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Negative Regulation of Insulin-induced Tetrahydrobiopterin Synthesis by Protein Kinase C in Vascular Endothelial Cells

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Abstract

We examined the role of protein kinase C (PKC) in the insulin-induced biosynthesis of tetrahydrobiopterin (BH_4), which is one of the cofactors of nitric oxide (NO) synthase (NOS), in vascular endothelial cells. The BH_4 level was determined as biopterin by reversed-phase high performance liquid chromatography with fluorometric detection. Measurement of the level of mRNA for GTP cyclohydrolase I (GTPCH), which is the first and the rate-limiting enzyme for the novo pathway for BH_4 synthesis, was performed by reverse transcription-polymerase chain reaction (RT-PCR). Treatment with insulin increased the BH_4 level and the GTPCH mRNA level in endothelial cells. The insulin-induced increases of both GTPCH mRNA and BH_4 levels were enhanced by co-treatment with bisindolylmaleimide I or Ro 31-8220, inhibitors of PKC. On the other hand, the insulin-induced increases of BH_4 synthesis and expression of GTPCH mRNA were also enhanced by co-incubation with a PKC activator, phorbol 12-myristate 13-acetate (PMA) for 24 h. Long-term treatment with PMA is known to down-regulate PKC. Addition of insulin after pretreatment with PMA for 24 h markedly enhanced BH_4 synthesis and expression of GTPCH mRNA. These findings suggest that PKC negatively regulates the induction of BH_4 synthesis by insulin endothelial cells. Insulin-induced BH_4 synthesis may be suppressed in diabetes mellitus, since PKC has been shown to be up-regulated in diabetes mellitus.

Key words: insulin, tetrahydrobiopterin, protein kinase C, GTP cyclohydrolase I, endothelial cells

Introduction

Insulin is produced in and secreted from the pancreas, and regulates carbohydrate, protein and fat metabolism in mammals; it is used for the treatment of diabetes mellitus to decrease the blood sugar level. In addition, insulin is known to stimulate nitric oxide (NO) production in endothelial cells, and to elicit vasodilation (1-3). Zeng and Quon (4) showed that insulin induces NO production by endothelial NO synthase (eNOS) within a few minutes. We recently showed that insulin increases the synthesis of tetrahydrobiopterin (BH_4), one of the cofactors of NOS, through the induction of GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme of BH_4 synthesis, in endothelial cells (5,6). Shinozaki et al. (7) also suggested that insulin increases not only eNOS expression and NO production but also synthesis of BH_4 and expression of GTPCH in rat aorta. Therefore, the increase in BH_4 levels is likely to be involved in insulin-induced NO production in endothelial cells.

Endothelium-dependent relaxation of the aorta in response to acetylcholine is known to be decreased in rats with streptozotocin-induced diabetes (8,9). Pieper (9) reported that BH_4 availability can play a key role in the regulation of NO production in diabetic endothelial cells, and exogenous BH_4 restores endothelium-dependent relaxation in diabetic rat aorta. Moreover, Hamon et al. (10) suggested that the BH_4 content in diabetic rat brain is decreased. Recently, Shinozaki et al. (7) also reported that the BH_4 content of diabetic rat aorta is decreased, and the decrease in BH_4 content may cause eNOS dysfunction, which results in an

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increase of reactive oxygen production and a decrease of NO production. Thus, the tissue BH_4 levels in diabetic conditions are likely to be decreased, but the mechanism of the effects on the BH_4 level have not been elucidated.

Kuboki et al. (11) reported that insulin can regulate the expression of eNOS by the activation of phosphatidylinositol 3-kinase (PI3-kinase). Moreover, they showed that activation of protein kinase C (PKC) negatively regulates the insulin action. Therefore, they speculated that the effect of insulin on eNOS expression was blunted in the vasculature, under the conditions of hyperglycemia and insulin resistance, which are known to activate PKC and to inhibit PI3-kinase activities (12,13). In fact, there are many reports showing that the activation of PKC negatively regulates the PI3-kinase activity (11-14). Recently, we reported that the insulin-induced increases of GTPCH expression and BH_4 synthesis are mediated by the activation of PI3-kinase (6), but we did not determine the role of PKC in insulin-induced GTPCH expression and BH_4 synthesis in endothelial cells.

In the present study, we examined the role of PKC in BH_4 synthesis during insulin treatment in endothelial cells.

Materials and Methods

Cell culture

Mouse brain microvascular endothelial cells (MBMECs, second passage) were purchased from Toyobo Co. (Tokyo, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were used for the experiments at 5 to 10 passages after they were purchased.

Measurement of biopterin

Confluent cells in 6-well plates were washed twice with physiological saline solution (PSS, pH 7.4) containing 118.5 mM NaCl, 4.74 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 2.5 mM $NaHCO_3$, 11 mM glucose and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and incubated with insulin at 37°C in 2 ml of PSS. After the treatment with insulin, the supernatant and the remaining cells were separately treated with 10% trichloroacetic acid. Each sample was oxidized with MnO_2, and then subjected to reversed-phase high performance liquid chromatography (5,6). Results were expressed as total biopterin (intracellular plus extracellular biopterin), because all the drugs used showed similar patterns of effects on the intracellular and extracellular BH_4 levels.

Measurement of GTPCH mRNA Levels by Reverse Transcriptase (RT)-PCR

Confluent cells in 10-cm culture dishes were treated with insulin for 8 h, and then total RNA was extracted by a modified guanidinum isothiocyanate method using ISOGEN Reagent (Nippon Gene Co., Ltd., Tokyo, Japan). Reverse transcription and PCR amplification from 0.1 mg of total RNA were performed using r*Tth* DNA polymerase (RT-PCR high Plus, Toyobo Co., Tokyo, Japan). The pair of primers used for amplification of GTPCH sequences was 5'-GGATACCAGGAGACCATCTCA-3' and 5'-TAGCATGGTGCTAGTGACAGT-3'. The thermocycler was programmed to give an initial cycle consisting of reverse transcription at 60°C for 30 min, and denaturation at 94°C for 2 min, followed by 28 cycles of denaturation at 94oC for 1 min and annealing/extension at 58°C for 1.5 min. To control for the amounts of total RNA, parallel RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed as a reference, using the pair of primers 5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3'. PCR products were electrophoresed on a 3% NuSieve® 3:1 agarose (FMC Co., Rockland, ME, USA) gel containing ethidium bromide and visualized by UV-induced fluorescence (6).

Materials

Insulin (from bovine pancreas) and bisindolylmaleimide I were from Sigma Chemical Co. (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) was from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Ro 31-8220 was obtained from Calbiochem Novabiochem (San Diego, CA, USA). All other reagents were of the highest grade commercially available.

Statistical analysis

Data are presented as means ± S.E.M. of n observations. The statistical significance of observed differences was determined by analysis of variance followed by Bonferroni's method. Differences between means were considered significant when P was less than 0.05.

Results

We previously reported that insulin stimulates BH_4 synthesis through the induction of GTPCH in mouse brain microvascular endothelial cells (5,6). In the present study, to determine the role of PKC in the induction of BH_4 synthesis by insulin, we first examined the effects of bisindolylmaleimide I and Ro 31-8220,

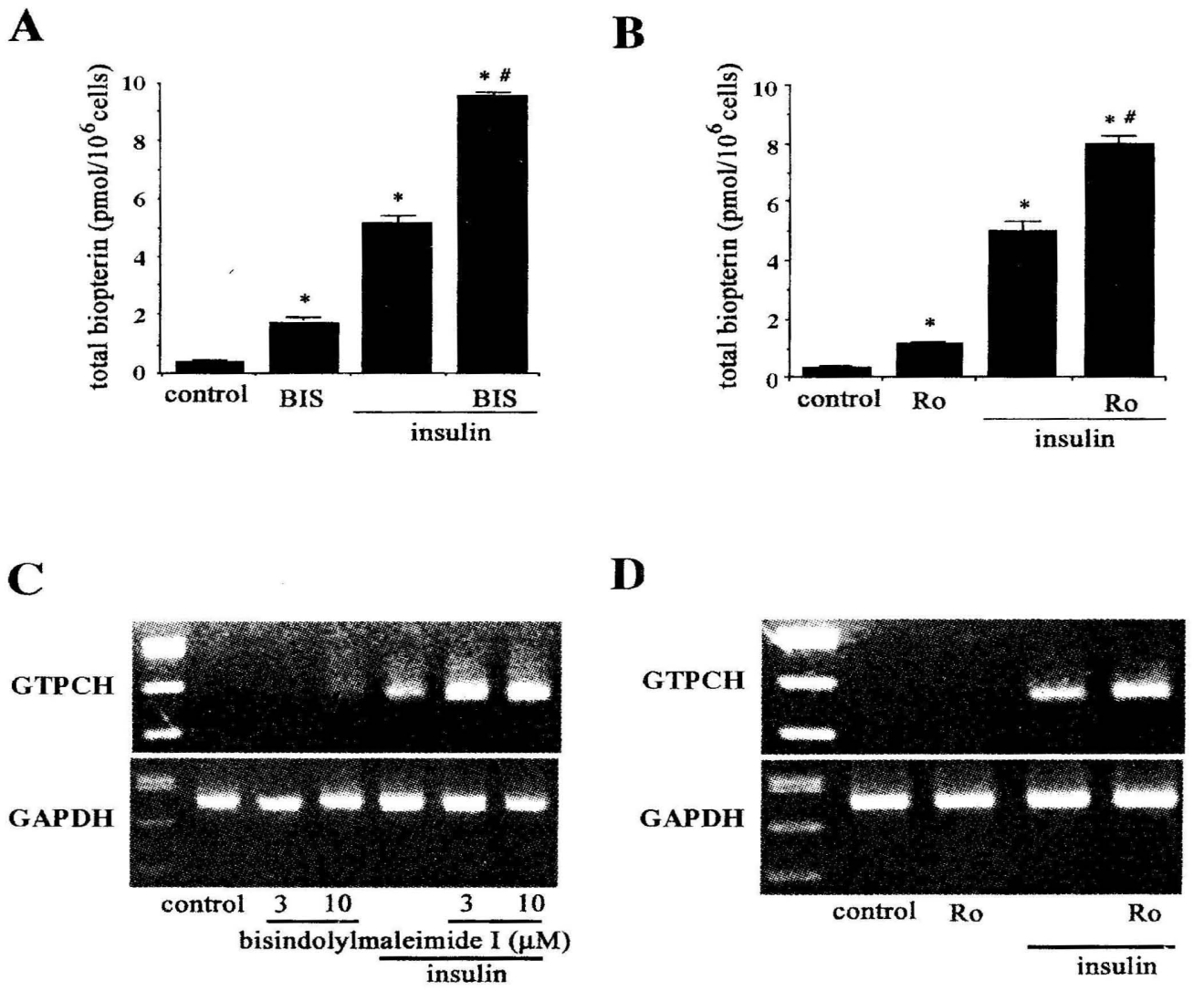

Figure 1. Effects of bisindolylmaleimide I and Ro 31-8220 on insulin-induced BH4 synthesis and expression of GTPCH mRNA in endothelial cells. A,B: Cells were pretreated with bisindolylmaleimide I (10 μM; A) or Ro 31-8220 (2 μM; B) for 30 min, and then incubated with insulin (10 μg/ml) for 24 h. Results are the means ± S.E.M. of 8 assays. *Significantly different from control ($p<0.05$). #Significantly different from insulin-treated group ($p<0.05$). C,D: Cells were pretreated with bisindolylmaleimide I (3 or 10 μM; C) or Ro 31-8220 (2 μM; D) for 30 min, and then incubated with insulin (10 μg/ml) for 8 h. Total RNA was prepared from the cells, and was amplified by RT-PCR using primers for GTPCH or GAPDH. DNA size markers in the left-most lane correspond to (bp): 525, 500, 400 and 300.

which are both inhibitors of PKC, on the insulin (10 μg/ml)-induced increase in BH_4 level and expression of GTPCH mRNA in endothelial cells. Significantly enhanced increases of BH_4 levels during insulin treatment were observed in endothelial cells treated with both PKC inhibitors (Fig. 1A & 1B). Both PKC inhibitors also increased the basal BH_4 levels in endothelial cells not treated with insulin (Fig. 1A & 1B). Moreover, the expression of GTPCH mRNA in insulin-treated endothelial cells was further stimulated by the treatment with both PKC inhibitors (Fig. 1C & 1D).

We next studied the effect of PMA, a PKC activator, on insulin-induced BH_4 synthesis. The insulin-induced increases in BH_4 levels and expression of GTPCH mRNA were strongly enhanced by the co-treatment

with PMA for 24 h (Fig. 2). PMA also increased the basal BH_4 levels and expression of GTPCH mRNA in endothelial cells (Fig. 2). When the cells were pre-

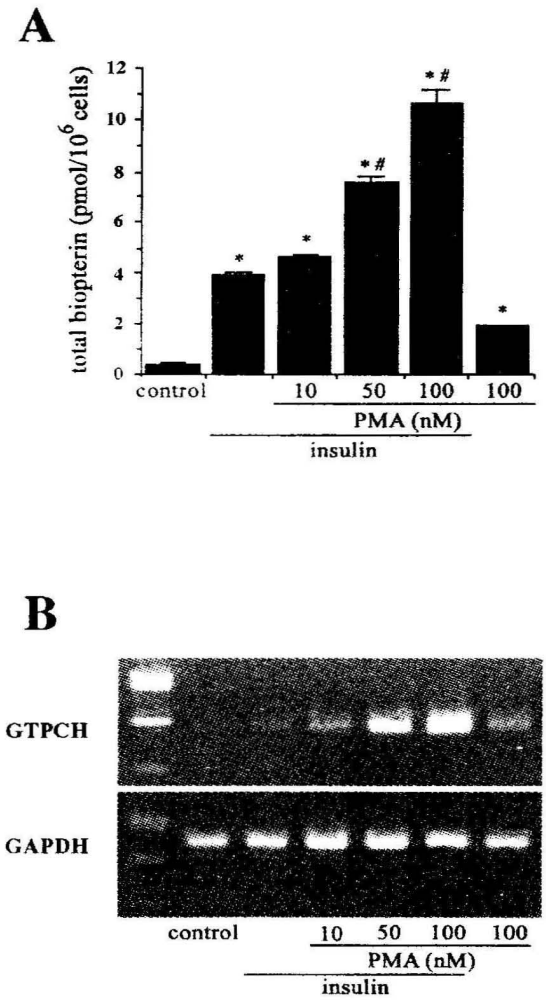

Figure. 2. Effects of PMA on insulin-induced BH4 synthesis (A) and expression of GTPCH mRNA (B) in endothelial cells. A: Cells were incubated with insulin (10 μg/ml) in the presence or absence of PMA (10-100 nM) for 24 h. Results are the means ± S.E.M. of 8 assays. *Significantly different from control ($p<0.05$). #Significantly different from insulin-treated group ($p<0.05$). B: Cells were incubated with insulin (10 μg/ml) in the presence or absence of PMA (10-100 nM) for 8 h. Total RNA was prepared from the cells, and was amplified by RT-PCR using primers for GTPCH or GAPDH. DNA size markers in the left-most lane correspond to (bp): 525, 500, 400 and 300.

treated with PMA for 24 h, and then treated with insulin or PMA for 24 h, BH_4 levels were significantly increased compared to those of an insulin-treated group or PMA-treated group, respectively, without PMA pretreatment (Fig. 3).

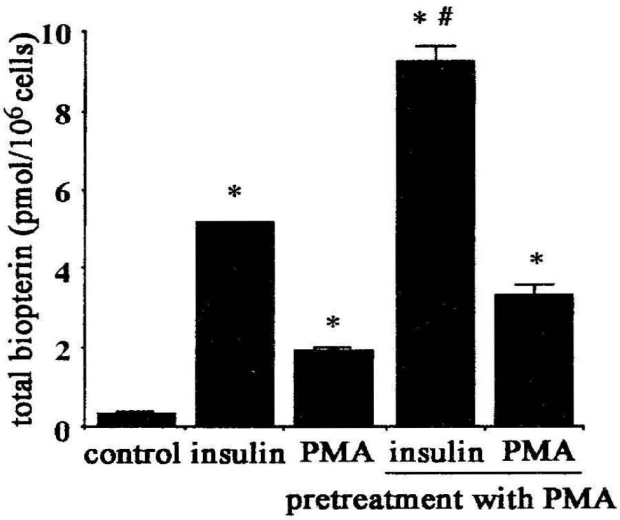

Figure. 3. Effects of pretreatment with PMA for 24 h on insulin-induced BH4 synthesis in endothelial cells. Cells were pretreated with PMA (100 nM) for 24 h, and then incubated with insulin (10 μg/ml) in the absence or presence of PMA (100 nM) for 24 h. Results are the means ± S.E.M. of 4 assays. *Significantly different from control ($p<0.05$). #Significantly different from insulin-treated group ($p<0.05$).

Discussion

We previously reported that insulin stimulates the BH_4 synthesis in brain microvascular endothelial cells through the de novo pathway via induction of GTPCH (6). In the present study, we observed that the inhibition of PKC by PKC inhibitors further increased the BH_4 level and the expression of GTPCH mRNA induced by insulin treatment. On the other hand, co-treatment with PMA (a PKC activator) for 24 h also strongly stimulated the insulin-induced BH_4 synthesis. Long-term treatment with PMA is known to down-regulate PKC activity in various types of cells (11,15,16). In fact, long-term treatment with PMA markedly stimulated the insulin-induced BH_4 synthesis. These findings strongly suggested that insulin-

induced BH_4 synthesis is negatively regulated by the activation of PKC.

In diabetes mellitus, it is known that PKC is activated (12,13), and the activation of PKC reduces eNOS activity (11,18,19). High glucose also suppresses NO production and eNOS expression in endothelial cells (20). Recently, Kuboki et al. (11) reported that insulin could stimulate the expression of eNOS via the activation of PI3-kinase. Moreover, the activation of PKC down-regulated these insulin actions. Therefore, those authors speculated that the effect of insulin on eNOS expression was blunted in the vasculature under the conditions of hyperglycemia and insulin resistance, which are known to activate PKC activity and to inhibit PI3-kinase activity (12,13). In the present study, we found that the inhibition of PKC stimulates BH_4 synthesis and enhances the induction of GTPCH during insulin treatment. In diabetes mellitus, the activation of PKC may reduce not only insulin-induced eNOS expression but also BH_4 synthesis and GTPCH expression in endothelial cells. Reduction of the BH_4 content of the brain and aorta has been observed in diabetic rats (7,10). The activation of PKC may be one of the causes of the low levels of BH_4 in the brain and blood vessels in diabetes mellitus.

Kuboki et al. (11) showed that both a general PKC inhibitor, GF109203X, and a specific PKCß isoform inhibitor, LY333531, increased the basal eNOS level. Moreover, overexpression of the PKCß isoform in endothelial cells inhibited the insulin-induced eNOS expression in endothelial cells, suggesting that PKC, especially PKCß, activity may regulate the eNOS mRNA level in endothelial cells. It is possible that the activation of PKCß affects not only the insulin-induced eNOS expression and NO production, but also GTPCH expression and BH_4 synthesis, in diabetic endothelial cells.

We previously showed that insulin stimulates BH_4 synthesis via induction of GTPCH expression in MBMECs, and that the signaling pathway involves the activation of PI3-kinase (6). Many other investigators have reported that the activation of PKC reduces PI3-kinase activity (13,14). In diabetes mellitus, PI3-kinase is negatively regulated by PKC (12,13). Although we did not determine whether the activation of PKC reduces PI3-kinase activity in the present study, it is possible that induction of BH_4 synthesis by insulin may be inhibited by the reduction of PI3-kinase activity through the activation of PKC.

In summary, PKC negatively regulates the induction of BH_4 synthesis by insulin in endothelial cells. The activation of PKC in diabetes mellitus may suppress the induction of BH_4 synthesis by insulin through the reduction of GTPCH expression, since PKC is known to be activated in diabetes mellitus. The decrease in the BH_4 level in various tissues in diabetes mellitus may be caused by the activation of PKC.

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