A Role for the Aryl Hydrocarbon Receptor in Regulation of Ischemia-Induced Angiogenesis

Sahoko Ichihara, Yoshiji Yamada, Gaku Ichihara, Tamie Nakajima, Ping Li, Takahisa Kondo, Frank J. Gonzalez, Toyoaki Murohara

Objective—The aryl hydrocarbon receptor (AHR) is a transcription factor that binds to DNA as a heterodimer with the AHR nuclear translocator (ARNT) after interaction with ligands such as polycyclic and halogenated aromatic hydrocarbons found in tobacco smoke and the environment. We have investigated the interaction between AHR and hypoxia signaling pathways in regulation of angiogenesis with the use of a surgical model of ischemia.

Methods and Results—Ischemia was induced by femoral artery occlusion in wild-type and AHR-null mice. Ischemia-induced angiogenesis was markedly enhanced in AHR-null mice compared with that in wild-type animals. Ischemia-induced upregulation of the expression of hypoxia-inducible factor–1α (HIF-1α) and ARNT as well as that of target genes for these transcription factors, such as that for vascular endothelial growth factor (VEGF), were also enhanced in AHR-null mice. Furthermore, the DNA binding activity of the HIF-1α–ARNT complex as well as the association of HIF-1α and ARNT with the VEGF gene promoter were increased by ischemia to a greater extent in AHR-null mice than in wild-type animals.

Conclusions—Ablation of AHR resulted in enhancement of ischemia-induced angiogenesis. This effect was likely attributable in part to the associated enhancement of ischemia-induced VEGF expression, which in turn may be caused by an increased abundance and activity of the HIF-1α–ARNT heterodimer. (Arterioscler Thromb Vasc Biol. 2007;27:1297-1304.)

Key Words: angiogenesis • hypoxia • ischemia • peripheral vascular disease • smoking

Angiogenesis, the development of new blood vessels from preexisting vessels, is a tightly controlled physiological process that is also associated with pathological conditions such as tumor growth, diabetic retinopathy, and ischemic diseases. Both hypoxia and inflammation contribute to the regulation of new vessel growth in ischemic diseases. Vascular endothelial growth factor (VEGF) is a key angiogenic factor produced by ischemic tissues and growing tumors, and upregulation of VEGF expression at the transcriptional level is thought to be responsible for the progressive development of the collateral circulation. Activation of the VEGF gene is mediated by the binding of hypoxia-inducible factor–1α (HIF-1α) to the hypoxia response elements (HREs) present in the promoter region of the gene. HIF-1α belongs to the Per-Arnt-Sim (PAS) family of basic helix-loop-helix transcription factors and dimerizes with another basic helix-loop-helix transcription factor, HIF-1β. The expression of HIF-1α is maintained at low levels in most cells under normoxic conditions. However, hypoxia results in loss of prolyl hydroxylation of HIF-1α and a consequent reduction both in the level of HIF-1α ubiquitination and in its degradation by the proteasome, leading to an increase in HIF-1α abundance and HIF-mediated transcriptional responses.

A wide range of biological responses to and toxic effects of exogenous compounds, such as polycyclic and halogenated aromatic hydrocarbons and dioxins found in tobacco smoke and the environment, is mediated by the aryl hydrocarbon receptor (AHR). The AHR also contains a basic helix-loop-helix motif and functions together with the AHR nuclear translocator (ARNT), which is identical to HIF-1β. The ligand-bound AHR and ARNT form a heterodimeric transcription factor that binds to xenobiotic response elements (XREs) in the promoter regions of target genes such as that encoding cytochrome P4501A1 (CYP1A1). Transcriptional activation of the CYP1A1 gene results in oxidative metabolism of polycyclic aromatic hydrocarbons which, depending on the structure of the compound, can result either in the generation of active electrophilic derivatives that are able to bind to cellular macromolecules such as DNA or, alternatively, in detoxification. Given that AHR contributes to the harmful effects of toxic environmental chemicals, its function...
in the regulation of xenobiotic metabolism and carcinogenesis has been extensively studied. Moreover, a role for AHR in cardiac and vascular development and homeostasis has been revealed by the generation of AHR-null mice. However, whether AHR plays a role in ischemia-induced angiogenesis has remained unclear.

We hypothesized that AHR might interact with hypoxia signaling in the regulation of angiogenesis, given that smoking is a risk factor for both ischemic heart disease and peripheral vascular disease and that tobacco smoke contains AHR activators. We have now investigated this hypothesis with AHR-null mice and a surgical model of ischemia.

Methods

Animals
Twelve-week-old male wild-type (C57BL/6N) mice or AHR-null mice backcrossed with C57BL/6N mice for more than 8 generations were studied. AHR-null mice were obtained from the National Cancer Institute colony and maintained by the Animal Resource Facility at Nagoya University Graduate School of Medicine. Animals were subjected to left femoral artery ligation as described previously (see online supplement, available at http://atvb.ahajournals.org). All experimental procedures were performed in accordance with institutional guidelines for animal research, and the study was approved by the animal ethics committee of Nagoya University Graduate School of Medicine.

Systolic Blood Pressure and Laser Doppler Perfusion Imaging
Systolic blood pressure of conscious mice was measured by the tail-cuff method (BP-98A, MCP-1; Softron) immediately before and each week after arterial ligation. Ischemia-induced functional changes in vascularization were detected by laser Doppler perfusion imaging with a laser Doppler flow analyzer (Moor LDI; Moor Instruments) immediately as well as 3, 7, 14, and 21 days after surgery, as previously described (see online supplement).

Histological Analysis
Ischemic or nonischemic adductor skeletal muscles isolated from mice 3 weeks after surgery were fixed with methanol, embedded in paraffin, and sectioned at a thickness of 5 μm. Leukocyte infiltration was examined by hematoxylin-eosin staining. Capillary endothelial cells were identified by immunohistochemical staining with goat polyclonal antibodies specific for mouse CD31 (Santa Cruz Biotechnology) as described. Capillaries defined by positive staining for CD31 were then counted in 10 randomly chosen fields of defined area. Capillary density was expressed as the number of capillaries per square millimeter.

Quantitative RT-PCR and Immunoblot Analysis
Total RNA was extracted from ischemic or nonischemic thigh muscles isolated 3 days after surgery and was subjected to quantitative reverse transcription and polymerase chain reaction (RT-PCR) analysis. Nuclear extracts of ischemic or nonischemic thigh muscles isolated 1 week after surgery were prepared and according to the instructions for a nuclear extraction kit (Active Motif). The nuclear and protein extracts were subjected to immunoblot analysis (see online supplement).

Analysis of the DNA Binding Activity of Transcription Factors
The DNA binding activity of HIF-1α–ARNT was determined by electrophoretic mobility-shift assay (EMSA) as described (see online supplement). We also performed chromatin immunoprecipitation (ChIP) as described (see online supplement).

Statistical Analysis
Data are presented as means±SEM. Statistical significance of differences was evaluated with the unpaired Student t test for comparisons between 2 means and with one-way analysis of variance.
followed by Dunnett post hoc test for comparisons among 4 means. A probability value of \( P < 0.05 \) was considered statistically significant.

**Results**

**Body Weight and Systolic Blood Pressure**

Body weight did not differ between AHR-null mice and wild-type mice either before (12 weeks of age) or for up to 3 weeks after arterial ligation to induce hypoxia in the left hindlimb (data not shown). Systolic blood pressure remained at basal levels for up to 3 weeks after surgery and also did not differ between AHR-null and wild-type mice (data not shown).

**Serial Laser Doppler Perfusion Imaging**

Blood flow recovered in the ischemic hindlimb of both wild-type and AHR-null mice (Figure 1A). However, 3 days after surgery, the ischemic/nonischemic blood flow ratio of AHR-null mice was significantly greater than that of wild-type mice (Figure 1B). This difference between AHR-null and wild-type mice remained apparent for up to 3 weeks.

**Capillary Density**

Capillary density was evaluated as a measure of vascularity at the level of the microcirculation. Adductor muscles from the ischemic and nonischemic hindlimbs of mice 3 weeks postsurgery were immunostained for CD31 (Figure 2A). Collateral capillary density in the ischemic hindlimb was significantly greater for AHR-null mice than for wild-type animals (Figure 2B). Capillary density in the nonischemic hindlimb did not differ between the two mouse strains. Histological analysis revealed that, whereas the number of infiltrating leukocytes in skeletal muscle was significantly greater for the ischemic hindlimb than for the nonischemic hindlimb of AHR-null or wild-type mice, there was no significant difference in this parameter between the ischemic hindlimb of AHR-null mice and that of wild-type mice (data not shown).

**Blood Flow Ratio in Mice Exposed to B[a]P**

Exposure to benzo[a]pyrene (B[a]P) did not affect body weight (supplemental Figure 1A) or blood pressure (data not shown) in wild-type mice. Blood flow recovered in the ischemic hindlimb of wild-type mice exposed to B[a]P (supplemental Figure 1B); however, at 1 to 3 weeks after surgery, the ischemic/nonischemic blood flow ratio of wild-type mice exposed to B[a]P at a dose of 125 mg/kg per week was significantly reduced compared with that of control wild-type mice. Furthermore, the blood flow ratio was significantly greater in AHR-null mice exposed to B[a]P at 125 mg/kg per week than in wild-type animals exposed to B[a]P at the same dose (supplemental Figure 1C).

**Expression of AHR, HIF-1α, and ARNT**

Quantitative RT-PCR analysis showed that the amounts of both HIF-1α and ARNT mRNAs were increased in skeletal muscle of the ischemic hindlimb of both AHR-null and wild-type mice, compared with those in the corresponding nonischemic hindlimb, at 3 days after surgery (Figure 3A). These increases were significantly greater in AHR-null mice than in wild-type mice. Immunoblot analysis also revealed similar changes in the nuclear abundance of HIF-1α and ARNT proteins at 1 week after surgery (Figure 3B). The amount of AHR mRNA in skeletal muscle did not differ
between the ischemic hindlimb and the nonischemic hindlimb in wild-type mice at 3 days after surgery (Figure 3A).

Expression of VEGF, VEGF Receptors, PDGF-B, TGF-β1, Ang-1, and Ang-2

Quantitative RT-PCR analysis revealed that, whereas the amounts of VEGF and PDGF-B mRNAs in skeletal muscle were significantly greater for the ischemic hindlimb than for the nonischemic hindlimb of AHR-null or wild-type mice at 3 days after surgery, they were also significantly greater for the ischemic hindlimb of AHR-null mice than for that of wild-type mice (Figure 4A). The amounts of plasminogen activator inhibitor (PAI)-1 and TGF-β1 mRNAs in skeletal muscle were similar in the ischemic and nonischemic hindlimbs of AHR-null or wild-type mice (Figure 4A). Immuno blot analysis also revealed results for the abundance of VEGF, PDGF-B, PAI-1, and TGF-β1 proteins at 1 week after surgery similar to those obtained for the corresponding mRNAs (Figure 4B). The amounts of mRNAs for the VEGF receptors Flt-1, Flk-1, and neuropilin-1 in skeletal muscle were significantly greater for the ischemic hindlimb than for the nonischemic hindlimb of AHR-null or wild-type mice at 3 days after surgery (supplemental Figure II); those for Flt-1 and Flk-1 mRNAs were also significantly greater for the ischemic hindlimb of AHR-null mice than for that of wild-type mice. The amounts of mRNAs for Ang-1 and Ang-2 in skeletal muscle were significantly greater for the ischemic hindlimb than for the nonischemic hindlimb of AHR-null or wild-type mice at 3 days after surgery (supplemental Figure II); that for angiopoietin-2 (Ang-2) mRNA was also significantly greater for the ischemic hindlimb of AHR-null mice than for that of wild-type mice.

DNA Binding Activity of HIF-1α–ARNT

To investigate whether the lack of the AHR affects the DNA binding activity of HIF-1α–ARNT in nuclear extracts of skeletal muscle, we performed an EMSA with an oligonucleotide containing an HRE. Controls for the EMSA experiments, including the addition of excess unlabeled wild-type or mutant probes and supershift analysis with antibodies to HIF-1α or to ARNT, demonstrated that the DNA binding activity observed was indeed attributable to HIF-1α–ARNT. The DNA binding activity of HIF-1α–ARNT was significantly increased in the ischemic hindlimb of both AHR-null and wild-type mice at 1 week after arterial ligation; however, it was significantly greater in the ischemic limb of AHR-null mice than in that of wild-type mice (Figure 5A). ChIP analysis revealed that the amount of DNA corresponding to
the promoter of the VEGF gene that was precipitated with antibodies to ARNT or to HIF-1α was greater for the ischemic hindlimb of both AHR-null and wild-type mice at 1 week after arterial ligation than for the corresponding nonischemic limb; however, it was also greater for the ischemic limb of AHR-null mice than for that of wild-type mice (Figure 5B). These results are thus consistent with the corresponding levels of HIF-1α and ARNT detected by immunoblot analysis (Figure 3B).

**Figure 4.** Expression of VEGF, PDGF-B, PAI-1, and TGF-β1 in skeletal muscle of the ischemic and nonischemic hindlimbs of AHR-null or wild-type mice. A, Quantitative RT-PCR analysis of VEGF, PDGF-B, PAI-1, and TGF-β1 mRNAs in tissue isolated 3 days after surgery. Data are normalized by the abundance of β-actin mRNA and are means±SEM of values from 8 mice per group. *P<0.05 vs nonischemic hindlimb of wild-type mice; †P<0.05 vs nonischemic hindlimb of AHR-null mice; ‡P<0.05 vs ischemic hindlimb of wild-type mice. B, Representative immunoblot analysis of VEGF, PDGF-B, PAI-1, and TGF-β1 in ischemic or nonischemic tissue at 1 week after surgery.

**Discussion**

Although AHR serves as a receptor for polycyclic and halogenated aromatic hydrocarbons in tobacco smoke and for dioxins in the environment, one or more endogenous ligands for AHR has been proposed to play important roles in embryonic development, homeostasis, apoptosis, immunosuppression, and cell proliferation.19 AHR-null mice are relatively unaffected by the potent AHR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at doses that induce severe toxic and pathological effects in wild-type mice.20 Moreover, B[a]P-induced genotoxic and carcinogenic responses are greatly diminished in AHR-null mice.21 It had remained unclear, however, whether AHR plays a role in ischemia-induced angiogenesis. We have now shown that both angiogenesis and the recovery of blood flow after the induction of ischemia in the mouse hindlimb were markedly potentiated by AHR deficiency.

In the present study, we demonstrated that ischemia-induced upregulation of the expression of HIF-1α and ARNT as well as that of the VEGF gene, a target of these transcription factors, was enhanced in AHR-null mice. Transcriptional activation of the VEGF gene and other HIF-1 target genes by a HIF-1α–VP16 hybrid transcription factor was previously shown to facilitate recovery of perfusion in the rabbit ischemic hindlimb, suggesting that hypoxia-induced angiogenesis is mediated in large part by upregulation of proteins encoded by HIF-1α target genes.22 Furthermore, VEGF and Ang-1 are thought to have potential as therapeutic angiogenic factors for
promotion of progressive development of the collateral circulation,\textsuperscript{2,23} given that their receptors are expressed specifically by endothelial cells. In the present study, the expression of HIF-1\textalpha and ARNT at the mRNA and protein levels was significantly greater in the ischemic hindlimb of AHR-null mice than in that of wild-type animals. Moreover, the DNA binding activity of the HIF-1\textalpha–ARNT complex and the amounts of ARNT and HIF-1\textalpha associated with the VEGF gene promoter were markedly greater in the ischemic hindlimb of wild-type mice. Specificity of HIF-1\textalpha–ARNT binding activity was analyzed in the representative blot shown in the upper right panel. Lane 1, reaction mixture without nuclear extract; lane 2, reaction mixture with unlabeled probe instead of labeled probe; lane 3, control complete reaction mixture; lane 4, reaction mixture with 66-fold excess of unlabeled probe; lane 5, reaction mixture with mutant probe instead of wild-type probe; lanes 6 and 7, supershift analysis with antibodies to HIF-1\textalpha or to ARNT, respectively. Arrows indicate supershifted bands. B, Representative ChIP assay of the binding of HIF-1\textalpha or ARNT to the VEGF gene promoter in tissue extracts of tissue isolated 1 week after surgery. The upper panels represent immunoprecipitates prepared with antibodies to ARNT and 10\% of the input for immunoprecipitation; the lower panels represent immunoprecipitates prepared with antibodies to HIF-1\textalpha and 10\% of the input for immunoprecipitation. Arrows indicate PCR products corresponding to the HREs of the VEGF gene promoter, and the leftmost lanes contain molecular size markers.

Although VEGF is a powerful growth factor for angiogenesis or arteriogenesis in animals and humans with limb or myocardial ischemia, various angiogenic factors and inhibitors regulate the proliferation of endothelial cells and capillary permeability.\textsuperscript{1} In the present study, we also detected a marked increase in the expression of PDGF-B, Ang-1, Ang-2, and multiple receptors that bind VEGF in the ischemic hindlimb of both AHR-null and wild-type mice. Furthermore, the ischemia-induced increase in the expression of PDGF-B, Flt-1, Flk-1, and Ang-2 was significantly greater in AHR-null mice than in wild-type mice. Given that the expression of VEGF receptors correlates with capillary cell proliferation,\textsuperscript{24} the greater ischemia-induced increase in Flt-1 and Flk-1 expression in AHR-null mice may result from the greater ischemia-induced increase in capillary growth in these animals. Moreover, the expression of Ang-1 and Ang-2, both of which are ligands for the receptor Tie2, is differentially controlled. Ang-2 expression is induced by various growth factors, including VEGF and fibroblast growth factor–2, and by hypoxia.\textsuperscript{25} The expression of Ang-2 might therefore be upregulated to a greater extent in the ischemic hindlimb of AHR-null mice than in that of wild-type mice as a result of the associated greater increase in the expression of angiogenic growth factors such as VEGF or PDGF-B.
To determine the effect of smoking on angiogenesis, we treated mice with B[α]P, one of the polycyclic and halo- nated aromatic hydrocarbons found in tobacco smoke. At a dose of 125 mg/kg per week, oral exposure to B[α]P resulted in significant inhibition of the increase in blood flow induced by hypoxia in wild-type mice. This observation is consistent with previous results showing that mice exposed to cigarette smoke exhibited a marked impairment of angiogenesis in response to surgically induced hindlimb ischemia. This inhibition of angiogenesis by cigarette smoke was associated with inhibition of hypoxia-induced upregulation of HIF-1α and VEGF expression. Thackaberry et al showed that AHR-null mice developed cardiac hypertrophy at 5 months of age and that the abundance of HIF-1α protein and VEGF mRNA was significantly increased in the heart of AHR-null mice. In the present study, whereas the expression of HIF-1α, ARNT, or VEGF in the nonischemic hindlimb did not differ between AHR-null and wild-type mice, the expression of these proteins and the DNA binding activity of HIF-1α–ARNT were increased by ischemia to a greater extent in AHR-null mice than in wild-type animals. An increased availability of transcriptional cofactors common to both the HIF-1α pathway and the disrupted AHR signaling cascade, including ARNT, the 90-kDa heat shock protein (Hsp90), the cAMP response element–binding protein (CREB)–binding protein (CBP), and steroid receptor coactivator–1 (Src-1), might contribute to the greater ischemia-induced increase in the transactivation activity of HIF-1α–ARNT apparent in AHR-null mice. Given that the hypoxia and dioxin response pathways compete for limiting cellular factors, the dimerization of ARNT with HIF-1α and consequent activation of genes involved in the regulation of adaptation to low oxygen tension are likely facilitated in AHR-null mice. However, for a single intraarterial bolus of vascular endothelial growth factor augmented revascularization in a rabbit ischemic hind limb model. The AHR pathway interferes with other ARNT-independent pathways, such as the progesterone- and estrogen-responsive pathways, suggesting that competition for other cofactors might also account for this cross-talk. The overexpression of AHR might be able to clarify the mechanisms of the cross-talk between the AHR and HIF-1α signaling pathways.

There are some limitations to the present study. (1) Evidence for ischemia-induced functional changes in vascu larization was obtained by laser Doppler perfusion imaging. The data yielded by this technique can be affected by anesthesia, ambient light, and body temperature. To avoid interference from such effects, we expressed blood flow in the ischemic leg relative to that in the nonischemic leg. However, this technique also largely reflects changes in surface blood flow. We placed mice on a heating plate at 37°C immediately before measurement of blood flow and obvious surface blood flow was excluded from the analysis. (2) Whereas it remains controversial as to whether the amount of HIF-1α mRNA increases in response to hypoxia, ARNT is thought to be expressed constitutively regardless of oxygen tension. The availability of HIF-1α is thus thought to be rate limiting in formation of the HIF-1α–ARNT heterodimer. In the present study, the expression of HIF-1α and ARNT at the mRNA and protein levels as well as the amounts of HIF-1α and ARNT associated with the VEGF gene promoter were greater in the ischemic hindlimb than in the nonischemic hindlimb of both AHR-null and wild-type mice, and these effects of hypoxia were more pronounced in the former animals. The mechanisms of these effects of hypoxia and AHR deficiency remain to be determined.

In conclusion, angiogenesis as well as upregulation of the expression of HIF-1α, ARNT, and VEGF elicited by ischemia were found to be enhanced in AHR-null mice. These effects of AHR deficiency were also associated with an increase in the DNA binding activity of the HIF-1α–ARNT complex in the ischemic hindlimb of AHR-null mice. Our observations suggest that the enhancement of ischemia-induced angiogenesis in AHR-null mice may be attributable, in part, to an increased association of HIF-1α with ARNT that results from the lack of AHR and leads to increased production of VEGF.

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Disclosures
None.

References


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Methods:

Animals

Twelve-week-old male wild-type (C57BL/6N) mice or AHR-null mice backcrossed with C57BL/6N mice for more than eight generations were studied. AHR-null mice were obtained from the National Cancer Institute colony and maintained by the Animal Resource Facility at Nagoya University Graduate School of Medicine. Animals were subjected to left femoral artery ligation as described previously (see online supplement). Ischemia was induced in the left hindlimb with mice anesthetized by intraperitoneal injection of sodium pentobarbital (10 mg per kilogram of body mass). After careful dissection of the vein and nerve, the proximal and distal portions of the femoral artery and the distal portion of the saphenous artery were ligated. All side branches were dissected and ligated, and the arteries were then excised. All experimental procedures were performed in accordance with institutional guidelines for animal research, and the study was approved by the animal ethics committee of Nagoya University Graduate School of Medicine.

Systolic Blood Pressure and Laser Doppler Perfusion Imaging

Systolic blood pressure of conscious mice was measured by the tail-cuff method (BP-98A, MCP-1; Softron, Tokyo, Japan) immediately before and each week after arterial ligation. Ischemia-induced functional changes in vascularization were detected by laser Doppler perfusion imaging with a laser Doppler blood flow analyzer (Moor LDI; Moor Instruments, Axminster, UK) immediately as well as 3, 7, 14, and 21 days after surgery, as previously described (see online supplement). Blood flow was measured for all parts of the limb (including the thigh, calf, and foot) and was displayed as changes in the laser frequency
with different colored pixels. After scanning, the stored images were analyzed to quantify blood flow, and the average flows of the ischemic and nonischemic hindlimbs were calculated. To avoid interference from ambient light and body temperature, we expressed blood flow in the ischemic leg relative to that in the nonischemic leg.

**Quantitative RT-PCR Analysis**

Total RNA was extracted from ischemic or nonischemic thigh muscles isolated 3 days after surgery and was subjected to quantitative reverse transcription and polymerase chain reaction (RT-PCR) analysis with primers specific for mRNAs encoding AHR, HIF-1α, ARNT, platelet-derived growth factor B (PDGF-B), transforming growth factor-β1 (TGF-β1), plasminogen activator inhibitor–1 (PAI-1), VEGF, receptors that bind VEGF (Flt-1 [VEGFR1], Flk-1 [VEGFR2], and neuropilin-1), angiopoietin-1 (Ang-1), and Ang-2 (Applied Biosystems, Foster City, CA). The mRNA for β-actin was used as an internal standard.

**Immunoblot Analysis**

Nuclear extracts of ischemic or nonischemic thigh muscles isolated 1 week after surgery were prepared as described and according to the instructions for a nuclear extraction kit (Active Motif, Carlsbad, CA). The nuclear extracts were subjected to immunoblot analysis with rabbit polyclonal antibodies to HIF-1α and goat polyclonal antibodies to ARNT (both at 1:200 dilutions and from Santa Cruz Biotechnology) as well as with a mouse monoclonal antibody to lamin B (1:500 dilution; Chemicon, Santa Monica, CA). Immune complexes were detected with enhanced chemiluminescence (ECL) reagents (GE Healthcare Bio-Science, Piscataway, NJ). Band intensity was quantified with the use of Quantity One Image software (Bio-Rad, Hercules, CA). Protein extracts prepared from homogenates of ischemic or nonischemic thigh muscles isolated 1 week after surgery were also subjected to
immunoblot analysis with goat polyclonal antibodies to VEGF or rabbit polyclonal antibodies to PDGF-B, to PAI-1, and to TGF-β1 (all at dilutions of 1:200 and from Santa Cruz Biotechnology) as well as with a mouse monoclonal antibody to β-actin (1:500 dilution, Chemicon).

Analysis of the DNA Binding Activity of Transcription Factors

The DNA binding activity of HIF-1α–ARNT was determined by electrophoretic mobility-shift assay (EMSA) with a kit (Panomics, Redwood City, CA) as described. In brief, nuclear extracts were prepared from skeletal muscle isolated 1 week after surgery as described above and were incubated with a biotin-labeled double-stranded oligonucleotide containing a binding site for HIF-1α–ARNT. Protein-DNA complexes were then separated from the free DNA probe by electrophoresis on a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer under nondenaturing conditions and were stained with ECL reagents. “Supershift” analysis with antibodies to HIF-1α or to ARNT was performed to identify the binding of HIF-1α and ARNT to the HRE as described. Chromatin immunoprecipitation (ChIP) was performed with a kit (Active Motif) as described. In brief, nuclear extracts were prepared from skeletal muscle isolated 1 week after surgery, and DNA and proteins were cross-linked by exposure to 1% formaldehyde. After fragmentation of DNA with an ultrasonic disrupter (Sonicator VP-5S; Taitec, Saitama, Japan), DNA-ARNT or DNA–HIF-1α complexes were immunoprecipitated with antibodies to ARNT or to HIF-1α, protein was removed from the complexes, and the remaining DNA was used as a template for amplification of the HREs in the VEGF gene promoter by PCR with the primers 5′-GTCTGCCCAGCAGTTGTCTCT-3′ (forward) and 5′-GTGAGACGACCTGTGGAAACC-3′ (reverse).

Oral Exposure to Benzo[a]pyrene
The effect of benzo[a]pyrene (B[a]P) treatment on angiogenesis induced by femoral artery occlusion was examined in wild-type and AHR-null mice. B[a]P (Sigma-Aldrich Japan, Tokyo, Japan) was first administered to wild-type mice by oral gavage in corn oil (10 ml/kg) at 125, 25, or 5 mg/kg once per week beginning at 12 weeks of age as described. Ischemia-induced functional changes in vascularization were evaluated by laser Doppler perfusion imaging immediately as well as 7, 14, and 21 days after surgery performed at 12 weeks of age. Given that B[a]P at the dose of 125 mg/kg per week induced a significant decrease in the blood flow ratio in wild-type mice, the effects of B[a]P were compared between wild-type and AHR-null mice at this concentration.

References:


Results:
Figure I. Changes in body weight and in blood flow in the ischemic hindlimb of AHR-null or wild-type mice exposed to B[a]P. (A) Body weight before (time 0) and for up to 3 weeks after surgery in wild-type mice exposed to B[a]P at 0, 5, 25, or 125 mg/kg per week. (B) The ratio of blood flow in the ischemic hindlimb to that in the normal hindlimb measured immediately (time 0) as well as 1, 2, and 3 weeks after surgery in wild-type mice exposed to B[a]P. (C) The ratio of blood flow in the ischemic hindlimb to that in the normal hindlimb of AHR-null or wild-type mice exposed to B[a]P at a dose of 125 mg/kg per week. All data are means ± SEM of values from five or six animals per group. *P < 0.05 versus corresponding value for vehicle-treated wild-type mice; †P < 0.05 versus corresponding value for wild-type mice exposed to B[a]P at 125 mg/kg per week.
**Figure II.** Quantitative RT-PCR analysis of Flt-1, Flk-1, neuropilin-1, Ang-1, and Ang-2 mRNAs in skeletal muscle of the ischemic and nonischemic hindlimbs of AHR-null or wild-type mice 3 days after surgery. Data are normalized by the abundance of β-actin mRNA and are means ± SEM of values from eight mice per group. *P < 0.05 versus nonischemic hindlimb of wild-type mice; †P < 0.05 versus nonischemic hindlimb of AHR-null mice; ‡P < 0.05 versus ischemic hindlimb of wild-type mice.