AH RECEPTOR-MEDIATED INDUCTION OF THE CELL STRESS RESPONSE SIGNALING PATHWAY

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Abstract
One of the major functions of the Ah receptor (AhR) is mediation and coordination of cell stress responses. Here we describe the basic processes through which TCDD-bound AhR triggers an inflammatory signal transduction pathway by activating cytosolic phospholipase A2 (cPLA2) and Cox-2 through a "nongenomic" action route in many types of cells. The subsequent down-stream events taking place are largely dependent on the type of cells. In the case of MCF10A mammary epithelial cells, Src kinase activation is a prominent feature, while in U937 macrophages, the production of TNFα as a paracrine/autocrine factor plays the dominant role at the early stage of TCDD's action. In due course, however, all transient signals generated by AhR through such a "nongenomic" action route must be converted to longer-lasting "genomic" signaling, which is aided by several protein kinases in a cell-specific manner. Those kinases are capable of affecting nuclear translocation as well as functions of major nuclear transcription factors. In the case of IL-8, a chemokine found in lymphoid cells and several tissue-specific cells, its expression is clearly stimulated by cAMP-dependent protein kinase, which also promotes nuclear translocation of AhR. The active form of AhR in nucleus promoting IL-8 gene expression will be discussed.

Materials and Methods
The sources as well as the cultural conditions employed for the following cell lines used for this study have already been published: e.g. MCF10A (1,2), U937 (3), and 3T3-L1 (4). A lung epithelial carcinoma cell line, A549 was originally purchased from ATCC, and cultured according to the method of Martinez et al. (5). The methods for quantitative RT-PCR (4), U937 macrophage differentiation (6), 3T3-L1 adipocyte differentiation (4), assessment of membrane translocation of Src kinase (1) have also been described in our previous publications. Assessment of cPLA2 activity was carried out through measurement of tritium-labeled arachidonic acid release (7), and that on ATP concentration was done through the method originally developed by Crouch et al. (8) and modified by Farfan et al. (9). The IL-8 gene promoter analysis was performed according to the methods of Freund et al. (10) and Henriquet et al. (11).

Introduction
It is well known that dioxin and related chemicals act as direct agonists for the Ah receptor (AhR) and thereby activate a number of genes, particularly those encoding proteins involved in detoxification of xenobiotics through the classic DRE-dependent action pathway. However, in recent years tremendous amounts of information have been generated on this subject suggesting that AhR serves for a variety of cellular functions that are not explainable from the above classic model alone. In this presentation we have decided to address the functions of AhR from a specific viewpoint of its cell stress response signaling pathway. The background information leading us to propose that at least one of the major functions of AhR is to evoke cell stress responses has already been published from this research group (3,12). Briefly, we have noted that chronic symptoms induced by TCDD, particularly the wasting syndrome, bear great similarities to those produced in many animals through chronic poisoning by bacterial endotoxins. Subsequent molecular investigations have uncovered a variety of cell stress symptoms TCDD causes in several tissue specific cell types. As will be shown below, cell stress response signaling could differ among individual tissue- and differentiation-specific types of cells even within the same animal, and therefore it is important to consider many different types of cells in gaining the perspectives on the nature of coordinated cellular responses. Fortunately, such a challenging task has been made somewhat easier by the recent progress in the science of stress signaling mechanisms and the pathways that are generated by this type of stress response receptors as are the cases of Toll-like receptors. As a

result we have gained new perspectives on the role of AhR on this specific function in coordinating cell stress responses, and now wish to provide the essence of our most recent study results and their interpretation.

Results and Discussion

Earliest events occurring upon binding of TCDD to AhR

The main reason why we have been interested in the earliest events of TCDD's action is that in conducting any signal transduction studies the knowledge in the earliest event helps greatly in assessing most of the down-stream events, since at least in theory blocking the most up-stream event should abrogate all subsequent signaling activities. In the case of the functional analysis of AhR there is an extra advantage of recognizing the hitherto unexplainable events occurring apart from its action results through the classic DRE-mediated pathway as the latter process takes some time, since it involves *de novo* transcription, translation and protein synthesis and protein modification activities.

To date the earliest event we could observe was TCDD-induced release of ATP from mitochondria, which starts taking place as early as 1 min in U937 human macrophage model. This observation has brought our attention to an earlier report by Hanneman et al. (13) that in the case of rat hippocampus neuronal cells TCDD causes rapid reduction of the mitochondrial membrane potential. The resulting increase in the intracellular free Ca2+ concentration,[Ca2+]i occurs as early as in 30 sec. They concluded that the source of [Ca2+]i is likely extra cellular judging by the inhibitory action of EGTA (which does not penetrate into the cell) and nifedipine. It has also reminded us of the original finding of Puga et al. (14) that in mouse hepatoma cells TCDD causes a rapid transient rise in Ca2+ influx, which leads to activation of AP-1 nuclear transcription factors. The key point is that such early events are not likely to be caused by the classic DRE pathway, judging by the speed of its occurrence. As for the source of [Ca2+]i, we now consider that in most types of cells the initial release of Ca2+ from endoplasmic reticulum that is induced by mitochondrial membrane destabilization is the earliest trigger which further activate the Ca2+ influx from outside based on our latest observation in U937 macrophages. This conclusion agrees well with that of a recent report by Shertzer et al. (15), who studied the details of this subject in mice in vivo, and concluded that such mitochondrial effects of TCDD is a prominent effect, which is not carried out through the classic DRE-mediated pathway.

Early events triggered by the increase in [Ca2+]i

Earlier our research team found in MCF10A, a normal human mammary epithelial cell line that TCDD causes functional activation of Src tyrosine kinase (1,2) as well as ERK 1 and 2 both of which take place within 15 min, when there is no sign of induction of CYP1A1 in that cell line. Subsequent studies on this cell material yielded a wealth of information, indicating that such an effect of TCDD is accompanied with activation of cytosolic form of phospholipase A2 (cPLA2), which can be blocked by a number of agents inhibiting the rise in intracellular Ca2+ concentration. The enzymatic activities of cPLA2 was assessed through 3H-arachidonic acid (AA) release from cells pre-labeled with 3H-AA in the presence and the absence of cPLA2-specific inhibitors such as AACOCF3 and MAFP at appropriate concentrations and timing. The most prominent inflammatory gene activation was observed to be Cox-2, of which induction starts taking place in 30 min in the case of MCF10A cells and continue to intensify over the next 2 hrs. During this time span there was no sign of activation of the TNFα/NFkB axis in this cell line. These findings helped us to delineate the outline of this Cox-2 mediated nongenomic, inflammation pathway. Furthermore, we found that the process of arachidonic acid-induced activation of the Cox-2 inflammation pathway is depending on activation of Src kinase in this cell line, the finding helped us to understand finally the reason for the involvement of Src kinase in this early action of TCDD. It was also found that Cox-2 activation occurs at early stage in every type of cell lines we have observed so far, and therefore we may be safe to project that this Cox-2 mediated pathway is likely to be the early determinant of the inflammatory responses in most types of cells treated with TCDD.

How the messages of inflammatory signaling-triggered by cPLA2 and Cox-2 are propagated?

Having found that cPLA2 and Cox-2 play an important up-stream mediator role for the inflammation pathway in several types of cells, the question we must address next is how that message is passed on to other major inflammatory pathways, particularly to the NFkB pathway. In this regard we noted the activation of TNFα mRNA by the action of TCDD on U937 macrophages takes place within 1 to 6 hrs, but not in MCF10A, 3T3-

L1 adipocytes or HegG2 cells (all took longer than 6 hrs to up-regulate the NFkB pathway). Thus, it is possible that upon receiving an inflammation signaling the tissue types of cells needing helps must rely on certain paracrine factors such as chemokines/cytokines to recruit macrophages as well as other types of hematopoietic cells. The earliest markers for production of paracrine factors identified by us through qRT-PCR are IL-8, VEGF (MCF10A, 3T3-L1, A549 lung carcinoma cells) and MCP-1 (3T3-L1,A549). These are well recognized paracrine factors known to enhance monocyte/macrophage recruitment to the site of inflammation. Examination on their gene expression mechanisms revealed that IL-8 is predominantly activated by cAMP-dependent protein kinase (i.e. PKA), MCP-1 by PKC and tyrosine kinases, and VEGF by both PKA and PKC (studied in 3T3-L1 and MCF10A). In the case of 3T3-L1 adipocytes, which are capable of producing TNFα as an autocrine factor in response to the action of TCDD, on the other hand, it takes 24 hrs to reach the statistically significant level as judged by qRT-PCR assessment (4).

Studies on the pattern of inflammatory responses in U937 macrophages

As shown above this human macrophage cell line seems to be capable of quickly activating the NFkB mediated inflammatory responses at an earlier stage of TCDD's action than MCF10A cells. Another characteristic of U937 macrophages is that induction of CYP1A1 takes place at a slower pace than MCF10A cells in that within the first 1 hr there is no sign of induction of CYP1A1 in U937 macrophages. This allowed us to investigate the nongenomic action of TCDD on both Cox-2-mediated and NFkB mediated-pathways at early time points of the action of TCDD. The most conspicuous feature of their cell responses is clear cut activation of Cox-2 by 1 hr followed by activation of TNFα by 3 to 6 hrs. This capability of U937 macrophages to elicit such a rapid inflammatory response appears to represent the stereotypic pattern of responses of this type of cells, basing on our observations that even several physical stress inducers such as NaCl-induced hyper-osmotic shock, oxidative stress as well as oxidized LDL-induced phagocytotic events trigger rapid activation of Cox-2 as well as TNFα release.

How do cells convert the initial [Ca2+]i- triggered "nongenomic signaling" of ligand-activate AhR to genomic signals?

It must be reminded that the initial effect of TCDD on [Ca2+]i is actually transient. Therefore, to maintain the state of inflammation cells must somehow convert this initial message and/or those of the immediate down-stream inflammation triggering events into relatively longer lasting "genomic" expressions. We have noted that initial transient increase in the mRNA expression of cPLA2 by TCDD in the case of MCF10A cells, is followed by a rise in its functional expression by 24 hrs as judged by its-promoter-Luc reporter assay. A survey of literature has indicated that cPLA2 gene is well known for its up-regulation through post-transcriptional factors, particularly by interferon-γ (16). While the process of "fixation" of initial "nongenomic" signals into "genomic" messages are cell specific as well as complex, regularly requiring several transcription factors including hormone receptors in nucleus, the above example on cPLA2 gene activation illustrates a point that even a transient initial event can be fixed through subsequent post-translational factors that can promote "genomic" activation of the appropriate gene engaged in cell stress responses.

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Activation of AhR through PKA and its role in mediation of cell inflammatory responses

Among several chemokines, we have been most interested in IL-8, since the expression of its mRNA (it is called KC in mice) is significantly induced in various tissues of mice after treatment with TCDD (17). IL-8 is well known to recruit neutrophils and macrophages and plays a critical role in inflammation. Recently, it was reported by Oesch-Bartlomowicz et al. (18) that exogenous cAMP triggers rapid nuclear translocation of AhR. Furthermore, the recent discovery by de Oliveira et al. (19) indicates that the process of the regulation of intracellular levels of cAMP is tightly regulated by the formation of a dimer between XAP-2, a chaperone protein attached to the cytosolic complex of AhR and PDE2A, a phosphodiesterase known to degrade cAMP. This pivotal finding shows that cAMP, through activation of PKA, plays an integral role in the functional activation of AhR, which includes its nuclear translocation. We have studied the phenomenon of TCDD-induced rapid activation of IL-8 gene expressions, and found that this process is also activated by cAMP through PKA as judged by the effectiveness of forskolin and H89, a specific inhibitor of PKA to modulate this process. Through a detailed promoter analysis of the IL-8 gene, moreover, we were able to identify the specific AhR binding site on the promoter of human IL-8 gene close to the start site (0 to -50 bp). While activation of

the IL-8 gene through AhR binding to this site is enhanced by TCDD treatment, this action of TCDD is not significantly attenuated by the treatment with siRNA against ARNT. By running a series of EMSA/supershift assay studies as well as antibody dependent co-precipitation assays we were able to identify a unique form of AhR, which acts as the dominant nuclear transcription factor activating this gene expression in 3 different cell types. Such findings help to delineate the unique process through which those cells are utilizing AhR in generating the original nongenomic inflammatory signaling through ligand binding as well as its translocation into nucleus through the action of PKA. It is important to point out that PKA can also assist nuclear translocation of non-liganded form of AhR (18) as well as participates in the process of fixing that transient signal into a more stable "genomic" signaling of IL-8 gene. This example illustrates that at least in this case, AhR as assisted by a protein kinase functions for a dual purpose of mediating the original "nongenomic" signaling as well as assisting, the conversion of the former signaling to a *de novo* "genomic" signal. It is interesting to note that this example also helps to describe a process of a paracrine factor, which is capable of contributing to the promotion of macrophage-host cell communications and thereby assisting overall cellular stress response coordination.

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References

1. Mazina O, Park S, Sano H, Wong P, Matsumura F. *J Biochem Mol Toxicol* 2004; 18:313.
2. Park S, Matsumura F. *Toxicology* 2006; 217:139.
3. Matsumura F, Vogel CF. *Biol Chem* 2006; 387:1189.
4. Li W, Vogel CF, Matsumura F. *J Cell Biochem* 2007; In press.
5. Martinez JM, Afshari CA, Bushel PR, Masuda A, Takahashi T, Walker NJ. *Toxicol Sci* 2002; 69:409.
6. Vogel CF, Sciullo E, Wong P, Kuzmicky P, Kado N, Matsumura F. *Environ Health Perspect* 2005; 113:1536.
7. Viu E, Zapata A, Capdevila JL, Fossom LH, Skolnick P, Trullas R. *J Pharmacol Exp Ther* 1998; 285:527.
8. Crouch SP, Kozlowski R, Slater KJ, Fletcher J. *J Immunol Methods* 1993; 160:81.
9. Farfan A, et al. *Cell Notes* 2004; 10:2.
10. Freund A, Jolivel V, Durand S, Kerusal N, Chalbos D, Chavey C, Vignon F, Lazennec G. *Oncogene* 2004; 23:6105.
11. Henriquet C, Gougat C, Combes A, Lazennec G, Mathieu M. *Lung Cancer* 2007; 56:167.
12. Matsumura F. *Biochem Pharmacol* 2003; 66:527.
13. Hanneman WH, Legare ME, Barhoumi R, Burghardt RC, Safe S, Tiffany-Castiglioni E. *Toxicology* 1996; 112:19.
14. Puga A, Nebert DW, Carrier F. *DNA Cell Biol* 1992; 11:269.
15. Shertzer HG, Genter MB, Shen D, Nebert DW, Chen Y, Dalton TP. *Toxicol Appl Pharmacol* 2006; 217:363.
16. Wu T, Levine SJ, Lawrence MG, Logun C, Angus CW, Shelhamer JH. *J Clin Invest* 1994; 93:571.
17. Vogel CF, Nishimura N, Sciullo E, Wong P, Li W, Matsumura F. *Arch Biochem Biophys* 2007; In press.
18. Oesch-Bartlomowicz B, Huestler A, Wiss O, Antoniou- Lipfert P, Dietrich C, Arand M, Weiss C, Bockamp E, Oesch F. *Proc Natl Acad Sci U S A* 2005; 102:9218.
19. de Oliviera SK, Hoffmeister M, Gambaryan S, Muller-Esterl W, Guimaraes JA, Smolenski AP. *J Biol Chem* 2007; 282:13656.