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cAMP inhibits cytokine-induced biosynthesis of tetrahydrobiopterin in human umbilical vein endothelial cells

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Abstract

We studied the effects of cAMP on cytokine (interferon- γ plus tumor necrosis factor- α)-induced stimulation of tetrahydrobiopterin (BH₄) synthesis in human umbilical vein endothelial cells (HUVEC). The cytokine mixture caused a marked increase in the biosynthesis and release of BH₄ by HUVEC. Dibutyryl-cAMP produced a dose-dependent inhibition of this cytokine-induced stimulation of synthesis and release of BH₄ by these cells. 8-Bromo-cAMP also caused a significant inhibition, although the effects were less marked than those of dibutyryl-cAMP. Both forskolin and the stable analog of prostacyclin, iloprost, caused cAMP accumulation and a concomitant diminution of the cytokine-induced BH₄ synthesis in HUVEC. Dibutyryl-cAMP and iloprost also significantly inhibited the cytokine-induced stimulation of GTP cyclohydrolase I (GCHI) activity and mRNA production. We concluded that the suppression by the cAMP messenger system of cytokine-induced

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stimulation of synthesis and release of BH₄ by HUVEC can be attributed to the inhibition of the activity of GCHI, the rate-limiting enzyme in BH₄ biosynthetic pathway, in HUVEC. The data also suggest that the cAMP-mediated reduction in the GCHI mRNA level may at least partially explain the decline in GCHI activity. It is reasoned that under inflammatory conditions, cAMP-elevating agents such as prostacyclin exert regulatory effects on circulation by inhibiting cytokine-induced synthesis and release of BH₄ by HUVEC. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Tetrahydrobiopterin (BH₄); cAMP; Cytokine; Human umbilical vein endothelial cells (HUVEC); GTP cyclohydrolase I (GCHI); mRNA

Introduction

Proinflammatory cytokine such as interferon (IFN)- γ and tumor necrosis factor (TNF)- α have been reported to strongly stimulate the de novo synthesis of tetrahydrobiopterin (BH₄) in human and murine cells such as macrophages [1,2], fibroblasts [3], endothelial cells [4–6], glial cells [7], C6 glioma cells [8], vascular smooth muscle [9], pancreatic β cells [10], cardiac myocytes [11], and osteoblastic cells [12]. It has been suggested that this increased production of BH₄ is required to ensure a sufficient supply of the cofactor for the massive production of nitric oxide by inducible nitric oxide synthase (iNOS) [13]. It was also demonstrated that cytokines stimulated the secretion of BH₄ by human vascular endothelial cells [14]. The cofactor BH₄ is synthesized de novo from GTP, and the first and rate-limiting step in the biosynthetic pathway is catalyzed by GTP cyclohydrolase I (GCHI, EC 3.5.4.16). Evidence is accumulating that cytokines enhance GCHI activity [3,5,6] and increase GCHI protein levels [8] as well as mRNA levels of this enzyme [12,15] in various tissues. The effects of cAMP on cytokine-induced BH₄ biosynthesis in vascular endothelial cells has not been clarified. Katusic et al. [16] documented that cAMP alone did not affect the expression of GCHI mRNA in human umbilical vein endothelial cells (HUVEC). Prostacyclin induces vasodilation by activating adenylate cyclase to generate cAMP. Also, cytokine-induced expression of cyclooxygenase has been reported [17]. Thus, it is likely that prostacyclin and its cAMP signaling system are involved in the regulation of cytokine actions in vascular endothelial cells. The present study was designed to determine the effects of cAMP on cytokine-induced stimulation of BH₄ synthesis and secretion by HUVEC. Our results indicate that the cAMP signaling system is possibly involved in the regulation of cytokine-induced BH₄ synthesis in these cells.

Materials and Methods

Materials

The sources of materials used in this work were as follow: 8-bromoadenosine 3': 5'-cyclic monophosphate (8-bromo-cAMP), N², 2'-O-dibutyryl-adenosine 3': 5'-cyclic monophosphate

(dibutyryl-cAMP), 3-isobutyl-1-methylxanthine (IBMX), and forskolin from Sigma Chemical Co. (St. Louis, MO); collagenase type II, penicillin–streptomycin, trypsin-0.53 mM Na₄EDTA, fetal bovine serum, and medium 199 from Life Technologies (Rockville, MD); IFN- γ and TNF- α from Pepro Tech EC (London, England); human endothelial cell culture medium prepared for endothelial cells including 5 % fetal bovine serum from Nissui Co. (Tokyo, Japan). Iloprost, the stable analog of prostacyclin, was a generous gift from Nihon Schering Co. (Osaka, Japan). Dibutyryl-cAMP, 8-bromo-cAMP, and iloprost were dissolved in culture medium. Forskolin was dissolved in dimethyl sulfoxide.

Endothelial cell isolation and culture

HUVEC were isolated by collagenase digestion from normal umbilical cords, as described by Jaffe et al [18]. The experimental protocol for obtaining human umbilical cord cells was approved by the institutional review board of Fujita Health University. Cells were grown to confluence in 0.2 % gelatin-coated dishes (Iwaki Glass, Japan) containing human endothelial cell culture medium including 25 μ g/ml endothelial cell growth supplement (Nissui Co., Tokyo), 50 mg/ml heparin, and 100 U/ml penicillin–streptomycin at 37 °C in a humidified atmosphere of 5 % carbon dioxide in air. Studies were conducted on cells from passages 3–6. Confluent HUVEC monolayers were treated with TNF- α and IFN- γ for the times indicated in the figure legends. When the effects of exogenous cAMP and cAMP-elevating agents (forskolin and iloprost) on cytokine action were being studied, the agents were added 2 h prior to the addition of the cytokines. In the present study, the effects of forskolin and iloprost were investigated in the presence of phosphodiesterase inhibitor IBMX (100 μ M). Control cultures always contained the same amount of vehicle.

Determination of BH₄, GCHI activity and NO_x (nitrite and nitrate)

Synthesis and release of BH₄ by HUVEC was estimated by measuring intracellular and extracellular BH₄ levels in HUVEC monolayers, respectively. At the end of the treatment period, supernatants were collected. The cells were detached from dishes with trypsin, and pelleted by centrifugation. Both the cell supernatants and pellets were frozen at –80 °C. Measurements of BH₄ were performed by HPLC analysis as described by Fukushima and Nixon [19]. Both the intracellular and extracellular BH₄ levels were expressed in terms of pmoles per mg protein of the cell pellet. GCHI activity was assayed based on the quantification of D-erythro-neopterin by HPLC after the conversion of enzymatically formed D-erythro-7, 8-dihydroneopterin triphosphate into D-erythro-neopterin by sequential reaction of iodine oxidation and dephosphorylation [20,21]. Nitric oxide production by HUVEC was assessed by the determination, based on the Griess reaction [22], of the stable end products of nitric oxide, NO_x (nitrite and nitrate), in the culture medium. The culture medium to be measured was applied to TCI-NOX 1000 system (Tokyo Kasei Kogyo, Tokyo, Japan) after treatment according to the manufacturer's instructions. Protein concentration of cell lysates was determined according to Bradford, with bovine serum albumin used as a standard [23].

Determination of cAMP levels

cAMP levels in cell pellets were determined by enzyme-linked immunosorbent assay (Amersham Pharmacia Biotech, NJ) in KOH-neutralized HClO₄ extracts of HUVEC.

Northern blot analysis

Northern blot analysis of mRNA from HUVEC was performed by using a fragment of human GCHI cDNA [24] as a probe. Briefly, total RNA was extracted with a total RNA isolation kit (RNeasy Mini Kit, Qiagen) according to the manufacturer's instructions. Samples of total RNA (20 µg) from HUVEC were subjected to electrophoresis on a 1.0 % agarose gel containing formaldehyde and transblotted onto a nylon membrane (Hybond-N, Amersham). Hybridization was carried out for 16 h in 5×SSPE (1×SSPE contains 0.18 M NaCl, 10 mM NaH₂PO₄ [pH 7.7], and 1 mM ethylenediaminetetraacetic acid, EDTA) containing 50 % formamide, 0.3 % SDS, 5×Denhardt's solution, 250 µg/ml of salmon sperm DNA, and cDNA fragment labeled with [α -³²P] dCTP with a Megaprime DNA labeling system (Amersham-Pharmacia). The membrane was washed twice with 2×SSPE containing 0.1 % SDS at room temperature for 15 min, twice with 0.1×SSPE containing 0.1 % SDS at 37 °C for 15 min, and then autoradiographed. The densities of the bands were determined with an imaging analyzer (BAS-3000II, FUJIX).

Statistical analysis

The results were expressed as means \pm S.E.M. Statistical evaluation of the data was made by means of Student's *t*-test for paired data. A *P* value < 0.05 was considered significant.

Results

Effects of dibutyryl-cAMP and 8-bromo-cAMP on the cytokine-induced synthesis and release of BH₄ by HUVEC

In a preliminary study, we found that the combination of respective 300 units per ml of IFN- γ and TNF- α produced the maximal effect on synthesis and release of BH₄ by HUVEC (data not shown). Thus we decided to use this concentration of cytokine mixture for studying the effects of cAMP and cAMP-elevating agents on cytokine-induced synthesis and release of BH₄ by HUVEC. The data presented in Fig. 1A clearly indicate that exogenously applied cell-permeable cAMP, dibutyryl-cAMP, produced a dose-dependent inhibition of cytokine-induced BH₄ synthesis during a 24h. incubation of HUVEC. 3 mM dibutyryl-cAMP reduced intracellular BH₄ levels by 78.3 % (intracellular BH₄ level at 24 h: control, 94.1 \pm 17.9 pmoles/mg protein; 3 mM dibutyryl-cAMP, 20.4 \pm 5.5 pmoles/mg protein, N = 3). The dose-response effect of dibutyryl-cAMP on BH₄ release correlated very closely with that on BH₄ synthesis (Fig. 1A). BH₄ release was decreased by 62.8 % with 3 mM dibutyryl-cAMP (extracellular

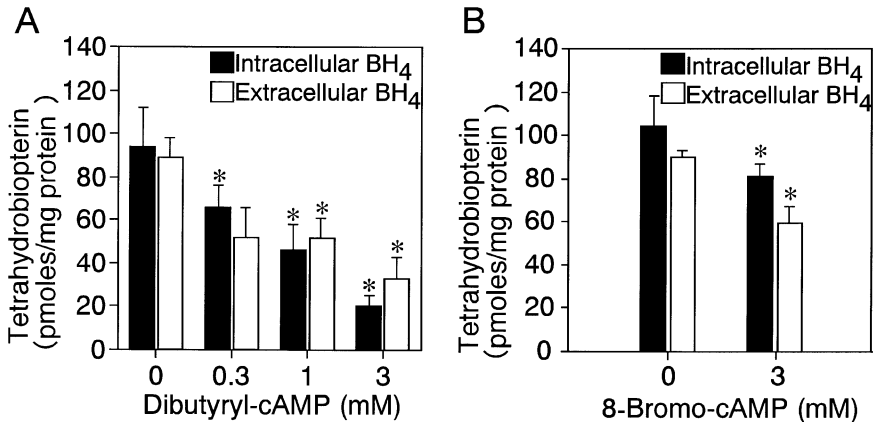


Fig. 1. Effects of dibutyryl-cAMP (A) and 8-bromo-cAMP (B) on cytokine-induced stimulation of intracellular and extracellular accumulation of BH₄ in HUVEC. HUVEC monolayers were treated with IFN- γ (300 units/ml) plus TNF- α (300 units/ml) for 24 h. Exogenous cAMP was added 2 h prior to the addition of cytokine mixture. Results are expressed as means \pm S.E.M. for three or four independent experiments. * $P < 0.05$, compared with the control values (i.e., no exogenous cAMP).

BH₄ levels at 24 h: control, 88.7 ± 9.3 pmoles/mg protein; 3 mM dibutyryl-cAMP, 33.0 ± 9.5 pmoles/mg protein, $N = 3$). 8-Bromo-cAMP (3 mM), another cell-permeable cAMP, also caused significant inhibition of both cytokine-induced synthesis and release of BH₄, although the effects were less marked than those of dibutyryl-cAMP (Fig. 1B). Neither dibutyryl-cAMP nor 8-bromo-cAMP itself affected BH₄ synthesis in HUVEC in the absence of the cytokine mixture (data not shown).

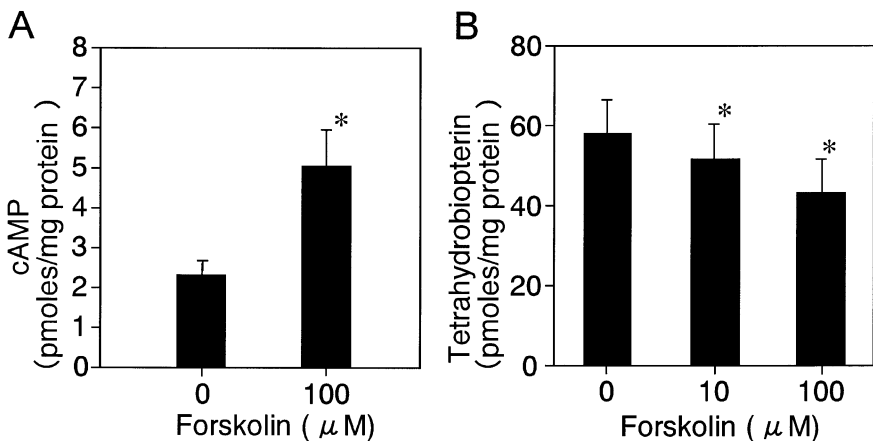


Fig. 2. Effects of forskolin on cAMP accumulation (A) and cytokine-induced stimulation of intracellular BH₄ accumulation (B) in HUVEC. HUVEC monolayers were treated with IFN- γ (300 units/ml) plus TNF- α (300 units/ml) for 16 h. Forskolin was added 2 h prior to the addition of the cytokines. The effects of forskolin were investigated in the presence of phosphodiesterase inhibitor IBMX (100 μ M). Results are expressed as means \pm S.E.M. for four independent experiments. * $P < 0.05$, compared with the control values (i.e., no forskolin).

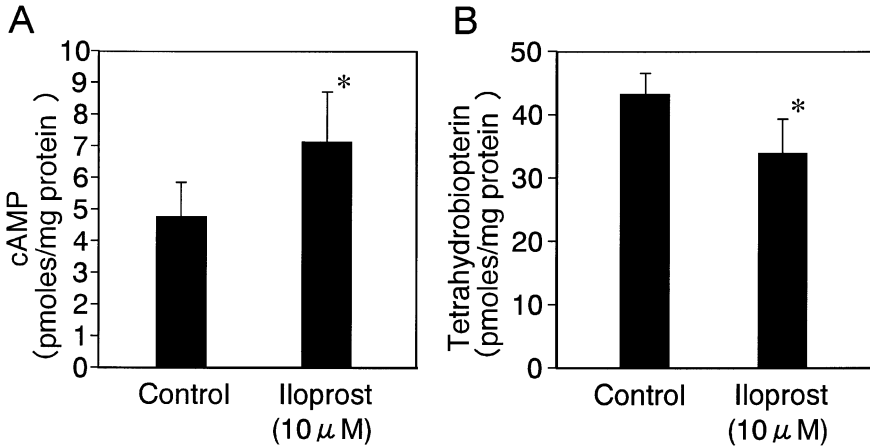


Fig. 3. Effects of iloprost on cAMP accumulation (A) and cytokine-induced stimulation of intracellular BH₄ accumulation (B) in HUVEC. HUVEC monolayers were treated with IFN- γ (300 units/ml) plus TNF- α (300 units/ml) for 16 h. Iloprost (10 μ M) was added 2 h prior to the addition of the cytokines. The effects of iloprost were investigated in the presence of phosphodiesterase inhibitor IBMX (100 μ M). Results are expressed as means \pm S.E.M. for four independent experiments. * P < 0.05, compared with the control values (i.e., no iloprost).

Effects of forskolin and iloprost on the cytokine-induced BH₄ synthesis in HUVEC

We tested the effect of forskolin, an activator of adenylate cyclase, on the cytokine-induced BH₄ biosynthesis in HUVEC. Fig. 2A illustrates that the intracellular cAMP level after 16 h

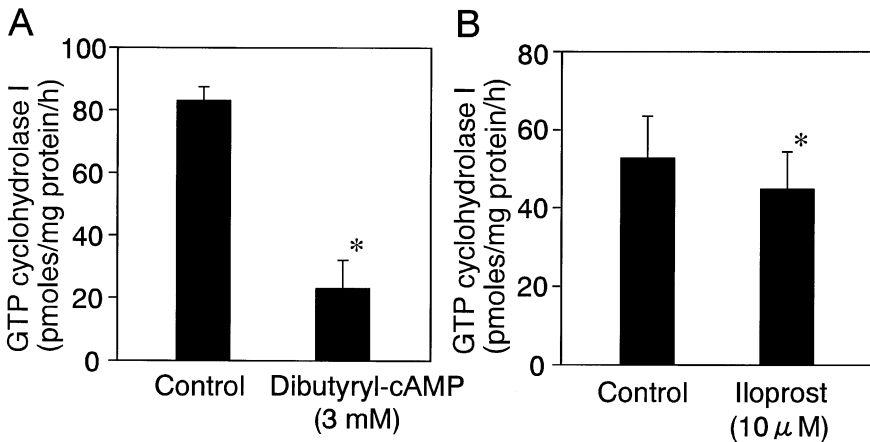


Fig. 4. Effects of dibutyryl-cAMP (A) and iloprost (B) on GCHI activity in HUVEC. HUVEC monolayers were treated with IFN- γ (300 units/ml) plus TNF- α (300 units/ml) for 24 h for the experiment with dibutyryl-cAMP and for 16 h for the experiment with iloprost. Dibutyryl-cAMP (3 mM) or iloprost (10 μ M) was added 2 h prior to the addition of the cytokines. The effects of iloprost were investigated in the presence of phosphodiesterase inhibitor IBMX (100 μ M). Results are expressed as means \pm S.E.M. for three or four independent experiments. * P < 0.05, compared with the control values (i.e., no dibutyryl-cAMP or iloprost).

of incubation was indeed increased by forskolin (100 μ M). In accordance with the stimulation of cAMP accumulation in HUVEC, the level of cytokine-induced BH₄ synthesis significantly dropped. The data in Fig. 2B show that forskolin inhibited cytokine-induced BH₄ synthesis in a dose-dependent manner. To investigate the effects of prostacyclin on the cytokine-induced BH₄ synthesis, we employed the stable prostacyclin analog iloprost. Iloprost (10 μ M) also caused a stimulation of cAMP accumulation in HUVEC (Fig. 3A) and a concomitant suppression of cytokine-induced BH₄ synthesis (Fig. 3B).

Effects of dibutyryl-cAMP and iloprost on the cytokine-induced stimulation of GCHI activity in HUVEC

Next, we conducted experiments to see whether cAMP-elevating agents would affect the cytokine-induced increase in GCHI activity in HUVEC. In a preliminary study, we confirmed that cytokine mixture caused a marked stimulation of GCHI activity in HUVEC (data not shown). Fig. 4A shows that the cytokine-induced GCHI activity in HUVEC was markedly inhibited by 3 mM dibutyryl-cAMP (GCHI activity at 24 h: control, 82.8 ± 4.7 pmoles/mg protein/h; 3 mM dibutyryl-cAMP, 22.7 ± 8.0 pmoles/mg protein/h, N = 4). Percent inhibition (72.6 %) by 3 mM dibutyryl-cAMP of the cytokine-induced GCHI activity was almost comparable to that of the cytokine-induced BH₄ synthesis. Iloprost (10 μ M) was also found to

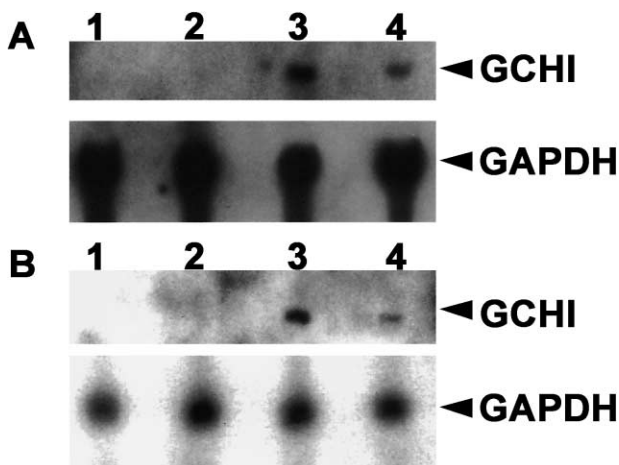


Fig. 5. Effects of dibutyryl-cAMP (A) and iloprost (B) on the expression of GCHI mRNA by Northern blot analysis in HUVEC. HUVEC monolayers were treated with IFN- γ (300 units/ml) plus TNF- α (300 units/ml) during 24 h. Dibutyryl-cAMP or iloprost was added 2 h prior to the addition of cytokines. Panel A: lane 1, control (i.e., no additive); lane 2, dibutyryl-cAMP (3 mM); lane 3, IFN- γ (300 units/ml) plus TNF- α (300 units/ml); lane 4, dibutyryl-cAMP (3 mM) plus IFN- γ (300 units/ml) plus TNF- α (300 units/ml). Dibutyryl-cAMP was added 2 h prior to the addition of the cytokines. Panel B: lane 1, control (i.e., no additive); lane 2, iloprost (10 μ M); lane 3, IFN- γ (300 units/ml) plus TNF- α (300 units/ml); lane 4, iloprost (10 μ M) plus IFN- γ (300 units/ml) plus TNF- α (300 units/ml). The effects of iloprost were investigated in the presence of phosphodiesterase inhibitor IBMX (100 μ M). For standardization, mRNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected with a human GAPDH cDNA probe.

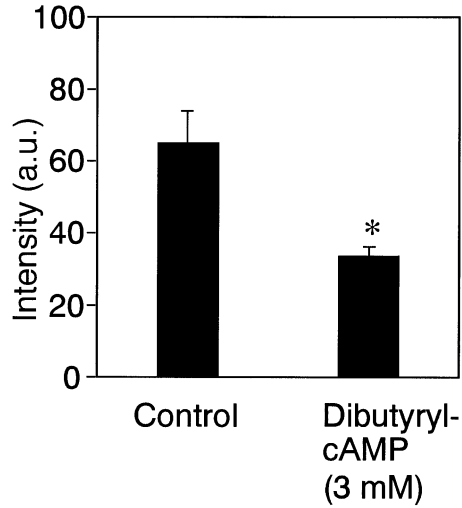


Fig. 6. Effects of dibutyryl-cAMP on cytokine-induced GCHI mRNA expression in HUVEC. HUVEC monolayers were treated with IFN- γ (300 units/ml) plus TNF- α (300 units/ml) for 24 h. Dibutyryl-cAMP (3 mM) was added 2 h prior to the addition of the cytokines. GCHI mRNA levels were quantified by densitometric scanning of the autoradiographs after normalization with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Results are expressed as means \pm S.E.M. for three independent experiments. * $P < 0.05$, compared with the control value (i.e., no dibutyryl-cAMP).

produce a significant, though less marked, inhibition of the cytokine-induced GCHI activity (Fig. 4B).

Effects of dibutyryl-cAMP and iloprost on the cytokine-induced GCHI mRNA expression in HUVEC

In the present study, the Northern blot analysis detected a single GCHI mRNA band with an approximate size of 3.6 kb in HUVEC treated with the cytokine mixture. The size of GCHI mRNA was almost identical to that for other human tissues including lymphocytes and liver cells [25] and pheochromocytoma [24]. Fig. 5 (A and B) clearly illustrates that both dibutyryl-cAMP (3 mM) and iloprost (10 μ M) suppressed the cytokine-induced GCHI mRNA expression in the cells. Neither dibutyryl-cAMP nor iloprost itself affected GCHI mRNA expression in the absence of the cytokine mixture. The densitometric analysis of GCHI mRNA content shows that the addition of dibutyryl-cAMP (3 mM) resulted in a 48.0 % decrease in the cytokine-induced GCHI mRNA content in the HUVEC (Fig. 6).

Discussion

In the present study, we found, for the first time, that not only exogenously applied cell-permeable cAMP but also forskolin and iloprost which elevate cellular levels of cAMP,

inhibited cytokine-induced BH₄ synthesis in HUVEC. The present results strongly suggest that the cAMP signaling pathway is at least partially responsible for the inhibition of the cytokine-induced BH₄ synthesis and release by HUVEC. As the magnitude of the inhibition was large when dibutyryl-cAMP was used, it might be likely that butyrate, the breakdown product of dibutyryl-cAMP, is also acting to modify the cytokine induction of GCHI. The biological effects of butyrate on cellular signal transduction systems [26] or mRNA expression [27] have been reported. The possible roles of BH₄ synthesized in vascular endothelial cells have been investigated in several laboratories. It has been assumed that the co-induction of BH₄ synthesis is prerequisite for the induction of iNOS in various cells including murine macrophage cell line RAW 264 [2], rat glial cells [7], human mesangial cells [28], chicken macrophages [29], rat pancreatic-β cells [10] and mouse osteoblastic cells [12]. In the present study, however, we found that the cytokine mixture failed to increase nitrite and nitrate production in the HUVEC (NO_x concentration in the culture medium at 24 h: control (i.e., none), 6.4 ± 0.2 μM; IFN-γ (300 units/ml) plus TNF-α (300 units/ml), 6.8 ± 0.4 μM, N = 3), although it induced a marked increase in BH₄ synthesis. As the treatment of HUVEC with the cytokine mixture did not lead to the high output of nitric oxide, i.e., induction of iNOS expression, it is thus conceivable that cytokines do not always produce a simultaneous induction of BH₄ synthesis and iNOS expression in the same cells. BH₄ released from HUVEC may serve as an endothelium-derived relaxing factor augmenting the activity of cytokine-inducible nitric oxide synthase in vascular smooth muscle cells [4]. Also, it has been demonstrated that BH₄ is secreted from endothelial cells vectorially in the basal direction, i.e., toward the underlying smooth muscle cells [14]. MacNaul and Hutchinson [30] reported that the locus of nitric oxide production shifted from endothelial cells to vascular smooth muscle cells in inflammatory vessels. Furthermore, it is also likely that BH₄ exerts the action unrelated to that of a cofactor of nitric oxide synthase. For example, BH₄ has been suggested to act as a self-protecting factor against nitric oxide toxicity [31]. It has also been demonstrated to stimulate angiogenesis [32]. Thus, our results strongly suggest that under inflammatory conditions, an increase in cAMP levels in endothelial cells may exert a regulatory effect on the circulatory system by inhibiting BH₄ synthesis and release by endothelial cells.

Cytokine-induced expression of cyclooxygenase has been reported [17]. Prostacyclin, one of the cyclooxygenase products, is an endothelial vasodilator product. Our present study clearly demonstrate that prostacyclin causes significant inhibition of BH₄ synthesis as well as stimulation of cAMP accumulation in the cytokines-treated HUVEC. It is suggested that prostacyclin prevents excessive vasodilation by suppressing BH₄ synthesis in endothelial cells under inflammatory conditions or in septic shock. The possible regulation of cytokine-induced BH₄ synthesis and nitric oxide production by cAMP elevating agents in the vascular system may provide new insights into the treatment of septic shock.

Our present data suggest that the suppression by the cAMP signaling system of the cytokine-induced stimulation of synthesis and release of BH₄ by HUVEC can be attributed to the inhibition of GCHI activity. The down-regulation of GCHI mRNA levels by cAMP can at least partially explain the decline in GCHI activity in these cells. Cytokine-induced BH₄ synthesis in rat neonatal cardiac myocytes is regulated at least in part by NF-κB activation

[33]. TNF and IFN- γ have also been reported to activate MAP kinase [34,35]. The cloning of the murine and human GCHI genes yielded data indicating the presence of cis-acting elements within the promoter region that are typically associated with cytokine regulation of gene expression [36]. cAMP response elements in the promoter of the rat GCHI gene have also been identified and characterized [37]. Post-transcriptional modulation of the enzyme activity by protein kinase C has been postulated [38,39]. At present, it is not clear whether GCHI is possibly regulated by covalent modification through the cAMP signaling pathway. The molecular events mediating the suppression by cAMP signaling of BH₄ biosynthesis induced by TNF- α and IFN- γ in HUVEC remain to be clarified.

Acknowledgments

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