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PPAR δ agonist GW501516 prevents uncoupling of endothelial nitric oxide synthase in cerebral microvessels of hph-1 mice

Anantha Vijay R. Santhanam, Livius V. d'Uscio, Tongrong He, and Zvonimir S. Katusic*
Departments of Anesthesiology, and Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55905

Abstract

Peroxisome proliferator-activated receptor delta (PPAR δ) is ubiquitously expressed in the vasculature, including cerebral circulation. The role of PPAR δ in metabolism of tetrahydrobiopterin (BH₄) has not been studied in the cerebral microvasculature. In the present study, the effects of PPAR δ agonist GW501516 on uncoupling of endothelial nitric oxide synthase (eNOS) were determined in cerebral microvessels of BH₄-deficient hph-1 mice. Wild-type (B6CBA) and hph-1 mice were orally gavaged with a selective PPAR δ activator, GW501516 (2 mg/kg/day) for 14 days, and thereafter, cerebral microvessels were isolated and studied. Treatment of hph-1 mice with GW501516 significantly reduced oxidation of BH₄ and increased the ratio of BH₄ to 7,8-BH₂ (P<0.05, n=6–9). Attenuation of L-NAME-inhibitable superoxide anion levels by GW501516 demonstrated that activation of PPAR δ might prevent uncoupling of endothelial nitric oxide synthase (eNOS, P<0.05, n=6–9). Western blotting studies demonstrated that GW501516 selectively increased the endothelial expressions of CuZn superoxide dismutase (P<0.05, n=6–9) and catalase (P<0.05, n=6–8). PPAR δ activation increased the total nitrite and nitrate (NO₂ + NO₃) content in cerebral microvessels (P<0.05, n=6). Obtained results suggest that *in vivo* activation of PPAR δ prevents eNOS uncoupling, restores bioavailability of NO and may help preserve endothelial function in the BH₄-deficient cerebral circulation.

Keywords

eNOS uncoupling; cerebral microvessels; GTP cyclohydrolase I; nitric oxide; antioxidant

1. Introduction

Peroxisome proliferator-activated receptor delta (PPAR δ) is a member of the ligand-dependent family of PPAR nuclear receptors, and is ubiquitously expressed in the vasculature. Loss of PPAR δ in smooth muscle cells of cerebral vasculature resulted in larger infarcts, blood brain barrier breakdown, inflammatory responses and neuronal loss in mice brain exposed to focal cerebral ischemia (Yin et al., 2011). Prior studies have indicated that long-term treatment with PPAR δ agonists suppress the expression of pro-inflammatory

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Corresponding author: Zvonimir S. Katusic, M.D., Ph.D., Department of Anesthesiology, Mayo Clinic College of Medicine, 200 1st St SW, Rochester, MN. 55905, Phone: 507-255-5156, Fax: 507-255-7300, Katusic.zvonimir@mayo.edu.

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The authors disclose no conflict of interest.

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genes (Barish et al., 2008; Fan et al., 2008; Takata et al., 2008) and exerts anti-atherosclerotic effects. PPAR δ activation in cultured endothelial cells also increased expression of antioxidant enzymes – catalase, CuZn superoxide dismutase (CuZn SOD), Mn superoxide dismutase (Mn SOD), and thereby inhibited apoptosis (Inoue et al., 2001; Liou et al., 2006; Piqueras et al., 2007; Jiang et al., 2009).

In a recent study from our laboratory (He et al., 2011), we reported that selective PPAR δ agonist GW501516 increased production of tetrahydrobiopterin (BH $_4$), an essential co-factor of endothelial nitric oxide synthase (eNOS) in endothelial progenitor cells. Loss of BH $_4$ and subsequent uncoupling of eNOS causes production of superoxide anions (Vasquez-Vivar et al., 1998; Katusic, 2001; d'Uscio et al., 2011). Relevant to the present study, the *in vivo* effects of PPAR δ activation on cerebrovascular pathologies associated with BH $_4$ deficiency have not been systematically investigated. In this regard, using the GTP cyclohydrolase I (rate-limiting enzyme in BH $_4$ biosynthesis)-deficient hph-1 mice (Canevari et al., 1999; Cosentino et al., 2001; Hyland et al., 2003; Lam and Heales, 2007; d'Uscio et al., 2011), we demonstrated that loss of BH $_4$ resulted in uncoupling of eNOS and elevated concentration of eNOS-derived superoxide anion in the cerebral microvessels (d'Uscio et al., 2011; Santhanam et al., 2012). In the present study, we tested the hypothesis that selective PPAR δ agonist GW501516 exerts protective effects in cerebral microvessels of BH $_4$ -deficient mice by preventing eNOS uncoupling.

2. Results

2.1. Effect of GW501516 on physiological parameters

We and others have shown that hph-1 mice were normotensive (Nandi et al., 2005; d'Uscio et al., 2011). Treatment of hph-1 mice with GW501516 did not alter systolic blood pressure, body weight, plasma cholesterol, blood glucose and blood cell counts (Table 1).

2.2. Effect of PPAR δ agonist on BH $_4$

Consistent with our previous studies on hph-1 mice (d'Uscio et al., 2011; Santhanam et al., 2012), we observed that oxidation of BH $_4$ and the ratio of BH $_4$ to 7,8-BH $_2$ were significantly reduced in the cerebral microvessels of hph-1 mice used in the present study. Treatment of wild-type mice with GW501516 did not alter BH $_4$ or 7,8-BH $_2$ levels (Figure 1). In hph-1 mice treated with GW501516, BH $_4$ levels in cerebral microvessels (Figure 1A) were significantly increased ($P < 0.05$), while the levels of 7,8-BH $_2$ were significantly reduced (Figure 1B, $P < 0.05$). The ratio of BH $_4$ to 7,8-BH $_2$ was significantly elevated in cerebral microvessels of hph-1 mice treated with GW501516 (Figure 1C, $P < 0.05$).

Enzymatic activity of GTP cyclohydrolase I was also significantly increased by GW501516 in the cerebral microvessels of hph-1 mice (Figure 1D, $P < 0.05$).

2.3. Effect of GW501516 on superoxide anions

Consistent with findings from other laboratories (Cosentino et al., 2001; Khoo et al., 2005) as well as with our prior studies on hph-1 mice (d'Uscio et al., 2011; Santhanam et al., 2012), we confirmed that loss of BH $_4$ caused increase in formation of superoxide anion, as indicated by increased formation of 2-hydroxyethidium, the product generated by chemical reaction of superoxide with dihydroethidium. Treatment with L-NAME, a NOS inhibitor, reduced superoxide anion levels selectively in hph-1 mice, indicative of increased superoxide anion production by eNOS activity (Figure 2). Treatment of hph-1 mice with PPAR δ agonist GW501516 also significantly attenuated the increased superoxide anion levels in the cerebral microvessels (Figure 2, $P < 0.05$). Importantly, in cerebral microvessels

of hph-1 mice treated with GW501516, L-NAME did not further reduce this attenuated superoxide anion production (Figure 2).

2.4. Effect of PPAR δ activation on expression of antioxidant proteins

Expression of antioxidant proteins, namely CuZn SOD, Mn SOD and catalase were not different between wild-type and hph-1 mice (data not shown). GW501516 did not affect these antioxidant enzymes in wild-type mice (data not shown). Treatment of hph-1 mice with GW501516 selectively increased the expression of CuZn SOD in cerebral microvessels (Figure 3A), while expression of Mn SOD remained unchanged (Figure 3B). Protein expression of EC SOD was undetectable in the cerebral microvessels of hph-1 mice (data not shown).

Expression of catalase, a H₂O₂ scavenging enzyme, was also significantly increased by GW501516 in cerebral microvessels of hph-1 mice (Figure 3C, $P < 0.05$).

2.5. Effect of GW501516 on bioavailability of NO

GW501516 did not affect expression of eNOS in the cerebral microvessels of hph-1 mice (Figure 4A). However, levels of nitrite and nitrate ($\text{NO}_x = \text{NO}_2 + \text{NO}_3$) were significantly increased in the cerebral microvessels of hph-1 mice treated with GW501516 (Figure 4B).

3. Discussion

In the present study, we report several novel findings. First, treatment with PPAR δ agonist GW501516 increased the bioavailability of BH₄ in the cerebral microvessels of hph-1 mice. Second, activation of PPAR δ decreased oxidative stress as demonstrated by the reduced production of superoxide anions. Third, GW501516 increased the expression of antioxidant enzymes, CuZn SOD and catalase. Finally, PPAR δ agonist GW501516 significantly increased the levels of NO, as evidenced by increased content of nitrite and nitrate in cerebral microvessels.

To the best of our knowledge, this is the first *in vivo* study to demonstrate the cerebrovascular protection conferred by a PPAR δ agonist in mouse model of eNOS uncoupling. In the present study, we used GW501516 to selectively activate PPAR δ in the hph-1 deficient mice. Indeed, GW501516 at the dose (of 2 mg/kg per day for 14 days) used in the present study has previously been demonstrated to selectively activate PPAR δ (Oliver et al., 2001; Brunelli et al., 2007; Barish et al., 2008; He et al., 2011). Recently, we reported that PPAR δ activation stimulated biosynthesis of BH₄, an essential co-factor for eNOS activation in endothelial progenitor cells (He et al., 2011). In the present study, we expanded understanding of the benefits of PPAR δ activation to include protection of cerebral microvessels under conditions of oxidative stress induced by eNOS uncoupling. Lack of effects on plasma cholesterol and blood glucose by GW501516 ruled out the contribution of metabolic effects of PPAR δ activation to the vascular protective effects observed in the present study.

Activation of PPAR δ in hph-1 deficient mice increased the ratio of BH₄ to 7,8-BH₂, by two mechanisms: (a) increasing the biosynthesis of BH₄, mediated by activation of GTP cyclohydrolase I, and (b) reducing the levels of 7,8-BH₂, the oxidized derivative of BH₄ that competitively binds and inactivates eNOS (Vasquez-Vivar et al., 2002; d'Uscio et al., 2003). The protective effects of GW501516 on attenuation of 7,8-BH₂ levels are explained by up-regulation of antioxidant enzymes and subsequent protection of BH₄ against oxidation by peroxynitrite, generated by chemical reaction between superoxide anion and nitric oxide (Milstien and Katusic, 1999). Indeed in a previous study, we demonstrated that peroxynitrite is a potent oxidant of BH₄ (Milstien and Katusic, 1999).

Further characterization of the biochemical mechanism responsible for attenuated superoxide anion production by GW501516 identified selective up-regulation of CuZn SOD in the cerebral microvasculature hph-1 mice. CuZn SOD gene is the predominant and primary antioxidant defense enzyme of the vasculature. Promoter of CuZn SOD gene has a peroxisome-proliferator activated receptor response element (PPRE), and our results are consistent with published reports on other PPAR ligands that demonstrate binding to the PPREs and activation of CuZn SOD transcription (Yoo et al., 1999; Fan et al., 2008). In addition, PPAR δ agonist seems to prevent the accumulation of H₂O₂ by increasing the expression of catalase.

Treatment with GW501516 increased availability of NO, as reflected in elevation of NO₂/NO₃. This effect is most likely caused by prevention of eNOS uncoupling and protection of NO from inactivation by superoxide anion. Since NO is a key vascular protective molecule, our findings support the concept that, besides reported anti-atherosclerotic and neuroprotective effects, PPAR δ agonists may prevent endothelial dysfunction induced by uncoupling of eNOS. The effects of PPAR δ are mediated by enhanced activity of GTP cyclohydrolase I, increased expression of CuZn SOD and subsequent inactivation of superoxide anion. In aggregate, presented findings suggest that PPAR δ is a potential therapeutic target for prevention of endothelial dysfunction in the cerebral microvasculature.

4. Experimental procedures

4.1. Mice

Breeder pairs of homozygous hph-1 mutant mice, on B6CBA background, were provided by Dr. Alex Chen (Michigan State University, East Lansing, MI). Age-matched wild-type (B6CBA) mice were procured from The Jackson Laboratory (Bar Harbor, ME). Prior studies established that wild-type (B6CBA) mice are appropriate control animals (Nandi et al., 2005; d'Uscio et al., 2011). All experiments were performed on 3–4 months old (20 – 25 g) male mice according to the guidelines of the Institutional Animal Care and Use Committee of Mayo Clinic. Mice were orally gavaged with a selective PPAR δ activator GW501516 (2 mg/kg/day) for 14 days. Prior studies established that this dosing regimen selectively activated PPAR δ (Oliver et al., 2001; Barish et al., 2008). Control mice were treated with the appropriate vehicle (0.5% carboxy methyl cellulose in 2% dimethylsulfoxide). Following treatment with GW501516, mice were killed by injection of an overdose of pentobarbital (250 mg/kg body weight, i.p.).

4.2. Physiological parameters in mice

Systolic blood pressure was measured in non-anesthetized mice by tail-cuff method, as described earlier (d'Uscio et al., 2011). For blood cell counts, mice were anesthetized in a bell jar containing isoflurane (1%) and 150 μ L blood was drawn from the orbital venous sinus and transferred immediately to EDTA containing tubes (Microtainer®; Becton Dickinson; Franklin Lakes, NJ). For measurement of glucose and cholesterol levels, blood was obtained as previously reported (d'Uscio et al., 2011).

4.3. Isolation of cerebral microvessels

Brain were removed and placed in cold (4° C) modified Krebs-Ringer bicarbonate solution (in mmol/l: NaCl 118.6; KCl 4.7; CaCl₂ 2.5; MgSO₄ 1.2; KH₂PO₄ 1.2; NaHCO₃ 25.1; glucose 10.1; EDTA 0.026). Mice brains were exposed under a surgical microscope, anterior and posterior cerebral, middle cerebral and basilar arteries were isolated and discarded. Cerebral microvessels were subsequently isolated from the brains by Dextran centrifugation, as described earlier (Austin et al., 2010; Santhanam et al., 2012). The isolated microvessels

contained a heterogeneous mixture of small arteries, arterioles, small veins, venules and capillaries.

4.4. Measurement of biopterin levels and GTP cyclohydrolase I activity

Cerebral microvessels were homogenized in extraction buffer containing 50 mmol/l Tris (pH 7.4), 1 mmol/L dithiothreitol, and 1 mmol/L EDTA at 4°C and were centrifuged at 10,000 *g* (8 min at 4°C). Tetrahydrobiopterin (BH₄), 7,8-dihydrobiopterin (7,8-BH₂) and enzymatic activity of GTPCH I were measured by reverse phase HPLC, as reported previously (d'Uscio et al., 2003; d'Uscio et al., 2011; Santhanam et al., 2012).

4.5. Detection of intracellular superoxide anion

Intracellular superoxide anion levels in cerebral microvessels were quantified using a HPLC-based fluorescence detection of the oxidation of dihydroethidium. Briefly, microvessels were incubated in Krebs-HEPES buffer containing 50 μmol/L of dihydroethidium (Molecular Probes) at 37°C for 15 minutes. In some experiments, microvessels were incubated with L-NAME (30 μmol/L) for 30 minutes prior to addition of dihydroethidium (d'Uscio et al., 2010; d'Uscio et al., 2011; Santhanam et al., 2012). The samples were washed to remove the free dihydroethidium and incubated in Krebs-HEPES buffer for 1 additional hour at 37°C. Microvessels were homogenized in cold methanol and centrifuged at 12,000 rpm. The supernatant was analyzed by fluorescence detection by HPLC (Beckman Coulter) in 37% acetonitrile in 0.1% trifluoroacetic acid aqueous solution. Data were quantified using 2-hydroxyethidium standard from the reaction between dihydroethidium and Fremy's salt and normalized against tissue levels (d'Uscio et al., 2010; d'Uscio et al., 2011; Santhanam et al., 2012).

4.6. Western blot analysis

Cerebral microvessels were homogenized in lysis buffer containing [50 mmol/L NaCl, 50 mmol/L NaF, 50 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, 5 mmol/L EGTA, 0.1 mmol/L Na₃VO₄, 1% Triton X-100, 10 mmol/L HEPES, pH 7.4, and protease inhibitor cocktail (Sigma)]. The experimental methods for protein expression studies are published elsewhere (Santhanam et al., 2007; Santhanam et al., 2012). Monoclonal antibodies against eNOS (1:500) [BD Transduction], catalase (1:1000), β-actin (1:5000) [Sigma] and polyclonal antibodies (1:1000 dilution) against CuZn superoxide dismutase (CuZn SOD), manganese superoxide dismutase (Mn SOD), extracellular SOD (EC SOD) [Stressgen] were used, and bands were visualized by enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific, Rockford, IL).

4.7. Measurement of nitric oxide

Nitric oxide in the cerebral microvessels were measured as total nitrite and nitrate (NO_x = NO₃ + NO₂) using a commercially available fluorometric nitrite/nitrate assay kit according to manufacturer's instructions (Cayman Chemical Co.) (Austin et al., 2010; Santhanam et al., 2012).

4.8. Statistical analysis

Data are represented as mean ± SEM, *n* represents the number of mice used in each group. Un-paired Student's 't' test was used to determine statistical difference between two groups, and multiple comparisons were performed by two-way ANOVA. A value of *P* < 0.05 was considered statistically significant.

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Highlights

- We studied the effects of GW501516 in cerebral microvessels of hph-1 mice.
- Selective PPAR δ agonist GW501516 significantly increased bioavailability of BH $_4$.
- GW501516 reversed eNOS uncoupling and increased nitrite/nitrate content.
- GW501516 may preserve endothelial function in BH $_4$ -deficient cerebral circulation.

Figure 1.

GW501516 increases bioavailability of BH₄ in hph-1 mice. **(A)** Levels of BH₄ were decreased in cerebral microvessels (* P<0.05, n=9) of hph-1 mice. Treatment with GW501516 tended to decrease BH₄ levels in cerebral microvessels of wild-type mice, but GW501516 significantly increased BH₄ only in cerebral microvessels of hph-1 mice (# P<0.05, n=9). **(B)** Levels of 7,8-BH₂ were significantly increased in hph-1 mice, as compared to wild-type mice (* P<0.05, n=9). Treatment with PPAR δ agonist GW501516 inhibited this increase in 7,8-BH₂ levels (# P<0.05, n=9). **(C)** The ratio of BH₄ to 7,8-BH₂ were significantly decreased in cerebral microvessels of hph-1 mice (* P<0.05, n=9) and treatment with GW501516 significantly increased the BH₄ to 7,8-BH₂ ratio (# P<0.05, n=9). **(D)** The reduced enzymatic activity of GTP cyclohydrolase I in cerebral microvessels of hph-1 mice (* P<0.05, n=6) were reversed on treatment with GW501516 (# P<0.05, n=6).

Figure 2.

GW501516 reduced superoxide anion generation in hph-1 mice. Superoxide anion levels were significantly increased in cerebral microvessels of vehicle-treated hph-1 mice (* $P < 0.05$, $n = 8-9$, as compared to wild-type mice). Treatment of hph-1 mice with GW501516 significantly attenuated this increased production of superoxide anions (# $P < 0.05$, $n = 8-9$, as compared to vehicle-treated hph-1 mice). The increased superoxide anion production in cerebral microvessels of hph-1 mice was attenuated selectively by NOS inhibitor L-NAME ($30 \mu\text{mol/L}$; 30 min; # $P < 0.05$; $n = 8-9$, as compared to vehicle-treated hph-1 mice).

Figure 3.

GW501516 increased protein expressions of CuZn SOD and catalase in hph-1 mice. Representative Western blot and densitometric analysis demonstrating selectively increased expression of CuZn SOD (* $P < 0.05$, $n = 6-9$) in cerebral microvessels (A), while Mn SOD (B) expression remained unchanged. Expression of catalase was significantly increased by GW501516 in cerebral microvessels (C) of hph-1 mice.

Figure 4.

GW501516 increased total nitrite content in hph-1 mice. **(A)** Representative Western blot and densitometric analysis demonstrating that expression of eNOS remained unaffected in cerebral microvessels of hph-1 mice treated with GW501516 ($P=0.83$, $n=4$). **(B)** Levels of total nitrate and nitrite ($\text{NO}_x = \text{NO}_2 + \text{NO}_3$) was significantly increased in the cerebral microvessels of hph-1 mice treated with GW501516 (* $P<0.05$, $n=6$).

Table 1

Characteristics of wild-type and hph-1 mice treated without or with GW501516.

Parameters	Wild-type	Wild-type + GW501516	hph-1	hph-1 + GW501516
SBP (mmHg)	118±1	117±1	120±1	116±1
Body weight (g)	31±1.0	30±1	31±1	30±1
Glucose (mg/dL)	161±8	160±10	183±12	182±11
Cholesterol (mmol/L)	2.0±0.1	2.2±0.1	2.3±0.2	2.2±0.1
HDL (mmol/L)	1.6±0.1	1.8±0.1	1.8±0.2	1.7±0.1
White blood cells (10 ³ /mm ³)	6.8±0.4	6.8±0.6	9.9±0.8	9.7±1.1
Red blood cells (10 ⁶ /mm ³)	9.9±0.1	9.8±0.1	10.2±0.2	10.1±0.2
Platelets (10 ³ /mm ³)	682±38	712±31	823±24	792±62

SBP indicates systolic blood pressure. Data are means ± SEM (n=6–9). P= not significant (ANOVA with Bonferroni's).