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Characterization of the pattern of the nongenomic signaling pathway through which TCDD-induces early inflammatory responses in U937 human macrophages

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Abstract

2,3,7,8-Tetrachlorodibenzo(*p*)dioxin (TCDD) has been known to induce inflammatory signaling in a number of cell types and tissues. We found that in U937 macrophages TCDD causes rapid activation of cytosolic phospholipase A2 (cPLA2) within 30 min as judged by the increase in the serine 505 phosphorylated form of cPLA2 protein and the increased cellular release of free arachidonic acid. This initial action of TCDD is accompanied with the up-regulation of an important inflammation marker, COX-2 mRNA expression within 1 h, and by 3 h, several other markers become up-regulated. These effects appear to be dependent on the initial increase in the intracellular concentration of Ca²⁺, and activation of cPLA2 and COX-2. A comparative study among three different human cell lines showed that activation of COX-2 within 1 h of action of TCDD is a common feature exhibited by all cell lines. On the other hand, the U937 macrophage line appears to be unique among them with respect to its ability to activate TNF- α and IL-8 mRNA expressions, and not requiring Src kinase in propagating the initial signaling of cPLA2. Based on the rapidity of activation of cPLA2 and COX-2, which occurs within 1 h of cell exposure to TCDD, when no change in mRNA expression of CYP1A1 has been observed, it is apparent that this unique action of TCDD is carried out through a distinct “nongenomic” pathway which, is clearly discernable from the classical, “genomic” action pathway of the AhR by not requiring the participation of ARNT.

Keywords

AhR; TCDD; CPLA2; Inflammation; Macrophage; Calcium

1. Introduction

TCDD and its specific receptor, the aryl hydrocarbon receptor (AhR), have been the focus of intense research activities to elucidate the mechanism of its action and toxic outcomes for the last 30 y. Even though a tremendous amount of work has greatly contributed to the understanding of the essential role of AhR in inducing activation of many of its target genes, there are still serious questions remaining regarding the fundamental mechanisms through which many of the toxic effects are elicited by this environmental pollutant. The focus of this paper is to specifically address the early inflammatory signaling events initiated by TCDD in human macrophage cells, since it is becoming increasingly evident that TCDD evokes a potent AhR-dependent cellular inflammatory response, which appears to be associated with a number

of serious toxic end-results known to be induced by this compound (Matsumura, 2003; National Toxicology Program, 2006). Such an inflammatory effect of TCDD is particularly evident in macrophages (Matsumura and Vogel, 2006). Macrophages are key regulators of the innate immune response, as well as one of the first types of cells to respond to biological, chemical, and physical stress, and therefore it is important to study the action of TCDD in these types of cells. Several studies have shown that TCDD acts as a stimulator of inflammatory cytokines such as IL-1 β (Sutter et al., 1991), TNF- α (Clark et al., 1991), the inflammatory enzyme cyclooxygenase-2 (COX-2) (Kraemer et al., 1996), and IL-8 (Vogel et al., 2006) in various tissues and cell types. Further evidence exists that implicates aspects of TCDD toxicity with its pro-inflammatory mechanisms of action. For example, the role of IL-1 β in hepatocellular damage by TCDD (Pande et al., 2005), and the activation of a host of inflammatory mediators in the differentiation of macrophage cells to potentially plaque-forming foam cells (Vogel et al., 2004).

While the existence of these publications shows the general interest on this topic by many scientists, the main problem hindering the progress of this field is the lack of a clear-cut theoretical framework of the action of TCDD to induce a variety of cellular stress responses including inflammatory reactions. Furthermore, most publications dealing with cell stress reactions in this field are aimed at studying the secondary effects of oxidative stress that result from the induction of cytochrome P450s and other detoxification enzymes, found particularly in hepatocytes. Thus, it is very important to clearly delineate the pro-inflammatory mechanism of action of TCDD from that of the well established action of TCDD to induce detoxification enzymes. Recently it has been reported from this laboratory that TCDD rapidly activates inflammatory signaling in a mammary epithelial cell line, MCF10A. This effect was shown to be mediated by an increase in intracellular free Ca²⁺ concentration, [Ca²⁺]_i, followed by enzymatic activation of cPLA2 and COX-2 (Dong and Matsumura, 2008). Since this inflammatory signaling of TCDD-activated AhR in MCF10A cells is clearly discernable from its classical signaling to induce CYP1A1, by not involving ARNT, it has been proposed that this inflammation inducing route of action of TCDD be designated as the nongenomic pathway. However, the patterns of inflammatory responses of cells to external stimuli are known to be specific to the type of cells and tissues that are responding to the external stress stimuli: i.e. it is quite possible that a pattern identified in one type of cells may not be applicable to other types of cells. Accordingly, we have set our two major objectives as (a) to determine the main cause of early action of TCDD to induce inflammation in U937 macrophages, and (b) to recognize any difference in the pattern of this inflammation inducing signaling pathway of TCDD in this macrophage cell line from what has already been identified in MCF10A cells.

2. Materials and methods

2.1. Chemicals

TCDD (>99.99% purity) was originally obtained from Dow Chemicals Co. (Midland, MI). Dimethyl sulfoxide (Me₂SO) was obtained from Aldrich. [γ -³²P]ATP (6000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). Phorbol-12-myristate-13-acetate (TPA), 1-5-(isoquinolinyI-sulfoxyI) 2-methylpiperazine (H7), p-dioxane, and ethylene glycol tetraacetic acid (EGTA), were purchased from Sigma-Aldrich (St. Louis, MO). 3'methoxy-4'-nitroflavone (MNF) was a kind gift from Dr. Josef Abel (University of Duesseldorf, Institute of Environmental Research, Germany). Arachidonyl trifluoromethyl ketone (AACOCF₃), nifedipine, 2-aminoethoxydiphenyl borate (2-APB), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-d]pyrimidine (PP-2), ethyleneglycol-bis(β -aminoethyl)-N,N,N',N'-tetraacetoxymethyl ester (EGTA-AM), and calcimycin (A23187) were purchased from Calbiochem. Other molecular biological reagents were purchased from Qiagen (Valencia, CA) and Roche (Indianapolis, IN).

2.2. Cell culture and siRNA transfection experiments

Human U937 monocytic cells were obtained from A.T.C.C. (Manassas, VA) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (invitrogen), supplemented with 4.5 gL⁻¹ glucose (sigma), 1 mM sodium pyruvate (invitrogen), and 10 mM HEPES (invitrogen). Cells were maintained at a concentration between 2 × 10⁵ and 2 × 10⁶ cells/mL. For differentiation into monocytes/macrophages, U937 cells were treated with TPA (5 µg mL⁻¹) and allowed to adhere for 48 h in a 5% CO₂ tissue culture incubator at 37 °C, after which they were fed with TPA-free medium. For inhibitor studies, AACOCF₃ was pre-treated prior to TCDD treatment for 1 h, nifedipine, EGTA, EGTA-AM and 2-APB for 30 min, PP-2 and H7 for 15 min, while MNF, was co-treated simultaneously with TCDD. Transfection of short interfering RNA (siRNA) into U937 macrophages was performed via Nucleofector technology, 10⁶ cells were resuspended in 100 µl Nucleofector Solution V (Amaxa GmbH, Köln, Germany) and nucleofected with 1.5 µg of the corresponding siRNA for 16 h using program V-001, which is preprogrammed into the Nucleofector device (Amaxa GmbH). Cells were then treated accordingly. For siRNA studies 21 nucleotide RNA for AhR (M-004990-00), and ARNT (1027020) with 3'-dTdT overhangs were synthesized by Dharmacon Research (Lafayette, CO) and Qiagen (Valencia, CA), respectively. Corresponding control cells were transfected with AllStar negative control siRNA (1027281) by Qiagen.

2.3. Quantitative real-time reverse transcriptase PCR (qRT-PCR)

Total RNA was isolated from U937 macrophages using a high pure RNA isolation kit (Qiagen) and cDNA synthesis was carried out as previously described (Vogel et al., 2006). Quantitative detection of target gene mRNAs was performed with a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The primers for each gene were designed using OLIGO primer analysis software, provided by Steve Rosen and Whitehead institute/MIT center for genome research. All PCR assays were performed in duplicate or triplicate. The intra-assay variability was <7%. The designs of all RT-PCR primers have already been published (Vogel et al., 2006).

2.4. Antibodies and western blotting

Monoclonal antibody against human ARNT (SC-17812), a polyclonal anti-human AhR (SC-5579), a polyclonal antibody against human β-ACTIN (SC-47778), a horseradish peroxidase conjugated secondary antibody, and pre-stained standard markers (SC-2361) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody against human Phospho-cPLA₂ (P-cPLA₂) was purchased from Cell Signal (Cell Signal, Danvers, MA) (2831). Whole cell lysates (30 µg) were separated on a 10% SDS-polyacrylamide gel and blotted onto a PVDF membrane (Immuno-Blot, Bio-Rad, Hercules, CA). The antigen-antibody complexes were visualized using the chemiluminescence substrate SuperSignal®, West Pico (Pierce, Rockford, IL) as recommended by the manufacturer. For quantitative analysis, respective bands were quantified using a ChemiImager™4400 (Alpha Innotech Corporation, San Leandro, CA).

2.5. Radionuclide assays

Tritiated arachidonic acid [5,6,8,9,11,12,14,15-³H(N)] (PerkinElmer, Boston, MA) was added to U937 macrophages and allowed to culture for 16 h in a 37 °C cell incubator in standard medium conditions. Cells were then washed twice with fresh medium and new medium was added. The cells were then left for 30 min at 37 °C to allow for stabilization. They were then exposed to the appropriate treatment for the times indicated. The medium was then collected

and spun down briefly to pellet any cells that may have been present. The medium was diluted in scintillation fluid and the CPMs were measured using a Beckman liquid scintillation counter.

2.6. Statistical analysis

All experiments were repeated a minimum of three times and results were expressed as mean $\pm$ standard error. Statistical differences were determined by student's *t*-test, and for the analysis of the significance between pairs of mean values, the Bonferroni post hoc test was applied.

3. Results

Preliminary studies using quantitative RT-PCR assays identified two inflammatory mRNA expression markers that are significantly up-regulated by the early action of TCDD within 1 h. They are cytosolic phospholipase A2 (cPLA2) within 30 min and COX-2 by 1 h. In contrast, during this short time span there was no sign of induction of CYP1A1 mRNA in this line of macrophages, unlike in the case of MCF10A cells. In view of this initial indication of the cell specific difference in the pattern of early responses to the action of TCDD, we have conducted a more thorough comparison among MCF10A, U937, and HepG2 human cell lines (Table 1). Unlike the data obtained studying the MCF10A and HepG2 cell lines, the results show that the TCDD-induced up-regulation of mRNA expression of CYP1A1 before 1 h exposure is characteristically absent in U937 macrophages. Also, U937 macrophages were found to differ from the MCF10A cell line in that the former clearly shows up-regulation of the expression of TNF- α , while the latter showed no such response. Nevertheless, up-regulation of COX-2, which was also observed in the other two cell lines, was also evident in U937 macrophages. At later points in time, the inflammatory response of U937 cells to TCDD has become more apparent; which include the up-regulation of mRNA expressions of TNF- α and IL-8 that were not seen in MCF10A and HepG2 cells. In contrast, HepG2 did not show any of these inflammatory signs, except activation of COX-2, and as expected, significant levels of induction of CYP1A1 mRNAs. The interim diagnosis we have reached is that U937 macrophages are more prone to activate inflammatory responses to TCDD exposure than the other two cell lines, and that COX-2 activation could serve as the common denominator of early cell responses to TCDD in all three different types of cell lines.

In view of the above finding on COX-2, we have initiated a search process for its probable cause by following the research approaches employed in our previous studies on MCF10A cells (Dong and Matsumura, 2008). As the first step in searching the probable cause, we tested the possibility of the involvement of a Ca²⁺ stimulated protein kinase. We could find that the early action of TCDD to up-regulate COX-2 mRNA expression in 1 h was found to be sensitive to H7, an inhibitor of protein kinase C (PKC), which totally attenuates this effect (Fig. 1). Since it is well known that Ca²⁺ is an early trigger of cPLA2 and COX-2, the above observations have provided the initial hint for the involvement of an inflammatory signaling pathway mediated by [Ca²⁺]_i, cPLA2, and COX-2 in the early action of TCDD on this cell material independently from that of the classical genomic action of TCDD to cause induction of CYP1A1, judging by the absence of CYP1A1 induction at this early point in time (Table 1).

3.1. Increased serine 505 phosphorylated form of cPLA2 protein detected after 15 min exposure to TCDD

It is well known that cPLA2 is basically a Ca²⁺ activated enzyme and that such an effect of Ca²⁺ is mediated by its action to trigger phosphorylation on serine residue 505 of cPLA2 protein (Qiu et al., 1998). Fig. 2 shows that 10 nM TCDD treatment for 15–30 min resulted in a significant increase in the serine phosphorylated form of cPLA2 protein. This is known to be the active form (Alexander et al., 2004), which provides indication that in this short time period cPLA2 is likely already activated by TCDD.

3.2. Enhanced release of ^3H -arachidonic acid (AA) following treatment with TCDD is AhR-dependent, calcium-dependent, and ARNT-independent

We could find a significant Ca^{2+} -sensitive increase of free extracellular AA by U937 macrophages treated with 10 nM TCDD as early as 30 min (Fig. 3). A short time-course study was performed comparing the effects of 10 nM TCDD against a typical activator of cPLA2, A23187, a calcium ionophore. Using 1 μM A23187, a significant release of AA was measured at 30 min that was comparable to exposure with 10 nM TCDD (Fig. 3A). By 2 h, TCDD was found to be still significantly active in increasing the release of labeled AA, when the effect of A23187 on this enzyme activity had already subsided (Fig. 3A). AhR-dependency of TCDD-induced cPLA2 activation was also tested by using siRNA preparations targeted against the AhR. After 30 min treatment with 10 nM TCDD, the samples receiving siRNA targeting the AhR (siAhR) showed a reduced ^3H -AA release (Fig. 3B). This blocking effect remained significant up to 2 h following treatment with TCDD (Fig. 3B). In addition, samples were also transfected with siRNA targeting ARNT, the dimerization partner for the AhR. This treatment did not suppress the increase of released ^3H -AA by TCDD under this test condition (Fig. 3B). To be sure, an Allstar negative control siRNA was also employed as a procedural control for all of the above nucleofection treatments. Western blot confirmation of the effectiveness of siARNT on this cell material indicated that the extent of suppression of ARNT protein expression after 24 h of its transfection treatment was 55% reduction (data not shown). To confirm the result of this siAhR molecular blocking test, a chemical inhibitor study was then performed using 10 μM 3'-methoxy-4'-nitroflavone (MNF), a potent antagonist of the AhR. For this purpose cells were co-treated with TCDD and MNF and the amounts of ^3H -released AA after 30 min were assessed. MNF co-treatment significantly prevented the TCDD-mediated release of AA after 30 min exposure (Fig. 3C).

Because of the well known background information that cPLA2 is essentially a calcium-dependent enzyme, we tested the effects of two calcium inhibitors on TCDD-mediated AA release (Fig. 3D). For this purpose, we pre-treated cells with nifedipine, an L-type calcium channel blocker, and EGTA, an inhibitor of extracellular calcium, in order to assess calcium-dependency as well as the possible source of calcium entry. Treatment with both nifedipine and EGTA resulted in a significant attenuation of TCDD-mediated AA release after 30 min exposure.

3.3. TCDD-induces inflammatory gene expressions after 3 h

Based on the above finding in Fig. 3D that EGTA treatment prevented the increase in AA release during the initial 30 min period of exposure to TCDD, additional experiments were conducted to test the continuity of calcium-dependency on TCDD-mediated cPLA2 mRNA induction. For that purpose cells were pre-treated with 50 μM EGTA-AM (a form of EGTA, which does penetrate into cells) for 30 min, and were then treated with 10 nM TCDD for 3 h. The results indicate that EGTA-AM treatment completely blocks TCDD-mediated cPLA2 mRNA induction at this time point (Fig. 4). So far as we could detect in the current study, the changes in the expression of mRNA markers for inflammation occurring as a result of the action of TCDD within 1 h in this cell line are limited to those related to cPLA2 and COX-2. However, by 3 h a host of mRNA expressions are altered in this cell material. Therefore, the role of calcium and cPLA2 was investigated by monitoring changes in mRNA expression occurring after 3 h of action of TCDD. The results (Table 2) have shown that TCDD treatment at this timing caused significant up-regulation of not only the mRNA expression of cPLA2 and COX-2, but also that of VEGF and IL-8. Furthermore, TCDD-induced expressions of all of these markers were found to be significantly antagonized by the action of 20 μM AACOCF3, a selective blocker of cPLA2, and nifedipine and 2-APB, two well accepted modulators of calcium signaling (Table 2). More specifically, the data indicate that up-regulation of COX-2 and cPLA2 mRNA expressions induced by the 3 h action of TCDD are significantly

antagonized by pre-treatment of cells with 20 μ M AACOCF₃ for 1 h for both mRNAs. In particular, TCDD-induced cPLA2 mRNA expression was completely suppressed. It must be noted that the combination of these two $[Ca^{2+}]_i$ blockers was also effective in suppressing both of these mRNAs, although when tested alone neither of these could accomplish complete suppression, suggesting probably that each agent can block only one of two major sources of $[Ca^{2+}]_i$ (Table 2).

To support the likely involvement of PKC α in the process of Ca^{2+} triggered up-regulation of cPLA2 and COX-2, we assessed the inhibitory effects of nifedipine and 2-APB (added 30 min prior to the treatment of TCDD for 1 h) on the mRNA expression of PKC α . The expression values for PKC α mRNA (all have been normalized to β -actin expression and then compared to control as 1.00) were: (A) control = 1.00 ± 0.226 (four replicates); (B) TCDD treated, 1.02 ± 0.285 (4); (C) treated with NIFEDIPINE + TCDD, 0.526 ± 0.0750 (4); and (D) with 2-APB + TCDD, 0.495 ± 0.00600 (2). The difference between (B) versus (C) or (B) versus (D) were statistically highly significant, indicating that the same Ca^{2+} blockers used in the previous experiment (Table 2) were also effective in preventing the action of TCDD to induce the expression of PKC α . In contrast, unlike in the case of MCF10A cells (Dong and Matsumura, 2008), we failed to observe any consistent inhibitory action of PP-2, a specific Src kinase inhibitor, on COX-2 mRNA expression following exposure to TCDD for 3 h (Fig. 5), indicating that Src kinase is not likely involved in the early action of TCDD in U937 macrophages. Additionally PP-2 treatment also failed to affect TCDD-induced CYP1A1 mRNA expression (data not shown).

4. Discussion

In the current study, we have clearly established that TCDD, at its very early stage of action, stimulates the activity of cPLA2 in human U937 macrophage cells. This conclusion is supported by our observations on the increase in the level of its protein phosphorylation (Fig. 2), as well as the release of free radio-labeled arachidonic acid (AA) as early as 30 min following TCDD treatment. Such an early action of TCDD to activate cPLA2 is likely to be mediated by a rapid rise in $[Ca^{2+}]_i$. This is based on the effectiveness of several blockers of Ca^{2+} transport in preventing the activation of the enzymatic activity of cPLA2 (Fig. 3D), and the effectiveness of A23187 in simulating this action of TCDD (Fig. 3A). In view of the existing knowledge that cPLA2 is essentially a Ca^{2+} stimulated enzyme (Qiu et al., 1998), the data from our current study showing the effectiveness of Ca^{2+} blockers clearly supports the calcium-dependent nature of cPLA2 enzymatic activity in this cell line (Table 2, Fig. 3). The early phosphorylation of cPLA2 has been well studied, and the literature shows that in nearly all cases cPLA2 requires an increase in intracellular calcium in addition to MAPK phosphorylation (i.e. activation of ERK, Hiller and Sundler, 1999; Alexander et al., 2004). As for the involvement of PKC in this process, the data shown in Fig. 1 (i.e. the effectiveness of H7, a specific inhibitor of PKC in significantly suppressing COX-2 expression after 1 h exposure to TCDD) clearly support this view. As for the identity of the actual isoform of the PKC involved, PKC α is likely the best candidate. It is known that in U937 macrophages, PKC- α is abundantly expressed and rapidly activated in response to stressors that initiate pro-inflammatory and pro-survival cell signaling. In the case of this early induction of COX-2 mRNA by TCDD, PKC- α is the most likely suspect due to its association with cPLA2 activation and pro-inflammatory signaling in U937 macrophages (Cantoni and Guidarelli, 2008). Furthermore, we could obtain some supporting data that nifedipine and 2-APB, which are so effective in antagonizing this nongenomic action of TCDD are also effective inhibitors of PKC α mRNA expression under the identical test condition.

It is important to point out that the TCDD-induced early activation of Ca^{2+} signaling followed by the up-regulation of mRNA expressions of cPLA2, COX-2 and VEGF appears to be

identical to the pattern of early responses to TCDD observed in MCF10A cells (Dong and Matsumura, 2008). This indicates that there are likely some common features of this nongenomic action of TCDD among different cells, despite the known specificities of inflammatory responses among different types of cells. The observation that TCDD-induced COX-2 mRNA activation could be observed in HepG2 hepatocytes (Table 1) also supports this diagnosis.

While much more work would be needed to fully address this topic, the current study has already revealed a unique feature of U937 macrophages in response to TCDD; that is they respond to TCDD by up-regulation of mRNA expressions of TNF- α and IL-8 (Table 1). Such a finding infers that an ability of macrophages is to quickly connect the TCDD-induced inflammatory messages generated by cPLA2 and COX-2 to other major inflammation pathways. In particular, those operated by the members of the NF κ B family of nuclear transcription factors which depend on their production of inflammatory cytokines and chemokines as their autocrine factors. However, much more work would be needed to confirm this possibility, which should include studies on other types of cells in the future.

The early action of TCDD to cause a rapid increase, within several min, in the cytosolic concentration of Ca²⁺ has initially been demonstrated by Hanneman et al. (1996) and Puga et al. (1997). Other laboratories have confirmed such an early rise in intracellular calcium by TCDD in various cell types and tissues (Tanneheimer et al., 1997; N'Diaye et al., 2006; Dale and Eltom, 2006; Xie et al., 2006; Piaggi et al., 2007; Kim et al., 2007). Taken together these initial findings on the rapid sequence of events provide the initial solid foundation for our hypothesis that there is likely a unique signaling pathway for the ligand-activated AhR to transmit its early message to call for cellular inflammatory responses.

As for the mechanism of activating COX-2 and other inflammatory genes at the later stages of action of TCDD, the involvement of AP-1 proteins such as Fos and Jun is likely since phosphorylation on the serine residue of cPLA2 protein is known to be carried out by ERK. In the case of COX-2, however, it is likely that another nuclear transcription factor, C/EBP β (CCAAT-enhancer binding protein beta) is also involved, as shown previously by Vogel et al. (2000).

The key concept emerging from our current study is that TCDD exposure is responsible for the calcium influx in a manner independent of the classic action pathway of DRE-driven transcriptional mechanisms. While the question of AhR-dependency in the TCDD-mediated influx of calcium has yet to be thoroughly studied in the U937 cell model, there is a solid line of evidence supporting this concept. For instance, Puga et al., 1997 have already shown that, by utilizing wild-type Hepa-1 cells, in comparison to its AhR deficient C2 cell line, the AhR is absolutely essential in the action of TCDD to induce an influx of calcium.

The second main objective of this study has been to clearly distinguish this newly found, inflammation inducing action pathway of TCDD from that of the well known "classical pathway". Our observation is that the most noticeable difference of this inflammatory pathway from the classical pathway is that the former does not require the participation of ARNT (Fig. 3B). While it requires much more confirmatory work to ascertain the non-involvement of ARNT in this action of TCDD in U937 cells, the observation made in our previous study (Dong and Matsumura, 2008) as well as the data presented in Fig. 3B support this notion so far. Along with the information that at this early time point (i.e. within 60 min of action of TCDD) there is no induction of CYP1A1 mRNA in this cell line (Table 1), the data obtained in the current work support our hypothesis that, in activating this inflammation pathway, the ligand-bound AhR may not have to travel into the nucleus. Although it would require much

more work to confirm this hypothesis, this way of interpretation helps to explain why such an inflammatory response can take place so rapidly.

5. Conclusion

In conclusion, we have clearly established that TCDD-induces activation of the nongenomic, inflammatory signaling pathway in U937 macrophages through the increase in $[Ca^{2+}]_i$, which leads to activation of cPLA2. This agrees with the previous finding from this laboratory in the MCF10A mammary epithelial cell line (Dong and Matsumura, 2008). The existence of such a nongenomic pathway which is mediated by a rapid increase in $[Ca^{2+}]_i$ that is distinctly different from the well known genomic signaling pathway has been well documented in the case of ligand-activated steroid receptors such as the estrogen receptor (Bulayeva et al., 2005) in a pituitary cell line, and the androgen receptor (Guo et al., 2002) in macrophages. Therefore, the above conclusion derived from this study is adequately supported by those precedents. The additional significance of the current work, at the same time, is unlike the case of MCF10A cells, or the newly studied HepG2 cells (Table 1). U937 macrophages are capable of activating the mRNA expression of TNF- α , an important factor in linking the initial action of TCDD to activate cPLA2 and COX-2 to another major inflammation pathway, which is mediated by the TNF- α /NF κ B axis. The observation that TCDD-induced activation of IL-8 mRNA expression could be found only in U937 macrophages (Table 1) further supports this diagnosis: i.e., macrophages are the type of cells specialized to assist the action of TCDD to induce inflammation. Such findings provide enough impetus to continue our efforts in the future to address the toxicological significance of this newly found action pathway in the toxic action of TCDD in other types of cells.

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Abbreviations

AhR	aryl hydrocarbon receptor
ARNT	AhR nuclear translocator
cPLA2	cytosolic phospholipase A2
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
COX-2	cyclooxygenase-2
VEGF	vascular endothelial growth factor
2-APB	2-aminoethoxydiphenyl borate

References

- Alexander LD, Alagarsamy S, Douglas JG. Cyclic stretch-induced cPLA2 mediates ERK 1/2 signaling in rabbit proximal tubule cells. *Kidney Int* 2004;65:551–563. [PubMed: 14717925]
- Bulayeva NN, Wozniak AL, Lash LL, Watson CS. Mechanisms of membrane estrogen receptor- α -mediated rapid stimulation of Ca^{2+} levels and prolactin release in a pituitary cell line. *Am. J. Physiol. Endocrinol. Metab* 2005;288:E388–E397. [PubMed: 15494610]
- Cantoni O, Guidarelli A. Peroxynitrite damages U937 cell DNA via the intermediate formation of mitochondrial oxidants. *IUBMB Life*. 2008 doi: 310.1002/iub.116.

- Clark GC, Taylor MJ, Tritscher AM, Lucier GW. Tumor necrosis factor involvement in 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated endotoxin hypersensitivity in C57BL/6J mice congenic at the Ah locus. *Toxicol. Appl. Pharmacol* 1991;111:422–431. [PubMed: 1660630]
- Dale YR, Eltom SE. Calpain mediates the dioxin-induced activation and down-regulation of the aryl hydrocarbon receptor. *Mol. Pharmacol* 2006;70:1481–1487. [PubMed: 16891617]
- Dong B, Matsumura F. Roles of cytosolic phospholipase a2 and src kinase in the early action of TCDD through a nongenomic pathway in MCF10A cells. *Mol. Pharmacol.* 2008 doi: 10.1124/mol.107.044669.
- Guo Z, Benten WP, Krücken J, Wunderlich F. Nongenomic testosterone calcium signaling. Genotropic actions in androgen receptor-free macrophages. *J. Biol. Chem* 2002;277:29600–29607. [PubMed: 12048191]
- 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. [PubMed: 8792845]
- Hiller G, Sundler R. Activation of arachidonate release and cytosolic phospholipase A2 via extracellular signal-regulated kinase and p38 mitogenactivated protein kinase in macrophages stimulated by bacteria or zymosan. *Cell. Signal* 1999;11:863–869. [PubMed: 10659994]
- Kim SY, Lee HG, Choi EJ, Park KY, Yang JH. TCDD alters PKC signaling pathways in developing neuronal cells in culture. *Chemosphere* 2007;9:S421–S427. [PubMed: 17222441]
- Kraemer SA, Arthur KA, Denison MS, Smith WL, DeWitt DL. Regulation of prostaglandin endoperoxide H synthase-2 expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Arch. Biochem. Biophys* 1996;330:319–328.
- Matsumura F. On the significance of the role of cellular stress response reactions in the toxic actions of dioxin. *Biochem. Pharmacol* 2003;66:527–540. (Review).
- 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;387:1189–1194. [PubMed: 16972785]
- N'Diaye M, Le Ferrec E, Lagadic-Gossmann D, Corre S, Gilot D, Lecureur V, Monteiro P, Rauch C, Galibert MD, Fardel O. Aryl hydrocarbon receptor and calcium-dependent induction of the chemokine CCL1 by the environmental contaminant benzo[a]pyrene. *J. Biol. Chem* 2006;281:19906–19915. [PubMed: 16679317]
- National Toxicology Program. NTP toxicology and carcinogenesis studies of a mixture of 2,3,7,8-tetrachlorodibenzo-p-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). *Natl. Toxicol. Program Tech. Rep. Ser* 2006;526:1–180. [PubMed: 17342195]
- Pande K, Moran SM, Bradfield CA. Aspects of dioxin toxicity are mediated by interleukin 1-like cytokines. *Mol. Pharmacol* 2005;67:1393–1398. [PubMed: 15722456]
- Piaggi S, Novelli M, Martino L, Masini M, Raggi C, Orciuolo E, Masiello P, Casini A, De Tata V. Cell death and impairment of glucose-stimulated insulin secretion induced by tetrachlorodibenzo-p-dioxin (TCDD) in beta-cell line INS-1E. *Toxicol. Appl. Pharmacol* 2007;220:333–340.
- Puga A, Hoffer A, Zhou S, Bohm JM, Leikauf GD, Shertzer HG. Sustained increase in intracellular free calcium and activation of cyclooxygenase-2 expression in mouse hepatoma cells treated with dioxin. *Biochem. Pharmacol* 1997;54:1287–1296.
- Qiu ZH, Gijon MA, de Carylho MS, Spencer DM, Leslie CC. The role of calcium and phosphorylation of cytosolic phospholipase A2 in regulating arachidonic acid release in macrophages. *J. Biol. Chem* 1998;273:8203–8211. [PubMed: 9525925]
- Sutter TR, Guzman K, Dold KM, Greenlee WF. Targets for dioxin: genes for plasminogen activator inhibitor-2 and interleukin-1 beta. *Science* 1991;254:415–418. [PubMed: 1925598]
- 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;18:1177–1182. [PubMed: 9214600]

- Vogel C, Boerboom AM, Baechle C, El-Bahay C, Kahl R, Degen GH, Abel J. Regulation of prostaglandin endoperoxide H synthase-2 induction by dioxin in rat hepatocytes: possible c-Src-mediated pathway. *Carcinogenesis* 2000;21:2267–2274. [PubMed: 11133817]
- Vogel CF, Sciullo E, Matsumura F. Activation of inflammatory mediators and potential role of ah-receptor ligands in foam cell formation. *Cardiovasc. Toxicol* 2004;4:363–373. [PubMed: 15531779]
- Vogel CF, Sciullo E, Wong P, Kuzmicky P, Kado N, Matsumura F. Induction of proinflammatory cytokines and C-reactive protein in human macrophage cell line U937 exposed to air pollution particulates. *Environ. Health Perspect* 2006;113:1536–1541. [PubMed: 16263508]
- Xie A, Walker NJ, Wang D. Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) enhances triggered after depolarizations in rat ventricular myocytes. *Cardiovasc. Toxicol* 2006;6:99–110. [PubMed: 17303918]

Fig. 1.

Effect of treatment of U937 macrophages with TCDD (for 1 h) and H7, a specific inhibitor of PKC on mRNA expression of COX-2. U937 cells were treated with 10 nM TCDD for 1 h and compared to 0.1% DMSO-treated controls on COX-2 mRNA expression with those pre-treated with 20 μ M H7 for 15 min prior to TCDD exposure. *, treatment significantly different from controls $p < 0.05$. ψ , treatment significantly different from TCDD alone $p < 0.05$. All data is normalized to β -actin.

Fig. 2. Effect of TCDD on the levels of serine phosphorylation on cPLA2 protein as detected by western blotting. U937 macrophages were treated with 10 nM TCDD for 15 and 30 min and compared against 0.1% DMSO-treated controls. Loading controls were verified using a specific probe for β -actin.

Fig. 3.

Assessment of the effects of several diagnostic agents on the action of TCDD to stimulate ^3H -Arachidonic acid (AA) release for 30 min or 2 h. Test agents used were: (A) 10 nM TCDD or 1 μM A23187, (B) siRNAs targeting AhR (C). 10 μM MNF, (D) 10 μM Nifedipine, and 250 μM EGTA. *, treatments significantly different from controls $p < 0.05$. ψ , TCDD treatments significantly different from inhibitors or siRNA approaches plus TCDD $p < 0.05$.

Fig. 4.

Effect of 3 h exposure of U937 macrophages to TCDD in the presence and the absence of EGTA-AM on mRNA expression of cPLA2. For this purpose cells were pretreated with 50 μ M EGTA-AM for 30 min and the results were compared to 0.1% DMSO-treated controls. *, treatments significantly different from controls $p < 0.05$. ψ , TCDD treatments significantly different from inhibitor approaches plus TCDD $p < 0.05$. Data was obtained using real-time RT-PCR and normalized to β -actin.

Fig. 5. The lack of inhibitory action of PP2, a specific inhibitor of Src kinase on the action of TCDD to induce the expression of COX-2 mRNA. *, treatments significantly different from controls $p < 0.05$. Data was obtained using real-time RT-PCR and normalized to β -actin.

Table 1

Comparison of the early effects of TCDD on mRNA expressions in MCF10A cells, U937 macrophages and HepG2 cells. Cells were treated with 10 nM TCDD for 1, 2 or 6 h; control cells received only the vehicle solvent (p-dioxane). The levels of mRNA expressions of the genes were measured by qRT-PCR and normalized to the expression of GAPDH. The levels of induction of mRNA expressions are given relative to the values of the control. Results are shown as mean $\pm$ standard error.

mRNA expressions						
Cell line	Time	CYP1A1	COX-2	IL-1β	IL-8	TNF-α
MCF10A	1 h	21.7 $\pm$ 1.1**	1.86 $\pm$ 0.080**	2.38 $\pm$ 0.17*	0.330 $\pm$ 0.030	0.710 $\pm$ 0.26
	2 h	89.8 $\pm$ 3.4**	1.56 $\pm$ 0.040**	2.97 $\pm$ 0.13**	0.310 $\pm$ 0.13	1.29 $\pm$ 0.070
	6h	71.6 $\pm$ 6.2**	1.29 $\pm$ 0.080	2.49 $\pm$ 0.19*	0.220 $\pm$ 0.060	0.730 $\pm$ 0.25
U937	1 h	1.15 $\pm$ 0.020	4.18 $\pm$ 0.73*	0.990 $\pm$ 0.030	1.32 $\pm$ 0.23	1.59 $\pm$ 0.070*
	2 h	2.52 $\pm$ 0.10**	5.85 $\pm$ 0.87*	1.22 $\pm$ 0.060	0.800 $\pm$ 0.12	2.10 $\pm$ 0.040*
	6 h	5.94 $\pm$ 0.25**	12.8 $\pm$ 1.4*	7.56 $\pm$ 1.2*	5.46 $\pm$ 0.90*	1.71 $\pm$ 0.070*
HepG2	1 h	11.6 $\pm$ 2.1**	1.54 $\pm$ 0.070*	0.990 $\pm$ 0.010	0.980 $\pm$ 0.12	1.12 $\pm$ 0.28
	2 h	36.4 $\pm$ 5.4**	1.46 $\pm$ 0.11*	0.860 $\pm$ 0.39	0.920 $\pm$ 0.11	1.08 $\pm$ 0.10
	6 h	116 $\pm$ 3.2**	1.15 $\pm$ 0.36	0.760 $\pm$ 0.27	0.660 $\pm$ 0.10	0.860 $\pm$ 0.20

* The statistically significant difference between the treatment and the control is indicated by ($p < 0.05$).

** The statistically significant difference between the treatment and the control is indicated by ($p < 0.01$).

Table 2

Effects of AACOCF₃ (a cPLA₂ inhibitor), and nifedipine and 2-ABP (two Ca²⁺ blockers) on the action of TCDD to induce mRNA expressions of COX-2, cPLA₂, IL-8, and VEGF in 3 h.

Treatment	COX-2	cPLA ₂	IL-8	VEGF
Control (DMSO)	1.00 ± 0.040	1.00 ± 0.080	1.00 ± 0.020	1.00 ± 0.34
+TCDD	4.29 ± 0.010 [*]	2.05 ± 0.080 [*]	2.28 ± 0.080 [*]	3.29 ± 0.48 [*]
+AACOCF ₃	0.830 ± 0.10	1.22 ± 0.050	0.960 ± 0.11	1.40 ± 0.090
+AACOCF ₃ + TCDD	1.67 ± 0.52 ^{**}	1.08 ± 0.090 ^{**}	0.690 ± 0.12 ^{**}	1.37 ± 0.24 ^{**}
+Nif	1.82 ± 0.24	1.03 ± 0.15	1.15 ± 0.12	1.26 ± 0.27
+Nif + TCDD	4.92 ± 0.060	1.22 ± 0.57 ^{**}	1.78 ± 0.32	1.37 ± 0.16 ^{**}
+2-APB	1.61 ± 0.47	1.28 ± 0.22	1.43 ± 0.32	1.22 ± 0.31
+2-APB + TCDD	5.26 ± 0.93	1.72 ± 0.19	2.32 ± 0.050	1.41 ± 0.26 ^{**}
+Nif + 2-APB	1.57 ± 0.58	1.04 ± 0.24	1.81 ± 0.080	1.18 ± 0.24
+Nif + 2-APB + T	1.12 ± 0.16 ^{**}	1.29 ± 0.82 ^{**}	1.26 ± 0.18 ^{**}	0.830 ± 0.39 ^{**}

* The treatment significantly different from controls is indicated by $p < 0.05$.

** The treatment significantly different from TCDD alone is indicated by $p < 0.05$.