Insulin Stimulates Tetrahydrobiopterin Synthesis in Mouse Brain Microvascular Endothelial Cells

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Summary

We examined the effects of insulin on tetrahydrobiopterin (BH4) synthesis in mouse brain microvascular endothelial cells (MBMECs). Treatment of MBMECs with insulin increased the intracellular BH4 content in a time- and concentration-dependent manner. The insulin-induced increase in BH4 content was inhibited by treatment with 2,4-diamino-6-hydroxypyrimidine, a selective inhibitor of GTP cyclohydrolase I, and N-acetylserotonin, a selective inhibitor of sepiapterin reductase. These findings indicate that insulin stimulates BH4 synthesis in MBMECs through a *de novo* synthetic pathway of BH4.

Key words: Tetrahydrobiopterin, Insulin, Endothelial cell

Introduction

Insulin is produced and secreted from the pancreas, and regulates carbohydrate, protein and fat metabolism in mammals. Moreover, insulin has been shown to stimulate the production of nitric oxide (NO) in endothelial cells, and to elicit vasodilation (1-3). Verma *et al.* (4) indicated that insulin-induced vasodilation is attenuated by inhibition of BH4 synthesis. BH4 is known to be an essential cofactor of NO synthase, and regulates NO synthase function (5,6). Therefore, they speculated that insulin may regulate NO production by increasing BH4 synthesis in endothelial cells is still not determined. As another

function, insulin is known to have a protective effect against ischemic injury in various tissues including brain and heart (7,8). We recently reported that BH4 has a protective effect against vascular endothelial cell injury induced by H₂O₂, NO and a combination of superoxide and NO, which are implicated in a development of reperfusion injury following ischemia (9-14). More recently, *in vivo*, we showed that BH4 attenuates gastric ischemia-reperfusion injury (15). The protection mechanisms of BH4 are likely to involve the scavenging activity of reactive oxygen species and antioxidative activity (10,16). If insulin stimulates BH4 synthesis in endothelial cells, it is possible that the increase in BH4 content may be involved in the protection mechanisms of insulin against ischemic injury. In this study, we examined whether insulin increases in BH4 synthesis in MBMECs.

Materials and Methods

Cell culture

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MBMECs were obtained from Toyobo Co. (Tokyo, Japan). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Measurement of intracellular biopterin content

Confluent endothelial cells in 6-well plates were washed 2 times with physiological saline solution (PSS, pH 7.4) containing 118.5 mM NaCl, 4.74 mM KCl, 2.5 mM CaCl₂, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 2.5 mM NaHCO₃, 11 mM glucose and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and incubated with insulin at 37°C in 2 ml PSS. After treatment with insulin for various periods (0-24 h), intracellular BH4 was released by addition of 10% trichloroacetic acid on ice for 30 min. BH4 content was measured by oxidation in acid conditions as biopterin, with reversed-phase high performance liquid chromatography with fluorometric detection, as previously described (9).

Materials

Insulin (from bovine pancreas) and N-acetylserotonin (NAS) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2,4-Diamino-6-hydroxypyrimidine (DAHP) was obtained from Aldrich Chemical Co. (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Statistical analysis

Data are presented as means ± S.E.M. of n obser-

vations. 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

Induction of BH4 synthesis by insulin

We examined whether insulin (10 µg/ml) induces BH4 synthesis in MBMECs. Significant increase in BH4 content was observed in MBMECs from 8 h after the addition of insulin (Fig. 1A). The insulin-induced increase in of BH4 content was observed in a concentration-dependent manner (Fig. 1B).

Effects of DAHP and NAS on the synthesis of BH4

To determine whether insulin-induced increase in BH4 content is due to the de novo synthesis, the effects of DAHP and NAS, both inhibitors of the de novo synthetic pathway of BH4, were examined. Treatment with DAHP (Fig. 2A), a GTP cyclohydrolase I (GTPCH) inhibitor, or with NAS (Fig. 2B), a sepiapterin reductase inhibitor, inhibited BH4 synthesis induced by insulin in a concentration-dependent manner.

Discussion

Addition of insulin to endothelial cells increased intracellular BH4 content. The biosynthesis of BH4 can occur via a de novo pathway from GTP to dihy-

Figure 1. Effect of insulin on intracellular BH4 content in endothelial cells. A; Cells were incubated with insulin (10 µg/ml) for various periods. B; Cells were incubated with insulin (0.01-100 µg/ml) for 24 h. Results are the means ± S.E.M. of 10-22 assays. *Significant change in comparison to control group ($p < 0.05$).

Figure 2. Effects of DAHP and NAS on increase in BH4 content induced by insulin. Cells were incubated with insulin (10 μg/ml) in the absence or presence of DAHP (10-1000 μM: A) or NAS (10-1000 μM: B) for 24 h. Results are the means ± S.E.M. of 8-14 assays. *Significant change in comparison to insulin alone group ($p < 0.05$).

droneopterin triphosphate, in which the enzyme GTPCH catalyzes the first and rate-limiting step. DAHP, a selective inhibitor of GTPCH, inhibited the formation of BH4 induced by insulin in endothelial cells. Moreover, NAS, an inhibitor of sepiapterin reductase, which converts 6-pyruvoyl tetrahydropterin to BH4, the last step of the *de novo* pathway of BH4 synthesis, also inhibited the insulin-induced increase in BH4 content. Therefore, insulin may induce the biosynthesis of BH4 in endothelial cells through the *de novo* pathway. The mechanisms of BH4 synthesis induced by insulin in MBMECs are needed to examine in further detail in the future.

Insulin has been shown to elicit vasodilation by NO-dependent mechanism in various vessels (1-3). Verma *et al.* (4) indicated that inhibition of BH4 synthesis results in an attenuation in the insulin-induced vasodilation of rat femoral artery. Therefore, they speculated that insulin may regulate NO production by increasing BH4 availability for activation of NO synthase. In the present study, we showed that insulin stimulates BH4 synthesis and increases BH4 content in endothelial cells. Moreover, we previously reported that the increase in BH4 content in endothelial cells stimulates calcium ionophore-induced L-citrulline formation, which is a marker for NO production (10). The increase in BH4 content may be involved in insulin-induced NO production in endothelial cells.

Endothelium-dependent relaxation of aorta by acetylcholine has been known to be decreased in streptozotocin-induced diabetic rats (17-19). Pieper (19) reported that BH4 availability can play a key role of the regulation of NO production in diabetic

endothelial cells, and exogenous BH4 restores endothelium-dependent relaxation in diabetic rat aorta. Moreover, Hamon *et al.* (20) suggested that BH4 content in diabetic rat brain is decreased. Under diabetic mellitus, BH4 content of vessels is likely to be decreased. Therefore, it is possible that insulin may improve endothelium-dependent relaxation by increasing BH4 content in diabetic rats.

For several years, we reported that BH4 has a protective effect against vascular endothelial cell injury induced by H₂O₂, NO and a combination of superoxide and NO, which are implicated in a development of reperfusion injury following ischemia (9-14). More recently, *in vivo* study, we showed that BH4 attenuates gastric ischemia-reperfusion injury (15). The protection mechanisms of BH4 are likely to involve a scavenging activity for reactive oxygen species and antioxidative activity (10,16). Insulin is known to have a protective effect for ischemic injury in various tissues including brain and heart (7,8). It is possible that the increase in BH4 content induced by insulin may be involved in insulin-induced cell protection. In the future, we will examine whether cell protection mechanisms of insulin against ischemic injury involve increase in BH4 content.

Recently, we suggested that BH4 stimulates the proliferation and angiogenesis in bovine endothelial cells (21). Other investigators also showed that BH4 stimulates cell proliferation in various cells including PC12, SV40-transfected human fibroblasts and rat C6 glioma cells (22). Furthermore, growth factors including epidermal growth factor and nerve growth factor are known to increase in synthesis of BH4 (23,24). Insulin is known to stimulate the prolifer-

ation and angiogenesis in endothelial cells (25). Therefore, it is possible that the increase in BH4 content in MBMECs may be involved in insulin-induced proliferation of MBMECs.

In conclusion, our findings indicate that insulin induces the increase in BH4 content through a de novo pathway from GTP in MBMECs. The physiological role of increase in BH4 content induced by insulin will be needed to examine in detail.

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