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Role of prostacyclin signaling in endothelial production of soluble amyloid precursor protein-α in cerebral microvessels

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Abstract

We tested hypothesis that activation of the prostacyclin (PGI_2) receptor (IP receptor) signaling pathway in cerebral microvessels plays an important role in the metabolism of amyloid precursor protein (APP). In human brain microvascular endothelial cells activation of IP receptor with the stable analogue of PGI_2, iloprost, stimulated expression of amyloid precursor protein and a disintegrin and metalloprotease 10 (ADAM10), resulting in an increased production of the neuroprotective and anticoagulant molecule, soluble APPα (sAPPα). Selective agonist of IP receptor, cicaprost, and adenylyl cyclase activator, forskolin, also enhanced expression of amyloid precursor protein and ADAM10. Notably, in cerebral microvessels of IP receptor knockout mice, protein levels of APP and ADAM10 were reduced. In addition, iloprost increased protein levels of peroxisome proliferator-activated receptor δ (PPARδ) in human brain microvascular endothelial cells. PPARδ-siRNA abolished iloprost-augmented protein expression of ADAM10. In contrast, GW501516 (a selective agonist of PPARδ) upregulated ADAM10 and increased production of sAPPα. Genetic deletion of endothelial PPARδ (ePPARδ$^{-/-}$) in mice significantly reduced cerebral microvascular expression of ADAM10 and production of sAPPα. In vivo treatment with GW501516 increased sAPPα content in hippocampus of wild type mice but not in hippocampus of ePPARδ$^{-/-}$ mice. Our findings identified previously unrecognized role of IP-PPARδ signal transduction pathway in the production of sAPPα in cerebral microvasculature.

Keywords

ADAM10, APP, PPARδ, sAPPα, endothelium

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Introduction

Vascular protective effects of prostacyclin (PGI_2) are essential for the preservation of cerebrovascular homeostasis and hemostasis.[1–4] The endothelium is a major source of PGI_2 which is generated in response to a number of different stimuli including vascular injury.[5–7] PGI_2 is a potent vasodilator; it stimulates angiogenesis and preserves barrier function of endothelial cells.[8,9] Furthermore, PGI_2 analogues attenuate brain damage caused by ischemic stroke,[10] suggesting that activation of PGI_2 receptors (IP receptors) is an important neuroprotective mechanism. In addition, PGI_2 also exerts an inhibitory effect on blood coagulation by activation of IP receptors coupled to adenylyl cyclase and increased formation of cyclic AMP in circulating platelets.[6,9]

Amyloid precursor protein (APP) is a member of a family of evolutionarily conserved single-pass type I transmembrane glycoproteins.[11] Three major isoforms

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of APP (APP695, APP751, and APP770) have been identified. APP 751 and APP770 contain a domain homologous to Kunitz-type protein inhibitors (KPI).[12] We have reported that APP is highly expressed in endothelium.[13] It has also been demonstrated that brain microvascular endothelial cells primarily express very high levels of APP770 containing KPI domain.[12] APP770 and soluble APP770 (sAPP770) that contains the KPI domain may protect the brain from cerebrovascular thrombosis by inhibition of prothrombotic proteinase factor Xla.[12,14–16] Thus, both PGI_2 and APP protect cerebral vasculature against thrombosis; however, to date the functional relationship between these two molecules has not been studied. In the present study we tested the hypothesis that activation of the IP receptor signal transduction pathway in cerebral microvessels plays an important role in metabolism of APP.

Once expressed, APP is subsequently cleaved by two different pathways. The amyloidogenic pathway generates cytotoxic Aβ, which is considered a major culprit in the pathogenesis of Alzheimer's disease. In the non-amyloidogenic pathway APP is cleaved by α-secretases within the Aβ sequence thereby generating soluble APPα (sAPPα) ectodomain. Under physiological conditions, α-processing of APP is a dominant pathway, producing sAPPα, a well-known neurotrophic and neuroprotective molecule.[17–21] Of note, sAPP770 has stronger in vitro neurotrophic effect than neuronal isoform sAPP695.[22] In vivo studies have also revealed an important role of sAPPα in cognitive function such as memory, and maintenance of normal brain structure and electrophysiological function.[17,23,24] Interestingly, elevated levels of KPI-containing APP have been observed in brain tissue exposed to ischemia and neurotoxic damage,[25–28] indicating a possible neuro-vascular protective effect of endothelial APP under pathological conditions. Indeed this concept has been reinforced by the findings that inflammatory cytokines stimulate release of sAPPα770 from endothelial cells.[29] Thus, endothelial sAPPα might play a critical role in neurovascular protection in response to ischemia and inflammatory insults. Therefore, understanding mechanisms underlying regulation of sAPPα production in cerebral vascular endothelial cells is of major importance for the development of therapeutic targets in prevention and treatment of stroke and brain injury.

Peroxisome proliferator-activated receptor δ (PPARδ) is a ligand-activated transcription factor that belongs to the nuclear receptor super-family which includes two additional isoforms PPARα and PPARγ.[30] Besides their role in the control of metabolism, PPAR isoforms are essential regulators of cardiovascular system.[30] Prior studies suggest that systemic activation of PPARδ is protective against major cardiovascular risk factors including dyslipidemia, insulin resistance, and obesity.[30] In peripheral blood vessels, pharmacological analysis demonstrated that PPARδ agonists exert vascular protective effects[13,31,32]; however, the role of PPARδ in cerebral vascular function is not fully understood. In our previous study we demonstrated that in the cerebral microvessels, activation of PPARδ prevented endothelial dysfunction thereby suggesting that endothelial PPARδ might be an important vasoprotective molecule.[33] We also established that in endothelial specific PPARδ knockout ($ePPAR\delta^{-/-}$) mice oxidative stress is a major factor leading to peripheral vascular dysfunction.[32] Since our prior studies suggest that PPARδ could mediate PGI_2-induced endothelial regeneration,[31] the present study was also designed to determine the role of PPARδ in PGI_2-dependent regulation of APP expression and metabolism.

Material and methods

Mice

Use of the animals and protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. Mice homozygous for deletion of IP receptor ($IP^{-/-}$ mice) were generously provided by Dr. Garret A. FitzGerald (University of Pennsylvania, Philadelphia, PA),[34] and bred with C57BL/6 J mice (Jackson Laboratory) to generate mice heterozygous for deletion of IP receptor. The $IP^{-/-}$ mice and wild type littermates (2–3 months old, males and females) generated from breeder mice heterozygous for deletion of IP receptor were used in experiments. Mice with endothelial-specific knock-down PPARδ ($ePPAR\delta^{-/-}$ mice) and their wild type littermates were kindly provided to us by Dr. Ronald. M. Evans (The Salk Institute, La Jolla, CA) and bred in our laboratory.[32] Male mice (3–5 months old) were maintained on standard chow with free access to drinking water. $ePPAR\delta^{-/-}$ and wild type mice were orally gavaged with GW501516 (2 mg/kg/day) or vehicle (0.5% carboxy methyl cellulose in 2% dimethylsulfoxide, control group) for 14 days. This dose was chosen based on the results of the previous in vivo studies demonstrating selectivity of GW501516 as a PPARδ agonist.[33,35] Investigator was not blinded as to which mouse belonged to control group and which belonged to treated group. Brain tissues were collected after mice were killed by injection of an overdose of pentobarbital (250 mg/kg body weight, i.p.). The experiments were performed in accordance with the ARRIVE guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Vascular reactivity

Organ chambers were used for recording isometric force of isolated aortic rings as described previously.[32] Relaxations to cicaprost were obtained in aortas of wild-type and $IP^{-/-}$ mice during submaximal contractions to phenylephrine.

Isolation of cerebral microvessels

Isolation of cerebral microvessels was performed as described in our previous studies.[33] Briefly, mouse brain was removed and placed in cold (4°C) modified Krebs–Ringer bicarbonate solution (in mmol/L: NaCl 118.6; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$25.1; glucose 10.1; EDTA 0.026). Under a surgical microscope, anterior and posterior cerebral, middle cerebral and basilar arteries were removed. Cerebral microvessels were then isolated from whole brain or brain hemisphere using 15% Dextran (Sigma-Aldrich, St. Louis, MO) centrifugation. The isolated microvessels contained a heterogeneous mixture of small arteries, arterioles, capillaries, small veins, and venules.[33]

Isolation of mouse cerebral microvascular endothelial cells

Primary mouse brain endothelial cells were obtained as described[36] with minor modification. After brain microvessels were isolated, the pellet was suspended in endothelial basal medium 2 (EBM2) (Lonza, Allendale, NJ) containing 1 mg/ml collagenase/dispase (Roche, Indianapolis, IN), 1000 U/ml DNase 1 (Roche), and 0.147 μg/ml tosyllysine chloromethyl ketone (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 75 min with occasional shaking. The digest was centrifuged at 2000 *g* for 5 min, and cells were washed with endothelial growth medium 2 (EGM2, Lonza), and seeded in 24-well plates coated with mouse collagen IV (BD Biosciences, San Jose, CA). Cells were cultured in EGM2 +15% FCS. To deplete the cells of non-endothelial origin, puromycin (4 μg/ml, Sigma-Aldrich St. Louis, MO) was added to the culture for the first 48 h. After culturing for 8 days, cells were collected for characterization. For fluorescent confocal microscopy, cells were seeded on 4-well glass chamber slide system. Immunofluorescent confocal microscopy analysis was performed as described in our previous studies,[37] using mouse antibody against PECAM-1 (1:100 dilution, Santa Cruz Biotechnology, Dallas, TX).

Western blot analysis

Western blotting was performed as previously described.[38] 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 Na_3VO_4, 1% Triton X-100, 10 mmol/L HEPES, pH 7.4, and protease inhibitor cocktail (Sigma, St. Louis, MO)). Rabbit antibodies against APP, and β-site APP cleaving enzyme 1 (BACE1) were obtained from Invitrogen (Carlsbad, CA), and Abcam (Cambridge, MA), respectively. Rabbit antibody against a disintegrin and metalloprotease 9 (ADAM9), and mouse antibody against sirtuin 1 (SIRT1) were obtained from Cell Signaling Technology (Danvers, MA). Rabbit antibodies against ADAM10 and ADAM17 were obtained from Chemicon International (Temecula, CA). Rabbit-anti-PPARδ was obtained from Santa Cruz Biotechnology. Blots probed with goat-anti-actin (Santa Cruz Biotechnology) were used as loading controls. Protein expression was normalized to actin. For α-secretase, the protein levels of mature ADAM10, mature ADAM17, and mature ADAM9 (lower bands on the blots) were quantified. Mouse anti-soluble APPα antibody (2B3) was purchased from Immuno-Biological Laboratories (Minneapolis, MN).

cAMP measurement

Cerebral microvessels were isolated and incubated in Krebs–Ringer bicarbonate solution containing 5 μM cicaprost (for microvessels of right hemisphere) or DMSO (vehicle, for microvessels of left hemisphere) at 37°C for 30 min. The microvessel lysates were prepared for measuring cAMP levels using colorimetric cAMP ELISA Kit (Cell Biolabs, San Diego, CA). The samples were acetylated before the assay according to manufacturer's protocol.

Quantitative real-time polymerase chain reaction and RT-PCR

Quantitative real time polymerase chain reaction (PCR) was performed as previously described.[39] For in vitro experiments, primary human brain cerebral cortex microvascular endothelial cells (BMECs, Applied Cell Biology Research Institute, Kirkland, WA) were used at passages 4–6. After human BMECs were treated with iloprost (Cayman Chemical, Ann Arbor MI) or GW501516 (Cayman Chemical) for 57 h, total cellular RNA was isolated using RNeasy Plus Mini kit (Qiagen, Redwood City, CA), and reverse transcription of RNA to cDNA was performed using SuperScript III First-Strand Synthesis System kit (Invitrogen). The amounts mRNA were determined (in triplicate for each sample) using iCycler IQ Real Time Detection System (Bio-Rad, Hercules, CA). The primers[40] corresponding to human ADAM10 mRNA were forward

(5′TTTGAAGGATTCATCCAGACTC3′) and reverse (5′ACACCAGTCATCTGGTATTTCC3′). Internal control human GAPDH mRNA was amplified with forward (5′AAAACCTGCCAAATATGATGAC3′) and reverse (5′CAGGAAATGAGCTTGACAAAG T3′) primers. To determine mRNA levels in cerebral microvessels of ePPAR$^{-/-}$ mice and wild type mice in vivo, primers for mouse ADAM10[41] were used: forward (5′AATTCTGCTCCTCTCCTGGG3′), and reverse (5′ACGCTGGTGTTTTTGGTGTA3′). Mouse GAPDH primers are: forward (TGCCAAGGCT GTGGGCAAGG3′), and reverse (5′TGGGCCCTCA GATGCCTGCT3′).

RT-PCR was performed to confirm endothelial deletion of PPARδ in ePPAR$^{-/-}$ mice. Primers for mouse PPARδ, eNOS, and GAPDH were used as described in our previous publication.[32]

Knockdown of PPARδ and SIRT1 by small interfering RNA (siRNA)

Experiments were performed as described in our previous studies by use of Lipofectamine 2000 (Invitrogen).[39] PPARδ-siRNA targeting human PPARδ mRNA (ON-TARGETplus SMART pool), and Control-siRNA (ON-TARGETplus Non-targeting siRNA#1) were obtained from Dharmacon (Lafayette, CO). Human BMECs were treated with PPARδ-siRNA or Ct-siRNA 30 nM for five days. The protein samples were then collected for Western blot. In some experiments, cells were treated with PPARδ-siRNA or Ct-siRNA for 2 days, followed by addition of iloprost to the culture for 3 days. For knock-down of SIRT1, cells were treated with SIRT1-siRNA (ON-TARGETplus human SIRT1 siRNA-SMART pool, from Thermo Scientific), or Control-siRNA-A (Santa Cruz Biotechnology) (50 nM) for 2 days. Then cells were incubated in GW501516 (100 nM) for 3 days, and protein samples were collected.

ELISA for sAPPα, Aβ42, and Aβ40

Human BMECs were incubated with EGM2 containing iloprost or GW501516, or EGM2 alone for 48 h. Then cells were incubated in EBM2+0.5% FCS containing iloprost or GW501516, or EBM2 + 0.5% FCS for 24 h. Supernatants were collected for assay of sAPPα, using human sAPPα high sensitive assay kit (Immuno-Biological Laboratories-America, Minneapolis, MN). The supernatants were also subjected to assay for Aβ40 and Aβ42 using human Aβ40 ELISA kit and human Aβ42 ELISA kit (Invitrogen, Camarillo, CA). Microvessels isolated from whole brain were incubated in 200 µl EBM2 containing 0.1% BSA for 24 h. The supernatants were then collected and subjected to measurement of sAPPα using mouse/rat sAPPα high sensitive assay kit (Immuno-Biological Laboratories-America) according to the manufacturer's protocol.

Statistical analysis

Data are presented as mean ± SEM. Differences between mean values of multiple groups were analyzed using 1-way ANOVA followed by the Tukey test (SigmaStat 12.0 for Windows). Unpaired Student *t*-test was used to analyze comparison between two groups. $P < 0.05$ was considered statistically significant.

Results

To determine the role of PGI_2 in APP processing, we first examined the effects of the stable analogue of PGI_2, iloprost, in cultured human BMECs. Iloprost treatment caused concentration-dependent increase in protein expression of APP (Figure 1(a)). The time course experiments demonstrated that treatment with iloprost for 48–72 h, but not 24 h, increased APP protein levels (Figure 1(b)). Iloprost also significantly augmented APP mRNA levels (Figure 1(c)). We further determined expressions of ADAM9, 10, and 17, since these three metalloproteases of the ADAM family have been suggested to exert α-secretase activity in metabolism of APP.[17,40–42] Iloprost treatment for three days increased ADAM10 protein expression in a concentration dependent manner (Figure 1(d) and (e)). Incubation of cells with iloprost enhanced mRNA levels of ADAM10 (Figure 1(f)). The expression of ADAM 9, ADAM17, or BACE1 was not significantly altered in response to iloprost treatment (Suppl. Figure 1(A) to (C)). Effects of iloprost on expression and processing of APP led to increased sAPPα formation, but did not affect production of Aβ 40 and Aβ 42 (Figure 1(g) and (h)).

Since IP receptors are coupled to activation of adenylyl cyclase and generation of cyclic AMP, in the next series of experiments we examined the effects of forskolin (an adenylyl cyclase activator) and cicaprost (a selective agonist of IP receptor) on expression and processing of APP. Treatment of human BMECs with forskolin or cicaprost stimulated expression of APP and ADAM10 (Figure 2(a) to (d)). To validate the in vivo relevance of the findings observed in cultured human BMECs, we next performed studies in cerebral microvessels derived from IP$^{-/-}$ mice. The phenotype of IP$^{-/-}$ mice was confirmed by the experiment demonstrating that relaxation of aorta to cicarprost was abolished in IP$^{-/-}$ mice (Suppl. Figure 2(A) and (B)). In addition, in isolated brain microvessels of WT mice, cicaprost significantly increased cAMP levels; however,

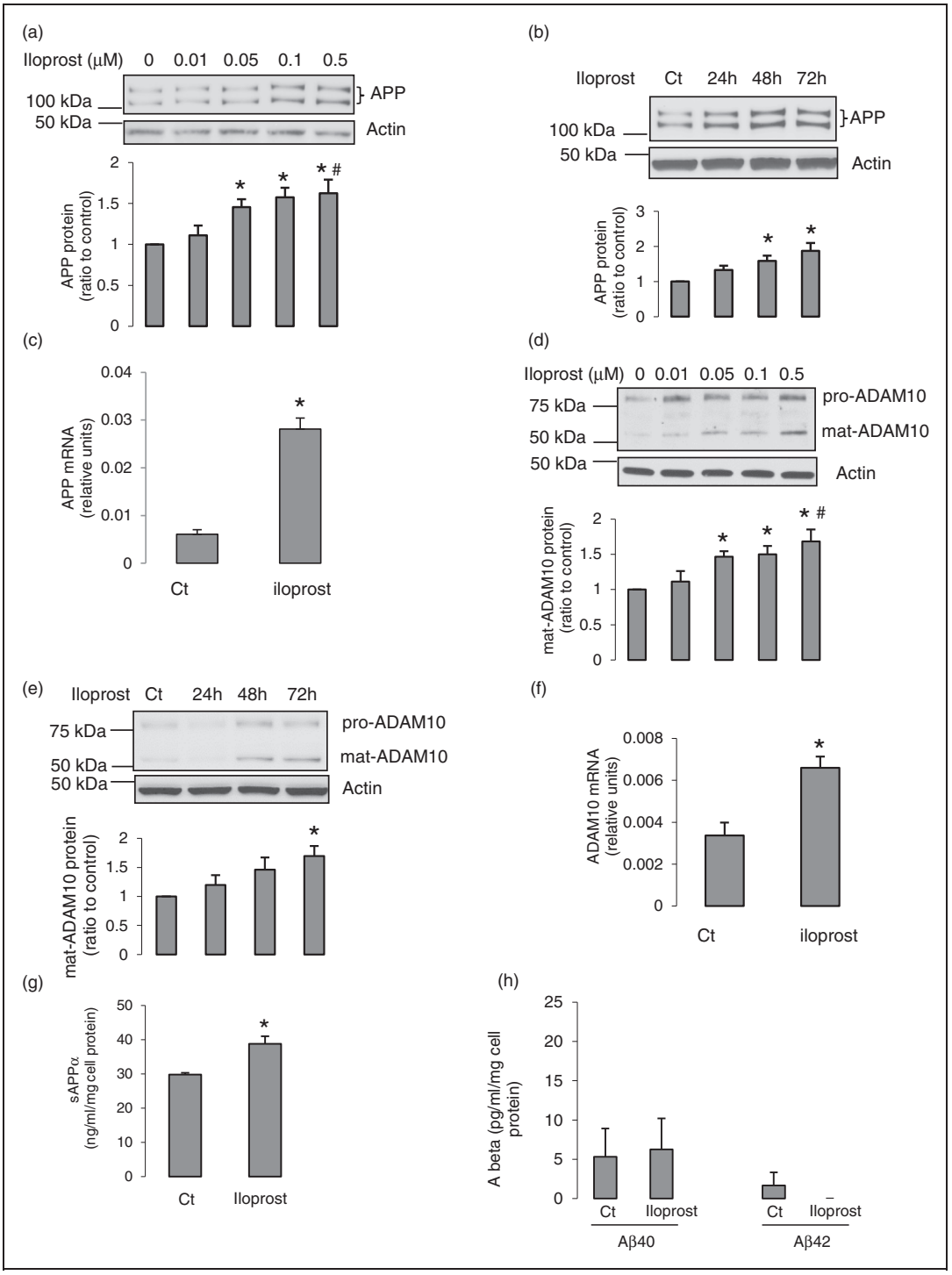

Figure 1. Iloprost stimulated production of sAPPα in human BMECs. (a) Cells were treated with iloprost with indicated concentrations for three days. Protein samples were subjected to Western blot. n = 6–8, *P < 0.05, compared to control; #P < 0.05 compared to iloporst 0.01 μM. (b) After cells were incubated with iloprost (0.5 μM) for indicated durations, APP protein expression was assayed. n = 6, *P < 0.05, compared to control. (c) Human BMECs were treated with iloprost (0.5 μM) for 57 h; cells were collected for quantitative real-time PCR. n = 7, *P < 0.05, compared to control. (d) Cells were treated with iloprost for three days. n = 5-7, *P < 0.05, compared to control; #P < 0.05 compared to 0.01 μM iloprost (pro-ADAM10 is ~ 85 kDa, mat-ADAM10 is ~ 60 kDa). (e) Time course of ADAM10 expression in response to 0.5 μM iloprost treatment. n = 6, *P < 0.05, compared to control. (f) mRNA levels of ADAM10 after treatment with 0.5 μM iloprost for 57 h. n = 4, *P < 0.05, compared to control. (g) and (h) Cells were treated with iloprost (0.5 μM) or EGM2 alone (Ct) for 48 h. Then cells were incubated in EBM2 + 0.5% FCS containing iloprost (0.5 μM), or EBM2 + 0.5% FCS for 24 h. Conditioned media were collected for measuring sAPPα (g), and Aβ42 and Aβ40 (H). n = 6, *P < 0.05, compared to control.

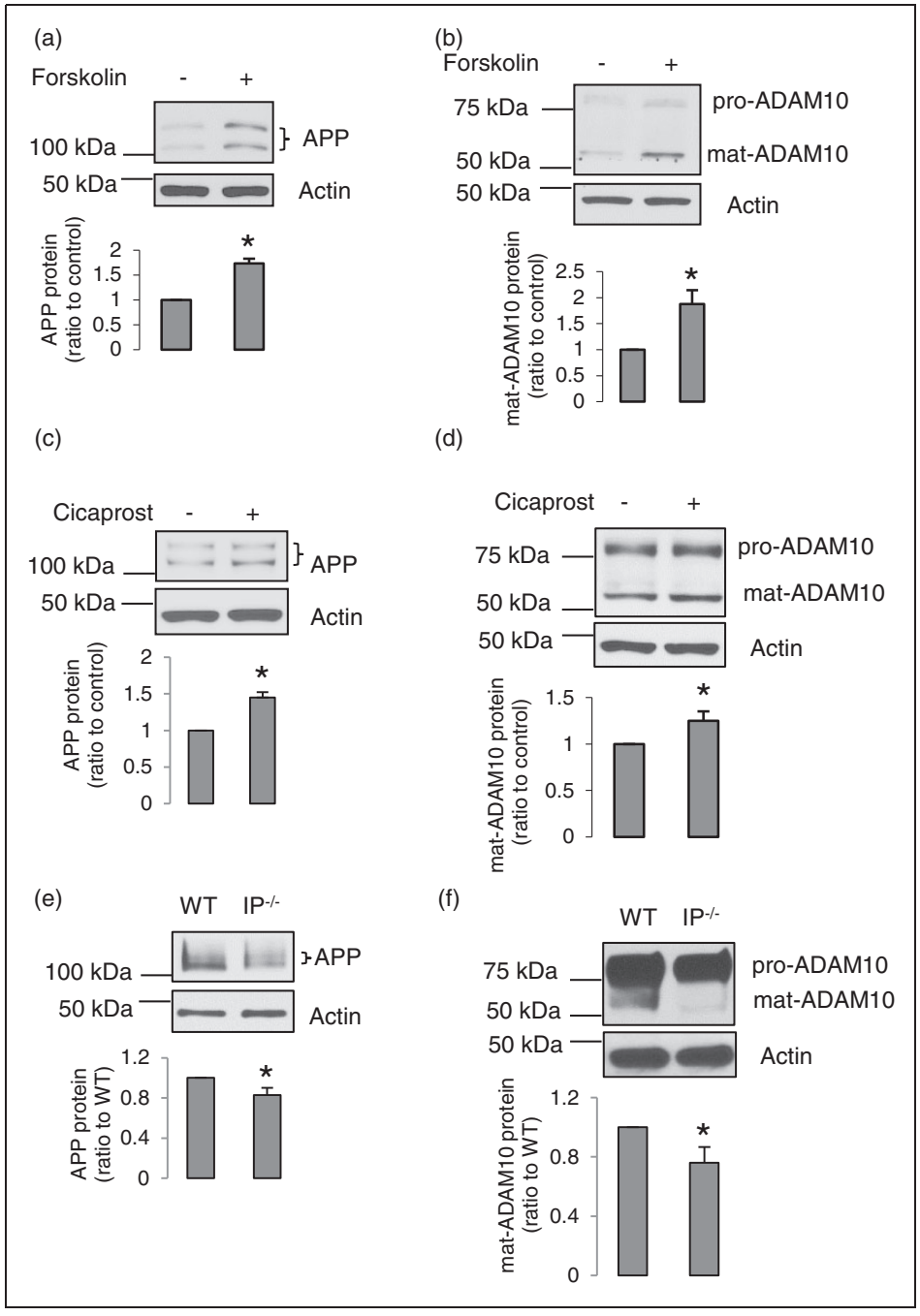

Figure 2. IP-cAMP signaling regulated expression of APP and ADAM10. (a)–(d) Activation of IP-cAMP signaling pathway stimulated protein expression of APP and ADAM10 in human BMECs. (a) and (b) Cells were treated with 40 μM forskolin for 3 days. (a), $n = 3$, $^*P < 0.05$, compared to control; (b) $n = 6$, $^*P < 0.05$, compared to control. (c) and (d) Cells were treated with 1 μM cicaprost for three days. $n = 8$, $^*P < 0.05$, compared to control. (e) and (f) Genetic deletion of IP receptor reduced cerebral microvascular expression of APP and ADAM10. Cerebral microvessels were isolated from $IP^{-/-}$ and littermate WT mice, and protein samples were subjected to Western blot. $n = 10$, $^*P < 0.05$, compared to WT mice.

brain microvessels of $IP^{-/-}$ mice failed to respond to cicaprost stimulation (Suppl. Figure 2 (C) and (D)). Consistent with findings in cultured human BMECs, protein levels of APP and ADAM10 were significantly reduced in the brain microvessels of $IP^{-/-}$ mice (Figure 2(e) and (f)). Protein expression of BACE1, ADAM9, and ADAM17 were not changed (Suppl. Figure 3(A) to (C)). Since hippocampal neuronal loss

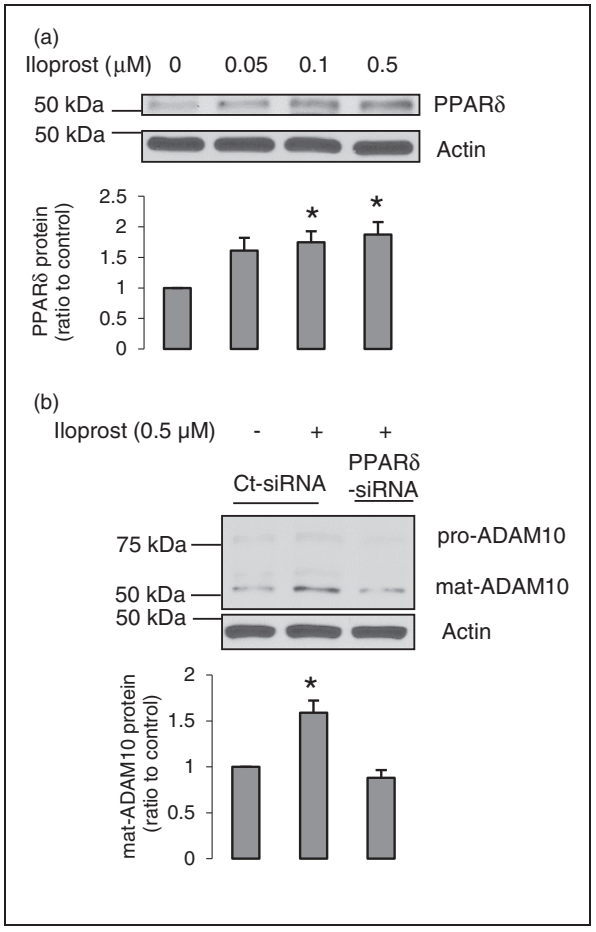

Figure 3. PPARδ-mediated iloprost-induced expression of ADAM10. (a) Human BMECs were treated with iloprost for three days. $n = 4$, $P < 0.05$, compared to control. (b) Human BMECs were treated with PPARδ-siRNA or Ct-siRNA (30 nM) for two days, then cells were treated with iloprost for three days; $n = 4$, $^*P < 0.05$ compared to other two groups.

and cognitive dysfunction have been observed in $IP^{-/-}$ mice after brain ischemic injury,[43,44] we further examined the sAPPα levels in hippocampus. As shown in Suppl. Figure 3(D), sAPPα protein was significantly reduced in hippocampus of $IP^{-/-}$ mice. Thus, our findings suggest that activation of IP receptors and the subsequent increase in cAMP stimulate expression and α-processing of APP.

Because cAMP signaling has been shown to activate PPARδ,[45,46] it is possible that PPARδ plays a role in the effect of iloprost on APP processing. Indeed, iloprost treatment increased PPARδ protein expression (Figure 3(a)). Moreover, PPARδ-siRNA abolished the stimulatory effect of iloprost on expression of ADAM10 (Figure 3(b)), suggesting that PPARδ plays a major role in mediating the iloprost-induced expression of ADAM10. To further examine the contribution of PPARδ in regulation of expression of APP and its processing enzymes, we treated human BMECs with the PPARδ selective agonist, GW501516, for three days. We observed that GW501516 stimulated ADAM10 expression (Figure 4(a) and (b)), but did not affect APP protein levels (Figure 4(c)). Moreover, the sAPPα production was increased in response to GW501516 treatment (Figure 4(d)). Shorter duration of treatment with GW501516 (for 24–48 h) did not significantly change ADAM10 expression ($n = 5$–7, data not shown), consistent with time lines of the effects of iloprost on ADAM10. GW501516 did not affect protein levels of ADAM9, ADAM17, and BACE1 (Suppl. Figure 4(a) to (c)). Deletion of PPARδ using siRNA reduced ADAM10 protein levels (Figure 4(e)), but did not significantly change expression of APP (Figure 4(f)), ADAM9, ADAM17, or BACE1 (Suppl. Figure 4(D) to (F)). Therefore, activity of PPARδ in human BMECs appears to be involved only in regulation of ADAM10 but not APP expression. In addition, we tested the effect of PPARα agonist WY14643 and PPARγ agonist rosiglitazone on ADAM10 expression in human BMECs. As shown in Suppl. Figure 5(A), WY14643 had no effect on ADAM10 protein expression, whereas rosiglitazone significantly reduced ADAM10 protein expression. Moreover, PPARδ-siRNA did not affect protein expression of PPARα and PPARγ (Suppl. Figure 5(B) and (C)). These results indicate that the regulatory role of PPARδ in expression of ADAM10 is not dependent on other two isoforms.

To further investigate the role of PPARδ in APP processing in vivo, we used $ePPAR\delta^{-/-}$ mice, in which PPARδ is exclusively deleted in the endothelium.[32] Here we confirmed that PPARδ was knocked-down in the endothelium of cerebral microvessels derived from $ePPAR\delta^{-/-}$ mice. In cultured cerebral microvascular endothelial cells of $ePPAR\delta^{-/-}$ mice PPARδ mRNA was deleted (Suppl. Figure 6(A) and (B)). Furthermore, in isolated cerebral microvessels of $ePPAR\delta^{-/-}$ mice, the mRNA levels of PPARδ were also decreased, as compared to that of cerebral microvessels derived from WT littermates (Suppl. Figure 6(C)). The incomplete deletion of PPARδ in microvessels of $ePPAR\delta^{-/-}$ mice most likely reflects presence of other cells such as smooth muscle cells in the isolated cerebral microvessel preparation. Most importantly, in isolated microvessels of $ePPAR\delta^{-/-}$ mice, the levels of ADAM10 protein and mRNA were significantly reduced, as compared to WT littermates (Figure 5(a) and (b)). Interestingly, the protein level of ADAM17 was increased in isolated microvessels of $ePPAR\delta^{-/-}$ mice (Figure 5(c)). But, protein expression of ADAM9, BACE1, and APP was not significantly changed by deletion of PPARδ in cerebrovascular endothelium (Figure 5(d) to (f)). Importantly,

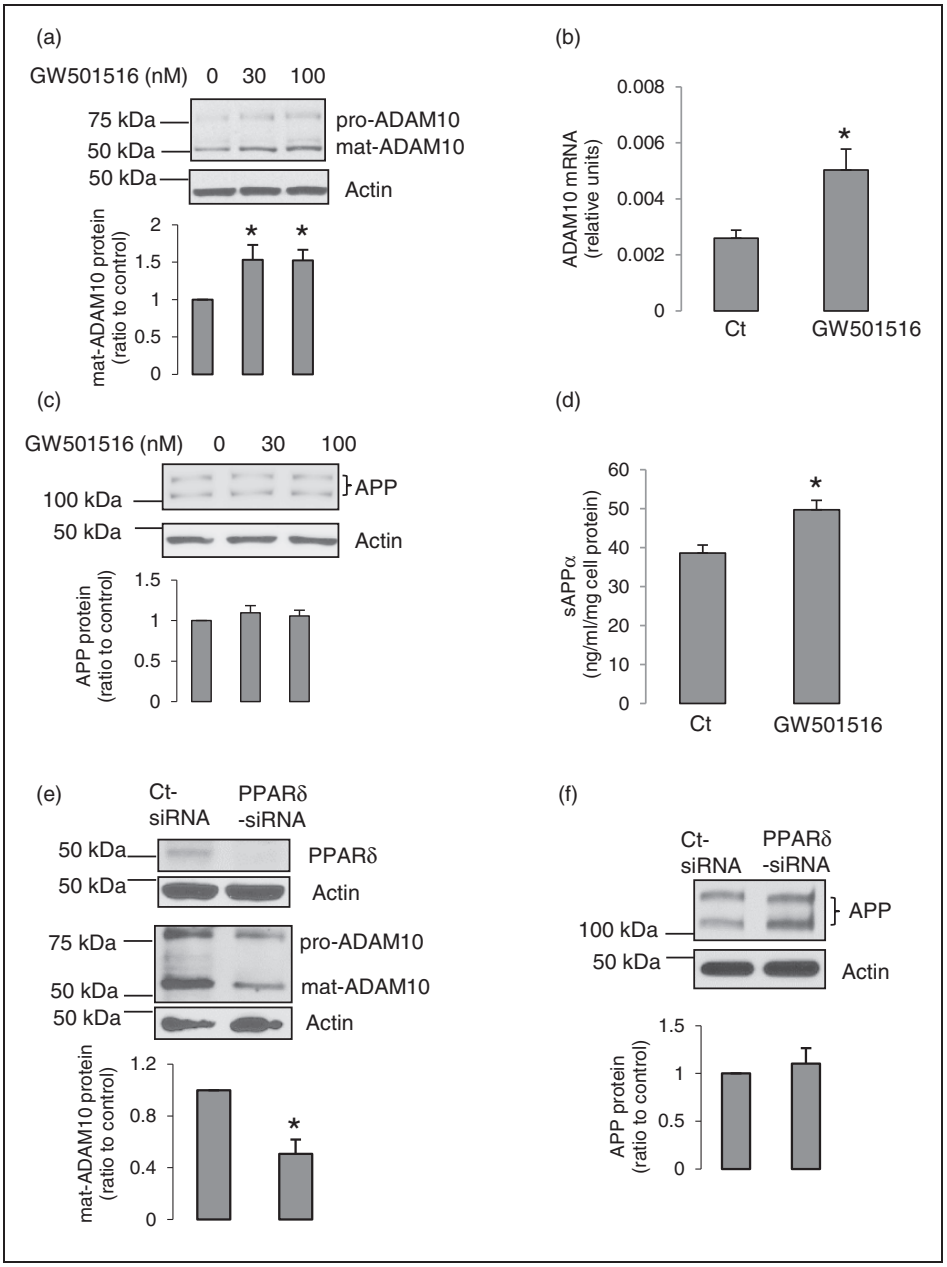

Figure 4. PPARδ regulated ADAM10 expression and sAPPα production in human BMECs. (a) Cells were cultured in EGM2 with indicated concentrations of GW501516 for 3 days. Cells were collected for Western blot. n = 6, *P < 0.05, compared to control (no GW501516 treatment). (b) Human BMECs were cultured in EGM2 with GW501516 (100 nM) or EGM-2 (Ct) for 48 h, then in EBM2 + 0.5%FCS + GW501516 or EBM2 + 0.5%FCS (Ct) for 9 h. Cells were collected for quantitative real-time PCR. n = 4, *P < 0.05, compared to control. (c) APP protein levels in response to GW501516 treatment for three days, n = 6. (d) Cells were cultured in EGM2 with GW501516 (100 nM) or EGM2 alone for 48 h, then in EBM2 + 0.5%FCS with or without GW501516 (100 nM) for 24 h. The conditioned media were collected for measuring sAPPα n = 9–10, *P < 0.05, compared to control. (e) and (f) Human BMECs were treated with PPARδ-siRNA or Ct-siRNA (30 nM) for 5 days. n = 4, *P < 0.05 compared to Ct-siRNA.

release of sAPPα from isolated brain microvessels of ePPARδ$^{-/-}$ mice was significantly reduced (Figure 5(g)). Most notably, sAPPα protein level was significantly reduced in hippocampus of ePPARδ$^{-/-}$ mice, as compared to WT mice (Figure 5(h)). Endothelial knockdown of PPARδ did not affect sAPPα levels in cortex and cerebellum (Figure 5(h)). In vivo treatment of WT mice with GW501516 for two weeks significantly increased sAPPα content in hippocampus, but not in cortex and cerebellum (Figure 5(i)). GW501516 had no effect on sAPPα levels in all three brain regions derived from ePPARδ$^{-/-}$ mice (Figure 5(j)). These results

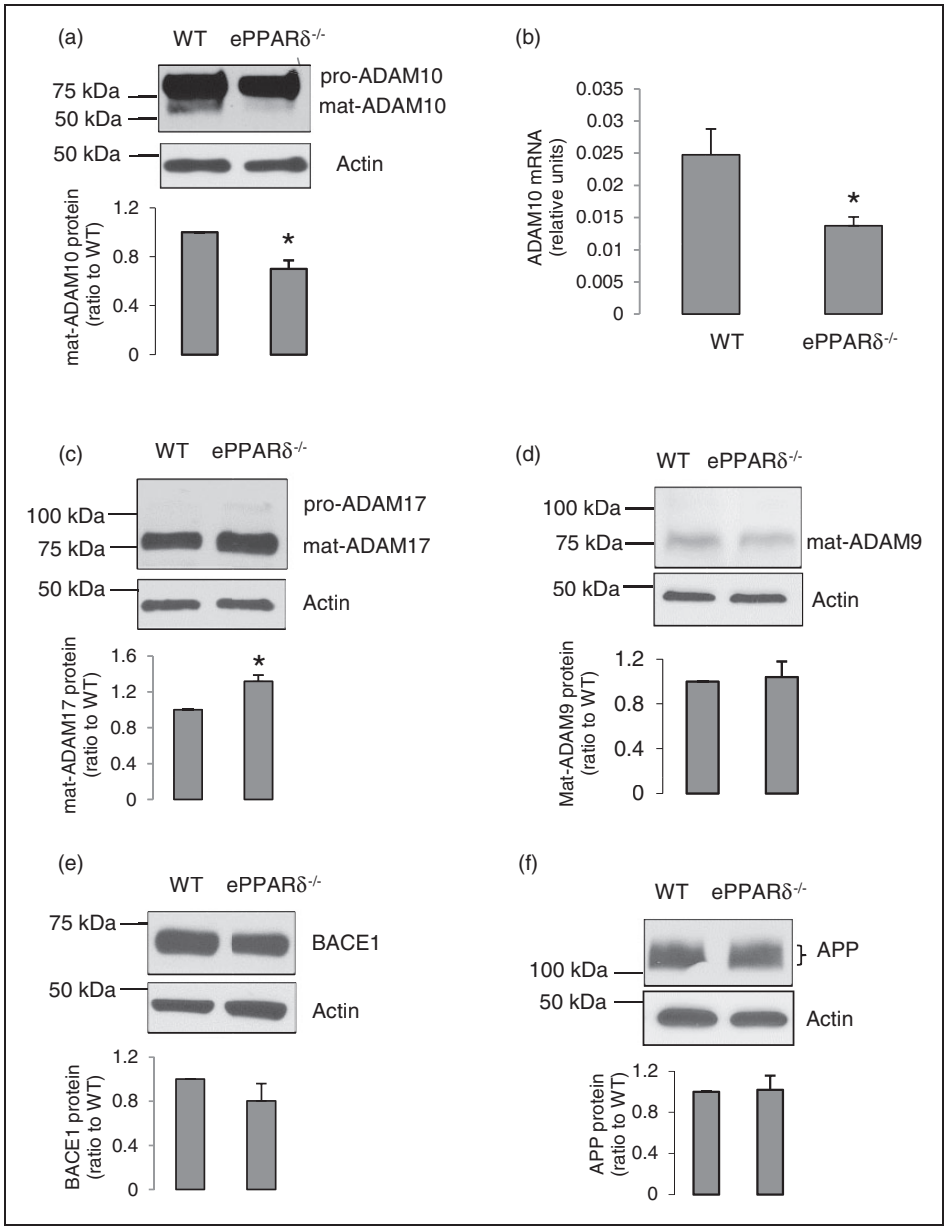

Figure 5. PPARδ regulated ADAM10 expression and sAPPα production in vivo. (a) ADAM10 protein expression in brain microvasculature of ePPARδ$^{-/-}$ mice is decreased. n = 18, *P < 0.05, compared to wild type. (b) mRNA levels of ADAM10 in brain microvasculature of ePPARδ$^{-/-}$ mice is reduced, in compared to wild type mice, n = 5, *P < 0.05. (c) Protein levels of ADAM17 were increased in brain microvasculature of ePPARδ$^{-/-}$ mice, n = 4, *P < 0.05, compared to wild type. (d)–(f) The cerebral microvascular expression of ADAM9 (D, n = 13), BACE1 (E, n = 4), and APP (f, n = 19) are not significantly different between WT and ePPARδ$^{-/-}$ mice. (g) sAPPα production was decreased in cerebral microvessels of ePPARδ$^{-/-}$ mice. Brain microvessels isolated from whole brain of ePPARδ$^{-/-}$ and wild type mice were incubated in EBM2 containing 0.1% BSA for 24 h. The supernatants were collected for measuring sAPPα. n = 17–19, P < 0.05. (h) Protein levels of sAPPα in brain regions. Note that in hippocampus of ePPARδ$^{-/-}$ mice sAPPα level was decreased compared to WT mice (n = 11, *P < 0.05). (i) In wild type mice, GW501516 treatment increased sAPPα protein level in hippocampus (n = 10, *P < 0.05, compared to vehicle treatment). (j) In ePPARδ$^{-/-}$ mice, GW501516 treatment did not affect sAPP α levels in all three brain regions (n = 5).

indicated that endothelial PPARδ was critical for ADAM10 expression and sAPPα production in mouse cerebral microvessels, as well as sAPPα content in hippocampus.

It has been suggested that ADAM10 gene transcription is up-regulated by SIRT1 via activation of retinoid acid receptor/retinoid X receptor (RAR/RXR) heterodimers in neuroblastoma and neuroglioma cells.[47,48]

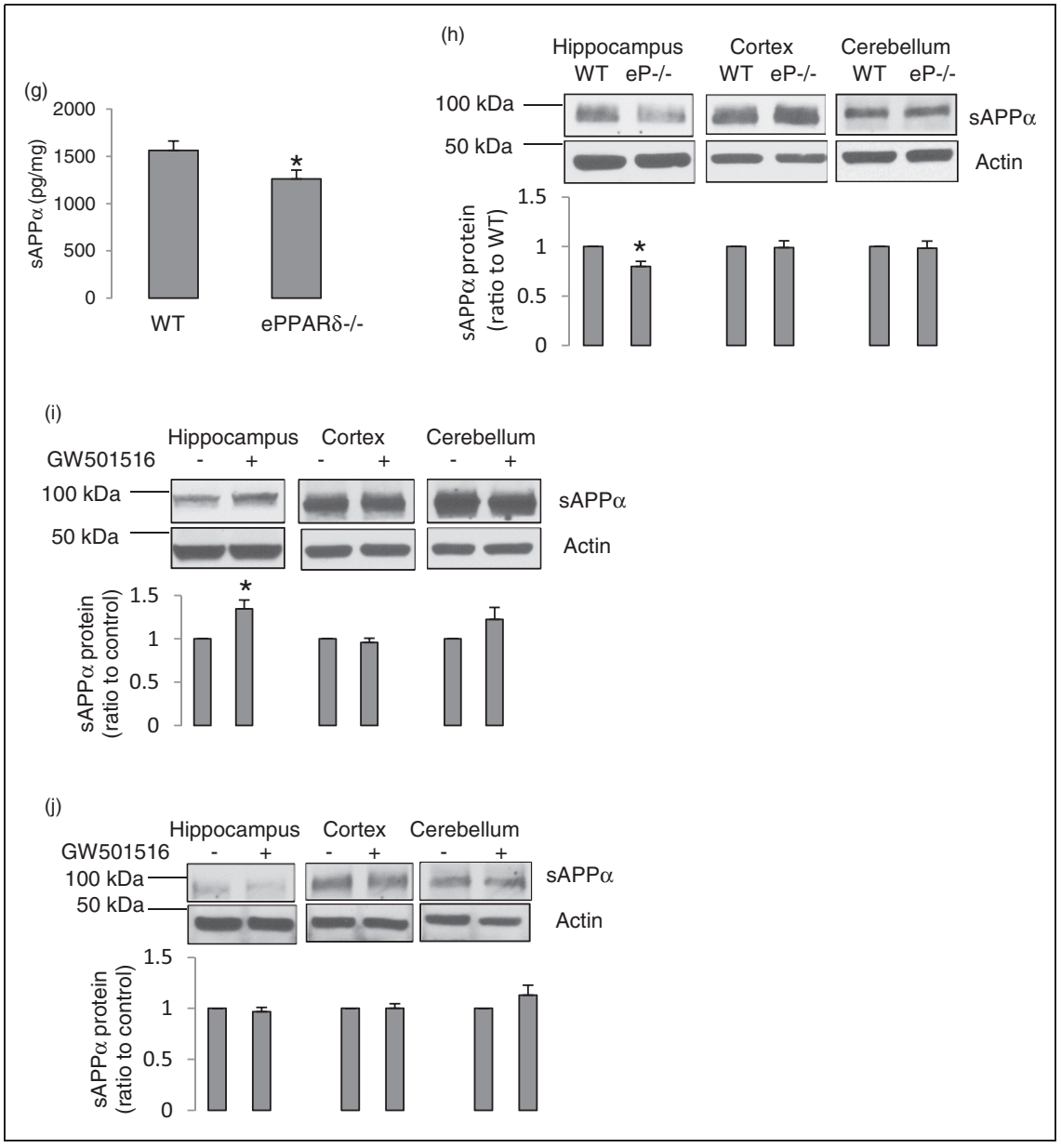

Figure 5. Continued.

It was also observed that SIRT1 gene transcription could be regulated by PPARδ.[49,50] Therefore, we further explored the relationship between PPARδ, SIRT1, and ADAM10. In human BMECs, both iloprost and forskolin up-regulated SIRT1 protein levels (Figure 6(a) and (b)), indicating that IP-cAMP signaling induced expression of ADAM10 might be mediated by SIRT1. Activation of PPARδ using GW501516 also enhanced SIRT1 protein expression (Figure 6(c)), while PPARδ-siRNA reduced SIRT1 protein levels (Figure 6(d)). PPARδ-siRNA also blocked stimulatory effect of iloprost on expression of SIRT1 (Figure 6(e)), suggesting that PPARδ mediates iloprost-induced SIRT1 expression. Notably, SIRT1-siRNA did not affect PPARδ protein expression (Suppl. Figure 7); however, SIRT1-siRNA inhibited GW501516-induced expression of ADAM10 (Figure 6(f)), indicating that SIRT1 was downstream of PPARδ in the ADAM10 regulation pathway (Figure 7).

Discussion

In the present study, we report several novel findings. First, activation of IP-cAMP signaling pathway up-regulates expression of APP and ADAM10 and stimulates production of sAPPα in human BMECs and

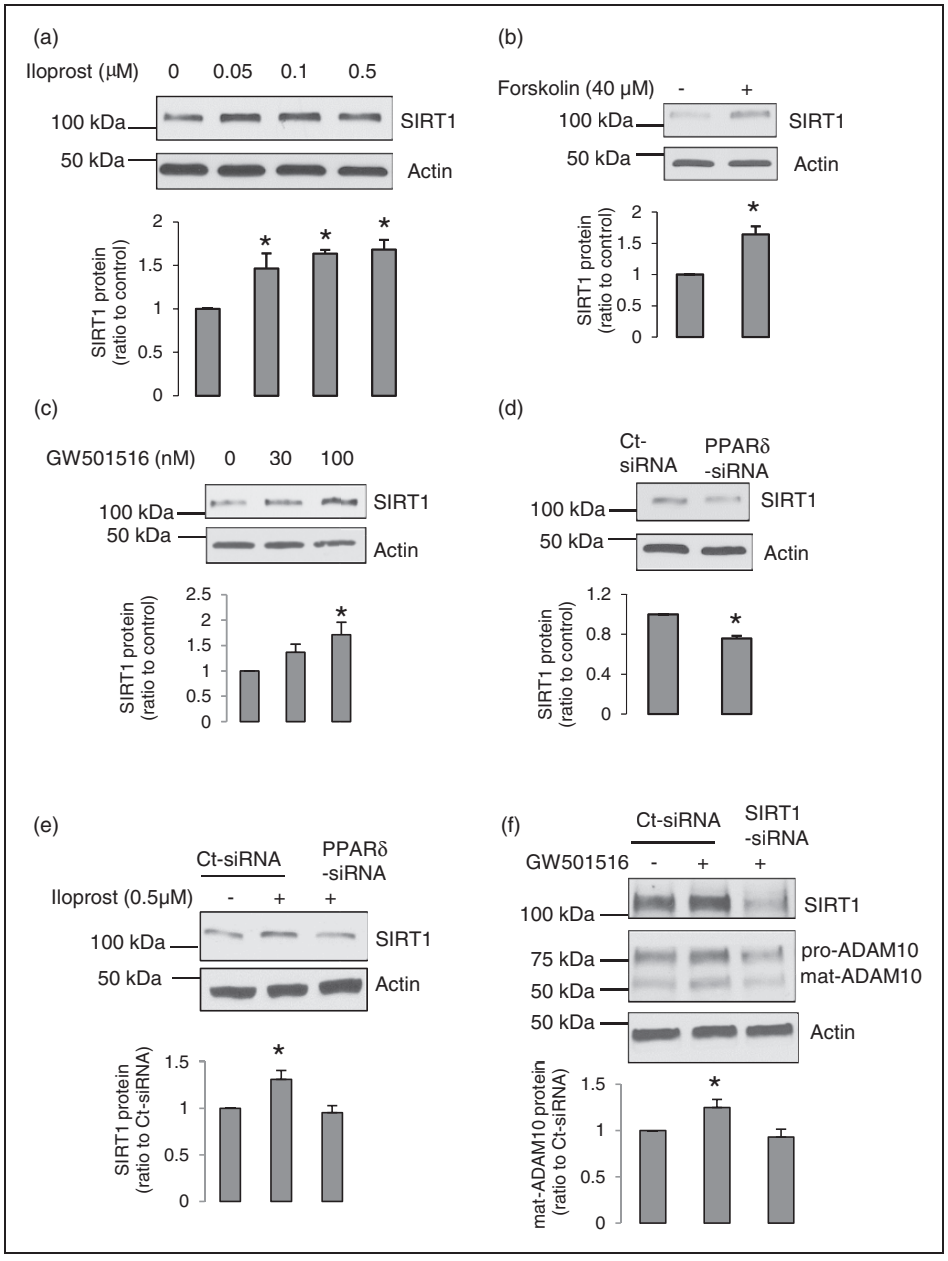

Figure 6. Role of SIRT1 in the regulation of ADAM10. (a)–(c) Protein levels of SIRT1 in human BMECs in response to iloprost ($n = 4$), forskolin ($n = 7$), or GW501516 ($n = 6$) treatment for three days. *$P < 0.05$, compared to control. (d) Cells were treated with PPARδ-siRNA or Ct-siRNA (30 nM) for five days. $n = 4$, *$P < 0.05$, compared to Ct-siRNA. E, human BMECs were treated with PPARδ-siRNA or Ct-siRNA (30 nM) for two days, then treated with iloprost for three days. $n = 11$, *$P < 0.05$, compared to other two groups. (f) Cells were incubated with SIRT1-siRNA or Ct-siRNA (50 nM) for two days, then treated with GW501516 (100 nM) for three days. $n = 12$, *$P < 0.05$, compared to two other groups.

murine cerebral microvasculature. Second, PPARδ plays a critical role in regulation of ADAM10 expression and production of sAPPα stimulated by iloprost. Third, PPARδ is coupled to activation of SIRT1 and subsequent increase in expression of ADAM10 in human BMECs. Fourth, endothelial PPARδ activity is a critical determinant of sAPPα level in mouse hippocampus.

Our previous studies have demonstrated that APP is highly expressed in vascular endothelium[13] thereby suggesting that intraluminal release of the products of APP processing from the endothelium may play an important role in vascular homeostasis and hemostasis. Most notably, previous studies have provided evidence that sAPPα released from the endothelium contains a KPI

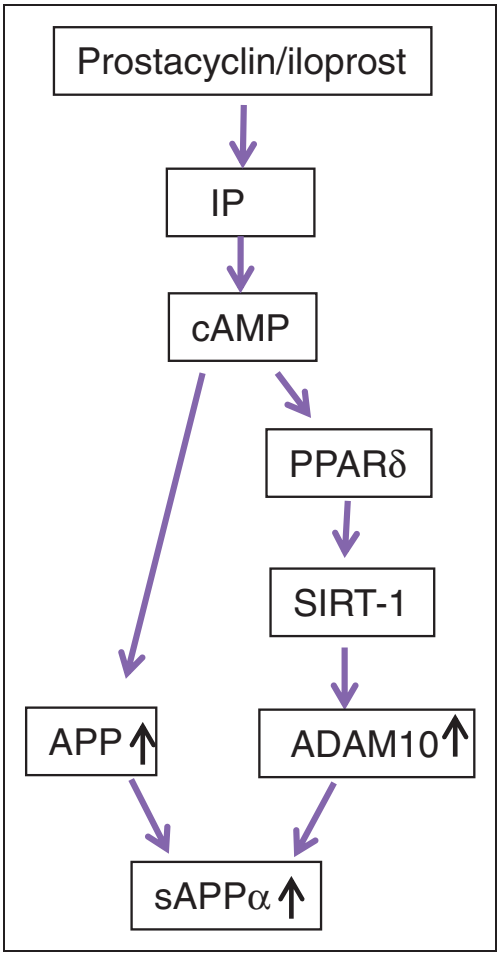

Figure 7. Schematic summary of possible mechanism of PGI_2 signaling responsible for sAPPα production.

domain, an established anticoagulation factor.[12,14] Our studies are the first to demonstrate that in human BMECs, activation of the IP-cAMP signaling pathway stimulates expression of APP and production of sAPPα, an anticoagulant and neuroprotective molecule derived from non-amyloidogenic processing of APP. In contrast, deletion of IP receptor in mouse reduces APP protein levels in cerebral microvessels, indicating an important in vivo role of IP-cAMP signaling in regulation of cerebrovascular expression of APP.

The ADAMs are type 1 transmembrane proteins with endoproteolytic activities. By shedding the ectodomains of various membrane proteins, they are involved in physiological and pathological processes such as cell growth, adhesion, and migration.[17] Among all ADAMs, ADAM9, 10, and 17 participate in α-secretase activity-mediated shedding of APP.[17] ADAM10 is responsible for the constitutive α-secretase activity in primary neurons,[51] and contributes to both constitutive and inducible α-secretase activity in the central nervous system.[52,53] In vivo and in vitro studies suggest that the α-secretase activity of ADAM17 is primarily PKC-regulated (inducible activity).[54–56] It has been suggested that the ADAM9 is not able to directly cleave APP protein at α-secretase site, but that its α-secretase activity could be related to ADAM9 regulation of ADAM10 activity.[17,57] We have detected all three ADAMs in mouse brain microvessels and human BMECs. Interestingly, endothelial deletion of PPARδ in mouse decreased sAPPα production, accompanied by a significant reduction of ADAM10 expression and increased ADAM17 protein level, indicating that in brain microvessels sAPPα production is primarily determined by activity of ADAM10. The mechanisms underlying increased ADAM17 expression in brain microvessels of ePPARδ$^{-/-}$ mice is unknown and remains to be determined. Of note, in vitro knockdown of PPARδ in human BMECs did not change ADAM17 expression. We do not have an explanation for discrepancy between in vivo and in vitro results, other than the possibility that species difference or experimental conditions may account for our observations.

PPARδ belongs to the nuclear hormone receptor family of ligand-activated transcription factors.[8] PPARδ is ubiquitously expressed in various tissues, including the vasculature, and plays important roles in the regulation of lipid and glucose metabolism, inhibition of inflammation, and improvement of insulin sensitivity.[8] Preclinical experiments also demonstrated strong vascular protective effects of PPARδ agonists.[8] Our previous studies have demonstrated that endogenous PGI_2-dependent angiogenesis is at least in part mediated by activation of PPARδ.[31] In addition, activation of PPARδ protects cerebral microvasculature from oxidative stress,[33] indicating that activity of PPARδ may play a critical vascular protective role in the cerebral circulation. Our current findings revealed a previously unrecognized role of PPARδ in the regulation of ADAM10 in the cerebral microvessels. We demonstrated that PPARδ-siRNA abolished iloprost-induced expression of ADAM10, indicating that PPARδ was a major mediator of the iloprost-induced expression of ADAM10. Since cAMP signaling has been shown to activate PPARδ,[8,45,46] it is likely that activation of IP-cAMP signaling augments ADAM10 expression via activation of PPARδ (Figure 7). It has also been reported that in vitro both PGI_2 and iloprost could directly bind to PPARδ,[8,58,59] therefore, we cannot rule out the possibility that direct activation of PPARδ by iloprost might contribute to increase in expression of ADAM10.

We also observed that iloprost and forskolin augmented SIRT1 protein levels in endothelial cells. This finding is consistent with the results of a previous study demonstrating that in mouse carotid arteries activation

of IP receptors activates SIRT1.[60] Our findings indicate that SIRT1 may also mediate iloprost and cAMP signaling-induced up-regulation of ADAM10. Since iloprost augmented ADAM10 protein expression via PPARδ-SIRT1 pathway, and activation of PPARδ by GW501516 also increased SIRT1 protein levels, it is likely that IP-cAMP activation stimulated PPARδ-SIRT1 leading to up-regulation of ADAM10. Though it has been reported that cAMP/PKA signaling phosphorylates and activates SIRT1,[61] our results demonstrating that deletion of PPARδ completely inhibit the effect of iloprost on expression of ADAM10 suggest that direct activation of SIRT1 by cAMP/PKA leading to stimulation of ADAM10 expression is unlikely a dominant pathway in our experimental model. Rather, our observations suggest that PPARδ activated by IP-cAMP signaling or directly by binding of IP ligands (such as iloprost and PGI_2), stimulates SIRT1 activity and consequently up-regulates expression of ADAM10 (Figure 7).

We do not have an explanation as to why activation of PPARδ by GW501516 increases production of sAPPα but does not affect protein expression of APP. It has been reported that both ubiquitin dependent and independent mechanisms of proteasome mediate APP degradation,[62] and that PPAR isoforms regulate proteosomal degradation.[63] Therefore, it is possible that GW501516 may also affect metabolism of APP via proteasome system[62,63] thereby preserving apparently normal protein levels of APP despite increased α-processing of APP. We also wish to point out that similar to our findings in endothelium, previous studies in neuronal tissue reported that activation of protein kinase C increased production of sAPPα without affecting expression of APP protein.[64,65] The exact mechanisms responsible for discrepancy between increased generation of sAPPα and preservation of APP protein expression are currently unknown and remain to be determined.

Relevant to our study, it has been shown that reduction of cholesterol promotes APP processing via the non-amyloidogenic α-secretase pathway, increasing the formation of sAPPα, and decreasing Aβ production.[66] Our previous studies have revealed that ePPARδ$^{-/-}$ mice have normal lipid profile,[32] therefore ruling out the possibility that suppressed ADAM10 expression in cerebral microvessels of ePPARδ$^{-/-}$ mice is caused by changes in lipid profile. Moreover, experiments in cultured human BMECs indicated that PPARδ up-regulated ADAM10 expression, thus minimizing the possibility that systemic factors were involved in reduced expression of ADAM10 in ePPARδ$^{-/-}$ mice.

Endothelium-derived PGI_2 is an essential molecule responsible for preservation of cerebrovascular homeostasis under pathological conditions when blood vessels are exposed to injury induced by stroke, inflammation, and brain trauma.[1–3,6,7,43,44] Indeed, PGI_2 has been identified as a major protective product of arachidonic acid metabolism within the vasculature.[6] In the cerebral circulation, elevated production of PGI_2 in response to vascular injury is designed to prevent aggregation of platelets, to preserve blood flow, and to inhibit aberrant remodeling of injured vascular wall.[3,34] However, no previous studies have addressed the role of PGI_2 in regulation of APP processing. Recognition of the ability of PGI_2 to regulate expression and non-amyloidogenic processing of APP suggests that this mechanism might be an important contributor to overall neurovascular protective functions of PGI_2.

Prior studies established that activation of IP receptors exerts neuro-protective effects during focal cerebral ischemia, transient global cerebral ischemia, and traumatic brain injury.[3,43,44,67] Moreover, systemic administration of PGI_2 analogue, beraprost, attenuated neuronal damage caused by ischemic stroke[67] thereby suggesting that activation of vascular IP receptors may contribute to tissue protective effects of beraprost. In the present study, we provide in vitro evidence that activation of IP receptors increases expression and α-processing of APP and generation of neuroprotective and anticoagulant cleavage product sAPPα. Moreover, we found that hippocampal sAPPα was significantly reduced in IP$^{-/-}$ mice. These findings are in agreement with previous observations that impairment of PGI_2 signaling enhances loss of neuronal cell in hippocampus exposed to brain ischemia or traumatic brain injury,[43,44] conditions known to be associated with neuro-protective activation of COX2-PGI_2 signaling.[3,7,68]

In line with our findings regarding an important role of PPARδ in production of sAPPα, previous studies also demonstrated that activation of PPARδ reduces ischemia-induced brain vascular endothelial injury,[69] and attenuates injury induced by ischemic stroke.[70] Moreover, previous study established important role of PPARδ/SIRT1 activation in anticoagulant effect of PGI_2.[71] Another notable prior observation was that PPARδ agonist, GW501516, improved spatial memory in wild type mice under physiological conditions.[72] Consistent with this observation, we found that systemic administration of GW501516 increased levels of sAPPα in hippocampus. Since sAPPα level was decreased in hippocampus of in ePPARδ$^{-/-}$ mice, and loss of endothelial PPARδ abolished GW501516-induced increase of hippocampal sAPPα, PPARδ signaling in cerebrovascular endothelium appears to be responsible for the changes of sAPPα content in hippocampus. This explanation is also consistent with

prior findings demonstrating that GW501516 does not cross blood-brain barrier.[73]

At the present time, the reasons for selective elevation of sAPPα in hippocampus are unknown. However, findings from several prior studies are relevant for understanding of our observations: (a) vascular architecture and function may vary in different brain regions,[74] (b) albumin can access the parenchyma of the hippocampus under normal conditions,[75] and (c) different transport rates of chemokine in different brain regions have been reported.[76] Moreover, a distinguished feature of subgranular zone of hippocampus is that angiogenesis and neurogenesis are tightly coupled[74] thereby maintaining strong interaction between vascular and neuronal compartment. These findings imply that in hippocampus, large proteins including sAPPα produced in endothelium might have access to neuronal tissue. In addition, activation of PPARδ may exert more pronounced effect in hippocampal microvessels as compared to microvessels derived from other brain regions. Of note, it has been reported that in hippocampus exercise stimulates angiogenesis and neurogenesis.[74,77,78] Since activation of PPARδ mimics beneficial effects of exercise,[79,80] it is possible that hippocampal vasculature is generally more dependent on activation of PPARδ for production of sAPPα. In aggregate, our findings provide new insights into signal transduction mechanisms that might help to explain therapeutic effects of PPARδ agonists.[81]

In the present study, we provide in vitro and in vivo evidence that in cerebral microvessels activation of IP receptors promotes expression and α-processing of APP. Our results suggest that in hippocampus, endothelial PPARδ mediates IP signaling-induced production of sAPPα. These findings have important implications for understanding the mechanisms underlying neurovascular protective function of PGI_2.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

Tongrong He: experiments design and performance, data collection and analysis, and manuscript writing.

Anantha Vijay R. Santhanam: isolation of brain microvessels.

Tong Lu: performed quantitative real-time PCR and data analysis.

Livius V. d'Uscio: performed vascular reactivity experiments and data analysis.

Zvonimir S. Katusic: experiment design and manuscript writing.

Supplementary material

Supplementary material for this paper can be found at http://jcbfm.sagepub.com/content/by/supplemental-data

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