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COMMENTARY

HOW IMPORTANT IS THE PROTEIN PHOSPHORYLATION PATHWAY IN THE TOXIC EXPRESSION OF DIOXIN-TYPE CHEMICALS?

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Dioxin-type chemicals include polyhalogenated dibenzo-*p*-dioxins, dibenzofurans, polychlorinated biphenyls, naphthalenes, and polyaromatic hydrocarbons, all of which possess toxicologically very similar action properties [1–3]. There have been very intensive studies on this type of chemical, as attested to by the fact that the most recent Dioxin '92 conference in North Carolina attracted 448 platform and poster presentations. The most likely reasons that so many scientists are interested in studying the mode of action of this group of chemicals are: (a) some of them are very toxic, (b) most of them are real world pollutants, (c) they cause all sorts of toxic symptoms in many different vertebrate species, and (d) they are good molecular probes to study basic cellular mechanisms of toxic interactions. Particularly popular among those scientists interested in the last aspect (d) is the use of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD†) as a molecular probe. Their interest in TCDD is understandable, since it is the most toxic congener of all dioxin-type chemicals and is very metabolically stable, itself being the acknowledged toxicant. Furthermore, a chemically very pure isomer is now available and, most importantly, it appears to represent the action mechanism of the entire range of toxic polyhalogenated aromatics and polyaromatic hydrocarbons.

Despite the fact that TCDD alone is capable of causing a wide variety of toxic symptoms in many animal species, there is a near-unanimous consensus on its initial action site, i.e. its binding to the cytosolic Ah-receptor [3–5]. Upon TCDD binding, this cytosolic Ah-receptor detaches itself from the heat shock protein complex, and travels into the nucleus. In the case of cytochrome P4501A1 induction, the ligand-bound receptor is known to form a complex with another helix-loop-helix protein, called "arnt," in the nucleus and eventually interacts with the promoter DNA regions of specific genes [6–8]. It has been demonstrated clearly, in the results of many scientists, that all of the active congeners belonging to the family of "dioxin-type" chemicals show significant affinities to the Ah-receptor, and that their interactions to this cytosolic receptor represent the essential first step in expressing their toxic actions [9]. For instance, mouse strains possessing Ah-receptor with low affinities to TCDD show uniformly low susceptibilities to all of these active dioxin-type chemicals in terms of *in vivo* toxic responses, such as thymic involution and lethality, as compared with mouse strains known to possess similar Ah-receptors with higher TCDD affinities [9, 10].

The most common action of all dioxin-type chemicals is their "inducing action" on xenobiotic-metabolizing enzymes in various tissues, particularly in the liver [11]. Among them, the most studied induction phenomenon occurs on cytochrome P4501A1 (CYP1A1) and to a lesser extent 1A2 [12–14]. These CYP1A1 genes have been shown to possess a specific DNA sequence, 5′-GCGTG-3′, often termed "XRE" or "DRE" (xenobiotic-or dioxin-responsive element, respectively), to which the ligand-bound Ah-receptor–"arnt" complex is capable of binding, thereby causing the activation of their expression to produce more mRNAs for CYP1A1 protein synthesis. Again, there is unanimous acceptance for such an action pattern of dioxin-type chemicals.

Is induction of cytochrome P450s related to other toxic actions of dioxin-type chemicals?

If everybody seems to agree that the main course

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† Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CYP1A1, cytochrome P4501A1; XRE, xenobiotic-responsive element; HLH helix-loop-helix type transcription factor; EGF and EGFR, epidermal growth factor and its receptor; CRE, cyclic AMP responsive element; GRE, glucocorticoid hormone responsive element; TCDF, tetrachlorodibenzofuran; DCDD, dichlorodibenzo-*p*-dioxin; IP_3 and IP_4, inositol tri- and tetraphosphate; HSP90, heat shock protein 90; GR, glucocorticoid receptor; ERE, estrogen responsive element; SRC, Rous sarcoma gene product (60 kDa protein tyrosine kinase); RAS, 21 kDa G-protein; and IE genes, immediate early genes (="primary response" genes).

of action of TCDD eventually activates cytochrome P450 types of genes with XREs (e.g. CYP1A1) through the Ah-receptor, why is there a question on other types of toxic action mechanisms? While there could be many reasons why several scientists are raising such a question, one of the main reasons must be the lack of a direct correlation between the above liver microsomal induction phenomenon and the lethal actions of TCDD among various experimental animal species *in vivo*. For instance, such an induction phenomenon is hardly noticeable in the most TCDD-susceptible species, guinea pigs (LD_{50} = 0.6 μg/kg in males) [15], whereas it is very marked in hamsters, the species regarded as the most tolerant in terms of lethality (LD_{50} 1000 → 3000 μg/kg). Since TCDD is a very stable chemical which is not degraded readily by monooxygenases, this high induction response of hamsters is not likely to be providing this species any extra advantage. If such is the case, rats, which also show extreme induction sensitivity and responsiveness, should be the most tolerant species, rather than being a relatively sensitive species to TCDD (LD_{50} = 25 μg/kg).

Another major reason why people suspect the presence of other types of action mechanisms is the qualitative difference between induction of liver detoxification enzymes and several of the major TCDD-specific toxic expressions, such as symptoms of vitamin A deficiency, hyperthyroidism, hyperlipidemia, hypoinsulinemia, wasting syndrome, thymic involution, reduced immunocompetency, and hyperkeratinization. It is very hard to attribute all of these toxic expressions solely to the TCDD-caused rise in CYP1A1 expression. Nor is it realistic to expect that these are the secondary or tertiary responses of the tissues to induction. On the other hand, inasmuch as almost all of those events are the eventual results of the initial interaction of TCDD with the cytosolic form of the Ah-receptor, one could argue that the induction phenomenon represents a typical example of the way TCDD acts, i.e. TCDD-bound Ah-receptor/"arnt" complex activating various genes in the nucleus.

The scheme regarded more likely by most scientists is, therefore, scenario A (Fig. 1). This scheme also agrees well with the generally held view that this is the way pleiotropism proceeds after the action of hormones, such as steroid and thyroid hormones, to transactivate their responsive genes. Thus, most scientists would accept that the action pattern of TCDD on CYP1A1 is a good molecular model and that by elucidating the precise mechanisms involved in these Ah-receptor–DNA interactions, the true mechanism of toxic action of this class of chemicals would be elucidated eventually. It must be made absolutely clear at this point that the work done in this line of approach has been very solid and fruitful, and that as the result of hard work of many scientists employing modern techniques and creativity, a number of superb contributions have been made in this line of research, which have revealed many eye-opening, hitherto unknown toxicological molecular mechanisms, such as the discovery of Ah-receptor [1–3], arnt [16], identification of the HLH configuration [17], XREs [7, 8, 18], and the composition of an XRE interacting form of Ah-receptor heteropolymer [16], to name a few. What I am trying to point out

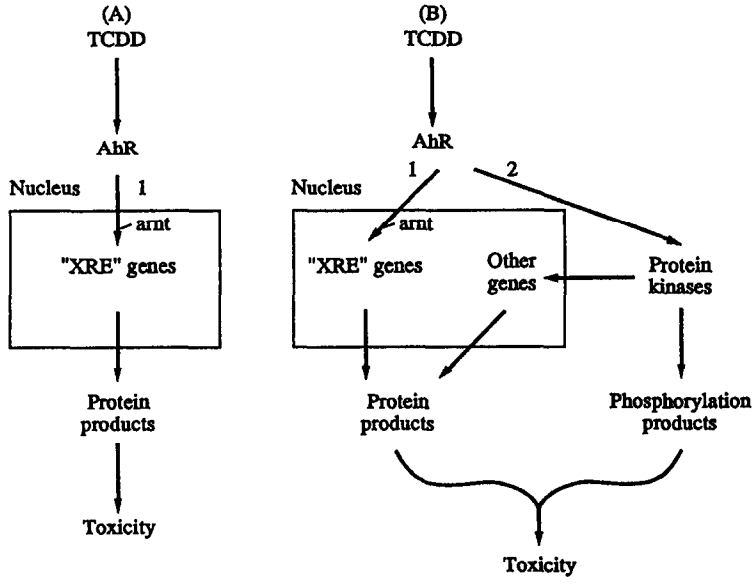

Fig. 1. Schematic diagram illustrating the conventionally accepted route of toxic action of dioxin-type chemicals (A) and the newly proposed action pathways in this paper (B). The major difference between these two is the presence of the second pathway marked as pathway 2 in scheme B. This new pathway, termed the "protein phosphorylation pathway," is mediated by the Ah-receptor, but not by "arnt" or "XRE" (i.e. pathway 1). It must be noted that both pathways could eventually affect transcriptional processes in the nucleus; therefore, in this paper the conventional pathway (1) is termed the "XRE" pathway.

in this commentary is the possibility of the presence of some *additional* routes of action for this type of chemical, rather than questioning any of the above work to date.

Activation of various protein kinases and down-regulation of epidermal growth factor receptor (EGFR) by TCDD

In 1984, we reported that TCDD given to rats *in vivo* causes a spectacular rise in cAMP-independent and -dependent protein kinases in the hepatic plasma membrane. At a dose of 25 μg/kg (i.p. single injection), TCDD caused increases of 267 and 374% of control values of the above kinases, respectively. Such an activation of kinases was found to be accompanied by down-regulation of a number of enzymes and receptors bound to the plasma membrane of hepatocytes. Particularly affected was the EGFR [19–22]. At about the same time, two other groups found that TCDD also causes down-regulation of EGFR in cultured cells *in vitro* [23, 24]. At that stage the roles and the classification of protein kinases were not well understood. Therefore, the attention of our research group, as well as that of others, was focussed on EGFR, which is a definable entity with known cell regulatory roles [25]. In brief, all subsequent experimental results have confirmed that TCDD causes *in vivo* [20, 21] and *in vitro* [22–24] down-regulation of this receptor in a variety of tissues and cell types, and that this phenomenon is clearly Ah-receptor-mediated, as judged by the differential responses by low and high affinity Ah-receptor mouse strains as well as the structure–activity relationships shown by various analogs of dioxin-type chemicals. Furthermore, several similarities between the actions of EGF and TCDD *in vivo* were also noted, e.g. early eye opening and incisor eruption, fatty liver, skin cell proliferation and hyperkeratinization, and the existence of a good correlation between *in vivo* susceptibilities of animal species and their sensitivities to show EGFR down-regulation to TCDD [21, 26, 27].

The weaknesses of the theory of EGFR-related toxicity in satisfactorily explaining all of the action mechanisms of TCDD are: (a) there are types of cells that do not express EGFR, but are capable of responding to TCDD, (b) TCDD is slow to cause down-regulation of EGFR, which in the case of rats typically starts on day 3 and fully develops only by day 10, (c) in some tissues, the effect of EGF on cellular responses is not identical to TCDD, and (d) there is no logical connection between the action of TCDD and EGFR down-regulation. While the presence of such questions is distracting, the important messages of TCDD's action in causing EGFR down-regulation should not be ignored. They are: first, it is definitely mediated by the Ah-receptor [6, 20, 21, 28]; second, it is always accompanied by a rise of protein tyrosine kinases [29] as expected from its ligand-dependent (EGF) activation of the signal transduction pathway; and third, many types of affected cells behave as though they have received a growth-factor and are in the process of activating signal transduction.

It is now well established that the initial activation of EGFR by its ligands involves the activation of its own protein tyrosine kinase [25, 30, 31]. Subsequent to such receptor activation, EGFR is down-regulated by protein kinase C via phosphorylation on the threonine residue located near the transmembrane region of the receptor [31]. This general sequence of events also has been shown to take place in the case of TCDD-triggered changes in protein kinase activities, i.e. the initial type of protein kinases induced by TCDD appears to be protein tyrosine kinases followed by protein kinase C [32]. One protein tyrosine kinase that we studied initially in 1987 was the c-*src* product SRC (sometimes called $pp60^{src}$) [33]. This cellular oncogene in guinea pig hepatocytes was found to be quite responsive to TCDD, and the effect was long-lasting, e.g. the effect of a single dose of 1 μg/kg TCDD in this regard was clearly visible even after 28 days. In addition, in the same study it was shown that both wild-type and v-*src* transfected NIH 3T3 fibroblast cell lines responded to *in vitro* administered TCDD by increasing the titer of $pp60^{src}$ proteins. The *src* family of protein tyrosine kinases are known to be involved somehow in the signal transduction pathways of growth factors. Two likely possibilities are: (a) they act as the triggering protein tyrosine kinase for those growth factor receptors lacking their own built-in kinase, and (b) they act as a part of the tyrosine phosphorylation cascade [34, 35].

One key member of the growth factor signal transduction pathway is the *ras* family of G proteins (Fig. 2). For them to act as transducers, it is essential that they bind to GTP upon the arrival of the growth factor signal. At the same time the intracellular levels of RAS proteins, $pp21^{ras}$, are usually raised in cells engaged in active growth factor signal transduction. In subsequent studies we demonstrated that TCDD indeed causes a rise in RAS protein titers and GTP binding activities [36–38]. Thus, it is clear from these studies that TCDD eventually causes the activation of the growth factor signal transduction pathway and that such a cellular response is induced by an Ah-receptor-mediated process.

Furthermore, there is now other evidence that such a growth factor signal transduction message is transmitted to nuclear transcriptional factors. For instance, it has been shown recently by Ma and Babish [39] that TCDD causes a rise in tyrosine phosphorylation of murine hepatic p34 (cdc2) kinase. The same research group had also shown earlier that protein tyrosine phosphorylation is a sensitive and reliable biochemical indicator of the exposure of animals to TCDD [40]. Numerous studies have demonstrated that $p34^{cdc2}$ or its homologs are indispensable mediators of cell cycle events that are regulated at the G_2M and probably G_1/S transition phase [41, 42]. It is noteworthy that activation of c-*src* protein tyrosine kinase is also intimately associated in cdc2 activation and mitosis [43].

The growth factor signal transduction pathway itself induces mitogenic responses in cells by directly activating "primary response genes" through a series of protein phosphorylations and dephosphorylations of cytosolic and eventually nuclear proteins. This transduction message eventually results in

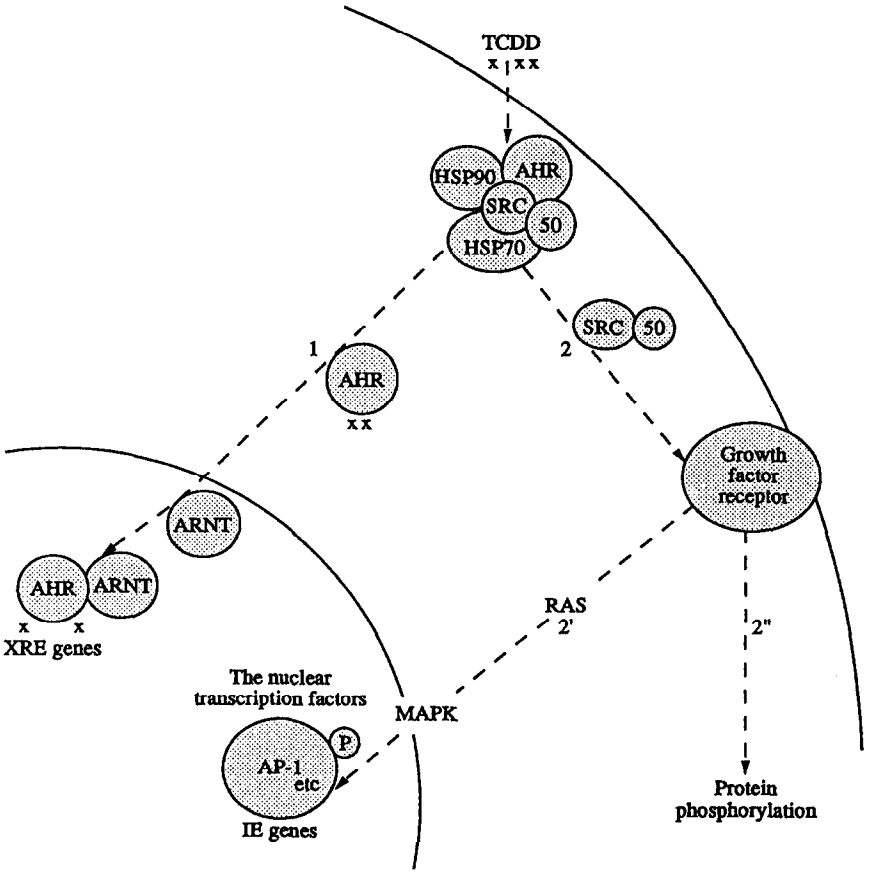

Fig. 2. Details of the hypothetical scheme of the TCDD-activated "protein phosphorylation pathway." TCDD upon entering into the cell through the plasma membrane (outer circle) finds the Ah-receptor (AHR) complex consisting of heat shock protein (HSP) 90, 70, and 50, a protein kinase, SRC and AHR. The composition of this complex is by no means certain at this stage, with the exception of AHR and HSP90. After the binding reaction, the Ah-receptor with TCDD leaves the complex and moves into the nucleus (inner circle) with the aid of a second transcription factor, arnt (pathway 1). At the same time, this departure of the ligand bound AHR from the HSP complex frees up the SRC and thereby activates its own protein kinase activity (pathway 2), which acts as the trigger for the well defined "growth factor signal transduction" activities (pathway 2') through RAS protein and MAP-kinase (MAPK) activation to eventually phosphorylate nuclear transcription factors, such as AP-1. The latter factors, in turn, activate "immediate early" (IE) genes (= "primary response genes"). The activation of SRC in cytosol, at the same time, could result in direct phosphorylation actions on other important proteins in the cytosol, plasma membrane (e.g. growth factor receptors) and other intracellular organelles (pathway 2'') to cause their functional changes.

phosphorylational changes on nuclear transcriptional factors that are capable of directly reacting with primary response genes (sometimes designated as "immediate early genes" or IE genes) through AP-1- and AP-2-responsive, CRE, MYC-responsive, and other responsive DNA sites on the promoter regions of these genes, thereby regulating their expressions [44]. Therefore, once it is activated, this pathway itself does not require any ligand-induced receptor interaction with DNA (e.g. "XRE" or "GRE" interactions for TCDD or glucocorticoid agonists) to modulate gene expression of c-*fos*, c-*jun*, *junD*, and c-*myc*. This point will become an important issue in the following section.

Recent controversy regarding the relationship between protein kinase C and functions of the Ah-receptor

Recently, Puga *et al.* [45] and Carrier *et al.* [46] have produced experimental evidence that TCDD induction of CYP1A1 mRNA, as well as CYP1A1 enzyme activity, is abolished by protein kinase C inhibitors, such as staurosporin and 2-aminopurine, in cultured Hepa-1 cells. They also showed that depletion of protein kinase C by prolonged treatment with phorbol ester leads to complete suppression of CYP1A1 mRNA. They concluded that: (1) protein kinase C is necessary for the formation of Ah-receptor complex for transcriptional activation of CYP1A1; (2) the site of phosphorylation is likely on the Ah-receptor; and (3) protein kinase C is responsible for this phosphorylation action. While phosphorylation on nuclear transcriptional factors is a very reasonable assumption, the work of Puga *et al.* [45] also revealed several unexplainable phenomena associated with the action sequence of TCDD. First, TCDD caused a rise in Ca^{2+} influx as

early as 2 min from the time of treatment, and second, this phenomenon could be observed even in a C4 cell line, which is known to be deficient in the nuclear complex factor "arnt" [47], or in C2 line, which has a very low level of Ah-receptor. Furthermore, according to Puga *et al.* [45], TCDD, as well as benzo[*a*]pyrene, increases the level of mRNAs for *junB*, *junD* and c-*fos* in C4 cells, as in Hepa-1 cells, indicating that the lack of "arnt" is not really affecting certain responses of C4 cells in the nucleus. It is likely, therefore, that "arnt" is not required for the induction of *jun* and *fos* gene expression in this cell line. Staurosporin was tested only on Hepa-1 cells, and it certainly inhibited the induction effect of TCDD on CYP1A1. Curiously enough, however, this inhibitor also totally abolished transcription of mRNAs, including c-*myc*, *junD* and *junB*, below the level of their background expression, but it did not affect that of β-actin. Their conclusion was: "Our experiments using receptorless and nuclear translocation-defective lines suggest that the induction process is independent of the Ah-receptor, but as indicated before, there are other possible explanations. For example, the Ah-receptor might not be involved in the earlier events (<1–2 hr) of the TCDD response, but might have a function at later times." It is possible that this TCDD-triggered early rise in Ca^{2+} influx is totally unrelated to the later "induction" events.

While one could argue for the possibility that staurosporin is a very powerful inhibitor affecting many transcriptional activities at this dose [48], including the available titer of Ah-receptor itself, we should not ignore the larger issue raised by these and other workers, i.e. is there a positive relationship between Ah-receptor and protein kinases in the course of action of TCDD? Otherwise, we will be baffled continuously by conflicting and unexplainable observations.

The central issues I must now raise are: (a) whether TCDD-induced changes in protein phosphorylation are absolutely Ah-receptor mediated, (b) if so, whether there is a way to logically explain some of these unusual observations, and (c) whether these protein phosphorylation changes cause serious toxicological consequences.

Proofs that significant portions of TCDD-induced protein phosphorylation changes are mediated by its interaction with Ah-receptor

It is noteworthy that in an earlier experiment [32] we clearly demonstrated, by using the TCDD-responsive C57BL/6J and the less responsive DBA/2J strain of mice, that the effects of TCDD *in vivo* on both $pp60^{src}$ and protein tyrosine kinase activities measured after 2 days were much more pronounced in the former strain than in the latter, which has been shown to have an Ah-receptor with a very low affinity. Thus, there is already an indication that at least some of the TCDD-induced protein kinase changes are clearly Ah-receptor dependent. The TCDD-induced increases in RAS proteins and their GTP binding activities are also known to be mediated by Ah-receptor [37], again based on the differential effect seen between responsive and less responsive mouse strains.

How about protein kinase C? Unfortunately, no published data have indicated that a critical examination has been made of the relationship of Ah-receptor to protein kinase C *per se* before these two publications from Nebert's laboratory [45, 46]. However, recent data shown by Weber *et al.* [49] indicate that protein kinase C in aortic smooth muscle cells in culture responds to TCDD, TCDF and DCDD in the order expected from their action on Ah-receptor. In our own laboratory we were able to demonstrate, using cultured adipose tissue from male guinea pigs, that TCDD causes a significant increase in protein kinase C 3 hr after its administration into the culture medium [50]; and this effect of TCDD is abolished by simultaneous treatment with 100 μM neomycin or 1 μM 4,7-phenanthroline [51]. The former is an agent known to specifically bind to phosphyatidylinositol bisphosphate (IP_2) [52, 53], at concentrations of less than 0.6 mM, thereby preventing active IP_3 and IP_4 from forming. This compound is also known to inhibit the release of internally stored Ca^{2+}, and so to prevent both phospholipase C and protein kinase C from functioning normally. Furthermore, such an action blocks the intracellular production of diacyl glycerol needed for protein kinase C activity. The latter compound has been shown to specifically block Ah-receptor so that TCDD could not attach to this receptor. All these data support the view that TCDD-induced changes in protein kinase C activity are also mediated by the Ah-receptor. It must be stressed again that it is not possible for a direct action of TCDD on protein kinase C proper (e.g. TCDD binding to this enzyme molecule) to cause all of these changes, as pointed out by Kramer *et al.* [54]. Therefore, the above conclusion that TCDD initially binds with the Ah-receptor, and that somehow this interaction leads to the eventual changes in protein phosphorylation activities, becomes a very important concept to establish at this stage to allow further pursuit of this topic. The proposed scheme of action sequences of protein phosphorylation activation following the initial binding of TCDD to Ah-receptor is shown in scenario B of Fig. 1.

Organization of cytosolic hormone receptor: A hint of the role of Ah-receptor as a protein kinase trigger

Thus far the key points of this investigation have been: (a) to establish that a significant part of TCDD-induced protein phosphorylation changes appears to be Ah-receptor-mediated, (b) to analyze the controversy on the subject of Ah-receptor functions in relation to protein kinases, and (c) to make an effort to explain these phenomena by using conventional theories.

Regarding the last point, however, it does not appear to be possible to satisfactorily explain the major discrepancies, such as why TCDD causes c-*fos*, *junB* and *junD* mRNA changes in the "arnt"-free cell line, how TCDD could cause a rapid change in Ca^{2+} influx within a few minutes of its addition to the medium, and how some protein kinase inhibitors could abolish most of the action of TCDD.

To obtain some clues to solve these puzzles, we have started to look for examples in other cytosolic

receptors. The best example we could find was the ligand-induced receptor activation of glucocorticoid receptors. Note, however, that there is a major difference between this type of receptor and the Ah-receptor. That is, the former belongs to an erb-A type zinc finger protein family and the latter is a helix-loop-helix type protein [17]. Therefore, one must be careful not to blindly regard these receptor systems as totally analogous. Another difference is the requirement of a nuclear helper protein, "arnt," for the Ah-receptor, but not the glucocorticoid receptor. On the other hand, there are two critical similarities that may be of great importance in this comparative study. One is the state of the cytosolic, quiescent form of these receptors complexed with heat shock proteins, and the other is their absolute requirement that their ligand-bound forms be translocated into the nucleus to eventually modulate gene expressions by interacting with specific hormone-responsive elements of DNA sequences at their promoter regions.

The fact that Ah-receptor in cytosol is stored in the form of a nonactive complex with heat shock protein 90 (HSP90) in mouse cells is well established [55]. The glucocorticoid receptor (GR) in the non-activated state is also known to be complexed with the same type of (HSP90) [56]. This appears to be the general pattern for these cytosolic hormone receptors, as the progesterone receptor complex also has been found to contain HSP90 as well as HSP70 proteins. Furthermore, this complex is formed and stabilized by the action of ATP and Mg^{2+} [57].

In addition, there appear to be other proteins that are closely associated with GR and HSP90 and 70. Perdew and Whitelaw [58] have identified three other proteins with molecular weights of 63, 56 and 50 kDa. It is generally acknowledged that HSP90 itself is the component that directly binds with the receptor, at the ligand binding region in the case of GR agonist, and that upon ligand binding the receptor subunit dissociates from the rest of the complex and travels into the nucleus.

The most intriguing aspect of the association of these heat shock proteins with the cytosolic hormone receptor is that the same complex appears to be capable of associating with the v-*src* product protein, $pp60^{v\text{-}src}$ [58]. In fact, this appears to be the way newly transcribed and translated $pp60^{v\text{-}src}$ is initially assembled and eventually inserted into the plasma membrane. There has been a question as to whether this is the way $pp60^{c\text{-}src}$, the native protooncogene counterpart of $pp60^{v\text{-}src}$, is also normally folded properly and transported into a plasma membrane location by the aid of HSP 90, 70, 50, etc. However, Hutchison *et al.* [59] were able recently to reconstruct the entire complex in a cell-free system with the aid of a rabbit reticulocyte lysate system and were successful in incorporating both $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$, indicating that the association of $pp60^{c\text{-}src}$ with the heat shock protein complex in normal cells is highly likely. Thus, the basic scenario appears to be that as soon as $pp60^{src}$ proteins are synthesized, they become associated with HSP90 and 50 with help from HSP70, which properly folds this newly synthesized protein kinase (see Fig. 2). They are associated more specifically with the 50 kDa protein, and during the translocation and insertion into the membrane site for myristylation, HSP70 again helps the process by acting as an unfoldase. $pp60^{src}$ Proteins are well known protein tyrosine kinases, capable of activating the growth factor signal transduction pathway [60] and altering cell cycle activities [43]. Thus, if one can assume that the departure of the receptor subunit, upon its ligand binding, from the heat shock protein complex could affect the conformation of HSP90 so as to block $pp60^{src}$ (or any other protein kinases associated with HSP90 for that matter), the increases in protein phosphorylation activities at the time of ligand binding begin to make sense. In this scheme, it is the act of TCDD binding to Ah-receptor, itself, and not the subsequent nuclear translocation, dimerization with "arnt" or binding to "XRE", that could activate the protein kinase that is originally associated with the heat shock protein complex. The idea that some heat shock proteins could play a facilitating role in modulating protein kinases as a part of the action pathway of TCDD has also been mentioned in a recent speech by Linda Birnbaum (U.S. Environmental Protection Agency).

To be sure, no one has shown, to our knowledge, that such a phenomenon takes place. However, there is some supporting evidence. It has been shown that Ah-receptor itself must be phosphorylated upon binding to TCDD before it enters into the nucleus to dimerize with "arnt." The data produced by Poellinger *et al.* [61] have demonstrated that dephosphorylated Ah-receptor does not bind well with "arnt" and is not able to interact with DNA. Therefore, it makes sense for the responding cell to coordinate the act of ligand binding and the phosphorylation activity. The above workers also have treated animals *in vivo* with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to down-regulate protein kinase C and showed that both DNA binding of the ligand-activated receptor and the function of "dioxin response element" (XRE) were inhibited, indicating a critical role of protein phosphorylation, in agreement with the conclusion of Puga *et al.* [45] and Carrier *et al.* [46].

It must be pointed out that at this stage I am not making a special effort to identify the protein kinase(s) whose activity could be triggered by the ligand binding action. Protein kinase C and $pp60^{src}$ are two main candidates, but, knowing that the activation of a protein kinase could induce a cascade of changes in other kinases, this subject must be considered later in a more meticulous fashion. For the time being, and for the sake of this investigation, I intend to emphasize the concept of the ligand binding event itself being the trigger of activation of protein kinases.

Analysis of hitherto unexplainable observations using this new viewpoint/hypothesis

The first phenomenon that becomes readily explainable by this hypothesis would be the *in vitro* action of TCDD on the C4 cell line to cause changes in Ca^{2+} uptake, which takes place within a few minutes, and the subsequent stimulation of AP-1 mRNA production in 2 hr [45]. C4 cells do possess a normal amount of Ah-receptor. Therefore, upon

receiving TCDD by Ah-receptor, according to this hypothesis, the heat shock protein-bound protein kinase must be activated. Since it does not require DNA interaction and subsequent *de novo* protein synthesis, this initial action of TCDD could occur within a few minutes. In comparison, Puga *et al.* [45] also tested the C2 line, which has a low titer of Ah-receptor with "arnt" system intact. The extent of stimulation of $^{45}Ca^{2+}$ uptake by the same treatment of TCDD was much less, as expected by this hypothesis. On the other hand, the stimulatory effect of TCDD on c-*fos* mRNA was high in C4 cells and insignificant in C2 cells. Again, assuming that this aspect of the action of TCDD is not carried out by the XRE-mediated transcriptional pathway but by the ligand-induced protein kinase activation pathway, the event becomes fully explainable.

Another phenomenon that can be explained from this viewpoint is the speed with which TCDD causes certain biochemical changes. For instance, in addition to the above case, we have found that the TCDD-induced rise in c-*ras* GTP-binding activities takes place within 15 min from the time of TCDD administration in isolated guinea pig adipose tissue in culture (Enan E and Matsumura F, unpublished data). Moreover, the rise in *junD* mRNA in Hepa 1 has been observed to take place within 15 min from the time of TCDD administration [45]. The quickest activation time of primary responsive genes (immediate early genes), which are not mediated through *de novo* protein synthesis, is approximately 15 min. Thus, any Ah-receptor-dependent changes occurring in less than this time span are not likely to be mediated through the action of this receptor on XRE-DNA to initiate *de novo* gene expression and protein synthesis.

By the same logic, the above interpretation also helps to explain why the level of *junD* mRNA rises within 15 min from the time of TCDD administration [45]. This particular gene for an AP-1 protein has been shown to possess a "CRE" (cAMP responsive element), unlike c-*jun* and *junB* [62], and is capable of responding to the changing protein phosphorylation activities in its environment. Again, 15 min is a short time period for *de novo* synthesis of proteins. In fact, in the experiment shown in their Fig. 2B [45], the stimulatory effect of cAMP on *junD* mRNA is clearly recognizable even in the presence of cycloheximide (a protein synthesis inhibitor); furthermore, TCDD also acted in the same way as cAMP in the presence of cycloheximide, indicating that this aspect of TCDD's action is not likely to require *de novo* synthesis of protein or the power of protein phosphorylation change to affect AP-1 protein actions on the expression of the primary responsive genes (IE genes).

Toxicological significance of the protein phosphorylation pathway; future research needs and summary

With regard to the importance of TCDD-induced changes in protein phosphorylation activities in the toxic expression of this compound, it has been shown by Bombick and Matsumura [33] that quercetin, an inhibitor of protein kinases, can prevent the action of TCDD *in vivo* and counteract thymic involution in a TCDD-responsive strain (C57) of mice. That TCDD-induced thymic involution is mediated by Ah-receptor in this strain is well documented [1, 10]. Therefore, the involvement of some Ah-receptor-mediated protein phosphorylation process that is sensitive to quercetin in the process of the development of this toxic lesion is likely. Although the specificity of this bioflavonoid on protein tyrosine kinases as opposed to protein kinase C [63] is not as specific as previously thought, the above observation must be considered along with the evidence of antagonistic effects of other protein kinase inhibitors such as staurosporin [45], 2-aminopurine [46], neomycin and genistein,* indicating a close association of protein kinase activities and Ah-receptor-mediated processes or toxic end-results. On the other hand, it must be pointed out that the use of protein kinase inhibitors has one shortcoming at this stage, i.e. as long as we do not know the exact identity of the protein kinase that phosphorylates Ah-receptor itself, we cannot distinguish inhibition of the kinase that affects nuclear translocation and "XRE" binding from those directly affecting other target proteins involved in important cellular functions relevant to the toxic expression of the effect of TCDD. However, there are two lines of evidence to indicate that the former route is not the only route of action. One is the fact that some specific inhibitors, such as genistein, which is well known to affect protein tyrosine kinases but not protein kinase C, block some of the toxic action of TCDD. Another line of evidence is the action of TCDD on *junD* mRNA in C4 cells, which lack arnt [45], as discussed above.

Furthermore, there are additional reports indicating the TCDD-induced changes in protein phosphorylation in vital cells and tissues. In the case of B lymphocytes, it has been shown by Clark *et al.* [64] that TCDD clearly stimulates protein tyrosine phosphorylation. We have shown previously that in the case of mouse thymus, a src family protein tyrosine kinase, $p56^{lck}$, is elevated as a result of TCDD administration *in vivo* [33]. Two very recent reports from our laboratory have shown that TCDD causes profound changes in protein phosphorylation patterns in adipocytes [65] and in pancreatic cells [66]. Beebe *et al.* [67] have found recently that glucagon-stimulated adenyl cyclase and cAMP-dependent protein kinase levels are elevated in the plasma membrane fraction of hepatocytes from Ah-responsive C57BL/6 mice 24 hr after TCDD treatment. While this assortment of evidence represents various efforts on different cellular materials, together they constitute overall supporting data to indicate that TCDD-induced protein phosphorylation changes may be found in many types of cells in many animal species.

What are the future research needs along this line of approach? The first item that we must clearly establish is the phenomenon of TCDD-induced Ah-receptor-mediated protein kinase activation in totally nucleus-free systems. The second question we must ask would be the nature of the protein kinase(s) activated by this system, followed by the mechanism

* Enan E and Matsumura F, manuscript in preparation.

of activation, which must involve interactions with heat shock proteins. Thereafter, we must raise the larger issue of the relative significance of the "XRE" mediated pathway versus the protein phosporylation pathway in the process of expressing toxic symptoms initiated by TCDD interaction with the Ah-receptor. Knowing that TCDD-induced cellular changes are often very interactive and vary among different cell types, sex and species, the last issue is a very difficult one to address. However, as in the case of studies on the growth factor signal transduction pathway, there are several approaches to use, e.g. sequential events (separation of primary and secondary response genes), the use of specific blockers, and cell mutants lacking certain key components.

One clear-cut difference between the above "XRE"-mediated pathway and the protein phosphorylation pathway is that the former is very well suited for the direct activation of independent genes that possess "XRE" but could be functionally unrelated to each other. In contrast, the latter pathway offers a convenient means to trigger major cellular changes in cell programs that require tight coordination among many different cell regulators. Thus, TCDD-induced changes in hormone and growth factor levels and receptor functions, nuclear transcription factor activities, and cellular oncogene functions are more likely to be good candidates for toxic effects that are mediated by the protein phosphorylation pathway. For instance, TCDD at the 1 nM level has been reported to cause down-regulation of estrogen receptor activities in the MCF-7 human breast cancer cell line [68]. Such a change occurs within 3 hr after TCDD treatment without the involvement of changes in the titer of mRNA for the estrogen receptor (ER) protein. Along a similar line of logic, Doucas *et al.* [69] have shown in the same cell line that overexpression of c-Jun or c-Fos proteins could also cause repression of ER activities. Interestingly both studies have indicated that the main cause of the suppression of ER activities by these treatments is inhibition of ER binding to "ERE" (estrogen responsive element) of transactivated genes. Judging by the fact that in the latter study these workers could also produce the same end-results using TPA, a modulator of protein kinase C, the above phenomenon appears to be a good example of protein phosphorylation-mediated coordination of transcription factor activities (e.g. AP-1 vs steroid hormone receptor proteins in the nucleus) through this "cross-talking" mechanism.

The flip side of the above argument on the "XRE" versus the phosphorylation pathway is that the latter eventually gives rise to some very generic changes, their only specificity being controlled at the stage of the Ah-receptor binding. Therefore, one would expect that some of these TCDD-induced cellular changes in this regard may be indistinguishable from those caused by hormones and growth factors that happen to utilize the same components of the pathway, such as $p21^{ras}$ and mitogen activated protein kinase. At the same time, I must add a note of caution that the above hypothesis for the existence of the second major pathway does not rule out the possibility of the presence of additional major action pathways for TCDD, e.g. the ligand-bound Ah-receptor interacting with transcription factors other than "arnt," and the possibility of responsible elements other than "XRE" responding to the Ah-receptor. My intention is to stimulate future research activities along such a line of thought by pointing out the possibility of at least one nonconventional pathway.

While there are many unsolved questions remaining, the main point of this commentary may merit a re-emphasis. That is, the majority of TCDD-caused protein phosphorylation changes appear also to be mediated by the ligand-Ah-receptor interaction, and furthermore there is a strong indication that such a ligand binding process itself could serve as the major trigger for the activation of the second major pathway through protein kinase activation. By this scheme, as I have pointed out before, the possibility exists that at least some part of the action of TCDD does not require its direct transcriptional activation step through "XRE" interaction in the nucleus.

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