Short Communication

Reduction of GTP Cyclohydrolase I Feedback Regulating Protein Expression by Hydrogen Peroxide in Vascular Endothelial Cells

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Abstract. We examined the effect of H₂O₂ on the expression of GTP cyclohydrolase I (GTPCH) feedback regulating protein (GFRP). Addition of H₂O₂ to endothelial cells decreased GFRP mRNA levels, in contrast to the increase of tetrahydrobiopterin (BH₄) content and GTPCH mRNA levels. The inhibitors of nitric oxide (NO) synthase and GTPCH had no influence on the decrease of GFRP mRNA levels in H₂O₂-treated cells. It is suggested that H₂O₂ induces BH₄ synthesis through not only induction of GTPCH but also reduction of GFRP. The decrease of GFRP mRNA level appears to be independent of the produced NO and BH₄.

Keywords: GTP cyclohydrolase I, GTP cyclohydrolase I feedback regulating protein, hydrogen peroxide

Tetrahydrobiopterin (BH₄) is an essential cofactor for nitric oxide (NO) synthase (NOS) (1, 2), and also has a protective effect against toxicities of reactive oxygen species (ROS) (1, 2). It is generally accepted that BH₄ is an important molecular target for oxidative stress leading to vascular injury in various diseases, including hypercholesterolemia, diabetes mellitus, and hypertension, since BH₄ is easily oxidized by ROS (1, 2). The biosynthesis of BH₄ can occur via a de novo pathway from guanosine triphosphate (GTP), in which the enzyme GTP cyclohydrolase I (GTPCH) catalyzes the rate-limiting step (1, 2). Although the regulation mechanisms underlying the activation and expression of GTPCH in vascular endothelial cells are yet to be elucidated, the enzyme activity has been shown to be reduced by BH₄ through the formation of a ternary complex among BH₄, GTPCH, and GTPCH feedback regulatory protein (GFRP) by using the purified enzymes (3).

Interestingly, we recently reported that H₂O₂, one of the ROS, stimulates BH₄ synthesis through the induction of GTPCH in vascular endothelial cells (4). However, the mechanism of H₂O₂-induced BH₄ synthesis is not

clear. To understand the underlying mechanisms of vascular injury by oxidative stress, it is important to determine the stimulation mechanisms of BH₄ synthesis by H₂O₂. Proinflammatory cytokines and lipopolysaccharide (LPS), which are known to stimulate BH₄ synthesis with GTPCH induction, decrease the expression of GFRP (5, 6).

In the present study, the effect of H₂O₂ on the expression of GFRP mRNA was examined, and the changes were compared with that of GTPCH mRNA, in vascular endothelial cells. We found that H₂O₂ decreases GFRP mRNA levels, in contrast to the increases of GTPCH mRNA levels. The reduction of GFRP expression is likely to be implicated in the increases of BH₄ content by H₂O₂.

Mouse brain microvascular endothelial cells (MBMECs, 2nd passage) were purchased from Toyobo Co. (Osaka). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were used at 5 to 20 passages after purchase. Confluent MBMECs in 6-well plates or 100-mm culture dishes were washed twice with physiological saline solution (PSS, pH 7.4) containing 118.5 mM NaCl, 4.74 mM KCl, 2.5 mM CaCl₂,

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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 then they were treated with H_2O_2 at 37°C in 2 ml (6-well plates) or 10 ml (100-mm dishes) of PSS.

The total biopterin (BH_4 plus 7,8-dihydrobiopterin (BH_2) plus biopterin) content in MBMECs was measured by oxidizing in 0.02 M KI/I_2 in 0.1 M HCl , as previously described (4).

The preparation of cDNA probes for GTPCH and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) and Northern blot analysis for GTPCH and GAPDH mRNAs were performed as previously described (7). GFRP cDNA probe was prepared by RT-PCR, using pairs of primers, 5'-GGAGTCCTTGGACCGGAGAAG AAC-3' and 5'-CCGAGAGTTTGGAACTTTCCACG-3', and GFRP mRNA levels were measured by Northern blot analysis.

Addition of H_2O_2 (100 μM) to MBMECs increased the total biopterin content from 9 h after its treatment (Fig. 1). We recently reported that the treatment with H_2O_2 increases BH_4 without significant increasing of its oxidized forms (4). To determine whether H_2O_2 affects the expression of GTPCH and GFRP mRNA levels, Northern blot analysis was employed. The endothelial cells expressed low levels of GTPCH mRNA and high levels of GFRP mRNA in the basal conditions. Hydrogen peroxide (100 μM) induced the expression of the GTPCH mRNA levels from 3 h after its treatment (Fig. 2A). In contrast to GTPCH mRNA, Northern blot analysis revealed that the basal GFRP mRNA levels were decreased by the treatment with H_2O_2 (Fig. 2: A and B). We previously showed that BH_4 synthesis and GTPCH mRNA levels were concentration-dependently

Fig. 1. Effects of H_2O_2 on total biopterin content in MBMECs. Cells were incubated with H_2O_2 (100 μM) for various periods (0–24 h) at 37°C. Values are the means $\pm$ S.E.M. of 3 experiments.

increased by H_2O_2 , and both the increases were reduced by the co-treatment with catalase (4, 8). As shown in Fig. 2C, the reduction of GFRP mRNA levels by H_2O_2 was also inhibited by the co-treatment with catalase (100 U/ml).

To determine whether the NO and BH_4 produced by H_2O_2 treatment were involved in the reduction of GFRP mRNA expression, the effects of L-*N*⁶-(1-iminoethyl) lysine (L-NIL), an iNOS inhibitor, and 2,4-diamino-6-hydroxypyrimidine (DAHP), a GTPCH inhibitor, were examined. Both L-NIL and DAHP did not affect the decrease of GFRP mRNA expression by H_2O_2 (Fig. 3: A

Fig. 2. Changes in GTPCH and GFRP mRNA levels by H_2O_2 treatment. A: Cells were incubated with H_2O_2 (100 μM) for various periods (0–24 h). B: Cells were incubated with H_2O_2 (1–100 μM) for 12 h. C: Cells were pretreated with catalase (100 U/ml) for 1 h and then treated with H_2O_2 (100 μM) for 12 h. After incubation, the total RNA was extracted, and then the expression of GTPCH, GFRP, and GAPDH mRNA levels were determined by Northern blot analysis.

and B). Moreover, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, 0.01–1 mM), a NO donor, did not reduce GFRP mRNA levels (Fig. 3C).

In the present study, we observed the expression of GFRP mRNA in basal conditions, and the expression was decreased by the treatment with H₂O₂, in contrast to the increase of GTPCH mRNA levels and BH₄ content. Previous reports showed the involvement of GFRP in BH₄ synthesis by treatment with LPS and/or cytokines in various types of cells. Werner et al. (5) reported that LPS, which is known to stimulate BH₄ synthesis through a *de novo* pathway with the induction of GTPCH, down-regulates the expression of GFRP mRNA level in human

THP-1 myelomonocytoma. Gesierich et al. (6) also showed that interferon- γ up-regulates the expression of GTPCH mRNA and down-regulates GFRP mRNA in human umbilical vein endothelial cells. Thus, the decrease of GFRP seems to be implicated in the stimulation of BH₄ synthesis by not only LPS and/or cytokines but also H₂O₂.

Park et al. (9) recently suggested that NO decreases GFRP mRNA levels. However, an iNOS inhibitor, L-NIL, which completely blocked NO production in H₂O₂-treated cells (4), had no effect on the reduction of GFRP mRNA expression. The NO donor SNAP also did not reduce the expression of GFRP mRNA levels in our experimental conditions. The discrepancy may reflect the effect of NO on GFRP mRNA expression in different cell types and species. Moreover, although we previously showed that the GTPCH inhibitor DAHP (1 mM) completely inhibited H₂O₂-induced BH₄ synthesis (4), it had no effect on the reduction of GFRP expression. These observations suggest that endogenously produced NO and BH₄ are not implicated in the underlying mechanisms of GFRP mRNA reduction by H₂O₂. Recently, H₂O₂ has been shown to stimulate various signaling cascades including extracellular signal-regulated protein kinase (ERK), leading to cell survival and proliferation (10, 11). In the future, we will need to clarify the mechanism by which H₂O₂ decreases GFRP mRNA.

In conclusion, our findings suggest that H₂O₂ induces BH₄ synthesis through not only the induction of GTPCH but also the decrease of GFRP. The decrease of GFRP mRNA level appears to be independent of the produced NO and BH₄. The stimulation of BH₄ synthesis by decreasing GFRP might be one of the defense systems against oxidative stress since BH₄ has an antioxidative activity.

Fig. 3. Effects of GTPCH and iNOS inhibitors on the expression of GFRP mRNA expression during H₂O₂ treatment in MBMECs. Cells were pretreated with DAHP (0.01–1 mM, A) or L-NIL (0.01–1 mM, B) for 15 min and then incubated with H₂O₂ (100 μM) for 12 h. C: Cells were incubated with SNAP (0.01–1 mM) for 12 h. After incubation, the total RNA was extracted, and then the expression of GFRP and GAPDH mRNA levels were determined by Northern blot analysis.

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