Biochemical and Biophysical Research Communications **259,** 50–55 (1999)
Article ID bbrc.1999.0723, available online at http://www.idealibrary.com on IDEAL®

The Requirement of both Intracellular Reactive Oxygen Species and Intracellular Calcium Elevation for the Induction of Heparin-Binding EGF-like Growth Factor in Vascular Endothelial Cells and Smooth Muscle Cells[1]

Yoshiro Kayanoki,*,† Shigeki Higashiyama,* Keiichiro Suzuki,* Michio Asahi,* Sumio Kawata,† Yuji Matsuzawa,† and Naoyuki Taniguchi*

**Department of Biochemistry and †Second Department of Internal Medicine, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

Received March 20, 1999

Heparin-binding EGF-like growth factor (HB-EGF), which is a potent mitogen for vascular smooth muscle cells (SMC) and fibroblasts, has been reported to be strongly implicated in atherosclerosis and wound healing. HB-EGF mRNA is known to be induced by thrombin, angiotensin-II, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and HB-EGF itself in SMC. In vascular endothelial cells (EC), its mRNA is induced by tumor necrosis factor-α and interleukin-1β. Only phorbol 12-myristate 13-acetate is a common inducer for HB-EGF mRNA. The present study shows that calcium ionophore A23187 also induced HB-EGF mRNA in both SMC and in EC and that both intracellular reactive oxygen species (ROS) and an increase in calcium levels were essential for the induction of this growth factor mRNA. While HB-EGF caused an increase in both intracellular ROS and calcium in SMC, it increased only calcium, but not the intracellular ROS in EC. When the intracellular ROS was elevated by treatment with hydrogen peroxide (H_2O_2) or by depletion of glutathione by buthionine sulfoxamine, both HB-EGF and thrombin were observed to upregulate HB-EGF mRNA in EC. These data suggest that H_2O_2, produced by activated leukocytes in inflammatory lesions, upregulates HB-EGF mRNA by cooperating with thrombin, angiotensin-II, and the above growth factors. Since activated macrophages under the EC are thought to elevate the ROS in neighboring EC, this mechanism might play a major role in the progression of atherosclerosis and for wound healing.

Heparin-binding EGF-like growth factor (HB-EGF) was originally identified as a secretory product of human monocytes, macrophages, and a macrophage-like cell line, U937 (1, 2). The secreted form of HB-EGF, as purified from U937-conditioned medium is a 20-22-kDa glycoprotein which binds to and stimulates the phosphorylation of the EGF receptor. The C-terminal portion of the primary sequence of HB-EGF is 35-40% structurally identical to EGF and to transforming growth factor-α (TGF-α). Unlike EGF and TGF-α, HB-EGF contains an extended N-terminal sequence which is capable of interacting with heparin/heparan sulfate proteoglycans. It has been reported that the heparin-binding property of HB-EGF facilitates its interaction with cell surface proteoglycans, thus modulating its biological activity (1, 2). HB-EGF is mitogenic for fibroblasts and vascular smooth muscle cells (SMC) but not for endothelial cells (EC) (1). HB-EGF, more potent SMC mitogen than EGF (3), is comparable to platelet derived growth factor (PDGF). Since HB-EGF is a potent mitogen for SMC and fibloblasts, it has been suggested that it plays an important role in atherosclerosis (4) and wound healing (5). Recently, we reported that significant levels of HB-EGF protein can be immunohistologically detected in SMC and macrophages of atherosclerotic plaques, suggesting that HB-EGF might be involved in the migration and proliferation of

[1] This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas and Specific Project Research on Cancer Bio-Science by the Ministry of Education, Science and Culture, Japan and from the Tokyo Biochemical Foundation.

Abbreviations: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; TGF-α, transforming growth factor-α; SMC, smooth muscle cells; EC, endothelial cells; PDGF, platelet derived growth factor; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PMA, phorbol 12-myristate 13-acetate; H_2O_2, hydrogen peroxide; BSO, buthionine sulfoxamine; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; TGF-β, transforming growth factor-β; DMEM, Dulbecco's modified Eagle's medium.

0006-291X/99 $30.00

SMC that is involved in the formation of atherosclerotic plaques (4).

In recent years, a considerable number of evidence has been published which suggests that reactive oxygen species (ROS) are involved in gene expression. For example, the production of interleukin-8, which is a potent neutrophil chemoattractant and activator, is upregulated by ROS (6). Monocyte chemoattractant protein-1 and monocyte colony-stimulating factor are also induced by ROS (7). Since activated leukocytes produce considerable amounts of ROS at inflammatory sites, these chemoattractants may be released in order to gather both neutrophils and monocytes at such sites. Since HB-EGF is also a chemoattractant for SMC (3), it is conceivable that ROS are involved in the induction of HB-EGF at inflammatory sites, as well.

HB-EGF mRNA is known to be induced by thrombin, angiotensin-II, bFGF, PDGF, and HB-EGF itself in SMC (8–10). In contrast, its mRNA is induced by tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in EC (11). Only phorbol 12-myristate 13-acetate (PMA) is a common inducer for HB-EGF mRNA (9, 11). In this study, we examined some factors which alter the expression of HB-EGF mRNA in SMC and EC. The inducers are all calcium agonists, which elevate intracellular calcium. In addition, TNF-α, IL-1β, and PMA have been reported to produce intracellular ROS, thus elevating oxidant status (12–14). Therefore, our first investigation addressed the issue of whether calcium ionophore induced HB-EGF mRNA in both SMC and EC. Subsequently, since both calcium and oxidant status appear to be involved in the induction of HB-EGF, we examined both intracellular calcium and intracellular ROS, after the exposure of EC and SMC to HB-EGF. We also modified the intracellular redox status using hydrogen peroxide (H_2O_2) or buthionine sulfoxamine (BSO) in order to induce the expression of HB-EGF mRNA in EC.

MATERIALS AND METHODS

Materials. H_2O_2 and calcium ionophore A23187 were all obtained from Wako Pure Chemical Industries Ltd., Osaka. TNF-α was a gift from Ube Industry. 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular probe Co. Ltd. Buthionine sulfoxamine (BSO), and bovine thrombin were obtained from Sigma.

Cell culture. EC were prepared from human umbilical cord veins using dispase (Godo-shusei Co.) (15) and were cultured in E-GM UV medium, modified MCDB131 medium containing 10% fetal bovine serum, 10 ng/ml bFGF, 1 mg/ml hydrocortisone, 50 μg/ml gentamycin, and 0.25 mg/ml amphotericin B (Kurabo Co.). The cells at passage 4 to 5 were used in these experiments. Rat aortic vascular SMC were harvested from the thoracic aortas of male Sprague–Dawley rats (250 g) by enzymatic dissociation according to the method of Gunther *et al.* (16). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml). SMC from passages 4-8 were used in the experiments.

RNA isolation and Northern analysis. After incubation with the above agents for several periods of time, the cells were collected by centrifugation. Total RNA was isolated by extraction with acidic guanidinium thiocyanate-phenol-chloroform (17) and quantitated by measuring the absorbance at 260 nm. Twenty mg of total RNA were heat-denatured at 65°C for 15 min in the presence of 50% formamide and gel running buffer (40 mM morpholinopropanesulfonic acid, 10 mM sodium acetate, and 1 mM EDTA, pH 7.0) and electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNAs were transferred onto a Zeta-Probe membrane (Bio Rad) for 20 to 40 h by capillary action, and the blotted RNAs immobilized on the membrane by incubation at 80°C for 60 min. After hybridization with a ^{32}P-labeled human HB-EGF cDNA (1) probe at 42°C in the presence of 50% formamide, the membranes were washed twice for 80 min at 55°C with 30 mM sodium citrate, 300 mM NaCl, pH 7.5, and 0.1% sodium dodecyl sulfate. Kodak XAR films were exposed for 1-3 days with an intensifying screen at −80°C. To normalize mRNA contents, blots were stripped and reprobed with a human β-actin cDNA using the hybridization and washing conditions described above. All other DNA and RNA manipulations were conducted according to methodology reported by Maniatis *et al.* (18).

Measurement of intracellular ROS by flow cytometry. Intracellular ROS levels were estimated fluorometrically by using an oxidation-sensitive probe DCFH-DA (19). In the presence of a variety of intracellular ROS, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells, treated with or without 10 ng/ml HB-EGF, were incubated with 5 μM DCFH-DA. The cellular fluorescence intensity, which was directly proportional to levels of intracellular ROS after 30 min of DCFH-DA oxidation, was measured using FACScan (Becton Dickinson, Mountain View, CA). For each analysis, 10,000 events were recorded. For image analysis, cells were analyzed for fluorescence intensity using a lysis cell analysis system.

RESULTS

HB-EGF mRNA Expression Induced by Calcium Influx in both EC and SMC

PMA was a common inducer of HB-EGF mRNA for SMC and EC as was previously reported. TNF-α induced HB-EGF mRNA in EC but not in SMC. In contrast, HB-EGF upregulated its own mRNA in SMC but not in EC. Calcium ionophore, A23187 was also a common inducer of HB-EGF for SMC and EC (Fig. 1). Since calcium ionophore elevates cytosolic calcium, intracellular peroxides are thought to be subsequently produced (20). Moreover, it has been reported that PMA elevates intracellular calcium and peroxides as well (14). Therefore, it was assumed that both intracellular calcium and peroxides could be involved in the induction of HB-EGF mRNA.

Differences in Intracellular ROS Production in EC and SMC after Treatment with HB-EGF

It is known that EC are protected but prevented from proliferating when the EGF receptor ligand binds to the EGF receptor of the cells (21). An increase in intracellular calcium occurred at that time. We therefore investigated whether or not intracellular ROS are elevated after incubation with HB-EGF in SMC and EC using flow cytometry. Figure 2 shows that intracellular

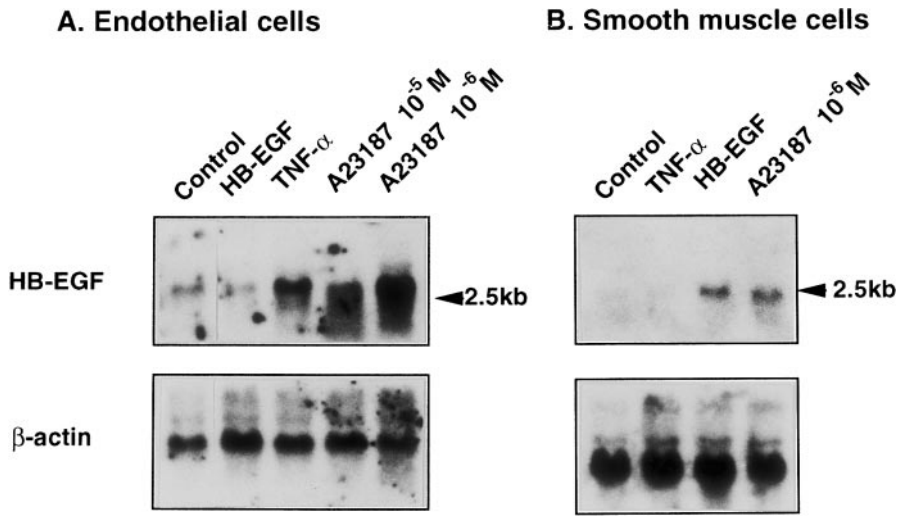

FIG. 1. Differential induction of HB-EGF mRNA between SMC and EC. HUVEC (A) and SMC (B) were stimulated with 10 ng/ml TNF-α, 10 ng/ml HB-EGF, and then given doses of calcium ionophore A23187 for 3 h. Total RNA was isolated and Northern blots were performed for ^{32}P-labeled HB-EGF cDNA and ^{32}P-labeled β-actin cDNA.

ROS are increased in SMC, but not in EC 1 h after incubation with 10 ng/ml HB-EGF. HB-EGF has been shown to induce HB-EGF mRNA and increase intracellular ROS only in SMC. Thus, an increase in intracellular ROS levels might be involved in the induction of HB-EGF mRNA.

Cooperation of H_2O_2 with HB-EGF in the Induction of HB-EGF mRNA in EC

It is possible that HB-EGF failed to induce HB-EGF mRNA in EC because intracellular ROS were not increased by HB-EGF itself in the cells. If so, then if H_2O_2 were added exogeneously, HB-EGF might be capable of inducing HB-EGF mRNA in EC. Figure 3 shows that H_2O_2 does, in fact, cooperate with HB-EGF in the induction of HB-EGF mRNA in EC.

Induction of HB-EGF mRNA by both HB-EGF and Thrombin in the Case of Decreased Intracellular Glutathione Levels in EC

Thrombin, also an HB-EGF inducer for SMC (8), has been reported to elevate intracellular calcium levels (22). Thrombin, as well as HB-EGF itself, induced HB-EGF mRNA in EC when H_2O_2 was added exogeneously (Fig. 4). Since the intracellular ROS was elevated when glutathione levels were depressed 1 day after incubation with BSO (23), we also examined whether or not BSO pretreatment had the same effect as exogenous H_2O_2. Figure 4 shows that pretreatment by BSO also (by cooperating with either HB-EGF or thrombin) induced HB-EGF mRNA in EC. These results demonstrated that the intracellular ROS can be strongly implicated in the induction of HB-EGF mRNA.

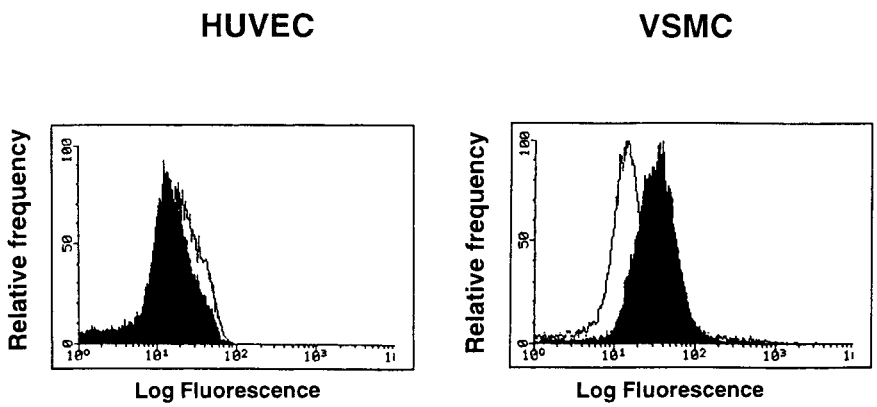

FIG. 2. Differences in intracellular ROS levels for EC and SMC after incubation with HB-EGF. Intracellular ROS levels of each cell were quantitated by flow cytometry using a peroxide-sensitive dye DCFH-DA after EC (left) and SMC (right) were stimulated with 10 ng/ml HB-EGF for 30 min. Cells were treated with (black area) or without (white area) 10 ng/ml HB-EGF.

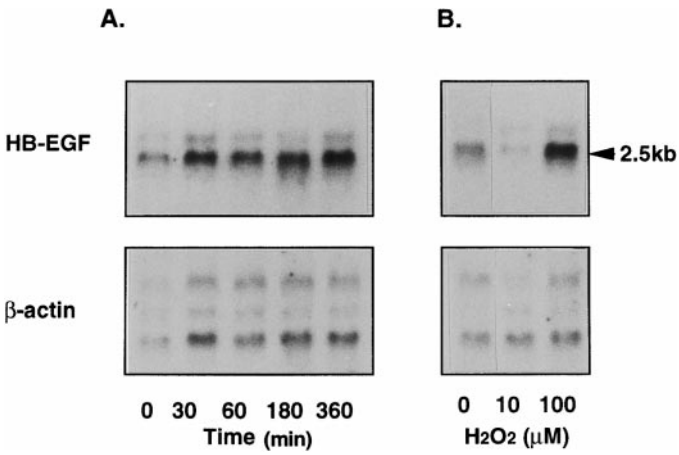

FIG. 3. Time course and dose dependency of the gene expression of HB-EGF by H_2O_2 cooperated with HB-EGF in HUVEC. (A) HUVEC were incubated with both 100 μM H_2O_2 and 10 ng/ml HB-EGF for several time intervals. At the time points indicated, total RNA was isolated and analyzed by Northern blotting using radiolabeled HB-EGF and β-actin cDNAs. (B) HUVEC were incubated with 10 ng/ml HB-EGF and given doses of H_2O_2 for 3 h. RNA was isolated and analyzed by Northern blotting.

Stimulation of HB-EGF mRNA Transcription by H_2O_2 in the Absence of Added Calcium Agonists and the Necessity of Calcium Ion in the Medium

H_2O_2 upregulates HB-EGF mRNA transcription in a dose-dependent manner in the absence of calcium agonists (Fig. 5A). This could be the result of other growth factors in the medium. Alternatively, this could be due to the increased permeability of cell membrane caused by H_2O_2. To clarify this hypothesis, we examined the ability of 100 μM H_2O_2 to induce HB-EGF mRNA when calcium ions in the medium are chelated by 5mM EDTA. Figure 5B shows that 5 mM EDTA blocked the upregulation of HB-EGF mRNA by 100 μM H_2O_2 as well as 1 μM calcium ionophore. These data suggest that extracellular calcium is likely responsible for the induction of HB-EGF by H_2O_2 and that the role of excess extracellular H_2O_2 is to sufficiently damage the cells, so as to render them more permeable to calcium.

DISCUSSION

Low levels of ROS are naturally produced as a normal part of cellular metabolism, and cells contain several enzymes, including catalase, glutathione peroxidase, and superoxide dismutases, which function to detoxify these radicals (24). Higher levels of ROS are generated upon the activation of xanthine oxidase or during the respiratory burst of phagocytic cells, and these higher concentrations have been associated with tissue damage (25, 26). A new role for free radicals has been proposed with the discovery that nitric oxide (NO), a nitrogen-containing radical, is important in intracellular signaling (27). It satisfies many of the criterion for a second messenger in that it is ubiquitous, it can be rapidly produced in a regulated manner, and is rapidly inactivated. Recently, Schreck and Bauerle (28) have hypothesized that certain oxygen-centered radicals may play a similar role as intracellular messengers. The present study indicates that ROS, as well as calcium, are important in regulating HB-EGF gene expression in both SMC and EC. When SMC are incubated with HB-EGF, both intracellular calcium and the ROS are increased, resulting in the induction of HB-EGF mRNA. On the other hand, since

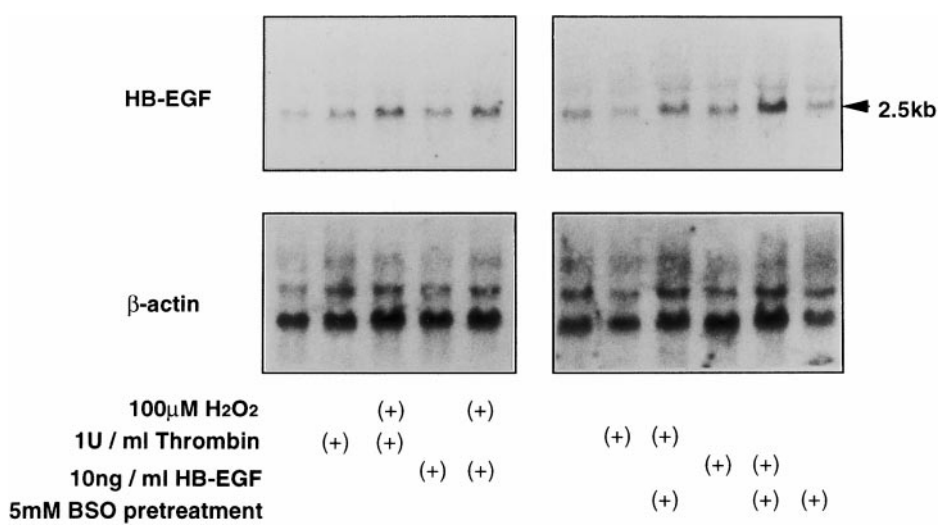

FIG. 4. Induction of HB-EGF mRNA by thrombin or HB-EGF cooperated with H_2O_2 or BSO pretreatment. HUVEC were incubated with 1 U/ml thrombin or 10 ng/ml HB-EGF with or without 100 μM H_2O_2 for 3 h (left panel). After cells were incubated with 5 mM BSO for 1 day, the medium was exchanged with fresh media. The cells were then exposed to 1 U/ml thrombin or 10 ng/ml HB-EGF for 3 h (right panel). Total RNA was prepared and analyzed by Northern blotting using radiolabeled HB-EGF and β-actin cDNAs.

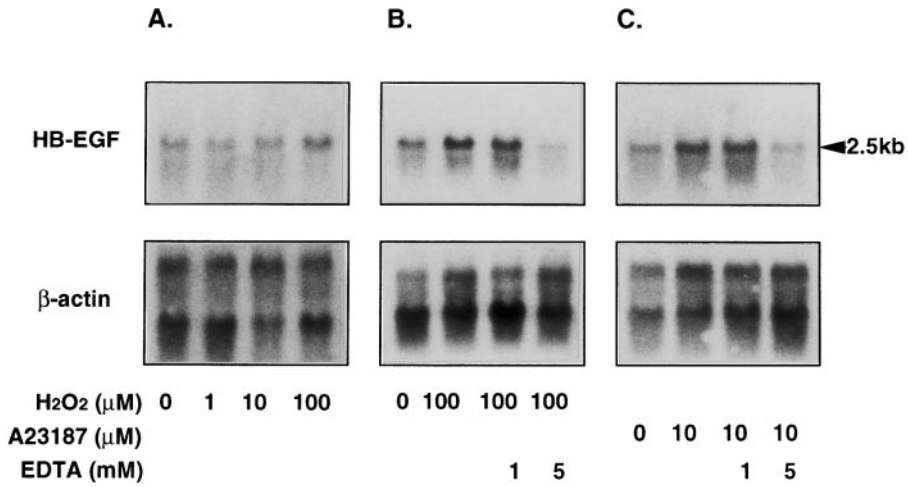

FIG. 5. Induction of HB-EGF mRNA by H_2O_2 alone and suppression of HB-EGF mRNA expression by calcium removal from the medium by EDTA. HUVEC were incubated with given doses of H_2O_2 for 3 h (A). Cells were incubated with 100 μM H_2O_2 with or without EDTA for 3 h (B). Cells were incubated with 10 μM calcium ionophore A23187 with or without EDTA for 3 h (C). Total RNA was prepared and analyzed by Northern blotting using radiolabeled HB-EGF and β-actin cDNAs.

the intracellular ROS levels were not risen by HB-EGF in EC, HB-EGF mRNA was not upregulated by the growth factor in EC. EC might contain higher levels of antioxidative enzymes than SMC, in order to protect against ROS produced at inflammation sites. Therefore, the higher levels of these enzymes appear to prevent the cells from elevating their intracellular ROS. This resistance of EC to oxidation might be implicated in HB-EGF mRNA not being induced by thrombin, AT-II, and HB-EGF in such cells. However, if the intracellular ROS levels are elevated by means of H_2O_2 or BSO pretreatment, HB-EGF and thrombin were also able to induce HB-EGF mRNA, even for the case of EC. These results suggest that, when inflammation occurs, ROS produced by activated leukocytes are capable of upregulating HB-EGF mRNA in the surrounding EC by cooperating with thrombin, AT-II, or HB-EGF. It is possible that the increased amount of HB-EGF in the EC could stimulate the proliferation of neighboring SMC or fibroblasts. This hypothesis is consistent with the progression of atherosclerosis, since activated macrophages under the EC could elevate the intracellular ROS of the neighboring EC.

It has been proposed that the formation of ROS may be a common denominator for the diversity of NF-κB-inducing signals. This integrative role of ROS is suggested because NF-κB can be induced by H_2O_2 and activation by a variety of stimuli is commonly inhibited by antioxidants (29–31). Another broad mediator of immediate-early gene expression is the transcription factor AP-1, which couples extracellular signals to gene-activating events, which are associated with growth, differentiation, and cellular stress (32, 33). AP-1 is composed of the jun and fos gene products which form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexs. Interestingly, several prooxidant conditions, such as H_2O_2 and UV irradiation, are capable of inducing AP-1 activation (34–36). The activation of AP-1 is regulated by complex mechanisms consisting of postranscriptional events acting on preexisting AP-1 molecules and transcriptional activation, ultimately leading to increased levels of AP-1 binding proteins. In addition, redox modification of a conserved cysteine residue in the DNA-binding domain of Fos and Jun may constitute another mechanism for controlling DNA binding protein of AP-1 (37, 38). Taken together our data and these findings, either NF-κB or AP-1 might be responsible for the transcriptional regulation of the HB-EGF gene. It was recently reported that the promoter of HB-EGF contains the AP-1 consensus sequence (39). Our data are supported by this recent finding. It also has been reported that H_2O_2 is involved in PDGF signaling in SMC (40). Collectively, these data suggest that oxidative stress may well be a more important factor in the progression of atherosclerosis than has previously been thought.

REFERENCES

1. Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C., and Klagsbrun, M. (1991) *Science* **251,** 936–939.
2. Higashiyama, S., Lau, K., Besner, G. E., Abraham, J. A., and Klagsbrun, M. (1992) *J. Biol. Chem.* **267,** 6205–6212.
3. Higashiyama, S., Abraham, J. A., and Klagsbrun, M. (1993) *J. Cell Biol.* **122,** 933–940.
4. Miyagawa, J., Higashiyama, S., Kawata S., Inui, Y., Tamura, S., Yamamoto, K., Nishida, M., Nakamura, T., Yamashita, S., Matsuzawa, Y., and Taniguchi, N. (1995) *J. Clin. Invest.* **95,** 401–411.
5. Marikovsky, M., Breuing, K., Liu, P. Y., Eriksson, E., Higashi-

yama, S., Farber, P., Abraham, J. A. and Klagsbrun, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3889–3893.

6. Deforge, L. E., Preston, A. M., Takeuchi, E., Kenney, J., Boxer, L. A., and Remick, D. G. (1993) *J. Biol. Chem.* **268,** 25568–25576.
7. Satriano, J. A., Schuldiner, M., Hora, K., Xing, Y., Shan, Z., and Schlondorff, D. (1993) *J. Clin. Invest.* **92,** 1564–1571.
8. Nakano, T., Raines, E. W., Abraham, J. A., Wenzel IV, F. G., Higashiyama, S., Klagsbrun, M., and Ross, R. (1993) *J. Biol. Chem.* **268,** 22941–22947.
9. Temizer, D. H., Yoshizumi, M., Perrella, M. A., Susanni, E. E., Quertermous, T., and Lee, M-E. (1992) *J. Biol. Chem.* **267,** 24892–24896.
10. Dluz, S., Higashiyama, S., Damm, D., Abraham, J. A., and Klagsbrun, M. (1993) *J. Biol. Chem.* **268,** 18330–18334.
11. Yoshizumi, M., Kourembanas, S., Temizer, D. H., Cambria, R. P., Quertermous, T., and Lee, M-E. (1992) *J. Biol. Chem.* **267,** 9467–9469.
12. Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyaert, R., Jacob, W. A., and Fiers, W. (1991) *J. Biol. Chem.* **267,** 5317–5323.
13. Meier, B., Radeke, H. H., Selle, S., Younes, M., Sies, Resch, K., and Habermehl, G. G. (1989) *Biochem. J.* **263,** 539–545.
14. Robertson, F. M., Beavis, A. J., Oberyszyn, T. M., O'Connell, S. M., Dokidos, A., Laskin, D. L., Laskin, J. D., and Reiners, Jr., J. J. (1990) *Cancer Res.* **50,** 6062–6067.
15. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) *J. Clin. Invest.* **52,** 2745–2756.
16. Gunther, S., Alexander, R. W., Atkinson, W. J., and Gimbrone, M. A. Jr. (1982) *J. Cell Biol.* **92,** 289–298.
17. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
18. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C., and Thomas, M. (1983) *J. Immunol.* **130,** 1910–1917.
20. Kass, G. E. N., Nicotera, P., and Orrenius, S. (1992) *Cell. Mol. Mech. Inflam.* **4,** 133–156.
21. Etoh, T., Takehara, K., Igarashi, A., and Ishibashi, Y. (1989) *Biochem. Biophys. Res. Commun.* **162,** 1010–1016.
22. Lyons, R. M., and Sham, J. D. (1980) *J. Clin. Invest.* **46,** 309–320.
23. Meister, A. (1992) *Chemtracts Biochem. Mol. Biol.* **3,** 75–106.
24. Freeman, B. A., and Crapo, J. D. (1982) *Lab. Invest.* **47,** 412–426.
25. Granger, D. N. (1988) *Am. J. Physiol.* **255,** H1269-H1275.
26. McCord, J. M. (1987) *Fed. Proc.* **46,** 2402–2406.
27. Collier, J., and Vallance, P. (1989) *Trends Pharmacol. Sci.* **10,** 427–431.
28. Schreck, R., and Baeuerle, P. A. (1991) *Trends Cell. Biol.* **1,** 39–42.
29. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 2247–2258.
30. Schreck, R., Meier, B., Mannel, D., Droge, W., and Baeuerle, P. (1992) *J. Exp. Med.* **175,** 1181–1194.
31. Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G., and Fiers, W. (1993) *EMBO J.* **12,** 3095–3104.
32. Angel, P, and Karin, M. (1991) *Biochim. Biophys. Acta* **1072,** 129–157.
33. Herrlich, P., Ponta, H., and Rahmsdorf, H. J. (1992) *Rev. Physiol. Biochem. Pharmacol.* **119,** 187–223.
34. Stein, B., Rahmsdorf, H. J., Steffen, A., LItfin, M., and Herrlich, P. (1989) *Mol. Cell. Biol.* **9,** 5169–5181.
35. Devary, Y., Gottlieb, R. A., Lau, L. F., and Karin, M. (1991) *Mol. Cell. Biol.* **11,** 2804–2811.
36. Nose, K., Shibanuma, M., Kikuchi, K., Kageyama, H., Sakiyama, S., and Kuroki, T. (1991) *Eur. J. Biochem.* **201,** 99–106.
37. Abate, C., Patel, L., Rauscher, F. J., III, and Curran, T. (1990) *Science* **249,** 1157–1161.
38. Xanthoudakis, S., Miao, G., Wang, F., Pan, Yu-C., E., and Curran, T. (1992) *EMBO J.* **11,** 3323–3335.
39. Fen, Z., Dhadly, M. S., Yoshizumi, M., Hilkert, R. J., Quertermous, T., Eddy, R. L., Shows, T. B., and Lee, M-E. (1993) *Biochemistry* **32,** 7932–7938.
40. Sundaresan, M., Yu, Z-X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science* **270,** 296–299.