Volume 238, number 2, 249–252 FEB 06371 October 1988

Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells

Issei Komuro, Hiroki Kurihara, Takao Sugiyama, Fumimaro Takaku and Yoshio Yazaki

The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

Received 16 August 1988

Recently, a potent vasoconstrictor peptide, endothelin (EDT), was isolated from vascular endothelial cells. We examined its effect on rat vascular smooth muscle cells (VSMCs). EDT induced the elevation of intracellular calcium, which was dependent on extracellular calcium and inhibited by a calcium-channel antagonist in a competitive manner. EDT caused a rapid and transient increase in the c-fos and c-myc mRNA levels and stimulated the DNA synthesis of VSMCs in a dose-dependent manner. This effect of EDT on the proliferation of VSMCs might be related to the development of atherosclerosis.

Endothelin; Ca^{2+}; c-myc gene; c-fos gene; Atherosclerosis; (Vascular smooth muscle)

1. INTRODUCTION

Vascular smooth muscle cells are the major cellular constituent of mammalian arteries, their growth being a critical event in the development of atherosclerosis [1,2]. In response to endothelial injury, the migration of VSMCs from the medial layer to the intima triggers abnormal VSMC proliferation. Several growth factors, which are released from platelets in the injured region of the arteries, are thought to play important roles in the proliferation of VSMCs. Although endothelial cell-derived growth factors are also thought to be involved in the proliferation of VSMCs, there have been quite a few reports about them.

Recently, an endothelium-derived vasoconstrictor peptide, endothelin, was isolated from the culture supernatant of porcine aortic ECs [3]. EDT is the most potent mammalian vasoconstrictor peptide known to date, and may be an endogenous modulator of voltage-dependent Ca^{2+} channels. In the present study, we examined the proliferative effects of EDT on rat VSMCs. EDT increased the levels of $[Ca^{2+}]_i$, caused a rapid and transient increase in the c-fos and c-myc mRNA levels and stimulated the DNA synthesis of VSMCs.

Correspondence address: I. Komuro, The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

Abbreviations: EDT, endothelin; VSMC, vascular smooth muscle cell; $[Ca^{2+}]_i$, intracellular calcium; EC, endothelial cell; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1. *Materials*

The following materials were used: endothelin (Peptide Research Laboratory); [*methyl*-^{3}H]thymidine (Amersham); PDGF (Collaborative Research Inc.); EGF (Earth Chemical Company); FCS (Gibco); Indo-1 AM (Molecular Probe); and nicardipine (Sigma). The oncogene probes were obtained from the Japanese Cancer Research Resources Bank. v-myc, *Pst*I-*Pst*I fragment [4]; v-fos, *Bgl*II-*Pvu*II fragment [5]. Other materials and chemicals were purchased from commercial sources.

2.2. *Cell culture*

Rat aortic medial VSMCs were prepared from explants of the thoracic aorta of 3-month-old male Wistar Kyoto rats according to the modified method of Chamley [6]. The VSMCs were seeded in 100-mm dishes or 24-well plates at a density of 1×10^4

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

cells/cm^2 with DMEM containing 10% FCS. After confluence was reached, the growth was arrested by transfer to DMEM containing insulin (1 μM) and transferrin (5 μg/ml) [7].

2.3. *$[Ca^{2+}]_i$ measurement*

Intracellular calcium was measured using the method described previously [8].

2.4. *Induction of c-myc and c-fos*

Quiescent cultures of rat VSMCs were incubated with either vehicle (phosphate-buffered saline [PBS]) or various concentrations of EDT. Cells were then lysed in situ, and total cellular RNA was extracted using the lithium urea method [9]. Northern blot analyses were performed as described previously [10].

2.5. *DNA synthesis*

After exchanging the medium for fresh DMEM, the quiescent cells were stimulated with various concentrations of EDT, 10% FCS, synthetic EGF (25 ng/ml) or partially purified PDGF (25 ng/ml) for 24 h at 37°C. 1 μCi of [*methyl*-^{3}H]thymidine per well was added during the final 4 h and trichloroacetic acid-insoluble radioactivity was measured as described by Dicker and Rozengurt [11].

3. RESULTS

Fig.1A illustrates a typical example of the $[Ca^{2+}]_i$ transient which has been induced by 10^{-7} M EDT with 1 mM extracellular Ca^{2+}. In contrast, no increase in the Indo-1 fluorescence signal was detectable when extracellular Ca^{2+} was not present. As shown in fig.1B, the cytoplasmic free Ca^{2+} concentration increased in response to EDT in a dose-dependent manner and the dihydropyridine-sensitive Ca^{2+}-channel antagonist, nicardipine (10^{-6} M), competitively inhibited the elevation of $[Ca^{2+}]_i$ by EDT.

As shown in fig.2A, when serum-deprived cultures of VSMCs were incubated with EDT (10^{-7} M), the c-myc and c-fos mRNA levels increased and reached maximal levels 120 min and 30 min, respectively, after the addition of EDT and then declined to the basal levels. The increases in c-myc and c-fos mRNA were found to be concentration-dependent and maximal expression (ten times the basal level in c-myc mRNA) was reached under stimulation by 10^{-6} M EDT (fig.2B).

EDT stimulated the [*methyl*-^{3}H]thymidine incorporation in a concentration-dependent manner in the presence of insulin. The concentration required to induce half the maximal proliferation was 3×10^{-8} M and maximal stimulation was obtained using 10^{-6} M (fig.3A). These doses were similar to those required for induction of a $[Ca^{2+}]_i$ increase and protooncogene expression. The maximal level was about 35% and 65% compared with

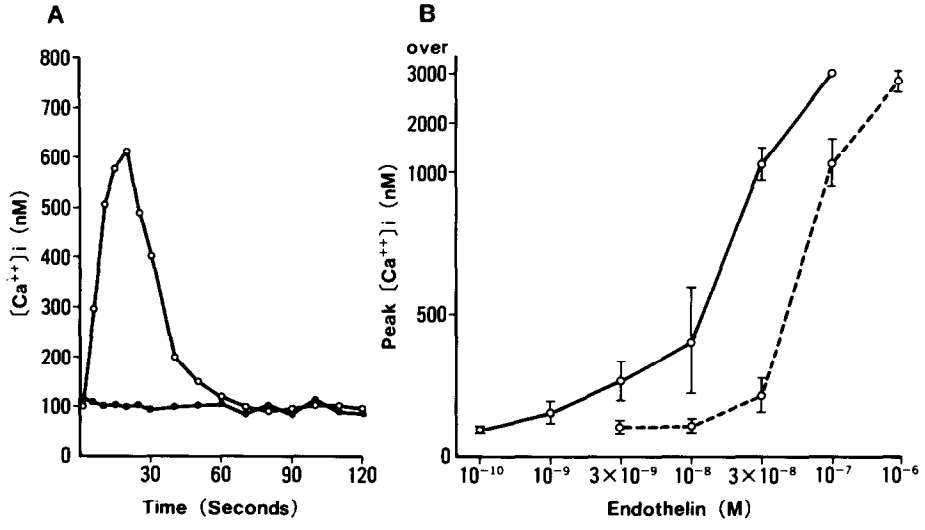

Fig.1. Effect of EDT on the elevation of $[Ca^{2+}]_i$. (A) The cells were stimulated by 10^{-7} M EDT in the presence (○) or absence (●) of 1 mM extracellular Ca^{2+}. (B) The cells were stimulated by various doses of EDT in the presence (broken line) or absence (solid line) of 10^{-6} M nicardipine. Cytoplasmic free calcium concentrations of a single cell were measured by using ultra-violet laser and Indo-1 [8]. Each point represents the mean ± SE of eight experiments.

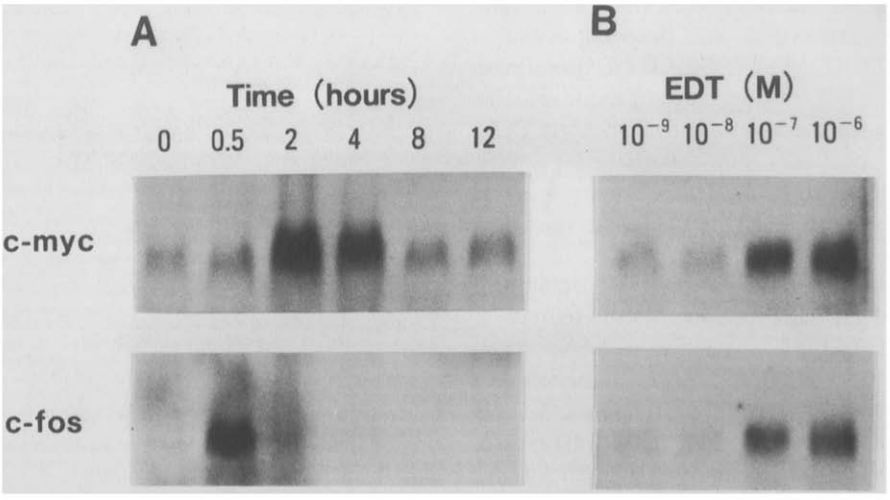

Fig.2. Induction of c-myc and c-fos expression by EDT. (A) The quiescent cultures were incubated with 10^{-7} M EDT for various periods of time. (B) The cells were incubated with various concentrations of EDT for 120 min (c-myc) or 30 min (c-fos) at 37°C. 10 μg of total RNA was hybridized with the oncogene probes.

10% FCS and 25 ng/ml of PDGF, respectively (fig.4B). The increase of [*methyl*-^{3}H]thymidine incorporation began 12–16 h after the addition of EDT (not shown), and DNA synthesis barely occurred in the absence of insulin (fig.4A).

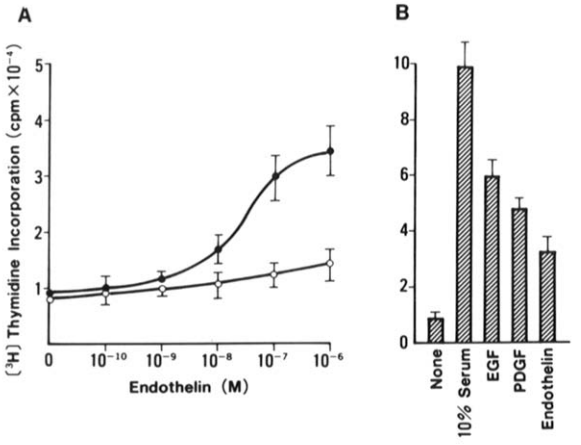

Fig.3. Stimulation of DNA synthesis by EDT in cultured VSMCs. The quiescent cultures of VSMCs were incubated with various concentrations of EDT in the presence (●) or absence (○) of insulin (A) or with 10% FCS, synthetic EGF (25 ng/ml), PDGF (25 ng/ml) and EDT (10^{-6} M) (B) for 24 h at 37°C. Each point represents the mean ± SE of triplicate determinations from five experiments.

4. DISCUSSION

Many studies have suggested the important role of $[Ca^{2+}]_i$ in cell proliferation [12], but decisive evidence for the idea has not been obtained. A23187 and ionomysin also induce the Ca^{2+} influx and the expression of these protooncogenes [13,14], but they fail to stimulate DNA synthesis (not shown). In the present study, we first demonstrated that calcium influx, probably via dihydropyridine-sensitive Ca^{2+}-channels, itself might be a signal of VSMC proliferation.

Data have been accumulated indicating that both c-myc and c-fos proteins were involved in DNA replication [15]. In the study presented here, EDT induced the expression of these competent genes, c-myc and c-fos. The expression kinetics of these protooncogenes agree with those observed in stimulation by other growth factors, hence suggesting that EDT directly stimulates the expression of these genes as opposed to via the induction of other growth factors. Taken together with the results that DNA synthesis barely occurred in the absence of insulin, EDT might function as 'a competent factor' by inducing Ca^{2+} influx.

EC injury could provide opportunities for interaction with platelets, which release several biologically active substances. Using Northern blot

analysis, it has been observed that thrombin stimulates the expression of preproendothelin mRNA [3]. These data suggest a paracrine mechanism of EDT in the development of the atherosclerosis. Furthermore, since cultured ECs, which actively produce and secrete EDT, may more closely approximate a state of 'injury' in vivo [2], injured ECs might produce EDT without other agents.

Ross hypothesized that even when ECs remained intact, they could still produce and secrete a growth factor causing the induction of VSMC proliferation [2]. Especially under these conditions, endothelium-derived growth factors are assumed to play a key role in atherosclerosis. Except a mitogen resembling platelet-derived growth factor, only Gajdusek et al. have reported on an EC-derived growth factor, a polypeptide of 10000 to 30000 Da [16]. The present study suggests that the novel potent vasoconstrictor peptide, endothelin, is one of these EC-derived growth factors, the molecular mass of which was 2492 Da, quite different from that reported earlier. Whether or not EDT plays an important role in atherosclerosis in vivo, however, requires further investigation.

Acknowledgements: This investigation was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, and a grant from the research program on cell calcium signals in the cardiovascular system, Japan.

REFERENCES

[1] Ross, R. and Glomset, J.A. (1976) N. Engl. J. Med. 295, 369–377; 420–425.
[2] Ross, R. (1986) N. Engl. J. Med. 314, 488–500.
[3] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[4] Vennstrom, B., Moscovici, C., Goodman, H.M. and Bishop, J.M. (1981) J. Virol. 39, 625–631.
[5] Curran, T., Peters, G., Beveren, C.V., Teich, N. and Verma, I.M. (1982) J. Virol. 44, 674–682.
[6] Chamley-Campbell, J., Campbell, G.R. and Ross, R. (1979) Physiol. Rev. 59, 1–61.
[7] Libby, P. and O'Brien, K.V. (1983) J. Cell. Physiol. 115, 217–223.
[8] Sugiyama, T., Yoshizumi, M., Takaku, F., Urabe, H., Tsukakoshi, M., Kasuya, T. and Yazaki, Y. (1986) Biochem. Biophys. Res. Commun. 141, 340–345.
[9] Auffray, C. and Rougeon, F. (1980) Eur. J. Biochem. 107, 303–314.
[10] Komuro, I., Kurabayashi, M., Takaku, F. and Yazaki, Y. (1988) Circ. Res. 62, 1075–1079.
[11] Dicker, P. and Rozengurt, E. (1980) Nature 287, 607–612.
[12] Rozengurt, E. (1986) Science 234, 161–166.
[13] Kaibuchi, K., Tsuda, T., Kikuchi, A., Tanimoto, T., Yamashita, T. and Takai, Y. (1986) J. Biol. Chem. 261, 1187–1192.
[14] Tsuda, T., Hamamori, Y., Yamashita, T., Fukumoto, Y. and Takai, Y. (1986) FEBS Lett. 208, 39–42.
[15] Callahan, M., Cochran, G.H. and Stiles, C.D. (1985) in: Growth Factors in Biology and Medicine, Ciba Foundation Symposium 116 (Evered, D. et al. eds) pp.87–96, Pitman, London.
[16] Gajdusek, C., Dicorleto, P., Ross, R. and Schwartz, S.M. (1980) J. Cell Biol. 85, 467–472.