Original Article

Cilostazol Inhibits Cytokine-Induced Tetrahydrobiopterin Biosynthesis in Human Umbilical Vein Endothelial Cells

Hiroaki Shiraishi¹, Kazuhisa Ikemoto¹, Shin Tada², Yasuhiro Udagawa², Masatsugu Ohtsuki³, Chiho Sumi-Ichinose¹, Kazunao Kondo¹, and Takahide Nomura¹

Hiroaki Shiraishi and Kazuhisa Ikemoto equally contributed to this work.

¹Department of Pharmacology, Fujita Health University School of Medicine, Aichi, Japan

²Department of Obstetrics and Gynecology, Fujita Health University School of Medicine, Aichi, Japan

³Department of Internal Medicine, Fujita Health University School of Medicine, Aichi, Japan

Aims: Cilostazol, a type III phosphodiesterase inhibitor, is utilized for the treatment of intermittent claudication and is considered to have the beneficial effects against the atherogenic process. In the present study, we examined the effects of cilostazol on BH₄ biosynthesis in HUVEC treated with a mixture of the pro-inflammatory cytokines IFN- γ and TNF- α .

Methods: Isolated HUVECs were grown to confluence and treated with IFN- γ (300 units/mL) and TNF- α (300 units/mL) for 16 h in order to stimulate BH₄ biosynthesis. The BH₄ levels were measured by HPLC. The mRNA expression of GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme of BH₄ biosynthesis, and GTPCH feedback regulatory protein (GFRP) were quantified by real-time PCR. The GTPCH protein expression was assessed by western blot analysis.

Results: Cilostazol significantly reduced the BH₄ levels in cytokine-stimulated HUVEC. Cilostazol produced a concomitant increase in the cAMP levels in HUVEC. Cilostazol decreased the GTPCH activity as well as the expression of GTPCH mRNA and protein. 8-bromo-cAMP (8Br-cAMP), a cell-permeable cAMP analogue, did not reproduce the effects of cilostazol. Cilostazol did not affect the cytokine-induced inhibition of GFRP mRNA expression.

Conclusions: We conclude that cilostazol inhibited cytokine-stimulated BH₄ biosynthesis via a cAMP-independent mechanism in HUVEC. Our data indicate that cilostazol reduced GTPCH activity and did so by suppressing the GTPCH protein levels.

J Atheroscler Thromb, 2011; 18:312-317.

Key words; Cilostazol, Tetrahydrobiopterin, GTP cyclohydrolase I, HUVEC

Introduction

Recent pathophysiologic observations in numerous laboratories indicate that atherosclerosis is an inflammatory disease¹⁾. The earliest changes in the formation of atherosclerosis take place in the endothelium. It is thus suggested that endothelial dysfunction induced by inflammatory injury leads to the promo-

tion of atherosclerosis. Cilostazol, a type III phosphodiesterase (PDE[III]) inhibitor, is a potent inhibitor of platelet aggregation²⁻⁴⁾. This agent was approved by the United States Food and Drug Administration for the treatment of intermittent claudication⁵⁾. Recently, cilostazol was demonstrated to exert a protective action against apoptotic cell death^{6, 7)} and to repress vascular cell adhesion molecule expression⁸⁾ in human umbilical vein endothelial cells (HUVEC). Thus, it is reasonable that cilostazol has the potential to elicit some beneficial effects against the atherogenic process.

It has been well established that pro-inflammatory cytokines enhance tetrahydrobiopterin (BH₄) biosynthesis by increasing GTP cyclohydrolase I

Address for correspondence: Kazuhisa Ikemoto, Department of Pharmacology, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Toyoake, Aichi 470-1192, Japan
E-mail: ikeike@fujita-hu.ac.jp

Received: July 1, 2010

Accepted for publication: November 5, 2010

(GTPCH) activity^{9, 10)} and GTPCH protein levels¹¹⁾ through the expression of mRNA for this enzyme¹²⁾ in various tissues. Lipopolysaccharide and cytokines such as interferon (IFN)- γ and tumor necrosis factor (TNF)- α strongly induce the *de novo* synthesis of BH₄, and concomitantly increase the *de novo* synthesis of nitric oxide by inducible nitric oxide synthase (iNOS) in various cells¹³⁻¹⁵⁾. It is assumed that the increased production of BH₄ is required to ensure a sufficient supply of the co-factor for the massive production of nitric oxide by iNOS. It is known that an excessive amount of nitric oxide in the vascular tissues is involved in the pathogenesis of various vascular diseases, such as atherosclerosis and septic shock.

In the present study, we tested the effect of cilostazol on BH₄ biosynthesis as well as on nitric oxide production in HUVEC treated with pro-inflammatory cytokines. We found for the first time that cilostazol repressed the induction of BH₄ without affecting nitric oxide levels in HUVEC treated with a cytokine mixture. The possible mechanism whereby cilostazol inhibited BH₄ production in HUVEC is discussed.

Materials and Methods

Endothelial Cell Isolation and Culture

HUVEC were isolated by collagenase digestion from normal human umbilical cord as described previously¹⁶⁾ based on the method by Jaffe *et al.*¹⁷⁾. 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, Tokyo, Japan) containing human endothelial cell culture medium supplemented with 25 μ g/mL endothelial cell growth supplement (Nissui Co., Tokyo, Japan), 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.

Assay of BH₄ and GTPCH activity

Confluent HUVEC monolayers were treated with a mixture of IFN- γ (300 units/mL) and TNF- α (300 units/mL) for 16 h in order to induce GTPCH activity. When the effects of cilostazol were being studied, the cells were simultaneously treated with the cytokine mixture and the agent. Control cultures always contained the same amount of vehicle. After treatment, the cells were sequentially harvested by trypsinization and pelleted by centrifugation. BH₄ was measured by HPLC analysis according to the method by Fukushima and Nixon¹⁸⁾. GTPCH activity was

assayed by the quantification of D-*erythro*-neopterin by HPLC¹⁹⁾.

Determination of mRNA Levels of GTPCH and GTPCH Feedback Regulatory Protein (GFRP)

GTPCH and GFRP mRNA levels were determined by real-time PCR using fluorescent TaqMan probes. Total RNA was extracted with a total RNA isolation kit (RNeasy Mini Kit; Qiagen, Hilden, Germany). The isolated total RNA (200 ng) was reverse-transcribed using a High Capacity cDNA Reverse transcription Kit (Applied Biosystems, Foster City, CA), and then the cDNA was subjected to real-time PCR using a GeneAmp 5700 sequence detection system (Applied Biosystems). The primer and probe sets were selected from TaqMan® Gene Expression Assays (Applied Biosystems). The assay identification numbers used in this study were as follows: GTPCH, Hs00609198_m1; GFRP, Hs00193360_m1; GAPDH, Hs99999905_m1.

Determination of GTPCH Protein Expression

For measurement of GTPCH protein expression, cytoplasmic protein was extracted according to the method by Schreiber *et al.*²⁰⁾. A 20- μ g amount of protein was subjected to 10% SDS-PAGE. The separated protein bands were electrophoretically transferred to a PVDF membrane (Immun-Blot™PVDF Membrane; Bio-Rad, Hercules, CA). GTPCH protein was probed with an antibody against a portion of the C-terminal sequence of human GTPCH (TREEFLTLIRS-5)²¹⁾. PVDF membranes were incubated with 1000 times-diluted anti-GTPCH antibody, followed by incubation with 5000 times-diluted horseradish peroxidase-linked anti-rabbit IgG. Immunoreactive proteins were detected with luminol as a substrate using an ECL Advance Western Blotting Detection Kit (GE Healthcare, Amersham, England). The membranes were imaged and analyzed with a Lumino Image Analyzer LAS-4000UVmini (Fujifilm, Tokyo, Japan).

Determination of STAT1 and NF- κ B Levels in the Nucleus

Nuclear protein was extracted as described previously¹⁶⁾. In order to determine the levels of STAT1 and p65, a component of NF- κ B in the nucleus, we subjected 5 μ g of the nuclear protein to 7.5% SDS-PAGE. The separated proteins were transferred to PVDF membranes, and then incubated with anti-phosphorylated (Tyr 701) STAT1 (p-STAT1) or anti-p65 polyclonal antibody, which had been diluted 500 times and 5000 times, respectively. The secondary antibodies, horseradish peroxidase-conjugated

anti-rabbit antibodies, were diluted 2000 times and 15000 times, respectively. The bands of p-STAT1 and p65 were detected as described in "Determination of GTPCH Protein Expression".

Determination of cAMP and Nitric Oxide Levels

cAMP levels in KOH-neutralized HClO₄ extracts of HUVEC cell pellets were determined using a commercially available enzyme immunoassay kit (Amersham Biosciences Corp., Piscataway, NJ). Nitric oxide production was assessed by determination of the stable end product of nitric oxide nitrite in the culture medium based on the Griess reaction. The content of nitrite in the culture medium was analyzed using a Nitric Oxide Assay Kit (Assay Designs, Inc., MI, USA).

Materials

The materials used in this work, and their sources, were as follows: Cilostazol was generously provided by Otsuka Pharmaceutical Co. Ltd (Tokyo, Japan); collagenase type II, penicillin-streptomycin, trypsin, 0.05% with EDTA 4Na, fetal bovine serum, and medium 199 were from Invitrogen Corp. (Grand Island, NY); PBS was from Takara Bio Inc (Shiga, Japan); IFN- γ and TNF- α were from Pepro Tech EC (London, UK); 8-bromoadenosine 3',5'-cyclic monophosphate (8Br-cAMP) was from Sigma Chemical Co. (St. Louis, MO); the primary antibody against β -actin was from Cell Signaling Technology, Inc. (Danvers, MA); anti phosphorylated (Tyr 701) STAT1 polyclonal antibody (p-STAT1) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-p65 polyclonal antibody was from Stressgen (BC, Canada); horseradish peroxidase-linked anti-rabbit IgG was from Amersham Biosciences Corp. Cilostazol was dissolved in dimethyl sulfoxide. 8Br-cAMP was dissolved in water.

Statistical Analysis

The results are expressed as the means $\pm$ S.E.M. Data were evaluated using Student's *t* test for paired data and one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple data in the group. *P* < 0.05 was considered significant.

Results

The data presented in **Fig. 1A** clearly indicate that cilostazol dose-dependently suppressed cytokine-induced BH₄ biosynthesis in HUVEC treated with a mixture of IFN- γ and TNF- α for 16 h. The addition of 30 μ M cilostazol resulted in a 44.4% decrease in cytokine-induced BH₄ biosynthesis in the cells. We found that cilostazol produced a concomitant increase

Fig. 1. Effects of cilostazol on tetrahydrobiopterin (A) and cAMP (B) levels in HUVEC. HUVEC monolayers were treated with a mixture of IFN- γ (300 units/mL) and TNF- α (300 units/mL) for 16 h in the absence or presence of various concentrations of cilostazol. Results are expressed as the means $\pm$ S.E.M. of 5 independent experiments. **p* < 0.05, compared with the control value (i.e., cytokine mixture only).

in the cAMP level in the HUVEC, although the stimulation was statistically significant only at concentrations of 10 μ M and higher (**Fig. 1B**).

GTPCH is known to be the rate-limiting enzyme for BH₄ biosynthesis²². The data presented in **Fig. 2A** clearly show that cilostazol (30 μ M) significantly reduced cytokine-stimulated GTPCH activity by 31.2%. The data illustrated in **Fig. 2B** show that cilostazol also inhibited the cytokine-stimulated increase in the GTPCH mRNA level. The results of Western blot analysis shown in **Fig. 2C** and **2D** indicate that cilostazol actually decreased the expression of GTPCH protein itself (data with a wider range of molecular weight are indicated in **Supplementary Fig. 1**). These results are in good agreement with the effect of cilostazol on the BH₄ level (**Fig. 1A**).

Harada *et al.*²³ documented that GFRP inhibited GTPCH when it was bound with BH₄, thus demonstrating that it mediated the feedback inhibition of BH₄ biosynthesis. In the present study, we first

Fig. 2. Effects of cilostazol and 8-bromo-cAMP (8Br-cAMP) on GTPCH activity (A), GTPCH mRNA expression (B), and GTPCH protein expression in HUVEC (C, D). HUVEC monolayers were treated with a mixture of IFN- γ (300 units/mL) and TNF- α (300 units/mL) for 16 h. Cilostazol (30 μ M) or 8Br-cAMP (3 mM) was added at time zero. GTPCH mRNA levels (B) were quantified by real-time PCR and normalized with respect to glyceraldehyde 3-phosphate dehydrogenase mRNA levels. Representative image of Western blot analysis (C) and its densitometric analysis (D) are shown. GTPCH protein expression levels were normalized with the level of β -actin protein expression. Results in (A) and (B) are expressed as the means $\pm$ S.E.M. of 4 independent experiments. Results in (D) are expressed as the means $\pm$ S.E.M. of 3 independent experiments. [#] $p < 0.05$, compared with the value for untreated cells (i.e., no cytokine mixture). ^{*} $p < 0.05$, compared with the control value (i.e., cytokine mixture only).

found that the expression of GFRP mRNA in cytokine-untreated HUVEC was decreased by 90.0% by the mixture of IFN- γ and TNF- α (**Fig. 3**). Cilostazol failed to affect this cytokine-induced inhibition of GFRP mRNA levels.

In order to clarify whether the inhibition by cilostazol of the cytokine-induced up-regulation of GTPCH activity originated from the increase in the cAMP level, we tested the effect of 8Br-cAMP, a cell-permeable cAMP analogue, on cytokine-induced BH₄ production. 8Br-cAMP was found to exert no significant effect on BH₄ levels (BH₄ level at 16 h in the presence of the cytokine mixture: control, 62.3 ± 7.8 pmoles/mg protein; 3 mM 8Br-cAMP, 52.9 ± 4.0 pmoles/mg protein, $n=4$, $p < 0.05$). Furthermore, 8Br-cAMP (3 mM) failed to reproduce any effect of

cilostazol on GTPCH activity, mRNA and protein expression (**Fig. 2**). 8Br-cAMP (3 mM) also did not influence cytokine-induced inhibition of GFRP mRNA levels (**Fig. 3**).

We conducted experiments to see whether cilostazol exerts significant effects on STAT1 levels as well as NF- κ B levels in HUVEC nuclei by Western blot analysis. Cilostazol failed to affect the cytokine-induced accumulation of STAT1 and NF- κ B in HUVEC nuclei. Negative results of the experiments conducted with cilostazol are not shown.

In the present study, cilostazol did not affect the accumulation of nitrite, the stable end product of NO, in culture medium (nitrite levels at 16 h in the presence of cytokine mixture: control, 6.7 ± 1.0 μ M, 30 μ M cilostazol, 6.9 ± 1.0 μ M, $n=3$).

Fig. 3. Effects of cilostazol and 8-bromo-cAMP (8Br-cAMP) on GFRP mRNA expression in HUVEC. HUVEC monolayers were treated with a mixture of IFN- γ (300 units/mL) and TNF- α (300 units/mL) for 16 h in the absence or presence of cilostazol (30 μ M). GFRP mRNA levels were quantified by real-time PCR and normalized with respect to glyceraldehyde 3-phosphate dehydrogenase mRNA levels. Results are expressed as the means $\pm$ S.E.M. of 4 independent experiments. [#] $p < 0.05$, compared with value for untreated cells (i.e., no cytokine mixture).

Discussion

It is conceivable that the accelerated production of BH₄ in endothelial cells under inflammatory stimulation is a manifestation of endothelial dysfunction during the pathogenesis of atherosclerosis and is pivotal for the progress of atherosclerosis. BH₄ is synthesized *de novo* from GTP, and the first and rate-limiting step in the biosynthetic pathways is catalyzed by GTPCH²²⁾. The present data strongly suggest that the cytokine mixture stimulated GTPCH activity in HUVEC at least by 2 mechanisms: the enhancement of GTPCH protein levels due to increased GTPCH mRNA expression, and the obliteration of GFRP mRNA expression. The new and important finding in the present study is that cilostazol significantly inhibited cytokine-induced BH₄ biosynthesis in HUVEC. Our results clearly indicate that cilostazol blocked cytokine-stimulated GTPCH activity along with inhibition of the GTPCH protein level as well as GTPCH mRNA expression. Cilostazol did not release the suppression by the cytokine mixture of the expression of GFRP mRNA in HUVEC. We thus conclude that the cilostazol-mediated inhibition of cytokine-induced BH₄ production in HUVEC was principally exerted through the inhibition of GTPCH activity due to the suppression of GTPCH mRNA expression and the consequent reduction in GTPCH enzyme levels in HUVEC. We also found in the present study that the

inhibition of BH₄ biosynthesis by cilostazol was accompanied by the accumulation of intracellular cAMP in cytokine-stimulated HUVEC. Nevertheless, we observed that exogenously applied 8Br-cAMP did not reproduce any effects of cilostazol on BH₄ production, GTPCH activity, GTPCH mRNA level or GTPCH protein expression in the present experimental condition. We also observed that forskolin did not affect BH₄ levels in cytokine-stimulated HUVEC (data not shown). It is thus suggested that cilostazol is not likely to inhibit BH₄ biosynthesis via a cAMP mediated intracellular signal transduction system in HUVEC. Huang *et al.*²⁴⁾ reported that both STAT1 and NF- κ B were indispensable for the induction of GTPCH in HUVEC. Otsuki *et al.*⁸⁾ reported that cilostazol repressed vascular cell adhesion molecule-1 gene transcription in cultured HUVEC by inhibiting NF- κ B binding to its recognition sequence. Recently, Hattori *et al.*²⁵⁾ documented that cilostazol inhibited cytokine-induced NF- κ B activation via AMP-activated protein kinase activation in HUVEC; however, our results indicated that cilostazol failed to affect the cytokine-induced accumulation of STAT1 and NF- κ B in HUVEC nuclei.

In the present study, we found that cilostazol did not affect nitric oxide production in cytokine-stimulated HUVEC, although it caused a significant inhibition of BH₄ production. Further study needs to be carried out to elucidate the biological significance and the mechanism of cilostazol action.

Acknowledgement

This work is supported by a grant-in-aid from Fujita Health University.

References

- 1) Ross R: Atherosclerosis an inflammatory disease. *N Engl J Med*, 1999; 340: 115-126
- 2) Mori D, Ishii H, Kojima C, Nitta N, Nakajima K, Yoshida M: Cilostazol inhibits monocytic cell adhesion to vascular endothelium via upregulation of cAMP. *J Atheroscler Thromb*, 2007; 14: 213-218
- 3) Iwamoto T, Kin K, Miyazaki K, Shin K, Takasaki M: Recovery of platelet function after withdrawal of cilostazol administered orally for a long period. *J Atheroscler Thromb*, 2003; 10: 348-354
- 4) Oida K, Ebata K, Kanehara H, Suzuki J, Miyamori I: Effect of cilostazol on impaired vasodilatory response of the brachial artery to ischemia in smokers. *J Atheroscler Thromb*, 2003; 10: 93-98
- 5) Aoki C, Hattori Y, Tomizawa A, Jojima T, Kasai K: Anti-inflammatory role of cilostazol in vascular smooth muscle cells in vitro and in vivo. *J Atheroscler Thromb*, 2010; 17:

- 503-509
- 6) Kim KY, Shin HK, Choi JM, Hong KW: Inhibition of lipopolysaccharide-induced apoptosis by cilostazol in human umbilical vein endothelial cells. *J Pharmacol Exp Ther*, 2002; 300: 709-715
 - 7) Shin HK, Kim YK, Kim KY, Lee JH, Hong KW: Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cytokines via lectin-like oxidized low-density lipoprotein receptor-1 activation: Prevention by cilostazol. *Circulation*, 2004; 109: 1022-1028
 - 8) Otsuki M, Saito H, Xu X, Sumitani S, Kouhara, H, Kurabayashi M, Kasayama S: Cilostazol represses vascular cell adhesion molecule-1 gene transcription via inhibiting NF- κ B binding to its recognition sequence. *Atherosclerosis*, 2001; 158: 121-128
 - 9) Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Schmidt K, Weiss G, Wachter H: Pteridine biosynthesis in human endothelial cells: Impact on nitric oxide-mediated formation of cyclic GMP. *J Biol Chem*, 1993; 268: 1842-1846
 - 10) Rosenkranz-Weiss P, Sessa WC, Milstien S, Kaufman S, Watson CA, Pober JS: Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells: Elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. *J Clin Invest*, 1994; 93: 2236-2243
 - 11) D'Sa, C, Hirayama K, West A, Hahn M, Zhu M, Kapatos G: Tetrahydrobiopterin biosynthesis in C6 glioma cells: induction of GTP cyclohydrolase I gene expression by lipopolysaccharide and cytokine treatment. *Mol Brain Res*, 1996; 41: 105-110
 - 12) Hattori Y, Nakanishi N, Kasai K, Shimoda S: GTP cyclohydrolase I mRNA induction and tetrahydrobiopterin synthesis in human endothelial cells. *Biochim Biophys Acta*, 1997; 1358: 61-66
 - 13) Nakayama DK, Celler DA, DiSilvio M, Bloomgarden G, Davies P, Pitt BR, Hatakeyama K, Kagamiyama H: Tetrahydrobiopterin synthesis and inducible nitric oxide production in pulmonary artery smooth muscle. *Am J Physiol*, 1994; 266: L455-460
 - 14) Sakai N, Kaufman S, Milstien S: Parallel induction of nitric oxide and tetrahydrobiopterin synthesis by cytokines in rat glial cells. *J Neurochem*, 1995; 65: 895-902
 - 15) Togari A, Arai M, Mogi M, Kondo A, Nagatsu T: Coexpression of GTP cyclohydrolase I and inducible nitric oxide synthase mRNAs in mouse osteoblastic cells activated by proinflammatory cytokines. *FEBS Lett*, 1998; 428: 212-216
 - 16) Ikemoto K, Matsumoto T, Ohtsuki M, Itoh M, Tada S, Udagawa Y, Sumi-Ichinose C, Kondo K, Nomura T: 2,4-Diamino-6-hydroxypyrimidine (DAHP) suppresses cytokine-induced VCAM-1 expression on the cell surface of human umbilical vein endothelial cells in a BH4-independent manner. *Biochim Biophys Acta*, 2008; 1780: 960-965
 - 17) Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins: Identification by morphologic and immunologic criteria. *J Clin Invest*, 1973; 52: 2745-2756
 - 18) Fukushima T, Nixon JC: Analysis of reduced forms of biopterin in biological tissues and fluids. *Anal Biochem*, 1980; 102: 176-188
 - 19) Sawada M, Horikoshi T, Masada M, Akino M, Sugimoto T, Matsuura S, Nagatsu T: A sensitive assay of GTP cyclohydrolase I activity in rat and human tissues using radioimmunoassay of neopterin. *Anal Biochem*, 1986; 154: 361-366
 - 20) Schreiber E, Matthias P, Müller MM, Schaffner W: Rapid detection of octamer proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res*, 1989; 17: 6419
 - 21) Nomura T, Ohtsuki M, Matsui S, Sumi-Ichinose C, Nomura H, Hagino Y, Iwase K, Ichinose H, Fujita K, Nagatsu T: Isolation of a full-length cDNA clone for human GTP cyclohydrolase I type 1 from pheochromocytoma. *J Neural Transm*, 1995; 101: 237-242
 - 22) Nichol CA, Smith GK, Duch DS: Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annu Rev Biochem*, 1985; 54: 729-764
 - 23) Harada T, Kagamiyama H, Hatakeyama K: Feedback regulation mechanisms for the control of GTP cyclohydrolase I activity. *Science*, 1993; 260: 1507-1510
 - 24) Huang A, Zhang Y-Y, Chen K, Hatakeyama K, Keaney JF Jr: Cytokine-stimulated GTP cyclohydrolase I expression in endothelial cells requires coordinated activation of nuclear factor- κ B and Stat1/Stat3. *Circ Res*, 2005; 96: 164-171
 - 25) Hattori Y, Suzuki K, Tomizawa A, Hirama N, Okayasu T, Hattori S, Satoh H, Akimoto K, Kasai K: Cilostazol inhibits cytokine-induced nuclear factor- κ B activation via AMP-activated protein kinase activation in vascular endothelial cells. *Cardiovasc Res*, 2009; 81: 133-139

Supplementary Fig. 1. Representative image of Western blot analysis of GTPCH protein expression with a wider range of molecular weight.