Aryl hydrocarbon receptor mediates laminar fluid shear stress-induced CYP1A1 activation and cell cycle arrest in vascular endothelial cells

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Aims We investigated the mechanisms of shear stress (SS)-induced activation of cytochrome P450 (CYP) 1A1 and cell cycle arrest with regard to the role of the aryl hydrocarbon receptor (AhR), since AhR mediates the expression of CYP1A1 induced by polycyclic aromatic hydrocarbons (PAHs) and is thought to be involved in the regulation of cell growth and differentiation.

Methods and results Human umbilical vein endothelial cells (ECs) were exposed to laminar SS and thereafter collected to evaluate the expression, activity, and transcription of CYP1A1 and the expression of AhR and cell cycle-related proteins. A physiological level of laminar SS (15 dynes/cm²) markedly increased the expression level and enzymatic activity of CYP1A1. SS stimulated CYP1A1 promoter activity without influencing mRNA stability. Loss of two functional xenobiotic response elements (XREs) in the 5' flanking region of the CYP1A1 gene suppressed the SS-induced transcription of CYP1A1. Laminar SS stimulated the expression and nuclear translocation of AhR. α-Naphthoflavone, an AhR antagonist, and a small interfering RNA (siRNA) for AhR significantly suppressed SS-induced CYP1A1 expression. The siRNA also abolished SS-induced cell cycle arrest, the expression of the cyclin-dependent kinase inhibitor p21Cip1, and dephosphorylation of retinoblastoma protein.

Conclusion Laminar SS stimulated the transcription of CYP1A1 through the activation of AhR in a way that is similar to the effects of PAHs. AhR was also involved in cell cycle arrest induced by SS. Our results suggest that sustained activation of AhR exposed to blood flow plays an important role in the regulation of EC functions.

1. Introduction

Aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, plays a central role in the induction of cytochrome P450 (CYP) 1A1, an enzyme produced on exposure to polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).² On binding to AhR, PAHs translocate the receptor from the cytoplasm into the nucleus, where it forms a heterodimer with AhR nuclear translocator protein (ARNT). The dimer binds to the xenobiotic response elements (XREs) in the promoter region of the CYP1A1 gene to activate CYP1A1 transcription.³ CYP1A1 generates harmful bioactive substances by metabolizing PAHs, and therefore, it is believed that AhR activation and subsequent CYP1A1 induction are aetiological factors for diseases such as cancers and atherosclerosis.⁴

In contrast, AhR is reported to contribute to normal physiological processes during cell growth and differentiation.⁵ AhR-knockout mice exhibit a number of phenotypic abnormalities such as peripheral immune system deficiency, liver defects, and hypertrophic and fibrotic changes of the cardiovascular system, although the mice are resistant to TCDD toxicity.⁶ In cultured cells, several studies suggest that the activation of AhR is involved in cell cycle arrest in the absence or presence of exogenous agonists.⁷–⁹ These observations suggest that AhR may have important roles in physiological functions. CYP1A1 is constitutively expressed in vascular endothelium in vivo, however, the involvement of AhR has not been fully investigated. Vascular endothelial cells (ECs) are constantly exposed to haemodynamic forces, such as fluid shear stress (SS). Laminar SS plays an essential
role in the maintenance of the structure and function of blood vessels by regulating the expression of numerous genes and proteins. Recent studies show that laminar SS strongly induces the expression of CYP1A1 in vascular ECs. However, in spite of this, evidence suggests that laminar blood flow is athero-protective. Atherosclerosis tends to occur in areas exposed to disturbed flow or low SS, and a lack of SS induces apoptosis in ECs. Therefore, we believe that the SS-induced sustained expression of the cyclin-dependent kinase inhibitor reported that SS inhibits EC proliferation by inducing the involvement of AhR in vascular ECs. Since we have identified that CYP1A1 is strongly induced by laminar SS in relation to the involvement of AhR in vascular ECs. Since we have reported that SS inhibits EC proliferation by inducing the expression of the cyclin-dependent kinase inhibitor p27kip1, we also investigated the relationship between AhR and SS-induced cell cycle inhibition.

2. Methods

2.1 Chemicals

Actinomycin D (Act D), alpha-naphthoflavone (α-NF), and SB203580 were purchased from Sigma. U0126 was purchased from Cell Signaling Technology. SP600125 was purchased from LC Laboratories.

2.2 Cell culture

Human umbilical vein ECs (HUVECs) were isolated by collagenase digestion and cultured as described previously. Human umbilical cord vessels were obtained from healthy women who underwent uncomplicated term pregnancies. This investigation conforms to the principles outlined in the Declaration of Helsinki. Informed consent was obtained from each donor. Bovine arterial ECs (BAECs) were obtained from each donor. Bovine arterial ECs (BAECs) were purchased from Amershams Biosciences. The primers for human CYP1A1 and GAPDH were synthesized based on information obtained from the GenBank database: CYP1A1, 5′-GGACTCTTCCTCAGACCGGTA-3′ and 5′-AGCATGTCCCTCAGCCAGA-3′; GAPDH, 5′-TCCACCACCTGTGTTCTGTA-3′ and 5′-ACCAACAGTCGACCATCA-3′; bovine CYP1A1, 5′-TCGGCCATGCTGTGTTG-3′ and 5′-GCAAGATAGCAGTGGCCACTG-3′; and bovine GAPDH 5′-AAGGCAGAAGGGAAGACT-3′ and 5′-TCCCTCCAGATGCCAAAGT-3′.

2.3 Shear stress experiments

The laminar flow experiments were performed using a parallel plate chamber as described previously. Briefly, cells were grown on gelatin-coated polyester sheets (Plastic Suppliers) until confluent. Control cells (static cells) were grown on the same polyester sheets in the same medium as sheared cells until confluent, and were transferred into fresh medium before being maintained in an incubator. A confluent monolayer of ECs on a polyester sheet was placed in a parallel-plate flow chamber and subjected to steady laminar shear stress. The flow loop with reservoirs and the flow chamber were filled with DMEM containing 10% foetal bovine serum.

To compare the effects of laminar and turbulent SS on the expression of CYP1A1 and AhR, a cone-plate type apparatus was used as described previously. Cells were grown on a 10 cm dish until confluent and then exposed to laminar or turbulent SS using 0.5° or 5° cones, respectively, with a rotational velocity of 120 r.p.m. From the calculated modified Reynolds number (R), turbulent flow was established at radii ≥2.4 cm, which corresponded to an R > 5 and represented an average SS strength of 1.5 dyne/cm². Laminar SS strength was 6 dyne/cm². Cells were harvested from only the outer portion of the glass plate (≥2.4 cm).

2.4 Western blot analysis

Western blotting was performed as described previously, using a polyclonal anti-CYP1A1 antibody (Santa Cruz Biotechnology), polyclonal anti-AhR antibody (Santa Cruz Biotechnology), polyclonal anti-ERK/phospho-ERK antibody (Cell Signaling Technology), monoclonal anti-JNK antibody and polyclonal anti-phospho-JNK antibody (Cell Signaling Technology), polyclonal anti-p38 mitogen-activated protein kinase (MAPK)/phospho-p38 MAPK antibody (Santa Cruz Biotechnology), polyclonal anti-p21Cip1 antibody (Santa Cruz), and monoclonal anti-pRb antibody (Pharmingen). Nucleic and cytoplasmic proteins were purified using a Nuclear and Cytoplasmic Extraction Reagents kit (PIERCE), following the manufacturer’s instructions. Membranes were re-probed with anti-β-actin monoclonal antibody to normalize the amounts of proteins applied to the SDS-PAGE gel, (Calbiochem).

2.5 Reverse transcription–polymerase chain reaction

Total cellular RNA was extracted with TRIzol Reagent (Invitrogen). The expression of mRNA was analysed by reverse transcription–polymerase chain reaction (RT-PCR) using Ready-To-Go RT-PCR Beads (Amershams Biosciences). The primers for human CYP1A1 and GAPDH were synthesized based on information obtained from the GenBank database: CYP1A1, 5′-GGACTCTTCCTCAGACCGGTA-3′ and 5′-AGCATGTCCCTCAGCCAGA-3′; GAPDH, 5′-TCCACCACCTGTGTTCTGTA-3′ and 5′-ACCAACAGTCGACCATCA-3′; bovine CYP1A1, 5′-TCGGCCATGCTGTGTTG-3′ and 5′-GCAAGATAGCAGTGGCCACTG-3′; and bovine GAPDH 5′-AAGGCAGAAGGGAAGACT-3′ and 5′-TCCCTCCAGATGCCAAAGT-3′.

2.6 Measurement of CYP1A1 activity

CYP1A1 activity was evaluated with the ethoxyresorufin-O-dealkylase (EROD) assay, which measures the ability to convert ethoxyresorufin to resorufin, as described previously.

2.7 Construction of plasmids and mutagenesis

The 5′-flanking region of rat CYP1A1 gene (−1166/+18) was amplified by PCR with 5′-GGTGAGATCTGCGCCCTTGCAAAGCTTAAAGACTA-3′ as an antisense primer, and 5′-GGGAGGCTTGGCACCAACCACCTTTATATG-3′ as an antisense primer, and subcloned into the SacI/XhoI sites of a PGL3-basic luciferase-expressing reporter vector (Promega), a firefly luciferase reporter vector. For the two deletion constructs (DM-1 and DM-2), two restriction enzyme-digested fragments (DM-1, SacI/Bst1107I −859/+18; DM-2, SacI/BstEII −206/+18) were blunt-end ligated using a DNA Blunting Kit (Takara). Site-directed mutagenesis was performed using QuickChange Site-directed Mutagenesis Kit (Stratagene), as instructed by the manufacturer. The luciferase reporter construct containing the −1166/+18 region of the CYP1A1 promoter was used as a DNA template. The mutations introduced into the putative binding sites for XRE were as follows: Mut-1 (−1069/-1052) CCCCCAGCTAGTGTA −CCCCCAGCATGTGGACAA; Mut-2 (−987/-970), TCTCAG CAACACTTCGGG −TCTCTAACAACCTTCCGGG. Mut-3 has both mutations. The structure of every DNA construct was verified by sequencing.

siRNA duplexes were prepared by SAMCHULLY and targeted the coding regions of bovine AhR mRNA (770–778). The siRNA duplexes used in this study were as follows: siRNA for AhR (si-AhR), 5′-UACUUCCACUGAUGUUUGGTT-3′ and 5′-GCCAUCGUGGUAGUAU-3′; Scramble (si-Scr), 5′-GCCGCUUUGUUGGAAUUGC-3′ and 5′-CGAUAUCUAAAACCCCGCGTT-3′, respectively.

2.8 Transfection and luciferase reporter assay

Transfection and luciferase reporter assays were performed as described. Since we failed to amplify the fragment of human
CYP1A1 promoter, we used rat CYP1A1 promoter in these assays because it has a high homology with the human CYP1A1 promoter. This is especially true of the four XRE motifs which show 93.3% homology because it has a high homology with the human CYP1A1 promoter. We used rat CYP1A1 promoter in these assays because it has a high homology with the human CYP1A1 promoter.

2.9 Fluorescence microscopy

After stimulation, the cells were fixed in ice-cold methanol/acetone (1:1) for 15 min at −20°C and then washed twice with phosphate-buffered saline. After blocking with 2% bovine serum albumin in phosphate-buffered saline for 30 min, cells were incubated overnight with polyclonal anti-AhR antibody (Santa Cruz Biotechnology, 1:200) at 4°C. Thereafter, cells were washed twice with phosphate-buffered saline and incubated with anti-rabbit IgG+IgA+IgM-biotin (Nichirei) for 1 h at room temperature, followed by streptavidin-fluorescein isothiocyanate conjugate (1:50 dilution; Invitrogen) for 1 h at room temperature. The cells were examined under a fluorescence microscope (Olympus).

2.10 Cell proliferation assay

To assess EC proliferation, we used the BrdU Cell Proliferation Assay (Exalpha Biologicals, Inc., MA, USA) according to the manufacturer’s instructions. After stimulation, ECs were incubated overnight in culture medium containing BrdU (2 μl/ml). The uptake of BrdU was determined with a spectrophotometer (Bio-Tek Instruments, Highland Park, VT, USA) and normalized to the amount of cellular protein, which was measured in parallel samples according to the method of Lowry.

2.11 Statistics

Results are expressed as the mean ± SD of a number of the observations. Statistical significance was assessed by Student’s t-test for paired or unpaired values.

3. Results

3.1 The induction and activation of CYP1A1 in response to shear stress

We first confirmed the effect of SS on the expression of CYP1A1 using HUVECs. Although the level of CYP1A1 mRNA expression was analysed by western blotting. Error bars represent the SD values obtained from four independent experiments. *P < 0.01 vs. SC. (E) HUVECs were exposed to SS for 4 h and thereafter treated with actinomycin D (Act D, 3 μmol/L), and incubated in static conditions (SC + Act D), or exposed to SS (SS + Act D). Expression levels of CYP1A1 mRNA normalized to those of GAPDH mRNA were standardized to the values obtained at the start time (−4 h), and the time-course after the administration of Act D is plotted in the lower panel. Error bars represent the SD values obtained from four independent experiments. (F) HUVECs (upper panel) or bovine arterial endothelial cells (BAECs) (lower panel) transfected with the plasmid containing the −1116/−18 region of the rat CYP1A1 promoter together with pRL-SV40 were incubated in SC, or exposed to laminar SS for 8 h. Mean raw values of firefly (CYP1A1) or renilla (control) luciferase activity are indicated below the panel. Relative luciferase activity is presented as the fold increase against the value obtained under SC. Data represent means ± SD of four independent experiments. *P < 0.01. (G) BAECs were maintained in SC or exposed to laminar SS (SS, 15 dyne/cm²) for the periods indicated. Upper panel: CYP1A1 mRNA expression was analysed by RT–PCR. Lower panel: CYP1A1 protein expression was analysed by western blotting. Error bars represent the SD values obtained from four independent experiments. *P < 0.01 vs. SC. (H) CYP1A1 enzymatic activity in BAECs maintained in SC or exposed to laminar SS (SS, 15 dyne/cm²) was analysed and plotted as described in D. *P < 0.01 vs. SC.
was very low in static control cells, it rose markedly on exposure to a physiological level of laminar SS (15 dyne/cm²) (Figure 1A), consistent with previous reports. The expression of CYP1A1 protein was also increased by SS and sustained over 24 h (Figure 1B). This CYP1A1 induction was SS strength-dependent (Figure 1C) and the expression level reached a maximal at 15 dyne/cm². As shown in Figure 1D, CYP1A1 activity determined by EROD assay was also increased by SS and reached a plateau after 2 h.

To investigate the mechanism of SS-induced CYP1A1 expression, we measured the rate of decay of the CYP1A1 mRNA after the addition of actinomycin D (Act D, 3 μmol/L), a transcription inhibitor. The degradation rates did not differ between the cells exposed to SS and those cultured in static conditions (Figure 1E). We also examined the effect of SS on CYP1A1 gene transcription in HUVECs transfected with a luciferase reporter construct driven by the 5'-flanking region of the rat CYP1A1 gene (−1116/+18 bp). Although the obtained raw values of luciferase activity were relatively low, it was significantly enhanced in HUVECs after exposure to SS for 8 h (Figure 1F, upper panel). When we did the same experiment using BAECs, the values of luciferase activity were much higher than those in HUVECs and furthermore, relative luciferase activity was strongly enhanced by SS (10.9-fold) (Figure 1F, lower panel). In BAECs, laminar SS induced mRNA and protein expressions (Figure 1G) and increased enzymatic activity of CYP1A1 (Figure 1H) as well as in HUVECs. These results suggest that SS induces CYP1A1 expression by activating gene transcription. The following transfection experiments were done using BAECs because of their higher transfection efficiency.
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3.2 Xenobiotic response element mediates shear stress-induced CYP1A1 transcription

A number of studies have shown that PAHs stimulate CYP1A1 gene transcription through the XREs in the gene’s promoter. SS activates the transcription of a variety of genes through several specific sequences; however, the SS-response element in the CYP1A1 promoter has not been identified. Therefore, we attempted to uncover the precise mechanism of SS-induced CYP1A1 expression by analysing the CYP1A1 gene promoter. Since there were four XREs in the rat CYP1A1 gene (−1116/+18 bp), we generated two deletion mutant constructs: DM-1 (−859/+18 bp) and DM-2 (−206/+18 bp). In BAECs transfected with these deletion mutants, the increase in luciferase activity was almost completely suppressed in comparison with the marked elevation in wild type (WT) (Figure 2A). This suggests that the main elements responsive to SS were located between bp −1116 and −859 in the 5'-flanking region, where two XREs exist.

Subsequently, we introduced mutations (Mut-1, Mut-2, and Mut-3) into these two upstream XREs to determine whether these sequences are responsible for SS-induced CYP1A1 expression. Mut1 and Mut2 significantly suppressed SS-induced CYP1A1 promoter activity, and Mut3, where both of the XREs were mutated, almost eliminated the response to SS (Figure 2B). These results suggest that both of these XREs are essential for CYP1A1 induction by SS.

3.3 Laminar shear stress induced the expression and nuclear translocation of aryl hydrocarbon receptor

The results suggested that SS activates CYP1A1 gene transcription through XREs in a similar way to PAHs. AhR is known to mediate PAH-induced transcriptional activation of the CYP1A1 gene. Therefore, we subsequently examined the effect of SS on AhR expression. As shown in Figure 3A, SS significantly increased the expression of AhR protein, as well as CYP1A1, and this induction was sustained over 24 h. Immunoblotting of the subcellular fractions revealed that AhR was predominantly in the cytoplasm with only a small amount present in the nucleus in static ECs; however, it was translocated from the cytoplasm into the nucleus after stimulation with SS (Figure 3B). The nuclear translocation of AhR by SS was confirmed by immunofluorescence staining (Figure 3C).

We also compared the effects of the physiological level of laminar and turbulent SS on the expression of AhR and CYP1A1, because atherosclerotic lesions tend to develop at arterial bifurcations and curvatures where laminar blood flow is often turbulent. As shown in Figure 3D, the physiological level of turbulent SS (average strength: 1.5 dyne/cm²) induced AhR and CYP1A1, however; the induction was weaker than that by laminar SS (6 dyne/cm²).

3.4 The aryl hydrocarbon receptor inhibitor alpha-naphthoflavone and aryl hydrocarbon receptor small interfering RNA suppressed laminar shear stress-induced CYP1A1 expression

We examined the effect of AhR inhibition to determine whether AhR is essential for the SS-induced expression of CYP1A1. Alpha–NF (10 μmol/L), an AhR inhibitor, strongly suppressed SS-induced CYP1A1 expression (Figure 4A). We also examined the effect of siRNA for AhR (si-AhR) and found that si-AhR significantly suppressed SS-induced expression of AhR, whereas scramble siRNA (si-Scr) did not (Figure 4B). When the AhR si-RNA was transfected into BAECs, SS-induced expression of CYP1A1 was strongly suppressed (Figure 4C).
3.5 Involvement of the mitogen-activated protein kinase pathways in shear stress-induced aryl hydrocarbon receptor expression

Several reports have suggested that MAPKs modulate AhR expression. It has also been reported that SS activates MAPKs. To elucidate the mechanism by which SS induces the expression of AhR, we examined the effect of SS on the phosphorylation of MAPKs, i.e. ERK, JNK, and p38. As shown in Figure 5A, the expression levels of these MAPKs were not altered by SS. However, the levels of phosphorylated JNK and p38 but not ERK were markedly elevated by SS. Importantly, these time-courses were similar to those of AhR and CYP1A1 expression induced by SS. We subsequently examined the effects of the MAPK inhibitors U0126 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) on SS-induced expression of AhR and CYP1A1. Both SP600125 (Figure 5B) and SB203580 (Figure 5C) markedly reduced SS-induced expression of AhR and CYP1A1, whereas U0126 did not (Figure 5D). This suggests that SS induces expression of AhR through phosphorylation of JNK and p38, resulting in an increase in CYP1A1 expression.

Figure 3  Effects of laminar shear stress (SS) on aryl hydrocarbon receptor (AhR) expression and nuclear translocation of AhR. (A) Human umbilical vein endothelial cells (HUVECs) were maintained in static conditions (SC) or exposed to laminar SS (15 dyne/cm²) (SS) for the periods indicated. Expression levels of AhR protein normalized to those of β-actin protein were standardized to the values obtained at 0 h and shown as means ± SD of four independent experiments in the bar graph. *P < 0.01. (B) HUVECs were maintained in SC or exposed to laminar SS (15 dyne/cm²) (SS) for 8 h. Nuclear (N) and cytoplasmic (C) proteins were purified as described under ‘Methods’. (C) HUVECs were maintained in SC or exposed to laminar SS (15 dyne/cm²) (SS) for 8 h. After stimulation, immunofluorescent staining with an anti-AhR antibody was performed as described under ‘Methods’. Bar graph represents the percentages of cells positive for nuclear AhR staining. *P < 0.01. (D) HUVECs were exposed to laminar and turbulent SS for 4 h using a cone-plate type apparatus as described in ‘Methods’. Expression levels of AhR protein or CYP1A1 protein normalized to those of β-actin protein were standardized to the values obtained from cells maintained in SC, and shown as means ± SD of three independent experiments in the bar graph. *P < 0.01.
3.6 Aryl hydrocarbon receptor mediates shear stress-induced cell cycle arrest

Previous studies suggest that there is a relationship between AhR and cell growth inhibition.22,26 We reported that SS inhibits EC proliferation by inducing expression of the cyclin-dependent kinase inhibitor p21Cip1.18 To investigate whether AhR is involved in SS-induced cell cycle arrest in ECs, we examined the effects of si-AhR on the arrest. As shown in Figure 6A, si-AhR restored the ability to proliferate. SS induced p21Cip1 expression and suppressed phosphorylation of the retinoblastoma tumour suppressor protein (pRb) required for G1/S transition consistent with our previous report.18 However, both of these changes were reversed by treatment with si-AhR (Figure 6B).

4. Discussion

In this study, we showed for the first time that the expression of AhR is induced by laminar fluid SS, probably through activation of the JNK/p38 pathways, and leads to expression of the CYP1A1 gene through XRE-dependent transcription. Furthermore, we found that AhR is also involved in SS-induced cell cycle arrest. The induction of AhR and CYP1A1 by turbulent SS was weaker than that by laminar SS, suggesting that the expression of these proteins is involved in the maintenance of vascular homeostasis.

A physiological level of laminar SS strongly induced CYP1A1 expression in vascular ECs, consistent with previous reports,12,13 and enhanced CYP1A1 activity. SS increased the activity of the CYP1A1 gene promoter; furthermore, deletion of parts of the 5′-flanking region of the CYP1A1 gene showed that the response to SS depended on the region between bp –1116 and –859, which contains two XREs. Mutations introduced into these two XREs almost completely abolished the SS-mediated response, suggesting that these XREs are essential for SS-induced transcription of the CYP1A1 gene.

Laminar SS induced the expression of AhR and facilitated its nuclear translocation required for binding to XREs (Figure 3B and C). The inhibition of AhR by α-NF or siRNA suppressed CYP1A1 induction by SS, suggesting that AhR mediates CYP1A1 induction by SS. Importantly, the induction patterns of AhR and CYP1A1 by SS were different from those by PAHs. Previous reports indicated that the expressions of AhR and CYP1A1 by SS were different from those by PAHs.20,21 However, laminar SS showed sustained induction of AhR and CYP1A1 until at least after 24 h in the present study. SS may continuously induce an unknown endogenous ligand that can bind and activate AhR. Considering the very low expression of AhR in ECs in static condition, AhR seems to be mainly regulated by SS in the vascular wall. Furthermore, the sustained induction of AhR and subsequent activation of CYP1A1 by SS may essentially indicate a physiological role, which is distinct from the effects of the vast majority of PAHs.

In addition, SS-induced AhR expression was probably mediated through the activation of the JNK/p38 pathways, since SS induced sustained activation of JNK and p38 (Figure 5A and D) and the inhibition of JNK or p38 suppressed the SS-induced expression of AhR and CYP1A1 (Figure 5B and C). However, SS did not activate ERK and U0126 failed to inhibit SS-induced expression of AhR and CYP1A1, suggesting that the ERK pathway is not required for this process, although several papers have reported an association between ERK and AhR activation.23,24

We previously reported that laminar SS induces cell cycle arrest in vascular ECs by inhibiting the transition from G1 to S phase.18 Since frequent cell division seems to increase the vascular wall permeability,25 restricting the proliferation of EC would achieve the stabilization of ECs. In fact, early
studies demonstrated that EC turnover and DNA synthesis are increased in areas around branch orifices where ECs are exposed to turbulent blood flow and atherosclerotic change is often initiated. Therefore, the anti-proliferative effect of laminar SS may be essential for the maintenance of vascular homeostasis. In the present study, si-AhR recovered DNA synthesis that had been suppressed by SS, and also prevented SS from inducing expression of the Cdk inhibitor p21Cip1 and de-phosphorylating pRb (Figure 6B). Recent studies suggested that by directly interacting with pRb, AhR inhibits the E2F-dependent transcription that initiates G1/S transition, resulting in inhibition of the cell cycle. Our results strongly agree with these results. TCDD was reported to induce cell cycle arrest in G1 phase through AhR-mediated induction of the Cdk inhibitor p27Kip1 in rat hepatoma cell line. However, we previously showed that SS did not change the expression level of p27Kip1 in ECs, and therefore, the action of AhR may differ among cell species and stimulants.

There are several limitations in this study. We used BAECs instead of HUVECs for the promoter analysis because of their higher transfection efficiency. Furthermore, we used the rat CYP1A1 promoter sequence to examine promoter activity. Although rat CYP1A1 promoter had a high homology with human CYP1A1 promoter, the differences between species in response to SS cannot be denied.

Our results suggested that the constitutive activation of AhR and CYP1A1 in response to blood flow is a normal physiological mechanism to protect the vascular wall from toxic stimuli, distinct from the pathological role of these proteins in atherosclerosis.

Figure 5  Effect of shear stress (SS) on the phosphorylation of mitogen-activated protein kinase (MAPKs). (A) Human umbilical vein endothelial cells (HUVECs) were maintained in static conditions (SC) or exposed to laminar SS (15 dyne/cm²) (SS) for the periods indicated. Levels of MAPKs and phosphorylated (p-) MAPKs were analysed by western blotting. (B) HUVECs were exposed to laminar SS (15 dyne/cm²) in the absence or presence of SP600125 for the periods indicated. Expression levels of the Cdk inhibitor p21Cip1 protein normalized to those of β-actin protein were standardized to the values obtained at 0 h and shown as means ± SD of four independent experiments in the bar graph. *P < 0.01. (C) HUVECs were exposed to laminar SS (15 dyne/cm²) in the absence or presence of U0126 for the periods indicated. Protein levels of AhR, CYP1A1, and β-actin were analysed by western blotting. Expression levels of AhR protein or CYP1A1 protein normalized to those of β-actin protein were standardized to the values obtained at 0 h and shown as means ± SD of four independent experiments in the bar graph.
molecules as generators of toxic metabolites. Even though, AhR and CYP1A1 expressed in response to PAHs results in the generation of harmful substances, their initially assumed roles may have been different, since few environmental pollutants such as PAHs existed when these molecules first evolved. Further study is needed to precisely determine the physiological and pathological roles of AhR and CYP1A1 expressed in the vascular wall.

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