Increasing cAMP antagonizes hypertrophic response to angiotensin II without affecting Ras and MAP kinase activation in vascular smooth muscle cells

Tomosaburo Takahashi, Yasuhiro Kawahara*, Masanori Okuda, Mitsuhiro Yokoyama

Department of Internal Medicine (1st Division), Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650, Japan

Received 10 September 1996

Abstract **Angiotensin II (Ang II), a potent hypertrophic factor for vascular smooth muscle cells (VSMC), induces activation of the *ras* proto-oncogene product (Ras) and mitogen-activated protein (MAP) kinases, and tyrosine phosphorylation of a focal adhesion-associated protein, paxillin. Forskolin, a direct activator of adenylate cyclase, and dibutyryl cAMP (Bt_2 cAMP), a membrane permeable cAMP analogue, potently inhibited Ang II-stimulated protein synthesis. However, they did not inhibit Ang II-induced activation of Ras and MAP kinases. Although both forskolin and Bt_2 cAMP potently reduced background tyrosine phosphorylation of paxillin, they allowed Ang II to induce the same reaction. These results indicate that increasing cAMP antagonizes the hypertrophic response to Ang II without affecting Ras and MAP kinase activation in VSMC and suggest that it does not interrupt signaling from the Ang II receptor to focal adhesions.**

Key words: Angiotensin II; Hypertrophy; cAMP; Ras; MAP kinase; Vascular smooth muscle cell

1. Introduction

Angiotensin II (Ang II) has been shown to induce cell hypertrophy as a result of increased protein synthesis rather than cell proliferation in cultured vascular smooth muscle cells (VSMC) [1,2]. Since this growth promoting action of Ang II is considered to contribute to the development of various vascular diseases, particularly, vascular remodeling observed in hypertension, it is important to clarify how the hypertrophic action of Ang II is regulated.

Ang II acts via a high affinity cell surface receptor called the AT1 receptor. Although this receptor is a seven transmembrane, heterotrimeric G protein-coupled receptor, many of the intracellular signals mediated by the AT1 receptor are similar to the signaling pathways activated by receptor tyrosine kinases such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors. For instance, Ang II induces activation of the *ras* proto-oncogene product (Ras) [3–5], tyrosine and threonine phosphorylation and activation of mitogen-activated protein (MAP) kinases [6], expressing early growth response genes such as c-*fos*, c-*jun* and c-*myc* [7–9] and tyrosine phosphorylation of a focal adhesion-associated protein, paxillin [10–12]. These intracellular events are considered to be involved in the growth promoting action of Ang II. Less is known, however, about negative regulatory mechanisms for the hypertrophic response to Ang II.

Elevation in intracellular cAMP potently inhibits growth factor-induced proliferation of VSMC [13–15]. Recently, Graves et al. [16] reported that increasing intracellular cAMP attenuates PDGF-induced MAP kinase activation in human VSMC. Since the MAP kinase cascade plays a critical role in growth factor-induced cell proliferation [17], the inhibition of MAP kinase activation by cAMP is considered to be one of the mechanisms of anti-proliferative action of this nucleotide. Moreover, cAMP-mediated inhibition of MAP kinase activation has been observed in various cell types stimulated by their respective agonists [17]. Therefore, in the present study, we addressed the question as to whether elevation in intracellular cAMP analogously attenuates Ang II-induced MAP kinase activation and then inhibits the hypertrophic response of VSMC. This paper reports that increasing cAMP potently inhibits Ang II-stimulated hypertrophy as assessed by protein synthesis but does not affect Ang II-induced activation of Ras and MAP kinases in rat VSMC. The effect of increasing cAMP on tyrosine phosphorylation of paxillin is also described.

2. Materials and methods

2.1. Materials

Ang II, forskolin, dibutyryl cAMP (Bt_2 cAMP) and myelin basic protein (MBP) were obtained from Sigma (St. Louis, MO, USA). The anti-Ras rat monoclonal antibody Y13-259, anti-MAP kinase mouse monoclonal antibody, anti-paxillin mouse monoclonal antibody, and anti-phosphotyrosine monoclonal antibody PY-20 were obtained from Oncogene Science (Cambridge, MA, USA), Zymed Laboratories (San Francisco, CA, USA), Transduction Laboratories (Lexington, KY, USA), and ICN (Costa Mesa, CA, USA), respectively. L-[4,5-^{3}H]Leucine (139 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol) and peroxidase-labeled sheep anti-mouse IgG were from Amersham Life Science (Tokyo, Japan). [^{32}P]Orthophosphate was from Du Pont (Boston, MA, USA). Protein A-Sepharose 4 Fast Flow was from Pharmacia (Uppsala, Sweden). Rabbit antiserum to rat IgG and affinity-purified rabbit anti-mouse IgG were from Cappel (Durham, NC, USA) and Jackson ImmunoResearch Laboratories (West Grove, PA, USA), respectively. Other materials and chemicals were obtained from commercial sources.

2.2. Cell culture

VSMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously [18]. Cells were grown and passaged as described previously [19] and used at passage levels 8–16.

2.3. Protein synthesis assay

Protein synthesis was measured by [^{3}H]leucine incorporation as described previously [2]. The quiescent VSMC in triplicate wells of 24-well plates were stimulated with Ang II in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 0.5 μCi/ml [^{3}H]leucine. After 24 h, the medium was aspirated. Cells were washed three times with ice-cold phosphate-buffered saline, and incubated with 5% trichloroacetic acid at 4°C for 30 min. Trichloroacetic acid precipitable materials were washed twice with 5% trichloroacetic acid and solubilized in 0.1 N NaOH at 37°C for 30 min. The radioactivity was measured by liquid scintillation spectrometry.

*Corresponding author. Fax: (81) (78) 341 1390.

PII S0014-5793(96)01145-3

2.4. Analysis of Ras-bound GDP and GTP

Detection and quantification of Ras-bound GDP and GTP were performed as described previously [3]. Briefly, the quiescent VSMC on 60-mm dishes were incubated with phosphate-free DMEM supplemented with 0.2 mCi/ml [^{32}P]orthophosphate for 12 h. 50 μM sodium orthovanadate was added to the cells during the last 30 min. After stimulation with Ang II, the cells were lysed. Ras was immunoprecipitated from the cell lysates with anti-Ras monoclonal antibody Y13-259 and analyzed by thin-layer chromatography.

2.5. MAP kinase assay in polyacrylamide gel containing MBP

MAP kinase activity was measured by a MAP kinase renaturation assay in MBP-containing polyacrylamide gels as described previously [3]. Briefly, the cell lysates from VSMC stimulated with Ang II were subjected to SDS-polyacrylamide gel electrophoresis (10% gel containing 0.5 mg/ml MBP). After washing the gel with 50 mM Tris-Cl, pH 8.0, and 20% 2-propanol, and then with 50 mM Tris-Cl, pH 8.0, and 5 mM 2-mercaptoethanol (buffer A) to remove SDS, the enzymes were denatured by treating the gel with 6 M guanidine HCl and then renatured with buffer A containing 0.04% Tween 40. The gel was incubated at 22°C for 1 h with 40 mM HEPES, pH 8.0, 2 mM 1,4-dithiothreitol, 0.1 mM EGTA, 5 mM $MgCl_2$, and 25 mM [γ-^{32}P]ATP (1×10^5 cpm/nmol) for kinase reactions. The gel was extensively washed with 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried, and the radioactivity was analyzed using a Fujix BAS2000 bio-imaging analyzer.

2.6. Immunoprecipitation with anti-paxillin antibody and immunoblot with anti-phosphotyrosine antibody

Detection and quantification of tyrosine phosphorylation of paxillin was performed as described previously [10] with slight modifications. Briefly, VSMC stimulated with Ang II were lysed in a buffer containing 50 mM HEPES, pH 7.6, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 100 KIU/ml aprotinin. After brief centrifugation, the supernatant was mixed with anti-paxillin antibody for 2 h at 4°C. Immunocomplexes were precipitated for 1 h at 4°C with Sepharose A-linked anti-mouse IgG. Paxillin immunoprecipitates were separated on SDS-polyacrylamide gel electrophoresis (10% gel). The separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes. Blots were incubated with the anti-phosphotyrosine antibody and then incubated with peroxidase-labeled donkey anti-mouse IgG. Peroxidase-labeled proteins were visualized by enhanced chemiluminescence (Amersham Life Science). Stained protein bands were scanned by NIH image software interfaced with a personal computer for quantification.

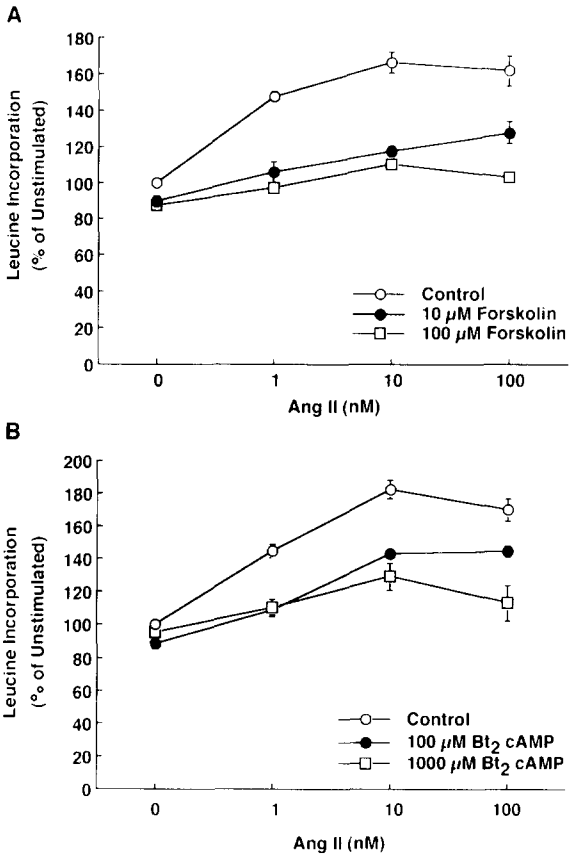

Fig. 1. Effect of increasing cAMP on Ang II-induced protein synthesis. Cultured VSMC were pretreated for 15 min with or without forskolin or Bt_2 cAMP and then stimulated with the indicated concentrations of Ang II for 24 h. Protein synthesis was measured by [^{3}H]leucine incorporation. Values shown are the mean ± standard error of the mean (S.E.M.) of three independent trials. (A) Cells were pretreated with vehicle (○), 10 μM (●) or 100 μM (□) forskolin. (B) Cells were pretreated with vehicle (○), 100 μM (●), 1000 μM (□) Bt_2 cAMP.

3. Results

In agreement with previous observations [2], Ang II stimulated protein synthesis in VSMC with a half-maximal effect observed at ~1 nM (Fig. 1A). No effect on DNA synthesis as measured by [^{3}H]thymidine incorporation was observed in these cells (data not shown). Pretreatment of cells with forskolin, a direct activator of adenylate cyclase, potently inhibited Ang II-induced protein synthesis (Fig. 1A). Bt_2 cAMP, a membrane permeable cAMP analogue, also inhibited Ang II-induced protein synthesis (Fig. 1B). These results indicate that elevation in intracellular cAMP antagonizes the hypertrophic response to Ang II in VSMC.

To gain insight into the mechanisms of this anti-hypertrophic action of cAMP, we next examined the effects of forskolin and Bt_2 cAMP on Ang II-induced activation of Ras and MAP kinases, both of which are considered to be involved in the growth-promoting action of Ang II. As has been previously described [3], Ang II induced Ras activation as demonstrated by an increase in the GTP-bound form of Ras (Fig. 2). Forskolin alone had no effect on Ras activation. In contrast to its inhibitory effect on protein synthesis, forskolin did not affect the Ang II-induced increase in the GTP-bound form of Ras. Ang II also stimulated the enzymatic activity of MAP kinases in a dose-dependent manner (Fig. 3). Also, forskolin did not influence the dose dependence of Ang II to stimulate MAP kinase activity. Pretreatment of the cells with Bt_2 cAMP affected neither Ang II-induced Ras activation nor Ang II-induced MAP kinase activation (data not shown).

We [10] and others [11,12] have previously shown that Ang II transduces its signals not only to the nucleus but also to focal adhesions and stimulates tyrosine phosphorylation of paxillin. In accord with previously published results [10], tyrosine phosphorylation of paxillin was significantly elevated in control unstimulated VSMC (Fig. 4). Ang II consistently increased tyrosine phosphorylation levels of this protein. Pretreatment of the cells with forskolin potently reduced background tyrosine phosphorylation of paxillin. Ang II, however, could stimulate its tyrosine phosphorylation even in the presence of forskolin. Pretreatment of the cells with Bt_2 cAMP also reduced background tyrosine phosphorylation of paxillin but allowed Ang II to stimulate the same reaction (data not shown).

4. Discussion

Since VSMC are capable of two distinct growth responses, hypertrophy and proliferation, it is important to clarify the regulatory mechanisms not only of proliferation but also of hypertrophy in order to understand the pathogenesis of various vascular diseases characterized by abnormal VSMC growth. It has long been appreciated that elevation of intracellular cAMP, a second messenger for VSMC relaxation, inhibits growth factor-induced proliferation of VSMC [13–15]. We [20] and others [21] have reported previously that elevation of intracellular cGMP, another second messenger for VSMC relaxation, also inhibits proliferation of VSMC. Itoh et al. [22] reported that increasing cGMP by atrial natriuretic peptide (ANP) potently inhibited Ang II-induced VSMC hypertrophy. However, it has not yet been reported whether increasing cAMP analogously inhibits Ang II-induced VSMC hypertrophy. In the present study, we first addressed this question and found that increasing cAMP potently inhibits Ang II-induced VSMC hypertrophy. Therefore, it is likely that both distinct growth responses of VSMC, hypertrophy and proliferation, may be negatively modulated not only by cGMP-elevating vasodilators such as nitric oxide and ANP but also by cAMP-elevating vasodilators such as prostacyclin and adrenomedullin.

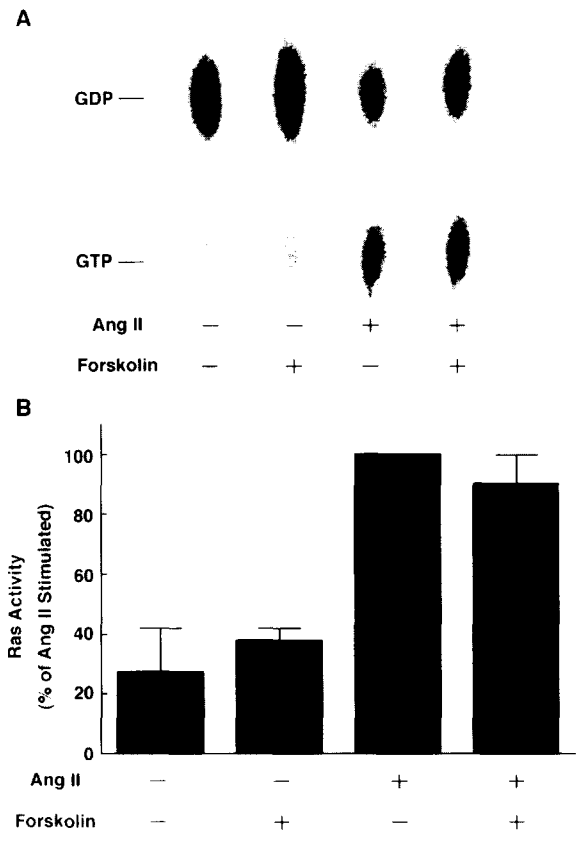

Fig. 2. Effect of forskolin on Ang II-induced Ras activation. VSMC labeled with [^{32}P]orthophosphate were pretreated for 15 min with or without 100 μM forskolin and then stimulated with the indicated concentrations of Ang II for 2 min. After lysis of the cells, Ras was immunoprecipitated and analyzed. (A) Positions of GDP and GTP are indicated. (B) Quantification of Ras activation (GTP/(GDP+GTP)) was performed using the Fujix BAS2000. Values shown are the mean ± S.E.M. of three independent trials and are expressed as the percentage of the response to 100 nM Ang II.

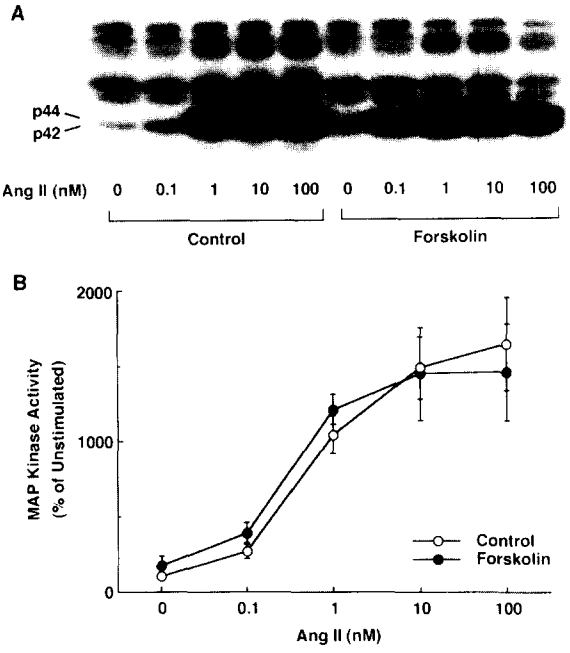

Fig. 3. Effect of forskolin on Ang II-induced MAP kinase activation. Cultured VSMC were pretreated for 15 min with or without 100 μM forskolin and then stimulated with the indicated concentrations of Ang II for 5 min. Cell extracts were subjected to the kinase assay in polyacrylamide gel containing MBP. (A) The enzyme activity was detected in both 42- and 44-kDa bands in the gel. (B) Cells were pretreated with vehicle (○) or 100 μM (●) of forskolin. The radioactivities of phosphorylated MBP at positions of MAP kinases were quantified using the Fujix BAS2000. Values shown are the mean ± S.E.M. of four independent trials and are expressed as the percentage of the unstimulated levels.

MAP kinases are a family of serine-threonine kinases that are rapidly activated in response to a variety of growth factors such as PDGF and EGF in various cell types and are believed to play a critical role in the control of cell growth [17]. Recent studies have shown that MAP kinase activation by growth factors involves sequential activation of Ras and the proto-oncogene serine kinase Raf. Activated Raf then phosphorylates MAP kinase kinase (MEK-1), which in turn phosphorylates and activates MAP kinase. Recently, we [3,6] and others [4,5] have shown that Ang II also induces activation of Ras and MAP kinases in VSMC. To determine whether the anti-hypertrophic action of cAMP might be mediated via inhibition of the MAP kinase pathway, we examined the effects of forskolin and Bt_2 cAMP on Ang II-induced activation of Ras and MAP kinases. Our observation that these cAMP-elevating agents had no effect on Ang II-induced activation of Ras and MAP kinases indicates that the inhibition of MAP kinases is not necessary for the anti-hypertrophic action of cAMP and suggest that increasing intracellular cAMP may inhibit the step(s) downstream of MAP kinases in the MAP kinase-mediated pathway or the step(s) in the parallel pathway(s) that is/are independent of MAP kinase activation.

Paxillin is a 68-kDa protein that binds to the cytoskeleton protein vinculin and localizes to focal adhesions. Although the precise role of tyrosine phosphorylation of paxillin is unknown at present, its localization suggests that tyrosine phosphorylation of paxillin has a potential role in the processes that regulate cell adhesion, motility and growth via the arrangement of the actin cytoskeleton [23]. In the last experi-

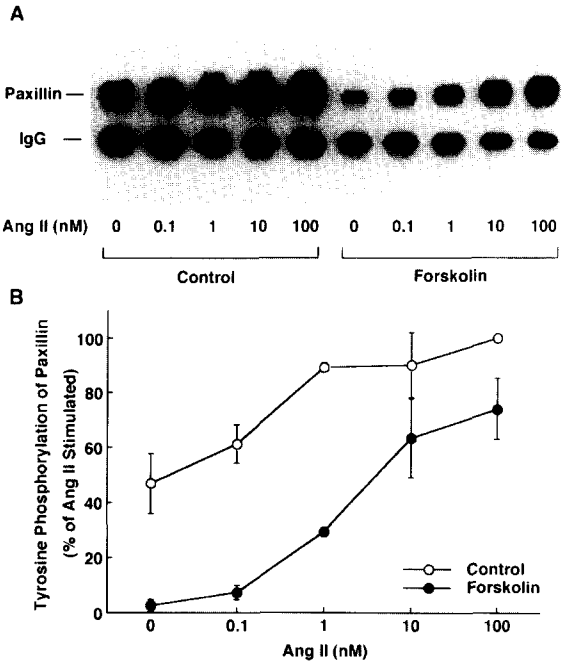

Fig. 4. Effect of forskolin on Ang II-induced tyrosine phosphorylation of paxillin. Cultured VSMC were pretreated for 15 min with or without 100 μM forskolin and then stimulated with the indicated concentrations of Ang II for 10 min. Cell extracts were subjected to immunoprecipitation analysis with anti-paxillin antibody. Immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibody. (A) Positions of paxillin and immunoglobulin G heavy chain (IgG) are indicated. (B) Cells were pretreated with vehicle (○) or 100 μM (●) of forskolin. Tyrosine phosphorylation of paxillin was quantified by scanning densitometry. Values shown are the mean ± S.E.M. of three independent trials and expressed as the percentage of the response to 100 nM Ang II.

ment of the present study, we showed that, although forskolin and Bt_2 cAMP potently inhibited the basal level of tyrosine phosphorylation of paxillin, they allowed Ang II-induced stimulation of the same reaction, suggesting that cAMP may not interrupt signaling from the AT1 receptor to focal adhesions. Since background tyrosine phosphorylation of paxillin may be maintained, at least in part, by integrin-mediated stimulation by the extracellular matrix, it is possible that increasing cAMP negatively modulates the hypertrophic response of VSMC by interrupting signaling from the extracellular matrix via integrins. Further studies are needed to clarify the mechanisms of the anti-hypertrophic action of cAMP.

Acknowledgements: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (1995).

References

[1] Geisterfer, A.A.T., Peach, M.J. and Owens, G.K. (1988) Circ. Res. 62, 749–756.
[2] Berk, B.C., Vekshtein, V., Gordon, H.M. and Tsuda, T. (1989) Hypertension 13, 305–314.
[3] Okuda, M., Kawahara, Y. and Yokoyama, M. (1996) Am. J. Physiol. (in press).
[4] Schieffer, B., Paxton, W.G., Chai, Q., Marrero, M.B. and Bernstein, K.E. (1996) J. Biol. Chem. 271, 10329–10333.
[5] Eguchi, S., Matsumoto, T., Motley, E.D., Utsunomiya, H. and Inagami, T. (1996) J. Biol. Chem. 271, 14169–14175.
[6] Tsuda, T., Kawahara, Y., Ishida, Y., Koide, M., Shii, K. and Yokoyama, M. (1992) Circ. Res. 71, 620–630.
[7] Kawahara, Y., Sunako, M., Tsuda, T., Fukuzaki, H., Fukumoto, Y. and Takai, Y. (1988) Biochem. Biophys. Res. Commun. 150, 52–59.
[8] Naftilan, A.J., Gilliland, G.K., Eldridge, C.S. and Kraft, A.S. (1990) Mol. Cell. Biol. 10, 5536–5540.
[9] Naftilan, A.J., Pratt, R.E. and Dzau, V.J. (1989) J. Clin. Invest. 83, 1419–1424.
[10] Okuda, M., Kawahara, Y., Nakayama, I., Hoshijima, M. and Yokoyama, M. (1995) FEBS Lett. 368, 343–347.
[11] Leduc, I. and Meloche, S. (1995) J. Biol. Chem. 270, 9, 4401–4404.
[12] Turner, C.E., Pietras, K.M., Taylor, D.S. and Molloy, C.J. (1995) J. Cell Sci. 108, 333–342.
[13] Nilsson, J. and Olsson, A.G. (1984) Atherosclerosis 53, 77–82.
[14] Loesberg, C., Van Wijk, R., Zandbergen, J., Van Aken, W.G., Van Mourik, J.A. and De Groot, P.G. (1985) Exp. Cell Res. 160, 117–125.
[15] Jonzon, B., Nilsson, J. and Fredholm, B.B. (1985) J. Cell. Physiol. 124, 451–456.
[16] Graves, L.M., Bornfeldt, K.E., Raines, E.W., Potts, B.C., Macdonald, S.G., Ross, R. and Krebs, E.G. (1993) Proc. Natl. Acad. Sci. USA 90, 10300–10304.
[17] Graves, J.D., Campbell, J.S. and Krebs, E.G. (1995) Ann. N.Y. Acad. Sci. 766, 320–343.
[18] Gunther, S., Alexander, R.W., Atkinson, W.J. and Gimbrone, M.A., Jr. (1982) J. Cell Biol. 92, 289–298.
[19] Nakayama, I., Kawahara, Y., Tsuda, T., Okuda, M. and Yokoyama, M. (1994) J. Biol. Chem. 269, 15, 11628–11633.
[20] Kariya, K., Kawahara, Y., Araki, S., Fukuzaki, H. and Takai, Y. (1989) Atherosclerosis 80, 143–147.
[21] Garg, U.C. and Hassid, A. (1989) J. Clin. Invest. 83, 1774–1777.
[22] Itoh, H., Pratt, R.E. and Dzau, V.J. (1990) J. Clin. Invest. 86, 1690–1697.
[23] Clark, E.A. and Brugge, J.S. (1995) Science 268, 233–239.