously hypertensive rats (SHR) have been proposed as a model of human essential hypertension, with hypertrophy of the vascular media [1] and dysregulation of immune function [2]. In culture, vascular smooth muscle cells (VSMC) from SHR proliferate more than those from normotensive Wistar Kyoto rats (WKY) [3,4], as demonstrated by increases of both leucine incorporation into cell proteins [5] and thymidine uptake during DNA synthesis [4].

Nitric oxide (NO), which is the endothelium-derived relaxing factor involved in the regulation of VSMC contraction [6,7], has been shown to inhibit thymidine incorporation and thus DNA synthesis [8,9]. All these properties suggest the possible involvement of NO in cell proliferation.

To examine this possibility, NO synthase (NOS) activities in cultured VSMC from WKY and SHR was evaluated. The hyperproliferation of the latter may be the result of decreased activity of the inducible NOS produced by these cells compared to that from WKY cells.

2. Materials and methods

2.1. Cell culture

VSMC were extracted from the thoracic aorta of WKY and SHR rats and cultured as described by Inoue et al. [10].

All assays were performed in flat-bottomed 96-well (0.33 cm^2 each) culture plates. 10^4 cells per well were plated in 100 μl of DMEM (Gibco, Cergy-Pontoise, France) containing 100 U/ml of penicillin, 100 U/ml of streptomycin, and 10% heat-inactivated fetal calf serum (FCS). After 24 h, the medium was replaced with serum-free DMEM and cells were cultured for 48 h so as to obtain homogeneous G_0 phase cells. The cells were then stimulated for various times with 5% FCS, with or without the following compounds: (i) lipopolysaccharide (LPS) or interleukin-1 beta (Il-1β), as inducers of NOS II; (ii) N^G-nitro-L-arginine methyl ester (L-NAME), as an inhibitor of NOS. Cells were counted according to the method of Kueng et al. [11]: briefly, cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet in MES buffer; the intracellular crystal violet was extracted with 100 μl of 10% acetic acid and the optical density (OD) was read at 590 nm, using an MR 7000 microplate reader (Dynatech, Elancourt, France).

2.2. Nitrite assay

100 μl of Griess reagent, constituted of 1 vol. of naphthylethylene diamine solution (0.2% in bidistilled water) and 1 vol. of sulfanylamide solution (2% in 5% phosphoric acid), were added to each well containing plated VSMC in 100 μl of culture medium. After 1 h at 4°C, the OD was read at 550 nm in the microplate reader.

2.3. Citrulline measurement

To start stimulation, 3.7 kBq of L-[U-^{14}C]arginine (12 GBq/mmol, Amersham-France, Les Ulis, France) were added to each well, containing 100 μl of medium and cultured VSMC. At each selected time, 10 μl of the cell medium were applied as a linear spot on precoated silica gel HPTLC 60 plates (Merck, Darmstadt, Germany). The different radiolabeled L-arginine metabolites were separated using the solvent system described by Ivengar et al. [12]. The plates were then scanned with an automatic TLC linear LB 283 analyzer (Berthold, Evry, France).

3. Results and discussion

Fig. 1A shows the time course of VSMC proliferation over 48 h. No significant differences between the numbers of SHR and WKY cells could be observed until 24 h. After this time, SHR VSMC increased markedly in number while the WKY cell number remained stable. On the other hand, Fig. 1B shows that WKY cells secreted much more citrulline into the culture medium after 24 h while citrulline release by SHR was unchanged. Uehara et al. [5] previously observed similar kinetics of leucine incorporation and thus protein synthesis. NOS generates both citrulline and NO which is an inhibitor of DNA synthesis. Thus, the time-dependent accumulation of citrulline in the cell medium can be used as a characteristic marker of NOS activity, as already demonstrated [12]. After its generation, NO is rapidly transformed into nitrites [12] and measurement of the nitrite level revealed (Fig. 1C) parallel accumulation of citrulline and nitrites, both being higher for WKY than SHR VSMC. Thus, NOS product secretion by SHR VSMC was lower than that of WKY cells and is in agreement with the higher proliferation level of SHR VSMC in the presence of FCS.

Fig. 2 gives the mean values of NOS activity for WKY and SHR VSMC measured on 3 separate cell preparations at 24, 30 or 48 h. For each individual experiment in the presence of 5% FCS alone, the ratio of SHR/WKY NOS products was between 31 and 49%. These results confirm the lower NOS activity of SHR VSMC in culture. Moreover, the addition of LPS, an inducer of NOS activity, characteristically enhanced this activity in WKY cells while that in SHR VSMC was unaffected. Similarly, WKY VSMC exhibited increased citrulline production in the presence of Il-1β and decreased citrulline secretion in the presence of L-NAME, while SHR citrul-

PII S0014-5793(96)00817-4

line values were unchanged by any modulator, thereby suggesting a weaker induction of NOS II activity in SHR than WKY VSMC. Nitrite level were modulated in the same manner in WKY cells, but this time SHR VSMC responses paralleled them, albeit to a lesser extent. In this study, the numbers of cells counted after the action of LPS, Il-1β or L-NAME were not significantly different when compared to the number of control cells, i.e. with only 5% FCS.

This smaller increase in NO secretion has already been reported for SHR endothelial cells after stimulation by bradykinin for constitutive NOS III, and for VSMC after induction by Il-1β in the absence of serum for NOS II [13] which is only present in VSMC [14]. On the other hand, Nava et al. [15] observed a lower specific activity of the inducible NOS II in SHR cardiac cells, compared to WKY cells.

The markedly lower nitrite and citrulline levels secreted into the cell medium by SHR VSMC compared to the WKY VSMC observed in this study occurred essentially during the M phase of the cell cycle. This decrease could partially explain the hyperproliferation of SHR cells. Three hypotheses can be proposed to explain this observation: (1) lower expression of the NOS II gene by the SHR cells, resulting in the action of other compounds, as was previously shown for Il-1β [16]; (2) decreased transfer of NOS II from the cytosolic storage vesicles to the plasma membrane where it becomes activated [17]; and (3) dysregulation of the transmembrane secretion of NOS products in SHR cells. The higher level of cell membrane peroxidation found for the SHR VSMC according to the method described in [18] is under investigation and could support the two latter hypotheses.

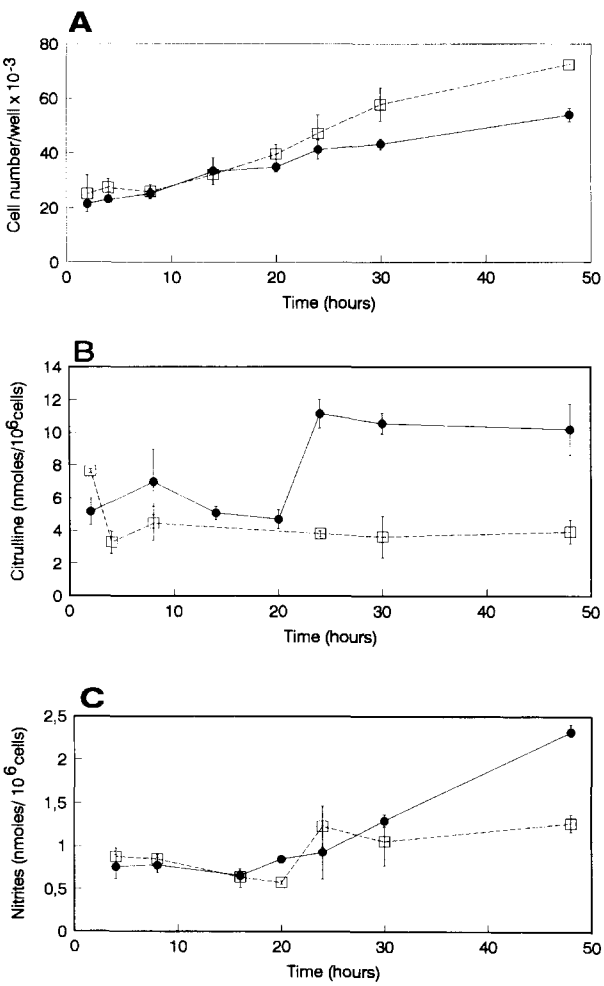

Fig. 1. Time course of cell number (A), citrulline (B) and nitrite (C) accumulation in the cell culture medium of VSMC from WKY (●) and SHR (□) after stimulation with 5% FCS. Each point represents the mean ± S.D. of 3 experiments.

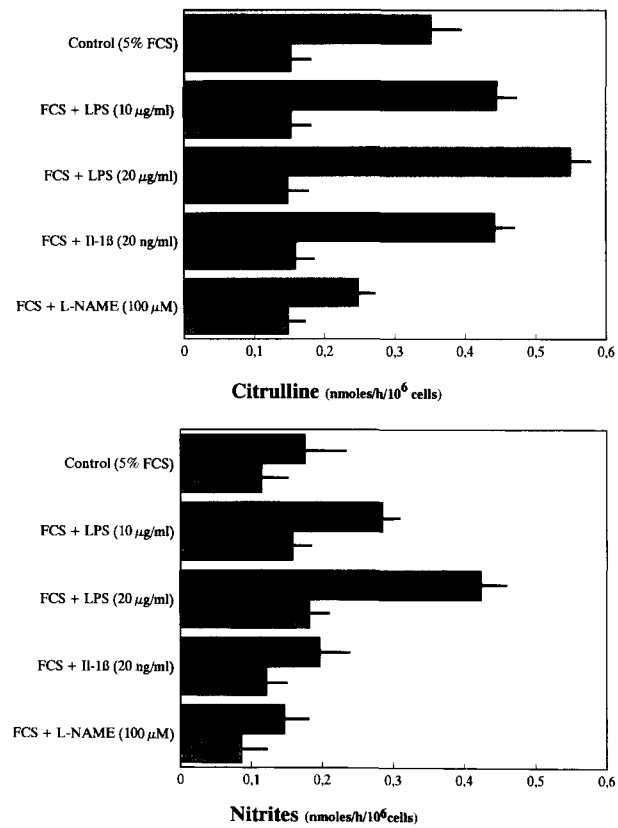

Fig. 2. Citrulline and nitrite accumulation in the culture medium of WKY (dark bars) and SHR (light bars) VSMC in the presence of 5% FCS alone (control) and in combination with LPS (10 and 20 μg/ml), Il-1β (20 ng/ml) or L-NAME (100 μM). Values are means ± S.D. ($n = 4$).

Acknowledgements: The author thanks Professor L.G. Alcindor for his contribution to the study of the peroxidation.

References

[1] Folkow, B. (1982) Physiol. Rev. 62, 347–504.
[2] Dzielak, D.J. (1991) Am. J. Physiol. 260, R459–R467.
[3] Berk, B.C., Vallega, G., Muslin, A., Gordon, H., Canessa, M. and Alexander, R. (1989) J. Clin. Invest. 83, 822–829.
[4] Hadrava, V. (1989) Hypertension 13, 589–597.
[5] Uehara, Y., Kawabata, Y., Hirawa, N., Takada, S., Numabe, A., Matsuoka, H., Ikeda, T., Takabatake, Y., Yagi, S. and Sugimoto, T. (1992) J. Cardiovasc. Pharmacol. 20, 408–415.
[6] Ignarro, L.J. (1989) Circ. Res. 65, 1–21.
[7] Arnal, J.F., Battle, T., Ménard, J. and Michel, J.B. (1993) J. Hypertens. 11, 945–950.
[8] Hibbs, J.B., Taintor, R.R. and Vavrin, Z. (1984) Biochem. Biophys. Res. Commun. 123, 716–723.
[9] Lepoivre, M., Chenais, B., Yapo, A., Lemaire, G., Thelander, L. and Tenu, J.P. (1990) J. Biol. Chem. 265, 14143–14149.
[10] Inoue, T., Fukuo, K., Morimoto, S., Koh, E. and Ogihara, T. (1993) Biochem. Biophys. Res. Commun. 194, 420–424.
[11] Kueng, W., Silber, E. and Eppenberger, U. (1983) Anal. Biochem. 182, 16–19.

[12] Ivengar, R., Stuehr, D. and Marletta, M.A. (1987) Proc. Natl. Acad. Sci. USA 84, 6369–6373.
[13] Malinsky, T., Kapturczak, M., Dayharsh, J. and Bohr, D. (1993) Biochem. Biophys. Res. Commun. 194, 654–658.
[14] Fleming, I., Gray, G.A., Schott, C. and Stoclet, J.C. (1991) Eur. J. Pharmacol. 200, 375–376.
[15] Nava, E., Noll, G. and Luscher, T.F. (1995) Circulation 91, 2310–2313.
[16] Koide, M., Kawahara, Y., Tsuda, T., Nakayama, I. and Yokoyama, M. (1994) Hypertension 23, 145–148.
[17] Ringheim, G.E. and Pan, J. (1995) Biochem. Biophys. Res. Commun. 210, 711–716.
[18] Zimmermann, L., Pages, N., Antebi, H., Hafi, A., Boudene, C. and Alcindor, L.G. (1993) Biol. Trace Elem. Res. 38, 311–318.