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cGMP Inhibits GTP Cyclohydrolase I Activity and Biosynthesis of Tetrahydrobiopterin in Human Umbilical Vein Endothelial Cells

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Abstract. Tetrahydrobiopterin (BH4) acts as an essential cofactor for the enzymatic activity of nitric oxide (NO) synthases. Biosynthesis of the cofactor BH4 starts from GTP and requires 3 enzymatic steps, which include GTP cyclohydrolase I (GCH I) catalysis of the first and rate-limiting step. In this study we examined the effects of cGMP on GCH I activity in human umbilical vein endothelial cells under inflammatory conditions. Exogenous application of the cGMP analogue 8-bromo-cGMP markedly inhibited GCH I activity in the short term, whereas an cAMP analogue had no effect on GCH I activity under the same condition. NO donors, NOR3 and sodium nitroprusside, elevated the intracellular cGMP level and reduced GCH I activity in the short term. This inhibition of GCH I activity was obliterated in the presence of an NO trapper carboxy-PTIO. NO donors had no effect on GCH I mRNA expression in the short term. Moreover, cycloheximide did not alter the inhibition by NO donors of GCH I activity. These findings suggest that stimulation of the cGMP signaling cascade down-regulates GCH I activity through post translational modification of the GCH I enzyme.

Keywords: GTP cyclohydrolase I, tetrahydrobiopterin, nitric oxide, endothelial cell, cGMP

Introduction

It is suggested that enhanced production of nitric oxide (NO) by the inducible form of nitric oxide synthase (iNOS) contributes importantly to a circulatory shock such as hypotension and vascular hyporeactivity to vasoconstrictors (1). Proinflammatory cytokines, such as interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α), as well as lipopolysaccharide, stimulate the production of NO by iNOS, and concomitantly increase the *de novo* synthesis of tetrahydrobiopterin (BH4) in several types of cells (2 – 5). BH4 acts as an essential cofactor for the enzymatic activity of nitric oxide synthases (6). Biosynthesis of the cofactor BH4 starts from GTP and requires 3 enzymatic steps, which include GTP cyclohydrolase I (GCH I; EC 3,5,4,16) catalysis of the first and rate-limiting step (for review, see reference 7). Hence, changes in GCH I activity directly influence BH4 biosynthesis. Evidence is accumulating that cytokine-stimulated enhancement of BH4 biosynthesis is critically caused by increases in GCH I protein and mRNA levels in various tissues (8 – 12).

It has been reported that in human umbilical vein endothelial cells (HUVEC), the cytokine mixture does not lead to the high output of nitric oxide, that is, induction of iNOS expression, although it induced a marked increase in BH4 synthesis (13). The results suggest that the cytokines do not always produce a simultaneous induction of BH4 synthesis and iNOS expression in the same cells. The biological significance of BH4 production induced in HUVEC under inflammatory conditions is obscure. It has been demonstrated that BH4 is secreted from endothelial cells vectorally in the basal direction, that is, toward the underlying smooth muscle cells (14). BH4 may serve as an endothelium-derived relaxing factor augmenting the activity of cyto-

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kine-inducible nitric oxide synthase in vascular smooth muscle cells (4, 15, 16). Thus, it is conceivable that elucidation of the regulatory mechanism of BH4 production in endothelial cells provides an new insight into the control of vascular tone. We previously reported that cAMP and prostacyclin inhibited cytokine stimulated BH4 biosynthesis and secretion by HUVEC (13). Our results strongly suggested that under inflammatory conditions, an increase in cAMP levels in endothelial cells may exert a regulatory effect on the circulatory system.

Under inflammatory conditions including septic shock, it is probable that the endothelium is exposed to a large quantity of NO derived from cells such as macrophages and smooth muscle cells in the surrounding tissues. In the present study, in order to clarify the regulation of BH4 biosynthesis by NO and its intracellular second messenger cGMP in HUVEC under inflammatory conditions, we examined the effects of a cell-permeable cGMP analogue and NO donors in HUVEC. In this report, we give the first documentation of the modification of GCH I activities by the NO-cGMP signaling cascade in HUVEC.

Materials and Methods

Preparation and culture of endothelial cells

HUVEC were isolated by collagenase digestion from normal umbilical cords, as described by Jaffe et al. (17). The experimental protocol for obtaining HUVEC was approved by the institutional review board of Fujita Health University. Cells were grown to confluence in 0.2% gelatin-coated dishes (Iwaki Glass, Tokyo) containing human endothelial cell culture medium including 25 $\mu g \cdot ml^{-1}$ endothelial cell growth supplement (Nissui Co., Tokyo), 50 $mg \cdot ml^{-1}$ heparin, and 100 $u \cdot ml^{-1}$ penicillin-streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Studies were conducted on cells from passages 3 – 6.

Assay of BH4 and GCH I activity

Confluent HUVEC monolayers were treated with IFN-γ and TNF-α (at a conc. of 300 $u \cdot ml^{-1}$) for 8 h in order to induce GCH I activity. After treatment with the cytokine mixture, the cells were washed with PBS buffer, and then reagents were added as indicated in the figure legends. The cells were harvested with trypsin and pelleted by centrifugation. Measurements of BH4 were performed by HPLC analysis as described by Fukushima and Nixon (18). GCH I activity was assayed as described by Sawada et al., (19) based on the quantification of D-erythro-neopterin by HPLC after the conversion of enzymaticaly formed D-erythro-7,8-dihydroneopterin triphosphate into D-erythro-neopterin by sequential iodine oxidation and dephosphorylation. Enzyme activity was expressed as pmoles of D-erythro-neopterin formed per mg protein per hour. Protein concentration of cell lysates was determined according to Bradford, with bovine serum albumin used as a standard (20).

Assay of cyclic GMP

cGMP levels in KOH-neutralized $HClO_4$ extracts of HUVEC were determined by using a commercially available cGMP kit (Biotrak; Amersham Pharmacia Biotech, Buckinghamshire, UK).

mRNA isolation and real time PCR

Total RNA was extracted with a total RNA isolation kit (Rneasy Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated total RNA (2 μg) was reverse-transcribed using oligo $(dT)_{12-18}$ primer. After a 1:25 dilution, quantitative PCR was performed in a GeneAmp 5700 sequence detection system using TaqMan fluorescent probes (PE Biosystems, Norwalk, CT, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was quantified as an internal control with TaqMan GAPDH control reagents (PE Biosystems). PCR primers and TaqMan probe used were 5'-CCTCGGCCATGCAGTTCTT-3' (sense) and 5'-TGGAACCAAGTGATGCTCACA-3' (antisense) for GCH I and 5'-CCAAGGGCTACCAG GAGACCATCTCAGA-3' as the TaqMan probe.

Statistical analyses

The results were expressed as the means ± S.E.M. obtained from at least 3 separate experiments. Statistical evaluations of the data were made by means of Student's *t*-test for paired data or analysis of variance (ANOVA) followed by Bonferroni's method. A value of $P<0.05$ was considered significant.

Materials

The materials used in this work and their sources were as follows: Collagenase type II, penicillin-streptomycin, trypsin-0.53 mM Na_4EDTA, fetal bovine serum, and medium 199 from Life Technologies (Rockvill, MD, USA); IFN-γ and TNF-α from Pepro Tech EC (London, UK); (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR3), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide, sodium salt (carboxy-PTIO), and 1*H*-[1,2,4-]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) from Dojindo Laboratories (Kumamoto); 3-isobutyl-1-methylxanthine (IBMX), 8-bromoguanosine 3',5'-cyclic monophosphate (8-br-cGMP), 8-bromoadenosine 3',5'-cyclic monophosphate (8-br-cAMP), and sodium nitroprusside (SNP) from

Sigma (St. Louis, MO, USA); and (Rp)-8-(*para*-chlorophenylthio)guanosine-3',5'-cyclic monophosphorothioate ((Rp)-8-pCPT-cGMPS) from Biomol Research Laboratories (Plymouth Meeting, PA, USA).

Results

Exogeneously applied cGMP analogue inhibited GCH I activity and BH4 biosynthesis

In order to investigate the modification of GCH I activity by cGMP, we firstly performed the experiments using 8br-cGMP, an analogue of cGMP. Figure 1A clearly illustrates that 8br-cGMP reduced the GCH I activity dose dependently during a 1-h incubation (GCH I activity [pmol · mg proteins^{-1} · h^{-1}]; control, 52.6 ± 3.3; 1 mM 8br-cGMP, 39.6 ± 3.0; 3 mM 8br-cGMP, 27.0 ± 3.6; n = 5). Intracellular levels of BH4 were also decreased by addition of 3 mM 8br-cGMP (BH4 [pmol · mg protein^{-1}]; control, 89.4 ± 5.7; 3 mM 8br-cGMP, 58.2 ± 2.5; n = 4) (Fig. 1B). Then we examined the time course of the effect of 3 mM 8br-cGMP on the activity. As indicated in Fig. 2A, 3 mM 8br-cGMP inhibited the GCH I activity significantly within 10 min (GCH I activity at 10 min: control, 42.8 ± 1.7; 3 mM 8br-cGMP, 23.2 ± 1.8; n = 4). We have previously reported that cAMP inhibits cytokine-induced GCH I activity in the long term experiments (13). In the present study, we found that 3 mM 8br-cAMP had no effect on the GCH I activity during a 1-h incubation (Fig. 2B).

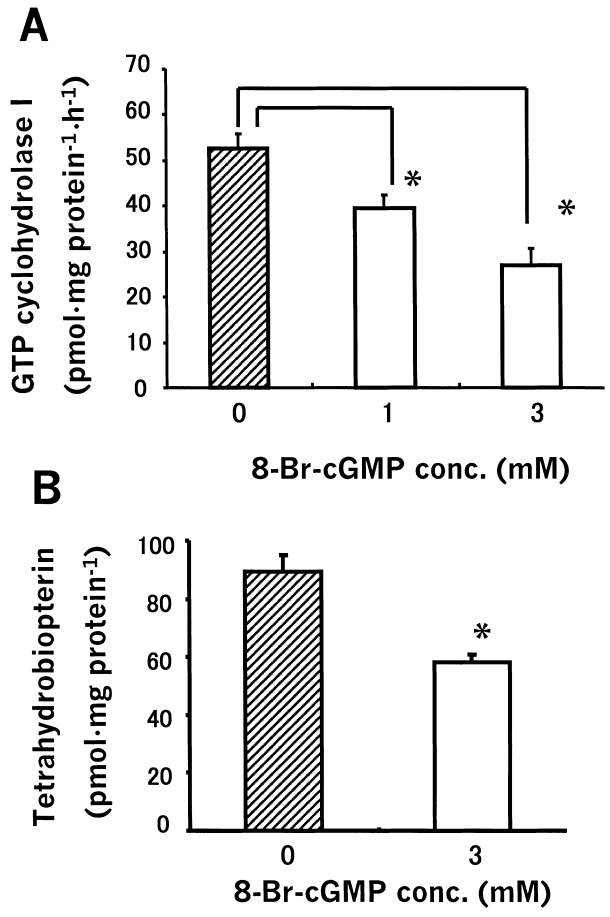

Fig. 1. Effects of cGMP analogue on GCH I activity (A) and accumulation of intracellular BH4 (B) in HUVEC. HUVEC monolayers were treated with a mixture of IFN-γ and TNF-α (final conc. of 300 u · ml^{-1} for each) for 8 h. After the treatment with the cytokine mixture, the cells were washed with PBS, and then reagents were added. Incubations were conducted for 1 h in the presence of IBMX (100 μM). A: Concentration-dependent effects of 8-br-cGMP on GCH I activities. Results are expressed as the mean ± S.E.M. of 5 separate experiments. Significance is calculated with one-way ANOVA, and denoted as *P<0.05. B: Effects of cGMP analogue on accumulation of intracellular BH4 in HUVEC. Results are expressed as the mean ± S.E.M. of 4 separate experiments. *P<0.05, compared with the control values (i.e., no cGMP analogue).

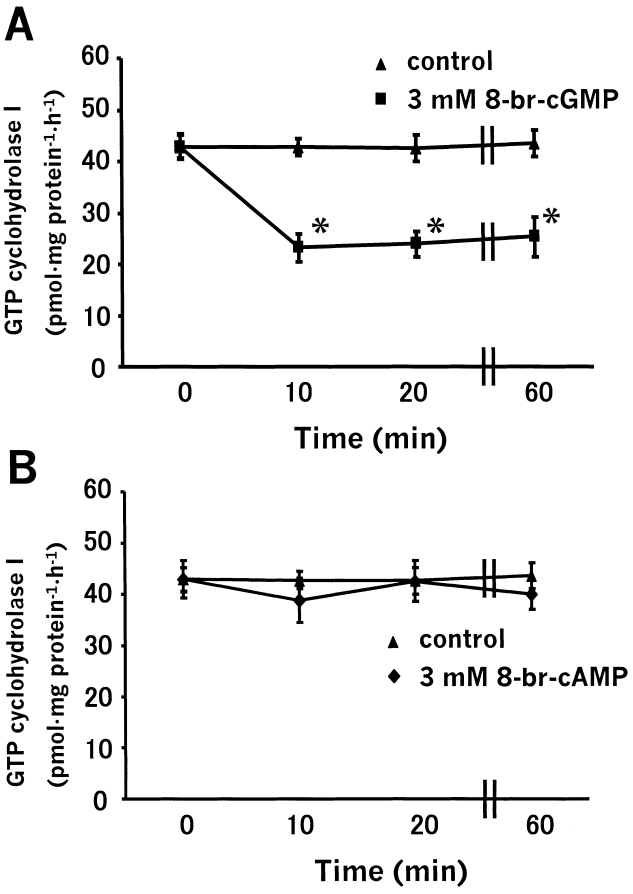

Fig. 2. Time course of the effects of cGMP (A) and cAMP (B) analogues on GCH I activity. HUVEC monolayers were treated as in Fig. 1 except the incubation time with cGMP or cAMP analogues were varied from 0 – 60 min. At the end of incubation, the cells were harvested and then assayed for GCH I activity. Results are expressed as the mean ± S.E.M. of 4 separate experiments. *P<0.05, compared with the control values of the corresponding time (i.e., no cGMP or cAMP analogues).

NO donors inhibited GCH I activity and BH4 biosynthesis

We next tested the effects of NO donors such as NOR3 and SNP, the stimulant of guanylate cyclase, on GCH I activity. The data in Fig. 3A show that NOR3 inhibited GCH I activity dose-dependently, although 10 μM NOR3 failed to cause statistically significant inhibition of the activity (GCH I activity [pmol·mg proteins^{-1}·h^{-1}]: control, 38.9 ± 2.0; 10 μM NOR3, 24.5 ± 5.0; 100 μM NOR3, 13.3 ± 5.2; n = 4). SNP also reduced the activity significantly (control, 44.7 ± 1.0; 100 μM SNP, 22.4 ± 3.1; n = 4) (Fig. 3B). Accumulation of intracellular BH4 was significantly decreased along with the inhibition of GCH I activity. (Fig. 4).

NOR3 inhibited GCH I activity in the short term and also elevated the intracellular level of cGMP

Figure 5 shows time courses of the effects of NOR3 on GCH I activity and the intracellular accumulation of cGMP. NOR3 at 100 μM significantly inhibited GCH I activity within 10 min and caused progressive inhibition of this activity during a 60-min incubation (Fig. 5A). On the other hand, 100 μM NOR3 increased the intracellular cGMP level 4 fold within 10 min, although the level declined there after (Fig. 5B).

NO trapper carboxy-PTIO blocked NO donor-mediated inhibition of GCH I activity

The antagonistic effect of carboxy-PTIO against NO has been documented (21). As indicated in Table 1, carboxy-PTIO indeed abolished the inhibition of GCH I activity by NOR3 or SNP in HUVEC.

Effects of ODQ and Rp-8-pCPT-cGMPS on SNP-mediated inhibition of GCH I activity

To clarify whether the inhibition of GCH I activity by NO donors was caused by the cGMP signaling system or not, we tested the effects of ODQ, a guanylate cyclase inhibitor and Rp-8-pCPT-cGMPS, an inhibitor of

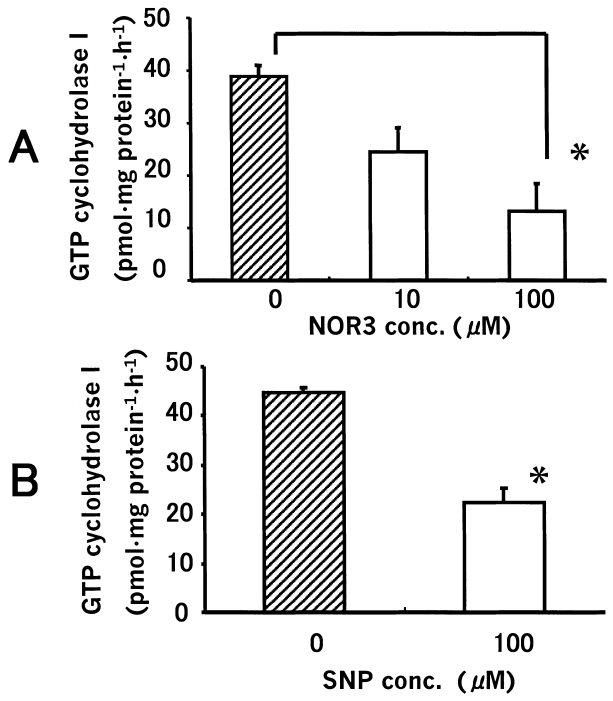

Fig. 3. Effects of NOR3 (A) and SNP (B) on cytokine-induced GCH I activity in HUVEC. HUVEC monolayers were treated with both IFN-γ and TNF-α, each at a final conc. of 300 u·ml^{-1}, for 8 h. After the treatment with the cytokine mixture, the cells were washed with PBS, and then NO donors were added. Incubations were conducted for 1 h in the presence of the phosphodiesterase inhibitor IBMX (100 μM). At the end of the incubation, the cells were harvested and then assayed for GCH I activity. Results are expressed as the mean ± S.E.M. of 4 separate experiments. Significance for the effects of NOR3 is calculated with one-way ANOVA and denoted as *P<0.05.

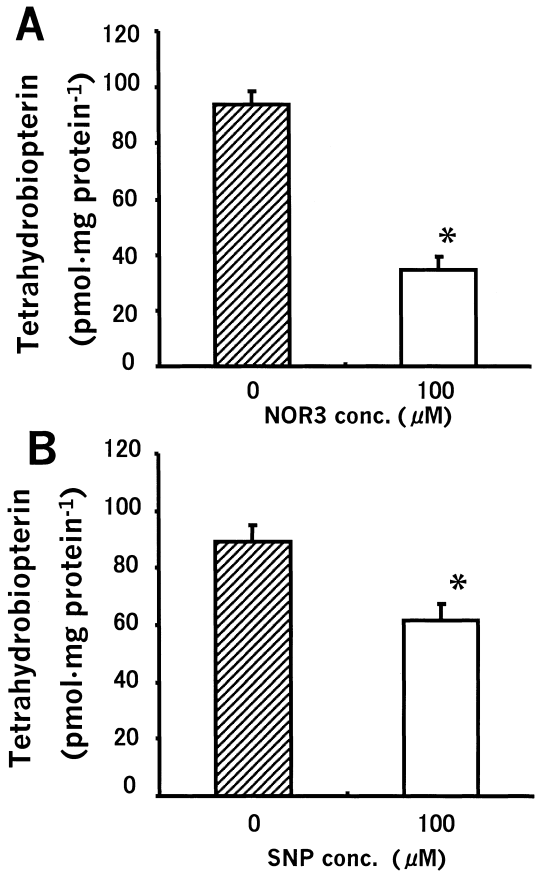

Fig. 4. Effects of NOR3 (A) and SNP (B) on accumulation of intracellular BH4 in HUVEC. HUVEC monolayers were treated as in Fig. 3. At the end of incubation, the cells were harvested and then assayed for BH4 accumulation. Results are expressed as the mean ± S.E.M. of 4 separate experiments. *P<0.05, compared with the control values (i.e., no NO donor).

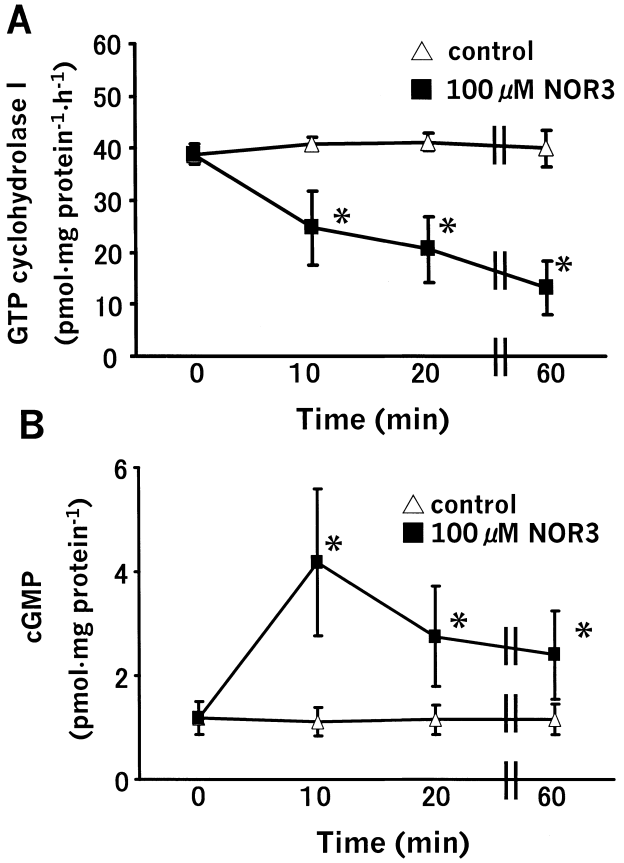

Fig. 5. Time course of the effects of NOR3 on GCH I activity (A) and cGMP accumulation (B) in HUVEC. HUVEC monolayers were treated as in Fig. 3 except the incubation time with NO donors was varied from 0 – 60 min. At the end of incubation, the cells were harvested and then assayed for GCH I activity and cGMP accumulation. Results are expressed as the mean ± S.E.M. of 4 separate experiments. *P<0.05, compared with the control values of the corresponding time (i.e., no NOR3).

Table 1. Effects of carboxy-PTIO on NO donor-induced inhibition of GCH I activity

Experimental conditions		GCH I activity (pmol · mg protein^{-1} · h^{-1})	
		Carboxy-PTIO (–)	Carboxy-PTIO (10 μM)
NOR3	0	40.7 ± 5.4	35.1 ± 3.0
	100 μM	24.4 ± 0.94*	35.2 ± 1.2
SNP	0	38.9 ± 3.1	37.9 ± 3.5
	100 μM	24.7 ± 2.4*	39.5 ± 4.8

The results are the mean ± S.E.M. of 4 independent experiments. HUVEC monolayers were treated with both IFN-γ and TNF-α, each at final conc. of 300 u · ml^{-1}, for 8 h prior to the addition of NO trapper (carboxy-PTIO) and NO donors (NOR3, SNP). Incubations were conducted for 1 h in the presence of phosphodiesterase inhibitor IBMX (100 μM). At the end of the incubation, the cells were harvested and then assayed for GCH I activity. *P<0.05, compared with the control values (i.e., no NO donor).

cGMP-dependent protein kinase, on SNP-mediated inhibition of GCH I activity. As indicated in Table 2, the inhibition of GCH I activity by SNP was completely blocked in the presence of 10 μM ODQ. The effect of SNP was also blocked by 100 μM Rp-8-pCPT-cGMPS.

Effect of NO donors on GCH I gene expression and mRNA translation

We checked the effects of NO donors on intracellular GCH I mRNA expression. As shown in Table 3, neither SNP nor NOR3 affected the GCH I mRNA expression ratio normalized with GAPDH during a 1-h incubation. Moreover, we tested the effects of cycloheximide, an inhibitor of mRNA translation, on SNP-mediated inhibition of GCH I activity in HUVEC. Cycloheximide at 30 μM did not alter SNP-mediated inhibition of GCH I activity (data not shown).

Table 2. Effects of ODQ and Rp-8-pCPT-cGMPS on SNP-mediated inhibition of GCH I activity

Experimental conditions		GCH I activity (pmol · mg protein^{-1} · h^{-1})	
		Control	SNP (100 μM)
ODQ	0	43.9 ± 4.0	29.6 ± 4.3*
	10 μM	44.3 ± 4.0	44.6 ± 5.2
Rp-8-pCPT-cGMPS	0	46.6 ± 2.9	26.8 ± 3.6*
	100 μM	48.7 ± 3.7	45.9 ± 5.8

The results are the mean ± S.E.M. of 6 (ODQ) or 5 (Rp-8-pCPT-cGMPS) independent experiments. HUVEC monolayers were treated with both IFN-γ and TNF-α, each at final conc. of 300 u · ml^{-1}, for 8 h prior to the addition of ODQ, Rp-8-pCPT-cGMPS, and SNP. After the treatment with the cytokine mixtures, the cells were washed with PBS, and then ODQ (inhibitor of guanylate cyclase), Rp-8-pCPT-cGMPS, or SNP was added. Incubations were conducted for 1 h in the presence of IBMX (100 μM). At the end of the incubation, the cells were harvested and then assayed for GCH I activity. *P<0.05, compared with the control values.

Table 3. Effects of NO donors on GCH I mRNA expression in the short term

Experimental conditions		Stimulation time (min)	
		0	60
NOR3	100 μM	1.00	0.94 ± 0.09
SNP	100 μM	1.00	1.09 ± 0.24

Expression of mRNA levels of GCH I and GAPDH were assayed with real-time PCR (ABI5700). Each ratio of GCH I/GAPDH was calculated and then demonstrated as relative expression ratio taking each control (0 time stimulation) as 1.00. The values are expressed as the mean ± S.E.M. of 3 separate experiments.

Discussion

In the present study we demonstrated that the NO donors tested, NOR3 and SNP, inhibited GCH I activity in the short term. These NO donors have been shown to release nitric oxide spontaneously into the medium (22 – 25). We found that the effects of NO donors were indeed abolished by the NO trapper carboxy-PTIO. It is thus conceivable that the inhibition of GCH I activity by NO donors was mediated by NO liberated from these NO donors. It is well known that NO liberated from NO donors is capable of modifying the functions of proteins through S-nitrosylation (26). The question then arises as to the involvement of S-nitrosylation in the inhibition of GCH I activity. Indeed we can not deny the possibility that S-nitrosylation may exert an inhibitory effect on GCH I activities. It may also be possible that superoxide radical generated by NO donors may cause the inhibition of GCH I activity and/or reduction of BH4 levels. In this study, however, we showed that exogeneously applied cGMP mimicked the effects of the NO donors. Furthermore, the inhibition of GCH I activity by NOR3 occurred in accordance with the elevation of the intracellular cGMP level. We found that another NO donor, SNP (100 μM), also caused accumulation of intracellular cGMP in HUVEC (intracellular cGMP: control, 2.4 ± 0.5 pmol·mg protein^{-1}, 100 μM SNP; 14.7 ± 4.0 pmol·mg protein^{-1}, $n = 6$). We demonstrated that the guanylate cyclase inhibitor ODQ eliminated NO donor-induced inhibition of GCH I activity. All these findings taken together clearly indicate that the NO-cGMP signaling pathway contributes to the inhibition of GCH I activity in HUVEC.

Recently, evidence is accumulating that GCH I activity is regulated by post translational modifications. Protein kinase C (PKC)-mediated modification of GCH I activity has been postulated (27 – 29). The primary amino acid sequence of GCH I reveals conserved phosphorylation sites for casein kinase II and for PKC (Ser-167 in the rat and mouse sequences) (30 – 32). However, no reports have been published with regards to PKG-mediated phosphorylation of the GCH I enzyme. In the present study, the PKG inhibitor Rp-8-pCPT-cGMPS blocked NO donor-induced inhibition of GCH I activity. From our finding, we suggest that PKG-mediated phosphorylation, in response to the NO-cGMP signaling pathway is involved in the regulation of GCH I activity. Further investigation is needed to clarify the possible regulation of GCH I activity through the phosphorylation by PKG. Our present data show that NO donors did not reduce the GCH I mRNA level. Furthermore, in the presence of the mRNA translation inhibitor cycloheximide, the effects of NO donors on GCH I activity were not altered. The results indicate that the inhibition of GCH I activity by NO donors is not related to the reduction of the level of either GCH I mRNA or protein in the short term. We previously reported that cAMP inhibited cytokine-stimulated GCH I activity through the suppression of GCH I mRNA expression in the long term, although contribution of post translational modification to regulation of GCH I activity through the cAMP signaling pathway remained unclear (13). The present data show that cAMP fails to affect the GCH I enzyme activity in the short term. This results suggests that the inhibition of GCH I activity by cAMP previously reported (13) was not caused by post translational modification of GCH I protein. It is therefore conceivable that the mechanism of the inhibition of GCH I enzyme activity in HUVEC is different between cAMP and cGMP signaling pathways. The regulation of GCH I enzyme activity and BH4 biosynthesis by the NO-cGMP signaling pathway in the long term remains unclear and is under investigation in our laboratory.

In vascular smooth muscle cells, both cAMP and cGMP signaling cascades lead to vasodilations. Our previous (13) and present data indicate that under inflammatory conditions, these 2 vasodilating signals reduce biosynthesis of BH4 in HUVEC although the mechanism is different. It is thus reasoned that the inhibition by cAMP and cGMP signaling pathways of BH4 biosynthesis in HUVEC could be a defense mechanism against over-stimulation of vasodilation under conditions such as septic shock. However, the reduction of BH4 level in response to the activation of cAMP and cGMP signaling pathways may lead to deterioration in the vascular function. It has been documented that under conditions when the intracellular concentration of BH4 is reduced, NOS generates superoxide anions instead of NO (33, 34). Furthermore, BH4 has been shown to have scavenging activity toward reactive oxygen species (ROS) and antioxidative activity (35). Thus we speculate that the inhibition of GCH I activity and BH4 production possibly causes an oxidative stress with an accumulation of ROS in vascular tissues. The pathophysiological significance of inhibition of BH4 biosynthesis needs to be elucidated.

In summary, we have demonstrated that stimulation of the NO-cGMP signaling pathway regulates GCH I activity. This is the first report of cGMP-mediated inhibitory regulation of BH4 biosynthesis in HUVEC.

Acknowledgments

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