Activation of PPARδ prevents endothelial dysfunction induced by overexpression of amyloid-β precursor protein

Livius V. d’Uscio¹,²*, Pritam Das³, Anantha V.R. Santhanam¹,², Tongrong He¹,², Steven G. Younkin³, and Zvonimir S. Katusic¹,²*

¹Department of Anesthesiology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA; ²Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA; and ³Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, FL, USA

Received 27 March 2012; revised 19 July 2012; accepted 3 August 2012; online publish-ahead-of-print 10 August 2012

Time for primary review: 26 days

Aims
Existing evidence suggests that amyloid-β precursor protein (APP) causes endothelial dysfunction and contributes to pathogenesis of atherosclerosis. In the present study, experiments were designed to: (1) determine the mechanisms underlying endothelial dysfunction and (2) define the effects of peroxisome proliferator-activated receptor delta (PPARδ) ligand on endothelial function in transgenic Tg2576 mice overexpressing mutated human APP.

Methods and results
Confocal microscopy and western blot analyses of wild-type mice aortas provided evidence that APP protein is mainly present in endothelial cells. Overexpression of APP significantly impaired endothelium-dependent relaxations to acetylcholine and phosphorylation of endothelial nitric oxide synthase at Ser1177 in aortas. HPLC analysis revealed that tetrahydrobiopterin (BH4) levels were reduced in Tg2576 mice aortas. This was caused by increased oxidation of BH4 and reduced expression and activity of GTP-cyclohydrolase I. Furthermore, gp91phox protein expression and superoxide anion production were increased in aortas of Tg2576 mice. This augmented superoxide formation was completely prevented by the NADPH oxidase inhibitor VAS2870. Expression of copper-/zinc-superoxide dismutase (Cu/ZnSOD) and extracellular SOD was downregulated. Treatment with PPARδ ligand GW501516 (2 mg/kg/day) for 14 days significantly increased BH4 bioavailability and improved endothelium-dependent relaxations in Tg2576 mice aortas. GW501516 also normalized protein expression of gp91phox and SODs, thereby reducing production of superoxide anion in the aortas.

Conclusion
Our results suggest that in APP transgenic mice loss of nitric oxide and increased oxidative stress are the major causes of endothelial dysfunction. The vascular protective effects of GW501516 in Tg2576 mice appear to be critically dependent on prevention of superoxide anion production.

Keywords
Amyloid-β precursor protein • Endothelial function • Superoxide anion • Tetrahydrobiopterin • Atherosclerosis

1. Introduction
The amyloid-β precursor protein (APP) is an integral membrane protein expressed abundantly in the brain. Consecutive proteolysis of APP by β- and γ-secretase generates amyloid-β (Aβ) peptides, which are major culprits in pathogenesis of Alzheimer’s disease.¹ Recent evidence suggests that APP is also present in vascular cells including endothelium and that it may play a role in pathogenesis of atherosclerosis.²,³ Indeed, several studies have demonstrated that APP and Aβ are present in advanced human carotid plaques and in human and apoE-deficient mice atherosclerotic aortas.⁴ The development of non-dietary induced early atherosclerotic lesions has been observed in transgenic Tg2576 mice overexpressing double Swedish mutated human APP (K670N/M671L).⁵ Moreover, APP overexpression accelerated aortic atherosclerotic development in apolipoprotein E-deficient mice.⁶ Conversely, genetic deletion of APP attenuated atherogenesis in apolipoprotein E-deficient mice.⁷ Nitric oxide (NO) plays a key role in the control of the cardiovascular system.⁸ In the blood vessel wall, NO is mainly formed in...
endothelial cells from L-arginine by enzymatic activity of endothelial NO synthase (eNOS). It is well established that tetrahydrobioperin (BH4) is an essential cofactor for allosteric and redox activation of eNOS enzymatic activity. Exposure of vascular endothelial cells to exogenous Aβ peptide results in cell damage and toxicity via oxidative injury. Likewise, previous studies showed that overexpression of APP impairs metabolism of BH4 and antioxidant capacity of the arterial wall, thereby causing endothelial dysfunction.

Peroxisome proliferator-activated receptor-delta (PPARδ), together with the other two isoforms, PPARα and PPARγ, constitute the PPAR subfamily of the nuclear receptor superfamily. While there are extensive studies on the vascular protective effects of PPARα and PPARγ, i.e. activation of PPARα or PPARγ increases eNOS expression and NO release in endothelial cells, there are only a few studies with regard to the vascular function of PPARδ. Animal studies have demonstrated that PPARδ activation exerts many favourable effects, including reduction of weight gain, increase in skeletal muscle metabolic rate, and improvement of insulin sensitivity. In addition, PPARδ is also expressed in endothelial cells although its function remains poorly understood. Recently, treatment with PPARδ agonist was shown to suppress vascular inflammation and to prevent development of atherosclerosis in apoE-deficient mice. Because of these effects of PPARδ on vascular homeostasis, we also hypothesized that PPARδ activation might protect endothelial function in APP transgenic Tg2576 mice.

2. Methods

2.1 Experimental animals

Transgenic Tg2576 mice overexpressing double Swedish mutated human APP (K670N/M671L) and non-transgenic littermates were provided by Dr Steven Younkin (Mayo Clinic, Jacksonville, FL, USA). Mice were maintained on standard chow with free access to drinking water. Housing facilities and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals. At 16–20 weeks of age, female Tg2576 transgenic and wild-type littermates were treated daily by gavages for 14 days with either vehicle (DMSO 2%) or PPARδ agonist ligand GW501516 (2 mg/kg of BW, Alexis Biochemical) suspended in 5% carboxymethylcellulose. The dose was selected on the basis of optimal serum GW501516 levels demonstrated in conduit arteries of Tg2576 mice. However, the exact mechanisms by which APP causes endothelial dysfunction in APP transgenic Tg2576 mice have not been defined. The central hypothesis of the present study is that overexpression of APP impairs metabolism of BH4 and antioxidant capacity of the arterial wall, thereby causing endothelial dysfunction.

2.2 Glucose and plasma lipid profile

Blood samples were transferred to a tube containing EDTA. Glucose levels were measured in whole blood with Accu Check® (Roche Diagnostics, Indianapolis, IN, USA). After centrifugation at 600 g (4°C, 10 min) supernatants were stored at −80°C until assayed. Plasma cholesterol and HDL levels were measured with a Hitachi 912 chemistry analyzer using Cholesterol CHOD-PAP and direct HDL-C plus reagents, respectively (Roche Diagnostics, Indianapolis, IN, USA).

2.3 Plasma levels of Aβ peptides

ELISA kits (Invitrogen) were used to perform the measurements of plasma Aβ40 and Aβ42 levels.

2.4 Protein expression

Western blot analyses were performed in aortas as described. Primary antibodies against APP (Invitrogen), ADAM 10, nicastrin (Millipore), BACE (Cell Signalling), Cu/ZnSOD, MnSOD, EcSOD (Enzo Live Sciences), catalase (Sigma), glutathione peroxidase I (GPx; AbFrontier), eNOS, Ser1177-phosphorylated eNOS, nNOS, iNOS, cyclooxygenase-2 (COX-2), gp91phox (BD Biosciences), prostacyclin synthase (PGIS), cyclooxygenase-1 (COX-1; Cayman), and GTPCH I were used. As a loading control, blots were rehybridized with β-actin (Sigma).

2.5 Immunofluorescent imaging

Aortic rings were fixed in a 4% paraformaldehyde solution and embedded in paraffin blocks. Cross-sections of 5 μm were permeabilized using 0.1% Triton X-100 in 10% normal goat serum. Sections were incubated with rabbit anti-APP and anti-VE-Cadherin antibodies overnight at 4°C. Sections were incubated with Cy5-conjugated anti-rabbit secondary antibody and Alexa-Fluor 488 conjugated anti-mouse secondary antibody (Jackson Immuno Research) for 2 h. Sections were visualized using a Zeiss LSM 510 laser scanning confocal microscope.

2.6 Vasomotor reactivity studies

Isometric force of aortic rings was studied in organ bath as described. The data were acquired using PowerLab data acquisition system and LabVIEW 7.1 software. Concentration-dependent response curves to acetylcholine (10−7−10−10 mol/L) and diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate; 10−10−10−3 mol/L) were cumulatively obtained during submaximal contractions to phenylephrine. Concentrations of phenylephrine (1–3 × 10−7 mol/L) were selected in order to obtain the same submaximal contraction in aortic rings from both wild-type and Tg2576 mice.

2.7 Bioppterin metabolism

Bioppterin levels and GTPCH I activity were measured in aortas by HPLC.

2.8 Detection of superoxide anion

An HPLC method was used to analyze intracellular superoxide levels in intact aortas in the absence or presence of polyethylene-glycolated superoxide dismutase (PEG-SOD, 10 U/mg) for 30 min, NADPH oxidase inhibitor VAS2870 (10 μM, Enzo Live Sciences) for 30 min, or NOS inhibitor L-nitro-l-arginine methyl ester (L-NAME, 30 μM) for 15 min.

2.9 Calculations and statistical analysis

All results are expressed as means ± SEM and ‘n’ indicates the number of animals from which tissues were harvested. Relaxations (expressed as percentage of contraction) were determined for each individual concentration–response curve by non-linear regression analysis. The concentration–response curves of the different groups were compared by ANOVA for repeated measurements followed by Bonferroni’s correction. Wild-type and Tg2576 mice treated with or without GW501516 were compared by ANOVA with Bonferroni’s. For simple comparisons, an unpaired Student’s t-test was used. A value of P < 0.05 was considered significant.
3. Results

3.1 Characteristics of Tg2576 mice
Plasma levels of HDL were significantly reduced in Tg2576 mice (P < 0.05; Table 1). In contrast, plasma levels of cholesterol, blood glucose, and body weight were not different between wild-type and Tg2575 mice (Table 1). Two weeks treatment with PPARδ ligand GW501516 normalized HDL levels in Tg2576 (Table 1) while it had no effect on other parameters in wild-type and Tg2575 mice (Table 1).

3.2 Expression of APP
Western blot analyses showed that APP protein expression is present in normal wild-type mice aortas (Figure 1A). In order to evaluate the cellular localization of APP protein, we determined APP levels in mouse aortas with and without endothelium. We found that about 72% of total APP protein is present in endothelial cells of wild-type mice (Figure 1A). Confocal microscopic analysis of mouse aorta slices demonstrated that APP staining was mainly localized to endothelial cells (Figure 1B).

In Tg2576 mice, protein expression of APP was elevated in the aorta (P < 0.05; Figure 2A). Treatment with GW501516 did not affect APP expression in wild-type and Tg2576 mice aortas (Figure 2A). Protein expression of non-amyloidogenic and amyloidogenic enzymes α-secretase ADAM10 as well as β-secretase BACE and γ-secretase nicastrin were not different between wild-type and Tg2576 mice and those treated without or with GW501516 (Figure 2B–D).

3.3 Plasma levels of Aβ peptides
Transgenic Tg2576 mice exhibit elevated circulating levels of Aβ-40 and Aβ-42, which were unaffected by PPARδ ligand GW501516 (Figure 2E and F, respectively).

3.4 Endothelial function
Endothelium-dependent relaxations to acetylcholine were significantly diminished in the aorta of Tg2576 mice when compared with wild-type littermates (Figure 3A). This impairment of endothelium-dependent relaxation was completely prevented by treatment with PPARδ ligand GW501516 (Figure 3A). In contrast, endothelium-independent relaxations to NO-donor DEA-NONOate were unchanged in wild-type and Tg2576 mice treated without or with GW501516 (Figure 3B).

3.5 Protein expression of enzymes involved in the production of prostanoids
Protein expression of COX-1, COX-2, and PGI2 was unaltered in aortas of Tg2576 mice when compared with wild-type mice and those treated with GW501516 (see Supplementary material online, Figure S1).

3.6 Phosphorylation of eNOS
Western blot analysis showed a significant decrease in protein expression of phosphorylated eNOS at Ser1177 in the aorta of Tg2576 mice when compared with wild-type mice (Figure 3C). Treatment with PPARδ agonist normalized eNOS phosphorylation in Tg2576 (Figure 3C). In contrast, total eNOS proteins did not differ between wild-type littermates and Tg2576 mice and those treated with or without GW501516 (Figure 3C). Protein expression of nNOS was present in mice aortas (see Supplementary material online, Figure S2). However, this was not significantly altered in Tg2576 mice or by GW501516 treatment (see Supplementary material online, Figure S2). Protein expression of iNOS was almost undetectable in all groups of mice (see Supplementary material online, Figure S2).

3.7 BH4 metabolism
Measurements of bioperotins indicated that BH4 levels were significantly reduced in the aortas of Tg2576 mice (Figure 4A) while oxidative products of BH4, 7,8-dihydrobiopterin (7,8-BH2), were increased in Tg2576 mice (Figure 4B). Consequently, the BH4 to 7,8-BH2 ratio was significantly reduced in Tg2576 mice (Figure 4C). Two weeks treatment with the PPARδ ligand normalized levels of BH4 and 7,8-BH2, and BH4 to 7,8-BH2 ratio in Tg2576 mice (Figure 4A–C). However, treatment with GW501516 significantly decreased BH4 levels in the aorta of non-transgenic mice (Figure 4A).

In order to evaluate further the mechanisms of decreased BH4 levels in Tg2576 mice, the vascular GTPCH I, the rate limiting enzyme for de novo biosynthesis of BH4, was determined. Interestingly, GTPCH I expression and activity were significantly reduced in Tg2576 mice aortas when compared with wild-type littermates (Figure 4D and E). GW501516 treatment normalized expression and activity of GTPCH I in Tg2576 aortas (Figure 4D and E). In addition, GW501516 treatment tended to increase GTPCH I protein expression in wild-type littermates (Figure 4D).

3.8 Production of superoxide anion
HPLC analysis revealed that PEG-SOD inhabitable production of superoxide anion was increased in the aorta of Tg2576 mice (P < 0.05; Figure 5A). In vitro incubation with L-NAME had no significant

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-type</th>
<th>Wild-type + GW501516</th>
<th>Tg2576</th>
<th>Tg2576 + GW501516</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>26 ± 2</td>
<td>27 ± 1</td>
<td>25 ± 1</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>Glucose (mg/mL)</td>
<td>138 ± 10</td>
<td>154 ± 19</td>
<td>122 ± 8</td>
<td>134 ± 11</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>54 ± 4</td>
<td>52 ± 3</td>
<td>48 ± 2</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>38 ± 3</td>
<td>40 ± 2</td>
<td>31 ± 1*</td>
<td>40 ± 2**</td>
</tr>
</tbody>
</table>

Data are means ± SEM (n = 5–10).

*P < 0.05 vs. wild-type mice.

**P < 0.05 vs. transgenic T2576 mice without GW501516 (ANOVA with Bonferroni’s).
Figure 1  (A) Effect of endothelial removal on APP expression in the aortas of wild-type mice. Please note that removal of the endothelium (E−) significantly reduced APP protein expression. eNOS expression is shown to control the experiments. Results are mean ± SEM (n = 4). *P < 0.05 vs. aortas with endothelium (E+). (B) Representative confocal images showing staining of thoracic aorta of wild-type mouse aorta with anti-APP (red) and anti-VE-cadherin (green) demonstrates colocalization of the two proteins mainly in vascular endothelium (yellow). 4′,6′-diamidino-2-phenylindole dilactate (DAPI) was used to visualize nuclei. Data are representative of at least three independent experiments. L, lumen; A, adventitia. Bar denotes 50 μm.

Figure 2  Protein expression of APP (A), α-secretase ADAM10 (B), β-secretase BACE (C), and γ-secretase nicastrin (D) in aortas of wild-type and Tg2576 mice treated without (−) or with (+) GW501516. The bar graphs indicated the results of the relative densitometry compared with β-actin protein. Plasma levels of Aβ-40 and Aβ-42 are shown in (E) and (F), respectively. Results are mean ± SEM (n = 6). *P < 0.05 vs. wild-type mice (ANOVA with Bonferroni’s).
effect on superoxide anion levels in Tg2576 mice aortas (0.48 ± 0.08 nmol/mg vs. 0.58 ± 0.09 nmol/mg for Tg2576 aortas without L-NAME; n = 5–6; * P < 0.05). Interestingly, protein expression of NADPH oxidase subunit gp91phox was increased in Tg2576 aortas (P < 0.05; Figure 5B). Treatment with GW501516 significantly decreased gp91phox protein expression and superoxide production in Tg2576 mice (P < 0.05; Figure 5). Incubation of aortic rings with NADPH oxidase inhibitor VAS2870 prevented increased superoxide anion production in APP Tg2576 mice while it had no effect in wild-type mice (Figure 5C).

3.9 Protein expression of antioxidants

In order to investigate further the mechanism responsible for increased oxidative stress, we performed western blot analyses of antioxidant profile. Protein expression of EcSOD and Cu/ZnSOD were significantly attenuated in the aortas of Tg2576 mice when compared with non-transgenic mice (Figure 6A and B). Treatment with PPARα ligand normalized EcSOD and Cu/ZnSOD protein expression in Tg2576 mice (Figure 6A and B) while it had no effect in wild-type mice. MnSOD was unaffected in all groups of treated mice (Figure 6C).

Protein expression of catalase and GPx were not different between wild-type and Tg2576 mice and those treated with GW501516 (see Supplementary material online, Figure S3).

4. Discussion

There are several new findings in the present study. First, endothelium-dependent relaxations and phosphorylation of eNOS at Ser1177 were significantly impaired in aortas of transgenic Tg2576 mice overexpressing APP. Secondly, BH4 levels and its rate-limiting enzyme GTPCH I were significantly reduced in Tg2576 mice aortas while 7,8-BH2 levels were increased. Thirdly, APP-overexpression significantly increased gp91phox expression and production of superoxide anion in the aorta while protein expression of Cu/ZnSOD and EcSOD were decreased. Fourthly, 2 weeks treatment with the PPARα ligand GW501516 normalized Ser1177-eNOS phosphorylation, BH4 biosynthesis, and significantly improved endothelial function in the aorta of Tg2576 mice. Fifthly, GW501516 normalized protein expression of gp91phox, Cu/ZnSOD, and EcSOD and significantly reduced superoxide anion production in APP transgenic mice. Our results demonstrate that endothelial cells and NO production are specifically susceptible to oxidative stress induced by overexpression of APP. Treatment with PPARα ligand reduced oxidative stress thereby improving endothelial function in Tg2576 mice.

Reduced NO bioavailability is a major mechanism responsible for initiation and progression of endothelial dysfunction in cardiovascular diseases such as hypercholesterolaemia, diabetes, ageing, and hypertension. All of these conditions are considered risks factors for development of atherosclerosis. Furthermore, both atherosclerosis and Alzheimer’s disease share many common pathogenetic mechanisms, such as inflammation and the generation of free radicals. It is well established that transgenic Tg2576 mice overexpressing double Swedish mutated human APP (K670N/M671L) are normotensive but exhibit several pathological features of Alzheimer’s disease. Relevant to our study, these mice develop spontaneous atherosclerosis in the aorta demonstrating that the detrimental vascular effects of overexpressed APP are not limited to the brain.
Moreover, recent study has reported that APP is expressed in the adipocyte tissue and is upregulated in obesity. In the present study, we observed that circulating levels of HDL were decreased in APP transgenic mice. The association between low levels of HDL and an increased risk for cardiovascular disease has been well established by epidemiological and clinical studies.

Overexpression of APP caused an impairment of endothelium-dependent relaxations to acetylcholine in aortas of Tg2576 mice. In contrast, endothelium-independent relaxations to NO-donor DEA NONOate were unaltered in Tg2576 mice demonstrating that the effects of overexpressed APP are endothelium specific. In addition, phosphorylation of eNOS at Ser1177 was significantly impaired in the aorta of Tg2576 mice suggesting that enzymatic activity of eNOS is diminished. This observation may help to explain endothelial dysfunction detected in Tg2576 mice. Although we did not detect any alteration of the APP processing enzymes α-secretase, β-secretase, as well as γ-secretase in Tg2576 mice aortas, circulating levels of Aβ peptides were significantly increased in APP transgenic mice consistent with the fact that this mutation causes increased secretion of Aβ peptides. It is very likely that elevated concentration of Aβ causes impairment of endothelium-dependent relaxations since several experimental studies have demonstrated that Aβ can directly induce endothelial dysfunction of both cerebral and peripheral blood vessels.

We have previously shown that under physiological conditions, the vascular endothelium of wild-type mouse aorta has a high rate of BH4 production while vascular smooth muscle cells do not contain detectable quantities of BH4. Our study is the first to show that overexpression of APP reduced BH4 levels and increased oxidation of BH4 to 7,8-BH2 in the aorta of transgenic mice. We also provided evidence that GTPCH I activity was decreased in Tg2576 mice aortas and that reduced activity of BH4-synthetizing enzyme was caused by reduced protein expression of GTPCH I. Decrease in BH4 to 7,8-BH2 ratio is considered an important index of reduced enzymatic activity of eNOS. Since both BH4 and 7,8-BH2 has been shown to bind eNOS with similar affinity, increased availability of 7,8-BH2 can efficiently replace BH4 thereby inhibiting eNOS enzymatic activity.

Evidence from experimental and human studies suggests that oxidative stress is an important mechanism of endothelial dysfunction and vascular injury as the local concentrations of NO in arterial wall are not only dependent on enzymatic activity of eNOS but are also determined by concentrations of superoxide anions. APP overexpression reduced protein expression of EcSOD and Cu/ZnSOD and increased PEG-SOD sensitive production of superoxide anion in the aorta of Tg2576 mice. This is consistent with reported study showing that endothelial dysfunction in cerebral arteries of Tg2576 mice was prevented by overexpression of Cu/ZnSOD. However, it is important to note that superoxide anion levels and endothelium-dependent...

Figure 4 Tetrahydrobiopterin (BH4) metabolism and GTP-cyclohydrolase I (GTPCH I) in aortas of wild-type and Tg2576 mice without (−) or with (+) GW501516 treatment. Bar graphs showing levels of BH4 (A), levels of 7,8-dihydrobiopterin (7,8-BH2) (B), and BH4 to 7,8-BH2 ratio (C). Representative western blot analysis showed GTPCH I protein expression of aortas, and bar graph indicated the results of the relative densitometry when compared with β-actin protein (D). Quantitative analysis of enzymatic activity of GTPCH I (E). Data are means ± SEM (6–10). *P < 0.05 vs. wild-type mice; **P < 0.05 vs. transgenic T2576 mice without GW501516 treatment (ANOVA with Bonferroni’s).
Figure 5  Quantitative analysis of intracellular superoxide anion (A) and western blot analysis of gp91phox protein expression (B) in aortas of wild-type littermates and Tg2576 mice without (−) or with (+) GW501516 treatment. Results are means ± SEM (n = 6–9 for superoxide anion and n = 4–5 for gp91phox). Effect of NADPH oxidase inhibition with VAS-2870 on superoxide anion levels in wild-type and Tg2576 mice aortas (C). *P < 0.05 vs. wild-type mice; **P < 0.05 vs. transgenic T2576 mice; ***P < 0.05 vs. control aortas (two-way ANOVA).

Figure 6  Representative western blot analysis of EC-SOD (A), CuZnSOD (B), and MnSOD (C) protein expression in aortas of wild-type and Tg2576 mice without (−) or with (+) treatment with GW501516. Bar graphs indicate the results of the relative densitometry when compared with β-actin protein. Results are mean ± SEM (n = 6–8). *P < 0.05 vs. wild-type mice; **P < 0.05 vs. transgenic T2576 mice without GW501516 (ANOVA with Bonferroni’s).
relaxations are comparable in young heterozygous Cu/ZnSOD and wild-type mice suggesting that partial reduction in SOD is not sufficient to impair endothelial function.35

We and others have shown that phosphorylated eNOS at Ser1177 is an important source for superoxide anion generation during BH4 deficiency.26,34 However, in Tg2576 mice, non-selective NOS inhibitor L-NAME did not affect increased superoxide anion production most likely because eNOS phosphorylation at Ser1177 was significantly reduced thereby minimizing production of superoxide anion by eNOS. In contrast, we demonstrated that protein expression of gp91phox was upregulated and that inhibition of NADPH oxidase completely prevented increased production of superoxide anion thereby suggesting that gp91phox-containing NADPH oxidase complex mainly contributed to the increased generation of superoxide anion in the aorta of Tg2576 mice. This observation is in line with the results of prior studies in peripheral and cerebral arteries.37,38

On the basis of the results obtained in human and animal studies it appears that APP and/or ββ play a role in the development of atherosclerosis.4–7 Recently, PPARα agonist has been shown to reduce vascular inflammation and development of atherosclerosis suggesting that activation of PPARα exerts vascular protective effects.7 In the present study, we detected several beneficial effects of PPARα agonist GW501516. We observed similar elevation of HDL levels by PPARα activation as reported by previous study.22 Remarkably, 2 weeks treatment with GW501516 normalized eNOS phosphorylation and improved endothelium-dependent relaxations to acetylcholine in aortas of Tg2576 mice without affecting ββ levels thus indicating that vascular protective effects of PPARα ligand are independent of APP processing. Moreover, the improvement in endothelial function of Tg2576 mice by GW501516 was associated with increased BH4 bioavailability, a critical cofactor required for enzymatic lial function of Tg2576 mice by GW501516 was associated with de novo synthesis of BH4 via GTPCH I, as protein expression and enzymatic activity of GTPCH I were augmented by GW501516. Our findings are consistent with reported study showing that the beneficial effects of GW501516 on re-endothelialization after vascular injury are dependent on increased production of BH4.39

Unexpectedly, BH4 levels were reduced in aortas of GW501516 treated wild-type mice. The decrease in BH4 levels by PPARα ligand was not caused by the decreased de novo biosynthesis of BH4 via GTPCH I or by the downregulation of GTPCH I protein expression. Furthermore, the decrease in BH4 levels were not caused by the increased oxidative stress as the production of superoxide anion was not elevated. We did not observe any increase in oxidation of BH4 to 7,8-BH2 that would compete with BH4 to bind eNOS and thus inhibit its enzymatic activity. Moreover, endothelium-dependent relaxations to acetylcholine were normal in GW501516 treated wild-type mice despite decreased BH4 levels. This finding suggests that reduced BH4 levels are not sufficient to cause impairment of endothelial function.33 The exact mechanism of selective reduction in BH4 levels by GW501516 remains to be determined.

We cannot rule out the possibility that endothelium-dependent relaxations in aortas of GW501516 treated mice are normal because of compensatory increase in production of other relaxing factors such as prostacyclin or hydrogen peroxide. However, we did not observe any effects of PPARα ligand on prostacyclin generating enzymes in transgenic Tg2576 mice and their wild-type littermates. On the other hand, our study also showed that expression and activity of eNOS was unchanged in GW501516 treated wild-type mice. It is thus unlikely that hydrogen peroxide is involved because hydrogen peroxide has been shown to increase expression and phosphorylation of eNOS.40

Interestingly, treatment with PPARα agonist decreased protein expression of gp91phox, normalized protein expression of both vascular Cu/ZnSOD and EcSOD isoforms, and prevented increased production of superoxide anion in the aorta of APP transgenic mice. The putative binding site for PPAR transcription factors has been found in the promoter region of Cu/ZnSOD gene suggesting that Cu/ZnSOD is regulated by PPARs.41,42 Indeed, PPARδ deficiency attenuated cardiac expression of Cu/ZnSOD in the adult mouse heart, leading to increased oxidative damage.43 Conversely, activation of PPARα enhances expression of Cu/ZnSOD in endothelial cells and increases endothelial cell resistance against oxidative stress.21,44 We did not investigate whether the vascular protective effects of PPARα agonist is blood pressure dependent. However, previous studies have demonstrated that in vivo treatment with GW501516 did not affect systolic blood pressure in healthy volunteers.45 Moreover, treatment with the other PPARδ ligand had no effect on blood pressure in angioten- sin II-infused mice.46

In conclusion, presented findings support the concept that APP causes endothelial dysfunction by increasing oxidative stress and reducing availability of NO. Activation of PPARα in Tg2576 mice exerts a number of vascular protective effects thereby leading to normalization of NO production and prevention of endothelial dysfunction.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest:** none declared.

**Funding**

This work was supported by National Institutes of Health grants HL-91867 and HL111062, by the American Heart Association Scientist Development grants #07-30133N (to L.V.d.) and #08-35436N (to A.V.R.S.), and by the Mayo Foundation.

**References**

1. De Strooper B, Vassar R, Golde T. The secretases: enzymes with therapeutic poten-


4. De Meyjer GR, De Cleen DM, Cooper S, Knaapen MW, Jans DM, Martinet W et al. Platelet phagocytosis and processing of beta-amyloid precursor protein as a mechan-

5. Austin SA, Sens MA, Combs CK. Amyloid precursor protein mediates a tyrosine kinase-dependent activation response in endothelial cells. J Neurosci 1999;29:


7. Tibolla G, Norata GD, Meda C, Armboldi L, Uboldi P, Piazza F et al. Increased ath-

8. Van de Parre TJ, Gunl PJ, Frensen P, Martinet W, Buhr H, Herman AG et al. Attenu-

9. De Strooper B, Vassar R, Golde T. The secretases: enzymes with therapeutic poten-


11. Austin SA, Sens MA, Combs CK. Amyloid precursor protein mediates a tyrosine kinase-dependent activation response in endothelial cells. J Neurosci 1999;29:


14. Van de Parre TJ, Gunl PJ, Frensen P, Martinet W, Buhr H, Herman AG et al. Attenu-

15. De Strooper B, Vassar R, Golde T. The secretases: enzymes with therapeutic poten-


