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Review

The significance of the nongenomic pathway in mediating inflammatory signaling of the dioxin-activated Ah receptor to cause toxic effects

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ABSTRACT

Evidence has been accumulating to indicate that the current classical model of dioxin's action based on the ligand-activated aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) dimer directly activating its target genes is not robust enough to explain many of the major toxic effects of this compound. In this review, efforts have been made to analyze the results of recent investigations in our laboratory in comparison to already existing evidence on the patterns of toxic actions of dioxin (=TCDD) from other laboratories from a specific viewpoint of elicitation of cellular inflammatory signaling by the ligand-activated AHR. The most salient features of the inflammatory action of TCDD are that its triggering events, such as the rapid increase in intracellular Ca^{2+} concentration, enzymatic activation of cytosolic phospholipase A2 (cPLA2) and that of Cox-2 are taking place through the nongenomic action of the ligand-activated AHR. This nongenomic pathway does not require ARNT. Therefore, this inflammation pathway is clearly discernable from the classical, genomic action pathway. The effect of such a nongenomic signaling persists for long time periods as shown by recent findings that artificial suppression of the early triggering events of this pathway, such as via suppression of cPLA2, Cox-2, or Src kinase indeed causes significant reduction of manifestations of hallmark toxicities of TCDD such as wasting syndrome and hydronephrosis. Together, the evidence strongly support the notion that the inflammatory action of the ligand-activated AHR that is mediated by the nongenomic pathway plays the major role in the inflammation inducing actions of dioxin-like chemicals.

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; Cox-2, cyclooxygenase-2; MMP-2, matrix metalloproteinase-2; CSF-1, colony stimulating factor-1; cPLA2, cytosolic phospholipase A2; DRE, dioxin responsive element; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TNFα, tumor necrosis facto alpha; AP-1, activator protein-1; C/EBP, CCAAT enhancer binding protein; PKA, protein kinase A; IBMX, isobutylmethylxanthine; CYP19, cytochrome P450 19 or aromatase; H89, N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; MAFP, methylarachidonyl fluorophosphonate; EGTA/AM, EGTA-acetoxymethyl ester; AACOCF3, arachidonyl trifluoromethyl ketone; $[Ca^{2+}]_i$, intracellular concentration of free calcium ion; NIEHS, National Institute of Environmental Health Sciences; TLR, Toll-like receptor; LPS, lipopolysaccharides; siRNA, small interfering RNA; GLUT4, glucose transporter 4; LPL, lipoproteinlipase; EMSA, gel electrophoresis mobility shift assay.

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Contents

1. Introduction

The history of the search for the toxic action mechanism of TCDD has been dominated by studies on induction of cytochrome P450s and other detoxification enzymes in many animal species (e.g. [1]). Therefore, most of the subsequent development of mechanistic studies has been based on the paradigm of ligand-induced activation of the AHR leading to direct activation of many target genes (referred hereafter as the ''genomic'' or classical action pathway). This process of the genomic action of TCDD takes place through binding of the AHR:ARNT dimer to the dioxin response element (DRE or XRE, their consensus sequence being ''GCGTG'') located on the promoter region of the target genes (e.g. [2]). This is particularly prominent in the case of liver, which has been used by many toxicologists in this field, including researchers in my own laboratory, as one of the standard test tissues. Certainly the importance of those detoxification enzymes in the overall responses of animals to the actions of this type of toxic chemicals cannot be questioned. Moreover, the phenomenon of induction of some of those enzymes is easy to recognize, particularly in the case of cytochrome P450s, such as CYP1A1, of which constitutive expression is so low in contrast to the spectacular rises in its expression induced by many dioxin-like chemicals. Assessments of the actions of dioxin-like chemicals based on this approach have therefore provided very unambiguous and well-definable markers for their specific actions. Thus, it is easy to understand why studies on this induction phenomenon have predominated in this field, and why many scientists consider that this phenomenon of induction of detoxification enzymes itself represents the typical toxic action mechanism of TCDD. However, we must now ask a serious question: if the above model based on the ''genomic'' action of ligand-activated AHR to directly activate its target genes is fully adequate, why scientists in this field have had the difficulty in finding major DRE-based target genes, which could fully account for the major toxic manifestation of TCDD in many species? This point will be elaborated later.

In view of the many new findings on the unexpected roles of AHR, not fully explainable on the basis of the classical action mode of ligand-activated AHR alone (as illustrated by other contributors in this special issue), the main objective of this review is (a) to analyze existing evidence on the patterns of toxic actions of TCDD from the specific viewpoint of its

initial elicitation of cellular inflammatory signaling mediated by the ligand-activated AHR, (b) to assess whether there is a need and the rationale to formally introduce the nongenomic pathway as an additional model for toxic signaling of TCDD and (c) to critically examine the existing and newly found evidence to assess whether such an initial inflammatory action of TCDD could be converted into more stable messages, which could mediate long-term effects of TCDD, leading to toxic consequences.

2. History and the background of studies on toxic actions of TCDD through "non-classical" action mechanisms

2.1. *Brief history of the processes of discovering the problems of directly applying the principles of the classical model of DRE-based action pathway to certain toxic effects of TCDD*

When one goes through studies on the action of TCDD conducted in early 1980s, from the very beginning, one can discern some critical questions about such a simplistic interpretation of toxic actions of TCDD basing only on gene activation. For instance, in the Cold Spring Harbor symposium held in April 1984 [3], although the overall tone of the symposium was clearly favoring the use of this newly introduced paradigm of the action of TCDD based on induction of cytochrome P450 as the tool for the assessment of its toxic actions, actually several questions were raised as to whether the model based on induction of detoxification enzymes could adequately explain other types of toxic effects occurring in different types of cells and tissues [3]. Richard Peterson, for instance, pointed out the large difference in the TCDD dose that is required to produce enzyme induction as compared to that required to cause immune effects. Joseph Voss commented that there is a large discrepancy between the concentration levels of TCDD and that of 3,4,3′,4′-tetrachlorobiphenyl in terms of causing reduction of cytotoxic lymphocytes as compared to their potencies in inducing detoxification enzymes. William Greelee also voiced his concern that the dose of TCDD causing epidermal hyperplasia in hairless mice is quite different from that is known to cause enzyme induction in liver. Similar comments have been made in later years, also pointing out the difficulty in explaining toxic actions of various ligands by relying solely on the model based on cytochrome P450. For instance, Delescluse et al. have pointed out the same type of difficulties and proposed the likely existence of an independent pathway for toxic action of dioxin-like chemicals from the classical genomic action pathway as well [4]. However, those comments have not attracted much attention from the majority of toxicologists, judging by the subsequent development of risk-assessment approaches in this field of science, and the world-wide development of regulatory guidelines based primarily on the classical model of action of the ligand-activated AHR.

One of the major problems of the classical model in relation to inflammatory responses of cells exposed to TCDD may be that, when it comes to the precise mechanism of action of TCDD, the genomic action model has not provided any consistent indication on how cells elicit inflammatory responses to the action of TCDD through a definable signaling cascade such as the one seen in the case of inflammatory signaling of the ligand-activated Toll-like receptors (TLRs) [5], particularly TLR4, which is known to mediate inflammatory signaling of cells exposed to bacterial endotoxin, lipopolysaccharides (LPS). Such typical toxic signaling pathways involving inflammatory responses of cells are known to clearly involve the NFκB-mediated cascade of changes in gene expressions that are well coordinated with other major cellular regulatory pathways such as immune responses [5,6], signaling of hormone receptors such as the estrogen receptor [7], and regulation of vitamin signaling [8]. Instead, searches for the patterns of DRE-based AHR target genes through DNA microarray approaches, etc. have often uncovered mostly genes encoding detoxification enzymes or proteins, or detection of only a few isolated genes of which functions are unrelated to each other. For instance, in one of most thoroughly conducted cross-species survey using DRE-based DNA microarray approach, comparing human, mouse and rat genes, Sun et al. [9] found 2437 DRE containing genes altogether. From those, 48 were identified to be human–mouse–rat orthologs. Further examination of those genes resulted in the identification of 19 genes with positionally conserved DREs. However, only seven of these 19 genes were found to be actually TCDD responsive by *in vivo* tests in mice. Of those only six (i.e. *Ugdh*, *Cyp1a1*, *Cyp1b1*, *Stc2*, *Znf l48*, *Rab10*) actually exhibited TCDD-induced changes in mouse liver, and only two were induced by TCDD treatment in mouse Hepa1c1c7 cells (i.e. *Cyp1a1* and *Cyp1b1*). Therefore, this extensive search resulted in the detection of only four genes that are unrelated to cytochrome P450. While some of those could be of interest individually (e.g. Rab 10 regulating insulin-dependent glucose transporter 4 (GLUT4) translocation), there is no cohesiveness among them in terms of their functional roles, making it very difficult to relate those to known toxic outcomes resulting from TCDD poisoning. As one can see, while there have been other sporadic DNA microarray findings of specific genes whose expected functions may be related to some independent toxic outcomes, their expressions have not been shown to be well coordinated with other signaling events. Furthermore, the findings on some of those target genes that have been shown to be activated by TCDD in a given tissue and/or species may not be applicable to different test materials from other species. A good example is the discovery of plasminogen activator inhibitor-2 and TGFα gene, as two of such DRE-based genes that have been discovered initially by Greenlee's group in human keratinocytes [10]. Certainly these are the type of genes which could logically contribute to toxic actions of TCDD on keratinocytes. However, when the same research group studied gene expression profiles of the livers of rats treated with TCDD through PCR, mRNA expression of these two genes was not found to be affected by TCDD [11]. This type of inherent complexity associated with the mechanisms of specific gene regulations, which could be different among various tissues and animal species at different developmental stages, makes it very difficult to pinpoint any specific target gene as the "master" determinant of the toxic action of TCDD, even within one species.

Having presented these problems associated with the classical genomic model as a sole paradigm for the toxic actions of TCDD, it is also important to emphasize here that it is not the intention of this review to minimize the contributions of

the DRE-based genomic action mechanism to the final expression of TCDD-induced toxicity. Certainly in selected cases, like the induction of human IGFBP-1 [12], DRE-dependent induction of some component of the growth factor signaling or that of an apoptosis resistance cascade could provide the logical prospect that this protein contributes to toxic consequences, and therefore it is not that the genomic action does not contribute to the toxic actions of TCDD at all. Furthermore, the classical model is a wonderful tool in assessing the exposure of many organisms to dioxin-like chemicals as well as that in discovering hitherto unknown ligands [13] including natural ligands, which are very important in toxicology [14]. Another example, in which a DRE-mediated activation of a given gene could cause a direct toxic effect within the confinement of that specific tissue of the defined species, may be the case of TGFα on human keratinocytes [10], which, if it can be proven to be acting as an autocrine, could logically explain the development of hyperkeratosis. Thus, genomic action results could indeed contribute to toxic actions of TCDD. In addition, it must be made clear that the difficulties of accepting the universal applicability of the classical model in the toxic action of TCDD is limited only to the cases of the AHR:ARNT dimer directly activating the toxicity-related ''target'' genes. This point is important, since elsewhere in this special issue, the reader would learn some solid examples indicating that the AHR:ARNT dimer does indeed interact with other nuclear transcription factors to indirectly affect the expression of ''non-target'' genes (see the contribution of AHR:ARNT on TGFβ by Pedro Fernandez-Salguero's group and on ERα by Fumiaki Ohtake's group).

Instead, the main goal of in this article is to offer an additional way to appreciate the ever expanding scope of the actions of the ligand-activated AHR, by presenting the evidence for the importance of the nongenomic pathway in eliciting inflammation. Moreover, inclusion of the inflammatory responses of cells affected by TCDD within the function of AHR will add a new dimension to the understanding of the role of this receptor in not only toxicology but also in cell physiology in terms of cells regulating inflammatory responses as a part of the cells' functions in managing cell stress responses through AHR [15,16]. The secondary objective of this article is therefore to stimulate this field of science so as to relate the functions of AHR to the regulation of cell stress responses.

In more practical terms, the most critical questions being addressed in this article are: (a) how activation of AHR causes toxicities that are unrelated to the induction of those metabolic enzymes, (b) how we can explain the rapid action of TCDD in evoking inflammatory responses from a number of cell types, and (c) whether such rapid action of TCDD could be related to any of the long-lasting toxic actions of TCDD?

2.2. *Previous studies in our laboratory on the toxic action mechanisms of TCDD*

Our interest on toxic action of TCDD started when we noticed the symptoms of severe body weight loss (i.e. ''wasting syndrome'') in male guinea pigs treated with single i.p. injection of 0.6 μg/kg of TCDD. Unlike the case of Golden Syrian hamsters, in which the response to CYP1A1 induction in the liver takes place at a dose of TCDD at least three orders of magnitude lower than that causing its lethal action. TCDD-evoked induction of CYP1A1 in liver of guinea pigs is not prominent even at the lethal dose to this species, and thus we judged that the main mechanism of lethal action of TCDD in guinea pigs is likely not related to induction of this cytochrome P450. Since this early symptom of body weight loss is eventually followed by a late manifestation of lethality, which in the case of male guinea pigs takes place on average in 40 days, we considered this phenomenon of body weight loss to be one of the most indicative markers of the toxic actions of TCDD. Subsequent studies by other scientists have shown that the phenomenon of TCDD-induced wasting syndrome can be observed in many experimental animals, though some species require relatively high doses. At that time, the most promising clue to the toxic action mechanism of TCDD in guinea pigs was the observation by Robert Neal's group that severe ''wasting syndrome'' (loss of body weight) is accompanied with prominent signs of serum ''hyperlipidemia'' (i.e. high levels of lipids, particularly triglycerides) and accumulation of fats in liver [17]. Based on the original report from Stanley Cohen (see a more recent review by Cohen [18]) indicating that exogenously added epidermal growth factor (EGF) causes hyperlipidemia and accumulation of liver triglycerides in mice, we realized that this phenomenon of activation of EGF receptor (EGFR) by its ligand or by TCDD could somehow be associated with the occurrence of ''wasting syndrome'' that precedes the death of guinea pigs.

When tested in the mouse neonate model, both TCDD and EGF induced practically indistinguishable symptoms such as early eye opening with pigmentation around eyes, early eruption of teeth, and lipid accumulation in liver, which is accompanied with hyperlipidemia [19]. Since EGF itself does not induce CYP1A1 or other detoxification enzymes that are typically activated by TCDD, this finding has provided enough impetus to continue investigation of this subject. Subsequently, we have learned that the action of TCDD on guinea pigs at 1 μg/kg is accompanied with profound down-regulation of EGFR (an after effect of functional activation of EGFR) in liver plasma membrane [20], and that of lipoprotein lipase (LPL) in adipose tissues [21].

We have also found that TCDD-induced down-regulation of EGFR occurring *in vivo* in rat hepatic plasma membrane is accompanied with changes in several ATP-utilizing biochemical parameters [22], including significant up-regulation of certain classes of protein kinases such as cAMP-dependent and -independent protein kinases and, at the same time, suppression of enzyme activities of three different types of ATPases. This phenomenon lasted for a period of at least 20 days following a single injection of TCDD (25 μg/kg in the case of male rats). Subsequently we could show that those activated protein kinases include protein kinase, such as cAMP-dependent protein kinases (PKA), protein kinase C (PKC) and tyrosine kinases [23,24]. One of the most prominent protein kinases that was clearly activated at the early stage of action of TCDD was Src kinase, $pp60^{c\text{-}src}$ [25]. Based on such observations, a commentary has been published pointing out that the ability of TCDD to elicit rapid activation of protein kinases deserves more attention than has been previously given [26].

Our subsequent efforts in this line of investigation resulted in the discovery that in both mouse hepatocytes [27], and guinea

pig adipocytes [28], Src kinase facilitates TCDD's action to activate the EGF receptor. In an effort to relate the role of Src kinase to *in vivo* toxicity of TCDD, we have subsequently developed a line of src-knockout C57BL/6J mice along with genetically matched wild-type strain, which was produced in our laboratory through six generations of back-crossing, and tested the toxicity of TCDD *in vivo* [29,30]. The results indicated that the src-null mice were indeed less susceptible to TCDD than their wild-type counterpart with respect to their development of signs of wasting syndrome, such as (a) hyperlipidemia, (b) fatty infiltration of liver, (c) reduction of body weight gain, (d) decrease in the adipose tissue weight, (e) down-regulation of PEPCK in liver and (f) loss of appetite. An interesting secondary finding in this line of investigation is that quercetin (Sigma–Aldrich Co, St Lous, MO), a flavinoid phytochemical that is known to act as a potent antioxidant and an anti-inflammatory agent as well as an inhibitor of protein kinases [31], could reduce some of the symptoms of TCDD in wild-type C57BL/6J mice [32], indicating that such an anti-stress agent can reduce certain toxic effects of TCDD. The involvement of the EGF receptor in this type of action of TCDD has been also shown by our more recent study using a mutant strain, wa1/wa1 (Jackson Laboratories, Bar Harbor, ME), of mice, which has a defective TGFα that cannot functionally activate the EGF receptor [33]. The similarity of the type of toxic end points of TCDD that were reduced in the src-null and this TGFα mutant mice has suggested that one of the most likely reasons for the action of TCDD on the EGF receptor signaling activity is activation of Src kinase. Despite the success of these studies in linking the manifestation of wasting syndrome and the action of TCDD to the pathway mediated by Src family kinases, the meaning of the mechanistic role of protein kinases in mediating toxicity of TCDD has remained unresolved until recently.

2.3. *Early indications of the existence of the "nongenomic" pathway mediated by protein kinases in the action of TCDD: contributions from other laboratories*

While no one has formally suggested the existence of the "nongenomic" pathway *per se* until our recent reporting on this pathway [34], there have been a number of reports contributed by other scientists suggesting that the occurrence of the toxic effects of TCDD is more compatible with the nongenomic type of actions of ligand-activated AHR, rather than with its genomic actions. For instance, the group of scientists at the National Institute of Environmental Health Sciences (NIEHS), who have been working on the cell and animal models of liver carcinoma, have reported that TCDD-induced activation of EGF receptor and accompanying elevation of tyrosine kinase activities in hepatocytes are very sensitive and reliable markers of a carcinogenic action of TCDD in liver, while CYP1A1 induction is not [35,36]. Kohle et al. [37] also found in WB-F344 cells (a stem cell like liver oval cells) that TCDD causes activation of Src kinase, which is accompanied by its translocation of Src protein into the plasma membrane (i.e. indicative of its functional activation), which appears to correlate well with its toxic effect in this cell line [37]. El-Sabeawy et al. found that both Src kinase and EGF receptor are activated by the action of TCDD in testis, resulting in acrosome reactions of sperm [38]. Vogel et al. have reported that in rat hepatocytes Src is an essential mediator of the action of TCDD to induce Cox-2 (PGHS-2) activation [39]. More recently it has been reported that in the process of adipocyte differentiation of C3H10T1/2 fibroblasts, both TCDD and EGF receptors act synergistically to suppress PPARγ-mediated adipogenesis through selective contributions from Src, Rho and ERK [40]. One of the possibilities they pointed out was an independent pathway being operated through the tyrosine kinase-mediated route.

As for the possible triggering event initiating the activation of the inflammatory signaling through the nongenomic pathway, there exist a number of pertinent publications that report the observation of rapid increase in intracellular concentration of Ca^{2+}, $[Ca^{2+}]_i$ triggered by TCDD usually within 30 min. This rapid effect of TCDD was initially reported by Puga et al. in Hepa1 cells [41]. This phenomenon has also been observed to occur in other types of cells [42,43]. Indeed, in 1993 Nebert et al. have already proposed that this phenomenon of TCDD-induced Ca^{2+} is directly connected to signal transduction activity of AHR to activate AP-1 proteins, as in the case of EGF triggered mitotic signaling [44].

The question is, if there have already been so many published study results indicating the existence of nongenomic actions of ligand-activated AHR, why has there not been a general acceptance of the legitimacy of this mode of the AHR signaling as at least one of the major routes through which TCDD exerts its toxicity? The main reasons for this lack of acceptance, among many, may be: (a) the overwhelming dominance of the classical model based on the AHR target gene activation mechanism, (b) the lack of a clear theoretical framework to explain this aspect of action of AHR, and (c) an insufficient amount of concrete experimental evidence produced through systematic searches that are solely dedicated to this subject to convince the rest of the scientific community. Based on these considerations, our research group has recently focused its efforts on obtaining experimental evidence that will be helpful in eventually establishing this route of AHR signaling, and we are now ready to present some of our recent findings below.

3. Recent findings in our laboratory: studies on the early action patterns of TCDD in several types of cells

3.1. *Studies on the early responses of MCF10A mammary epithelial cells to TCDD: how these initial events are connected to activation of Src kinase?*

The initial breakthrough in our endeavors to connect the above findings on the importance of TCDD-induced initial increase in $[Ca^{2+}]_i$ by other research groups [41,42] was made in MCF10A [34], an immortalized but otherwise normal human mammary epithelial cell line that has previously been shown to depend on Src kinase in transducing the toxic actions of TCDD. Here we were successful in clearly demonstrating that TCDD induces an early rise in the concentrations of free arachidonic acid (AA) released into the culture medium of those cells within 30 min. Since the enzyme responsible for AA release, upon stimulation by Ca^{2+}, is known to be cytosolic phospholipase (cPLA2), this discovery has given our research group the opening for this line of investigation. This was

followed by conducting the necessary confirmatory work to show that such an early effect of TCDD is clearly inhibited by specific cPLA2 inhibitors such as AACOCF3 and MAFP and by an siRNA preparation specifically aimed at blocking cPLA2. These results clearly confirmed that activation of cPLA2 is likely the cause for the increased release of AA from those affected cells. Furthermore, such TCDD-evoked AA release could be significantly suppressed by two well accepted Ca^{2+} blockers, nifedipine and 2-aminoethyldiphenyl borate (2-APB), as well as by AHR blockers such as MNF and siRNA against AHR, but not by that against ARNT or an inhibitor of Src kinase, PP-2. Such observations indicated that (a) this phenomenon is mediated by cPLA2, (b) its triggering is likely due to the increase in intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), (c) this route of action is different from that of the classical action pathway (because of the apparent lack of participation of ARNT), and (d) this very early action of TCDD to up-regulate cPLA2 itself in MCF10A does not depend on Src kinase. This last finding was surprising to us, since we have shown previously that activation of Src kinase also takes place within 15–30 min, and that this kinase is intimately involved in toxic action of TCDD in this cell line [45–48]. One of the possibilities we considered was that, in this nongenomic signaling of the AHR, activation of cPLA2 triggered by the increase in $[Ca^{2+}]_i$ serves as the most up-stream component, and that Src kinase activation might act as its immediate down-stream signaling component. Indeed, we could quickly find that both A23187, a Ca^{2+} ionophore known to increase $[Ca^{2+}]_i$ as well as exogenously added arachidonic acid at concentrations expected to be in the range produced as the result of cPLA2 activation, clearly induce Src kinase activity. In addition, the most quickly responsing, prominent pro-inflammatory gene turned out to be Cox-2, of which induction starts taking place in 30 min in the case of MCF10A cells and continues to intensify over the next 72 h. During this time period there was no sign of activation of any of the markers of activation of the TNFα/NFκB axis in this cell line, unlike the case of U937 human macrophage line as will be shown later. These findings helped us to delineate the outline of this Cox-2-mediated nongenomic pathway. Furthermore, we confirmed that the process of arachidonic acid-induced activation of the Cox-2 in this process clearly depends on the activation of Src kinase in this cell line, which agrees well with the previous finding of Vogel et al. that TCDD-induced activation of Cox-2 in rat hepatocytes is dependent on Src kinase [39].

Furthermore, we could demonstrate that the TCDD-induced expressions of all other markers of inflammation such as PAI-2 and VEGF, than cPLA2, clearly depend on Src kinase. These findings helped us to finally understand the position of Src kinase in the nongenomic signal transduction pathway (i.e. Src kinase occupies the immediate down-stream position from cPLA2, and the immediate up-stream transducer position to Cox-2, see Fig. 1). Thus, we now understand the reason for the involvement of Src kinase in this nongenomic action of TCDD, at least in this cell material.

Earlier, our research team found in MCF10A that TCDD causes functional activation of Src tyrosine kinase [45–48], which is accompanied with activation of ERK 1 and 2 that takes place within 15 min of action of TCDD [48], when there is no sign of induction of CYP1A1 in that cell line. Thus, there is no question that this pathway is different from the classical pathway. More importantly, this finding on the position of Src in the nongenomic pathway has given us the theoretical bases to explain our previous observations of the reduced action of TCDD found in src-null mice in terms of their expression of "wasting syndrome" [29,30].

Additionally, one important characteristic of this cell line worth re-emphasizing is that expressions of IL-8, TNFα, and NFκB (including RelA and RelB mRNAs) were not recognized during first 3 h of action of TCDD. Instead, up-regulation of mRNA expression of VEGF, PAI-2 and IL-1β were the most prominent features. It is well known that the NFκB family of nuclear transcription factors is a major regulator of inflammation [49]. Therefore, its absence in the inflammatory response of MCF10A cells to TCDD is very surprising. One way to explain this phenomenon could be that this mammary epithelial cell line totally depends on macrophages or other inflammation assisting hematopoietic cells for their secretion of TNFα or any other paracrines. Such findings have been reported by breast cancer specialists previously [50]. Another critical finding made in MCF10A cells that merits re-emphasis is that the ability of TCDD to elicit those inflammatory responses is not dependent on ARNT.

It must be cautioned that all of the data cited in this review have been obtained from cell lines rather than from primary cells, which would have been more desirable in detecting normal responses of mammary epithelial cells to TCDD. The use of cultured standard cell lines, on the other hand, offers the future possibilities of re-testing any of those studies by other scientists as those are widely available cells from well established commercial sources.

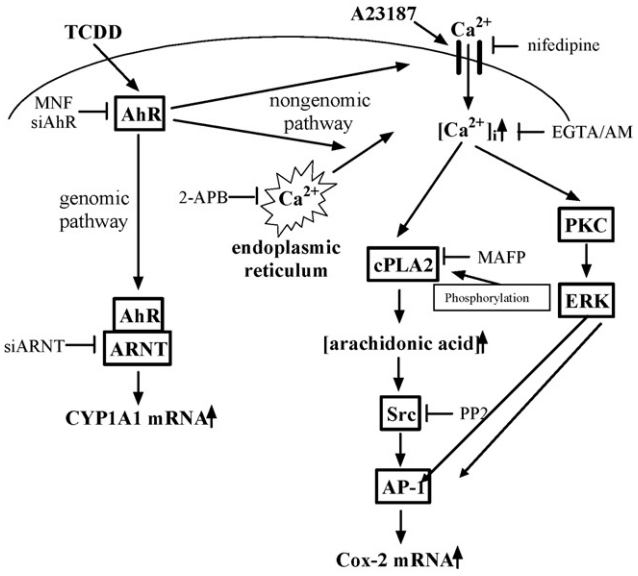

Fig. 1 – Simplified diagram illustrating the outline of the nongenomic inflammatory pathway of the ligand-activated AHR found in MCF10A cells [34]. The stop signs indicated are the expected action sites of those inhibitors (chemicals) and suppressors (siRNAs). A23187 is an ionophore inducing Ca2+ influx into the cell, and arachidonic acid produced by cPLA2 is envisioned to directly activate Src kinase.

3.2. Studies on the pattern of inflammatory responses in U937 monocytes-derived macrophages

Macrophages serve as the first line of defense for the animal body against invading foreign organisms or toxic chemicals [51,52]. They do so by utilizing their special ability to activate a number of defensive actions, against invading organisms and toxic substances. Such defensive actions include: phagocytosis, elicitation of oxidative reactions aimed at degrading the invading organisms or substances, production of chemical substances such as nitric oxide (which provides non-hospitable environments for invading organisms), cell migration to the site of inflammation and production of a host of cytokines and chemokines to assist the process of inflammatory and pro-survival reaction of tissues. Many of the features of this type of hematopoietic cells are not seen among most commonly studied target cells for TCDD such as hepatocytes. Furthermore, unlike hepatocytes, they are not particularly specialized to produce copious amounts of detoxification enzymes in response to TCDD and other xenobiotics. Earlier, we investigated the effect of TCDD, the most potent AHR ligand, on the differentiation process of U937 macrophages into foam cells, which is considered to be the sign of the development of early lesions of atherosclerosis [53]. To be sure U937 cells are an immortalized, monocytic cell culture line, and therefore macrophages derived from U937 monocytes may not reproduce all of the relevant actions of normal macrophages. Our findings showed, nevertheless, that, like oxidized low-density lipoprotein (oxLDL), TCDD promotes the differentiation of U937 macrophages to atherogenic ''foam cells'', as verified by increased lipid accumulation and extensive formation of blebs on the cell surface (i.e. the characteristics of foam cells), which have been observed by scanning electron microscopy [53]. By screening the expression patterns of typical genes involved in atherosclerosis and foam cell generation, we could demonstrate that the action of TCDD causes induction of mRNA levels of Cox-2, IL-1β and TNFα within 2–3 h in a dose-dependent manner in U937 macrophages, just like the action results of oxLDL, and that these changes were accompanied by significant up-regulation of matrix-degrading metalloproteinase (MMP)-1, MMP-3, MMP-12, and MMP-13. Increased levels of at least some of these MMPs are known to be associated with increased cell migration of U937 macrophages. Early up-regulation of TNFα in U937 macrophages in response to TCDD is one of the most conspicuous differences of this cell material from that was found in MCF10A cells. These findings clearly support the idea that AHR ligands, like TCDD, cause activation of inflammatory responses in this cell material, leading to the unique toxic manifestation that is very characteristic of this type of hematopoietic cells. One important feature of the response of U937 macrophages to TCDD appears to be the absence of the inhibitory action of PP-2, an inhibitor of Src on its early action to induce Cox-2 [54]. This is a markedly different pattern of response from the case of TCDD affected MCF10A cells [34]. This phenomenon is noteworthy, since there are publications indicating that Src is present in U937 macrophages. While the reason for this non-participation of Src kinase in the early nongenomic action of TCDD remains unknown, this finding illustrates one important lesson that one should not expect the same cellular stress responses from macrophages as those observed in other types of cells. Yet, as will be shown below, we could find that there are a number of early effects of TCDD that are qualitatively identical to those found to be occurring in other types of cells. In brief, this model of human macrophages seems to be capable of quickly responding to TCDD by activating the $[Ca^{2+}]_i$, cPLA2 and Cox-2 and thereby transducing the signal of the TCDD-activated AHR to inflammatory responses [54] as in the case of MCF10A cells [34].

Another characteristic of U937 macrophages is that induction of CYP1A1 takes place slowly (no signs of up-regulation of CYP1A1 mRNA in the initial 60 min period). This allowed us to investigate the nongenomic action of TCDD on both cPLA2- and Cox-2-mediated pathways at early time points of the action of TCDD. Based on the observation that this TCDD-induced activation of cPLA2 enzyme activity can be blocked by the same inhibitors of Ca^{2+} transport as shown above, we have reached the conclusion that the basic pattern of activation of the initial triggering events in this cell line is essentially identical to those occurring in other types of cells, except in the non-involvement of Src. Another characteristic is that, after 3 h of action of TCDD, marked up-regulations of mRNA expressions of IL-8 and RelB (a member of the NFκB family of nuclear transcription factors that are known for mediation of inflammatory responses) take place unlike the case of MCF10A cells [34]. Activation of RelB, a member of NFκB family of protein that is known to be capable of acting as a nuclear transcription factor, implicates this action of TCDD to functional activation of the inflammatory pathway mediated by the NFκB. This subject will be reviewed in depth in a separate chapter in this special issue by Chris F. Vogel and Fumio Matsumura. This capability of U937 macrophages to elicit such a rapid response involving NFκB-mediated type of inflammation signaling appears to represent the stereotypic pattern of responses of this type of cells, based on our observations that even several physical stress inducers such as NaCl-induced (150 mM) hyper-osmotic shock, oxidative stress as well as oxidized LDL- and zymosan-induced phagocytotic events trigger rapid activation of Cox-2 as well as TNFα release in macrophages. This finding on the rapid release of TNFα in response to the action of TCDD in this cell line offers at least one of the major mechanisms through which the initial signaling of cPLA2 and Cox-2 as seen in MCF10A cells can be linked to the other major inflammation pathway operated by the TNFα/NFκB axis through this autocrine (for macrophages) and paracrine (for mammary epithelial cells and many other cells that are known to recruit macrophages upon cell stress).

Another piece of key evidence obtained in this cell material delineating this inflammatory action pathway from the classical pathway of action of TCDD is that the former does not require ARNT [54].

3.3. Special cellular schemes of adipocyte differentiation and inflammatory stress responses toward ligand-activated AHR

It is clear by now that the pattern of action of TCDD that induces inflammatory responses depends largely on the type of cells it affects. This alone suggests that the various

functions of ligand-activated AHR are being tightly coordinated among different types of cells, some specifically associated with particular tissues, making it difficult to immediately find a simple universal principle that can be applied to all types of cells. On the other hand, as will be shown later, the pattern of very early cell responses to TCDD appears to be remarkably consistent among all cell materials studied by us so far.

Adipocytes are one such specialized type of cells associated with adipose tissues, which are becoming increasingly appreciated for the critical roles they play in regulation of not only fat storage (e.g. obesity), but also in nutritional homeostasis affecting the whole body, and in the etiology of human diseases such as diabetes and cardiovascular diseases. Despite the importance of adipocytes in the action of TCDD, particularly that is related to its toxic manifestation of wasting syndrome, the number of toxicologists specializing in the effect of TCDD on adipocytes has been surprisingly limited. Nevertheless, this study subject has yielded a wealth of information for us, thanks to the tremendous contributions made by a number of basic cell scientists who have been fascinated by the process of adipocyte differentiation from primitive fibroblasts [55] and stem cells [56]. Our interest in adipocytes started with the finding that TCDD causes tremendous biochemical changes in adipose tissues of guinea pigs, particularly noticeable was down-regulation of lipoprotein lipase [21]. This discovery was followed by an *in vitro* approach using explants guinea pig adipose tissues maintained in a culture medium, where TCDD was found to cause tremendous changes in protein phosphorylation activities and reduction of fat storage, which is accompanied with characteristic down-regulation of glucose transporter 4, which simulates what happens to TCDD affected adipose tissues in guinea pigs *in vivo* [57,58].

However, adipose tissue samples obtained from animals are known to be composed of immature adipocytes (preadipocytes) at varying stages of differentiation in addition to fully matured adipocytes, making detailed studies on the action site of TCDD difficult. Therefore at an early stage in our investigation we realized the need for an appropriate *in vitro* model, capable of yielding cells at various stages of adipocyte differentiation. After searching for a model we have settled on 3T3-L1 and its cognate mouse fibroblast cell lines for our studies because of the availability of their well studied background information.

By 1995 we were able to show the presence of the critical window of the timing of the action of TCDD in this cell line that prevents those fibroblasts from eventually differentiating into fully matured adipocytes. That is, TCDD must be added within the first 48 h of the addition of a mixture of differentiation inducers, consisting of insulin, dexamethasone (DEX) and IBMX (a phosphodiesterase inhibitor) [59]. This finding was quickly confirmed by Liu et al. [60] and Brodie et al. [61]. The intricate differentiation processes that are controlled by the sequential changes in the expression of the family of key nuclear transcription factor C/EBP proteins have become an important issue with regard to the action of TCDD [62]. Namely, upon stimulation of confluent cultures of primitive fibroblasts with the mixture of differentiation-inducing agents, the first isoform of C/EBP protein being up-regulated in the nucleus is the δ-isoform, which is quickly followed by that of the β-isoform and after a while by the steady rise in the titer of the α-isoform. Upon full maturation, occurring 7–12 days later, expressions of both the β- and the δ-isoform decline precipitously, but the expression of the α-isoform becomes fixed at the elevated level, making those adipocytes the fully ''committed'' state [55]. What we have found with respect to the action of TCDD was that during the early stage of adipocyte differentiation it promotes early up-regulation of C/EBPβ, and to a lesser extent C/EBPδ, which inevitably results in reduced up-regulation of C/EBPα (since the β- and the δ-isoform act as the priming factors for C/EBPα), and towards the later stage of differentiation TCDD prevents natural down-regulation of β and to a lesser extent that of δ [60–62]. From these findings we concluded that the most likely determinant of the action of TCDD to prevent adipocyte differentiation is its ability to significantly up-regulate the expression of C/EBPβ throughout the course of adipocyte differentiation.

This finding of TCDD's ability to antagonize the process of hormone-induced adipocyte differentiation has been confirmed later in other cell sources such as those of mouse embryonic fibroblasts (MEF) [63–65]. However, since this action of TCDD causes prevention of the appearance of many of adipocyte characteristic markers, there have been some arguments on the precise target of TCDD, some suggesting PPARγ or other major players on adipogenesis, being the target of TCDD [64]. Thus, this point has not yet been totally resolved. On the other hand, there is evidence of the involvement of Src in this action process, which shows that a cell line of mouse embryonic fibroblasts obtained from src-knockout mice (i.e. src−/− MEF) does not respond to TCDD, unlike the case of the matched wild-type (src+/+) MEF cell line, despite the fact that TCDD-induced CYP1A1 and 1B1 expresses equally in both lines [65]. In src−/− MEF, C/EBPβ was found to be constitutively expressed, suggesting that there is a connection between Src and C/EBPβ regulation, which is likely needed for the action of TCDD block adipocyte differentiation. The key point in understanding the meaning of C/EBPβ up-regulation is that activation of this nuclear transcription factor by the action of TCDD is closely associated with activation of PKA [66]. This agrees with the later observation that C/EBPβ activation is the most critical event in the ability of TCDD to prevent up-regulation of GLUT4 during adipocyte differentiation of 3T3-L1 preadipocytes to adipocytes [62]. The most impressive aspect of the latter finding is that a single exposure of preadipocytes causes sustained up-regulation of C/EBPβ over-expression throughout the entire process of differentiation, which takes place during next 7–12 days. More will be discussed later on this point.

While the above finding on the major critical stage of action of TCDD is a solid contribution to the fundamental knowledge on the action mechanism of TCDD, a major unsolved problem remains: i.e. the lack of an explanation for the ability of TCDD to reduce the lipid content of adipose tissue, which has been clearly demonstrated to take place *in vivo* by many scientists, or even *in vitro* with explanted guinea pig adipose tissues [67]. This phenomenon is sometimes referred as ''lipolysis'', but more precisely it represents the loss of stored lipids from individual matured adipocytes, which likely contributes to the loss of the weight of adipose tissues as well as the resulting

"hyperlipidemia" that is induced by TCDD, particularly in guinea pigs [21]. Certainly some of the loss of the total lipid storage of adipose tissues could be due to the decrease in the number of fully matured adipocytes (i.e. due to TCDD-induced inhibition of differentiation), but the speed through which "hyperlipidemia", particularly the increase in the quantity of total triglycerides in blood, following the administration of TCDD [21], and the recent acknowledgement that the percentage of adipocyte turnover in adipose tissue appears to be surprisingly low (approximately 10% per year in humans) do favor the view that TCDD must have the property to directly promote the release of stored lipids from adipocytes eventually into blood.

It was reported by Kern et al. that TCDD causes a 2-fold increase in TNFα secretion in F442A adipocytes when it was treated with TCDD for 48 h [68]. This phenomenon is accompanied by a 25% reduction in their lipid storage as well as in the activity of LPL. F442A cells share the same origin as 3T3-L1, though 442A cells show a higher propensity to become adipocytes, even in the absence of IBMX (probably due to constitutively expressed PKA), and therefore are less susceptible to the differentiation-inhibiting action of TCDD [69]. Accordingly, it was surprising to learn that matured adipocytes from this cell line respond to TCDD in this manner. This finding indicated that TCDD's lipids storage reducing action is likely carried out through a different mechanism from its known action of blocking adipocyte differentiation. Our group therefore re-focused its attention on the mechanism of action of TCDD on fully matured 3T3-L1 adipocytes. It was found that in this cell line the action of TCDD to induce actual reduction on the lipid storage of fully matured adipocytes takes place slowly over the period of at least 5 days [70]. Nevertheless, there were clear-cut signs of inflammation taking place at the early stages of this slow process: e.g. even after only 24 h of the action of TCDD, when there were no signs of down-regulation of adipocyte specific markers, the mRNA expression of Cox-2 increased by 2.9-fold, followed by that of NFκB (RelA, a marker for TNFα type inflammation), which showed an increase of 1.5-fold. Such an elevated state of inflammation lasted for the entire 5-day test period, which is accompanied by a steady rise in mRNA expression of KC (=an equivalent of human IL-8, an inflammatory chemokine) and that of TNFα. On the other hand, the incipient sign of modest down-regulation of adipocytes marker-expressions could be observed only by day 3, and the significant loss of lipids by these adipocytes (as judged by Oil Red O staining) occurred only after 5 days, which was accompanied by reduction of the mRNA expressions of GLUT4, LPL and C/EBPα. These findings indicate that the initial cause for TCDD's inducing lipid loss in matured adipocytes is inflammation induced by TCDD from the beginning. This conclusion coincides with the realization by many of scientists working in this field that fully matured adipocytes packed with lipids become susceptible to inflammation, which is greatly aided by inflammatory cytokines (such as TNFα) and chemokines (e.g. IL-8) produced by those adipocytes themselves (i.e. autocrine phenomenon). Furthermore, *in vivo* some of those cytokines and chemokines are utilized to recruit macrophages, which further enhance the state of cell inflammation. Thus, the finding that adipocytes exposed to TCDD show up-regulation of Cox-2 initially, which is followed by that of TNFα and KC (=IL-8), clearly supports our notion that inflammation is the initial response of adipocytes upon activation of AHR by TCDD binding. The additional finding that parthenolide (at 10 μM), a well known blocker of inflammation, as well as H89, a well known PKA inhibitor, clearly attenuate the ability of TCDD to cause reduction of stored lipids [61] also supports this view. The important point is that during the 5-day period of TCDD action, up-regulation of C/EBPβ is maintained continuously (as shown by both qRT-PCR and gel electrophoresis mobility assay, EMSA), which closely accompanies increased levels of expression of Cox-2 mRNA and its protein [71]. This finding gives us the reason to suspect that the elevated PKA activity is likely to be one of the means of converting the initial and transient signaling of $[Ca^{2+}]_i$ and cPLA2 into longer lasting cellular inflammatory activities in this cell line.

We recently confirmed essentially the same findings in a human adipocyte model, obtained through the use of adipocyte differentiation protocol applied to a human mesenchymal stem cell line (i.e. hMSC, originally from adult bone marrows) with new findings, that two additional cytokines/chemokines, IL-1β and MCP-1, in addition to IL-8, are involved in elevating the state of inflammatory status of those TCDD treated human adipocytes [71]. Again, the basic sequence of action of TCD in hMSC derived adipocytes was essentially identical: i.e. up-regulation of inflammatory markers seen in day 1 through day 3, followed by down-regulation of adipocyte markers by day 5. The main point of explaining the above works on adipocytes is that, despite the very unique and specialized nature of fully differentiated adipocytes, the main action of ligand-activated AHR is still to rapidly elicit the early inflammatory response from those adipocytes, as in the case of mammary epithelial cells and macrophages explained above.

4. Common features of early inflammatory responses among 3 different types of cells to early action of TCDD

The main reason why these different types of cells have been studied for their expression of the inflammation signaling of AHR through its nongenomic pathway is the known cell specificities of inflammatory responses among different cells. On the other hand, if we are going to clearly delineate this pathway from others, we must find what the essential requirements for this pathway are. The most direct approach in characterizing this newly found pathway would be first to find the common features occurring among all types of cells in response to TCDD, and then determine the differences among them to gain insight to the cellular specificities in inflammatory responses. Our findings along this line of approach are described below.

4.1. *Analysis on earliest events commonly occurring upon binding of TCDD to AHR*

The advantages of studying the earliest events of actions of TCDD is that in conducting any signal transduction study, the

knowledge of the earliest events is crucial in assessing the subsequent sequence of propagation of such signaling pathway, leading to stepwise changes in 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 signaling analysis of the ligand-activated AHR, there is the added advantage of recognizing that the hitherto unexplainable events occurring apart from its action results through the classical DRE-mediated pathway, since the latter process takes some time having to go through *de novo* transcription, translation and protein synthesis and post-translational modification activities, which are not needed in nongenomic actions that are carried out mainly through changes in the state of protein phosphorylation.

To date the earliest event we could observe in U937 macrophages was TCDD-induced release of ATP from mitochondria, which starts taking place as early as 1 min (Eric Sciullo et al., 2008, unpublished data). In this case, for *cellular ATP assay* U937 macrophages were plated on 96-well dishes and treated with 10 nM TCDD for 1, 2.5, 5, 10, and 20 min, while controls were treated with 0.1% DMSO. The CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) was used to determine ATP concentration according to their protocol, and samples were assessed using a Mithras LB 940 96-well plate reader (Berthold, Oak Ridge, TN). The percent increase in TCDD-induced release ATP were: 8% after 1 min, 15–16% in between 2.5 and 5 min, 13% after 10 min, and by 20 min returned to 0%.

This observation has brought our attention to an earlier report by Hanneman et al. that in the case of rat hippocampus neuronal cells TCDD causes rapid reduction of the mitochondrial membrane potential [42]. In their test system, the resulting increase in intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, occurs as soon as within 30 s. They concluded that the source of $[Ca^{2+}]_i$ is likely extra-cellular, judging by the inhibitory action of EGTA (which does not penetrate into the cell) and by the effectiveness of nifedipine (which affects the L-type Ca^{2+} channel on the plasma membrane). It has also reminded us of the original finding of Puga et al. that in mouse hepatoma cells TCDD causes a rapid transient rise in Ca^{2+} influx, which leads to the activation of AP-1 nuclear transcription factors [41]. Another report by Canga et al. also indicates that TCDD causes increases of $[Ca^{2+}]_i$ in myocytes from chick embryonic hearts [72]. So, one can ask which source of Ca^{2+} would be utilized by cells to respond to TCDD, mitochondria or outside? In our view, both may be correct in view of the effectiveness of both 2-APB (in directly supporting the intracellular source) and nifedipine (supporting the extracellular source). A hypothesis being proposed here is that the initial action of TCDD-activated AHR to affect mitochondria will lead to the release of stored Ca^{2+} from endoplasmic reticulum. Such an event could trigger influx of Ca^{2+} from outside of cells through the mechanism known as store-operated (also called ''capacitative'') Ca^{2+} entry, which in the case of macrophages is mediated through the L-type Ca^{2+} channel that is sensitive to nifedipine [73]. Indeed, we now consider that in most types of cells the initial release of Ca^{2+} from endoplasmic reticulum (judging by the effectiveness of 2-APB) that is initially induced by mitochondrial membrane destabilization is the earliest trigger, which further activates the Ca^{2+} influx from outside judging by the effectiveness of nifedipine (a blocker of an L-type Ca^{2+} channel), which might be the case in MCF10A cells, too. This conclusion agrees well with that of a recent report by Shertzer et al. [74], 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 classical DRE-mediated pathway.

It must be emphasized that the increase in $[Ca^{2+}]_i$ is the most consistently observed event during the very early stages of action (within 15–30 min of the time of addition of TCDD), among all types of cells studied by other groups, as well ours, thus far. Therefore, it appears reasonably safe to propose that this is likely the initial trigger for the inflammatory pathway of the nongenomic action of TCDD. Another point of reminder is that in all cell types examined thus far, activation of cPLA2 and Cox-2 follows the above triggering effect of Ca^{2+}. Thus, in spite of the knowledge that cell stress response programs are notoriously specific to each cell type – reflecting the specific role each cell must play in order to mount a well-orchestrated defensive action in the body – these initial events occurring in the nongenomic signaling in all these types of cells are surprisingly consistent. Thus, even though the subsequent down-stream events may vary among different cells, the above early events are indeed the common features occurring in all cells studied so far.

4.2. *Non-involvement of ARNT and its implications*

Another common feature we have found so far in our studies on the characterization of this nongenomic pathway, in MCF10A cells, U937 macrophages and 3T3-L1, has been the non-involvement of ARNT in assisting the nongenomic signaling of ligand-activated AHR. This lack of involvement of ARNT has been confirmed at several stages of the action of TCDD including the initial stage of signaling as well as later stages. This was done by using siRNAs against ARNT (siARNT) in comparison to that against AHR (siAHR). In all of the cell lines we have studied so far MCF10A, U937 macrophages and 3T3-L1 adipocytes, we used cPLA2 and Cox-2 mRNA expression as the standard markers of the activation of this nongenomic pathway and found that their expressions were clearly suppressed by siAHR, but not by siARNT. In contrast, the expression of CYP1A1, tested under the identical condition on the same batch of cells, was consistently suppressed by both siRNA treatments as expected. While much more work would be needed to elucidate the precise mechanism through which this nongenomic pathway is activated by the ligand-bound AHR without the help of ARNT, one possibility worth considering is that this pathway could take place without translocation of the ligand-activated AHR into the nucleus. Although no direct evidence has been obtained, some supporting evidence exists for this possibility. Recently it was reported from this laboratory that TCDD (single i.p. dosing of 100 μg/kg) causes modest, but clearly recognizable up-regulation in expression of MCP-1 and F4/80 in liver of AHR^{NLS} mice (a generous gift by Chris Bradfield) after 7 days [75]. This strain of mice lacks the capability of AHR to translocate into the nucleus, and therefore there is no

possibility of any direct involvement of the classical pathway in the above phenomenon of TCDD-induced up-regulation of those inflammation markers in this strain of mice. Assisting this diagnosis is the fact that these two markers are well known to be indicative of the activation of a typical inflammation that is assisted by macrophages. This way of interpretation also helps to explain why the process of inflammatory responses of animals generally involves intercellular interactions (e.g. macrophages and adipocytes) in addition to the initially affected cells (e.g. through infection of the hosting tissue). The case of TCDD-induced inflammation appears to follow this general pattern of progression of inflammation signaling, as F4/80 is a well-recognized marker of the arrival of macrophages to the site affected. While the above data would not provide any mechanistic insight, the information that these AHR^{NLS} mice are not capable of translocating those ligand-activated receptors still showed the sign of inflammatory response to TCDD is helpful information supporting our interpretation that the nongenomic pathway can take place without nuclear translocation of the ligand-bound AHR.

4.3. *The role of protein kinases and phosphatases mediating nongenomic signaling of AHR*

It must be mentioned that the idea of designating this newly found AHR mediated inflammatory pathway ''nongenomic'' comes from the well known example of the signaling activities of steroid receptors, which are operated by both the genomic and the nongenomic modes of operations in signaling processes of ligand-activated steroid receptors. While one must be careful in comparing the case of the cytosolic AHR signaling to that of some steroid receptors – which are often localized in other intracellular locations (e.g. in the case of the estrogen receptor, nongenomic signaling is mediated by the form residing in the plasma membrane location) – there are some common denominators among all these cases. One of them is the receptors' utilization of a series of protein phosphorylation activities mediated by a number of protein kinases and some phosphatases in propagating their nongenomic signaling [76]. Despite the generally held notion that signaling transmitted through nongenomic modes produces only short and acute effects, moves are now being made to re-evaluate the validity of such a notion [77].

In studies on the classical model of ligand-activated AHR action, the topic of the possible involvement of protein kinases and phosphatases has largely been centered around the effect of phosphorylation on the AHR protein proper [78]. This certainly is important particularly in the case of resolving the cause for the ligand-independent activation (LIA) of AHR [79], but it should not be confused with the case of nongenomic signaling of the ligand-activated AHR, since so far as known the consequence of the latter action is totally different from LIA. With respect to the operation of the steroid receptors, the most plausible explanation for the necessity of these seemingly redundant (i.e. genomic and nongenomic) action pathways for them is that any changes in major cellular programs, such as those induced by hormones, require careful coordination with the other major players involved in cellular activities and homeostasis – such as that with growth factor receptors, stress response receptors and nutritional-balancing receptors – through their ''cross-talking'' interactions, within which the protein phosphorylation processes could offer tremendous advantages over other methods of cross talks [80].

The second explanation may be that inducing changes in protein functions through activation or suppression of protein kinases and phosphatases offers faster cellular response to the given signaling than its genomic counterpart, since genomic actions take time to process a signal come up with a final product, such as *de novo* synthesized peptides or proteins and their post-translational modifications. In contrast, activation of protein kinases or phosphatases could take place literally within a few minutes of the arrival of given signaling. For instance, in the case of Toll-like receptor signaling for inflammatory responses that is induced by its ligand, bacterial lipopolysaccharides, it has been found recently that it is accompanied with rapid activation of the double-stranded RNA (dsRNA)-activated serine/threonine kinase R (PKR), which start within the initial 15-min period [81]. Therefore, it is not surprising that signaling of ligand-activated AHR, which could be viewed as the second major type of receptor triggering inflammatory responses, is also accompanied with rapid activation of protein kinases, such as Src kinase within 5–15 min of action of TCDD in MCF10A cells. Finally it would be prudent to point out the in the case of better studied cases of the steroid receptors, the ligand-induced signaling mediated by the nongenomic pathway is considered to be eventually integrated with that induced by the genomic pathway [82]. Therefore, there is a good possibility that some of the DRE-target genes activated by the action of TCDD through its classical pathway (i.e. genomic pathway) could be eventually ''integrated'' with those genes activated through the non-genomic pathway, depending on the specific cases such as tissues, species and timing of the action of TCDD.

4.3.1. *The role of protein kinase C in mediating early actions of TCDD*

That action of TCDD on a variety of cells is accompanied by the activation of protein kinase C has been reported by a number of research groups (e.g. [24,83,84]). The observation that PKC activation takes place rapidly after cell exposure to TCDD has also been reported by Weber et al. [85] in vascular smooth muscle cells (within 30 min). Hanneman et al. have shown that activation of PKCα takes place in primary culture of hippocampal neurons within 10 min of the addition of TCDD, which is accompanied by the rise in $[Ca^{2+}]_i$ as mentioned already. In our recent study on MCF10A cells, PKC activation takes place within 15 min of the action of TCDD, which is accompanied by activation of the serine phosphorylated form of cPLA2 protein [34]. These observations are consistent in indicating that activation of PKC is one of the earliest protein kinases activated by TCDD, and implicates the rise in $[Ca^{2+}]_i$ as the likely trigger for such an action of TCDD. Judging by the rapid activation of cPLA2 among all those cells, we should consider the possibility that activation of PKC is also a common feature among all susceptible cells to TCDD. It must be added that PKCα knockout mice (or those lacking other isoforms of PKC) are available, and hence those should be utilized in the future to confirm the role of PKCs in this nongenomic signaling of TCDD.

4.3.2. *Roles of other kinases: relatively early events showing distinct differences among cells*

As indicated previously, our studies on MCF10A cells have shown that the activation of Src kinase occurs as a result of the increase in the concentration of free arachidonic acid caused by the activation of cPLA2. On the other hand, Src is not involved in all cells, judging by its absence in U937 macrophages. Thus, Src serves as the first marker, indicating the cell specific nature of this nongenomic signaling of AHR. Nevertheless, it is clear that Src plays important roles in the etiology of TCDD-induced wasting syndrome *in vivo* [29,30].

On the other hand, we have found activation of PKA in all types of cells examined so far. PKA is known to be activated by both AA (likely due to TCDD-induced up-regulation of cPLA2) and PGE2 (likely due to Cox-2 up-regulation), and therefore, its activation takes place later than PKC, but the duration of activation of PKA by the action of TCDD appears to be very long, at least in the case of adipocytes (>5 days) assessed *in vitro* [70,71] and liver of rats *in vivo* (>40 days) [22]. While we are less certain that PKA activation is indeed a common feature of the nongenomic signaling of AHR among all cells, compared to cPLA2 and Cox-2, it is probable that PKA activation takes place in most of cells, because of its known immediate down-stream position of cPLA2 and Cox-2. Another kinase of great importance would be ERK, which is known to activate cPLA2 through phosphorylation of its protein [86]. However, ERK is only one of the MAP kinases, which serve as the key regulators of vital cellular activities. Thus, depending on the status of the cellular condition, activation of ERK may or may not take place. It is likely that there are many additional kinases, and phosphatase that have been reported to play some roles in aiding the process of propagating the nongenomic signaling of the ligand-activated AHR, but for the time being, the above examples are sufficient for this review to illustrate the point that signaling through directed changes in protein phosphorylation on its transduction pathway is the one of the features of any given ligand-induced nongenomic pathways of this type of receptor.

5. Mechanisms of conversion of the initial transient nongenomic signaling of TCDD-activated AHR into more stable messages

Despite the inherent complexity of this subject matter and the scarcity of reliable data, it must be emphasized that this is a tremendously important topic for this paper, because, if the initial message of nongenomic signaling of ligand-activated AHR remains transient as suggested to be the case of temporary increase in $[Ca^{2+}]_i$, there is little chance that this pathway would account for any of the long-lasting toxic effects of TCDD.

5.1. *How do cells convert the initial, transient $[Ca^{2+}]_i$ mediated "nongenomic signaling" of ligand-activate AHR to much longer lasting signals?*

It must be remembered that by all accounts the initial effect of TCDD on $[Ca^{2+}]_i$ appears to be transient [41,42]. Therefore, to maintain a 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 modes of signaling, which includes "fixation" of nongenomic messages in the form of genomic expressions, if the paradigms of the action mechanisms of growth factors and mitotic signaling of phorbol esters such as TPA (i.e. activation of immediate early genes by cell treatments with the above mitogenic stimulators) are applicable here. Indeed, it has already been suggested that such early signaling of $[Ca^{2+}]_i$ is transduced to the activation of AP-1 proteins [41,42], which is one logical way of converting those nongenomic signaling activities into mitogenic genomic messages.

5.2. *Role of protein kinases, particularly that of PKA in converting transient signaling to long-term messages*

At a very early stage of our investigation we learned that in hepatic plasma membrane preparation from TCDD treated (via single i.p. injection) rats – as compared to equivalent preparation from matched control rats – showed that the level of TCDD-induced activities of the total PKA activity steadily increased, reaching its peak around 20 days post-treatment, and stayed elevated until the end of that experiment (40 days) [22]. While the meaning of such a result from *in vivo* studies may be difficult to interpret (because of the complexity of *in vivo* interactions of cell regulatory factors), it at least gives a clue to the possibility of this mode of protein kinase-based mode of maintenance of initial signaling is one of the mechanisms through which the duration of nongenomic signaling could be prolonged. Main supporting data *in vitro* for this possibility came from our own work on 3T3-L1 cells. We have noted, during the process of studying the effect of TCDD on adipocyte differentiation, that a single treatment of 3T3-L1 fibroblasts at the beginning of differentiation is enough to cause sustained up-regulation of active form of C/EBPβ protein expression in the nucleus, which is observable even after completion of those differentiation experiments (8–12 days) [70,71]. Since we know that transcriptional activation of C/EBPβ gene, in a similar mouse fibroblast cell line, CH310T1/2, is predominantly affected by PKA [65], judging by the action of TCDD to induce stable activation of C/EBPβ (as judged by EMSA assay) for 24 h (the duration of this experiment), the above observation does lend support to the possibility of PKA's being one of the vehicles through which the signaling of nongenomic action of TCDD is performed. Although the evidence we obtained from the above 3T3-L1 differentiation studies [70,71] is indirect (i.e. we did not measure PKA directly), in our C/EBPβ gene promoter studies [66] we indeed measured PKA activities stimulated by TCDD in CH310T1/2 cells, along with the elevation of cAMP active DNA binding forms of CREB and C/EBPβ proteins in the nucleus. The possibility of the DRE-based action of TCDD in the activation of C/EBPβ gene was overruled since the DRE-like sequence present in the proximity of those active CREB sites turned out to be mostly inactive. It must be pointed out, however, that the likely involvement of PKA in stabilizing and sustaining the initial signaling of nongenomic signaling is only one of many possibilities.

5.3. *Direct fixation of nongenomic signaling of selected early markers of inflammation into their corresponding genomic messages*

Another possible mode of converting transient signaling to more stable one is the direct conversion of nongenomic signaling into a genomic one. We have noted that the initial transient increase in the mRNA expression of cPLA2 by TCDD, which subsides during the following 6 h in the case of U937 cells, is followed by a rise in its functional expression by 24 h, as judged by its-promoter-Luc reporter assay [54]. The expression vectors used in this study were: human $cPLA_2$-Luc (kindly provided by G. D'Orazi, Department of Oncology and Neurosciences, University G. d'Annunzio, Chieti, Italy [87] with the permission from its original creator, R.A. Nemenoff, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO). This reporter construct consists of 2.4 kb pairs of the 5′-region ligated into a host luciferase vector (PA3-Luc). The jetPEI^{TM}/NaCl solution was used as a transfection agent, and the transfection efficiency cells were assessed by co-transfecting with 0.1 μg per well β-galactosidase reporter construct.

A survey of literature has indicated that the cPLA2 gene is well known for its up-regulation through post-transcriptional factors, particularly by interferon-γ [88]. 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 of 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. Finally, one possibility for explaining the long-lasting effect of events that are originally triggered by the nongenomic signaling of TCDD-activated AHR may be due to the chemical persistence of TCDD itself. This possibility is logical in view of the known long half-life of TCDD, particularly in human tissues. However, we must also remind ourselves that some of the effects of TCDD are not long-lasting. For instance, up-regulation of IL-1β and PAI-2 mRNA expressions in MCF10A cells start declining after a few hours, while that of Cox-2 persists for a long period in that cell line. Thus, there are certainly some processes taking place to selectively convert the transient effects of nongenomic signaling into more long-lasting messages. The possibility of active integration processes taking place among the many changes induced by AHR, including those induced by the classical pathway, as in the case of steroid receptor signaling, must also be considered [77]. This subject needs much more future work in view of its importance to understanding the chronic toxicities of TCDD, though it is a very challenging subject.

Finally one more possibility to be considered viable is the continuous signaling of $[Ca^{2+}]_i$ being generated by the persisting TCDD despite the outward appearance of its initial transient signaling as assessed by the current technology. The reason for this possibility is that certain types of Ca^{2+} signaling is known for its oscillating nature, which is the results of interactions of finely tuned feedback controlling mechanisms. Much more work would be needed in the future to address this possibility.

5.4. *Roles of cytokines and chemokines in propagating the messages of initial inflammatory signaling-triggered by cPLA2 and Cox-2*

Cytokines and chemokine production by TCDD affected cells could also serve as one of the major means of propagation of inflammatory signaling affecting those cells themselves (i.e. autocrines), or other cells that are accessible physically or through circulation (i.e. paracrines). Indeed there is enough evidence that *in vivo* TCDD promotes production of many inflammation mediating cytokines and chemokines (e.g. [75]).

The nature of those extra-cellularly produced messengers largely depends on the specific types of cells affected by TCDD. Having found that cPLA2 and Cox-2 play an initial mediator role for the nongenomic inflammatory pathway in several types of cells, the first question we must address here is how their message is passed on to other major inflammatory pathways, particularly to the NFκB pathway. In this regard we noted that the activation of TNFα and IL-8 mRNA by the action of TCDD takes place within 3 h in U937 macrophages followed by production of IL-6 [54]. Therefore, there is no question about the readiness of macrophages to rapidly respond to the action of TCDD by producing those cytokines and chemokines as either autocrine or paracrine. However, in contrast, so far none of other cells studied by us could show the sign of such rapid activation of the TNFα/NFκB pathway. For instance, it takes HepG2 cells (Dong and Matsumura, unpublished data) or 3T3-L1 cells more than 6 h to up-regulate the NFκB pathway. Moreover, in the case of MCF10A, no sign of up-regulation of TNFα or NFκB could be observed even after 72 h [34]. At an early stage of action of TCDD, for instance in the case of MCF10A cells, IL-1β could be observed after 1 h, but expression of IL-1β did not last so long, and no induction of IL-8 could be observed within 24 h [29,34]. Thus, it is probable that certain tissue cells (particularly MCF10A cells in our study) must rely on paracrine factors such as chemokines/cytokines produced by themselves to recruit macrophages as well as by other types of hematopoietic cells, which are capable of producing inflammatory factors (e.g. TNFα) to activate the NFκB signaling. So far in the case of MCF10A cells the production of CSF-1 has been the only persistent paracrine observed to stay elevated during 72 h of observation (Dong and Matsumura, unpublished data). The role of CSF-1 in breast cancer in terms of recruiting macrophages is well known [89].

In the case of 3T3-L1 cells, on the other hand, the earliest markers of production of paracrine factors by 3T3-L1 adipocytes were IL-8 (called KC in murine cells) [70] and MCP-1 (Li and Matsumura, unpublished data). Furthermore we have found that 3T3-L1 adipocytes are capable of producing TNFα as an autocrine factor in response to the action of TCDD. However, it takes 24 h for 3T3-L1 adipocytes to up-regulate mRNA expression of TNFα to the level reaching the statistically significant as judged by qRT-PCR assessment [70].

These are well-recognized paracrine factors known to enhance monocyte/macrophage recruitment to the site of inflammation. IL-8, in particular, is a well-acknowledged chemokine that is very important to macrophage recruitment. Interestingly, IL-8 serves also as a pro-survival autocrine factor in addition to its well known role as an inflammatory chemokine [90]. 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 cells).

In summary we have found three different types of cells in the current study: (a) U937 macrophages which have the built in capability to quickly transduce the inflammatory signaling of cPLA2 and Cox-2 into NFκB-mediated inflammatory signaling, (b) 3T3-L1 adipocytes, which is initially incapable to do so, but after 24 h of continuous activation of Cox-2 those cells start producing TNFα as an autocrine to induce activation of NFκB, and (c) MCF10A cells which are incapable of transducing the inflammatory signaling of Cox-2 into activation of NFκB even after 72 h, which likely depend upon their production of paracrine factors to recruit macrophages and other inducers of the NFκB pathway.

6. Possible implication of AHR-dependent activation of stress responses in the etiology of toxic symptoms in animals and human diseases

Probably one of the most accepted cases of TCDD's action causing well-defined major disease-like symptoms in a number of species is that of wasting syndrome as explained above. The likeliness of a tight relationship between wasting syndrome and lethality has also been pointed out [91]. While no one has addressed the role of the nongenomic inflammatory signaling of TCDD-activated AHR *per se*, there are some indications that this syndrome/disease is significantly affected by this route of its signaling. For instance, it has been demonstrated by Taylor et al. [92] that *in vivo* treatment of mice that had been exposed to TCDD with either an antibody aimed against TNFα or with dexamethasone, a well known anti-inflammatory agent, greatly reduce the toxic expression of TCDD [92]. In more specific terms, TNFα antibody treatment resulted in a 54% reduction in TCDD-mediated mortality, while DEX treatment, a glucocorticoid receptor agonist that inhibits transcription of TNFα, reduced mortality by 92%. Furthermore, they observed that mice treated by TCDD along with either the TNFα antibody or by DEX co-treatment showed greatly reduced levels of development of wasting syndrome. At the same time they observed that CYP1A1 induction, the most commonly measured TCDD-mediated response, was not blocked by DEX, demonstrating a clear-cut separation of this biochemical effect from the detoxification enzyme-inducing action of TCDD. Judging by what we know today such an action of DEX should be characterized as a nongenomic action. Therefore, this original work should be credited as one of the earliest demonstrations of the causal link between the inflammatory signaling of TCDD and the etiology of wasting syndrome. Another early finding implicating a nongenomic origin of the wasting syndrome has been made by our group, which showed that the symptoms of wasting induced by a high dose (115 μg/kg, i.p., single dosing) of TCDD were much less expressed in src-knockout mice (src−/− mice) as compared to its matched wild-type strain of src+/+, or src−/+ strain of C57BL/6J mice [29,30,32]. The role of Src kinase in the toxic signaling of ligand-activated AHR through the route other than its classical action pathway has already been described [26]. Although this Src-mediated pathway was designated as the phosphorylation pathway at that time, there is little doubt that this non-classical action pathway is essentially identical to the nongenomic pathway, and therefore the above set of data confirms its significant contribution toward the etiology of TCDD-induced ''wasting syndrome''. Another supporting indication for this conclusion includes finding the effectiveness of anti-inflammatory natural products such as quercetin [32], curcumin and geranylgeranyl acetate [93]. While one must be careful not to assume that all the inflammatory and anti-apoptotic actions of TCDD are induced through activation of the nongenomic signaling of the ligand-activated AHR alone, the fact that in those studies such anti-inflammatory agents did not affect CYP1A1 induction supports the above interpretation that the influence of the cytochrome P450 activating action of the ligand-activated AHR through the classical route is not likely to be significant.

Another major human disease potentially affected by the inflammatory responses of cells to TCDD exposure is type 2 diabetes. Ebner et al. [94] originally found that blood glucose levels in rabbits treated with TCDD show transient increase, accompanied with the lowering of serum insulin levels. Subsequent human epidemiological studies have revealed that there are significant positive correlations between human exposure to TCDD and subsequent development of type 2 diabetes among Seveso populations [95] as well as veterans of the Vietnam War [96]. Our research group had the opportunity to assess the pattern of expression of several biomarkers in adipose tissue samples from those Vietnam veterans exposed to TCDD along with carefully matched comparison veterans who had served South East Asian tours of duty [97]. Among those biomarkers that showed the highest correlations were NFκB (an indicator of inflammation) and GLUT4 (an insulin-responsive glucose transporter). Particularly interesting is the tight correlation found between the levels of TCDD found in the veterans' serum lipids and the ratio of these two markers (GLUT4 divided by NFκB or G/N ratio). Since rising levels of inflammation (as shown by the increase in NFκB expression) and concomitant decreases in GLUT4 expression are the hallmark of obesity-induced type diabetes, we could quickly confirm the reliability of using their ratio in assessing the diabetogenic action of TCDD by correlating it to the degree of obesity and the levels of their exposure to TCDD among all study subjects (approximately 300 veterans altogether). We could also confirm that the essentially the same kind of changes in the G/N ratio in adipose tissues of mice as a result of TCDD exposure as well as in adipocytes cultured *in vitro* as described above. Thus, it has become apparent that inflammation induced by TCDD in adipocytes in culture could potentially serve as a cell model for the future studies on the TCDD-induced inflammation as a cause for type 2 diabetes as in the case of inflammation induced by obesity. While much more work would be needed to confirm the essential role of the nongenomic signaling of AHR, the above *in vitro* study results clearly support TCDD-induced suppression of GLUT4 in both 3T3-L1 adipocytes [70] and human mesenchymal stem cell-derived adipocytes [71] as due to early up-regulation of Cox-2 and other inflammation-

inducing actions of TCDD, which are mediated by the nongenomic signaling pathway, making it highly likely that the etiology of TCDD-induced type 2 diabetes is largely mediated through this route of action as well.

The third type of TCDD-induced disease that is likely mediated through this nongenomic route of action of ligand-activated AHR is nephropathy of the kidney, though in this case the only evidence on which we can base our judgment has been produced using a mouse model. In a recent paper Nishimura et al. [98] have shown that the symptom of hydronephrosis, which is frequently observed among mouse neonates exposed to TCDD through mothers' milk, is accompanied with significant activation of Cox-2 in kidney at early stages of action of TCDD: i.e. a sign of activation of the inflammatory action pathway. The most concrete evidence linking such an early inflammatory action of TCDD to the eventual development of hydronephrosis in those neonates is the effectiveness of *in vivo* treatments of those neonates with indomethacin N-octylamine, a selective inhibitor of Cox-2 enzyme, which totally eliminating the development of hydronephrosis. The effects of TCDD causing other types of nephrophathy has been also shown by the researchers of the National Toxicology Program, 2006, who studied the chronic effects (2 years) of TCDD and other homologs on female Harlan Sprague-Dawley rats [99]. The results of this extensive toxicological study have also revealed the occurrence of several additional long-term effects of TCDD-type chemicals that are clearly associated with chronic inflammation of target tissues such as the ones found in ovary, pancreas and mesenteric artery, etc.

7. Concluding remark

In this review efforts have been made to summarize the data indicating how inflammatory cellular responses are elicited by the action of TCDD-activated AHR in a selected list of different types of cells/tissues. The uniting factor among those cells is chronic inflammation known to cause serious consequences. Although the pattern of inflammatory responses could vary by types of cells, fortunately, the pattern of initial inflammatory signaling, its propagation and consequences of chronic inflammation in those cells has been relatively well studied [100], and hence in some cases one can recognize some stereotypic patterns in each type of cell. For instance, in the case of inflammation induced by LPS- [101] and TCDD, some of those stereotypic patterns are recognizable (see [13], regarding comparison of inflammatory effects of TCDD and those of lipopolysaccharides), although even in such a case it is prudent to pay a healthy respect for the details of their differences. In this regard, the selection of ''wasting syndrome'' as the main subject of study from the beginning was helpful, since the symptom of LPS-induced ''cachexia'' has been well studied using rodent models as well as in several domestic animals [99]. While there are many complications and details that are critical in the evaluation each action pattern of inflammatory signaling, the most significant finding is that there are a number of cases where treatment of test animals with agents known to ameliorate the effects of the inflammatory signaling of TCDD were successful in blocking some of the toxic manifestations of TCDD [26,29,33]. More recently our group had the opportunity to collaborate with Chiharu Tohyama's group on the subject on TCDD-caused hydronephrosis among mouse neonates exposed to TCDD through their mothers' milk [98]. During the course of our collaboration, we found that TCDD causes prominent activation of Cox-2 in kidney tubular epithelial cells in those mice. The importance of Cox-2 induced inflammation in the etiology of this disease has been shown when Noriko Nishimura found that treatment of those mice with indomethacin N-octylamine, a specific inhibitor of cox-2 *in vivo* completely eliminated the symptom of hydronephrosis. This is one of the most illustrative pieces of evidence, indicating that the nongenomic inflammatory signaling of ligand-activated AHR plays a pivotal role in the etiology of a TCDD-induced toxic symptom. Finally, for the sake of simplicity, in this review only the aspects of direct cellular inflammatory responses exhibited by the target cells have been emphasized. In reality, the process of converting those transient initial nongenomic signaling of ligand-activated AHR into long-term expression of chronic inflammation would require more than activation of protein kinases. In this regard, one of the most important vehicles of amplification of inflammatory signaling could be a number of inflammatory cytokines, chemokines and peptide growth factors (which are currently being actively studied by experts in the field [75,102,103]), some of which can be activated through non-classical routes of action. Some of those could act as autocrines to either protect those cells or amplify inflammatory signaling, or as paracrines to attract macrophages and other hematopoietic cells specialized for phagocytosis, or as other means of helping tissue cells fight invading organisms and substances. Certainly much more work remains to be done in order to clarify such interactions aiding in the cellular expression of inflammatory responses.

Nevertheless, there are now many pieces of evidence supporting the notion that the nongenomic inflammatory pathway of the ligand-activated AHR does indeed exist, and that activation of this pathway significantly contributes to the expression of toxicities of TCDD. In view of the apparent inability of the classical genomic action model to explain all of the major toxic actions of TCDD, this appears to be an ideal moment for us as toxicologists to re-address this very important topic from a fresh perspective.

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REFERENCES

[1] Poland A, Knutson J, Glover E. Studies on the mechanism of action of halogenated aromatic hydrocarbons. Clin Physiol Biochem 1985;3(2–3):147–54 [review].
[2] Whitlock Jr JP. Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 1999;39:103–25 [review].
[3] Poland A, Kimbrough RD. Cold Spring Harbor Laboratory. Biological mechanisms of dioxin action. Banbury Report 18. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1984 . p. 441–4.
[4] Delescluse C, Lemaire G, de Sousa G, Rahmani R. Is CYP1A1 induction always related to AHR signaling pathway? Toxicology 2000;153:73–82.
[5] Albiger B, Dahlberg S, Henriques-Normark B, Normark S. Role of the innate immune system in host defense against bacterial infections: focus on the Toll-like receptors. J Intern Med 2007;261:511–28.
[6] Koff JL, Shao MX, Ueki IF, Nadel JA. Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium. Am J Physiol 2008;294:L1068–75.
[7] Vegeto E, Ghisletti S, Meda C, Etteri S, Belcredito S, Maggi A. Regulation of the lipopolysaccharide signal transduction pathway by 17beta-estradiol in macrophage cells. J Steroid Biochem Mol Biol 2004;91:59–66.
[8] Liu PT, Krutzik SR, Kim J, Modlin RL. Cutting edge: all-trans retinoic acid down-regulates TLR2 expression and function. J Immunol 2005;174:2467–70.
[9] Sun YV, Boverhof DR, Burgoon LD, Fielden MR, Zacharewski TR. Comparative analysis of dioxin response elements in human, mouse and rat genomic sequences. Nucleic Acids Res 2004;32(15):4512–23.
[10] Sutter TR, Guzman K, Dold KM, Greenlee WF. Targets for dioxin: genes for plasminogen activator inhibitor-2 and interleukin-1 beta. Science (New York NY) 1991;254:415–8.
[11] Vanden Heuvel JP, Clark GC, Kohn MC, Tritscher AM, Greenlee WF, Lucier GW, et al. Dioxin-responsive genes: examination of dose–response relationships using quantitative reverse transcriptase-polymerase chain reaction. Cancer Res 1994;54:62–8.
[12] Murray IA, Perdew GH. Omeprazole stimulates the induction of human insulin-like growth factor binding protein-1 through aryl hydrocarbon receptor activation. J Pharmacol Exp Ther 2008;324:1102–10.
[13] Bohonowych JE, Zhao B, Timme-Laragy A, Jung D, Di Giulio RT, Denison MS. Newspapers and newspaper ink contain agonists for the ah receptor. Toxicol Sci 2008;102(2):278–90.
[14] Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 2003;43:309–34.
[15] Matsumura F. On the significance of the role of cellular stress response reactions in the toxic actions of dioxin. Biochem Pharmacol 2003;66:527–40.
[16] Matsumura F, Vogel CF. Evidence supporting the hypothesis that one of the main functions of the aryl hydrocarbon receptor is mediation of cell stress responses. Biol Chem 2006;387:1189–94.
[17] Swift LL, Gasiewicz TA, Dunn GD, Soule PD, Neal RA. Characterization of the hyperlipidemia in guinea pigs induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol 1981;59:489–99.
[18] Cohen S. Origins of growth factors: NGF and EGF. Ann N Y Acad Sci 2004;1038:98–102.
[19] Madhukar BV, Brewster DW, Matsumura F. Effects of in vivo-administered 2,3,7,8-tetrachlorodibenzo-p-dioxin on receptor binding of epidermal growth factor in the hepatic plasma membrane of rat, guinea pig, mouse, and hamster. Proc Natl Acad Sci U S A 1984;81:7407–11.
[20] Madhukar BV, Ebner K, Matsumura F, Bombick DW, Brewster DW, Kawamoto T. 2,3,7,8-Tetrachlorodibenzo-p-dioxin causes an increase in protein kinases associated with epidermal growth factor receptor in the hepatic plasma membrane. J Biochem Toxicol 1988;3:261–77.
[21] Brewster DW, Matsumura F. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) reduces lipoprotein lipase activity in the adipose tissue of the guinea pig. Biochem Biophys Res Commun 1984;122:810–7.
[22] Matsumura F, Brewster DW, Madhukar BV, Bombick DW. Alteration of rat hepatic plasma membrane functions by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Arch Environ Contam Toxicol 1984;13:509–15.
[23] Bombick DW, Jankun J, Tullis K, Matsumura F. 2,3,7,8-Tetrachlorodibenzo-p-dioxin causes increases in expression of c-erb-A and levels of protein-tyrosine kinases in selected tissues of responsive mouse strains. Proc Natl Acad Sci U S A 1988;85:4128–32.
[24] Bombick DW, Madhukar BV, Brewster DW, Matsumura F. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) causes increases in protein kinases particularly protein kinase C in the hepatic plasma membrane of the rat and the guinea pig. Biochem Biophys Res Commun 1985;127: 296–302.
[25] Bombick DW, Matsumura F. 2,3,7,8-Tetrachlorodibenzo-p-dioxin causes elevation of the levels of the protein tyrosine kinase pp60c-src. J Biochem Toxicol 1987;2:141–54.
[26] Matsumura F. How important is the protein phosphorylation pathway in the toxic expression of dioxin-type chemicals? Biochem Pharmacol 1994;48: 215–24.
[27] Bombick DW, Matsumura F. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) causes an increase in protein tyrosine kinase activities at an early stage of poisoning in vivo in rat hepatocyte membranes. Life Sci 1987;41:429–36.
[28] Enan E, Matsumura F. Identification of c-Src as the integral component of the cytosolic Ah receptor complex, transducing the signal of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) through the protein phosphorylation pathway. Biochem Pharmacol 1996;52:1599–612.
[29] Dunlap DY, Ikeda I, Nagashima H, Vogel CF, Matsumura F. Effects of src-deficiency on the expression of in vivo toxicity of TCDD in a strain of c-src knockout mice procured through six generations of backcrossings to C57BL/6 mice. Toxicology 2002;172:125–41.
[30] Dunlap DY, Matsumura F. Analysis of difference in vivo effects of TCDD between c-src +/+ mice, c-src deficient, −/+ and −/− B6, 129-Src(tm 1 sor) mice and their wild-type littermates. Chemosphere 2000;40:1241–6.
[31] Boots AW, Haenen GR, Bast A. Health effects of quercetin: from antioxidant to nutraceutical. Eur J Pharmacol 2008;585:325–37.
[32] Dunlap DY, Moreno-Aliaga MJ, Wu Z, Matsumura F. Differential toxicities of TCDD in vivo among normal, c-src knockout, geldanamycin- and quercetin-treated mice. Toxicology 1999;135:95–107.
[33] Kitamura N, Wong P, Matsumura F. Mechanistic investigation on the cause for reduced toxicity of TCDD in wa-1 homozygous TGFalpha mutant strain of mice as compared its matching wild-type counterpart, C57BL/6J mice. J Biochem Mol Toxicol 2006;20:151–8.

[34] Dong B, Matsumura F. Roles of cytosolic phospholipase A2 and Src kinase in the early action of 2,3,7,8-tetrachlorodibenzo-p-dioxin through a nongenomic pathway in MCF10A cells. Mol Pharmacol 2008;74:255–63.
[35] Kohn MC. Biochemical mechanisms and cancer risk assessment models for dioxin. Toxicology 1995;102:133–8.
[36] Lin FH, Clark G, Birnbaum LS, Lucier GW, Goldstein JA. Influence of the Ah locus on the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic epidermal growth factor receptor. Mol Pharmacol 1991;39:307–13.
[37] Kohle C, Gschaidmeier H, Lauth D, Topell S, Zitzer H, Bock KW. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-mediated membrane translocation of c-Src protein kinase in liver WB-F344 cells. Arch Toxicol 1999;73:152–8.
[38] el-Sabeawy F, Wang S, Overstreet J, Miller M, Lasley B, Enan E. Treatment of rats during pubertal development with 2,3,7,8-tetrachlorodibenzo-p-dioxin alters both signaling kinase activities and epidermal growth factor receptor binding in the testis and the motility and acrosomal reaction of sperm. Toxicol Appl Pharmacol 1998;150:427–42.
[39] Vogel C, Boerboom AM, Baechle C, El-Bahay C, Kahl R, Degen GH, et al. Regulation of prostaglandin endoperoxide H synthase-2 induction by dioxin in rat hepatocytes: possible c-Src-mediated pathway. Carcinogenesis 2000;21:2267–74.
[40] Liu X, Jefcoate C. 2,3,7,8-tetrachlorodibenzo-p-dioxin and epidermal growth factor cooperatively suppress peroxisome proliferator-activated receptor-gamma1 stimulation and restore focal adhesion complexes during adipogenesis: selective contributions of Src, Rho, and Erk distinguish these overlapping processes in C3H10T1/2 cells. Mol Pharmacol 2006;70:1902–15.
[41] Puga A, Nebert DW, Carrier F. Dioxin induces expression of c-fos and c-jun proto-oncogenes and a large increase in transcription factor AP-1. DNA Cell Biol 1992;11:269–81.
[42] Hanneman WH, Legare ME, Barhoumi R, Burghardt RC, Safe S, Tiffany-Castiglioni E. Stimulation of calcium uptake in cultured rat hippocampal neurons by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicology 1996;112:19–28.
[43] Tannheimer SL, Barton SL, Ethier SP, Burchiel SW. Carcinogenic polycyclic aromatic hydrocarbons increase intracellular Ca2+ and cell proliferation in primary human mammary epithelial cells. Carcinogenesis 1997;18:1177–82.
[44] Nebert DW, Puga A, Vasiliou V. Role of the Ah receptor and the dioxin-inducible [Ah] gene battery in toxicity, cancer, and signal transduction. Ann N Y Acad Sci 1993;685:624–40.
[45] Mazina O, Park S, Sano H, Wong P, Matsumura F. Studies on the mechanism of rapid activation of protein tyrosine phosphorylation activities, particularly c-Src kinase, by TCDD in MCF10A. J Biochem Mol Toxicol 2004;18:313–21.
[46] Park S, Dong B, Matsumura F. Rapid activation of c-Src kinase by dioxin is mediated by the Cdc37-HSP90 complex as part of Ah receptor signaling in MCF10A cells. Biochemistry 2007;46:899–908.
[47] Park S, Matsumura F. Characterization of anti-apoptotic action of TCDD as a defensive cellular stress response reaction against the cell damaging action of ultra-violet irradiation in an immortalized normal human mammary epithelial cell line, MCF10A. Toxicology 2006;217:139–46.
[48] Park S, Mazina O, Kitagawa A, Wong P, Matsumura F. TCDD causes suppression of growth and differentiation of MCF10A, human mammary epithelial cells by interfering with their insulin receptor signaling through c-Src kinase and ERK activation. J Biochem Mol Toxicol 2004;18:322–31.
[49] Wegener E, Krappmann D. Dynamic protein complexes regulate NF-kappaB signaling. Handb Exp Pharmacol 2008;186:237–59 [review].
[50] Gouon-Evans V, Rothenberg ME, Pollard JW. Postnatal mammary gland development requires macrophages and eosinophils. Development 2000;127(11):2269–82.
[51] Gordon S. The macrophage: past, present and future. Eur J Immunol 2007;37(Suppl. 1):S9–17 [review].
[52] Moonis M, Ahmad I, Bachhawat BK. Macrophages in host defence—an overview. Indian J Biochem Biophys 1992;29(2):115–22 [review].
[53] Vogel CF, Sciullo E, Matsumura F. Activation of inflammatory mediators and potential role of ah-receptor ligands in foam cell formation. Cardiovasc Toxicol 2004;4:363–73.
[54] Sciullo EM, Vogel FA, Li W. Initial and extended inflammatory messages of the nongenomic signaling pathway of the TCDD-activated Ah receptor in U937 macrophages. Arch Biochem Biophys 2008;480(2):143–55.
[55] MacDougald OA, Lane MD. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 1995;64:345–73.
[56] Lane MD, Tang QQ. From multipotent stem cell to adipocyte. Birth Defects Res 2005;73:476–7.
[57] Enan E, Matsumura F. 2,3,7,8-Tetrachlorodibenzo-P-dioxin induced alterations in protein phosphorylation in guinea pig adipose tissue. J Biochem Toxicol 1993;8:89–99.
[58] Enan E, Matsumura F. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-induced changes in glucose transporting activity in guinea pigs, mice, and rats in vivo and in vitro. J Biochem Toxicol 1994;9:97–106.
[59] Phillips M, Enan E, Liu PC, Matsumura F. Inhibition of 3T3-L1 adipose differentiation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Cell Sci 1995;108(Part 1):395–402.
[60] Liu PC, Phillips MA, Matsumura F. Alteration by 2,3,7,8-Tetrachlorodibenzo-p-dioxin of CCAAT/enhancer binding protein correlates with suppression of adipocyte differentiation in 3T3-L1 cells. Mol Pharmacol 1996;49:989–97.
[61] Brodie AE, Manning VA, Hu CY. Inhibitors of preadipocyte differentiation induce COUP-TF binding to a PPAR/RXR binding sequence. Biochem Biophys Res Commun 1996;228:655–61.
[62] Liu PC, Matsumura F. TCDD suppresses insulin-responsive glucose transporter (GLUT-4) gene expression through C/EBP nuclear transcription factors in 3T3-L1 adipocytes. J Biochem Mol Toxicol 2006;20:79–87.
[63] Alexander DL, Ganem LG, Fernandez-Salguero P, Gonzalez F, Jefcoate CR. Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. J Cell Sci 1998;111(Part 22):3311–22.
[64] Cho YC, Jefcoate CR. PPARgamma1 synthesis and adipogenesis in C3H10T1/2 cells depends on S-phase progression, but does not require mitotic clonal expansion. J Cell Biochem 2004;91:336–53.
[65] Vogel CF, Matsumura F. Interaction of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with induced adipocyte differentiation in mouse embryonic fibroblasts (MEFs) involves tyrosine kinase c-Src. Biochem Pharmacol 2003;66:1231–44.
[66] Vogel CF, Sciullo E, Park S, Liedtke C, Trautwein C, Matsumura F. Dioxin increases C/EBPbeta transcription by activating cAMP/protein kinase A. J Biol Chem 2004;279:8886–94.
[67] Enan E, Matsumura F. Significance of TCDD-induced changes in protein phosphorylation in the adipocyte of male guinea pigs. J Biochem Toxicol 1994;9:159–70.
[68] Kern PA, Dicker-Brown A, Said ST, Kennedy R, Fonseca VA. The stimulation of tumor necrosis factor and inhibition of glucose transport and lipoprotein lipase in adipose cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Metabolism 2002;51:65–8.

[69] Liu PC, Moreno-Aliaga MJ, Dunlap DY, Hu XM, Denison MS, Matsumura F. Correlation between the high expression of C/EBPbeta protein in F442A cells and their relative resistance to antiadipogenic action of TCDD in comparison to 3T3-L1 cells. J Biochem Mol Toxicol 2002;16:70–83.
[70] Li W, Vogel CF, Matsumura F. Studies on the cell treatment conditions to elicit lipolytic responses from 3T3-L1 adipocytes to TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Cell Biochem 2007;102:389–402.
[71] Li W, Vogel CF, Fujiyoshi P, Matsumura F. Development of a human adipocyte model derived from human mesenchymal stem cells (hMSC) as a tool for toxicological studies on the action of TCDD. Biol Chem 2008;389:169–77.
[72] Canga L, Paroli L, Blanck TJ, Silver RB, Rifkind AB. 2,3,7,8-tetrachlorodibenzo-p-dioxin increases cardiac myocyte intracellular calcium and progressively impairs ventricular contractile responses to isoproterenol and to calcium in chick embryo hearts. Mol Pharmacol 1993;44:1142–51.
[73] Azenabor AA, Chaudhry AU. 17 beta-estradiol induces L-type Ca2+ channel activation and regulates redox function in macrophages. J Reprod Immunol 2003;59:17–28.
[74] Shertzer HG, Genter MB, Shen D, Nebert DW, Chen Y, Dalton TP. TCDD decreases ATP levels and increases reactive oxygen production through changes in mitochondrial F(0)F(1)-ATP synthase and ubiquinone. Toxicol Appl Pharmacol 2006;217:363–74.
[75] Vogel CF, Nishimura N, Sciullo E, Wong P, Li W, Matsumura F. Modulation of the chemokines KC and MCP-1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice. Arch Biochem Biophys 2007;461:169–75.
[76] Coleman KM, Smith CL. Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. Front Biosci 2001;6:D1379–91.
[77] Mihailidou AS. Nongenomic actions of aldosterone: physiological or pathophysiological role? Steroids 2006;71:277–80.
[78] Schafer MW, Madhukar BV, Swanson HI, Tullis K, Denison MS. Protein kinase C is not involved in Ah receptor transformation and DNA binding. Arch Biochem Biophys 1993;307:267–71.
[79] Backlund M, Ingelman-Sundberg M. Regulation of aryl hydrocarbon receptor signal transduction by protein tyrosine kinases. Cell Signal 2005;17:39–48.
[80] Lange CA. Making sense of cross-talk between steroid hormone receptors and intracellular signaling pathways: who will have the last word? Mol Endocrinol 2004;18: 269–78.
[81] Cabanski M, Steinmuller M, Marsh LM, Surdziel E, Seeger W, Lohmeyer J. PKR regulates TLR2/TLR4-dependent signaling in murine alveolar macrophages. Am J Respir Cell Mol Biol 2008;38:26–31.
[82] Silva CM, Shupnik MA. Integration of steroid and growth factor pathways in breast cancer: focus on signal transducers and activators of transcription and their potential role in resistance. Mol Endocrinol 2007;21(7):1499–512 [review].
[83] DePetrillo PB, Kurl RN. Stimulation of protein kinase C by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rat thymocytes. Toxicol Lett 1993;69:31–6.
[84] Kramer CM, Johnson KW, Dooley RK, Holsapple MP. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) enhances antibody production and protein kinase activity in murine B cells. Biochem Biophys Res Commun 1987;145:25–33.
[85] Weber TJ, Ou X, Merchant M, Wang X, Safe SH, Ramos KS. Biphasic modulation of protein kinase C (PKC) activity by polychlorinated dibenzo-p-dioxins (PCDDs) in serum-deprived rat aortic smooth muscle cells. J Biochem Toxicol 1994;9:113–20.
[86] Gijon MA, Spencer DM, Siddiqi AR, Bonventre JV, Leslie CC. Cytosolic phospholipase A2 is required for macrophage arachidonic acid release by agonists that Do and Do not mobilize calcium. Novel role of mitogen-activated protein kinase pathways in cytosolic phospholipase A2 regulation. J Biol Chem 2000;275:20146–5.
[87] D'Orazi G, Sciulli MG, Di Stefano V, Riccioni S, Frattini M, Falcioni R, et al. Hmeodomain-interacting protein kinase-2 restrains cytosolic phospholipase A2-dependent prostaglandin E2 generation in human colorectal cancer cells. Clin Cancer Res 2006;12(3, Part 1):735–41.
[88] Wu T, Levine SJ, Lawrence MG, Logun C, Angus CW, Shelhamer JH. Interferon-gamma induces the synthesis and activation of cytosolic phospholipase A2. J Clin Invest 1994;93:571–7.
[89] Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 2004;64(19):7022–9.
[90] Laiakis EC, Baulch JE, Morgan WF. Interleukin 8 exhibits a pro-mitogenic and pro-survival role in radiation induced genomically unstable cells. Mutat Res 2008;640:74–81.
[91] Christian BJ, Inhorn SL, Peterson RE. Relationship of the wasting syndrome to lethality in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol 1986;82:239–55.
[92] Taylor MJ, Lucier GW, Mahler JF, Thompson M, Lockhart AC, Clark GC. Inhibition of acute TCDD toxicity by treatment with anti-tumor necrosis factor antibody or dexamethasone. Toxicol Appl Pharmacol 1992;117:126–32.
[93] Ishii Y, Ishida T, Mutoh J, Yamada H, Oguri K. Possible candidates for the compound which is expected to attenuate dioxin toxicity. Fukuoka Igaku Zasshi 2005;96:204–13.
[94] Ebner K, Brewster DW, Matsumura F. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on serum insulin and glucose levels in the rabbit. J Environ Sci Health Part 1988;23: 427–38.
[95] Consonni D, Pesatori AC, Zocchetti C, Sindaco R, D'Oro LC, Rubagotti M, et al. Mortality in a population exposed to dioxin after the Seveso, Italy, accident in 1976: 25 years of follow-up. Am J Epidemiol 2008;167:847–58.
[96] Michalek JE, Pavuk M. Diabetes and cancer in veterans of Operation Ranch Hand after adjustment for calendar period, days of spraying, and time spent in Southeast Asia. J Occup Environ Med 2008;50:330–40.
[97] Fujiyoshi PT, Michalek JE, Matsumura F. Molecular epidemiologic evidence for diabetogenic effects of dioxin exposure in U.S. Air force veterans of the Vietnam War. Environ Health Perspect 2006;114:1677–83.
[98] Nishimura N, Matsumura F, Vogel CF, Nishimura H, Yonemoto J, Yoshioka W, et al. Critical role of cyclooxygenase-2 activation in pathogenesis of hydronephrosis caused by lactational exposure of mice to dioxin. Toxicol Appl Pharmacol 2008;231(3): 374–83.
[99] National Toxicology Program (U.S.), United States. Department of Health and Human Services, United States. Public Health Service, National Institutes of Health (U.S.). NTP technical report on the toxicology and carcinogenesis studies of a mixture of 2,3,7,8-tetrachlorodibenzo-rho-dioxin (TCDD) (CAS no. 1746-01-6), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (CAS no. 57117-31-4), and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) (CAS no. 57465-28-8) in female Harlan Sprague-Dawley rats (gavage studies). Research Triangle Park, N.C.: National Toxicology

Program, National Institutes of Health Public Health Service U.S., Department of Health and Human Services; 2006.
[100] Beutler B. Cachectin in tissue injury, shock, and related states. Crit Care Clin 1989;5:353–67.
[101] Weiss U. (editor). Inflammation. Nature insight. Nature 2008;454(7203):427–77 [reviews].
[102] Beischlag TV, Luis Morales J, Hollingshead BD, Perdew GH. The aryl hydrocarbon receptor complex and the control of gene expression. Crit Rev Eukaryot Gene Expr 2008;18:207–50.
[103] Pande K, Moran SM, Bradfield CA. Aspects of dioxin toxicity are mediated by interleukin 1-like cytokines. Mol Pharmacol 2005;67:1393–8.