Initial and extended inflammatory messages of the nongenomic signaling pathway of the TCDD-activated Ah receptor in U937 macrophages

Eric M. Sciullo, Chris F. Vogel, Wen Li, Fumio Matsumura *

Department of Environmental Toxicology, Center for Health and the Environment, University of California, One Shields Avenue, Old Davis Road, Davis, CA 95616, USA

ARTICLE INFO

ABSTRACT

Using 2,3,7,8-tetrachlorodibenzo(*p*)dioxin (TCDD) we have investigated the mechanisms through which the AhR elicits inflammation through the nongenomic pathway. This AhR signaling depends on the initial action of TCDD to rapidly increase the intracellular concentration of free Ca^{2+}, which subsequently activates cPLA2 and additional inflammatory markers (e.g. COX-2 mRNA expression) lasting up to 72 h. Inhibition of cPLA2 activity resulted in attenuation of these inflammatory responses. We have hypothesized that specific protein kinases are responsible for further propagation of the initial transient nongenomic signaling into long-lasting cellular effects, and found protein kinase C (PKC) is activated at an early stage, followed by activation of cAMP-dependent protein kinase (PKA) at later stages. We clearly established in U937 macrophages cPLA2 activation is an essential initial step to activate the nongenomic inflammatory pathway of ligand-activated AhR. Furthermore, this pathway does not require the participation of ARNT, thus distinguishing itself from the classical genomic pathway.

© 2008 Elsevier Inc. All rights reserved.

It is well known that the toxic actions of TCDD (dioxin) are mediated by its specific receptor, the aryl hydrocarbon receptor (AhR)[1]. However, it is still not clear how the ligand-activated AhR sends its toxic signaling. Certainly the mechanism of TCDD to induce a variety of detoxification enzymes, particularly in liver, has been well studied. However, TCDD induces a host of toxic responses in types of cells that are not specialized for detoxification of xenobiotics. This is particularly true in the case of inflammatory responses TCDD is known to cause, which has been recorded to take place in cells lacking detoxification capacities such as lung alveolar epithelial cells. In studying the mechanism of inflammatory signaling of the ligand-activated AhR, we have chosen U937 human macrophages as an appropriate cell model, because of their significant role in aiding inflammatory responses of a host of tissues.

Recently we found that in MCF10A mammary epithelial cells, TCDD rapidly induces an Ah receptor (AhR)-dependent, nongenomic signaling to activate cytosolic phospholipase A2 (cPLA2) [1]. This signaling pathway appears to be distinct from its classical, genomic action pathway at least in that cell line, one of its main characteristics being the lack of participation of ARNT. Major

questions arising from the above finding are: (a) how prevalent is this phenomenon among different types of cells, (b) if so, what the minimum commonalities (i.e. essential characteristics) are and (c) whether such an early and transient nongenomic signaling could contribute to subsequent, long-lasting cellular states of inflammation.

Inflammation is an important mechanism of the defensive response of the body to infection, tissue damage and many other types of cellular stresses. Macrophages are known to play a vital role in eliminating pathogens through phagocytosis as well as coordinating the maintenance of the state of inflammation with the hosting tissues and organs through secretion of inflammatory cytokines and chemokines. It has been shown by a number of studies that TCDD acts as a stimulator of inflammatory cytokines such as IL-1ß [2], TNF-α [3], and IL-8 [4] in various tissues and cell types.

Further evidence exists that implicates close associations of dioxin toxicity with its pro-inflammatory mechanisms of action. For example, the role of IL-1β in hepatocellular damage by TCDD [5], and the activation of a host of inflammatory mediators in the differentiation of macrophage cells to potentially plaque-forming foam cells [6].

While the existence of these publications shows the general interest on this topic by many scientists, the main problem hindering the progress of this field is the lack of a clear-cut theoretical framework of the action of TCDD to induce a variety of cellular stress responses including inflammatory reactions. Furthermore, most publications dealing with cell stress reactions in this field are aimed at studying the secondary effects of oxidative stress that

---

result from the induction of cytochrome p450s and other detoxification enzymes. This may be particularly important in cells specialized for detoxification of xenobiotics such as hepatocytes, but may not be so in cells not particularly specialized for detoxification of xenobiotics, such as macrophages. Thus, it is very important to clearly delineate the pro-inflammatory mechanism of action of TCDD from that of the well established action of TCDD to induce detoxification enzymes. This is the main reason why we have chosen macrophages as our main study cell material, since macrophages are hematopoietic cells specialized to facilitate inflammatory responses, and since they are not particularly known for their roles in detoxification of xenobiotics. In our preliminary studies we have found that TCDD exposure results in rapid activation of both cPLA2 and COX-2 in U937 macrophages, which takes place at very early stages in this cell line, well ahead of its action to induce CYP1A1. These findings have provided the initial impetus for us to study the basic action mechanism of TCDD to elicit inflammatory responses from this chosen cell material. Accordingly, we have set our two major objectives as (a) to address the question how the nongenomic message of ligand-activated AhR is initially propagated and eventually transduced into its stable signaling in this cell material and (b) how important this route of action of TCDD is in maintaining the status of inflammation in macrophages, which is known to last for long-time periods. To meet these objectives we have formulated two hypotheses: i.e. first, the effects of the initial trigger of this nongenomic pathway, Ca^{2+}-induced activation of cPLA2 persist for a long-time period, and, second, such a process of converting the initial, transient message into long-term signaling is mediated by protein kinases.

Materials and methods

Chemicals

TCDD (>99.99% purity) was originally obtained from Dow Chemicals Co. (Midland, MI). Dimethyl sulfoxide (Me2SO) was obtained from Aldrich. $[\gamma\text{-}^{32}P]ATP$ (6000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). 1-5-(isoquinolinyl-sulfoxyl) 2-methyl-piperazine (H7), and phorbol-12-myristate-13-acetate (TPA) were purchased from Sigma–Aldrich (St. Louis, MO). 3′-Methoxy-4′-nitroflavone (MNF) was a kind gift from Dr. Josef Abel (University of Duesseldorf, Institute of Environmental Research, Germany). Arachidonyl trifluoromethyl ketone (AACOCF3), methyl arachidonyl fluorophosphates (MAFP), nifedipine, 2-aminoethoxydiphenyl borate (2-APB), and N-{2-[(p-bromocinnamyl amino) ethyl]}-5-isoquinolinesulfonamide·2HCl (H89) were purchased from Calbiochem. 6-Formylindolo [3,2-b] Carbazole (FICZ) was purchased from Biomol (Plymouth Meeting, PA). Other molecular biological reagents were purchased from Qiagen (Valencia, CA) and Roche (Indianapolis, IN).

Cell culture, transfection experiments, and luciferase assays

Human U937 monocytic cells were obtained from A.T.C.C. (Manassas, VA) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), supplemented with 4.5 g/L glucose (Sigma), 1 mM sodium pyruvate (Invitrogen), and 10 mM HEPES (Invitrogen). Cell culture was maintained at a cell concentration between 2×10^5 and 2×10^6 cells/ml. For differentiation into monocytes/macrophages, U937 cells were treated with TPA (5 μg/mL) and allowed to adhere for 48 h in a 5% CO_2 tissue culture incubator at 37 °C, after which they were fed with TPA-free medium. For transient transfection experiments, U937 macrophage cells were plated in 6-well culture plates (2×10^6 cells/2 ml) and were transfected using jetPEI™ (PolyTransfection, Qbiogene, Irvine, CA), according to the manu-

facturer's instructions in TPA-free culture medium. The expression vectors used in this study were: a human DRE-luc (kindly provided by Dr. Josef Abel (University of Duesseldorf, Institute of Environmental Research, Germany), human cPLA2-luc (kindly provided by G. D'Orazi, Department of Oncology and Neurosciences, University G. d'Annunzio, Chieti, Italy and R.A. Nemenoff, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO), and mouse COX-2-luc (TIS10 S) (kindly provided by H. Herschman, Department of Molecular and Medical Pharmacology, University of California-Los Angeles, Los Angeles, CA). Briefly, 0.7 μg of the DRE, cPLA2, or COX-2 construct was suspended in 100 μl of 150 mM sterile NaCl solution. Also 2 μl of jetPEI™ solution was suspended in 100 μl of 150 mM sterile NaCl solution. The jetPEI™/NaCl solution was then added to the DNA/NaCl solution and incubated at room temperature for 30 min. The medium in the wells was then changed to fresh medium, and 200 μl of the DNA/jetPEI™ was added to each well. The transfection was allowed to proceed for 16 h. Medium was replaced and the cells were then treated with 10 nM TCDD, inhibitors substances, or 0.1% Me2SO (control) for 24 h. To control the transfection efficiency cells were co-transfected with 0.1 μg per well β-galactosidase reporter construct. Cells were washed twice with PBS and lysed with 300 μl passive lyses buffer. Luciferase activities were measured with the Luciferase Reporter Assay System (Promega, Madison, WI) using a luminometer (Berthold Lumat LB 9501/16, Pittsburgh, PA). Relative light units were normalized to β-galactosidase activity and to protein concentration, using Bradford dye assay (Bio-Rad, Hercules, CA).

Transfection of short interfering RNA (siRNA) into U937 macrophages was performed via Nucleofector technology, 10^6 cells were re-suspended in 100 μl Nucleofector Solution V (Amaxa GmbH, Köln, Germany) and nucleofected with 1.5 μg of the corresponding siRNA using program V-001, which is preprogrammed into the Nucleofector device (Amaxa GmbH). Following nucleofection, the cells were immediately mixed with 500 μl of pre-warmed RPMI 1640 medium and transferred into 6-well plates containing 1.5 ml RPMI 1640 medium per well. After 48 h the reduction of the target RNA was detected by quantitative real-time RT-PCR. For siRNA studies twenty-one-nucleotide RNA for AhR (M-004990-00), ARNT (1027020), and cPLA2 (8100685699) with 3′-dTdT overhangs were synthesized by Dharmacon Research (Lafayette, CO) and Qiagen (Valencia, CA), respectively. Corresponding control cells were transfected with AllStar negative control siRNA (1027281) by Qiagen. Experiments were repeated three times. Three wells of cells were analyzed per experiment.

Quantitative real-time reverse transcriptase PCR

Total RNA was isolated from U937 macrophages using a high pure RNA isolation kit (Qiagen) and cDNA synthesis was carried out as previously described [4]. Quantitative detection of target gene mRNAs was performed with a LightCycler Instrument (Roche Diagnostics, Mannheim, Germany) using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA-free total RNA (1.0 μg) was reverse-transcribed using 4 U Omniscript reverse transcriptase (Qiagen, Valencia, CA) and 1 μg oligo(dT)15 in a final volume of 40 μl. The primers for each gene were designed on the basis of the respective cDNA or mRNA sequences using OLIGO primer analysis software, provided by Steve Rosen and Whitehead Institute/MIT Center for Genome Research. See Table 1 for a complete list of sequences designed according to this method. PCR amplification was carried out in a total volume of 20 μl, containing 2 μl of cDNA, 10 μl of 2× QuantiTect SYBR Green PCR Master Mix, and 0.2 μM of each primer. The PCR cycling conditions were 95 °C for 15 min followed by 30–40 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 10 s. Detection of the fluorescent product was performed at the end of the 72 °C