ANG II type 2 receptor regulates smooth muscle growth and force generation in late fetal mouse development

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Submitted 23 June 2004; accepted in final form 19 August 2004

Perlegas, Demetra, Hui Xie, Sanjay Sinha, Avril V. Somlyo, and Gary K. Owens. ANG II type 2 receptor regulates smooth muscle growth and force generation in late fetal mouse development. Am J Physiol Heart Circ Physiol 288: H96–H102, 2005. First published August 26, 2004; 10.1152/ajpheart.00620.2004.—Although evidence from culture studies implicates the angiotensin II (ANG II) type 2 receptor (AT2R) in the regulation of growth and differentiation of arterial smooth muscle (SM) cells (SMC), the lack of its expression in adult arteries has precluded direct investigation of its role in vivo. The goal of the present study was to determine the role of AT2R in the control of fetal SMC growth, contractility, and differentiation during vascular development. Determination of isometric tension in fetal aortas showed potentiated ANG II-induced contraction by treatment with the selective AT2R antagonist PD-123319, demonstrating the presence of functional AT2Rs that mediate reduced force development in vascular SMC. In direct contrast to numerous cell culture studies, proliferation indexes were decreased rather than increased in aortic SMC of fetal homozygous AT2R knockout compared with wild-type or heterozygous knockout mice. Experiments using SMC tissues from heterozygous female AT2R knockout mice, which are naturally occurring chimeras for AT2R expression, showed that AT2R mRNA expression was exactly 50% of that of wild type. This indicated that loss of AT2R expression did not confer a selective advantage or disadvantage for SMC lineage determination and expansion. Real time RT-PCR analyses showed no significant difference in expression of SM-α-actin, SM myosin heavy chain, and myocardin in various SM tissues from all three genotypes, suggesting that knockout of AT2R had no effect on subsequent SMC differentiation. Taken together, results indicate that functional AT2R are expressed in fetal aorta and mediate reduced force development but do not significantly contribute to regulation of SMC differentiation, proliferation, differentiation, contractility.

The smooth muscle (SM) cell (SMC) displays remarkable phenotypic plasticity during vascular development and disease (35, 36). Indeed, the model that has emerged is that SMC differentiation is dependent on the complex integration of a diverse range of local environmental cues that can either promote or repress SMC-selective gene expression (24, 36). The contractile agonists, such as angiotensin II (ANG II), are one class of environmental factors shown to contribute to the control of SMC differentiation by activating expression of multiple SMC differentiation marker genes including SM-α-actin (SMA), SM myosin heavy chain (SM-MHC), and tropomyosin at least in cultured SMC systems (12, 13). The primary, short-term function of these factors is the control of force development in the vasculature. However, they are also implicated in long-term control of SMC-selective protein expression and contractile mass as a means to promote SMC differentiation and to provide a mechanism to match force developing capacity with required work load (25, 35). There is also extensive evidence suggesting a potential role for ANG II in the control of vascular SMC growth (19). However, it is controversial whether ANG II promotes enlargement or hypertrophy of preexisting SMCs, versus hyperplasia, depending on the specific SMC culture conditions (34, 37). For example, our laboratory and others have shown that ANG II promotes hypertrophic growth of cultured rat aortic SMCs derived from Sprague-Dawley rats, but induces hyperplastic growth of those derived from spontaneously hypertensive rats, at least in part by stimulation of autocrine production of platelet-derived growth factor and transforming growth factor (10, 17, 43). Of interest, increased SMC mass in large arteries of animals with chronic hypertension is due to SMC hypertrophy not hyperplasia and can be selectively blocked by inhibition of angiotensin signaling (10, 38). SMC hypertrophy in hypertension is associated with coordinate upregulation of expression of multiple SMC contractile proteins (48), as is ANG II treatment of cultured rat aortic SMCs, suggesting a close association of SMC hypertrophy with increased contractile protein synthesis and the regulation of SMC growth, differentiation, and/or developmental remodeling both in vitro and in vivo. However, there are major controversies regarding the precise functions of ANG II in vivo versus cultured SMC systems, as well as the mechanisms by which ANG II manifests these effects.

The biological effects of ANG II are mediated by the stimulation of ANG II type 1 (AT1R) or type 2 receptors (AT2R) (3). Several studies indicate that functions of the AT1R and AT2R are antagonistic to one another (7, 18, 19, 22, 29, 32). For example, the AT1R promotes SMC proliferation, growth, and contractility, whereas the AT2R has been implicated in the suppression of proliferation and the mediation of vasodilation (3, 7). Although the functions of the AT1R have been clearly elucidated, there exists conflicting evidence for the precise functions and signaling pathways of the AT2R in the control of vascular SMC growth and proliferation. For example, several studies provide evidence showing that growth suppression is a major function of the AT2R in a variety of cell types, including vascular SMC (1, 19, 32), endothelial cells (4), cardiomyocytes (2), PC12W cells (44), and in mouse fibroblast cells (47), as well as in vascular disease models (2, 19). In contrast, other studies suggest that the AT2R exerts growth-promoting effects, specifically in the rat. For example, the AT2R was shown to promote hypertrophy of rat aortic SMCs during remodeling (33), neointimal formation after mechanical

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injury (6, 21, 21), cell proliferation in the rat mesenteric vasculature (6), and increased protein synthesis in cultured rat aortic SMCs (30). Furthermore, chronic AT2R blockade in the rat vasculature in vivo resulted in decreased vascular hypertrophy, indicating that the AT2R partially mediates vasotrophic effects of ANG II (26). Similarly, studies by Nakajima et al. (32) showed that systemic administration of an AT2R-selective antagonist to late-stage pregnant rats resulted in delayed reductions in SMC proliferation rates in fetuses during the perinatal period, but this may have reflected systemic or indirect effects of the pharmacological inhibitor.

Similar to the equivocal role of the AT2R in SMC growth regulation, very little is known regarding its role in SMC differentiation and lineage specification. However, it is known that expression of the AT2R in the mouse is highly abundant in the aorta and small resistance arteries during embryonic days (E) 16–21, is virtually eliminated from the vasculature by early adulthood, but is reexpressed in the adult vasculature after injury (16, 42). This temporally restricted expression pattern suggests a potential role of the AT2R in modulation of growth and remodeling of the fetal vasculature. Indeed, the results of some studies (41, 51) have suggested that the AT2R contributes to the control of SMC growth and/or differentiation during late fetal and postnatal development. A study by Yamada et al. (51) utilized the conventional AT2R knockout mouse to examine changes in SMCs, caldesmon, and calponin gene and protein expression during mouse embryonic vasculogenesis. At 2 and 4 wk of age, caldesmon and calponin protein expression in thoracic aortas was delayed in AT2R-null animals, whereas SMC protein levels remained unchanged. Although calponin and caldesmon are important proteins involved in SMC contractile function, their expression alone does not indicate the true SMC differentiated state. A more SMC-selective repertoire of contractile genes, as well as evidence supporting contractile function is required to demonstrate the properties of a mature, fully differentiated SMC. Furthermore, the use of a conventional knockout mouse poses the inherent limitation of the possibility of activation of compensatory genes and pathways that may indirectly alter SMC growth and/or differentiation. For example, compared with the wild-type mouse, the AT2R knockout mouse shows increased baseline systemic blood pressure (20), which could indirectly influence SMC growth and differentiation. In contrast, Hein et al. (15) showed that baseline systemic blood pressure was not significantly different in the AT2R knockout animal versus the wild type. Although these conflicting data could be attributed to different mouse genetic backgrounds, the induction of compensatory blood pressure signaling pathways independent of the AT2R could have resulted from the knockout, and in turn, normalize the baseline blood pressure in AT2R knockout mice found by Hein et al. It is again possible that such compensatory signaling pathways could also affect SMC growth and differentiation.

Finally, previous studies have not addressed the possible functional consequences of AT2R loss on SMC contractile function. Indeed, although the AT2R has widely been proposed to antagonize AT1R-dependent activation of SMC contraction, its role has not been directly examined in SMC of large arteries due to complete downregulation of AT2R expression in these blood vessels early after birth and into adulthood.

The goal of the present study was to test the hypothesis that the AT2R contributes to SMC differentiation and maturation within large arteries during late fetal development using a novel experimental approach that took advantage of the fact that female embryos heterozygous for the AT2R knockout allele are naturally occurring chimeras for AT3R expression, because the AT2R is present on the X chromosome (29) and undergoes random X chromosome inactivation during the late blastocyst stage (11, 31) before AT2R induction. As such, one can examine the effects of AT2R knockout on development of SMC in vivo in the context of a mouse that is phenotypically normal (14, 15), and where there is a population of wild-type cells that greatly reduces or eliminates activation of compensatory gene regulatory pathways as occurs routinely with conventional knockout mice (27). We thus exploited the naturally occurring chimerism evident in female heterozygous AT2R knockout mice to quantitatively assess whether cells lacking an AT2R have a selective advantage or disadvantage in forming SMC lineages. In addition, we developed a novel method for assessing contractility in fetal mouse blood vessels and, performed the first direct assessments of the functional role of the AT2R in vascular SMC force generation in large vessels during a developmental period when the receptor is abundantly expressed.

MATERIALS AND METHODS

Measurement of isometric tension. Pregnant mice were euthanized by CO2 inhalation and thoracic aortas from 18.5-day-old embryos were immediately removed and placed in ice-cold physiological salt solution (PSS; composition in mmol/l: 135 NaCl, 4.7 KCl, 1.6 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 15 NaHCO3, 11.1 dextrose, and 0.026 EDTA; pH 7.4). These vessels were dissected free of adipose and connective tissue with minimal mechanical trauma. They were cut into individual ring segments at an average diameter of ~120–150 μm and 0.5 mm in length. The segments were processed for force measurements by modification of previously described methods to assess 1–2 mm long veins and arterioles from adult animals (23, 50). Briefly, the aortic ring segments were mounted in chambers contain-
ing oxygenated PSS at 37°C on a bubble plate. Two tungsten wires were placed through the lumen of a segment where one wire was attached to a fixed support and the other connected to a force transducer (model AEG301, SensoNor). The aortic rings were stretched to 5 mg passive tension, which is optimal for embryonic tension development. During this process, it is highly probable that the mechanical trauma to the vessel lumen results in endothelial denudation, but the possibility that the endothelium could potentially contribute to vascular relaxation is not completely eliminated. Tissues were equilibrated in PSS for 1 h, during which the chamber PSS was replaced every 15–20 min. Changes in isometric tension were measured by a force transducer and displayed on a chart recorder. Animal handling followed the Institutional Animal Care and Use Committee protocol.

The ring segments were initially treated with 125 mM KCl (substituted for NaCl in PSS) to assess their maximal, voltage-dependent force developing capacity. Concentration response curves for ANG II, at doses between 0.01 nM and 1.0 μM were generated before and after treatment with either the AT1R-selective antagonist, losartan at 1 μM, or the AT2R-selective antagonist, PD-123319 at 1 μM for 30 min. Contractile responses were expressed as a percentage of the 125 mM KCl-induced contraction. To independently examine AT-R-mediated vasodilation effects without AT-R interference, all experiments were done in the presence of 1 μM losartan. Elevated vascular tone was based on myogenic tone, which lasted for 3–4 h without drug intervention in preliminary studies.

Measurement of DNA synthesis. To identify proliferative cells undergoing DNA replication in 18.5 day embryos, pregnant female mice were administered 50 mg/kg ip bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) 1 h before death. Mouse embryonic tissues were removed, fixed in 4% paraformaldehyde, paraffin-embedded, cut into 4–5 μm serial sections. Immunohistochemistry was performed using a peroxidase-conjugated, mouse monoclonal anti-BrdU antibody (Sigma). The sections’ nuclei were counterstained with hematoxylin. The BrdU-positive cells found in the embryonic aorta, intestine, and liver were quantified by counting in a blinded manner. At least two arbitrarily chosen fields per tissue section and three sections per tissue were examined under high magnification (×40). This was done for n = 4 or 5 embryos per AT-R genotype (wild-type, heterozygous, or homozygous knockout). The proliferation index was expressed as the number of proliferating cells per cross section.

Quantitative real-time RT-PCR for AT-R, AT-R, and SMC marker genes. The following tissues were harvested from 18.5-day-old embryos (n = 7 female embryos for wild-type and heterozygous AT-R knockout genotypes and n = 5 female embryos for homozygous knockout genotype) after the pregnant mothers were euthanized by CO2 inhalation: aorta, stomach, intestine, heart, bladder, brain, and liver. The tissues were immediately placed in ice-cold RNA Later buffer for tissue RNA stabilization (Ambion; Austin, TX). Total RNA was extracted from each tissue according to manufacturer protocol using the RNeasy RNA purification kit (Qiagen; Valencia, CA). Primers and probes for targeting the mouse AT-R were designed using Primer Select software (DNAStar, 2003). The following primers and probes were synthesized and used in these studies: (1) AT-R forward primer 5’ GATCGCTACCCCGCAATGTTCTG 3’; (2) AT-R reverse primer 5’ TGACTTTTGCCACCAACATGAT 3’ (3) AT-R probe 5’ CGGATAATGGCTCGCTCCGCGG 3’; (4) AT-R forward primer 5’ GTGGAGAACCCTGCTCCAAAC 3’; (5) AT-R reverse primer 5’ ATAGCTCTCTCTTGCTGTGGAGC 3’; and (6) AT-R probe 5’ AAGCTCCCAGTGTGGTTTAAGAGTCTCTCCTAATA 3’.

Statistical analysis. Results in all figures are expressed as means ± SE. Paired comparisons were performed with the use of Student’s t-test. One-way ANOVA was performed for multiple comparisons, followed by Tukey’s test when appropriate. A value of P < 0.05 was considered significant for all studies.

RESULTS

Functional AT-Rs in embryonic aortas reduced ANG II-induced contraction. The principle aim of the present study was to determine the functional role of the AT-R in SMC during vascular development. A widely accepted role of the AT-R involves the modulation of contractile responses to ANG II by antagonizing the effects mediated by the AT-R (5, 7, 16, 18, 19, 29, 32, 32). This dogma is based on studies using cultured adult rat aortic SMCs, and AT-R overexpression by in vivo gene transfer in adult rats and in adult mouse femoral arteries. However, in the adult animal systems, expression of the AT-R in the aorta and large conduit vessels is profoundly downregulated shortly after birth and completely absent by adulthood (18). Thus at present, there is no direct evidence of AT-R-AT-R antagonism for contractile function within large conduit vessels, especially in the period of peak AT-R expression between E16 and E21 (18, 39). Therefore, to directly determine the function of the AT-R during later embryonic
development, contractile studies were performed in freshly isolated aortas from E18.5 mouse embryos. Results of these studies showed that treatment of vessels with a selective AT2R antagonist PD-123319 potentiated ANG-induced contractile responses in E18.5 mouse aortas in a dose-dependent manner (Fig. 1). In contrast, administration of losartan alone, an AT1R antagonist, blocked ANG-induced contraction (data not shown). These results provide the first evidence supporting the presence of a functional AT2R within large conduit arteries in late mouse embryonic development. Furthermore, they indicate that a primary function of the AT2R at this stage is to reduce ANG II-induced contraction by antagonizing AT1R-mediated contractile effects.

Aortic SMC proliferation was decreased in AT2R-null embryonic aortas. Because of the apparent functional role of the AT2R in the aorta, another major goal of this study was to determine the role of the AT2R in SMC growth and proliferation during late embryonic development. BrdU labeling indexes were compared in aortic SMC, intestinal SMC, and hepatocytes from AT2R wild-type, heterozygous, or homozygous knockout 18.5-day embryos. Results showed that SMC proliferation in aortas of E18.5 mice was markedly reduced in AT2R knockout embryos compared with wild-type or heterozygous knockout embryos (Fig. 2). A similar reduction in cellular proliferation was observed in intestinal SMC, but not in hepatocytes (data not shown). Although these results implicate a positive role of the AT2R in SMC proliferation, the effects of AT2R knockout may be partially indirect.

Expression of functional AT2Rs in SMC tissues did not affect SMC lineage specification. To determine whether the presence or absence of the AT2R influences early stages of SMC development, or the subsequent expansion of SMC lineages, we performed real-time RT-PCR analyses of AT2R mRNA levels in SMC tissues of female heterozygous AT2R knockout mice that are naturally occurring chimeras for expression of the AT2R. Assuming a finite level of expression of the AT2R mRNA per allele, the proportion of AT2R wild type to knockout cells will be accurately reflected by the level of AT2R expression. That is, if the presence or absence of the AT2R in SMC has no role in the formation of SMC lineages, then the expression of the AT2R in SMC tissues of heterozygous female AT2R knockout mice should be 50% of that of wild-type control mice. In contrast, if the presence of the AT2R confers a selective advantage for SMC tissue formation or expansion, then the level of AT2R expression in heterozygotes should be >50%. However, if it confers a selective disadvantage for SMC tissue formation and expansion, then AT2R expression should be <50% of that of wild type. Real-time RT-PCR analysis of AT2R expression in aorta, bladder, and stomach from female heterozygous AT2R knockout embryos showed AT2R expression that was 50% of that of wild-type female embryos (P > 0.05). Thus results of these studies provide...
evidence showing that the presence or absence of the AT_{2R} did not confer a selective advantage or disadvantage for the formation of SMC lineages in multiple SMC-containing tissues (Fig. 3).

Expression of SM_{\alpha}A, SM-MHC, myocardin, and AT_{1R} was not significantly different in AT_{2R} knockout cells compared with wild type. Our laboratory and others (8, 12, 13, 17, 40, 48) have shown that ANG II induces AT_{1R}-dependent increases in the expression of multiple SMC marker genes in cultured SMCs, thus implicating a potential role for ANG II as a positive regulator of SMC differentiation. Although there is also evidence suggesting that knockout of the AT_{2R} is associated with delayed expression of SMC marker genes, such as calponin and caldesmon (51), the expression levels of more SMC-restricted genes, such as SM-MHC and myocardin, were not measured. Moreover, gene expression levels were not quantified, but determined by conventional RT-PCR. To determine the possible role of the AT_{2R} in the control of SMC differentiation in vivo, we used real-time RT-PCR analyses to determine the expression levels of the more restricted and selective SMC genes, SM-MHC, and myocardin, as well as SM_{\alpha}-actin in SMC-rich and non-SMC tissues from E18.5 old female embryos that were wild-type, heterozygous, and homozygous knockout for the AT_{2R} gene. We limited our studies to 18.5-day embryonic tissues because of highly abundant and temporally restricted AT_{2R} expression between E16 and E22 (18). Results of real time RT-PCR analysis in Figs. 4–6 showed no significant difference (P > 0.05) in the expression of SM-\alpha-actin, SM-MHC and myocardin among all three genotypes in E18.5 aorta, bladder, stomach, intestine, brain, liver, and heart, with the exception of the bladder, in which there were significantly greater SM-MHC message levels in AT_{2R} heterozygous knockout animals (P < 0.05). Furthermore, Fig. 7 shows that changes in the AT_{1R} genotype did not significantly affect expression levels of the AT_{1R} in aorta, heart, brain, and liver (P > 0.05). As such, it is unlikely that the observed changes in proliferation rate are secondary to increased AT_{2R} expression. However, we cannot rule out the possibility of a posttranscriptional-mediated effect on AT_{1R} expression or function. Thus we found no clear evidence that loss of the AT_{2R} had a significant effect on the level of expression of SMC-specific marker genes or the AT_{1R} in multiple vascular and nonvascular SMC tissues of the fetal mouse.

DISCUSSION

We have performed a unique combination of studies analyzing the potential functional role of the AT_{2R} in aortic force generation, as well as in SMC lineage determination during late stage mouse embryonic development. Of major interest, we found that functional AT_{2R}Rs in the embryonic aorta mediated reduced force generation based on evidence that an AT_{2R}-selective antagonist inhibited ANG II-induced force development in E18.5 mouse aortas (Fig. 1). These results are novel in that they directly show that AT_{2R}Rs mediate SMC relaxation in large arteries. Moreover, this study is the first to demonstrate that AT_{1R}-mediated regulation of force development is coupled to temporally restricted AT_{2R} expression in the fetal vasculature. The significance of abundantly expressed and functional AT_{2R}Rs in the vasculature exclusively between embryonic days 16 and 22 is unclear but suggests a potential role in remodeling during vascular development. It is interesting that AT_{2R}-mediated vasodilation would be important in a fetal, but not adult aorta, based on our knowledge of the respective AT_{2R} expression patterns (18). We speculate that embryo-restricted AT_{2R} expression in large vessels, such as the aorta, is likely necessary to regulate ANG II responsiveness during embryonic development. For example, among other cardiovascular alterations during gestation in both mice and humans, there are elevated levels of plasma ANG II and high resistance...
to pressor effects of endogenous and infused ANG II (9, 49). Because the fetus is also exposed to these elevated levels of ANG II in the circulation, the regulation of ANG II responsiveness may be required to prevent fetal hypertension. The results of the present study suggest that abundantly expressed AT2Rs could potentially mediate this regulation.

In the fetal aorta from an AT2R knockout animal, we observed reduced SMC proliferation, which suggests that the AT2R is a positive regulator of SMC proliferation in the late stages of mouse vascular development (Fig. 2). This finding was quite unexpected because a number of previous studies report that the AT2R is primarily a negative regulator of vascular SMC proliferation both in vivo and in cultured SMC systems (1, 32, 32). Hutchinson et al. (19) provide evidence implicating an important role of the AT2R in decreased SMC proliferation during embryonic vascular development in the rat between E14 and E21. However, it is difficult to distinguish whether the use of AT2R-selective antagonists used in this study directly or indirectly affected SMCs specifically in the fetal aorta. For example, the increased SMC proliferation indexes in fetal rats whose mothers were given AT2R antagonists could be attributed to effects of maternal rather than fetal receptor responsiveness. Therefore, to more directly determine whether there is a possible role of the AT2R in SMC growth regulation during late fetal mouse development, we analyzed aortic SMC proliferation in AT2R knockout mice compared with wild-type or heterozygous knockout mice (Fig. 2). Analysis of the complete loss of AT2R gene function targets the fetal vasculature more directly than an antagonist. We found a significant decrease in aortic SMC proliferation in AT2R knockout animals. Furthermore, regardless of this clear decrease in the proliferation index, results of our real time RT-PCR analysis of AT2R expression in chimeric heterozygous AT2R knockout female mice indicate that the frequency of AT2R-positive cells in SMC tissues of naturally occurring chimeric females was unaltered from that of wild-type mice (Fig. 3). In addition, there were no overall differences in SMC-specific lineage marker gene expression in the fetal aorta and other SMC-rich tissues (with the exception of SMMHC in bladder) from AT2R wild-type, heterozygous, and knockout animals, suggesting that the AT2R does not significantly affect SMC differentiation (Figs. 4–6). A potential explanation for these data is that the loss of the AT2R had only transient effects on SMC proliferation, and other dominant mechanisms exist to regulate overall SMC number and vascular morphogenesis. In addition, results suggest that loss of AT2R may have complex and indirect effects on SMC proliferation, but not differentiation. For example, embryonic SMCs exhibit a generalized, autonomous growth potential up to E18 in vivo, and replicate independent of serum in vitro (28). Thus aortic SMC within the AT2R-null mice may have shown a greater proliferation rate during earlier developmental stages. Also, they may have reached a more mature developmental stage by E18.5 than wild-type mice, and as a consequence, began the perinatal-associated drop in proliferation index previously shown by Hutchinson et al. (19). In any case, however, further extensive studies including SMC-selective knockouts of the AT2R are needed to directly test these various possibilities, and at present, the direct functions of the AT2R in regulation of SMC growth in vivo remain elusive.

In summary, our findings showed that functional AT2Rs are present in the fetal aorta and serve to mediate reduced force development but do not appear to have a clear role in the control of SMC differentiation during embryonic development. In contrast, results provided intriguing evidence that loss of the AT2R was associated with an unexpected drop in SMC proliferation index. However, further studies are needed to elucidate the precise role and mechanisms by which AT2R signaling pathways influence growth of SMC in vivo, as well as their potential role in diseases that exhibit both altered SMC AT2R expression and growth, such as hypertension and restenosis (29, 35, 36, 45, 46).

ACKNOWLEDGMENTS

We thank Dr. Tadashi Inagami (University of Tennessee) for generously providing the AT2R knockout mice for this study and Diane Raines for expert technical assistance.

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GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants PO1 HL-19242, R01 HL-38854, and HL-65958 (to G. K. Owens), and NIH Cardiovascular Training Grant T32 HL-072284 (to D. Perlegas).

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AJP-Heart Cir Physiol • VOL 288 • JANUARY 2005 • www.ajpheart.org