Significance of the Nongenomic, Inflammatory Pathway in Mediating the Toxic Action of TCDD To Induce Rapid and Long-Term Cellular Responses in 3T3-L1 Adipocytes[†]

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ABSTRACT: TCDD (dioxin) induces a rapid inflammatory response from 3T3-L1 adipocytes as judged by prominent induction of the mRNA expression of prostaglandin-endperoxide synthase 2 (Cox-2) along with other inflammation markers within 1 h. This action of TCDD is clearly antagonized by cell pretreatment with AACOCF3 (an inhibitor of cPLA2), nifedipine (a Ca^{2+} channel blocker), or 3′-methyl-4′-nitroflavone (MNF), an antagonist of the Ah receptor (AhR), suggesting the possible involvement of the nongenomic pathway of action of TCDD as shown previously in MCF10A cells [Dong, B., and Matsumura, F. (2008) *Mol. Pharmacol. 74* (1), 255−263]. This early inflammatory action of TCDD is clearly different from that mediated by its classical action pathway in that the former is mediated by protein kinases such as PKC, PKA, and tyrosine kinases, but not by ARNT. Furthermore, the former is not blocked by two "DRE-decoy" treatments. Such an inflammatory effect of TCDD on 3T3-L1 adipocyes persists at least for 5 days, when the affected adipocytes exhibit significant reduction in their adipocyte characteristics. To assess the cause for the long-lasting influence of this nongenomic action of TCDD, we tested the effects of AACOCF3, exogenous arachidonic acid (AA), and H89 (an inhibitor of PKA) on the 5 day action of TCDD. These agents clearly antagonized all the long-term actions of TCDD except that on CYP1A1 induction, indicating that the influence of the nongenomic action of TCDD lasts a long time in this cell material. One of the major factors mediating its long-lasting effects has been identified to be PKA.

Despite the tremendous progress made on the action mechanisms of TCDD[1] that induce CYP1A1 and other detoxification enzymes by many toxicologists (*1*−*3*), there are still serious research gaps in understanding the mechanisms through which this compound causes many toxic end results in animals. Certainly, many experts now agree that almost all of its toxicities are mediated by the aryl hydrocarbon receptor (AhR) and that to carry out the task of mediating toxic signaling of TCDD functional activation of this receptor is the key to understanding the toxic actions of TCDD (*4*). The most well studied action model includes the ligand-activated AhR traveling into the nucleus with the aid of x-protein-associated protein 2 (XAP2), a cochaperone protein (*5*), where it dimerizes with another helix−loop−helix protein called ARNT (*3*), and finally, it is this heterodimer that binds to the dioxin response element (DRE, or sometimes called XRE), which is present in the promoter region of AhR-controlled, "target" genes such as CYP1A1, to transactivate them. This well-established action model, hereafter termed the classical action pathway, has served superbly so far to explain many of the phenomena occurring as a result of exposure of animals to TCDD. However, there have been a number of recent reports indicating that there are events that cannot be satisfactorily explained by this classical action model alone. Good examples may the case of AhR dimerizing with the estrogen receptor (*6*) to influence its hormonal signaling activities and the case of direct interaction of AhR with the Rel A protein of the NFkB family which thereby affects the inflammatory signaling of the latter (*7*). The main point of bringing up such examples here is that at least there are some concrete precedents illustrating the principle that the classical action pathway is not the only way TCDD could induce cellular changes.

From a different point of view, our research group has been studying the phenomenon of TCDD-induced rapid activation of protein kinases (*8, 9*). On the basis of such observations, Matsumura (*10*) initially proposed that some of the early toxic actions of TCDD could mainly be mediated by nongenomic means through protein kinases and phosphatases. Indeed, in many cells, protein kinases are activated

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[1] Abbreviations: AA, arachidonic acid; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; Cox-2, prostaglandin-endperoxide synthase 2; cPLA2, cytosolic phospholipase A2; CYP1A1, cytochrome P450-1A1; Dex, dexamethasone; DMSO, dimethyl sulfoxide; DRE, dioxin response element; FBS, fetal bovine serum; FSK, forskolin; HSP90, heat shock protein 90; IBMX, 3-isobutyl-1-methylxanthine; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; MNF, 3′-methoxy-4′-nitroflavone; Nifedpine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl eater; PCR, polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; PTKs, protein tyrosine kinases; SOCS-3, suppressor of cytokine signaling; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TLR, Toll-like receptor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; VO4, sodium vanadate; XAP2, x-associated protein 2.

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Table 1: PCR Primers Used in This Study

gene	forward primer	reverse primer
CYP1A1	GGC CAC TTT GAC CCT TAC AA	CAG GTA ACG GAG GAC AGG AA
Cox-2	AGA AGG AAA TGG CTG CAG AA	GCT CGG CTT CCA GTA TTA AG
IL-8	CTT GAA GGF GTT GCC CTC AG	TGG GGA CAC CTT TTA GCA TC
MCP-1	TCT GGA CCC ATT CCT TCT TG	AGG TCC CTG TCA TGC TTC TG
VEGF	CAG GCT GCT GTA ACG ATG AA	AAT GCT TTC TCC GCT CTG AA
RelB	TGA TCC ACA TGG AAT CGA GA	CAG GAA GGG ATA TGG AAG CA
cPLA2	TGG CTC TGT GTG ATC AGG AG	GAG CCA GAA AGA CCA GCA AC
SOCS-3	GCG AGA AGA TTC CGC TGG TA	CCG TTG ACA GTC TTC CGA CAA
PPARγ	AAT CCT TGG CCC TCT GAG AT	TTT TCA AGG GTG CCA GTT TC
leptin	CTC ATG CCA GCA CTC AAA AA	AGC ACC ACA AAA CCT GAT CC
TNFα	ATG AGC ACA GAA AGC ATG ATC CGC	CCA AAG TAG ACC TGC CCG GAC TC
β-actin	AGC CAT GTA CGT AGC CAT CC	CTC TCA GCT GTG GTG GTG AA

by TCDD (*10*). Particularly noticeable is rapid activation of Src tyrosine kinase and ERK kinase that have been found to be activated by TCDD in MCF10A mammary epithelial cells within a short period of 5−15 min (*11, 12*), making it highly unlikely that such an action of TCDD is carried out through the classical action pathway, which requires de novo protein synthesis to accomplish its mission.

Recently, we have realized that certain TCDD-induced toxic responses such as "wasting syndrome" observed in experimental animals are actually very similar to the symptom called cachexia that is induced by bacterial endotoxins, such as lipopolysaccharides (LPSs) in animals (*13*). LPSs have been recognized to cause inflammatory cellular responses through their binding to the Toll-like receptors (more precisely TLR4). It is known that inflammation signaling mediated by this family of receptors is mediated by a series of activations of protein kinases. On the basis of these considerations, we have formulated a hypothesis that this type of protein kinase-mediated action of TCDD is closely related to "cellular stress responses", including inflammation. To test the hypothesis given above in a type of cell relevant to wasting syndrome, we have chosen 3T3-L1 adipocytes, since we have considerable experience with this cell material and since we recently could establish a long-term in vitro test protocol in inducing the typical wasting response, which is defined as the loss of stored lipids by those adipocytes, often termed the "lipolytic" action of TCDD occurring in vivo (*14*).

The primary objectives of this project are (a) to identify the upstream events occurring at every early stage of action of TCDD in 3T3-L1 adipocytes, (b) to distiguish this inflammation-inducing action of TCDD from that of its classical action to induce CYP1A1, and (c) to relate such an inflammatory action of TCDD to its long-term effect to cause the loss of lipids from those adipocytes.

EXPERIMENTAL PROCEDURES

Chemicals. ^{3}H-labeled arachidonic acid (AA) was purchased from Perkin-Elmer (Waltham, MA). TCDD (>99.99% purity) was originally obtained from Dow Chemical Co. (Midland, MI) and was prepared as 1000-fold concentrated stocks (10 μM) in dimethyl sulfoxide (DMSO). H89, forskolin (FSK), AACOCF3, Genistein, H7, VO4, 3-isobutyl-1-methylxanthine (IBMX), indomethacin, and dexamethasone (Dex) were purchased from Calbiochem (San Diego, CA). DMSO, arachidonic acid (AA), 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl eater (Nifedpine), insulin, and TNFα were obtained from Sigma-Aldrich Co. (St. Louis, MO). 3′-Methoxy-4′-nitroflavone (MNF) was a kind gift from J. Abel (University of Dusseldorf, Dusseldorf, Germany). Other molecular biological reagents for RT-PCR were purchased from Qiagen (Valencia, CA) and Roche Applied Science (Indianapolis, IN).

Cell Culture, Differentiation, and Chemical Treatments. 3T3-L1 fibroblasts were obtained from American type Culture Collection (ATCC) (Manassas, VA). Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS). Confluent cells were made to differentiate by incubation for 2 days with differentiation medium, containing 1 μM dexamethasone (Dex), 0.2 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μg/mL insulin, and 10% FBS in DMEM. After this time, cells were kept in the "maintenance medium", DMEM containing 10 μg/mL insulin and 10% FBS, and the medium was changed every 2−3 days (*15*). After differentiation in maintenance medium for an additional 5 days, colonies show the visible signs of mature adipocytes as confirmed by the appearance of rounded cells with numerous intracellular lipid droplets. These cells are designated as 3T3-L1 adipocytes (or sometimes called 7 day differentiated 3T3-L1 adipocytes) and used as the main source of this study.

To study the effects of TCDD or other chemicals, thus differentiated adipocytes were treated with TCDD alone or together with other chemicals for specified periods of time by addition of test compounds to fresh DMEM. Chemicals to be tested were prepared as 1000-fold concentrated stocks in DMSO. For the 5 day experiment, all chemical treatments (TCDD, AACOCF3, H89, and/or AA) were given at the time of the first medium change (given after differentiation for 7 days), and the same chemical treatments were renewed at the time of two additional medium changes (i.e., 48 and 96 h from the first chemical treatment). Control samples received an equal volume of the vehicle DMSO alone.

Quantitative Reverse Transcriptase PCR (real-time RT-PCR). Total RNA was isolated from 3T3 adipocyte cells using a highly pure RNA isolation kit (Qiagen), and cDNA synthesis was carried out as described by Vogel et al. (*16*). Briefly, DNA-free total RNA (1.0 μg) was reverse-transcribed using 4 units of Omniscript reverse transcriptase (Qiagen) and 1 μg of oligo(dT)$_{15}$ in a final volume of 40 μL. Mouse-specific primers of CYP1A1, Cox-2, KC (IL-8), SOCS-3, MCP-1, VEGF, RelB, cPLA2, PPARγ, leptin, and β-actin (as a normalization control) were selected from published sources and are described in Table 1. Quantitative detection of β-actin and the primers described above was

performed with a LightCycler Instrument (Roche Diagnostics) using the QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. PCR amplification was carried out in a total volume of 20 μL, containing 2 μL of cDNA, 10 μL of 2× QuantiTect SYBR Green PCR Master Mix, and the primers (0.2 μM each). The PCR cycling conditions were 95 °C for 15 min followed by 40−50 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 10 s. Detection of the fluorescent product was performed at the end of the 72 °C extension period. Negative controls were run concomitantly to confirm that the samples were not cross-contaminated. A sample with DNase- and RNase-free water instead of RNA was concomitantly examined for each of the reaction units described above. To confirm the amplification specificity, the PCR products were subjected to melting curve analysis. All PCR assays were performed in duplicate or triplicate. The intra-assay variability was <7%. For quantification, data were analyzed with the LightCycler analysis software according to the manufacturer's instructions.

Cell Transfection Experiments for DRE Decoy and Luciferase Assays. For transient transfection experiments, 3T3-L1 adipocytes were differentiated in 24-well plates. After being differentiated for 7 days, cells were washed once with PBS, and 0.45 mL of fresh growth medium was added before transfection complexes were applied drop by drop to the cells.

For DRE decoy experiments, cells were transiently transfected for 16 h by using 2 μL of jetPEI (PolyTransfection, Qbiogene, Irvine, CA) per well with 0.25 μg per well of DRE according to the manufacturer's instructions. In one experiment, cells were transfected with a DRE-Luc reporter plasmid, along with an identical reporter plasmid without the DRE sequence as a blank. The DRE luciferase reporter was kindly provided by J. Abel (University of Duesseldorf). In the other experiment, cells were transfected with a double-stranded oligonucleotide (dsOligo) containing DRE (5′-GCCCCGGAGTTGCGTGAGAAGAGCCTGG-3′), along with an equivalent ds oligonucleotide containing a mutant ds oligonucleotide (5′-GCCCCGGAGTTGCGCGAGAA-GAGCCTGG-3′) as a blank. These oligonucleotides were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). After transfection, cells were incubated for 24 h with 10 nM TCDD. Cells were then washed twice with PBS before RNA extraction and real-time RT-PCR analysis.

For luciferase assays, cells were transiently transfected for 16 h by using 2 μL of jetPEI (PolyTransfection, Qbiogene) per well with 0.25 μg per well of a *cPLA*$_2$-luc or *Cox-2*-luc reporter plasmid according to the manufacturer's instructions. An identical reporter plasmid without the *cPLA*$_2$ or *Cox-2* sequence was used as a blank. The *cPLA*$_2$ luciferase reporter plasmid was kindly provided by G. D'Orazi (University G. d'Annunzio, Chieti, Italy) and R. A. Nemenoff (University of Colorado Health Sciences Center, Denver, CO) (*17*). The *Cox-2* luciferase reporter was kindly provided by H. Herschman (University of California, Los Angeles, CA) (*18*). After transfection, cells were incubated for 24 h with 10 nM TCDD alone or together with 20 μM AACOCF3 and 10 μM Nifedipine. Cells were then washed twice with PBS and lysed with 100 μL of passive lysis buffer. Luciferase activities were measured with the Luciferase Reporter Assay System (Promega, Madison, WI) using a luminometer (Lumat LB 9501/16, Berthold, Pittsburgh, PA).

Arachidonic Acid Release Measurement. Arachidonic acid (AA) release was assessed according to the method of Viu et al. (*19*). 3T3-L1 adicypotes were differentiated in 12-well plated. After differentiation for 7 days, 0.2 μCi of [^{3}H]arachidonic acid (specific activity of 180 Ci/mmol) was added to each well and incubated overnight. After 24 h, the [^{3}H]arachidonic acid-containing medium was discarded and the cells were washed twice with complete growth medium to remove free [^{3}H]arachidonic acid which was not incorporated into the cells. The cells were then pretreated with AACOCF3 or MNF for 0.5 h before being treated with TCDD. After each incubation period, medium was collected and centrifuged to remove possible contamination by occasionally present, detached cells. The radioactivity released from the cells was measured using a liquid scintillation counter (Beckman, Coulter Inc., Fullerton, CA).

Protein Kinase A Assay. Protein kinase A (PKA) activity was determined in cell lysates using a PKA assay kit (Upstate Biotechnology Inc., Lake Placid, NY) as described by Vogel et al. (*20*). Briefly, 3T3-L1 adipocytes in 10 cm culture dishes were washed once with ice-cold PBS and scraped into 0.5 mL of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.1% Triton X-100. Samples were passed through a 21-gauge needle, and insoluble material was removed by centrifugation at 800*g* for 2 min. The supernatant was assayed in the presence of 5 μCi of [γ-^{32}P]ATP using synthetic Kemptide at 100 μM substrate. Total PKA activity was measured by the addition of 2 μM cAMP. The basal activity, a measure of active PKA at the time of harvest, was measured in the absence of exogenous cAMP. Unincorporated ^{32}P was removed by spotting samples on phosphocellulose paper and washing them three times with dilute H_3PO_4 (0.75%) followed by one acetone wash. The amount of ^{32}P was quantified by scintillation counting. Nonspecific activity, determined in the presence of PKA inhibitor peptide, was subtracted.

Statistical Analysis. All experiments were repeated a minimum of three times, and results are expressed as means ± standard deviations. Data were evaluated statistically by a one-way ANOVA followed by a Student's *t* test.

RESULTS

Studies on the Early Action of TCDD in 3T3-L1 Adipocytes. To gain insight into the process through which TCDD induces rapid inflammatory responses from 3T3-L1 adipocytes, we have examined the early time course (30 min to 3 h) of the action of TCDD to upregulate the expression of selected mRNA markers as shown in Figure 1. It was found that in this cell line, TCDD causes only a marginal sign (i.e., nonsignificant) of induction of mRNA of CYP1A1, during this initial 3 h. In contrast, there were clear signs of significant upregulation of mRNA expression of Cox-2, VEGF, TNFα, and IL-8, indicating that the pattern of early responses of this cell line to TCDD is qualitatively similar to that of MCF10A (*21*) except that in that cell line there was no induction of IL-8. Among those early responding markers, activation of Cox-2 mRNA expression was most clearly observed in 3T3-L1 adipocytes as in the case of U937 macrophages (*16*). We therefore examined the inhibitory actions of three diagnostic agents on TCDD-induced upregulation of Cox-2 mRNA (Figure 2). Of those, MNF, a

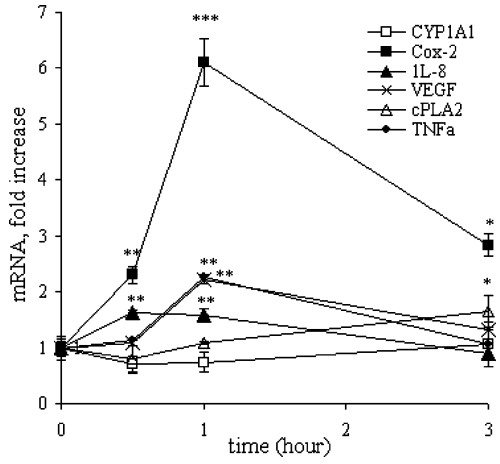

FIGURE 1: Early time course of action of TCDD (10 nM) in inducing the expression of selected mRNA markers in 3T3-L1 adipocytes as assessed by qRT-PCR. The data are expressed in fold increase over control (=1.0). The results are expressed as mean values from three to five independent tests. Statistically significant differences between the treatments and control (0 h) are indicated by one ($p < 0.05$), two ($p < 0.01$), or three ($p < 0.001$) asterisks.

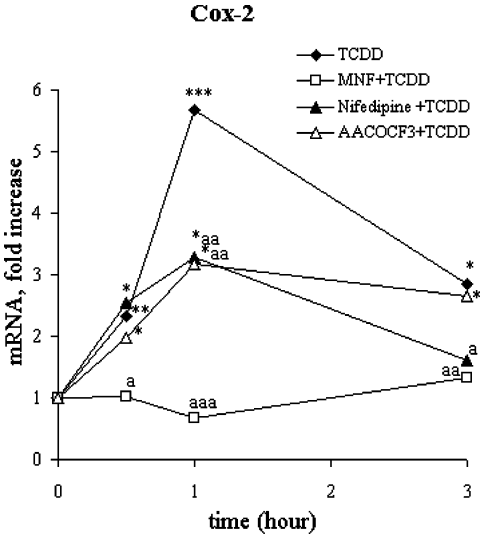

FIGURE 2: Assessment on the effect of diagnostic inhibitors on the action of TCDD during the early time course of its action to induce the Cox-2 mRNA expression in 3T3-L1 adipocytes. The results are expressed as fold increases over control (=1.0). 3T3 adipocytes were pretreated with MNF (10 μM), AACOCF3 (20 μM), or Nifedpine (10 μM) for 1 h before TCDD treatment. The results are shown as mean values from three to five independent tests. Statistically significant differences between treatments and control (0 h) are indicated by asterisks, and those between TCDD and TCDD with the inhibitor are shown with the letter a. The number of symbols indicates the following: $p < 0.05$ (one), $p < 0.01$ (two), or $p < 0.001$ (three).

specific antagonist of AhR, exhibited an outstanding inhibitory property, completely attenuating the action of TCDD on Cox-2, indicating that this phenomenon of early upregulation of these inflammation-related mRNAs is clearly dependent upon AhR. In addition, both AACOCF3, an inhibitor of cytosolic phospholipase A2 (cPLA2), and Nifedipine, a blocker of the L-type Ca^{2+} channel, were found to produce significant but incomplete inhibitions (each reducing the level of Cox-2 mRNA expression to approximately 45%).

Assessment of the Release of Arachidonic Acid from 3T3-L1 Adipocytes. Although we did not observe early induction

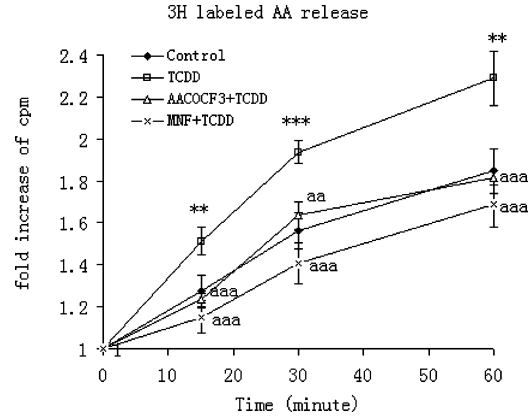

FIGURE 3: Effect of TCDD on the release of free ^{3}H-labeled arachidonic acid (AA) from 3T3 adipocytes and its inhibition by 20 μM MNF (an inhibitor of AhR) and 20 μM AACOCF3. [^{3}H[Arachidonic acid was incorporated into 3T3 adipocytes as described in Experimental Procedures. The results are mean values from three to five independent tests. Statistically significant differences between TCDD and matched control are indicated with asterisks, and those between TCDD and TCDD with the inhibitor are shown with the letter a. The number of symbols indicates $p < 0.05$ (one), $p < 0.01$ (two), or $p < 0.001$ (three).

of cPLA2 mRNA expression within 0.5 and 1 h of action of TCDD (Figure 1), this finding of the inhibitory effect of AACOCF3 and the Ca^{2+} signaling blocker on Cox-2 expression at this early stage of action of TCDD indicates that the enzymatic activity of cPLA2 is likely increased at this point, since it is well-known that the enzymatic activity of cPLA2 is stimulated by the increased intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), and since this process is likely antagonized by AACOCF3. To test this possibility, we have assessed directly the release of free arachidonic acid (AA) from these cells by using [^{3}H]AA-prelabeled 3T3-L1 adipocytes according to the protocol of Viu et al. (*19*). The amount of [^{3}H]AA released into the culture medium was indeed significantly higher in cells treated with TCDD within 1 h (Figure 3). Moreover, both AACOCF3 and MNF were found to be very effective in antagonizing the effect of TCDD in facilitating the release of [^{3}H]AA. This test result has clearly established that cPLA2 is one of the most upstream components of this nongenomic signaling pathway of TCDD in 3T3-L1 adipocytes as in the case of MCF10A cells (*21*).

Likely Involvement of Protein Kinases in the Early Action of TCDD. The finding of very rapid activation of cPLA2 and Cox-2 described above suggests that this aspect of the action of TCDD is likely mediated by the nongenomic signaling pathway, rather than through the classical, genomic action pathway. One of the common characteristics of "nongenomic pathways" is likely to be the early involvement of protein kinases, judging by the well-studied cases of ligand-activated steroid receptor signaling, which are transmitted through the genomic and nongenomic pathway. In the case of action of TCDD, for instance, it is known that mRNA activation of Cox-2 at a very early stage of action of TCDD in hepatocytes is mediated by protein kinases (*22*). Accordingly, we tested the effect of several class-specific kinase inhibitors along with their matched stimulators of each type of protein kinases on the expression of several mRNAs. The results summarized in Figure 4 show that the Cox-2 expression in 3T3-L1 adipocytes treated with TCDD for 1 h is indeed greatly affected by protein kinases. Judging by the

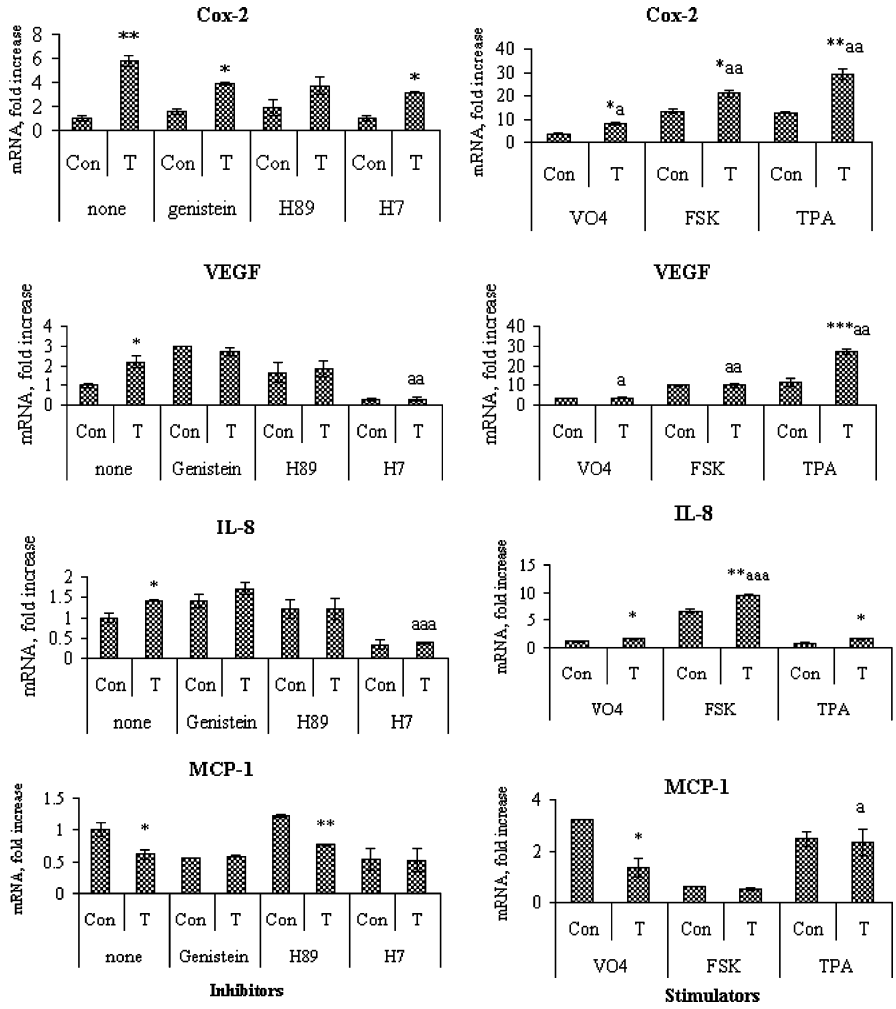

FIGURE 4: Effects of protein kinase class-specific inhibitors (i.e., Genistein, H89, and H7) and stimulators (VO4 and FSK TPA) on the 1 h action of TCDD (10 nM) to alter the mRNA expression of inflammation marker mRNAs in 3T3-L1 adipocytes differentiated for 7 days. 3T3-L1 adipocyte cells were pretreated with inhibitors or stimulators for 1 h. The results are shown as means ± standard deviations from three to five independent tests. Statistically significant differences between each pair of TCDD (T) and matched control (Con) (i.e., effect of TCDD regardless of the presence or absence of other chemicals) and that between TCDD (second colum from left) and TCDD with the inhibitor or stimulators (i.e., effect of inhibitor or stimulators on the action of TCDD) are indicated with asterisks and the letter a, respectively. The number of symbols indicates $p < 0.05$ (one) or $p < 0.01$ (two).

action of stimulators, for instance, Cox-2 expression is greatly enhanced by their specific activators of serine/threonine kinases (i.e., TPA for PKC and forskolin for PKA), and to a lesser degree by tyrosine kinases (activated by VO4, an inhibitor of protein tyrosine phosphatases). At the same time, their actions are generally antagonized by respective inhibitors of class-specific protein kinases (i.e., by Genistein for tyrosine kinases, H89 for PKAs, and H7 for PKCs), though none of the inhibitors could totally abrogate the action of TCDD on Cox-2 by itself. In contrast, although the pattern of activation of VEGF (vascular endothelial growth factor) by these stimulators was similar to that of Cox-2, all kinase inhibitors abolished the action of TCDD to upregulate VEGF mRNA. The patterns of activation of IL-8 and MCP-1 by kinase stimulators, on the other hand, were very different from the two mRNA expressions described above; e.g., the expression of IL-8 mRNA is mostly activated by forskolin (i.e., PKA-dependent), and that of MCP-1 is stimulated by VO4 and TPA. It is somewhat surprising to find that the cell responses to the effect of TCDD and kinase stimulators as well as inhibitors on IL-8 and MCP-1 were opposite from each other, since these two are generally considered to be inflammation mediators. However, in adipocytes, MCP-1 is known to act as a suppressor of insulin-stimulated uptake of glucose (*23*), unlike IL-8, and therefore, at this timing of action of TCDD, it is possible that MCP-1 is suppressed by TCDD as a negative feedback of the initial inflammatory action of TCDD through activation of tyrosine kinase. Nevertheless, the important information we obtained from this set of experiments is that, in all cases, the effect of TCDD in inducing these mRNAs was mostly attenuated by those kinase inhibitors, except in the case of MCP-1, which was not downregulated by TCDD in the presence of H89. Together, these results support our notion that the inflammatory responses taking place within 1 h of the action of TCDD in this cell material are strongly influenced by activation of a number of protein kinases.

Studies on the Role of cPLA2 and Ca^{2+} in the Nongenomic Action of TCDD. It was noted that in the case of Cox-2 none of these generic inhibitors of protein kinases could totally suppress the action of TCDD to upregulate its mRNA (Figure 4). Furthermore, in the experiment depicted in Figure 2, it was observed that, when each inhibitor was tested alone, Nifedipine (an L-type Ca^{2+} channel blocker) or AACOCF3 (a specific inhibitor cPLA2) caused significant but only partial inhibition (approximately 45%) at 1 h; we have

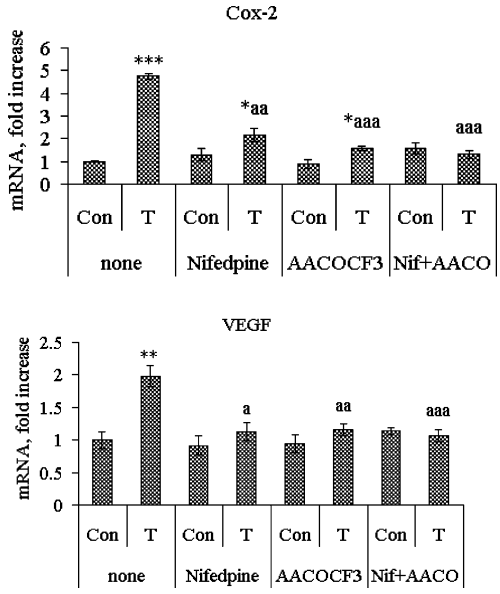

FIGURE 5: Action of TCDD over 1 h to induce Cox-2 and VEGF mRNA expressions in 3T3-L1 adipocytes in the presence of AACOCF3 (AACO) and Nifedpine (Nif). 3T3 adipocytes were pretreated with AACOCF3 (20 μM) or Nifedpine (10 μM) for 1 h before TCDD treatment. The results are shown as means ± standard deviations from three to five independent tests. Statistically significant differences between TCDD and matched control in each pair (regardless of the presence or absence of other chemicals) and that between TCDD (second column from the left) and TCDD with the inhibitor (i.e., effect of inhibitor on the action of TCDD) are indicated with asterisks and the letter a, respectively. The number of symbols indicates $p < 0.05$ (one), $p < 0.01$ (two), or $p < 0.001$ (three).

decided to examine the possibility of these two inhibitors having additive effects (i.e., AACOCF3 with Nifedipine). As shown in Figure 5, this turned out to be the case; i.e., in the presence of both inhibitors, the action of TCDD to upregulate Cox-2 or VEGF mRNA could not be observed.

DRE Decoy Experiments To Distinguish the Nongenomic Action of TCDD from Its Classical Action Pathway. To determine whether the action of TCDD to activate Cox-2 mRNA described above is qualitatively different from the classical action of TCDD to induce CYP1A1, we have decided to investigate the action of TCDD at a later time point of 24 h (Figure 6), when CYP1A1 induction becomes highly significant. For this purpose, we explored the possibility of employing two different "DRE [dioxin response element sequence (XRE)] decoy" treatment approaches (i.e., introducing excess amounts of oligonucleotides containing the DRE sequence, "GCGTG", and thereby to specifically block the classical pathway only). As shown in Figure 6, pretreatment of 3T3-L1 adipocytes with either (a) an excess amount of the "DRE-Luc" reporter plasmid (left two figures) or (b) a large quantity of double-stranded oligonucleotide (dsOligo) (right two figures), containing the consensus DRE sequence of oligonucleotides, taken from the promoter region of the CYP1A1 gene, clearly suppressed the action of TCDD to induce CYP1A1 mRNA expression over 24 h. It is likely, therefore, that these treatments successfully sequestered the bulk of the active AhR−ARNT protein complex in the nucleus away from the endogenous DRE sites present on the promoter region of the CYP1A1 gene in 3T3-L1 adipocytes. On the other hand, neither (a) an equivalent treatment with the matched empty Luc-reporter plasmid (i.e., the same reporter construct without DRE sequences) nor (b) a mutated and therefore inactive DRE ds oligonucleotide (GCG**C**G instead of GCG**T**G) was effective in suppressing CYP1A1 expression, supporting our interpretation. In contrast, neither of these two decoy treatments was effective in suppressing the expression of Cox-2; rather, both DRE decoy treatments actually enhanced the action of TCDD to upregulate Cox-2 mRNA. Such an observation may indicate the existence of a possible competitive relationship for the available AhR protein between these two pathways. To obtain supportive evidence, we further tested the effect of the Luc reporter-based decoy treatment on several other mRNA expressions. As expected, this DRE decoy treatment appears also to enhance the nongenomic action of TCDD to upregulate VEGF, IL-8, and MCP-1 (Figure 7).

Effect of Cell Treatment with siRNA against ARNT. To confirm the observation on the noninvolvement of the AhR−ARNT dimer in this nongenomic action of TCDD described above, we have assessed the effect of cell treatment with siRNA against ARNT (*21, 24*) on the effectiveness of TCDD in inducing mRNA expression of Cox-2 and IL-8 in comparison to that of CYP1A1 over 24 h (Figure 8). The result showed that this treatment clearly antagonized the effect of TCDD to induce CYP1A1 mRNA to the extent that, in the siARNT-treated pair of samples, no significant effect of TCDD could be observed. In contrast, the actions of TCDD to induce Cox-2 and IL-8 mRNA were still significant in those samples that have been treated with siARNT, although this siARNT treatment moderately reduced the extent of the action of TCDD on Cox-2 mRNA. It must be added that the effectiveness and the transfection efficiency of this siRNA preparation against ARNT have already been rigorously established in this laboratory (*21, 24*).

Studies on the Gene Activation Status of cPLA2 through a Luciferase Reporter Assay System. It is known that even in the cases of nongenomically induced signaling of steroid receptors (e.g., nongenomic signaling of the estrogen receptor) the message generated by its lignad must be eventually converted into genomic ones to have long-term cellular changes stabilized. For this purpose, we have selected the time point of 24 h to assess the effect of TCDD on *cPLA2* and *Cox-2* gene activation through the use of a luciferase reporter assay approach (Figure 9) as we have done previously in the U937 macrophage model (*24*). As a result, it was possible to demonstrate that indeed at this late stage of action of TCDD the initial nongenomic signaling of TCDD is already converted into the genomic actions of activated *cPLA2* and *Cox-2* gene expression (i.e., as assessed by induction of luciferase activity). Nevertheless, in the presence of either inhibitor, the action of TCDD to activate either gene expression became insignificant. Between these two inhibitors, AACOCF3 appears to exhibit a stronger antagonistic action against TCDD, suggesting that the initial nongenomic action of TCDD to activate cPLA2 enzyme is comparatively more important to dictating the later consequence of action of TCDD on activation of these two gene expressions.

Studies on the Long-Lasting Influence of Nongenomic Action of TCDD with Respect to Its Action in Causing the Loss of Adipocyte Characteristics in 5 Days. Finally, we conducted a study on the long-term effect of TCDD for 5 days according to the protocol of Li et al. (*14*) to see how such an initial nongenomic, inflammatory action of TCDD

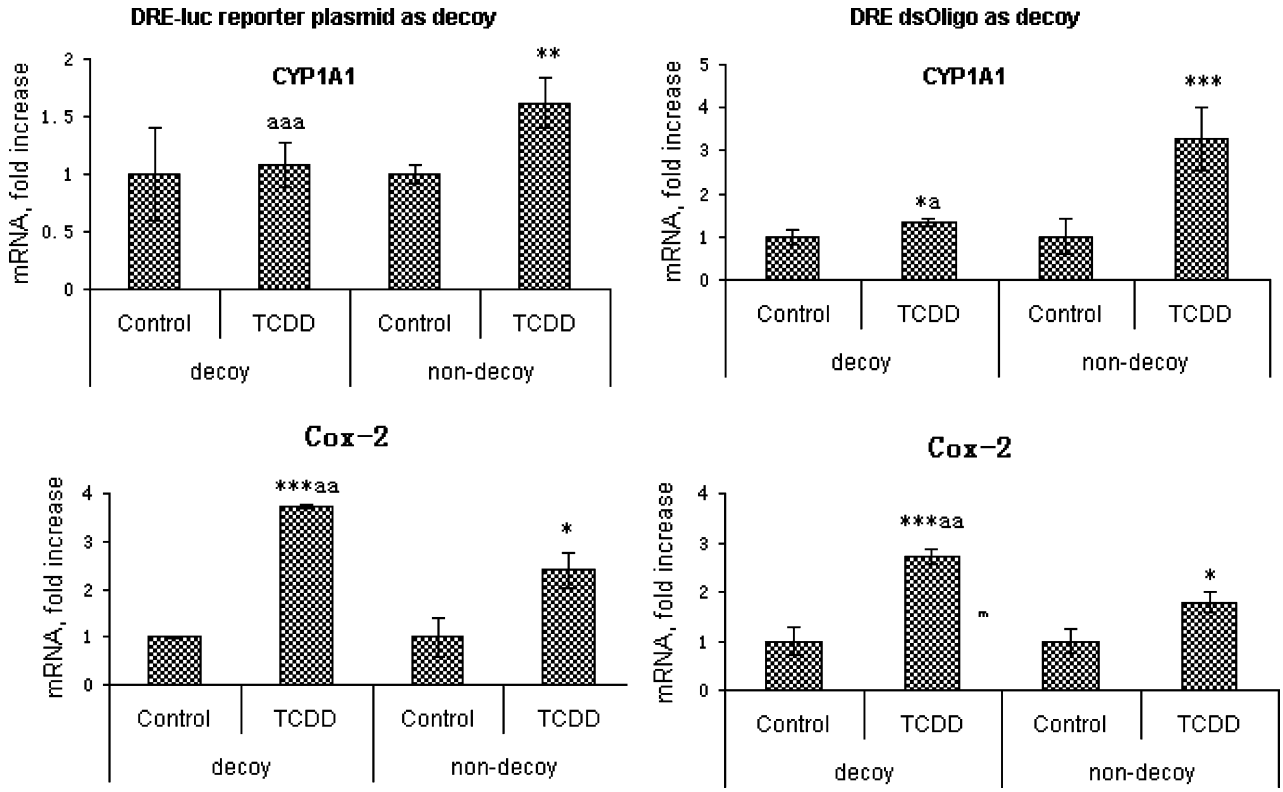

FIGURE 6: Effect of DRE decoy treatments on the action of TCDD (10 nM) to induce the mRNA expression of CYP1A1 vs Cox-2 in 3T3-L1 adipocytes over 24 h. In the first experiment (two figures on the left), cells were transfected with a DRE-Luc reporter plasmid, containing the DRE sequence (left two columns), along with an identical reporter plasmid without the DRE sequence as a blank (right two columns), for 24 h prior to the addition of TCDD, and thereafter, cells were incubated for an additional 24 h. In the second experiment (two figures on the right), cells were pretreated for 24 h with a preparation of double-stranded oligonucleotide (dsOligo) containing DRE (in place of the reporter plasmids), along with an equivalent dsOligo containing a mutant dsOligo. The results are shown as means ± standard deviations from three to five independent tests. Statistically significant differences between TCDD and matched control (i.e., the effect of TCDD regardless of treatment) and that between non-decoy-treated TCDD and decoy-treated TCDD (i.e., effect of decoy treatment) are indicated with asterisks and the letter a, respectively. The number of symbols indicates $p < 0.05$ (one), $p < 0.01$ (two), or $p < 0.001$ (three).

would affect the outcome of the long-term action of TCDD in causing the loss of adipocyte characteristics. This was done by examining the effect of AACOCF3, exogenous arachidonic acid (AA), and H89 (an inhibitor of PKA) on this process. We found as a result that this long-term cotreatment with AACOCF3 clearly antagonizes the action of TCDD to reduce the level of expression of several key adipocyte characteristic markers such as PPARγ, and leptin, but not that of CYP1A1 (Figure 10). AACOCF3 also generally antagonized the action of TCDD to upregulate the expression of these three inflammation markers (i.e., Cox-2, IL-8, and SOCS3), so that in the presence of this inhibitor, the action of TCDD became less significant in the case of Cox-2 or became insignificant in all other cases. The long-term effect of exogenous arachidonic acid (AA), tested at 20 μM, a physiologically realistic concentration, was studied here as a mimic for the result of activation of cPLA2 (since its action is to elevate the level of free AA). As expected, the results of exposure of adipocytes to AA were generally opposite from those of AACOCF3 as expected, except in the case of CYP1A1, judging by its effects on the expression of both inflammation markers (Cox-2, IL-8, and SOCS3) as well as adipocyte characteristic markers (i.e., PPARγ and leptin) (Figure 10). Another salient observation was that, despite causing the opposite effect from each other, both AACOCF3 and AA had the effect of eliminating the effect of TCDD on PPARγ or leptin mRNA expressions. At the same time, we noticed that in the presence of H89, a specific inhibitor of PKA, the 5 day effect of TCDD was not recognizable in any of the markers of action of TCDD, except in the case of CYP1A1 expression.

Direct Assessment of the Enzymatic Activities of PKA. In view of the long-term inhibitory effect of H89 on many of the actions of TCDD on inflammation as well as adipocyte markers found in the experiment described above, we decided to directly assess the enzymatic activity of PKA at two relevant time points of the action of TCDD. The results in Figure 11 clearly indicate that indeed the nongenomic signaling of TCDD to increase the enzymatic activity of PKA is long-lasting in this 3T3-L1 adipocyte line (e.g., even after 5 days), which is consistent with the effectiveness of H89 in attenuating the effect of TCDD on those markers.

DISCUSSION

The primary objectives of this study have been (a) to characterize the early action of TCDD to induce inflammatory changes in 3T3-L1 adipocytes, (b) to determine whether the mechanism of such an early action of TCDD is the same or different from its action to induce detoxification enzymes such as CYP1A1, and (c) to relate the above actions of TCDD to the loss of adipocyte characteristics, which accompany the loss of lipids from adipocytes, all of which take place over 5 days (*14*). To this end, we could clearly

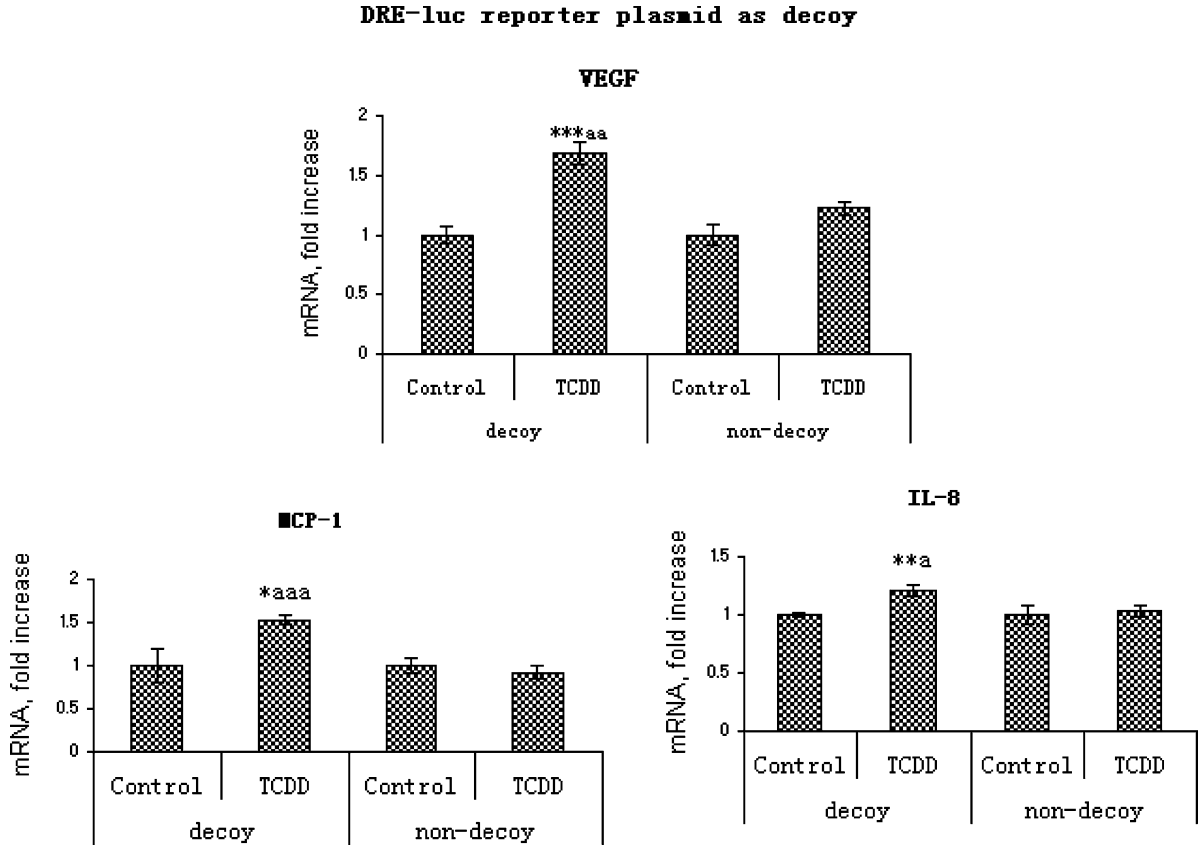

FIGURE 7: Effect of DRE decoy treatments on the action of TCDD (10 nM) to induce the mRNA expression of VEGF, MCP-1,and IL-8 in 3T3-L1 adipocytes over 24 h. In this experiment, cells were transfected only with the DRE-Luc reporter plasmid, along with an identical reporter plasmid without the DRE sequence as a blank, for 24 h prior to the addition of TCDD. The results are shown as means ± standard deviations from three to five independent tests. Statistically significant differences between TCDD and matched control (i.e., the effect of TCDD regardless of treatment) and that between non-decoy-treated TCDD and decoy-treated TCDD (i.e., effect of decoy treatment) are indicated with asterisks and the letter a, respectively. The number of symbols indicates $p < 0.05$ (one), $p < 0.01$ (two), or $p < 0.001$ (three).

show that TCDD-mediated rapid induction of several inflammation-related mRNAs such as Cox-2, VEGF, IL-8, and cPLA2 is carried out through an independent but AhR-dependent pathway within the initial period of 60 min, when induction of CYP1A1 mRNA is not apparent in this cell line, yet the difference in the timing of their induction is one of the criteria we could initially utilize in separating these two pathways.

The observation that early induction of Cox-2 and other inflammation-related mRNAs markers by TCDD is accompanied by activation of various protein kinases is consistent with the idea that such action of TCDD should be ascribed to its nongenomic action. cPLA2 is known as the phospholipase A2 of which activity is inducible with the increase in the intracellular concentration of free Ca^{2+} ion ($[Ca^{2+}]_i$). Knowing this, we are not surprised to find that PKC appears to be the most dominant protein kinase activating Cox-2 in this cell material at this early stage of action of TCDD. On the other hand, other types of protein kinases were also found to be activated by TCDD at this early stage of its action, and depending upon the mechanism of regulation of mRNA expression of individual genes, different classes of kinases have been found to contribute to activation of each of these mRNA expressions. A good example is the case of IL-8 mRNA, which was greatly affected by PKA, but not much by PKC or Genistein-sensitive PTKs (protein tyrosine kinases), whereas that of MCP-1 was clearly stimulated by PKC or PTKs but not by PKA (Figure 4).

The observation that TCDD-induced activation of the enzymatic activity of cPLA2 is one of the earliest signs of its nongenomic action to induce inflammation in this cell line is consistent with our recent finding in MCF10A cells (*21*) as well as U937 macrophages (*24*). Although in the past a limited number of scientists have noticed induction of PLA2 by TCDD, such an effect of TCCD has been considered to be due to its action to induce cytochrome P450s and/or oxidative stress, including lipid peroxidation activities (*25*). Thus, the main point of this finding is that rapid TCDD-induced activation of cPLA2 serves as the trigger of the nongenomic pathway of the action of TCDD and that such an action of TCDD is not the result of its classical action to induce cytochrome P450s. It is very critical, therefore, that these two pathways are clearly differentiated.

This is the very reason why we had to conduct those DRE decoy experiments at the time (24 h) when CYP1A1 mRNA expression becomes clearly recognizable in this cell material to make absolutely sure that the nongenomic pathway is clearly different from the classical, genomic pathway. By introducing excess DRE-containing constructs as decoys (Figures 6 and 7), we were able to clearly suppress induction of CYP1A1 by TCDD without blocking its action to induce Cox-2, IL-8, MCP-1, and VEGF. Accordingly, it appears to be safe to conclude that the action of TCDD to sustain

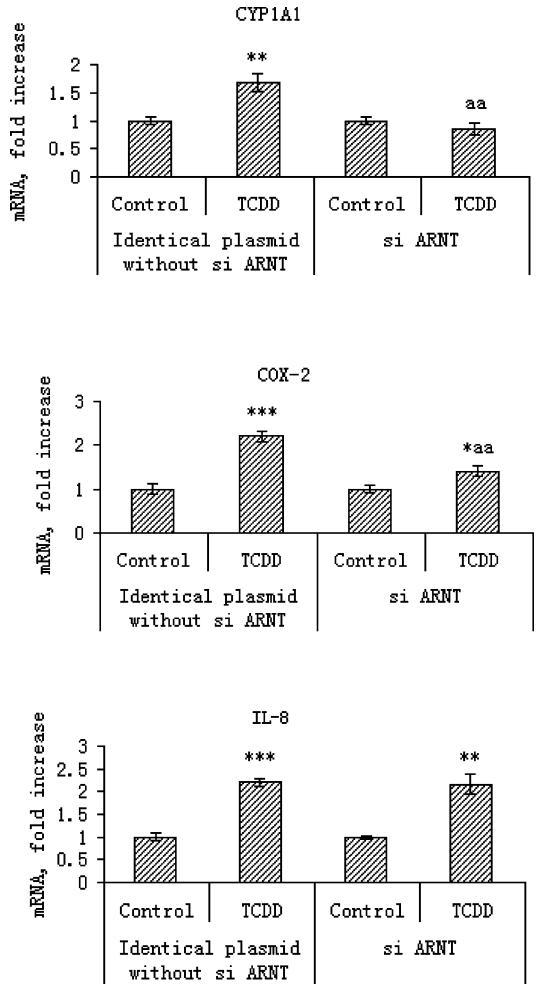

FIGURE 8: Suppression of 24 h TCDD-induced mRNA expression of CYP1A1, Cox-2, and IL-8 by treatment of cells with siRNA against ARNT (siARNT) in comparison to that with an inactive random RNA under the identical transfection and treatment conditions. Statistically significant differences between TCDD and matched control are indicated with asterisks, and those between TCDD and TCDD with siARNT are indicated with the letter a. The number of symbols indicates $p < 0.05$ (one), $p < 0.01$ (two), or $p < 0.001$ (three).

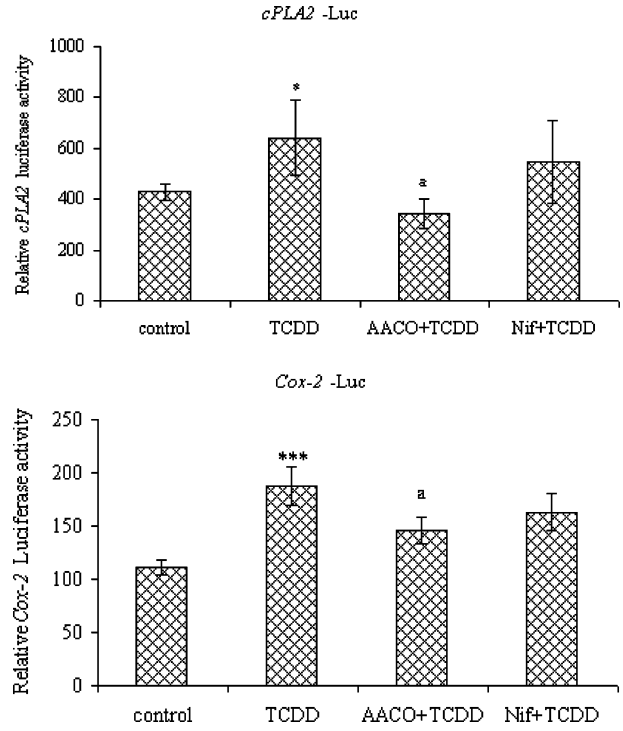

FIGURE 9: Action of TCDD to induce activation of *cPLA2* and *Cox-2* gene expression in 24 h and inhibitory effects of AACOCF3 (AACO) and Nifedipine (Nif) as assessed by using *cPLA2*-Luc and *Cox-2*-Luc plasmid reporter systems, respectively. The results are shown as means ± standard deviations from three to five independent tests. Statistically significant differences between TCDD and control and between TCDD and TCDD with the inhibitor are indicated with asterisks and the letter a, respectively. The number of symbols indicates $p < 0.05$ (one) or $p < 0.001$ (three).

elevated levels of expression of these inflammation-related mRNAs even after 24 h is still being influenced by its early nongenomic actions and not by the classical DRE-dependent pathway (i.e., the genomic pathway). An additional finding that ARNT is not contributing to this effect of TCDD to induce IL-8 and Cox-2 in this study cell material, just as in the case of MCF10A mammary epithelial cells (*21*) and that of U937 macrophages (*24*), further supports this conclusion.

While it is now clear that at the beginning TCDD activates inflammatory responses through its nongenomic action, we had to address another important question in the second part of this study: whether such a seemingly transient nongenomic signaling that is mediated by the increase in $[Ca^{2+}]_i$ (*26*) can have a sustained influence on the subsequent long-term consequence of toxic action of TCDD. The important point is that, if the nongenomic action is only transient, it would be of no interest to toxicologists, who are investigating long-term toxic action mechanisms of this compound. Another reason why we are interested in the further long-term effects of the 5 day action of TCDD in this project is to gain the perspective of the relative importance of such nongenomic actions of TCDD in relation to other types of actions of this compound, which occur at later stages of its action (*14, 27*). Namely, in the case of 3T3-L1 adipocytes, the action of TCDD to force these cells to reduce their lipid contents takes a long time period of 5 days (*14*) despite the fact that it clearly causes rapid inflammation in this cell material at early stages. In other words, the question we had to ask in this long-term study is whether the effect of the early, and hitherto considered to be a transient, nongenomic inflammatory signaling of TCDD would have any influence on the long-term action of TCDD to cause reduction of lipid storage, which is correlated to the suppression of selective markers of adipocyte characteristics (*14*). The results of this line of investigation clearly showed that cPLA2, which is initially activated through the nongenomic pathway, still plays an important role in the subsequent expression of the toxic action on TCDD as judged by the effectiveness of AACOCF3, exogenous AA, and H89 (Figure 10).

It must be pointed out that the action of TCDD to cause long-term effects of the loss of lipid storage from adipocytes is considered to be related to the wasting syndrome (*27*). Inflammation in adipocytes is considered to be the cause of this syndrome, which is consistent with the observation made in this study, i.e., upregulation observed in mRNA expressions of Cox-2, IL-8, and SOCS3 (inflammation markers), which is accompanied by downregulation of PPARγ and leptin (both are markers of mature adipocytes). In this regard, SOCS3, in particular, is known to respond to many inflammatory cytokines and also to play an important role in the etioloby of insulin reistance (*28*), and therefore, its upregu-

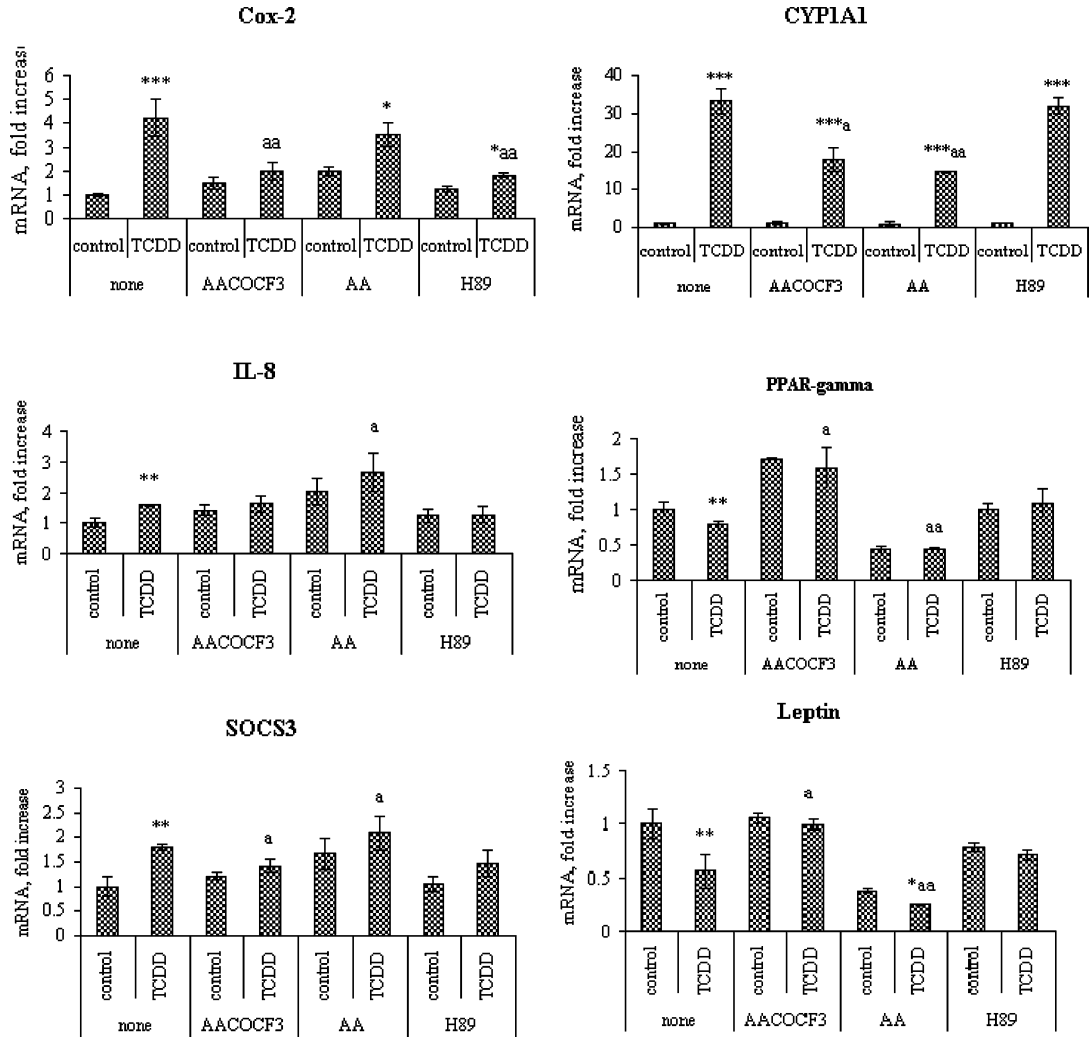

FIGURE 10: Effects of AACOCF3, arachidonic acid (AA), and H89 on the 5 day action of TCDD (10 nM) to alter the mRNA expression of inflammation and lipolysis markers in 3T3-L1 adipocytes differentiated for 7 days. Cells were pretreated with AACOCF3 (20 μM), AA (10 μM), and H89 (10 μM) for 1 h before TCDD treatment at the time of the first medium change following the 7 day differentiation, followed by their renewal at the second (2 days after the first one) and third (2 days after the second one) medium changes, and harvested after 5 days for qRT-PCR assays. The results are shown as means ± standard deviations from three to five independent tests. Statistically significant differences between the TCDD treatment and matched control (i.e., effect of TCDD) and between TCDD and TCDD with the inhibitor (i.e., effect of inhibitor) are indicated with asterisks and the letter a, respectively. The number of symbols indicates $p < 0.05$ (one), $p < 0.01$ (two), or $p < 0.001$ (three).

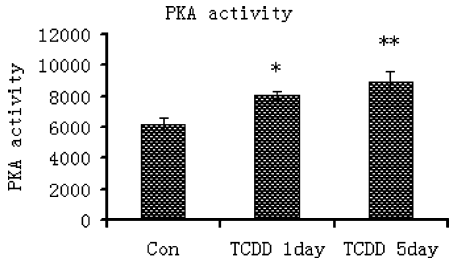

FIGURE 11: Assessment of the effect of TCDD after 24 h and 5 days on the status of the enzymatic activity of PKA in 3T3-L1 adipocytes. The method of TCDD treatment on fully mature adipocytes was identical to that of our previous report (*14*). The results are expressed as mean values from three to five independent tests. Statistically significant differences between the TCDD treatments and control (0 h) are indicated by one ($p < 0.05$) or two ($p < 0.01$) asterisks.

lation by the long-term action of TCDD through the activation of PKA is of great significance in its action to cause the loss of lipids from these adipocytes. While it may not be possible at this stage to ascribe all of those actions of these three inhibitors in antagonizing the late actions of TCDD that are transmitted through activation of the nongenomic pathway only, this result helps us to dispel the notion that the initial nongenomic action of TCDD is a transient and therefore a phenomenon observable only in a short window of the period of action of TCDD. Particularly significant in this regard is the action of H89, which has been used by many scientists to preferentially inhibit PKA. The fact that in H89-treated cells we could not observe any of the action of TCDD to induce inflammation as well as the fact its subsequent effect to lower the level of adipocyte-specific marker expressions was not affected, even under the same experimental condition for TCDD to induce CYP1A1 (Figure 9), supports our notion that PKA is likely one of the major players in converting the initial transient signaling of $[Ca^{2+}]_i$ (*29*) into a more stable signaling. What is more, we have already documented that this phenomenon is accompanied by persistent induction of binding activity of nuclear proteins to the labeled C/EBP response element oligonucleotide, particularly that of the C/EBPβ protein under the identical test condition as judged by an electrophoresis

gel mobility shift assay−supershift assay with the use of a specific antibody against the C/EBPβ isoform (*14*). It has been shown that induction of the C/EBPβ gene by TCDD is almost totally dependent on PKA (*20*).

A natural question raised by the observations described above is why and how TCDD-induced activation of PKA is sustained. One possibility is that the metabolic products of cPLA2, arachidonic acid (*30*), and/or Cox-2, prostaglandins (*31*), continue to be active in activating PKA, although much more work is needed to test this possibility. One factor contributing to such a long-lasting influence of nongenomic signaling of TCDD may be the chemical nature of TCDD to persist for long time periods, unlike the cases of estradiol or phorbol esters. However, this is speculation, and therefore, much more work is needed to address this question.

In conclusion, we could clearly show in this work that TCDD causes rapid activation of cPLA2 enzyme activity as well as induction of a number of inflammation-related mRNA expressions in 3T3-L1 adipocytes. Furthermore, we could establish that such rapid action of TCDD is mediated by AhR-dependent activation of its nongenomic signaling pathway, with the help of Ca^{2+}-triggered cPLA2 enzyme activation with accompanying elevation of the level of protein kinases in this cell line. The main evidence supporting this conclusion is that this nongenomic action of TCDD is clearly different from the classical, genomic action mechanism of TCDD, which takes place through binding of the AhR−ARNT dimer to DRE. The likelihood of such a nongenomic action of TCDD contributing to the long-term action of TCDD to cause the loss of lipid contents of adipocytes has also been demonstrated. Thus, although much more data are needed to determine how precisely these two pathways operate, jointly or separately, in producing the final toxic consequences of action of TCDD in adipocytes, the main achievement of this work may be that we could provide a concrete piece of evidence indicating the existence of the nongenomic pathway, which plays significant roles in the short term as well as long-lasting toxic action of TCDD in adipocytes.

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