Identification of the Aryl Hydrocarbon Receptor Target Gene TiPARP as a Mediator of Suppression of Hepatic Gluconeogenesis by 2,3,7,8-Tetrachlorodibenzo-p-dioxin and of Nicotinamide as a Corrective Agent for This Effect

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The environmental toxin TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin, dioxin) produces diverse toxic effects including a lethal wasting syndrome whose hallmark is suppressed hepatic gluconeogenesis. All TCDD toxicities require activation of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor. Whereas the mechanism for AHR induction of target genes is well understood, it is not known how AHR activation produces any TCDD toxicity. This report identifies for the first time an AHR target gene, TiPARP (TCDD-inducible poly(ADP-ribose) polymerase, PARP7) that can mediate a TCDD toxicity, i.e. suppression of hepatic gluconeogenesis. TCDD suppressed hepatic glucose production, expression of key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase), and NAD+ levels, and increased PARP activity and TiPARP expression. TCDD also increased acetylation and ubiquitin-dependent proteosomal degradation of the peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1α), a coactivator of PEPCK and G6Pase transcription. TiPARP overexpression reproduced TCDD effects on glucose output and NAD+ levels whereas TiPARP silencing diminished them. TiPARP overexpression also increased PGC1α acetylation and decreased PGC1α levels. In contrast, silencing of cytochromes P450 (CYP) 1A, main AHR-induced genes, did not alter TCDD suppression of gluconeogenesis. The vitamin B3 constituent, nicotinamide (NAM), prevented TCDD suppression of glucose output, NAD+, and gluconeogenic genes and stabilized PGC1α. The corrective effects of NAM could be attributed to increased NAD+ levels and suppression of AHR target gene induction. The results reveal that TiPARP can mediate a TCDD effect, that the AHR is linked to PGC1α function and stability and that NAM has novel AHR antagonist activity.

The aryl hydrocarbon receptor (AHR), a conserved basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) protein and ligand-activated transcription factor, is best known for mediating the pleiotropic toxicities of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin, dioxin), its most potent ligand (1–4). TCDD, a highly toxic by-product of certain high-temperature industrial processes and a component of the herbicide Agent Orange, is increasingly a source of global concern because of its high toxicity, resistance to metabolism, and environmental persistence. TCDD has diverse toxic effects all of which require binding of TCDD to the AHR (1, 3, 5, 6). These effects include dysregulated nutrient metabolism and energy production, immune system dysfunction, carcinogenesis, and cardiac contractile dysfunction (4, 7–10). Whereas the steps between ligand binding and transcriptional activation of AHR target genes are well understood (1, 3, 5), it is not known how AHR activation mediates TCDD toxicities, nor have any AHR target genes, i.e. cytochrome P450 (CYP)1A or others, been identified as mediating any TCDD toxic manifestations.

One of the most devastating effects of AHR activation by TCDD is a lethal wasting syndrome in which decreased hepatic gluconeogenesis is a prominent feature (4, 7). Whereas fasting or nutrient deprivation normally increases gluconeogenesis and stimulates food intake (11, 12), TCDD produces a fasting or starvation-like state in which gluconeogenesis and food intake are both decreased (7, 13). In mammalian liver TCDD is known to decrease the expression and activity of PEPCK and G6Pase, enzymes controlling gluconeogenic flux (7, 14–16), but the mechanism for these effects remains obscure (1, 3). As PGC1α is a critical transcriptional coactivator for PEPCK and G6Pase, we hypothesized that a negative interaction between AHR activation and PGC1α function, although not previously recognized, might help to explain TCDD suppression of gluconeogenesis.

The abbreviations used are: AHR, aryl hydrocarbon receptor; CE, chick embryo; CYP, cytochrome P450; DRE, dioxin responsive element; EROD, 7-ethoxyresorufin-O-deethylase; G6Pase, glucose-6-phosphatase; GO, glucose output; NAM, nicotinamide; PAR, poly(ADP-ribose) polymerase; PEPCK, phosphoenolpyruvate carboxykinase; qPCR, quantitative PCR; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1 α; SIRT1, sirtuin (silent mating type information regulation 2 homolog) 1; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TiPARP, TCDD-inducible poly(ADP-ribose) polymerase.
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We investigated this hypothesis using the chick embryo (CE) close to hatching, a well-established model for studying TCDD toxicity (4, 17). This model exhibits the metabolic effects of TCDD seen in mammalian liver and allows liver metabolism to be studied independently of confounding effects of changes in food intake (18). We discovered that dysregulation of glucose homeostasis by TCDD involves a previously unknown role of a PARP family member, TiPARP. In addition, the small molecule nicotinamide acts as a corrective agent for the negative effects of TCDD on glucose production and as an antagonist of AHR activation.

EXPERIMENTAL PROCEDURES

Chick Embryos, Hepatocyte Cultures, and Treatment—Fertilized white Leghorn eggs (Gallus gallus) (Burr Farm, Inc., Hampton, CT) were maintained at 37 °C at high humidity. Treatment and processing of livers for hepatocyte cultures was as previously described (17). For primary cultures, chick embryo hepatocytes (CEH) were plated in 24-well Cell Culture Cluster plates (Corning Inc., Corning, NY) at 0.5 × 10⁶ cells per well or in 6-well Cell Culture Cluster plates at 3 × 10⁶ cells per well and maintained in culture in the medium described (17) for 48 h before treatment. 16–18-day-old CE were used for 15- or 16-day-old CE for hepatocyte cultures. Rat H4IE cells (ATCC, Manassas, VA) were maintained in Eagle’s MEM supplemented with 10% FBS (ATCC) and 1% Pen Strep Solution (Invitrogen, Carlsbad, CA). Treatments with TCDD were at 1 nmol per egg in 0.005 ml dioxane (Sigma-Aldrich) incubated in 1N HCl under the same conditions was included in each assay. After incubation for 3 h at 37 °C, the medium was removed, centrifuged at 3,000 rpm for 10 min to remove cell debris and used to measure glucose using the same GO assay kit used for glycogen (above). The amount of glucose in the medium was calculated with reference to a glucose standard curve, and values were corrected for protein content.

For the rat hepatoma cell line H4IIE, GO was measured as described for CEH except that cells were plated at 0.75 × 10⁶ cells per well in 6-well plates in Eagle’s MEM (ATCC, Manassas, VA) medium supplemented with 10% FBS and 1% Pen Strep Solution. After 4 h, medium was removed and replaced with GO medium (1 ml) containing TCDD or dioxane. Following an overnight incubation, the medium was collected and used to measure GO.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)—Semiquantitative RT-PCR was performed as previously described (17). Primers, amplification conditions, and expected band sizes are listed in supplemental Experimental Procedures. The same primers and annealing temperatures were used for qPCR. Reaction mixtures contained: 10 μl of QuantiTect SYBR green PCR Mastermix (Qiagen Inc., Valencia, CA), 1 μl of each specific primer (10 μm), 6 μl of nuclease free water and 2 μl of cDNA. A Mastercycler epgradient machine (Eppendorf, Hauppauge, NY) was used. Amplification was for 40 cycles. mRNA fold change was calculated using the standard 2^−ΔΔCt method (20). All mRNA data shown have been corrected for GAPDH or 18S rRNA.

NAD⁺ and NADH—NAD⁺ and NADH were measured by fluorescence (21) in homogenates of CE liver (250 mg wet weight per ml of 1 × PBS) or cultured CEH (~3 × 10⁶ cells in 0.4 ml 1 × PBS). Samples (0.05 ml) were added to 0.05 ml of 7% perchloric acid for NAD⁺ or 0.05 M NaOH with 1 mM EDTA for NADH, vortexed for 30 s, sonicated, then incubated for 30 min at 60 °C to destroy endogenous enzyme activity. After centrifugation (3 min, 13,000 rpm) the supernatants were neutralized and 0.002 and 0.004 ml aliquots were added to black polystyrene 96-well plates (Corning Inc.). Reaction mixtures (0.130 ml total volume) contained cycling assay buffer (5 mM Tris, 5 mM MgCl₂, 50 mM KCl) 54 μM resazurin (Sigma-Aldrich), 0.4 units/ml lactate dehydrogenase (Sigma-Aldrich) and 2.25 mM lactate, with the pH adjusted to 7.5. Reactions (transformation of resazurin to resorufin) were initiated with 0.020 ml of diaphorase (Clostridium kluyveri, Sigma-Aldrich) per well for a final concentration of 0.7 units/well. Standard curves for NAD⁺ or NADH were included in each assay. Resorufin fluorescence was measured immediately after adding diaphorase, using an Analyst AD 96.384 plate reader (LJL Biosystems, Inc., Sunnyvale, CA) (Ex λ₅30 nm, Em λ₅80 nm), at 30 s intervals for 12.5 min. NAD⁺ and NADH concentrations were obtained by comparing slopes for samples and standards. Values were corrected for dilutions and protein content of the samples.

PARP Activity (ADP-Ribosylation)—CEH plated in 6-cm tissue culture dishes (BD Falcon, Franklin Lakes, NJ) at 5 × 10⁶ cells/dish were harvested by scraping in 1.5 ml of 1 × PBS and
transferred to pre-chilled microcentrifuge tubes. Tubes were centrifuged at 400 \times g for 10 min at 4 °C. Supernatants were discarded and PARP activity was measured in the cell pellets using Universal Chemiluminescent PARP Assay kits (Trevigen, Inc., Gaithersburg, MD). Luminescence was measured using an Analyst AD 96.384 plate reader (LJL Biosystems). ADP-ribosylation of proteins was measured by Western blotting using an anti-PAR (poly(ADP-ribose)) antibody (see supplemental Experimental Procedures).

Gene Silencing and Transfection of Primary CEH—CE livers were disaggregated with collagenase (17). CEH were collected by centrifugation at 50 \times g at 20 °C for 7 min and resuspended in Mouse ES Cell Nucleofector Solution (Lonza Walkersville, Inc., Walkersville, MD) at 7 \times 10^6 cells/0.1 ml. Hepatocyte suspensions were mixed with dsRNA (Invitrogen) (2 \muM, final concentration) against the target sequences listed in supplemental Experimental Procedures. Transfections were performed using the Lonza system following the manufacturer’s instructions. Transfection of pmxGFP (Lonza Walkersville) (2 \mug/transfection) was used to assess transfection efficiency. After transfection, hepatocytes were plated in 6-well plates previously coated with 0.1% gelatin at ~7 \times 10^6 cells per well in 2.5 ml of medium. Twenty-four hours later hepatocytes were treated with TCDD. After a further 24 h, cells were used to prepare RNA, for Western blot, or to measure GO or pyridine nucleotide levels as described.

PGC1α Overexpression and Acetylation—CEH obtained by collagenase digestion of CE livers were transfected with the plasmid pAd-Track Flag HA PGC1α (5 \mug Flag PGC1α plasmid 14426) (Addgene, Cambridge, MA) using the Lonza system, as described above. After treatment hepatocytes were used to measure GO or prepare cell lysates to measure PGC1α acetylation levels, using Cell Lysis buffer (Cell Signaling Technology, Inc., Beverly, MA). PGC1α was immunoprecipitated from lysates of the transfected hepatocytes with EZview Red ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich) following the manufacturer’s directions. Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis using 4–12% Tris-glycine gels (Invitrogen). PGC1α acetylation was measured in lysates or immunoprecipitates by Western blotting using antibodies to total PGC1α and acetylated lysine.

Chicken TiPARP Overexpression Construct—The chicken TiPARP was amplified by PCR. Primers were designed using the predicted chicken TiPARP mRNA sequence (GenBankTM XM_422828) to amplify the entire coding region (from +349 to +2358 bp). The stop codon was excluded to obtain a His-tagged TiPARP because the vector sequence contained a stop codon after the His-Tag. PCR amplification mixtures contained: Phusion High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA); forward primer, 5'-CACATGATGAGCAAAGAG-3'; reverse primer, 5'-CCTCTTTTCGATGAGAAG-3' and cDNA from TCDD-treated CEH as template. The 2,009 bp 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) following the manufactur-er’s directions. Sequencing confirmed the PCR product as chicken TiPARP.

SDS-PAGE/Western Blotting—CEH lysates were prepared in 1× PBS or Cell Lysis buffer (Cell Signaling Technology). Protein concentration was determined by Bio-Rad DC Protein Assay. Lysates were diluted 1:1 (v/v) in 2× sample buffer containing 10% β-mercaptoethanol (β-ME) and heated at 100 °C for 2 min. Proteins were separated on precast Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes. See supplemental Experimental Procedures for primary antibodies and dilutions used. The secondary antibodies were peroxidase-conjugated goat anti-rabbit (Sigma-Aldrich) or goat antimouse (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein bands were detected with ECL Western blotting Detection reagents (GE Healthcare, UK). Band intensities were measured by densitometry using AlphaEaseFC Software (Alpha Innotech, Santa Clara, CA).

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed as described (22) with modifications as follows: CEH were plated at 1.5 \times 10^7 cells per 10-cm dish. TCDD treatment (1 nm) was for 90 min. For each immunoprecipitation, cells from 4 dishes were combined. Cells were sonicated using a Sonifiser (Branson, Danbury, CT) for 10 min (alternating cycles of 30 s of sonication and 30 s of rest). Sonicated chromatin was immunoprecipitated with 5 \mug of a rabbit polyclonal anti-AHR antibody (Enzo Life Sciences International, Inc., Plymouth Meeting, PA) or normal rabbit IgG (Cell Signaling Technology). A CYP1A5 dioxin responsive element (DRE) at −262 bp, reported to be functional for AHR binding (23), was used as a positive control. Putative DREs for chicken TiPARP and PEPCK (see Fig. 4) were identified and all were tested for AHR binding. qPCRs were performed using the primers listed in supplemental Experimental Procedures.

His-Ub Ubiquitination Assay—CEH (3 \times 10^6 per well in 6-well plates) were cotransfected as described above with 3 \mug of pMT107-Ub (gift from Dr. Pengbo Zhou), coding for His-tagged ubiquitin, and with 3 \mug of Flag-PGC1α. Cells transfected with pMT107-Ub or Flag-PGC1α alone were used as negative controls to ascertain specificity of the signal in the Western blotting analysis. After 24 h cells were treated for 2 h with 1 nm TCDD or dioxane (control) in the presence of MG132 (25 \muM; Enzo Life Sciences International). Cells were scraped, resuspended in 200 \muL of a buffer containing: 0.1 mM NaH_2PO_4, 10 mM Tri-HCl, 6 mM guanidine-HCl, 10 mM imidazole, pH 8, and sonicated (2 s pulses, for a total of 30 s). Before sonication 5% of the cell suspension was removed, resuspended in 2× sample buffer containing 10% β-ME and boiled for 5 min, to use as input. For each sample of sonicated cells 30 \muL of Qiagen Ni-NTA-agarose were added and incubated for 2 h at room temperature. Ni-NTA-agarose resin was washed five times with a buffer containing: 0.1 mM NaH_2PO_4, 10 mM Tri-HCl, 8 mM urea, 10 mM imidazole, pH 6.3, and resuspended in 2× sample buffer, boiled for 5 min, and the supernatant used for Western blotting analysis.

Statistics—Differences between group means were evaluated by unpaired, two tailed t-tests; p values ≤ 0.05 were considered statistically significant.
**RESULTS**

Suppression of Hepatic Gluconeogenic Endpoints by TCDD—As previously reported for mammalian liver (24) TCDD suppressed G6Pase and PEPCK expression in CEH (Fig. 1A) and in liver in ovo (Fig. 1B). Concurrently, TCDD increased expression of major AHR target genes, CYP1A4 and CYP1A5, chick orthologs of mammalian CYP1A1 and 1A2, respectively (25). Effects in ovo were seen as early as 12 h after treatment (not shown) and were sustained for at least 96 h (Fig. 1B).

TCDD also suppressed glucose output (GO) in hepatocytes after treatment in culture (Fig. 1C) or in ovo (Fig. 1D). Further, TCDD decreased hepatic glycogen (Fig. 1E) establishing that TCDD decreases hepatic carbohydrate stores, consistent with observations in mammals (7, 13).

AHR Requirement for TCDD Effects on GO and Gluconeogenic Gene Expression—To confirm that the AHR is required for TCDD suppression of gluconeogenic endpoints we transfected CEH with AHR siRNA. AHR silencing (Fig. 2A) abrogated the decrease by TCDD in G6Pase expression (Fig. 2B) and GO (Fig. 2C), demonstrating that the AHR is required for these effects. Interestingly, AHR suppression also increased GO and G6Pase expression in the absence of TCDD, indicating that the AHR may participate in TCDD-independent regulation of glucose production, an inference consistent with evidence for higher PEPCK expression in AHR−/− than in AHR−/+ mice (26).

TCDD Suppresses Pyridine Nucleotide Levels and Increases PARP Activity and TiPARP Expression—As pyridine nucleotides have been implicated in regulating energy homeostasis and enhancing gluconeogenesis by stimulating deacetylation of PGC1α by NAD+–dependent SIRT1 (11, 12, 27, 28) and by regulating several steps in gluconeogenesis, we hypothesized that TCDD might decrease pyridine nucleotide levels as part of its antigluconeogenic effects. TCDD reduced hepatic NAD+ and NADH, both by about 35% (Fig. 3A). Because PARP enzymes are major consumers of NAD+, which they use as a substrate for ADP-ribosylation reactions (27, 29), we considered PARP activity as a possible source for NAD+ depletion by TCDD. TCDD increased hepatocyte PARP activity (Fig. 3B, left panel). Moreover, TCDD enhanced ADP-ribosylation of several protein bands in hepatocytes (Fig. 3B, right panel).

As TCDD increased PARP activity and reduced NAD+ levels (Fig. 3, A and B), we considered that TCDD induction of a specific PARP, TiPARP (TCDD-inducible PARP, PARP7),
might contribute to decreased NAD\(^+\) levels after TCDD treatment. \textit{TiPARP} is an AHR target gene without a known biological function which has PARP activity (29–31). Fig. 3C shows that TCDD increased mRNA for \textit{TiPARP} as well as \textit{CYP1A4}, but not for PARP1, the major PARP enzyme (29), evidence that the AHR selectively induces \textit{TiPARP}. AHR siRNA suppressed \textit{TiPARP} induction (Fig. 3D, left bar graph) and abrogated the suppression of NAD\(^+\) by TCDD (right bar graph). These results show that \textit{TiPARP} is an AHR target gene in the CE model as in other species and are consistent with a contribution of increased \textit{TiPARP} to the decrease in NAD\(^+\) after TCDD treatment.

To confirm \textit{TiPARP} as an AHR target gene in our model we identified the dioxin responsive elements (DREs) up to 10,000 bp upstream of the transcription start site in chicken \textit{TiPARP} (Fig. 4A) and tested them by ChIP assays for enhanced AHR occupancy. ChIP assays (Fig. 4B) showed that the TCDD-activated AHR binds to one of thirteen DREs in \textit{TiPARP} at \(-4952\) bp and exhibits enhanced binding to a DRE at \(-262\) bp for \textit{CYP1A5}, the positive control. In contrast, TCDD did not enhance AHR binding to either of the two DREs found in the \textit{PEPCK} promoter, indicating that TCDD suppression of \textit{PEPCK} expression is not a direct effect of AHR binding.

TCDD induction of \textit{TiPARP} in CE was observed at 90 min after TCDD treatment along with induction of \textit{CYP1A4} and suppression of \textit{PEPCK} (supplemental Fig. S1). These effects persisted at 3 and 6 h after TCDD treatment, when statistically significant suppression of NAD\(^+\) levels and GO were also observed, suggesting that the decreases in GO and NAD\(^+\) are rapidly occurring responses after TCDD activation of the AHR. Interestingly, \textit{TiPARP} induction was greatest at the earliest time point whereas \textit{CYP1A} induction increased over the 6 h period, suggesting that there may be some differences in the AHR regulation of \textit{TiPARP} and \textit{CYP1A} genes.

We investigated the role of CYP1A induction, a main target of AHR activation, in the effects of TCDD on glucose production. Silencing of \textit{CYP1A} genes did not affect GO (supplemental Fig. S2), excluding a
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**FIGURE 5.** NAM improves glucose output by repleting NAD\(^+\) and antagonizing AHR action. A, mean glucose output ± S.E. in CEH treated with TCDD or dioxane (C), with and without NAM for 24 h. B, qPCR results for PEPCK and G6Pase in CEH cotreated with 50 mM NAM and TCDD or dioxane (C) for 24 h. C, effects of NAM on NAD\(^+\) levels in CEH, treated as described in A, D, Western blots using antibodies that recognize CYP1A4/5 or β-actin for effect of cotreatment with 50 mM NAM (24 h) on TCDD induction of CYP1A protein. E, qPCR results for CYP1A4, CYP1A5 and TiPARP mRNA in CEH 24 h after treatment with TCDD, with and without 50 mM NAM. F, effect of cotreatment of CEH with NAM or nicotinamide riboside (NR) for 24 h with TCDD or dioxane (C) on glucose output (left panel), NAD\(^+\) levels, (middle panel), and EROD activity (right panel).

**A.** contribution of CYP1A enzymes to TCDD effects on gluconeogenesis.

**Nicotinamide (NAM) Corrects TCDD Effects on NAD\(^+\) Levels and Gluconeogenic Endpoints**—Because TCDD suppression of glucose production was associated with NAD\(^+\) depletion we asked whether NAD\(^+\) repletion could alter the effects of TCDD. We used nicotinamide (NAM), a known precursor of NAD\(^+\) (32), for this purpose. NAM enhanced GO in the presence or absence of TCDD and proved to be highly effective in correcting the suppression of GO by TCDD; 5 mM NAM was partially corrective and 50 mM fully corrective (Fig. 5A). This effect of NAM was extremely robust and was seen in many independent experiments. NAM also increased PEPCK and G6Pase mRNAs in control and TCDD-treated hepatocytes (Fig. 5B). Mannitol, at identical concentrations, did not improve GO (supplemental Fig. S3) excluding an osmotic basis for the effects of NAM. NAM could reverse as well as prevent TCDD suppression of GO as GO was no longer suppressed in hepatocytes treated with TCDD for 48 h and exposed to 50 mM NAM during the last 24 h (not shown).

NAM increased NAD\(^+\) levels in control and TCDD-treated hepatocytes (Fig. 5C), in parallel to its ability to increase GO, consistent with a role for NAD\(^+\) repletion in NAM reversal of TCDD toxicity. 5 mM NAM partly corrected TCDD depletion of NAD\(^+\) while 50 mM NAM was fully corrective. Treatment of CE with TCDD and NAM in ovo showed that the corrective effects of NAM could also be produced in the whole animal (supplemental Fig. S4).

In addition to increasing NAD\(^+\) levels, NAM suppressed TCDD induction of CYP1A4 and CYP1A5 mRNA and protein and TiPARP expression (Fig. 5, D and E), identifying NAM as an AHR antagonist and a potential in vivo modulator of AHR action, and suggesting that NAM suppression of AHR activation, and of TiPARP induction in particular, might have a role in the improvement by NAM of gluconeogenesis.

To explore the relative importance of increased NAD\(^+\) and suppression of AHR action in the corrective effects of NAM, we examined the effect on GO of nicotinamide riboside, another NAD\(^+\) precursor (21). Nicotinamide riboside increased GO (Fig. 5F, left panel), confirming a role for NAD\(^+\) in improving GO, but NR increased GO less than NAM at a concentration that produced even higher NAD\(^+\) levels (Fig. 5F, middle panel), suggesting that increased NAD\(^+\) could only partially account for the effectiveness of NAM. Nicotinamide riboside did not significantly decrease CYP1A-dependent EROD activity (Fig. 5F, right panel) possibly explaining the reduced effectiveness of nicotinamide riboside in improving GO as compared with NAM, and supporting an important role for AHR suppression in the effects of NAM.

The effects of TCDD on GO and the ameliorative effects of NAM, including the suppression of AHR induced CYP1A, were also seen in rat H4IIE cells (supplemental Fig. S5), confirming that the effects of TCDD and NAM in the avian model can be demonstrated in a mammalian cell type.

**TCDD Affects PGC1α Function, Acetylation, and Stability**—Since gluconeogenic genes are regulated by the transcriptional coactivator PGC1α and PGC1α in turn is activated by deacetylation by SIRT1 which, like PARP enzymes, uses NAD\(^+\) as a substrate (27), we investigated the effect of TCDD on PGC1α function. siRNA silencing of PGC1α mimicked TCDD effects, decreasing PEPCK expression and suppressing GO in CEH (Fig. 6A). PGC1α overexpression increased GO, and this effect was reduced by TCDD and improved by NAM (Fig. 6B). Whereas TCDD increased the relative amount of the acetylated (inactive) fraction of PGC1α (Fig. 6C, left panel and bar graphs), we found unexpectedly that TCDD also decreased PGC1α levels, indicating that TCDD affected both the active state and the amount of...
PGC1α. NAM stabilized PGC1α and decreased the acetylated fraction of PGC1α (Fig. 6C, right panel and bar graphs). These results support the hypotheses that PGC1α is a target of TCDD, that TCDD dysregulates PGC1α function and that the corrective effect of NAM involves mitigation of PGC1α dysregulation.

As TCDD did not affect PGC1α mRNA expression (Fig. 6A, left bar graph) it seemed likely that it might reduce PGC1α levels by enhancing PGC1α degradation. PGC1α levels were lower in TCDD-treated CEH cotreated for 2 h with cycloheximide to suppress new protein synthesis than in the controls.
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(Fig. 6D), supporting this hypothesis. Further, treatment of CEH with the proteasome inhibitor MG132 increased PGC1α levels (Fig. 6F), and TCDD enhanced ubiquitination of PGC1α (Fig. 6F). The novel finding that TCDD enhanced the proteasomal degradation of PGC1α is consistent with evidence that the TCDD activated AHR has E3 ubiquitin ligase activity (33) and that TCDD enhances degradation of estrogen receptor-α protein (34).

Evidence for a Role of TiPARP in TCDD Effects on Glucose Production—TiPARP overexpression mimicked the suppression by TCDD of GO (Fig. 7A), NAD⁺ levels (Fig. 7B), and PEPC expression (Fig. 7C), and increased PGC1α acetylation and decreased PGC1α protein (Fig. 7D) supporting a contribution of TiPARP to all of the effects of TCDD shown here, including the post-translational effects of TCDD on PGC1α acetylation and stability. TiPARP siRNA reduced TiPARP expression and increased GO and NAD⁺ levels (Fig. 7, E–G) resulting in loss of statistical significance for the suppression of GO and NAD⁺ by TCDD. TiPARP siRNA had variable results on gluconeogenic gene expression and did not increase PGC1α levels. These findings suggest that there are additional complexities in TiPARP actions that will require further exploration and that, as discussed below, TiPARP is not the sole contributor to TCDD effects on glucose production. The evidence that TiPARP siRNA also affected GO and NAD⁺ in the absence of TCDD is consistent, moreover, with a role for the AHR in glucose homeostasis independently of TCDD.

Finally, induction of TiPARP by TCDD would be expected to decrease SIRT1 activity by increasing NAD⁺ consumption. As SIRT1 is the sole factor identified to deacetylate PGC1α (35) and TCDD increased PGC1α acetylation (Fig. 6C) our findings implicate suppression of SIRT1 activity in TCDD effects on PGC1α. Supplemental Fig. S6 presents further evidence implicating SIRT1 in TCDD effects.

DISCUSSION

These studies identify TiPARP and nicotinamide as players in the theater of TCDD/AHR toxicity. The paper reports the following new findings: AHR-induced TiPARP is a mediator of TCDD suppression of hepatic glucose production; PGC1α is a target of TCDD action; NAM is a corrective agent for a major TCDD metabolic toxicity by a previously unrecognized AHR antagonist activity, and there is a link between signaling pathways for AHR toxicity and nutrient homeostasis. Additionally, the ability of AHR siRNA and of NAM to enhance GO and gluconeogenic gene expression in the absence as well as the presence of TCDD raises the new possibility that the
AHR may contribute to glucose homeostasis independently of TCDD.

Fig. 8 presents a possible pathway by which TiPARP might suppress glucose production. This hypothetical scheme unites our results and provides a framework for further research. AHR activation by TCDD induces TiPARP, which contributes to decreased NAD⁺ levels. Decreased NAD⁺ may suppress activity of SIRT1 (and possibly of other sirtuins), increasing PGC1α acetylation and decreasing expression of PGC1α gluconeogenic target genes. TCDD decreases PGC1α levels, an effect also observed for TiPARP, by enhancing PGC1α proteasomal degradation. Further studies will be needed to learn the extent to which TCDD-enhanced PGC1α degradation is related to TiPARP and coupled to increased acetylation or associated with other post-translational modifications marking PGC1α for ubiquitination. NAM prevents and reverses TCDD suppression of gluconeogenesis by cooperatively enhancing NAD⁺ production and suppressing AHR-mediated TiPARP induction. TCDD may also decrease glucose production by pathways other than those defined in Fig. 8.

It would be expected that TiPARP utilization of NAD⁺ would decrease SIRT1 activity, as evidenced by our findings that TCDD increased acetylation of SIRT1 targets PGC1α (Figs. 6C and 7D) and Histone H3 (supplemental Fig. S6B). A role for SIRT1 in these effects is supported by a report that SIRT1 knockdown in the rat increased hepatic PGC1α acetylation and decreased gluconeogenic gene expression and glucose production (37).

The ability of NAM to enhance GO and gluconeogenic gene expression (seen in both CEH and mammalian (H4IIE) cells) appears to involve both increased NAD⁺ production, and suppression of AHR action, resulting in decreased NAD⁺ utilization. The novel AHR antagonist action of NAM shown here is consistent with a report that NAM inhibits transcriptional activation by the progesterone and glucocorticoid receptors (38) and suggests that NAM may more generally suppress transcriptional activation by ligand-activated receptors. Whereas NAM is well recognized as a SIRT1 inhibitor (39), our findings suggest that the dual actions of NAM to enhance NAD⁺ production and decrease NAD⁺ utilization (by suppressing AHR induction of TiPARP (see Fig. 5E)), may overcome the inhibition of SIRT1 by NAM. This unorthodox hypothesis is consistent with our findings and the suggestion of others that in a variety of contexts, effects of NAM on NAD⁺ synthesis may overcome sirtuin inhibition with resulting net activation of NAD⁺-dependent sirtuin activity (27, 40–42). It has been reported, for example, that NAM improved suppression of neuronal SIRT1 deacetylase activity by G/N glutamate (43). A possible additional explanation of this observation is that NAM has suppressive effects on acetyltransferase activity, although this will require further investigation. A role for AHR suppression in NAM effects, even in the absence of TCDD, is consistent with our evidence that silencing AHR or TiPARP enhanced GO, as did NAM, with or without TCDD treatment (see Figs. 2, 5, and 7).

The experiments reported here suggest a novel route whereby TCDD can suppress PECK expression by impairing the function and levels of PGC1α, a transcriptional coactivator of PECK. Other PGC1α targets associated with glucose production, i.e. PDK4 (44) and TCA cycle enzymes (45) could also be affected and involved in suppression of glucose production by TCDD. The partial correction by TiPARP siRNA of TCDD effects on gluconeogenic endpoints could be attributable to incomplete silencing of TiPARP, and/or to the contribution of factors other than TiPARP, a possibility specifically included in the scheme in Fig. 8. Thus TCDD or NAM may well affect other factors known to modulate hepatic gluconeogenesis and PGC1α function (i.e. effects on transcriptional coactivators FOXO and HNF4α, or other signaling pathways that can impinge on PGC1α function (i.e. cAMP, PKA, Akt, AMPK)). It will be of interest to learn, for example whether (a) TiPARP affects the function of key gluconeogenic factors by pathways other than NAD⁺ depletion, i.e. by ADP-ribosylation; (b) NAM counteracts other toxic effects of TCDD, and (c) “natural” or endogenous AHR ligands (5, 17) affect hepatic glucose production.

As PGC1α has regulatory roles in organs other than liver which are targets for TCDD toxicities, i.e. muscle, adipose tissue and heart (28), these findings suggest a potential role for PGC1α dysregulation in other TCDD effects, for example, in the muscle atrophy and lipid depletion associated with the TCDD-wasting syndrome and impaired cardiac contractile responses to inotropic stimuli (8). Our evidence that TCDD reduced hepatic pyridine nucleotide levels, together with a report that TCDD reduces NAD⁺ levels in breast cancer cell lines (46) suggests that NAD⁺ depletion may be a generalized effect of AHR activation contributing to systemic PGC1α dysfunction.

In summary, these findings identify new cross talk between signaling pathways for environmental toxicity (AHR) and nutrient homeostasis (NAD⁺/PGC1α/SIRT1) via the aryl hydrocarbon receptor target gene TiPARP. They also identify
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PGC1α dysfunction as a potential factor in TCDD toxicities and nicotinamide as an aryl hydrocarbon receptor antagonist.

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