Peroxisome proliferator-activated receptor-γ protects against vascular aging

Mary L. Modrick,1 Dale A. Kinzenbaw,1 Yi Chu,1 Curt D. Sigmund,2 and Frank M. Faraci1,2

Departments of 1Internal Medicine and 2Pharmacology, Cardiovascular Center, Carver College of Medicine, University of Iowa, Iowa City, Iowa

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Modrick ML, Kinzenbaw DA, Chu Y, Sigmund CD, Faraci FM. Peroxisome proliferator-activated receptor-γ protects against vascular aging. Am J Physiol Regul Integr Comp Physiol 302: R1184–R1190, 2012. First published March 28, 2012; doi:10.1152/ajpregu.00557.2011.—Vascular disease occurs commonly during aging. Carotid artery and cerebrovascular disease are major causes of stroke and contributors to dementia. Recent evidence suggests that peroxisome proliferator-activated receptor-γ (PPARγ) may play a protective role in the vasculature, but the potential importance of PPARγ in vascular aging is unknown. To examine the hypothesis that PPARγ normally protects against vascular aging, we studied heterozygous knockin mice expressing a human dominant-negative mutation in PPARγ (P465L, designated L/+). Endothelial dysfunction, a major contributor to vascular disease, was studied using carotid arteries from adult (8 ± 1 mo) and old (24 ± 1 mo) L/+ mice and wild-type littermates. In arteries from wild-type mice, responses to endothelin-1 were reduced with age in both wild-type and U46619 was not affected by age or genotype, while maximal relaxation of arteries to nitroprusside (an NO donor) was similar in all groups. Contraction of arteries to scavenger of superoxide. Relaxation of arteries to nitroprusside (an

VASCULAR DISEASE OCCURS COMMONLY throughout the circulation with aging (4, 7, 16, 43, 48). Endothelial dysfunction is a key contributor to both the initiation and progression of vascular disease, including changes that occur with atherosclerosis (17, 38). Both endothelial dysfunction and atherosclerosis in carotid arteries (carotid artery disease) advance with age and greatly increase the risk for ischemic stroke and dementia (23, 25, 28, 33, 35). In relation to the goal of preventing the onset or slowing the progression of vascular disease, a major barrier has been our limited understanding of endogenous molecules that potentially protect against vascular aging.

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear hormone receptor superfamily that functions as a ligand-activated transcription factor (26, 42). PPARγ is present in many cell types (including the vasculature), where it regulates expression of target genes by binding to PPAR-response elements and other mechanisms (26, 42). Thiazolidinediones (TZDs) are synthetic activators of PPARγ used to treat type 2 diabetes (27). Varied protective effects of TZDs have been observed in the vasculature, including inhibition of oxidative stress and atherosclerosis (9, 13, 17, 26, 29). Oxidant-related mechanisms contribute to vascular dysfunction with aging (7, 16, 43, 48). Because endothelial dysfunction contributes to the initiation of vascular disease, we hypothesized that PPARγ normally protect against development of age-induced endothelial dysfunction. To address this question, we used heterozygous knockin mice expressing a human dominant-negative form of PPARγ [P467L (P465L in the mouse), designated L/+ ] that inhibits transcriptional activity of wild-type PPARγ (3, 5, 46). The P465L mutation in mouse PPARγ is equivalent to the P467L mutation that causes insulin resistance, type II diabetes, and early onset hypertension in humans (3, 46). Thus, these mice provide a novel genetic tool to interfere with the function of wild-type PPARγ and thus gain insight into the importance of PPARγ driven by endogenous ligands. With a goal of trying to phenocopy human disease, this was the best available genetic model to mimic patients carrying the same clinically relevant mutation. We used heterozygous mice because both homozygous knockin of the P465L mutation or full knockout of the PPARγ gene are lethal (46). We studied carotid arteries because this segment of the circulation is where some of the most important clinical consequences of vascular disease arise. Our findings indicate that age-related oxidative stress and endothelial dysfunction occur earlier, following genetic interference with PPARγ, suggesting a novel role for PPARγ to normally protect against age-induced endothelial dysfunction.

METHODS

Experimental animals. The animal protocol used was approved by the University of Iowa Animal Care and Use Committee. Experimental mice (and littermate controls) were generated by breeding 129/SvEv heterozygous P465L knockin mice with C57BL/6J mice, to produce control and heterozygous P465L mice on an F1 genetic background that is isogenic except for the mutation at the PPARγ locus (5). Thus, each L/+ mouse expresses one wild-type gene and one mutant PPARγ gene. Both male and female mice were used. Because we observed no apparent sex-related differences in these experiments, results were combined. Mice were fed standard chow and water ad libitum and studied at 8 ± 1 (adult) or 24 ± 1 mo of age (old). Body weight was similar in all groups; 38.7 ± 3.0 and 39.0 ± 2.9 g in adult and old WT compared with 39.9 ± 2.8 and 41.9 ± 3.7 g in adult and old L/+ mice. Care of mice met the standards set forth by the National Institutes of Health for the care and use of experimental animals.

Studies of vascular function. Methods used to measure vascular responses were described in detail previously (19). Briefly, mice were euthanized with pentobarbital sodium (100–150 mg/kg ip) followed by removal of carotid arteries and aorta. Loose connective tissue was removed, and arteries were cut into rings that were suspended in organ baths maintained at 37°C. The rings were connected to a force transducer to measure isometric tension (contraction and relaxation). Rest-
ing tension was increased stepwise to reach a final tension of 0.25 g. In our experience and in the literature, this level of resting tension is optimal for contraction in these arteries. Vessels were contracted submaximally (~50–60% of maximum) using U46619, a thromboxane A2 analog (9, 11-dideoxy-11a, 9a-epoxy-methanoprostaglandin F2α), prior to testing effects of vasodilators. We did not observe any effect of genotype on maximum contraction.

Experimental protocols. Responses to ACh [which produces endothelial nitric oxide synthase (eNOS)-dependent responses in this vessel (18)] and nitroprusside (an endothelium-independent nitric oxide donor) were measured following precontraction using U46619. Because endothelin-1 (ET-1) may contribute to vascular dysfunction during aging (43) and PPARγ can inhibit components of the endothelin system (21, 26), we also evaluated effects of ET-1. At the end of each experiment, a full-dose response curve to U46619 was obtained.

We examined the role of superoxide in mediating vascular dysfunction using a scavenger of superoxide (tempol, 1 mM). Poly (ADP-ribose) polymerase (PARP) can be activated during oxidative stress and contribute to vascular dysfunction (14). Thus, we also examined effects of an inhibitor of PARP (PJ34; 3 μM).

Quantitative real-time RT-PCR. RNA from aorta was prepared using the RNAeasy (Qiagen, Germantown, MD) method following extraction with TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were determined using a NanoDrop spectrophotometer, with an OD260/OD280 ratio of greater than 1.9 (indicating very high-quality RNA). Identical amounts of RNA (300 ng) were used for RT (10). Identical amounts of RT product were used for real-time PCR with a single well of a 96-well plate containing both TaqMan probes/primers (Applied Biosystems, Foster City, CA) for genes of interest (with FAM fluorophor) and β-actin (with VIC fluorophor) as a house-keeping gene. Expression levels were normalized to β-actin. Expression of superoxide dismutase-1, -2, and -3 [(SOD1, TaqMan primers/probe no. Mm01344233_g1), SOD2 (probe no. Mm01313000_m1), and SOD3 (probe no. Mm01213380_s1)], glutathione peroxidase-1 (Gpx1; probe no. Mm00656767_g1), catalase (Cat; probe no. Mm00437992_m1), nuclear factor E2-related factor-2 (Nrf2; no. Mm00432775_m1), Nox2 (probe no. Mm00432775_m1), Nox4 (probe no. Mm00479246_m1), and AT1a receptors for angiotensin II (AT1-R; probe no. Mm01166161_m1) were determined by quantitative real-time RT-PCR using the TaqMan method (10).

Drugs. ACh, nitroprusside, endothelin-1, tempol, and PJ34 were obtained from Sigma (St. Louis, MO) and were dissolved in saline. U46619 (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol with subsequent dilutions made in saline.

Statistical analysis. All data are expressed as means ± SE. Responses to specific agonists were expressed as percent relaxation to U46619-induced contraction. Comparisons of relaxation or contraction were made using two-way ANOVA followed by Bonferroni post hoc test. Statistical significance was accepted at P < 0.05.

RESULTS

ACh produced concentration-dependent relaxation of carotid arteries. Vascular responses to submaximal concentrations of ACh were not significantly altered in old wild-type mice or adult L+/+ mice compared with adult wild-type controls (Fig. 1). Maximal responses to ACh were similar in adult and old wild-type mice (Fig. 2) but were reduced by ~25% and ~50% in adult and old L+/+ mice, respectively (P < 0.05) (Fig. 2). The magnitude of endothelial dysfunction was significantly greater in old L/+ compared with adult or old wild-type mice and adult L+/+ mice. Thus, there was no evidence for endothelial dysfunction at this age in wild-type mice. In contrast, there was some vascular dysfunction in adult L/+ mice, and this impairment was increased substantially with age. Vasodilation to nitroprusside was similar in all groups and was not affected by age or genotype (Figs. 1 and 2) (responses to submaximal concentrations are not shown). The latter findings indicate the dysfunction occurred at the level of endothelium and not vascular muscle.

Tempol did not alter responses to ACh in adult wild-type or L/+ mice or in old wild-type mice (Fig. 2). In contrast, relaxation of carotid arteries to ACh in old L/+ mice was increased by tempol to levels seen in adult wild-type mice (Fig. 2). Vasodilation to nitroprusside was not affected by tempol, regardless of age or genotype (Fig. 2). Treatment of vessels with PJ34 (14), an inhibitor of PARP, did not improve responses to ACh in old L/+ mice (n = 5, data not shown). Lastly, contraction of carotid arteries to ET-1 and U46619 were unchanged in L/+ mice compared with wild-type at either age (Fig. 3). Maximal responses to ET-1 were reduced similarly in old wild-type and old L/+ mice, a finding consistent with previous reports (4, 44).

To gain additional insight into mechanisms that may contribute to changes following interference with PPARγ, we measured vascular expression of several genes thought to impact oxidative stress in models of vascular disease and aging. This included antioxidants (SOD1, SOD2, SOD3, Gpx1, Cat, and Nrf2), catalytic subunits of NADPH oxidases [Nox2 and Nox4, major sources of reactive oxygen species (ROS)], as
well as eNOS. Because ANG II contributes to vascular dysfunction with aging (32), we measured expression of the AT1-R. Compared with adult wild-type mice, gene expression was not significantly altered in adult L/H mice or in old wild-type mice (Fig. 4). In contrast, expression of Nox2 was increased significantly and expression of AT1-R tended to increase in old L/H mice (Fig. 4). This change in Nox2 was selective, as there was no increase in Nox4.

**DISCUSSION**

There are several new findings in this study. At the age studied, there were no significant differences in vascular gene expression or vascular function in old wild-type mice compared with controls. Genetic interference with PPAR/H had little overall effect on the same endpoints in adult mice. In contrast, there was substantial endothelial dysfunction in old L/H mice that was mediated by superoxide. Consistent with these functional data, vascular expression of Nox2 was increased following interference with PPARγ in aged mice. To our knowledge, this is the first evidence for any portion of the vasculature that PPARγ normally protects against oxidative stress and endothelial dysfunction during aging. Our findings also provide support for the concept that oxidative stress occurs with vascular aging and the emergence of PPARγ as a key protective molecule in relation to vascular disease in general (5, 7, 17, 21, 26, 29, 43). Importantly, our findings in this genetic model reveal that PPARγ exerts prominent vascular effects in the absence of treatment with a TZD. It remains unclear whether aging induces the production of an endogenous ligand, which then activates PPARγ to exert protective effects. If this is indeed the case, the presence of the dominant-negative mutation, which prevents ligand-induced activation of PPARγ, may impair its ability to respond to a stressor such as aging.

**Mouse models of vascular aging.** Endothelial cells play a major role in regulation of vascular structure and function (17). In the present study, we focused on effects of age on endothelial function, because endothelial dysfunction is a key element...
of mechanisms that underlie vascular disease (17). Endothelial dysfunction is also predictive of clinical events, including stroke (17, 25, 33). Thus, studies of endothelium-dependent vasodilation are important in understanding regulation of vascular tone, but also have broader implications in relation to mechanisms that promote vascular disease and contribute to major clinical complications.

We have previously shown that endothelial function in mouse aorta and carotid artery is unchanged or impaired only modestly at 22–24 mo of age (6, 13). The current finding that endothelial function and gene expression in carotid arteries were normal in 24-mo-old wild-type mice is consistent with this concept. Our finding that expression of eNOS was unchanged at this age is consistent with the functional data, as well as previous studies in mouse vessels (7). Additional studies have shown that mice 25 mo of age and older exhibit significant impairment of endothelial function (7, 15, 20).

Because we hypothesized that interference with PPARγ would augment vascular dysfunction with aging, we choose to study the aged group at 24 mo, when worsening of vascular function could potentially be detected.

**Impact of PPARγ in the vasculature.** PPARγ was initially thought to influence mainly adipocytes, as well as having effects on glucose and lipid metabolism (42). It is now clear that PPARγ has many other functions and is active in many cell types. PPARγ is expressed in endothelium, including in the carotid artery and cerebral blood vessels (26, 30, 39), where it regulates expression of a variety of target genes (26, 42).

Using TZDs as an exogenous ligand, some have attributed antioxidant effects to PPARγ in the vasculature. For example, TZDs reduce the expression of AT1 receptors and components of NADPH oxidase, while increasing the expression of SOD1 (24, 26, 29). In the carotid artery and cerebral vasculature, TZDs affect vascular structure [produce outward vascular remodeling under normal conditions, and prevent inward vascular remodeling during hypertension (11, 12)], and have beneficial effects in relation to regulation of vasomotor tone and vascular permeability (11, 12, 26, 34, 41). These findings suggest that pharmacological activation of PPARγ has protective effects on vascular structure and function. An underlying assumption of TZD-based studies is that the effects observed are mediated by PPARγ. However, in addition to activating PPARγ, TZDs can exert off-target or PPARγ-independent effects (37, 40). As an alternative, some laboratories have examined the role of PPARγ using a PPARγ antagonist (8).

Genetic variations in PPARγ genotypes associate with the extent of carotid artery disease in patients (1). Patients with select mutations in the ligand-binding domain of PPARγ (V290M or P467L) exhibit early-onset hypertension and diabetes (3). These mutations act in a dominant fashion to inhibit transcriptional activity of wild-type PPARγ (3, 26). The study of PPARγ using genetically altered mice expressing these same dominant-negative mutations (“humanized” mice) offers several advantages. First, this genetic manipulation is highly selective, avoiding off-target effects of pharmacological activators of PPARγ (37, 40). Second, genetic interference with...
wild-type PPARγ provides insight into the importance of PPARγ when driven by endogenous ligands, without the need for TZD treatment. Genetic interference with PPARγ mimics reductions in activity of PPARγ described in disease and with other genetic polymorphisms. Mice expressing these clinically relevant mutations share features of human disease, including abnormal fat distribution and elevated blood pressure but are not hyperglycemic and have normal insulin sensitivity (46). Direct recordings in conscious animals indicated that arterial pressure is modestly elevated in adult L/+ mice (5).

In our previous study, adult L/+ mice exhibited oxidative stress and endothelial dysfunction in the cerebral circulation but not aorta (5). The present findings in carotid artery of adult L/+ mice are generally consistent with the aortic phenotype and support the concept that interference with PPARγ has prominent effects in cerebral blood vessels but has little or more modest effects in aorta or other large muscular arteries in the absence of other risk factors (or stressors). Like the aorta, endothelium-dependent relaxation to submaximal concentrations of ACh was not significantly affected in carotid arteries from L/+ mice. In contrast to aorta, however, we did see some impairment of carotid artery responses to the maximal concentration of ACh in adult L/+ mice.

Oxidative stress and PPARγ in vascular aging. There has long been interest in the role of ROS in normal aging (22). Oxidative stress is the result of a shift in balance that favors the generation of ROS over antioxidant defense mechanisms (18). SODs and other antioxidants are key determinants of steady-state levels of superoxide in vascular cells (18). In relation to endothelial biology, NO is a major endothelium-derived signaling molecule but also a key molecular target of superoxide. NO reacts with superoxide at a near diffusion-limited rate, resulting in loss of normal NO-mediated signaling. Partial genetic deficiency in SOD1 or SOD2 increases vascular superoxide and accelerates age-induced vascular dysfunction (7, 14). In the present experiments, we found no change in vascular expression of SODs, as well as several other antioxidants in old wild-type mice. This lack of change in antioxidant expression is consistent with the observation that vascular function is still normal in wild-type mice at this age.

NADPH oxidase is a key source of superoxide in vascular cells (17). Several studies have highlighted the importance of oxidative stress and NADPH oxidase in vascular disease in models of aging and Alzheimer’s disease, as well as in humans (7, 14, 16, 31, 32, 34, 36, 48). Our finding that scavenging superoxide restores endothelial function in old L/+ mice is further evidence that oxidative stress is a key contributor to vascular abnormalities with aging. The finding that tempol had no effects on vascular responses to ACh in adult L/+ mice but restored these responses to normal in old L/+ mice suggests that more than one mechanism is involved in producing these changes and that oxidative stress contributes to the dysfunction seen in old L/+ mice. In addition to oxidative stress, other mechanisms may contribute to vascular abnormalities with aging. One such mechanism is cyclooxygenase-dependent production of an endothelium-derived contracting factor (EDCF) (47). Interactions between oxidative stress and EDCF formation exist (17). Although the data in the current study implicate oxidative stress, our findings do not rule out a potential contribution by other endothelium-dependent mechanisms in old L/+ mice. Although relatively little is known, a few studies have suggested PPARγ may play an important role as organisms age. PPARγ expression and activity decrease in kidney with age, while TZD treatment reduces Nox2 expression and oxidative stress in a model of age-related renal injury (45, 49). Expression of PPARγ is increased in a long-lived mouse strain (6), and genetic reductions in PPARγ expression reduce life span in mice (2). Consistent with these results, vascular function in an aged model of Alzheimer’s disease improves following treatment with a synthetic activator of PPARγ (34). The present finding that age-induced vascular dysfunction occurred to a much greater extent in old L/+ mice is consistent with the emerging concept that PPARγ may protect against aging in general. Vascular dysfunction old L/+ mice was superoxide-mediated and was accompanied by increased vascular expression of Nox2. Thus, these findings are consistent with previous reports that NADPH oxidase plays a key role in the vasculature during aging.

Perspectives and Significance

Data from experimental models and people suggest that oxidative stress is a major contributor to vascular aging. The present study supports this concept and provides evidence for accelerated endothelial dysfunction with age in a mouse globally expressing a human dominant-negative form of PPARγ. Vascular disease appears to result from highly interactive oxidant- and inflammation-related mechanisms that are often regulated though cell-specific processes. Continued study of PPARγ, along with other molecules that control these processes within endothelial cells, should provide better insight into vascular abnormalities that occur with aging or in the presence of other cardiovascular risk factors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.L.M., D.K., and Y.C. performed experiments; C.D.S. and F.M.F. analyzed data; C.D.S. and F.M.F. interpreted results of experiments; A.A.K. and R.S. performed genotyping; F.M.F. analyzed data; F.M.F. interpreted results of experiments; C.D.S. and F.M.F. approved final version of manuscript; A.A.K. and R.S. provided resources; F.M.F. provided resources; A.A.K. and R.S. provided resources; M.L.M., D.K., and Y.C. approved final version of manuscript. No conflicts of interest, financial or otherwise, are declared by the author(s).

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