Aryl Hydrocarbon Receptor Activation by Dioxin Targets Phosphoenolpyruvate Carboxykinase (PEPCK) for ADP-ribosylation via 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-inducible Poly(ADP-ribose) Polymerase (TiPARP)*§

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Background: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin) activates aryl hydrocarbon receptor (AHR) decreases gluconeogenesis by suppressing phosphoenolpyruvate carboxykinase (PEPCK) transcription via TCDD-inducible poly(ADP-ribose)-polymerase (TiPARP).

Results: AHR enhances PEPCK ADP-ribosylation through TiPARP, and AHR suppression increases ADP-ribosylation PARP-independently.

Conclusion: ADP-ribosylation, a new PEPCK posttranslational modification, is subject to complex regulation by the AHR.

Significance: AHR gene activation leads to ADP-ribosylation of PEPCK with implications for energy metabolism beyond TCDD toxicity.

The diverse toxic effects of the environmental toxin and carcinogen, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), an activator of the aryl hydrocarbon receptor (AHR), a ligand activated transcription factor (1-3), include metabolic dysregulation associated with suppressed gluconeogenesis and diminished mRNA, protein levels, and activity of hepatic phosphoenolpyruvate carboxykinase (PEPCK) (4, 5), a major regulator of hepatic gluconeogenesis (6). Impairment of gluconeogenesis has been associated with TCDD lethality by diminishing availability of glucose as an energy source. It has been noted that lethal exposure to TCDD causes rats to “behave as if they were satiated although they suffer from severe deficiency of energy” (7). TCDD treatment impairs hepatic glucose production and utilization in both mammalian and avian species (4, 5, 8, 9). The mechanism(s) by which TCDD produces its toxic effects and the relationship of TCDD toxicities to enhancement of AHR transcriptional effects remain central questions in AHR biology.

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Effects of the environmental toxin and carcinogen, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) include a wasting syndrome associated with decreased gluconeogenesis. TCDD is a potent activator of the aryl hydrocarbon receptor (AHR), a ligand activated transcription factor. The relationship between gene activation by the AHR and TCDD toxicities is not well understood. We recently identified a pathway by which the AHR target gene TiPARP (TCDD-inducible poly(ADP-ribose) polymerase) contributes to TCDD suppression of transcription of phosphoenolpyruvate carboxykinase (PEPCK), a key regulator of gluconeogenesis, by consuming NAD+ and decreasing Sirtuin 1 activation of the peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), a transcriptional activator of PEPCK. We report here that TCDD-induced TiPARP also targets PEPCK for ADP-ribosylation. Both cytosolic and mitochondrial forms of PEPCK were found to undergo ADP-ribosylation. Unexpectedly, AHR suppression also enhanced ADP-ribosylation and did so by a poly(ADP-ribose) polymerase-independent mechanism. This report 1) identifies ADP-ribosylation as a new posttranslational modification for PEPCK, 2) describes a pathway by which transcriptional induction of TiPARP by the AHR can lead to a downstream posttranslational change in a TCDD target protein (PEPCK), and 3) reveals that the AHR exerts complex, previously unidentified modulatory effects on ADP-ribosylation.

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ARTD14), contributes to TCDD suppression of PEPCK mRNA levels and hepatic glucose production (9). TiPARP is one of 17 members of the poly(ADP-ribose) polymerase (PARP) family of enzymes that catalyze poly- or mono-ADP-ribosylation of target proteins by NAD\(^+\)-dependent transfer of ADP-ribose to specific amino acids (10).

Since its discovery in 2001 (11) TiPARP has been repeatedly confirmed as an AHR target gene (12–15). As a result TiPARP is now included as a member of the AHR gene battery, a canonical group of genes co-induced by AHR activation in diverse cell types and species (CYP1A1, IA2, IB1, NQO1, ALDH3A1, UGT1A6, GSTA1 and now TiPARP (16–18)). We previously reported that TCDD-induced TiPARP suppresses PEPCK transcription by a mechanism in which utilization and consumption of NAD\(^+\) by TiPARP leads to decreased activity of the NAD\(^+\)-dependent deacetylase sirtuin 1 and, in turn, to decreased deacetylation (diminished activation) of peroxisome proliferator-activated receptor \(\gamma\) coactivator 1 \(\alpha\) (PGC1\(\alpha\)) (9), a transcriptional coactivator of PEPCK. The findings established a connection between an AHR-activated gene (TiPARP) and a particular effect of TCDD (suppression of gluconeogenesis).

PEPCK takes two forms: cytosolic (PEPCK-C) and mitochondrial (PEPCK-M), encoded by separate nuclear genes, PCK-1 and PCK-2, respectively (6). The two forms exhibit amino acid similarities of 70% for human, rat, and mouse and 62% for chicken. The relative amounts of PEPCK-C and -M in livers of different species vary. Although PEPCK-C constitutes >95% of the PEPCK in mouse and rat liver, livers of humans and many other mammalian species have equal amounts of both forms (6). We have continued to use the chick embryo (CE) close to hatching, a well established model for dioxin (TCDD) toxicity (8, 19–21) as our main animal model in these studies. Chick PEPCK-C declines through the embryonic period, and adult chick liver contains only PEPCK-M. However, the two forms are present to similar extents in the CE during the week before hatching (22–24), the age used in our studies, making the CE more representative of the human than typical rodent mammalian species with respect to distribution of the two forms.

Both PEPCK-C and PEPCK-M catalyze reversible GTP-dependent decarboxylation and conversion of oxaloacetate to phosphoenol-pyruvate (25). Although PEPCK-C is generally accepted to be the major form involved in regulation of gluconeogenesis, its role in glucose homeostasis is still not fully understood (26, 27). Even less is known about the function and regulation of PEPCK-M (28). It is thought that generation by PEPCK-M of phosphoenolpyruvate from lactate in mitochondria can help maintain redox balance (28). PEPCK-M is constitutively expressed in adult animals (29), but the extent of its contribution to gluconeogenesis or regulation by nutritional signals is unsettled (30). A possible role for PEPCK-M in insulin secretion has been suggested (31). A recent report (32) showed that overexpression of PEPCK-M in mice in which hepatic PEPCK-C was deleted partially rescued defects in glucose production. Furthermore, the two enzymes were shown to be independently regulated.

Although PEPCK-C levels are understood to be controlled largely by transcriptional changes in response to hormonal and nutrient signals (29), posttranslational modifications of PEPCK have begun to receive attention. PEPCK has been reported to be targeted for phosphorylation (33) and acetylation (34, 35). Furthermore, PEPCK-C acetylation has been reported to enhance its degradation (36).

We report here that PEPCK-C and PEPCK-M undergo ADP-ribosylation, extending the portfolio of posttranslational modifications for PEPCK. Furthermore, we show that the enhancement of ADP-ribosylation of PEPCK-C and PEPCK-M by TCDD is mediated by AHR-induced TiPARP, strengthening a role for TiPARP in TCDD dysregulation of nutrient signaling and energy balance and identifying a pathway by which AHR transcriptional activity can lead to a downstream posttranslational modification of a TCDD target protein. We also report the surprising finding that although AHR activation enhances ADP-ribosylation through TiPARP, AHR suppression also enhances ADP-ribosylation by a PARYP-independent mechanism.

EXPERIMENTAL PROCEDURES

Chick Embryos, Cell Cultures, and Treatments—Fertilized white Leghorn eggs (Gallus gallus) (Burr Farm, Inc., Hampton, CT) were incubated at 37°C at high humidity. Chick embryo hepatocytes (CEH) were obtained from chick embryo livers (CEL) as previously described (20). CEH were plated in 6-well Cell Culture Cluster plates (Costar, St Louis, MO) usually at 3 \(\times\) 10\(^6\) cells per well and maintained in culture medium for 48 h before treatment. Rat H4IIE, mouse Hepa1–6 and Hepa1c1c7, and human HepG2 and HEK293 cells were purchased from ATCC (Manassas, VA). Primary rat hepatocytes were from Invitrogen. H4IIE and HepG2 cells were cultured in minimum essential medium supplemented with 10% FBS (ATCC) and 1% Pen-Strep solution (Invitrogen); Hepa1–6 cells were cultured in DMEM with 10% FBS and 1% Pen-Strep solution; Hepa1c1c7 cells were cultured in a minimum essential medium with 10% FBS and 1% Pen-Strep; and HEK293 cells were cultured in DMEM with 10% FBS. Rat primary hepatocytes were maintained for 24 h after arrival in William’s Medium E without phenol red and supplementation according to the supplier’s instructions using the Maintenance Supplement Pack provided by the manufacturer. After 24 h the medium was replaced with William’s medium without further supplementation and treated with TCDD or dioxane. Chick embryos were used at 17 days of gestation for in ovo treatments and at 15 days for hepatocyte cultures before hatching at 21 days. Treatments with TCDD were at 1 nmol per egg in 0.005 ml dioxane in ovo or at 1 nm in CE hepatocyte cultures, 10 nm in cultures of rat primary hepatocytes, Hepa1c1c7, and Hepa1–6 cells, and 30 nm for HepG2 cells. All treatments were for 24 h unless otherwise indicated. Controls received dioxane in amounts equivalent to those used as solvent for the TCDD treatments.

Subcellular Fractionation of CEL and CEH—For cytosol from CEL, livers were isolated, weighed, and homogenized in 250 mM sucrose buffer (1:3, w/v). Homogenates were centrifuged at 10,000 \(\times\) g for 10 min at 4°C, and the supernatants were centrifuged at 100,000 \(\times\) g for 1 h at 4°C. The resulting supernatants, containing cytosol, were stored at \(-80°C. For CEH, \(\sim\)20 \(\times\) 10\(^6\) CEH (about 7 \(\times\) 10\(^6\) cells per 6-cm dish), were
scraped in 1× PBS and centrifuged at 500 × g for 2 min at 4 °C; the pellets containing nuclei were resuspended in 0.2 ml of Cell Lysis Buffer (Cell Signaling, Danvers, MA). The supernatants were centrifuged at 10,000 × g for 10 min. The resulting mitochondria-enriched pellets were resuspended in 0.1 ml of Cell Lysis Buffer, and the supernatants were centrifuged at 100,000 × g for 1 h at 4 °C to obtain cytosol. The fractions were stored at −80 °C.

Cell Lysate Preparation—Culture medium was aspirated, and cells were washed twice with 1× PBS and scraped. Lysates were prepared in 0.175 ml of Cell Lysis Buffer per well (Cell Signaling) supplemented with 1 mM 3-aminobenzamide and 5 μM adenosine 5′-diphosphate (hydroxymethyl)pyrrolidinediol, NH₂₂H₂O (ADP-HDP) (Enzo Life Sciences, Farmingdale, NY) to inhibit PARP and poly(ADP-ribose)glycohydrolase activity, respectively, during sample preparation.

Protein Concentrations—Protein was measured using Bio-Rad DC or RC DC Protein assays (Bio-Rad).

PEPCK-C Construct—The coding region of chick PEPCK-C was amplified by PCR. Primers were designed using the chick PEPCK-C mRNA sequence (GenBank™, NM_205471.1). The PCR amplification mixture contained: Phusion High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA); forward primer, 5′-CACCattggccccagctgaa-3′ (5′-CACC was added as a modification for cloning purposes); reverse primer, 5′-gctttatctctcaggg-3′ and cDNA from CH as template. The PCR amplification product was purified using Wizard SV Gel and PCR Clean-up System (Promega Co., Madison, WI) and cloned into pcDNA™3.1 using pcDNA™3.1 Directional TOPO Expression kit (Invitrogen/Invitrogen) following the manufacturer’s directions. Sequencing confirmed the PCR product as chicken PEPCK-C.

Transfections—For CEH, hepatocytes from CEL prepared as described above were collected by centrifugation (50 × g, 20 °C, 7 min) and resuspended in Mouse ES Cell Nucleofector Solution (Lonza Walkersville, Inc., Walkersville, MD) at 7 × 10⁶ cells/0.1 ml. Hepatocyte suspensions were mixed with 5 μg of a pcDNA-TiPARP-FLAG construct generated in our laboratory (9) or with an empty plasmid, pcDNA (Invitrogen/Invitrogen), or with pmaxGFP (Lonza). The following sequences were used for silencing TiPARP and the AHR: for TiPARP, SMARTpool dsRNA (Dharmacon/ThermoScientific, Waltham, MA) (5′-ggatacaccctggtgga-3′, 5′-gctgaatgctagaaag-3′, 5′-ggtactgtg-gaccttgtg-3′, and 5′-gccaagacgctgatt-3′); for AHR, a dsRNA targeting AHR sequence, 5′-gccttacgacgcca-3′ (Invitrogen); for control, siGENOME Non-Targeting siRNA #3 (Dharmacon, Lafayette CO). Transfections were performed using the Lonza system following the manufacturer’s instructions. Transfected CEH were plated in 6-well plates (Corning Inc., Corning, NY) in 2.5 ml of medium. After 24 h, lysates were prepared for Western blotting as described above. HEK293 cells were plated at 0.5 × 10⁶ cells/well in 6-well plates. PEPCK-C-His (5 μg) with or without TiPARP-FLAG (5 μg) or pmaxGFP (5 μg) was diluted with distilled H₂O to 87.6 μl. After adding 12 μl of 2 M CaCl₂, 100 μl of 2× Hepes-buffered saline were added dropwise while vortexing. The transfection solution was incubated for 1 min at room temperature, vortexed again, and added dropwise to wells containing the HEK293 cells. After 6 h, medium was removed, replaced with fresh medium, and incubated overnight.

Immunoprecipitation (IP)—For IP of ADP-ribosylated proteins, CEH lysates were precleared by incubation for 1 h at 4 °C with protein A-agarose beads to avoid nonspecific binding during the immunoprecipitation step. The agarose beads were removed by centrifugation, and lysates were incubated with 5 μg of an anti-PAR (polyADP-ribose) antibody (Trevisgen, Inc., Gaithersburg, MD) or normal rabbit IgG (Cell Signaling) overnight at 4 °C with gentle rocking. Protein A-agarose beads previously washed 3 times with Cell Lysis Buffer and incubated overnight with 1 mg/ml BSA were added to the samples for 2 h at 4 °C. The samples were then centrifuged. The beads were washed 5 times with Cell Lysis buffer (5 min per wash). After the last wash, 2× sample buffer (see below) was added. Samples were boiled for 5 min and stored at −80 °C before SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. For IP of PECK-C, anti-PECK-C IgG (Santa Cruz Biotechnology, Santa Cruz, CA), normal goat IgG (Santa Cruz), protein A/G-agarose beads, and washes with radioimmune precipitation assay buffer (Sigma) were used.

SDS-PAGE/Western Blotting—For CE, livers were homogenized in 2× sample buffer (125 mM TRIS-HCl, 4% SDS, 16% glycerol, 10% β-mercaptoethanol, 0.002% bromphenol blue) and boiled for 5 min. For primary CEH, rat hepatocytes, and cell lines, cells were scraped in 2× sample buffer, or lysates were prepared in Cell Lysis Buffer with PARP and poly(ADP-ribose)glycohydrolase inhibitors as indicated above. Lysates were diluted 1:1 (v:v) in 2× sample buffer containing 10% β-mercaptoethanol and heated (100 °C, 2 min). Proteins were separated on precast Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes. The following primary antibodies were used: Oct-A probe (Santa Cruz) for detection of FLAG-tagged TiPARP; anti-PAR, #4336-BPC (Trevisgen) or #96-10-04 (Enzo Life Sciences); anti-PCK1 (Sigma) (an antibody that was sold for PECK-C but recognizes both isoforms in chick); acetyl (K9) histone 3 and histone 3 (Millipore, Billerica, MA); AHR (Santa Cruz Biotechnology); α-tubulin (Cell Signaling); porin (Abcam, Cambridge, MA); β-actin (Sigma). The antibody detecting native TiPARP was custom-made (GenScript, Piscataway, NJ) using a peptide corresponding to amino acids 23–36, PSEDFFPQIRLSEK, in chicken TiPARP (XP_422828). The secondary antibodies were peroxidase-conjugated goat anti-rabbit (Sigma) or goat anti-mouse (Santa Cruz Biotechnology). Protein bands were detected using ECL or (for weaker signals) ECL Prime Western blotting detection reagents (GE Healthcare). Band intensities were measured by densitometry using Syngene Software (Syngene, Frederick, MD). Densitometry readings shown in graphs have been corrected for loading controls (β-actin, α-tubulin, or porin).

RT-Quantitative PCR—Total RNA was extracted from cultured CEH (3 × 10⁶ cells/well in 6-well plates) using RNA STAT-60 (Tel-Test “B,” Friendswood, TX) following the manufacturer’s directions. To prepare cDNA, 1 μg of total RNA, 4 μl of qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD), and water (final volume 20 μl) were mixed and then incubated at 25 °C (5′), 42 °C (40′), 85 °C (5′). The cDNA was diluted with 80 μl of water. For PCR, 2 μl of cDNA, 10 μl of...
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PerfeCTa SYBR Green FastMix (Quanta Biosciences), 1 µl of each primer (10 µM), and 6 µl of nuclease free water were combined. Primers and annealing temperatures (in parentheses) were as follows: 18 S, 5′-gaccattactagcgaccg-3′ and 5′-agaca-attctccgaacac-3′ (55 °C); PECK-C, 5′-actgtggactcaggtt-atg-3′ and 5′-tgctacgtaagtgagac-3′ (55 °C); PECK-M, 5′-acggtggaagcgttatc-3′ and 5′-cgttggtgaatgctttg-3′ (55 °C); TiPARP, 5′-ccagctcagctccaactac-3′ and 5′-cgtgtaagac-ccgcatcag-3′ (57 °C). An Eppendorf Mastercycler Eppgradient machine was used for 40 cycles of amplification. Fold changes in mRNA were calculated by the standard 2−ΔΔCt method (37) using 18 S mRNA as a reference for normalization.

Protein Identification by Nano-LC/MS/MS Analysis—CEH lysates overexpressing TiPARP or anti-PAR immunoprecipitates from that lysate as described above were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Bands spanning 63 kDa (about 4 mm) were excised and submitted for proteomic analysis (Cornell Proteomics Facility, Cornell University, Ithaca). The SDS-gel slices were subjected to in-gel digestion by trypsin and subsequent extraction (38). The digest was reconstituted in 10 µl of 2% acetonitrile with 0.5% formic acid for nano-LC-ESI-MS/MS analysis using a UltiMate3000 nano-LC system coupled to a LTQ-Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific, San Jose, CA) equipped with a “CorConneX” nano ion source device (CorSolutions LLC, Ithaca, NY). The tryptic peptides (5–10 µl) were injected onto a PepMap C18 trap column (5 µm, 300 µm × 5 mm, Dionex/Thermo Scientific, Sunnyvale CA) and separated on a PepMap C18 RP nano column (3 µm, 75 µm × 15 cm, Dionex) that had been installed in the “CorConneX” device with a 10-µm spray emitter (NewObjective, Woburn, MA) mounted in front of the Orbitrap orifice. Nano-LC conditions and Orbitrap instrument calibration/settings were as described previously (39). MS/MS data were acquired under parallel data-dependent acquisition mode. A Fourier Transform mass analyzer was used for a survey MS scan at a resolution of 60,000 followed by MS/MS scans on the 7 most intense peaks with multiple charged ions above a threshold ion count of 7500 in both a LTQ mass analyzer and high energy collision dissociation-based Fourier transform mass analyzer at 7500 resolution. Dynamic exclusion parameters were set at repeat count 1 for 20 s, exclusion list size of 500, and exclusion duration of 30 s. Collision-induced dissociation parameters were set at the following values: isolation width 2.0 m/z, normalized collision energy of 35%, activation Q at 0.25, and activation time of 10 ms. All data were acquired with Xcalibur 2.1 operation software (Thermo-Fisher Scientific).

MS Data Analysis—MS and MS/MS raw spectra were processed using Proteome Discoverer 1.2 software (Thermo-Fisher Scientific). The spectra from data-dependent acquisition files were converted to MGF files for subsequent database searches using in-house licensed Mascot Daemon (Version 2.3, Matrix Science, Boston, MA) against SwissProt, taxonomy G. gallus. Database searches were performed allowing two missed cleavage sites by trypsin. Peptide tolerance was set to 10 ppm, and MS/MS tolerance was set to 0.8 Da for collision-induced dissociation and 0.05 Da for higher energy collisional dissociation. Fixed carbamidomethyl modification of cysteine and variable modifications on methionine oxidation, ADP-ribosylation of lysine and deamidation of asparagines/glutamine were set. Only scores for the peptides defined by Mascot probability analysis significantly greater than “identity” were considered for peptide identification. The exponentially modified protein abundance index (emPAI) number outputted directly from Mascot results for each identified proteins was used for estimation of relative protein amounts within the same gel bands (38).

Statistical Analysis—Differences between group means were evaluated using GraphPad software by unpaired, two tailed t tests; p values ≤ 0.05 were considered statistically significant.

RESULTS

TCDD Increases ADP-ribosylation of a 63-kDa Protein Band in Hepatocytes, and TiPARP Can Mediate This Effect—TCDD suppressed PEPCK levels in CEH, consistent with evidence that TCDD decreases PEPCK transcription (9) (Fig. 1A, upper panel). At the same time TCDD increased ADP-ribosylation of a band at ∼63 kDa, the molecular weight at which PEPCK migrates (Fig. 1A, lower panel). TCDD suppressed PEPCK and increased ADP-ribosylation of a band at the same molecular weight also in rat primary hepatocytes (Fig. 1B), in mouse Hepa1c1c7 cells (Fig. 1C), and in other hepatocyte-derived cell lines, i.e. mouse Hepa1−6, rat H4IE, and human HepG2 (data not shown), indicating that these TCDD effects occur in mammalian species as well as in the chick model.

Cotreatment of CEH with TCDD and the PARP inhibitor PJ34 diminished the increase by TCDD in ADP-ribosylation of the 63-kDa band (Fig. 1D). Higher molecular weight ADP-ribosylated bands were also diminished by PJ34 treatment. The results are consistent with involvement of a PARP enzyme in TCDD-enhanced ADP-ribosylation. As TCDD decreased levels of PARP1, the major PARP enzyme (10) in CEH (Fig. 1E, upper panel), it seems unlikely that PARP1 mediates the increased ADP-ribosylation by TCDD. In contrast, TCDD increased TiPARP levels (Fig. 1E, lower panel).

Overexpression of TiPARP in CEH (Fig. 1F, top panel) increased ADP-ribosylation of the 63-kDa band in CEH (Fig. 1F, bottom panel). Diminishing TiPARP induction using TiPARP siRNA (9) suppressed the increase by TCDD of the 63-kDa ADP-ribosylated band (Fig. 1G). Together the findings in Fig. 1, D–G, support a role for TiPARP in the enhancement of ADP-ribosylation by TCDD.

Identification of PEPCK-M as a Target of ADP-ribosylation—Lysates of CEH transfected with TiPARP or GFP (Fig. 2, input, left panel) were immunoprecipitated with an anti-PAR antibody or normal IgG. Western blotting of the IP products (right panels) showed increased anti-PAR-specific ADP-ribosylated

3 In addition to the 63-kDa band, bands above 100 kDa were seen in some experiments. The differences are largely attributable to the use of two different anti-PAR (polyADP-ribose) antibodies, Enzo (#96-10-04) and Trevigen (#4336-BPC). The Enzo anti-PAR recognized a 63-kDa band preferentially and more strongly than the Trevigen antibody, which was better than the Enzo antibody at recognizing bands above 100 kDa.
bands at 63 kDa and at higher molecular masses in the lysates overexpressing TiPARP compared with the GFP control (right upper panel) (the 50-kDa band in both the normal IgG and anti-PAR IgG lanes is the expected IgG heavy chain). PEPCK was detected by Western blotting in the anti-PAR immunoprecipitates (bottom right panel) but not in the IgG IP products, showing that PEPCK was pulled down by an anti-PAR antibody and supporting PEPCK as a target of ADP-ribosylation.

A parallel set of experiments was performed for proteomic analysis. Aliquots of a lysate from CEH overexpressing TiPARP and of the anti-PAR IP product from that lysate (as shown in Fig. 2) were subjected to SDS-polyacrylamide gel electrophore-
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with the solvent alone (Fig. 4D). Furthermore, PEPCK-C cotransfected with TiPARP in HEK293 cells exhibited increased ADP-ribosylation compared with PEPCK-C transfected alone. PEPCK levels from the cells cotransfected with PEPCK and TiPARP were also lower than for PEPCK in HEK293 cells transfected with PEPCK alone (Fig. 4E).

**AHR Suppression and Inhibition of PARP Activity Increase PEPCK Levels**—We expected that TCDD effects on PEPCK levels would be dependent on the AHR given that TiPARP, an AHR activated gene, suppressed PEPCK transcription (9). Consistent with that expectation, AHR siRNA prevented the decrease in PEPCK-C levels by TCDD (Fig. 5A). Fig. 5B, left panel, further shows that AHR siRNA also prevented the decrease by TCDD in PEPCK-C mRNA, but neither TCDD nor AHR silencing affected PEPCK-M mRNA (right panel). Suppressing PARP activity by short term treatment with a PARP inhibitor (PJ34) suppressed ADP-ribosylation and ameliorated the suppression in PEPCK levels by TCDD (Fig. 5C) without modifying the effect of TCDD on PEPCK mRNA (Fig. 5D).

**AHR Suppression Enhances ADP-ribosylation**—As TCDD enhances ADP-ribosylation of PEPCK through AHR-induced TiPARP, we expected that AHR suppression would also diminish TCDD enhancement of ADP-ribosylation. Surprisingly, however, AHR suppression failed to diminish the activation of ADP-ribosylation by TCDD but, rather, increased it (Fig. 6A–C). This unexpected effect of AHR suppression was seen using three different approaches to diminish the AHR: geldanamycin (GA) (Fig. 6A), AHR siRNA (Fig. 6B), and nicotinamide (NAM, previously shown to antagonize AHR action in CEL (9)) (Fig. 6C) and in several experiments for each approach, indicating that this was a very robust effect. The same effect was also seen in the controls (Fig. 6D). The enhancement of ADP-ribosylation by AHR suppression was independent of TiPARP, as TCDD-induced TiPARP mRNA was much reduced by AHR suppression. (AHR siRNA, GA, and NAM suppressed AHR protein levels by means of 78, 86, and 60% and TiPARP mRNA by means of 55, 94, and 55%, respectively (data not shown)).

Inhibition of PARP activity by the PARP inhibitor PJ34 did not diminish the enhancement of ADP-ribosylation by AHR suppression (Fig. 6A–C), further evidence that the effect of AHR suppression was independent of PARPs. As some sirtuins (SIRTs 1 and 6) have been reported to have ADP-ribosylase as well as deacetylase activity (41), we examined the effect of TCDD and AHR suppression on acetylation of H3K9, an end point for the activity of SIRT1 and -6 (41–43). The experiments were conducted in the presence of trichostatin A, a class I/II deacetylase inhibitor, to ensure that only sirtuin (class III)-dependent deacetylation would be observed. TCDD increased H3K9 acetylation, whereas AHR suppression by GA or AHR siRNA diminished the increase in H3K9 acetylation by TCDD (Fig. 6, E and F), consistent with AHR suppression enhancing sirtuin activity.

**DISCUSSION**

This paper reports new findings addressing two distinct but connected subjects: (a) PEPCK, the major regulator of gluco-

sis and stained with Coomassie Blue. 63-kDa bands from the lysate and the IP product were excised and subjected to proteomic analysis. The 63-kDa band from the lysate (expected to contain proteins with and without ADP-ribosylation) yielded 122 proteins, including PEPCK-M (but not PEPCK-C) (supplemental Table S1). The 63-kDa band for the anti-PAR IP products yielded 21 proteins (Table 1). PEPCK-M was the top-hit protein followed by glutamate dehydrogenase, a protein previously shown to be ADP-ribosylated (40). These two proteins comprised nearly 50% of the total molar protein concentration in the SDS-polyacrylamide gel band and nearly ⅔ of the top 10 proteins with two or more peptides identified. The remaining 19 proteins including several keratins were present at much lower concentrations than PEPCK or glutamate dehydrogenase and were most likely to be contaminants. Consistent with the proteomic results, TiPARP overexpression increased an ADP-ribosylated protein band at the same molecular weight as PEPCK in mitochondria from CEH (Fig. 3). TiPARP did not lower PEPCK-M levels significantly.

**PEPCK-C Is Also an ADP-ribosylation Target of TCDD and TiPARP**—We conducted a series of experiments to examine whether TCDD also ADP-ribosylates PEPCK-C. TCDD decreased PEPCK levels and increased ADP-ribosylation of a 63-kDa band in cytosol from CEH (Fig. 4A) and in cytosol from livers of CE treated with TCDD in ovo (Fig. 4B). Overexpression of TiPARP in CEH reproduced TCDD effects in CEH cytosol (Fig. 4C) by suppressing PEPCK levels (upper panel) and enhancing ADP-ribosylation (lower panel). ADP-ribosylation of PEPCK immunoprecipitated from cytosol of TCDD-treated CEH was increased compared with ADP-ribosylation of PEPCK immunoprecipitated from cytosol of CEH treated...
neogenesis, and (b) AHR action. We report that 1) PEPCK can undergo ADP-ribosylation, a new posttranslational modification for this enzyme, 2) both the cytosolic and mitochondrial forms of PEPCK (PEPCK-C and PEPCK-M) can be ADP-ribosylated, 3) TCDD enhances ADP-ribosylation of PEPCK-C and PEPCK-M and does so through the AHR-activated target gene TiPARP, and 4) AHR suppression, paradoxically, also enhances ADP-ribosylation in a PARP-independent manner. The latter findings reveal new and complex actions for the AHR as a modulator of ADP-ribosylation.

**TABLE 1**

Proteomic analysis (G. gallus) of the 63-kDa band from anti-PAR immunoprecipitates of CEH lysates overexpressing TiPARP

| Hit no. | Accession no. | Protein identity | \(M_r\) | pI | Mascot score* | Peptide hit no. | Sequence coverage | emPAI | Molar |
|--------|----------------|-----------------|--------|----|--------------|----------------|------------------|-------|-------|
| 1      | gi 110591367   | Phosphoenolpyruvate carboxykinase (G. gallus) | 70.9   | 8.16 | 749          | 20             | 37.7            | 1.71  | 26.31 |
| 2      | gi 118534      | Glutamate dehydrogenase 1, mitochondrial (G. gallus) | 47.6   | 8.34 | 455          | 9              | 34.8            | 1.23  | 18.92 |
| 3      | gi 46399073    | Type II \(\alpha\)-keratin IIA (G. gallus) | 62.4   | 8.46 | 324          | 3              | 4.7             | 0.23  | 3.54  |
| 4      | gi 46399077    | Type II \(\alpha\)-keratin IIC (G. gallus) | 56.9   | 8.29 | 191          | 4              | 6.0             | 0.25  | 3.85  |
| 5      | gi 3746660     | Ototkeratin (G. gallus) | 53.8   | 5.97 | 160          | 3              | 5.9             | 0.20  | 3.08  |
| 6      | gi 118102980   | Predicted: keratin, type I cytoskeletal 12 (G. gallus) | 54.1   | 4.86 | 145          | 4              | 4.3             | 0.34  | 5.23  |
| 7      | gi 53127632    | Hypothetical protein RCJMBO4_2m8 (G. gallus) | 73.1   | 6.09 | 100          | 4              | 7.7             | 0.19  | 2.92  |
| 8      | gi 363730417   | Predicted: desmoplakin isoform2 (G. gallus) | 330.6  | 6.34 | 100          | 4              | 1.3             | 0.04  | 0.62  |
| 9      | gi 118103004   | Predicted: keratin, type I cytoskeletal 17-like (G. gallus) | 51.1   | 4.78 | 100          | 2              | 4.3             | 0.21  | 3.23  |
| 10     | gi 3345475     | Keratin-19 (G. gallus) | 46.1   | 4.94 | 99           | 3              | 7.1             | 0.23  | 3.54  |
| 11     | gi 302120406   | Plakoglobin (G. gallus) | 82.1   | 5.75 | 96           | 3              | 3.7             | 0.12  | 1.85  |
| 12     | gi 63413       | \(\beta\)-Globin (G. gallus) | 16.5   | 8.85 | 64           | 1              | 6.8             | 0.21  | 3.23  |
| 13     | gi 33130384    | Hypothetical protein RCJMBO4_7g5 (G. gallus) | 60.9   | 5.72 | 51           | 1              | 1.6             | 0.05  | 0.77  |
| 14     | gi 59797643    | RecName: Full \(\alpha\)-fetoprotein | 70.6   | 6.26 | 49           | 1              | 1.1             | 0.05  | 0.77  |
| 15     | gi 118085660   | Predicted: serine/threonine-protein kinase greatwall (G. gallus) | 98.8   | 5.34 | 42           | 1              | 1.8             | 0.03  | 0.46  |
| 16     | gi 833622      | Ubiquitin (34 AA) (1 is 2nd base in codon) (G. gallus) | 3.7    | 8.50 | 41           | 1              | 27.3            | 1.00  | 15.38 |
| 17     | gi 6706186     | Proprotein convertase PC7 (G. gallus) | 15.8   | 4.91 | 40           | 1              | 7.3             | 0.22  | 3.38  |
| 18     | gi 118099953   | Predicted: tRNA-splicing endonuclease subunit Sen54 (G. gallus) | 58.4   | 6.90 | 38           | 1              | 1.8             | 0.06  | 0.92  |
| 19     | gi 363732683   | Predicted: neuronal PAS domain-containing protein 2-like (G. gallus) | 94.9   | 9.42 | 37           | 1              | 1.0             | 0.03  | 0.46  |
| 20     | gi 363743387   | Predicted: G patch domain-containing protein8 (G. gallus) | 156.3  | 9.26 | 36           | 1              | 1.1             | 0.02  | 0.31  |
| 21     | gi 129293      | RecName: Full \(\alpha\)-Ovalbumin | 42.9   | 5.19 | 36           | 1              | 6.0             | 0.08  | 1.23  |

* Multidimensional Protein Identification Technology (MudPIT) scoring.

**FIGURE 3.** TiPARP increases a 63-kDa ADP-ribosylated band in CEH mitochondria. Western blots (anti-FLAG OctA probe, anti-PAR (Enzo), and anti-PEPCK) on a mitochondrial fraction from CEH transfected with TiPARP-FLAG or pcDNA (10 µg of protein/lane) are shown. *****, \(p < 0.001\) for PAR for TiPARP versus pcDNA. RDU, relative densitometry units.
tion products in CEH reflects the low levels of PEPCK-C in the hepatocyte lysates. Although both PEPCK-C and -M levels decrease toward the end of chick embryo development and are low before hatching, the stage studied here, TCDD and TiPARP suppress mRNA for PEPCK-C but not PEPCK-M (see Fig. 5B), leading to further decreases in levels of hepatic PEPCK-C (Fig. 4, A and B) compared with PEPCK-M (Fig. 3). Thus, PEPCK-C levels are likely to have been below the limit of detection by the proteomic analysis.

The experimental evidence in Fig. 4 demonstrates that PEPCK-C is also ADP-ribosylated by TCDD and TiPARP. This conclusion is further supported by the evidence (Fig. 1, B and C, and data not shown) that TCDD increased ADP-ribosylation of a band comigrating with PEPCK in rat primary hepatocytes and liver-derived cell lines from rat and mouse, species expressing hepatic PEPCK-C almost exclusively (6). We expect that the greater reduction by TCDD treatment of PEPCK-C mRNA than of PEPCK protein levels (i.e. Fig. 5, C and D) reflects the fact that TCDD suppresses mRNA for PEPCK-C (but not PEPCK-M), whereas hepatocyte PEPCK protein levels reflect the sum of PEPCK-C and PEPCK-M protein, with the latter unaffected by TCDD.

The effects of ADP-ribosylation on PEPCK activity and stability will merit investigation as ADP-ribosylation has been reported to affect both protein activity and stability (44). For example, ADP-ribosylation enhances transcriptional activation by histones (45) and increases activity of the cation channel P2X7 (44, 46) and of the transcriptional insulator CCCTC-binding factor (47) and decreases activity of glutamate dehydrogenase (48), glycogen synthase kinase 3β (49), and the circadian rhythm regulator CLOCK (50). ADP-ribosylation has also been reported to enhance protein ubiquitination and degradation (51, 52). Our findings that PEPCK-C levels were lower...
**AHR Modulates ADP-ribosylation of PEPCK**

![Image](https://via.placeholder.com/150)

**FIGURE 5. Effect of AHR suppression and PARP inhibition on PEPCK levels.**

A, Western blot (anti-PEPCK) on CEH transfected with non-targeting dsRNA (scr) or with siAHR dsRNA and treated with dioxane (C) or TCDD (T); p = 0.02 for both control versus TCDD and TCDD + siAHR versus TCDD + scr. B, quantitative PCR results for PEPCK-C and PEPCK-M in CEH treated as in A. For PEPCK-C, TCDD + scr versus control + scr, p = 0.008; C + siAHR versus C + scr and TCDD + siAHR versus TCDD + scr, p = 0.02 and 0.04, respectively. C, Western blot for PEPCK on control- and TCDD-treated CEH with and without PJ34 (1 μM for the last 3 h); p = 0.05 for TCDD versus control and p = 0.006 for TCDD + PJ34 versus TCDD. CEH scraped in sample buffer were used for Western blotting. D, quantitative PCR results for PEPCK-C mRNA in the same samples shown in C (p = 0.0001 for control versus TCDD; not significant (n.s.) for TCDD + PJ34 versus TCDD alone). *, p ≤ 0.05; **, p ≤ 0.01; ****, p ≤ 0.0001.

When PEPCK was co-expressed with TiPARP than when expressed alone in HEK293 cells (Fig. 4E) and that suppressing ADP-ribosylation with the PARP inhibitor PJ34 diminished TCDD reduction of PEPCK protein levels without affecting PEPCK-C mRNA (Fig. 5D) support an effect of ADP-ribosylation on PEPCK-C stability.

Samples enriched in PEPCK will be required to identify specific ADP-ribosylation sites on PEPCK-C and PEPCK-M. The labile nature of peptide-ADP-ribose bonds and bonds within ADP-ribose groups and potentially low abundance of ADP-ribosylation (53, 54) are well recognized technical barriers limiting detection of ADP-ribosylation sites by MS/MS. Use of recently reported precursor ion scanning MS analysis (55) for selectively detecting ADP-ribosylated peptides followed by MS/MS analysis will be required for this further step.

The evidence that TiPARP enhances ADP-ribosylation of PEPCK reveals a path by which TCDD can produce a posttranslational effect on a target protein downstream of AHR gene activation. The finding also strengthens a role for TiPARP activity in AHR action by showing that in addition to affecting PEPCK by consuming NAD⁺ and altering PEPCK transcription through PGC1α (9), TiPARP also targets PEPCK through its ADP-ribosylase activity. Thus our data demonstrate that the two aspects of TiPARP catalytic activity, consumption of NAD⁺ and ADP-ribose formation, both contribute to TCDD effects on PEPCK. TiPARP and TCDD enhancement of higher molecular weight ADP-ribosylated bands seen in Western blots (Figs. 1D and 2) suggests that TiPARP is also likely to ADP-ribosylate other proteins.

The lack of a mobility shift on SDS-polyacrylamide gel electrophoresis for PEPCK after TCDD or TiPARP treatment is consistent with other evidence that TiPARP is a mono- rather than a poly-ADP-ribosylase (56). Interestingly, several members of the PARP family previously considered to be poly-ADP-ribosylases are proving to catalyze mono-ADP-ribosylation (10). Human TiPARP was predicted to be a mono-ADP-ribosylase based on replacement of a critical glutamate by inosine in a triad of amino acids (HYE) needed for poly-ADP-ribosylation (57). Amino acid alignments of human, mouse, and chicken TiPARP show that all three species share the substitution of inosine for glutamate (58) (see supplemental Fig. S1) as well as a high degree of homology (89%) in the catalytic region. The term "PAR" is used here as a general term for ADP-ribosylation rather than to specify "poly-ADP-ribosylation."

Our unexpected finding that AHR suppression, a condition in which TiPARP is reduced, enhances ADP-ribosylation in a PARP-independent manner (as shown from experiments using the PARP inhibitor PJ34; see Fig. 6A–C) implies that the AHR has an underlying role in repressing an ADP-ribosylase distinct from PARPs. Although further investigation will be required to identify the ADP-ribosylase-enhancing factor suppressed by the AHR, our evidence is consistent with involvement of a sirtuin (Fig. 6, E and F). Alternatively, the AHR may activate an ADP-ribose glycohydrolase, in which case AHR suppression would diminish ADP-ribose hydrolyase activity and increase ADP-ribosylation levels.

Although H3K9 deacetylation was studied here as a tool to investigate involvement of a sirtuin in AHR effects on ADP-ribosylation, increased acetylation of H3K9 by TCDD and the reversion of that effect by AHR suppression could have additional implications. For example, enhanced transcriptional activity of acetylated H3K9 might contribute to transcriptional activation by the AHR.

The scheme in Fig. 7 summarizes our findings with respect to AHR actions on ADP-ribosylation. TCDD-induced TiPARP increases ADP-ribosylation of PEPCK (bold arrows). We hypothesize that the AHR also represses a different ADP-riboseylase or enhances an ADP-ribose hydrolyase. Although AHR suppression of ADP-ribosylation may temper the extent of
enhancement of ADP-ribosylation of PEPCK by TiPARP activation, the net effect of AHR activation by TCDD is to increase PEPCK ADP-ribosylation.

We have two final points. First, the activation of ADP-ribosylation by AHR suppression in control samples is consistent with our prior evidence that AHR suppression affected parameters related to glucose metabolism in control as well as TCDD treated CEH, i.e. NAD\(^{+}\) levels, gluconeogenic gene expression, and glucose output (9). It is also consistent with evidence that PEPCK mRNA was lower in AHR\(^{-/-}\) than in AHR\(^{+/+}\) mice even without TCDD treatment (12). Together the findings indicate that the AHR may repress PEPCK constitutively. Second, it seems likely that AHR effects on ADP-ribosylation shown here will have implications beyond TCDD toxicity. There is increasing interest in biologic effects of natural AHR ligands present in food, in the environment, or generated in the body (59–62) and in AHR effects on physiologic processes, most conspicuously.

**FIGURE 6.** AHR suppression increases ADP-ribosylation in a PARP-independent manner. A, Western blot (anti-PAR, Enzo) on CEH treated with dioxane (C), TCDD (T), TCDD + geldanamycin (GA, 2 \(\mu\)g/ml), or TCDD + GA + PJ34 (1 \(\mu\)M for the last 3 h); \(p = 0.002\) for TCDD versus control; \(p = 0.007\) for TCDD + GA versus control. *RDU, relative densitometry units. B, Western blot (anti-PAR, Enzo) on CEH transfected with non-targeting dsRNA (scr) or with siAHR dsRNA and treated with dioxane (C), TCDD, or TCDD + PJ34 (1 \(\mu\)M for the last 3 h); \(p = 0.02\) for TCDD + scr versus C + scr and \(p = 0.008\) for TCDD + siAHR versus C + scr. C, Western blot (anti-PAR, Trevigen) on CEH treated with dioxane, TCDD, TCDD + NAM (50 mM, 24 h), or TCDD + NAM + PJ34 (1 \(\mu\)M, for the last 3 h); \(p = 0.05\) for TCDD versus control; \(p = 0.04\) for TCDD + NAM versus TCDD; \(p = 0.02\) for TCDD + NAM + PJ34 versus TCDD + NAM. D, Western blots (anti-PAR, Enzo) on CEH treated with GA (2 \(\mu\)g/ml) (left panel) or CEH transfected with non-targeting dsRNA (scr) or with siAHR dsRNA (\(p = 0.006\)) (right panel). Vertical lines indicate deletion of irrelevant lanes from the blot. E and F, Western blots (anti-acetylated lysine 9 histone 3 (Ac(K9)H3 and anti-histone 3 (total H3)) on CEH treated with dioxane, TCDD, and TCDD + GA (2 \(\mu\)g/ml) (E) or transfected with non-targeting dsRNA (scr) or with siAHR dsRNA and treated with dioxane (C) or TCDD (T). The class I/II histone deacetylase inhibitor trichostatin A (30 \(\mu\)M) was added for the last 4 h; \(p = 0.01\) for TCDD versus control; \(p = 0.02\) for TCDD + GA; \(p < 0.001\) for TCDD + scr versus C + scr and \(p = 0.02\) for TCDD + siAHR versus TCDD + scr. *, \(p \leq 0.05\); **, \(p \leq 0.01\); ***, \(p \leq 0.001\).
immune and gastrointestinal system function (63–65). In fact, a role for TiPARP in TCDD modification of immune system function has been suggested (66).

Acknowledgments—We thank Brittany Baldwin-Hunter, Christopher Virunurm, and Prosenjit Mondal for reading the manuscript and providing helpful comments.

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