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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

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin) is known to induce rapid inflammatory cellular responses through the mechanism that has not yet been fully elucidated. In this report, we show that in MCF10A cells, an immortalized, normal mammary epithelial cell line, TCDD rapidly activates the enzymatic activity of cytosolic phospholipase A2 (cPLA2) as attested to by arachidonic acid release within 15 min, followed by activation of Src kinase and induction of several inflammation markers. Such an action of TCDD is clearly blocked by methylarachidonyl fluorophosphonate, a specific inhibitor of cPLA2, short interfering RNA against cPLA2, and several calcium signaling blockers, indicating that this action of TCDD is mediated by calcium-triggered activation of cPLA2. This action of TCDD is quite different from the classic action of TCDD to induce cytochrome P450 1A1 (CYP1A1) because blocking this newly identified pathway did not affect the induction of CYP1A1. Moreover, this newly identified pathway was found to depend only on aryl hydrocarbon receptor but not on aryl hydrocarbon receptor nuclear translocator. Together, these findings support the model that the early action of TCDD to induce rapid inflammatory responses is carried out through a characteristic "nongenomic" pathway, which is clearly different from the conventional model of action of TCDD through the "genomic" pathway.

Despite the tremendous progress made in the past several decades on the action mechanism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in terms of the understanding of the mediating role of the aryl hydrocarbon receptor (AhR) regarding its structure, intracellular organization, translocation into nucleus, and the process of genomic activation of genes encoding mostly detoxification enzymes (Poland and Knutson, 1982; Fisher et al., 1989; Fukunaga et al., 1995; Hankinson, 1995), the extent of our understanding on the mechanisms and the significance of its early action to induce inflammatory cellular responses lags far behind.

Earlier work in our laboratory has shown that protein kinases, particularly Src tyrosine kinase, play important roles in some of the toxic actions of TCDD (Matsumura, 1994), at least in certain types of cells. MCF10A cells have been shown to be quite responsive to dioxin-type chemicals (Reiners et al., 1997). Particularly interesting from the viewpoint of our current investigation is that in this cell line, TCDD has been shown to cause rapid up-regulation of tyrosine kinases, including those associated with growth factors (Davis et al., 2001, 2003). It is well known that Src kinase plays an important role in breast cancer (Hiscox et al., 2006); therefore, in studying this aspect of the action mechanism of TCDD, we found MCF10A to be well suited. For instance, it has been reported from this laboratory that TCDD causes rapid activation of Src kinase within 30 min, during which period inflammatory responses are also taking place in this cell line (Mazina et al., 2004).

Another reason that are interested in Src kinase is that the contribution of this Src-mediated pathway to the eventual toxic manifestations of TCDD in vivo has been well studied, particularly with regard to the manifestation of wasting syn-

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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; cPLA2, cytosolic phospholipase A2; IκB-α, Inhibitor κ B-α; IL, interleukin; NFκB, nuclear factor κB; PAI-2, plasminogen activator inhibitor-2; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; PVDF, polyvinylidene diflouride; MNF, 3′-methoxy-4′-nitroflavone; PCR, polymerase chain reaction; MAFP, methylarachidonyl fluorophosphonate; siRNA, short interfering RNA; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; AM, acetoxymethyl ester; 2-APB, 2-aminoethoxydiphenyl borate; A23187, calcimycin; si-AhR, short interfering RNA against the aryl hydrocarbon receptor; si-ARNT, short interfering RNA against the aryl hydrocarbon receptor nuclear translocator.

drome, through the use of an Src gene knockout mouse strain (Dunlap et al., 2002). Furthermore, a similar line of investigation recently conducted on a transforming growth factor-α mutant mouse strain with a defective form of transforming growth factor-α indicates that the lack of action of this Src-inducible autocrine factor also confers this strain of mice significant resistance to TCDD (Kitamura et al., 2006). Despite such an accomplishment, the role of Src kinase in the early action of TCDD to induce inflammatory cell responses has not been addressed systematically to date, although there are now a number of publications reporting such an action of TCDD both in vitro (Martinez et al., 2004; Singh et al., 2007) and in vivo (Yoon et al., 2006; Kitamura and Kasai, 2007).

A number of scientists have indicated that inflammation is an important environmental factor contributing to the etiology of breast cancer (Calogero et al., 2007; DeNardo and Coussens, 2007). In this regard, it is pertinent to point out that one of the major actions of TCDD is to elicit inflammatory cellular responses, which have been shown in a number of tissues and cells, including MCF10A cells, to be very intense and lasting for long time periods (Matsumura, 2003).

Therefore, this investigation has been initiated with two major objectives. The first is to identify the major triggering factor to induce the process of early action of TCDD to evoke rapid inflammatory responses from the affected mammary epithelial cells. The second objective is to clarify the difference between the nature of this TCDD-induced inflammatory pathway and that of the well-established classic action pathway of TCDD through AhR and ARNT. In planning this study, we have made a conscious effort to address the very early events occurring as a result of the initial cell exposure to TCDD within 2 h to identify the most upstream events occurring in this pathway.

Materials and Methods

Reagents and Antibodies. TCDD (>99% purity) was obtained from Dow Chemicals Co. (Midland, MI). 3′-Methoxy-4′-nitroflavone (MNF) was a kind gift from Dr. Josef Abel (University of Duesseldorf, Duesseldorf, Germany). Calcimycin (A23187), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), methylarachidonyl fluorophosphonate (MAFP), EGTA-acetoxymethyl ester (EGTA/AM), and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Calbiochem (San Diego, CA). Tumor necrosis factor-α (TNF-α), arachidonic acid, and 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl eater (nifedipine) were purchased from Sigma-Aldrich Co. (St. Louis, MO). [^{3}H]Arachidonic acid was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). siRNA against cPLA2, AhR, and ARNT were purchased from QIAGEN (Valencia, CA). Rabbit polyclonal anti-Src, anti-AhR, anti-ARNT, goat polyclonal anti-actin, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho-cPLA2 (Ser505) was purchased from Cell Signaling Technology (Danvers, MA).

Cells and Cell Culture. MCF10A human breast epithelial cells were purchased from the American Type Culture Collection (Manassas, VA). MCF10A cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 5% calf bovine serum (Sigma-Aldrich), 100 U of penicillin and 100 μg/ml streptomycin supplemented with 20 ng/ml epidermal growth factor, 10 μg/ml insulin, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone. Cells were incubated at 37°C with 5% CO_2 and medium was changed every 2 or 3 days.

Quantitative Reverse Transcriptional PCR. Total RNA was extracted from cells using RNeasy Mini kit (QIAGEN). Reverse transcription and quantitative reverse transcriptional-PCR was carried out as described previously (Park et al., 2007). In brief, 1 μg of total RNA was mixed with 40 pmol oligo(dT)$_{15}$ in a 10-μl total reaction volume. The mixture was run with an annealing program at 60°C for 5 min. After annealing, cDNA was synthesized using Omniscript Reverse Transcription kit (QIAGEN) with a reverse-transcription program (37°C for 60 min followed by 70°C for 10 min). To run PCR, 2 μl of cDNA was mixed with 10 μl of SYBRgreen (QIAGEN) and 10 pmol of each primer in a 20-μl total reaction volume. PCR was then performed using LightCycler (Roche Applied Science, Indianapolis, IN) with a PCR program (initial activation at 95°C for 15 min before first cycle, for each cycle, denaturation at 94°C for 15 s, annealing at 59°C for 20 s, and extension at 72°C for 20 s). The data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The primer sequences for each gene are listed in Table 1.

Preparation of Protein Extracts. Protein extracts were prepared as described previously (Park et al., 2007). MCF10A cells were seeded in 100-mm dishes. Whole-cell protein extracts were prepared as follows. Cells were rinsed twice with phosphate-buffered saline (PBS) and then lysed on ice with 200 μl of cold radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitor cocktail (Sigma-Aldrich) for 30 min. The lysates were then centrifuged at 16,000*g* at 4°C for 20 min, and the supernatants were stored at −80°C as whole-cell protein extracts. Membrane fraction protein extracts were prepared as follows. Cells were rinsed twice with PBS and then suspended with 200 μl of ice-cold homogenization buffer containing 50 mM HEPES, pH 7.5, 1.5 mM magnesium acetate, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and protease inhibitor cocktail. Cell suspensions were then homogenized in a Dounce homogenizer with 10 strokes. After centrifugation at 800*g* at 4°C for 10 min, supernatants were again centrifuged at 100,000*g* at 4°C for 1 h. Pellets were then extracted at 4°C for 1 h with 50 μl of homogenization buffer supplemented with 1% (v/v) Triton X-100 and stored at −80°C as membrane fraction protein extracts. Protein concentrations were determined by the Bradford method.

TABLE 1
Primer sequences

Gene	Primers
Cox-2	
Forward	5′-TGAGCATCTACGGTTTGCTG-3′
Reverse	5′-AACTGCTCATCACCCCATTC-3′
CYP1A1	
Forward	5′-TAGACACTGATCTGGCTGCA-3′
Reverse	5′-GGGAAGGCTCCATCAGCATC-3′
GAPDH	
Forward	5′-TGAAGGTCGGAGTCAACGGA-3′
Reverse	5′-CATGTGGGCCATGAGGTCCA-3′
IκB-α	
Forward	5′-GCAAAATCCTGACCTGGTGT-3′
Reverse	5′-GCTCGTCCTCTGTGAACTCC-3′
IL-1β	
Forward	5′-GGACAAGCTGAGGAAGATGC-3′
Reverse	5′-TCGTTATCCCATGTGTCGAA-3′
IL-8	
Forward	5′-CAGGAATTGAATGGGTTTGC-3′
Reverse	5′-AGCAGACTAGGGTTGCCAGA-3′
PAI-2	
Forward	5′-GTTCATGCAGCAGATCCAGA-3′
Reverse	5′-CGCAGACTTCTCACCAAACA-3′
VEGF	
Forward	5′-AAGGAGGAGGGCAGAATCAT-3′
Reverse	5′-ATCTGCATGGTGATGTTGGA-3′

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Western Blotting. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membrane. The PVDF membrane was blocked with 5% nonfat milk in Tris-buffered saline/Tween 20 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The PVDF membrane was then incubated with primary antibody (1:500 dilution) in blocking buffer at 4°C overnight. After incubation with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution) in Tris-buffered saline/Tween 20 for 3 h at room temperature, blots were developed using SuperSignal West Pico detection kit (Pierce, Rockford, IL). All Western blots were repeated at least three times for each experiment to confirm the reproducibility of the results.

siRNA Transfection. Cells were seeded in 12-well plates the day before transfection at a density of 10^5 cells/well in 1 ml of medium. After 24 h, 0.1 nmol siRNA was added to 90 μl of medium for each well and mixed by vortexing. Then 10 μl of RNAiFect Transfection Reagent (QIAGEN) was added to the diluted siRNA and mixed by pipetting up and down five times. The 100-μl mixture was incubated for 15 min at room temperature to allow the formation of transfection complexes, which was then added to each well with 900 μl of fresh medium to make a final siRNA concentration of 100 nM. The cells were incubated at 37°C for 24 h in a CO_2 incubator. After 24 h the medium was changed, and cells were incubated for additional 24 h before treatment.

Arachidonic Acid Release Measurement. Arachidonic acid release was measured as described previously (Viu et al., 1998). Cells were seeded in 12-well plates. When the cells reached 70% confluence, 0.2 μCi of [^{3}H]arachidonic acid (specific activity, 180 Ci/mmol) was added to each well and incubated overnight. The [^{3}H]arachidonic-containing medium was discarded the next day, and the cells were washed twice to remove residual [^{3}H]arachidonic acid that was not incorporated into the cells. The cells were then treated with TCDD and/or additional chemical agents for specific time periods, and thereafter the medium was collected and centrifuged to remove floating cells. The arachidonic acid released from inside the cells into the medium was calculated by measuring the radioactivity of the medium using a liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA).

Results

TCDD Induces Inflammatory Markers in a Qualitatively Different Way from TNF-α. We first studied the pattern of action of TCDD to induce inflammatory markers in comparison with exogenous TNF-α, a well known chemical to induce inflammatory responses, by focusing on their initial 2 h of action. As expected, exogenously administered TNF-α rapidly induced IκB-α (Fig. 1A) and IL-8 (Fig. 1B) expression in MCF10A cells, starting within 30 min, peaking at 60 min, and decreasing by 120 min, whereas TCDD induced neither of them. The observation that TNF-α rapidly induced IκB-α expression followed by a decrease is a typical sign for the activation of the NFκB pathway (Brown et al., 1993; Sun et al., 1993). Therefore the above finding indicates that the NFκB pathway is indeed operative in this cell line, and it can be activated by TNF-α but not by TCDD in this time span. In contrast, the induction of CYP1A1 (Fig. 1C) and Cox-2 (Fig. 1D) was only found in cells treated with TCDD but not at all in those affected by TNF-α. We could determine additionally that mRNA expressions of vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor-2 (PAI-2) were also induced by TCDD but not by TNF-α during the 120-min post-treatment period (Fig. 1, E and F). These two markers have been selected because their mRNA expressions are known to be activated by prostaglandins, the products of Cox-2 (Harada et al., 1994; Casibang et al., 2001). Even in the case of IL-1β, which was induced by TCDD and TNF-α, the progression of its up-regulation by the former was much slower than that by the latter (Fig. 1G). Together, the above results strongly suggest that the action of TCDD during this time span is not at all similar to that of TNF-α, which activates mainly the NFκB pathway.

PP2 and MAFP Inhibit the Action of TCDD to Induce Inflammation Markers and to Activate Src Kinase. Having verified the clearcut difference between the pattern of inflammatory responses induced by TCDD and those induced by TNF-α in this cell line, we then examined the effects of selected chemical inhibitors. Among them, we found that MNF (a specific AhR antagonist), PP2 (a specific inhibitor of Src kinases), and MAFP (a competitive inhibitor of cPLA2) are very effective in antagonizing the action of TCDD on all inflammation markers. MNF (10 μM) pretreatment almost completely abolished the ability of TCDD to induce CYP1A1, whereas PP2 (2 μM) and MAFP (20 μM) did not affect this marker at all. In contrast, the action of TCDD to induce other inflammation markers, including those for Cox-2, VEGF, and PAI-2, was almost uniformly attenuated by all of these three inhibitors (Fig. 2A). In view of our previous work that TCDD functionally activates Src kinase in 30 min by promoting its translocation to the plasma membrane (Park et al., 2007), we used this phenomenon to confirm the above effects of these chemical inhibitors on Src kinase activation. The results clearly show that MNF, PP2, and MAFP could totally abolish the ability of TCDD to activate Src kinase in 30 min (Fig. 2B).

Exogenous Arachidonic Acid Acts Similarly to TCDD. The above actions of PP2 were not totally surprising, in view of our previous work (Mazina et al., 2004), indicating the importance of Src kinase in the action of TCDD in this cell line. However, the effectiveness of MAFP, a competitive inhibitor of cPLA2, in suppressing the early action of TCDD to induce those inflammation markers and to activate Src kinase was new to us. The basic function of cPLA2 is to release, from the plasma membrane of cells, free arachidonic acid, which serves as the precursor of many inflammation-inducing metabolites such as prostaglandins and leukotrienes. Therefore, we formulated a hypothesis that free arachidonic acid released by activated cPLA2 serves as the upstream mediator of the action of TCDD to activate Src kinase, which in turn causes up-regulation of those inflammation markers. To test this hypothesis, exogenous arachidonic acid was examined for its effects in activating Src kinase and inducing those inflammation markers in MCF10A cells. The results show that arachidonic acid activated Src kinase in a dose-dependent manner. It significantly induced Src kinase to translocate to the plasma membrane at the concentrations of 10 and 50 μg/ml arachidonic acid after 30-min treatment (Fig. 3A). Arachidonic acid also activated Src kinase and induced those inflammation markers in a time-dependent manner. It activated Src kinase as early as 15 min and stayed activated for 30 min at the concentration of 10 μg/ml arachidonic acid (Fig. 3B). The expression of those inflammation markers, including Cox-2, VEGF, and PAI-2, was elevated after 1 h of treatment and stayed elevated after 2 h of treatment without affecting CYP1A1 expression (Fig. 3C). These results show the similarity of the action between TCDD and arachidonic acid except that the

action of arachidonic acid seems to take place earlier than that of TCDD. However, the ability of arachidonic acid to activate Src kinase (Fig. 3D) and induce those inflammation markers (Fig. 3E) is susceptible only to PP2, not to MAFP.

cPLA2 Is Activated by TCDD and Involved in the Action of TCDD. More direct evidence for the involvement of cPLA2 was obtained through the Western blotting approach to detect the activated form of cPLA2 using a specific antibody capable of reacting with a serine-phosphorylated, and therefore an activated, form of cPLA2 protein. By this approach, cPLA2 was found to be activated within 15 min after TCDD treatment and stayed activated for an additional 15 min. This sign of activation disappeared after 60 min of action of TCDD (Fig. 4A). As expected, activation of cPLA2 by TCDD was clearly blocked by MNF (Fig. 4B), which proves that AhR is responsible for the action of TCDD to activate cPLA2. To further confirm that cPLA2 is indispensable for the action of TCDD, a preparation of siRNA designed to silence cPLA2 (si-cPLA2) was used instead of the chemical inhibitor previously used to test its effects on the action of TCDD. This si-cPLA2 treatment significantly reduced the mRNA expression of cPLA2 as expected. More importantly, si-cPLA2 significantly antagonized the action of TCDD to induce Cox-2 and VEGF mRNA expression but not CYP1A1 (Fig. 4C).

TCDD Induces the Release of Arachidonic Acid. Another piece of evidence for the essential requirement of the involvement of cPLA2 in the inflammatory actions of TCDD has been obtained through the approach assessing arachidonic acid release from [^{3}H]arachidonic acid-labeled MCF10A cells. The results show that TCDD clearly stimulated arachidonic acid release from MCF10A cells, although not as strongly as A23187, which was used here as a positive control (Fig. 5A). It is significant that this action of TCDD to induce arachidonic acid release was found to start also as early as 15 min, which confirms the timeline of activation of

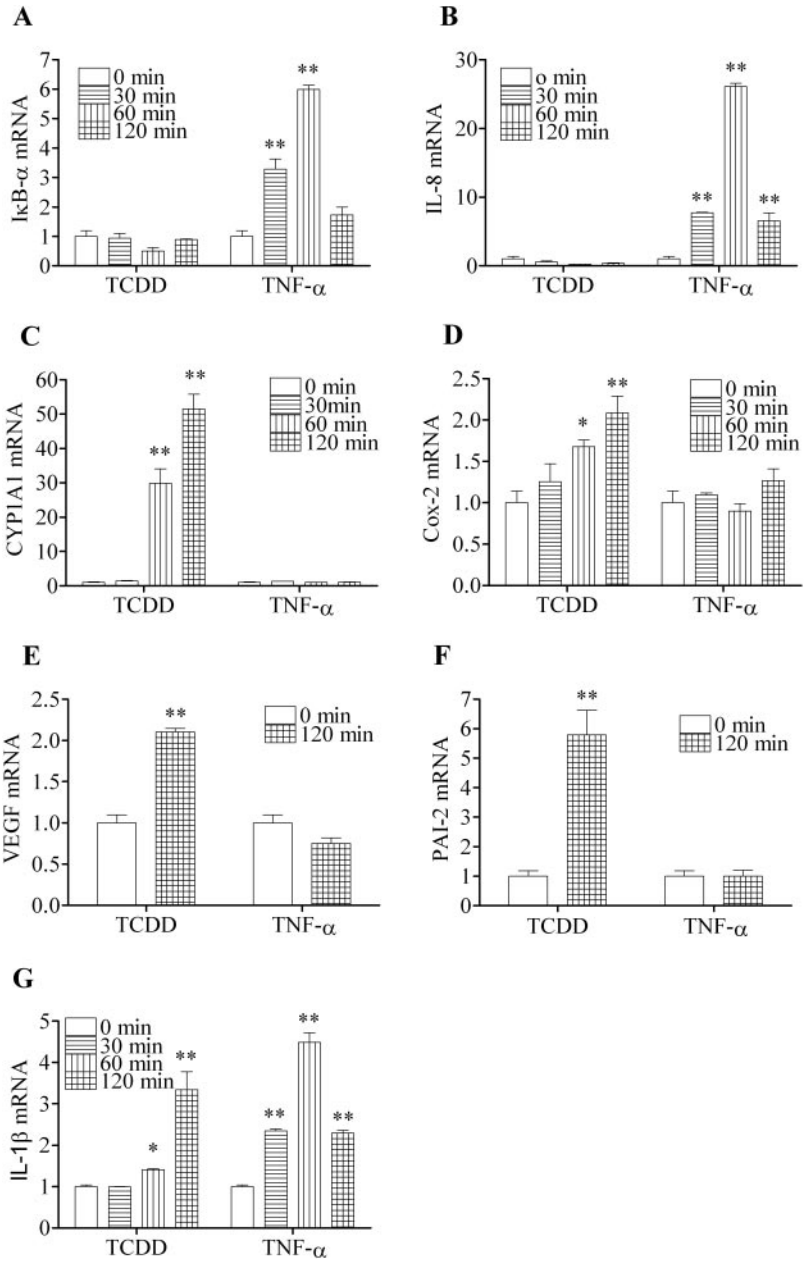

Fig. 1. Comparison of the effects of TCDD and TNF-α on mRNA expressions during the initial 120-min time period. MCF10A cells were treated with 10 nM TCDD or 10 ng/ml TNF-α for different time periods as indicated. The levels of mRNA expressions were assessed for IκB-α (A), IL-8 (B), CYP1A1 (C), Cox-2 (D), VEGF (E), PAI-2 (F), and IL-1β (G). The results are expressed as folds of induction over control, and the statistically significant differences between the 0 min group and other time period groups are indicated by *, $p < 0.05$, or **, $p < 0.01$.

cPLA2 by TCDD as judged by previous Western blotting results (Fig. 4A). Moreover, this action of TCDD is also clearly suppressed by both MNF (Fig. 5B) and MAFP (Fig. 5C) but not by PP2 at all (Fig. 5D). The last observation confirms our diagnosis that Src activation is a downstream consequence of cPLA2 activation by TCDD.

Calcium Is Involved in the Action of TCDD. One of the well known cPLA2 activators is calcium, the possible role of calcium being the initial trigger of this TCDD-induced inflammatory cellular responses was tested using several calcium-signaling blockers. Among them, we discovered that EGTA/AM (a calcium chelator capable of penetrating into cells), nifedipine (an L-type calcium-channel blocker), and 2-APB (a blocker of IP3 receptor-mediated release of calcium from endoplasmic reticulum) were most effective in antagonizing the action of TCDD to induce those inflammation markers such as Cox-2, VEGF, and PAI-2 without significantly affecting the CYP1A1 induction (Fig. 6A). It is important to note that all of these calcium blockers have been very effective in blocking the action of TCDD to release free arachidonic acid in MCF10A cells (Fig. 6B).

Induction of Inflammation Markers by TCDD is ARNT-Independent. So far, all of the above results have been consistent in showing that cPLA2 plays an indispensable role in the action of TCDD to activate Src kinase and to induce those inflammation markers, and that such an action of TCDD is qualitatively different to the action of TCDD to induce CYP1A1, based on the test results of chemical inhibitors and si-cPLA2 treatment. However, an additional concrete piece of evidence pinpointing the precise nature of the difference between this inflammation pathway and the classic genomic pathway would be very beneficial. To elucidate this difference, siRNA against AhR (si-AhR) and that against ARNT (si-ARNT) were used to silence AhR and ARNT expressions, respectively. Both si-AhR and si-ARNT treatment successfully suppressed the AhR and ARNT expression shown by Western blotting (Fig. 7A). In addition, as expected, both si-AhR and si-ARNT treatments significantly reduced the induction of CYP1A1 by more than 70%. However, si-ARNT treatment did not cause any significant suppression of the induction of those inflammation markers. In contrast, si-AhR treatment significantly suppressed the induction of all markers (e.g., 35% for Cox-2, 40% for VEGF and 60% for PAI-2) (Fig. 7B). The results of a parallel study on the arachidonic acid release have also shown that such an action of

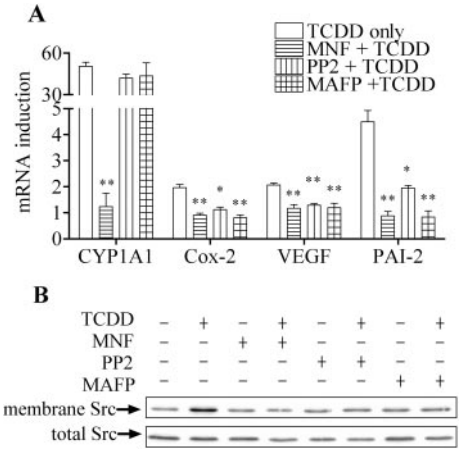

Fig. 2. Effects of inhibitors on the action of TCDD to induce the inflammation markers and to activate Src kinase. MCF10A cells were preincubated with 10 μM MNF, 2 μM PP2, or 20 μM MAFP for 30 min. A, for mRNA induction measurement, cells were then treated with 10 nM TCDD for 2 h. The results are expressed as folds of induction after TCDD treatment, and the statistically significant differences between the TCDD-only group and the groups preincubated with inhibitors are indicated by *, $p < 0.05$, or **, $p < 0.01$. B, for Src kinase activation test, cells were then treated with 10 nM TCDD for 30 min, and the activation of Src kinase was judged by its translocation to membrane.

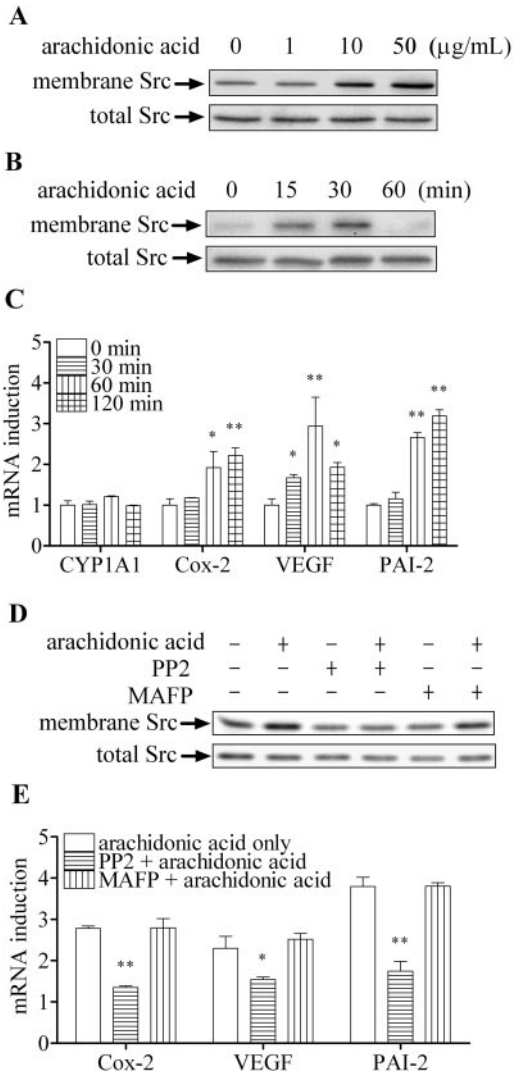

Fig. 3. Action of exogenous arachidonic acid to activate Src kinase and to induce inflammation markers. A, MCF10A cells were treated with different concentrations of arachidonic acid as indicated for 30 min, and the action of arachidonic acid to activate of Src kinase was judged by its translocation to membrane. B and C, MCF10A cells were treated with 10 μg/ml arachidonic acid for different time periods as indicated. The activation of Src kinase was judged by its translocation to membrane. The induction of those inflammation markers was expressed as folds of induction over control, and the statistically significant differences between the 0-min group and other time period groups are indicated by *, $p < 0.05$, or **, $p < 0.01$. D and E, MCF10A cells were preincubated with 2 μM PP2 or 20 μM MAFP for 30 min. For Src kinase activation test, cells were then treated with 10 μg/ml arachidonic acid for 15 min, and the activation of Src kinase was judged by its translocation to membrane. For mRNA induction measurement, cells were then treated with 10 μg/ml arachidonic acid for 2 h. The results are expressed as folds of induction after arachidonic acid treatment, and the statistically significant differences between the arachidonic acid-only group and the groups preincubated with inhibitors are indicated by *, $p < 0.05$, or **, $p < 0.01$.

TCDD is dependent on AhR only but not on ARNT, as judged by the suppressive effect of si-AhR but not si-ARNT (Fig. 7C).

Discussion

The main objectives of this study were first to identify the major triggering event for the action of TCDD to initiate this inflammation pathway and second to clearly differentiate this pathway from the classic pathway. To this end, we could identify the calcium-triggered cPLA2 activation as the most pivotal early event controlling subsequent activation of Src kinase and induction of several key inflammation markers. To our knowledge, this is the first report establishing that cPLA2 is directly activated by TCDD in such a short time span. The importance of PLA2 in cellular inflammatory responses is well known (Yedgar et al., 2006). It also has been indicated already that PLA2 is probably playing an important role in mediating the toxicities of many organochlorine pollutants (Mariussen and Fonnum, 2006). Therefore, it is surprising that there has not been any previous report suggesting that TCDD-activated AhR directly activates cPLA2 in the toxic action of TCDD. To be sure, there are reports indicating that PLA2 is associated with oxidative stress that is caused by the induction of cytochromes P450 by TCDD (Mufti and Shuler, 1996). However, such a cytochrome P450-dependent oxidative stress is a result of the genomic action of TCDD, which should be clearly differentiated from what we have found in the current study, based on the quick activation of cPLA2 (i.e., occurring almost immediately after TCDD treatment).

It is also important to note that the activation of cPLA2 by TCDD is closely related to our past findings on the role of Src kinase in this cell line. The fact of activation of Src kinase within 30 min after TCDD treatment and its essential role in mediating the action of TCDD to induce cell stress response in this cell line has already been established (Mazina et al., 2004; Park et al., 2004, 2007). The main research gap in connecting our previous findings on Src kinase to the action of TCDD has been that we have not provided the logic and the experimental evidence indicating the precise mechanism through which TCDD activates Src kinase. This research gap has also prevented us from providing the rational explanation of the reason why Src kinase plays an important role in the manifestation of some of the toxic effects of TCDD until now. Thus, it is significant that we could provide the concrete evidence that TCDD initially activates cPLA2 and promote the release of arachidonic acid, which in turn activates Src kinase. This conclusion is supported by our finding that PP2 blocks the action of exogenous arachidonic acid to induce those inflammation markers. There is a precedent in this regard that exogenous arachidonic acid indeed activates Src kinase (Alexander et al., 2006). According to these authors,

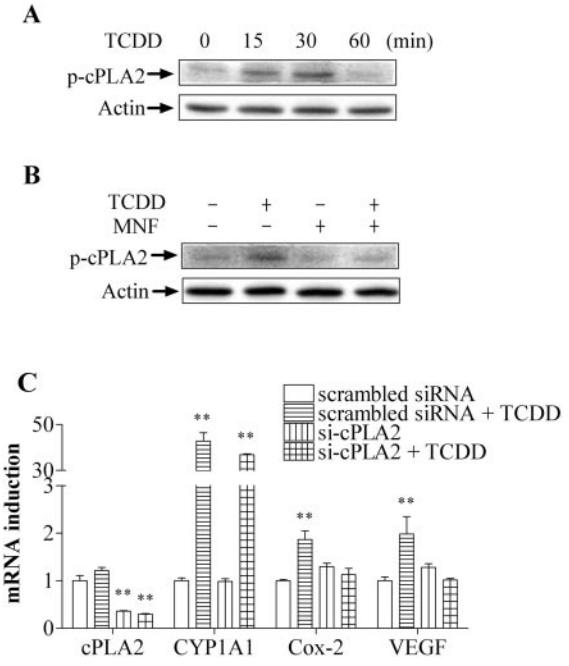

Fig. 4. Activation of cPLA2 by TCDD and the effect of si-cPLA2 to antagonize the action of TCDD to induce inflammation markers. A, MCF10A cells were treated with 10 nM TCDD acid for different time periods as indicated, and the titer of phospho-cPLA2 (Ser505) protein in comparison with that of actin was measured by Western blotting. B, MCF10A cells were preincubated with 10 μM MNF for 30 min followed by treatment of 10 nM TCDD for 15 min, and the resulting changes in protein titers were measured by Western blotting. C, MCF10A cells were treated with 100 nM scrambled siRNA (as control) or cPLA2 siRNA for 48 h followed by treatment with 10 nM TCDD for 2 h. The results are expressed relative to the values of the control, and the statistically significant differences between the control group and other groups are indicated by $*$, $p < 0.05$, or $**$, $p < 0.01$.

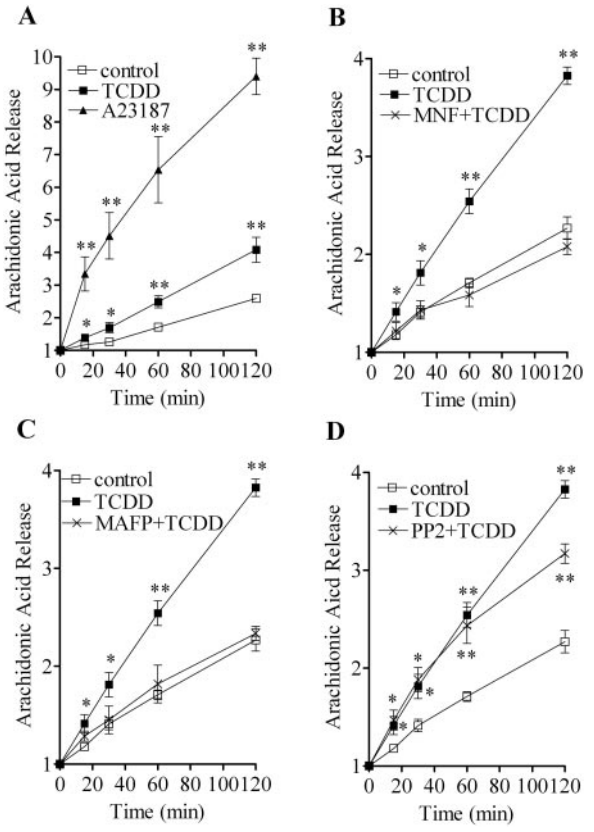

Fig. 5. Action of TCDD to promote [^{3}H]arachidonic acid release from MCF10A cells. The amount of [^{3}H]arachidonic acid release was measured at different time points after treatment as indicated. The results are expressed as relative values to the initial (0 min) background radioactivity for each treatment group. Control cells received only the vehicle solvent (*p*-dioxane). A, cells were treated with 10 nM TCDD or 2 μM A23187 as positive control. Cells were preincubated with 10 μM MNF (B), 20 μM MAFP (C), or 2 μM PP2 (D) for 30 min before the TCDD treatment. The statistically significant differences between the control group and other groups at the same time points are indicated by $*$, $p < 0.05$, or $**$, $p < 0.01$.

exogenous arachidonic acid mimics the action of angiotensin II on primary culture of rabbit proximal renal tubule cells by promoting Src protein association with EGFR through SH2, thereby activating Src kinase. Together, all of our findings in the current study suggest that the arachidonic acid release itself is likely to be the direct cause for Src kinase activation in this signaling pathway.

Our study also suggests that calcium probably serves as the initial trigger for this nongenomic action of TCDD. Calcium is well known to activate cPLA2. The involvement of calcium explains how TCDD activates cPLA2 in such a short time period. This conclusion indeed agrees well with the previous contributions from other research groups (Hanneman et al., 1996; Karras et al., 1996; Puga et al., 1997). For example, Hanneman et al. (1996) observed a significant increase in intracellular free calcium within a few seconds of addition of TCDD in rat hippocampal neuronal cells, and this action of TCDD could be antagonized by EDTA and by nifedipine. Puga et al. (1997) also found that TCDD treatment leads to a sustained elevation of intracellular free calcium and elevated levels of Cox-2 mRNA in mouse hepatoma cells. Karras et al. (1996) reached essentially the same conclusion in their studies on the action of TCDD on basal B-cells. All of these original works offer the consistent pattern of action of TCDD in other cell types to induce early increase in intracellular concentration of calcium.

Another objective of this study has been to clearly recognize this pathway through which TCDD activates this rapid inflammatory cellular responses apart from the well known classic, dioxin response element-mediated, genomic pathway. To this end, we have been successful in demonstrating the clearcut differences between these two pathways. In reaching this conclusion, we have relied on three important findings. The first finding is the effectiveness of specific inhibitors such as PP2, MAFP, and calcium-signaling blockers in blocking the early action of TCDD to induce several inflammation markers without affecting CYP1A1 induction. Second, we noted the ability of TCDD to induce arachidonic acid release at a very early time point, when the action of TCDD to induce CYP1A1 expression could not yet be observed (i.e., difference in timing of activation of these two pathways). The third evidence leading to this conclusion is the lack of blocking

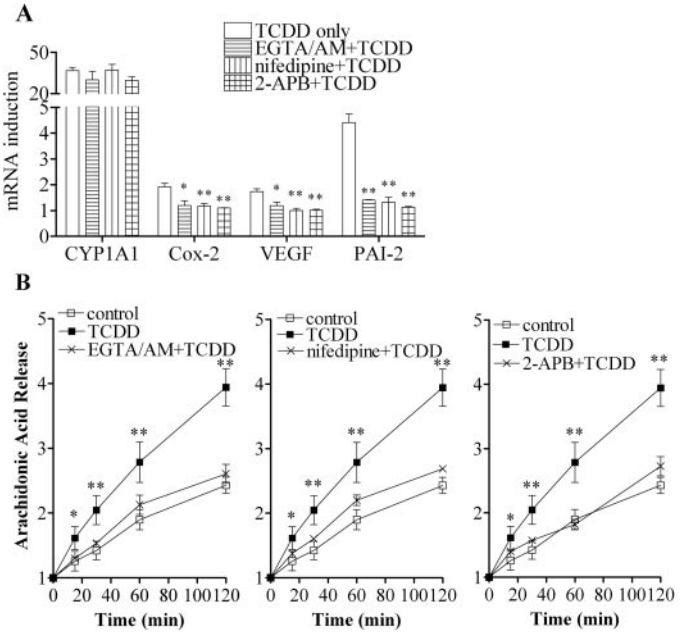

Fig. 6. Effects of calcium-signaling blockers on the action of TCDD to induce inflammation markers and to release arachidonic acid. MCF10A cells were preincubated with 10 μM EGTA/AM, 25 μM nifedipine, or 50 μM 2-APB for 30 min. A, for mRNA induction measurement, cells were then treated with 10 nM TCDD for 2 h. The results are expressed as folds of induction after TCDD treatment and the statistically significant differences between and the TCDD-only group and the groups preincubated with inhibitors are indicated by *, $p < 0.05$, or **, $p < 0.01$. B, for arachidonic acid release measurement, MCF10A cells were then treated with 10 nM TCDD, and the amount of arachidonic acid release was measured at different time points after treatment as indicated. The results are expressed as relative values to the initial (0 min) background radioactivity for each treatment group, and the statistically significant differences between the control group and other groups at the same time points are indicated by *, $p < 0.05$, or **, $p < 0.01$.

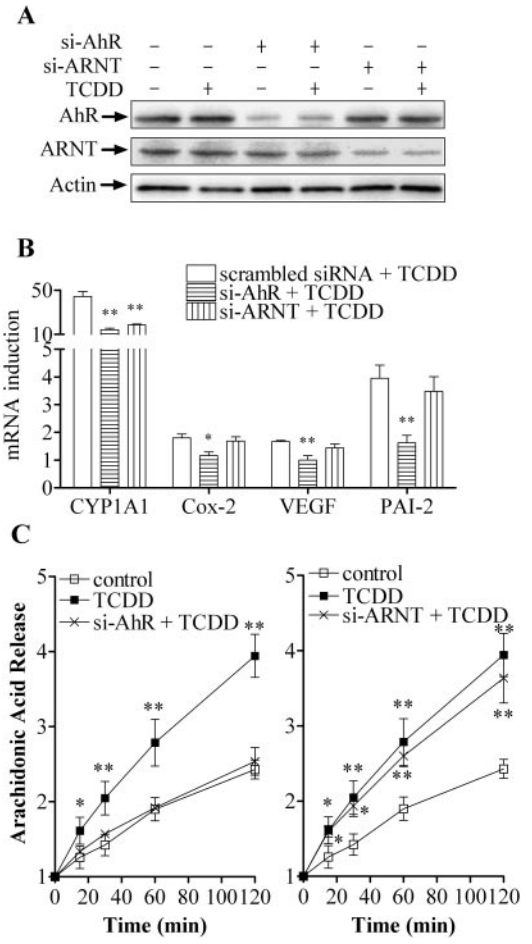

Fig. 7. Effects of siRNA transfection against AhR and ARNT on the action of TCDD to induce inflammation markers and to release arachidonic acid. MCF10A cells were treated with 100 nM scrambled siRNA (as control), AhR siRNA or ARNT siRNA for 48 h before TCDD treatment. A, AhR, ARNT, and actin protein levels were measured after siRNA treatment by Western blotting. B, for mRNA induction measurement, cells were treated with 10 nM TCDD for 2 h. The results are expressed as folds of induction after TCDD treatment, and the statistically significant differences between the scrambled siRNA + TCDD group and other groups are indicated by *, $p < 0.05$, or **, $p < 0.01$. C, for arachidonic acid release measurement, cells were then treated with 10 nM TCDD, and the amount of arachidonic acid arachidonic acid release was measured at different time points after treatment as indicated. The results are expressed as relative values to the initial (0 min) background radioactivity for each treatment group, and the statistically significant differences between the control group and other groups at the same time points are indicated by *, $p < 0.05$, or **, $p < 0.01$.

action of si-ARNT on TCDD-induced up-regulation of those inflammation markers in contrast to that of CYP1A1. These observations allowed us to designate this newly delineated pathway as the "nongenomic" pathway of the action of TCDD.

The term "nongenomic" used in this article has been adopted from the well known phenomenon of two distinct action pathways of steroid hormones through their respective receptors, particularly that of estrogen receptor signaling (Kim and Bender, 2005). It is now well acknowledged that some of the rapid cellular responses, elicited upon 17-β-estradiol binding to the estrogen receptor, are mediated through the nongenomic pathway that is aided by protein kinases, including extracellular signal-regulated kinase and Src kinase (Silva and Shupnik, 2007) and cPLA2 (Fiorini et al., 2003), as shown in several types of hormone-responsive cells. Although this analogy may not be totally applicable to the case of the TCDD-induced inflammatory cellular responses, the fact that such TCDD-induced responses also occur very rapidly through the activation of cPLA2, followed by the release of arachidonic acid and the activation of Src kinase, all of which occur within 30 min, clearly supports our notion that these are the typical signs of nongenomic action, which means there is no de novo protein synthesis involved. In fact, we have also assessed the induction of cPLA2 gene using a cPLA2-Luc reporter plasmid assay and found that there was no detectable sign of induction of cPLA2 gene during the initial period of 2 h after TCDD treatment (data not shown), which rules out the involvement of genomic action, at least during this early period of action of TCDD.

Having explained our rationale of using the term "nongenomic" for this particular pathway, however, it must be cautioned that there is a good possibility that at later time points of action of TCDD, many of the nongenomic actions will be converted into genomic messages to make the initial transient signaling of the receptor into more long-lasting messages. Furthermore, there might be more than one nongenomic pathway for the action of TCDD. We must also be careful not give the impression that this entire inflammation pathway found in MCF10A cells may be accepted as a good model for all "nongenomic" actions of TCDD, because the details of this type of protein phosphorylation-mediated pathway may vary among different types of cells, even within a single species. Much more work would be needed in the future, therefore, to study this phenomenon in many different types of cells to confirm or modify the intricate events of signaling pathway processing applicable to each type of cells to arrive at the finally established pathway and to further clarify the nature of this pathway apart from other similar pathways. Despite these reservations, nevertheless, we are confident in our assessment that the fundamental aspect of the current findings such as the initial calcium entry and subsequent activation of cPLA2 and Src kinase, which triggers this "nongenomic" pathway mediated by AhR (but not by ARNT), is probably applicable to many types of cells, judging by our results and those from other research groups (Hanneman et al., 1996; Karras et al., 1996; Puga et al., 1997).

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