ACTIVATION OF THE AH RECEPTOR SIGNALING PATHWAY BY VASCULAR SHEAR STRESS: GENERATION OF ENDOGENOUS LIGANDS AND/OR EXPERIMENTAL ARTIFACT?

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Introduction

The Ah receptor (AhR) is a ligand-dependent transcription factor that mediates many of the biochemical and toxic effects of a variety of persistent organic pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) and related dioxin-like chemicals (DLCs).[1,2] In addition, it has become well established that the AhR and AhR signal transduction pathway can be activated by a structurally diverse array of synthetic and naturally occurring chemicals[3,4]. Interestingly, while the existence of endogenous activators of the AhR have been proposed based on development effects obtained using AhR knockout animals and biochemical and genetic analysis, few endogenous substances have been identified that can stimulate the AhR at physiological concentrations[3,5]. Involvement of the AhR in cardiovascular and vascular development and disease and demonstration of AhR expression and activation in endothelial and hematopoetic cells have been described and suggest both the existence of endogenous activators of the AhR in normal biology and developmental processes and an avenue that TCDD and other AhR ligands adversely impact these normal processes[6-7]. Incubation of AhR-containing endothelial cells or continuous cell lines in fluid-flow, arterial sheer-stress conditions resulted in activation of the AhR and stimulation of AhR- and dioxin-responsive element (DRE)-dependent gene expression (most notably that of CYP1A1 and CYP1B1)7,9,10. An additional study demonstrated that shear stress conditions also stimulate AhR-dependent cell cycle arrest suggesting that sustained activation of the AhR by blood flow plays an important role in regulation of the functions of vascular endothelial cells[11]. Together, these studies suggested that shear-stress conditions in the vascular system stimulates the production of an ligand(s) that that can activate the AhR in cells of the vasculature, and it may represent a true physiological endogenous ligand of the AhR. While the identity of the responsible ligand(s) in most all of these studies was not examined, one study demonstrated that production of this AhR activator was serum-dependent and they identify the activator as shear-stress modified low density lipoprotein (LDL)[7]. However, the source of the shear-stress dependent AhR ligands is actually not clear. When we consider that typical shear stress studies with cells in culture are carried out using standard flow chambers that includes rubber tubing and gaskets that come into direct contact with the cell culture media, and when coupled with studies that demonstrate the existence of AhR ligands (agonists) that can easily be extracted out of rubber and plastic, the actual source of the AhR ligands in flow studies remain to be confirmed[12,13]. Here we describe studies examining the source of AhR ligands produced during exposure of cells to shear-stress conditions.

Materials and Methods

Cell culture and induction of EGFP/luciferase activity. Recombinant rat hepatoma (H4G1.1c2 and H4L1.1c4) cells which have been stably transfected with a DRE-driven enhanced green fluorescent protein (EGFP) or firefly luciferase reporter gene, respectively, were grown and maintained as previously described[14,15]. The reporter genes in these cells respond to AhR agonists with induction of expression in a time-, dose-, chemical- and AhR-specific manner[14,15]. Direct testing of normal, vigorously shaken and shear-stress treated media for effects on AhR-dependent gene expression was carried out using cells plated and grown in 96-well microplates. Cells were incubated with the indicated chemical/treated media for the indicated time period at 37°C (for luciferase) or 33°C (for EGFP) and luciferase/EGFP activity measured as we have previously described[16]. For flow cell, shear-stress analysis, H4G1.1c2 cells were plated and grown on slides and inserted into a parallel plate

continuous flow chamber (with peristaltic pump driven recirculation) and subjected to standard shear stress conditions as previously described in detail[17,18]. Time course studies of induction involved repetitive analysis of EGFP activity in the same cells at various times points by fluorescence microscopy. BNF (at 1 uM) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD at 1 nM) was included as a positive control in all studies.

Results and Discussion

To confirm the ability of laminar flow shear-stress to stimulate AhR-dependent gene expression, rat hepatoma (H4G1.1c2) cells, which contain a stably transfected AhR-response EGFP reporter gene were placed into a flow cells and incubated for up to 48 hours with continuous flow of standard culture ours incubation period; in static conditions, the negative control DMSO had no effect while the AhR agonist BNF induced EGFP activity (Figure 1). Since previous studies suggested that serum and sheared LDL were responsible for the shear-stress-induction of AhR-dependent gene expression, we examined the effect of added serum (10%) on the ability of static and sheared media to induce AhR-dependent luciferase gene expression in H4L1.1c4 cells (Figure 2). The results we obtained confirmed that serum was required for shear-stress stimulated AhR signaling, with sheared media without serum or static media (with or without serum) being unable to activate the AhR. The reduced induction response observed in cells incubated with these medium preparations for 24 hours, as compared to 4 hours of incubation, suggests that the responsible inducers are lost over time, likely as a result of metabolism by the enzymes present in the cells. Interestingly, shearing of medium/serum for as little as 30 minutes in the laminar flow system was sufficient to generate the AhR agonist (data not shown), suggesting that the response must be rapid.

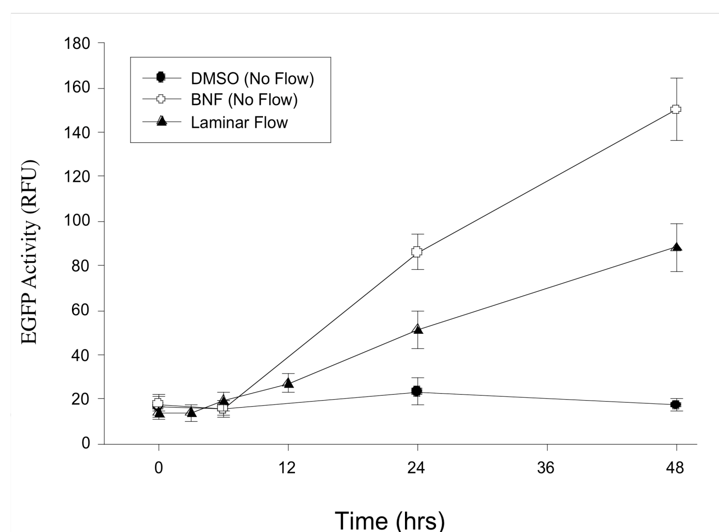

Fig. 1. Time course of laminar flow induction of EGFP in H4G1.1c2 cells. The EGFP activity induced by medium (DMSO) and beta-naphthoflavone (BNF) included as static controls. Values represent the mean ± SD of triplicate determinations.

To confirm that the generation of the agonist is a result of shearing of serum and not an simply an effect of medium/serum recirculation in the laminar flow system, media/serum was added to centrifuge tubes (to half full) and vigorously shaken overnight at room temperature to generate comparable shear forces; identical samples were stored in identical conditions without shaking as controls. Interestingly, media/serum sheared using this method did not induce AhR-dependent luciferase reporter gene in H4L1.1c4 cells at 4 or 24 hours of incubation (compared to DMSO and TCDD (data not shown)) even though this method generated comparable shearing forces. This suggested to us that there was something associated with the use of the laminar flow apparatus that is contributing to or is responsible for the induction response and since induction was only observed when serum was added to the media, the media must be responsible for the activity. Given our previous studies which demonstrated that potent hydrophobic AhR ligands (agonists) can easily be extracted out of rubber and plastic, we considered that the agonist activity observed in sheared media/serum might simply result from the ability of hydrophobic biological materials in serum (proteins/lipids/lipoproteins) to extract AhR agonist out of the tubing and gaskets of the flow apparatus[12,13]. To test this possibility, a small piece of the recirculation tubing was added

to a centrifuge tube containing medium, or medium/serum or serum only and sheared by vigorous shaking for 24 hours. Identical medium, medium/serum and serum samples without added tubing were also either sheared or stored without shaking as controls. The ability of these various medium and medium/serum mixtures to induce AhR-dependent luciferase gene expression is shown in Figure 3 and these studies clearly demonstrate that serum can extract AhR agonists out of the rubber tubing; shaker-sheared medium/serum and serum alone without added tubing were inactive. A small amount of induction was observed with the tubing-medium only sample.

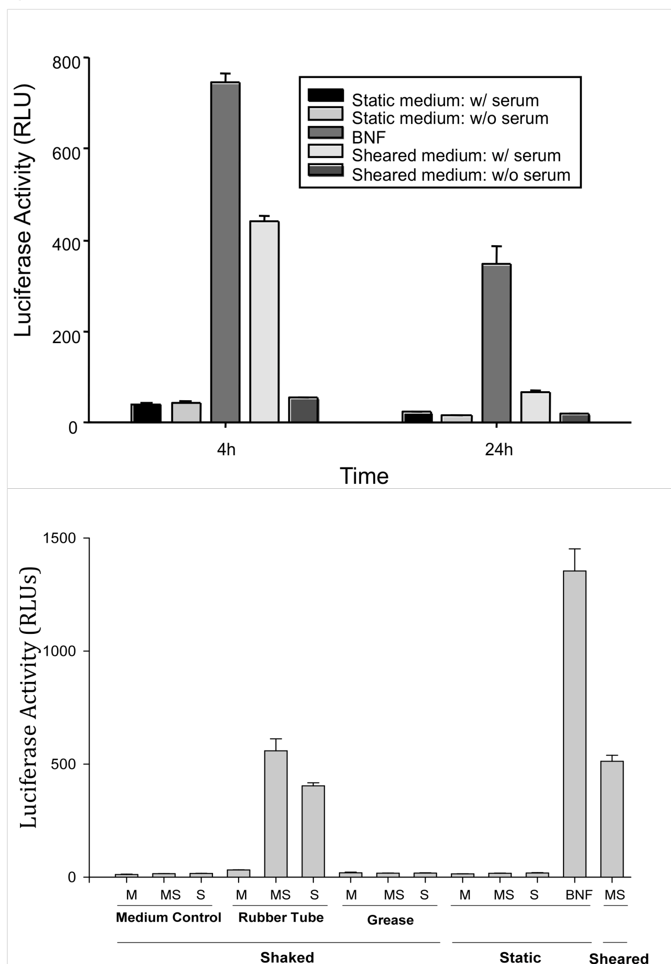

Fig. 2. Effect of static or sheared medium and the presence or absence of serum on the induction of AhR-dependent luciferase reporter gene expression in recombinant rat hepatoma (H4L1.1c4) cells. Sheared medium or medium/serum was prepared by recirculating it for 24 hours in the laminar flow system and then incubating it with H4L1.1c4 cells for the indicated time. Values represent the mean ± SD of at least triplicate determinations.

Fig. 3. The effect of conditioned medium on the induction of AhR-dependent luciferase reporter gene expression in rat hepatoma (H4L1.1c4) cells. The indicated incubation media was prepared by incubation of a piece of the peristaltic tubing or grease (used to seal the culture flow cell) in medium without added serum (M), medium with added 10% (v/v) serum (MS) or 100% serum (S) for 24 hours on a shaker. The conditioned medium was then added to wells containing H4L1.1c4 cells and luciferase activity determined after 4 hours of incubation. Values represent the mean ± SD of at least triplicate determinations.

The presence of AhR agonists in the various tubing and gaskets contained in the laminar flow recycling system was subsequently confirmed in gene expression studies (data not shown). In these experiments, small pieces of each tubing and gasket were incubated overnight in DMSO and their ability to stimulate AhR-dependent luciferase gene expression examined. DMSO extracts of all three types of tubing in the flow system and the gasket present in the flow chamber activated the AhR signaling pathway, to levels greater than that produced by incubation of the tubing in medium/serum (data not shown). Taken together, these results and those of additional studies question the conclusions of studies demonstrating that shear-stress activates the AhR signaling pathway and indicate that serum present in the media used in these studies can extract AhR agonist activity out of tubing and gaskets of the flow system apparatus that come in contact with the media and this has been interpreted to be an apparent shear-stress induction response. Rubber products are known to contain many AhR agonists, including polycyclic aromatic hydrocarbons and benzothiazoles and these compounds are likely contributors to the induction responses observed in many of these in these publications[13]. A previous study has suggested that the serum-dependent induction observed with shear-stress results from a modification of LDL in these flow

systems and additional experiments demonstrated the ability of NaOCl-treated LDLs to activate AhR-dependent gene expression[7]. However, although NaOCl-treated LDLs were AhR active and may represent novel endogenous AhR agonists, the induction response observed following the addition of LDL to medium in the flow apparatus may not be related to a shear-stress effect on the LDL, but simply from the ability of added LDL to extract AhR agonists from components of the flow system, similarly to that of what we observed with added serum. However, this remains to be confirmed. In conclusion, we demonstrate that the primary ligand(s) responsible for shear-stress-dependent activation of the AhR was not an endogenous ligand, but a chemical(s) extracted from components of the experimental flow-cell apparatus. Although many of the results obtained in previous shear-stress studies may actually result from the serum extracted AhR ligands, final confirmation will require detailed analysis of the materials used in these other flow systems and isolation and identification of the responsible AhR ligand(s).

Acknowledgements
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