Natural AHR Agonists from Human Serum

Xu S.[1], Rowlands J.C.[2], Budinsky R.A.[2], Fang N.[3], Wang C[1]

[1]School of Public Health, Tongji Medical College, Wuhan, P.R. China
[2]Toxicology and Environmental Research & Consulting, The Dow Chemical Company, Midland, MI, U.S.A.
[3]Hubei University of Traditional Chinese Medicine, Wuhan, Hubei, P.R. China

Abstract and Introduction

The aryl hydrocarbon receptor (AHR) mediates the biological effects of many different halogenated and non-halogenated aromatic hydrocarbons including polychlorinated-dibenzo-p-dioxins (PCDDs), -dibenzofurans (PCDFs) and -biphenyls (PCBs), collectively halogenated aromatic hydrocarbons of regulatory concern (HAHRCs). Numerous natural compounds found in the diet and endogenous compounds made in our bodies such as tryptophan derivatives are also known to activate the AHR. Research has identified that human serum contained significantly more AHR activation activity than explained by serum concentration of HAHRCs [1,2]. This increased AHR activation activity is believed to be made up of a combination of dietary phytochemcials and other endogenous compounds. However, the specific natural chemicals responsible for this AHR activation activity have not been identified. The research described below is designed to purify and identify the individual Natural AHR Agonists (NAHRAs) present in human serum which are expected to be an important source of AHR activity in human serum not explained by HAHRCs. By identifying these chemicals, important clues will be uncovered as to the normal role of the AHR in human physiology that may help shed light on the reasons for the lower human sensitivity to these HAHRCs relative to rodents.

Study Methods

Dietary Intervention Study

The following intervention study protocol has been approved by a Human Subject Review Board. Adult volunteer subjects will be recruited without preference to race into a dietary intervention study as previously described[1]. The subjects will be asked to avoid certain fruits, vegetables, and herbs, which have been found to contain other natural substances that interfere with the substances under investigation. These foods include grape and grape products, such as juice, wine, and red-wine vinegar; grapefruit, lime, or the peels of any citrus fruits; soybeans and soy products; herbs and herb beverages, such as sage and green tea. The meals that the subjects will consume include typical Chinese dishes with the only requirements being the types, amounts and day's specific dishes in order to comply with the baseline diet or special diet. Each subject will consume the baseline diet over a 4-day period (days 1–4), followed by a 2-day "interim" period during which there will be no dietary restrictions (days 5 and 6), followed by 4 days' consumption of the Special diet (days 7–10). Serving sizes will be determined by a nutritionist. The subject will complete a short

questionnaire and keep a record of their food consumption during the study. Two blood samples will be collected during the study, one after the 4 day baseline diet and the second blood draw will be at the end of the study following the special diet intervention.

Identification of NAHRAs in human serum

This study will develop an LC-MS/MS analytical method to identify phytochemicals in fruits and vegetables and small molecular components (not macromolecules e.g, proteins and polysaccharides). The isolated phytochemicals will be used as analytical standards to identify similar NAHRAs in human serum.

(1) The previously described method [3] will be used to prepare serum NAHRA concentrates. The method includes two steps: exhaustive extraction followed by direct analysis of the resulting crude extract by LC-MS/MS. The extraction procedure avoids any step that may cause loss and contamination of serum components. In order to avoid generation of artificial compounds during extraction, the extraction will be conducted at room temperature and completed in three hours without addition of any chemicals, such as acids.

(2) LC-MS/MS method will be developed to analyze NAHRAs concentrates and established fingerprints of NAHRAs concentrates. LC-MS/MS fingerprints include four wavelengths UV fingerprints from a diode-array detector, and positive and negative-ion fingerprints from electrospray ionization mass spectrometry (ESI-MS).

(3) Serum components will be identified by LC retention time and both positive and negative-ion spectra. Extract ion chromatogram (EIC), neutral loss (NL), and collision-induced dissociation (CID) programs will be employed to detect possible NAHRAs and their biotransformation products in serum. The structural information from LC-MS/MS can identify known chemicals and tentatively identify chemicals which are new to the literatures.

(4) NAHRAs in serum will be identified by comparison of the structures of NAHRAs identified from fruits and vegetables with the possible NAHRAs identified in serum.

(5) NAHRAs in serum will be quantified by direct comparison with their corresponding standards or their structure-related standards (pure NAHRAs isolated from fruits and vegetables). Precise quantification of a specific serum NAHRA will be performed by adding its isotope standard to the serum before extraction.

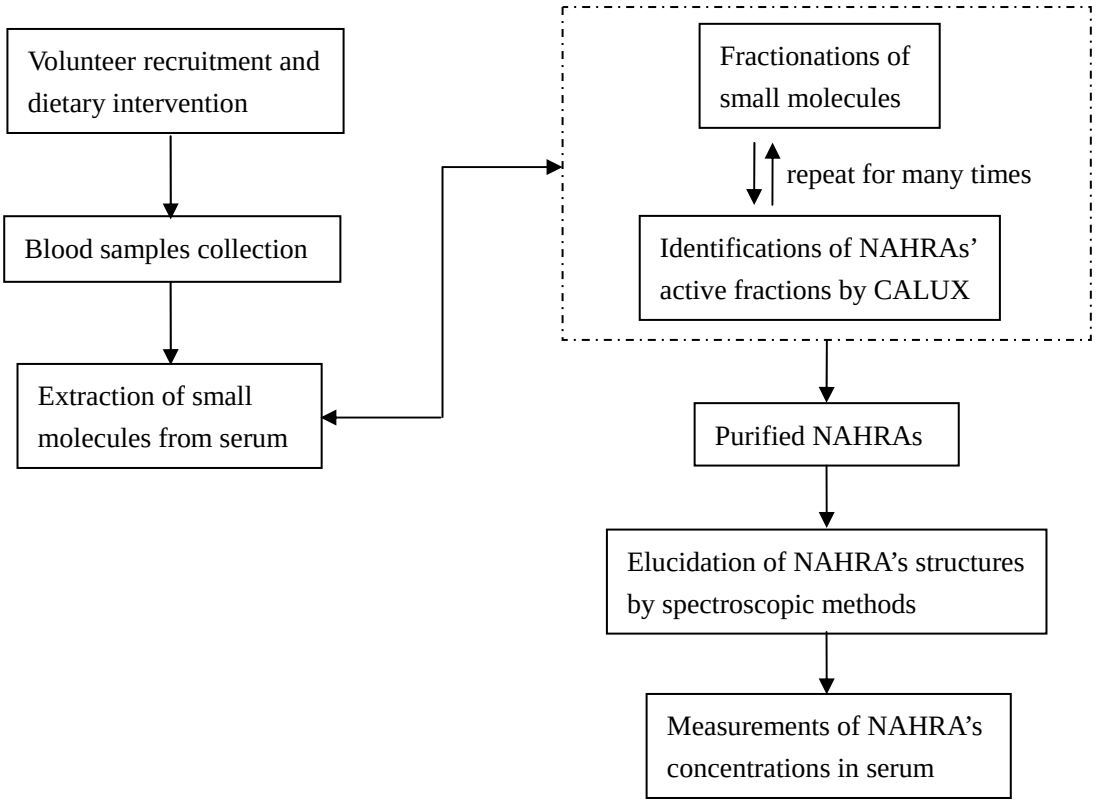
Screening for AHR Activity by CALUX

Bioassay-guided separation will be used to Isolate and purify NAHRAs from human serum. Extract of interest (NAHRAs concentrates) will be fractionated to several fractions and active fractions will be identified by CALUX,. The process will be repeated to isolat pure NAHRAs from serum.

CALUX activity will be measured after 24 h of incubation on a 96-well plate, using a standard 2,3,7,8-TCDD calibration curve. The conditions of the luciferase reporter gene bioassay will be as follows:

cells were cultured and maintained in 96-well plates. When cells reached 80–90% confluence, they will be harvested with trypsin and resuspended in fresh culture medium at a density of 7.5×105 cells/ml, and maintained for 16–24 h, before dosing. Dosing solutions will be prepared by diluting sample extracts in hexane and exchanging into DMSO by evaporation of the hexane in a vacuum centrifuge concentrator. Each DMSO extract will then be added to culture media and vortexed vigorously. Dosing of cells will be initiated by replacing cell culture media covering cells in microtiter plates with the media containing the extract (or standard/reference compound).

Figure 1. Identification of exogenous and endogenous AHR Agonists in serum



Structural elucidation of NAHRAs isolated from serum

Chemical structures of pure NAHRAs will be elucidated using different spectroscopic methods, such as HR-MS, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, UV, IR, optical rotation, X-ray crystallography. The ultraviolet spectroscopy (UV) and infrared spectroscopy (IR) will give the functionality; high resolution mass spectrometry (HR-MS) will give the formula; two-dimension nuclear magnetic resonance spectroscopy (2D-NMR) and MS will allow one to put together the molecular skeleton; X-ray crystallography will permit the detailed structural 'photograph' .

Method for isolation of NAHRAs from Plants

Since concentrations of natural AHR Agonists (NAHRAs) in human serum are very low, it may difficult to isolate enough pure NAHRAs from human serum for structural elucidation using spectroscopic methods including HR-MS, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, UV, IR, optical rotation, X-ray crystallography. Therefore, NAHRAs will be isolated from fruits and/or vegetables known to contain high amounts of NAHRAs. The expectation is that additional new NAHRAs will be isolated along with known NAHRAs. The approach will be similar to that used for isolation of NAHRAs from serum, namely bioassay-guided separation and identification of the NAHRAs by different spectroscopic methods. These plant isolated NAHRAs will be used as standards to aid in the identification of NAHRAs from human serum.

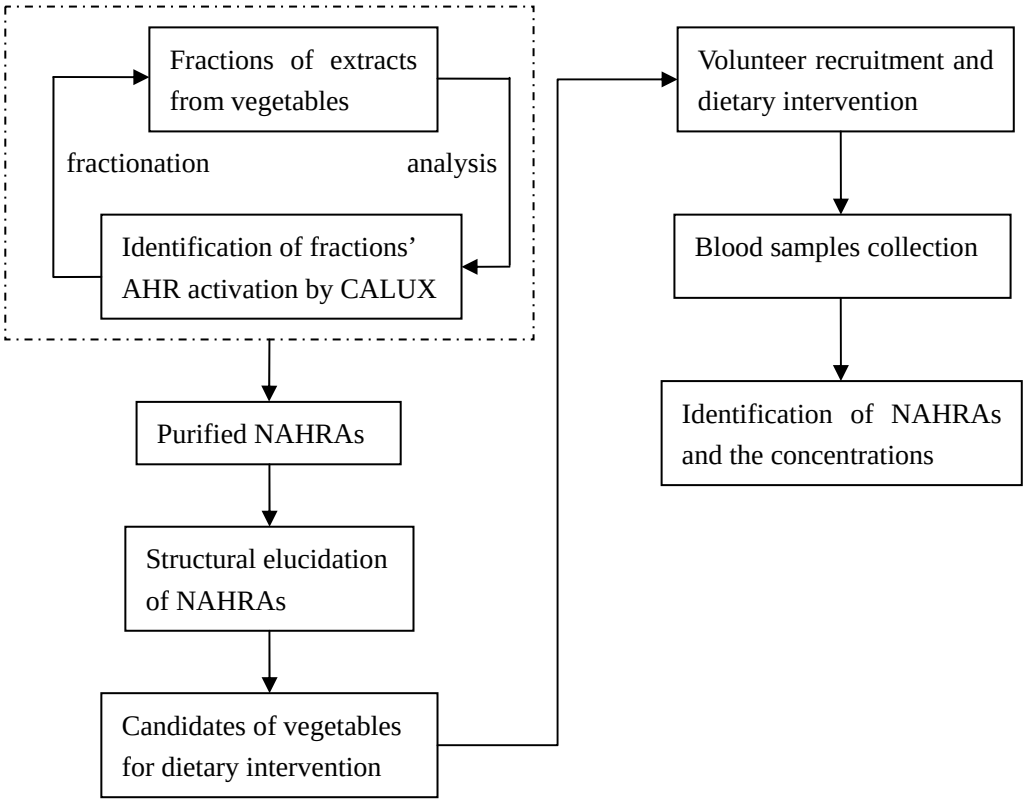
Selection of NAHRA-containing plants

NAHRA-containing fruits and vegetables (200 g/plant) used in the "special diet intervention" will be extracted with 300 mL of 80% aqueous methanol at 5 °C for 24 h with occasional stirring. The slurry is filtered through a #4 Whatman filter paper. The extraction process will be repeated with 100% (300 mL x 2) methanol and followed with 50% methanol (300 mL). The four extracts will be combined and concentrated on a rotary evaporator under reduced pressure at room temperature until the methanol is removed. The aqueous extract (200 mL) will be partitioned with ether (200 mL x 3). The ether phase and water phase will be evaporated under reduced pressure at room temperature followed by lyophilization. The dry ether and water fractions will be used for NAHRAs activity bioassay.

Discussion

Previous studies observed "background" blood levels of PCDD/Fs and PCBs that constituted only a trivial fraction of the total AHR agonist activity in human blood[1,2]. While the AHR activation by NAHRAs is likely to be relatively transient as compared to that induced by PCDD/Fs and PCBs, these previous observations of a net agonist activity in human blood of a magnitude that equated to TCDD equivalents (TEQs) of >100 ppt as measured *via* bioassays. The planned studies will further elucidate the relative contribution of NAHRAs and endogenous AHR ligands to the blood TEQ observed in the studies of Connor et al., 2007 and Shecter et al., 1999. This work may also provide important clues as to the normal physiological role of the AHR.

Figure 2. Schematic flow chart for isolation and use of plant NAHRAs



References

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