RT-PCR Quantification of AHR, ARNT, GR, and CYP1A1 mRNA in Craniofacial Tissues of Embryonic Mice Exposed to 2,3,7,8-Tetrachlorodibenzo-\textit{p}-dioxin and Hydrocortisone


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C57BL/6N mouse embryos exposed to hydrocortisone (HC) or 2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin (TCDD) develop cleft palate. An interaction between these agents produces clefts at doses which alone are not teratogenic. The glucocorticoid receptor (GR) and dioxin receptor (AhR) mediated these responses and their gene expression was altered by TCDD and/or HC in palates examined on gestation day (GD) 14 by Northern blot analysis and in situ hybridization. The present study quantifies AhR, AhR nuclear translocator (ARNT), and GR mRNA at 4, 12, 24, and 48 h after exposure (time 0 = dose administration at 8 A.M. on gestation day 12) on GD12 to TCDD (24 \( \mu \)g/kg), HC (100 mg/kg) or HC (25 mg/kg) + TCDD (3 \( \mu \)g/kg). The induction of CYP1A1 mRNA was also quantified at 2, 4, 6, 12, 24, and 48 h for control and TCDD-exposed samples. Total RNA was prepared from midfacial tissue of 4–6 embryos/litter at each time and dose. An RNA internal standard (IS) for each gene was synthesized, which included the gene’s primer sequences separated by a pUC19 plasmid sequence. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on total RNA + IS using a range of 5–7 IS concentrations across a constant level of total RNA. PCR products were separated in gels (mRNA and IS-amplified sequences differed by 30–50 bases), ethidium bromide-stained, imaged (Hamamatsu Photonics Systems, Bridgewater, NJ), and quantified with NIH Image. CYP1A1 mRNA was significantly induced in the TCDD-exposed samples at all time points examined (\( p = 0.005 \) at 2 h and 0.001 after 2 h). During palatal shelf outgrowth on GD12, AhR mRNA levels increased significantly and this was not affected by treatment with TCDD or HC + TCDD. A significant increase in GR was detected at 24 h (\( p < 0.05 \)) and this was unaffected by any of the exposures. Expression of ARNT increased at 12 h (\( p < 0.001 \)); however, treatment with HC or HC + TCDD blocked this increase (\( p < 0.05 \)). At 24 h, the TCDD-treated embryos had significantly lower ARNT mRNA compared with controls (\( p < 0.001 \)). The relative overall expression level of the genes was AhR > ARNT > GR. Within individuals, expression of AhR and/or ARNT was highly correlated with GR level. In conclusion, CYP1A1 mRNA was expressed in developing craniofacial tissue and was highly induced by TCDD exposure. AhR, ARNT, and GR mRNA are upregulated in early palatogenesis, although not on the same schedule. The TCDD-induced decrease in ARNT at 24 h after dosing and the HC and HC + TCDD-induced delay in upregulation of ARNT may affect the dynamics of heterodimer formation between AhR and ARNT. The changes in ARNT mRNA level could also affect availability of this transcriptional regulator to interact with other potential partners, and these effects, separately or in combination, may be involved in disruption of normal embryonic development.

Key Words: dioxin (Ah); palate; Ah receptor (AhR); AhR nuclear translocator (ARNT); glucocorticoid receptor; CYP1A1; hydrocortisone; quantitative RT-PCR.

Clefts of the secondary palate can be induced in embryonic mice by a wide range of physical and chemical exposures. The etiology of cleft-palate induction encompasses multiple morphological processes, including disruption of palatal shelf growth, elevation, adhesion of opposing shelves, and events critical to removal of the medial epithelial seam. A coordinated expression of genes that regulate cell proliferation, differentiation, transformation, and cell death is required for normal palatogenesis (Zimmerman, 1997). Teratogenic exposures that disturb the expression of one or more of the critical genes in these pathways would be expected to contribute to induction of cleft palate in the embryo. The environmental contaminant 2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin (TCDD) is developmentally toxic and induces cleft palate in the C57BL/6N mouse embryo (reviewed in Couture et al., 1990; Birnbaum, 1995).
The etiology of this clefting involves a failure of the palatal shelves to adhere and fuse, which is related to altered differentiation and proliferation of the medial epithelial cells (Pratt et al., 1984; Abbott and Birnbaum, 1989a, 1998b). The expression of growth factors involved in regulation of these processes in the epithelial cells was also found to be disturbed by TCDD exposure (Abbott and Birnbaum, 1990).

Our studies of the cellular and molecular pathways involved in the induction of cleft palate also included examination of interactions between TCDD and the glucocorticoid hormone pathway (reviewed in Abbott, 1995, 1997; Abbott et al., 1992, 1994b; Neubert et al., 1973; Birnbaum et al., 1986). The responses to TCDD and glucocorticoids such as hydrocortisone (HC) are mediated through binding of each of these agents to its cellular receptor: TCDD to the aryl hydrocarbon receptor (AhR) and HC to the glucocorticoid receptor (GR). These receptors enter the cellular nucleus, bind to transcriptional regulatory elements specific to each receptor, and alter transcription of target gene mRNA. The components of both of these receptor-mediated pathways are complex and include multiple regulatory peptides, including the heat-shock protein, HSP-90 (AhR and GR molecular pathways reviewed in Schmidt and Bradfield, 1996; and Muller and Renkawitz, 1991, respectively). AhR forms a complex with a second related basic helix-loop-helix (HLH) protein, the AhR nuclear translocator (ARNT), a member of the PER-ARNT-SIM family of transcriptional regulators (Hoffman et al., 1991; Reyes et al., 1992). AhR, ARNT, and GR are expressed in the developing embryo and mRNA and protein are expressed in both palatal epithelium and mesenchyme (Abbott et al., 1995; Abbott and Probst, 1995; Abbott et al., 1994a,c).

Synthetic glucocorticoids, including dexamethasone, triamcinolone, and hydrocortisone, bind to GR and, when administered at pharmacologic doses, are teratogenic and induce cleft palate (Baxter and Fraser, 1950). In the C57BL/6N embryonic mouse, exposure to glucocorticoids reduces the palatal shelf size such that the opposing shelves can not come into contact and fusion does not occur (Peterka and Jelinek, 1978). This morphological mechanism differed from that observed for TCDD, where palatal shelves were in contact but failed to fuse. The synergistic interaction between TCDD and HC occurs at doses that are too low to induce clefts if given alone, but when administered together, produce cleft palate in all of the exposed embryos. These clefts are glucocorticoid-like, as the primary cause of clefting is attributed to formation of small palatal shelves.

The mechanisms contributing to this synergistic interaction were examined in our laboratory, using the following dosing regimen: TCDD was given orally at 24 μg/kg on GD10, HC was given subcutaneously (sc) at 100 mg/kg from GD10–13, and TCDD + HC was administered once each day from GD10–13 at 3 μg TCDD/kg (orally) and 25 mg HC/kg (sc) (Birnbaum et al., 1986; Abbott et al., 1992). The effects of TCDD and HC on expression of AhR and GR were then examined immunohistochemically and by in situ hybridization on GD14, and palatal tissues from several GD14, embryos were pooled to quantify mRNA by Northern blot (Abbott et al., 1994b). AhR mRNA and protein were downregulated by the teratogenic dose of TCDD and similarly, GR was downregulated by the high dose of HC. However, in the TCDD-treated embryos GR was upregulated, in the HC-exposed embryos AhR was increased, and both GR and AhR were elevated in the embryos exposed to both HC and TCDD. This suggested that the interaction may be related to the increased expression of both receptors in embryos exposed to both HC and TCDD.

The observations of GR and AhR expression were made at a single time point (GD14) after dosing on GD10 or 10–13. The goal of the present study was to provide quantitative observations at several time points after a single dose and to begin observations as early as 2 h after the exposure began following events up to 48 h. The doses used for this study were based on the previous research as described above; however, instead of using repeated exposures from GD10–13 as was done previously, a single dose was given on GD12. This change in the dosing regimen was necessary because the palatal shelves do not exist as morphological structures that can be dissected before GD12, and a single dose given early on that day makes it possible to collect tissues beginning at 2 h post-exposure. An exposure to 24 μg TCDD/kg on GD12 produces a high incidence of cleft palate (85% of exposed embryos, compared to 100% after the same dose on GD10). The mechanism of clefting appeared similar for either day of exposure as palatal shelves elevate, come into contact, but fail to fuse (Abbott and Birnbaum, 1989a; Abbott et al., 1994a). The RT-PCR method allowed each individual embryonic palatal sample taken on GD12 to be evaluated for expression of all three genes, and thus correlations between AhR, ARNT and GR could be statistically evaluated. The mRNA levels for GR, AhR, ARNT, and CYP1A1 are expressed as molecules of mRNA relative to a constant amount of total RNA. With this standardized unit, the relative overall expression levels of each gene could also be evaluated. The induction of CYP1A1 was also examined in TCDD-exposed palates to confirm the responsiveness of the palatal cells. There is a substantial literature regarding the induction of CYP1A1 by TCDD through the AhR-mediated pathway (review in Whitlock et al., 1996) and the mRNA for CYP1A1 has been quantified with RT-PCR methods similar to the one used in our study (Vanden Heuvel et al., 1993; 1994).

This study demonstrates that CYP1A1 mRNA is expressed in the developing craniofacial tissue and is induced by exposure to TCDD. AhR, ARNT, and GR mRNA are shown to be upregulated in early palatogenesis, and ARNT expression was decreased by HC, HC + TCDD and TCDD. The patterns of effect on ARNT mRNA were similar for HC and HC + TCDD. This response may be a factor in the overall morphological response to exposure leading to cleft palate.
MATERIALS AND METHODS

Animals. Female C57BL/6N mice were mated overnight with male C57BL/6N mice, plug day = GD 0, and shipped by Charles River Laboratories (Raleigh, NC). On arrival, animals were housed under controlled conditions of temperature (72 ± 2°F), humidity (40–60%), and lighting (12/12 h light/dark cycle), and provided food (Agway rat, mouse and hamster 3000) and water ad libitum.

Dosing. A TCDD stock solution was prepared by dissolving the chemical (purity ≥98% by gas chromatography/mass spectroscopy, Radian Corp., Austin, TX) in acetone, adding corn oil, and removing the acetone under vacuum (Savant Speed Vac, Savant Instruments, Inc., Farmingdale, NY). Final concentrations in corn oil were 0.6 μg TCDD/ml and 4.8 μg TCDD/ml for dosing at 5 ml/kg. Hydrocortisone-21 acetate (Sigma Chemical Co., St. Louis, MO), suspended in 0.85% saline (50 mg/ml and 12.5 mg/ml), was prepared daily for subcutaneous (sc) dosing at 2 ml/kg.

Pregnant mice were assigned randomly to treatment groups and dosed at 7 a.m. on GD 12 (designated 0 h) with either vehicle: TCDD, HC, or HC + TCDD. TCDD in corn oil was given orally by gavage and HC in saline was administered subcutaneously (sc); (24 μg TCDD/kg, 100 mg HC/kg, or 25 mg HC/kg + 3 μg TCDD/kg). In experiment 1, 4 fetuses from each of 3 litters (12 fetuses per treatment group and time point) were collected from the control and TCDD-exposed (24 μg/kg) groups at 4,12, and 24 h after treatment. These samples were examined for expression of AhR and CYP1A1. In experiment 2, at least 6 fetuses were collected from each litter at 2, 4, 6, 12, 24, and 48 h after treatment (1–2 litters/time point/dose group, including vehicle control, TCDD, HC and HC + TCDD). The 6 samples, collected at 4, 12, 24 and 48 h, were examined for expression of AhR, ARNT, and GR. Samples collected in the total RNA samples were diluted to give working concentrations ranging from 20 ng/μl to 100 ng/μl for use in the quantitative RT-PCR.

Total RNA preparation. Total RNA was prepared by homogenizing tissues in 0.5 ml TRI® Reagent (Molecular Research Center, Inc., Cincinnati, OH), a reagent used for stabilization of RNA during homogenization and containing phenol for separation of RNA in the aqueous phase following centrifugation. The extraction and precipitation of the total RNA followed standard methods (Davis et al., 1986), and total RNA was resuspended in diethylpyrocarbonate (DEPC)-treated water. The small tissue sizes generally required that a low volume be used to resuspend RNA pellets. The volume of water required to give a final concentration of the range of 20–100 ng total RNA per μl was estimated for each sample. These estimates were based on a yield of total RNA that would be 0.2% of the tissue wet weight (data not presented; tissues were weighed before freezing and this estimation procedure was done simply to give a reasonable volume for resuspending each sample). The GeneQuant II (Pharmacia Biotech, Piscataway, NJ) was used to determine optical density at 260 and 280 nm wavelengths (OD260 and the OD260/OD280 ratios, using a very low volume (10 μl) cuvette. Dilutions of the total RNA were made as 1 μl RNA in 9 μl water. Following calculation of concentration, the total RNA samples were diluted to give working concentrations ranging from 20 ng/μl to 100 ng/μl for use in the quantitative RT-PCR.

Quantitative RT-PCR. The quantitative approach was similar to that described in Vanden Heuvel et al., (1993), and the RT and PCR procedures followed standard protocols (details in Held and Abbott, 1998). A brief summation of specific features of the approach and modifications to standard protocols is presented: Quantitative RT-PCR was performed, using an RNA internal standard (IS) specifically synthesized for each mRNA under study. Preparation of the internal standards (IS) involved selection of primers for each IS that included a T7 polymerase sequence, the gene primer sequences, and sequences that are homologous to regions of the pUC-19 plasmid. Linearized PUC plasmid provided the template for a PCR reaction with the IS primers, the subsequent cDNA was purified, and T7 polymerase was used to transcribe cRNA for use as an internal standard. Each IS incorporated the primer sequences of the gene, separated by a PUC sequence whose length was selected to result in a final RT-PCR product of a 30–50-base difference, compared to the amplified product from the mRNA. Preparation of internal standards required PUC-19 plasmid (Promega, Madison, WI); HIND III restriction enzyme, and buffer; columns for purification of PCR products (Wizard™ PCR Preps, Promega, Madison, WI); and the in vitro transcription kit for large scale synthesis of short transcript RNAs (Ambion T7-MEGASHortscript™, Ambion, Austin, TX).

For typical RT-PCR runs, the total RNA was added at a constant ng amount to each of 5 or 7 PCR tubes, but IS RNA was added at increasing pg amounts to the 5 or 7 tubes, creating an IS concentration range that included the expected level of mRNA in the sample. Total RNA and IS RNA were reverse transcribed in each PCR tube, using the reverse primer for the gene. Primer pairs for each gene and IS were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX), and PCR conditions were optimized for each primer pair (gene and IS pairs) by testing concentration gradients of MgCl2, and adjusting the dissociation, anneal, and extension times and temperatures. Reagents used for RT-PCR included AMV Reverse Transcriptase and 10X RT buffer (HC = 23,000 units/ml, Promega, Madison, WI), Recombinant RNAsin Ribonuclease Inhibitor (40 units/μl, Promega, Madison, WI), dNTPs (10 mM in water, pH 7.0), MgCl2 (25 mM), Taq DNA polymerase (Thermus aquaticus strain YTI, 5 units/μl, Promega, Madison, WI), and 10X Thermobuffer. The amplification was performed using 0.2 ml PCR tubes in strips of 8 and a thermocycler with heated lid and 96-well block for 0.2 ml tubes (MJ Research, Inc., Watertown, MA). PCR products were separated in Metaphor™ agarose (FMC, Rockland, ME) gels with DNA markers, (pGEM® Markers, Promega, Madison, WI). Gels were stained with ethidium bromide to reveal amplified DNA bands under UV illumination, and images were acquired with Argus-20 SC8 Software for the Hamamatsu Photonic System (Bridgewater, NJ), and processed using NIH Image for Densitometric Analysis.

Data were acquired as pg or fg of mRNA per 100 ng of total RNA and then converted to molecules of mRNA/100 ng total RNA (based on the molecular weight of the amplified cDNA as calculated from the base sequence). The data were expressed in this format, rather than as mass-based quantities such as pg or ng, in order to facilitate comparisons between studies. The pg or ng amount of IS will depend on the size of the transcript, which in turn will vary with the position of the primers selected. Conversion of ng to molecules provides a constant unit for comparison between different amplified sequences.

Primers and PCR conditions. AhR primers were selected based on the GenBank sequence M94623 (Schmidt et al., 1993). These primers produced a 317-nucleotide product from the C-terminus region involved in transactivation and transformation (between the basic and Q-rich domains), with the forward primer spanning bases 1406–1517 and the reverse spanning bases 1812–1789 (forward = CGC TGA AAC ATG AGC AAA TGG G; reverse = ACA GCT TAG GTG CTG AGT CAC AGG). The AhR IS primers incorporated a 295-base sequence of PUC9 (this is the same as the pUC19 sequence), and produced a 341-base product (forward primer = TAA TAC GAC TCA TCT TAG GCC CGT AAA CAT GAG CAA ATT GCC AGA AGT GCC GCT AAC ACT TTA TCC; reverse = TTT TTT TTT TTT TTT TTT ACA GCT TAG GTG CTG AGT CAC AGG). The primer was 455 bp in length, the reaction volume of 20 μl and total volume of the PCR reaction of 50 μl. PCR dissociation was at 94°C for 15 s, annealing at 56°C for 1 min, and extension at 72°C for 30 s; the process was repeated for 35 cycles total.

Selection of primers for ARNT were based on the GenBank sequence
U10325 (Reisz-Porszasz et al., 1994). ARNT primers produced a 300-nucleotide product from the region important as a dimerization interface between AhR and ARNT and involved in DNA binding. The forward primer spanned bases 193-214 and the reverse primer spanned bases 492-469 (forward = GAT GCG ATG ATG ACC AGA TGT G; reverse = CAG TGA GGA AAG ATG GCT TGT AGG). The ARNT IS primers incorporated a 226-base sequence of pUC9 and produced a 272-base product (forward primer = TAA TAC GAC TCA CTA TAG GGA TGC GAT GAC CAG ATG TGC ATT GCT ACA GCC ATC GTG GTG; reverse = TTT TTT TTT TTT TTT TTT CAG TGA GGA AAG ATG TGT AGG GCA TCT TAC GGA TGG CAT GAC AG). The reactions included 50 ng of total RNA. IS concentrations ranged from 20–0.5 pg, and MgCl₂ (25 mM) was added at 4 μl per 20-μl reaction in the RT step, and an additional 4 μl per 50-μl total in the PCR step. PCR required a hot start at 94°C and then dissociation occurred at 94°C for 15 s, annealing at 56°C for 1 min, and extending at 72°C for 30 s, with a total repetition of 30 cycles.

Primers for GR were selected from the GenBank sequence X04435 (Danielsen et al., 1986). GR primers produced a 299-nucleotide product from the C-terminal hormone-binding domain, with the forward primer spanning bases 1895–1917 and the reverse at bases 2193–2171 (forward = TGG TAT GCT TTG CTC CTC ATG TG; reverse = TGG CAG ATG ATA AAA CCG CTG CC). The GR IS primers incorporated a 295-base sequence of pUC9 and produced a 341-base product (forward primer = TAA TAC GAC TCA CTA TAG GTG CTA TGC TTG CTC CTT GCT CCT GTG CAT CAG TAG ATG TGC). The reactions included 50 ng of total RNA. IS concentrations ranged from 15 to 0.5 pg, and MgCl₂ (25 mM) was added at 4 μl in the RT step and 2 μl per reaction in the PCR step. PCR dissociation was at 94°C for 15 s, anneal at 55°C for 1 min, and extension at 72°C for 30 s, again repeating the process for 35 cycles total.

Mouse CYP1A1 primers were selected based on GenBank sequence M10021 (Gonzalez et al., 1984; Kimura et al., 1984) and these primers produced a 230-nucleotide product spanning intron C, with the forward primer from bases 1051–1070 and the reverse at bases 1280–1261 (forward primer = CCA CTT TGG AGA TGG GGT TG; reverse = TGG TGG GGA TGG TGA AG). The IS primers incorporated a 226-base sequence of pUC19 and produced a 265-base product (forward primer = TAA TAC GAC TCA CTA TAG GCC TCT TTG GAG CTG GTT CAT TGC TAC AGG CAT CTG GGT G; reverse = TTT TTT TTT TTT TTT TTT TGT CAG TTG ATA AAA CCG CTG CCC AGT GCC ACC ATA AGT G). The reactions included 20 to 100 ng of total RNA. IS concentrations ranged from 50–0.5 pg, and MgCl₂ (25 mM) was added at 4 μl in the RT step and 2 μl per reaction in the PCR step. PCR dissociation was at 94°C for 15 s, anneal at 55°C for 1 min, and extension at 72°C for 30 s, again repeating the process for 35 cycles total.

Mouse CYP1A1 samples were prepared based on GenBank sequence M10021 (Gonzalez et al., 1984; Kimura et al., 1984) and these primers produced a 230-nucleotide product spanning intron C, with the forward primer from bases 1051–1070 and the reverse at bases 1280–1261 (forward primer = CCA CTT TGG AGA TGG GGT TG; reverse = TGG TGG GGA TGG TGA AG). The IS primers incorporated a 226-base sequence of pUC19 and produced a 265-base product (forward primer = TAA TAC GAC TCA CTA TAG GCC TCT TTG GAG CTG GTT CAT TGC TAC AGG CAT CTG GGT G; reverse = TTT TTT TTT TTT TTT TTT TGT CAG TTG ATA AAA CCG CTG CCC AGT GCC ACC ATA AGT G). The reactions included 20 to 100 ng of total RNA (control and early time points for treated samples required more total RNA to detect the mRNA). IS concentrations were prepared in 3 sets (1000–50 pg, 250–10 pg, and 150–1 pg) to accommodate the wide range of mRNA expression. The selection of the range for each time point was based on trials of representative samples. The optimal IS range would provide amplification of total RNA as well as IS, giving 2 bands per lane on the gel, as shown in Figure 1. In the RT step, 4 μl of 25 mM MgCl₂ was added on the reaction and no additional MgCl₂ was added to the PCR buffer. Stratagene Buffer 2 and Master Mix from the Opti-Prime™ PCR Optimization Kit (Stratagene, Menasha, WI) were used for RT-PCR with this primer pair, with dissociation at 94°C for 15 s, anneal at 55°C for 1 min, extension at 72°C for 30 s, and repeating the process for a total of 35 cycles.

**Statistical analysis.** Data were examined for treatment effects, correlations between genes, changes in gene expression across time, and the relative scales of expression of AhR, ARNT, and GR were compared. All statistical testing was done in SAS with log transformation of the data (SAS Institute, 1989, 1990, 1992, Cary, NC). Mean differences between treatment*time groups were compared by pairwise tests within univariate and multivariate analyses of variance. In experiment 1, the analysis of variance was expanded to adjust for any variation due to PCR run, litter, and embryos nested within litter, with the addition to the model of these three variables as random effects. In experiment 2, variance due to the PCR run was negligible, and the others were not applicable. The Ansari-Bradley test, a rank-based test of scale, was used to examine differences in the ranges of data values between treated and control groups, and permutation tests were used to determine the exact p-values for each test (Hollander and Wolfe, 1973; Randles and Wolfe, 1979). Data sets are presented as back-transformed means having 95% confidence intervals with p-values from univariate tests. Data were presented graphically as means and standard errors calculated from original, non-transformed data sets.

**RESULTS**

**RT-PCR Sensitivity, Experimental Design and Sources of Data Variance**

Representative ethidium bromide-stained gels, in which the amplified mRNA and IS bands were electrophoretically separated, are shown in Figure 1 for each of the genes. For the CYP1A1, AhR and GR series (1A, 1B and 1D), the intensity of the RNA band increased (lower band proceeding from left to right in the gel lanes) as the intensity of the IS band (upper band) and concentration of IS added to each RT-PCR reaction decreased. For the amplification of ARNT (1D), the mRNA band was the upper band and the IS was the lower-molecular-weight band; the intensity of the IS band increased from left to right on the image. The quantitative RT-PCR method used in these studies was highly sensitive, and with appropriate internal standard concentrations, the mRNA detection range (GR, AhR, ARNT, and CYP1A1) spanned <10 to >60,000 mole-
cules per 100 fg of total RNA. Analysis of sources of variance that could affect interpretation of the data was an important determinant of experimental design and execution. The experiments included multiple treatments across several time points, and the PCR runs should ideally include representatives of each time/treatment combination. However, due to the large number of samples and time/treatment combinations, PCR runs could only include partial blocks of samples. Therefore, it was important to identify the significant contributors to variance in the data sets and particularly to determine if variability between PCR runs or differences between litters would affect the ability to identify treatment- or time-related differences in gene expression. Statistical analysis showed no significant PCR experiment-related variance. The degree of variance due to differences between litters (litter effect) was examined in samples of 4 embryos/litter across 3 litters for each time and treatment. There was no litter effect as variance between litters of the same treatment-time combination was essentially zero.

**CYP1A1**

A representative quantitative RT-PCR gel for CYP1A1 is shown in Figure 1A. There were two major experiments in which tissue was collected, and these are described in the Materials and Methods section as experiments 1 and 2. The expression of CYP1A1 in control and TCDD-exposed palates was examined in RT-PCR runs, which simultaneously included samples from both of these experiments. The means and 95% confidence intervals are presented in Table 1, and means and standard errors are plotted in Figure 2A. The GD12–14 embryonic craniofacial tissues expressed CYP1A1 mRNA, and levels in control tissue remained fairly constant across the timepoints (2–48 h), with the exception of a low point at 6 h (p < 0.01 compared to 4 or 12 h). The difference at 6 h seems relatively unimportant compared to the effects of TCDD on expression of the mRNA. As early as 2 h after treatment, a significant increase (p = 0.005) was detected and at all later time points the induction was significant (TCDD vs controls, p < 0.001). From 2 to 4 h, the mRNA levels rise significantly (p = 0.02); however, a plateau was observed from 4 to 48 h in which no further significant increase occurred. The higher value at 48 h was not statistically significant compared to the 24-h value (p = 0.069), but may indicate a further induction in some of the embryos.

**AhR**

An example of a quantitative AhR gel is shown in Figure 1B. AhR mRNA was quantified in samples collected from both of the experiments; however, since the RT-PCR runs for experiments 1 and 2 were separated by an extensive time period, and since experiment 2 included more treatments and a 48-h time point, the statistical analyses were performed separately, as shown in Table 1 (experiment 1) and Table 2 and Figure 2B (experiment 2). In both data sets, the expression of AhR was not significantly altered by TCDD exposure at any time point. The expression in experiment 1 was similar at 4 and 12 h, but increased significantly in both control and TCDD-treated samples (p < 0.05) at 24 h. The increase in AhR (Table 1) at 24 h was not detected in data set 2 and this is likely due to a smaller sample number in the second experiments. However, in experiment 2 (Table 2 and Fig. 2B) there was a pattern of significantly increased AhR expression at 12 h in all groups except the HC-treated tissues (control and TCDD: p < 0.001; HC + TCDD: p = 0.005; HC: p = 0.067).

**ARNT**

An RT-PCR run for quantification of ARNT mRNA, using 7 IS levels, is shown in Fig. 1C. The ARNT mRNA

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**TABLE 1**

Mean Estimates with 95% Confidence Intervals in Molecules of mRNA/100 fg Total RNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Hour</th>
<th>n</th>
<th>Control</th>
<th>TCDD*</th>
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</thead>
<tbody>
<tr>
<td>CYP1A1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>7</td>
<td>614.9 (201.3, 1879)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>428.6 (157.3, 1167)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>25.88 (7.6, 87.9)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>8</td>
<td>636.1 (233.4, 1733)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>6</td>
<td>304.6 (111.6, 831.1)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>7</td>
<td>684.0 (187.4, 2497)</td>
<td>7</td>
</tr>
<tr>
<td>AhR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
<td>11</td>
<td>781.4 (397.0, 1537.9)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>12</td>
<td>801.5 (419.2, 1532.5)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>12</td>
<td>1,994.8&lt;sup&gt;*&lt;/sup&gt; (1022.8, 3890.3)</td>
<td>11</td>
</tr>
</tbody>
</table>

Note. Embryos collected at 4, 12, and 24 hours examined for both AhR and CYP1A1 with additional embryos collected at 2, 6, and 48 h for CYP1A1. n = number of embryos. *p < 0.05, 24 vs. 4 & 12 h, comparison between times within treatment; **p = 0.005; ***p < 0.001, comparisons between control and treated within time points.

<sup>a</sup> Samples from experiment #1.

<sup>b</sup> Samples from experiments #1 & 2.
levels in control tissues increased significantly \((p < 0.001)\) from 4 to 12 h, but further change was not detected after 12 h. TCDD exposure did not affect this upregulation of ARNT, as the significant increase was present here as well \((p < 0.001)\). However, embryos exposed to HC or HC + TCDD exhibited significant delay in the developmental up-regulation of ARNT and levels of mRNA equivalent to the control were not achieved until 48 h after exposure (Fig. 2C and Table 2). Exposure to HC or HC + TCDD significantly reduced mRNA for ARNT at 12 h \((p = 0.016 \text{ and } 0.013, \text{ respectively})\), and although a difference was apparently present at 24 h, the data were not significantly different from control. The levels of ARNT gradually increased from 4 to 48 h, and there was no significant difference between control and HC or HC + TCDD values at either 4 or 48 h. The exposure to TCDD produced a significant decrease in the level of mRNA at 24 h \((p < 0.001)\) compared to controls at that time point. This is also reflected in a significant difference in TCDD-treated samples between the 24-h value and those at 12 and 48 h \((p = 0.007 \text{ and } p < 0.001, \text{ respectively})\).

**GR**

One of the gels for GR is shown in Figure 1D. As shown in Table 2, the expression of GR did not differ between 4 and 12 h, but a significant increase was detected between 12 and 24 h in all groups, and the degree of significance ranged from \(p < 0.05\) to 0.001. Exposure to a single dose of TCDD, HC, or HC + TCDD on GD12 did not significantly affect the level of mRNA for GR at any of the times examined (Table 2, Fig. 2D). The apparent decline in expression between 24 and 48 h in some groups was not statistically significant.
AhR and GR, and ARNT and GR remained highly correlated (correlation coefficients \( cc \) = 0.54 and 0.48, \( p < 0.001, 64 \ df \); however, AhR and ARNT were less strongly correlated (partial \( cc \) = 0.23, \( p = 0.069 \)). After partialling out time, treatment and GR, AhR and ARNT were no longer correlated. Thus, there is a statistically significant positive correlation such that an embryo with high AhR and/or high ARNT status (regardless of the amount collected or treatment received) will also have high GR status. Conversely, this association also applies to low AhR or ARNT status being associated with low GR status.

**DISCUSSION**

Quantification of mRNA for multiple genes in individual embryonic samples was accomplished through an RT-PCR reaction incorporating synthetic RNA internal standards. RT-PCR sensitivity and reproducibility were high, and although the levels of expression were quite different for the genes, adjusting the amount of total RNA and the range of the IS provided reproducible determinations across a wide range of values. The measurement of several mRNAs in each embryonic sample also provided a unique opportunity to look for correlations, and a significant correlation was found for expression of AhR and/or ARNT and GR. The correlation of GR and AhR is interesting, as the pathways of these genes appear to interact not only in the synergism of cleft palate induction, but also in regulation of hepatic glucocorticoid binding and induction of P450 enzymes. TCDD exposure is known to affect the hepatic GR pathways (Goldstein *et al.*, 1990) and in rats TCDD modified the ability of GR in hepatic cells to bind ligand without changing the level of the GR protein (Sunahara *et al.*, 1990). Glucocorticoids also potentiate the induction of CYP1A1 in hepatic cells by AhR agonists and the induction in endothelial cells was believed to involve GR-dependent mechanisms (Celander *et al.*, 1989). Glucocorticoids also potentiate the induction of CYP1A1 in mammalian cell types (Whitlock *et al.*, 1996, review).
HC with TCDD on GD10, or dosing from GD10–13 with HC or TCDD, or HC exposure on GD12 to HC, TCDD, or HC. The response to TCDD appeared to be more restricted in time, occurring only at 24 h post-exposure. It should also be noted that palates of embryos exposed to 24 μg TCDD/kg on either GD10 or GD12 are of normal size, and clefting is attributed to altered proliferation and differentiation of the epithelial cells, a response quite different from the reduced size observed after HC or HC + TCDD (Abbott et al., 1992).

GR mRNA expression was not significantly affected by exposure on GD12 to HC, TCDD, or HC + TCDD. Treatment with TCDD on GD10, or dosing from GD10–13 with HC or HC + TCDD, altered GR mRNA expression in the HC- and HC + TCDD-exposed groups and the direction of the effect depended on the specific treatment (Abbott et al., 1994b). Multiple doses of HC (GD10–13) downregulated GR mRNA on Northern blots of GD14 tissue. Exposure to TCDD on GD10 or HC + TCDD on GD10–13 upregulated GR expression on GD14 (in situ hybridization data). The induction of cleft palate in C57BL/6N embryos requires multiple exposures at 100 mg HC/kg exposure (GD10–13), and this regimen only produces clefts in approximately 30% of the embryos (Abbott et al., 1994b). The sensitivity to glucocorticoid-induced cleft palate is strain-dependent, linked to expression of a high affinity GR, and the C57BL/6N strain is relatively resistant to HC-induced clefting (Baxter and Fraser, 1950; Biddle and Fraser, 1976). The failure to detect and quantify changes in GR mRNA levels may be due in part to the overall resistance of this strain to HC and further efforts to characterize the responses would require adopting a multiple-dose regimen. However, since palates do not exist as morphological structures that can be dissected prior to GD12, the present study used a single dose on that day.

AhR expression was also not significantly affected by exposure to HC on GD12, although the mean appeared elevated at 24 and 48 h. However, AhR mRNA levels were elevated on GD14 following exposure to HC from GD10–13, as determined from Northern blots and in situ hybridization (Abbott et al., 1994b). Also in that study, TCDD downregulated both AhR protein and mRNA after exposure to 24 μg/kg on GD10 or 3 μg/kg from GD10–13. The GD12 exposure to 24 μg TCDD/kg appeared to reduce AhR values at 24 h, but the effect was not significant, and at 48 h, no effect of treatment could be detected. A single administration on GD12 of HC + TCDD also did not affect AhR mRNA expression. These outcomes suggest that alterations in AhR mRNA require early (GD10) or multiple exposures to TCDD, HC and HC + TCDD.

Thus the effects of TCDD and/or HC on AhR and GR mRNA expression appear to depend on one or more of the following: exposure of an earlier developmental stage (GD10), repeated exposures to maintain a presence of HC, or an extended duration of exposure to TCDD (4 days vs. only 2). For example, dosing on GD10 with TCDD not only allows the compound to be present at earlier stages of craniofacial development, but allows a relatively uniform tissue concentration to be maintained for 4 days (GD10–14). Persistence of TCDD and a relatively uniform tissue concentration during craniofacial development was demonstrated in embryos dosed on either GD11 or 12 (Abbott et al., 1989; Abbott et al., 1996).

AhR, ARNT, and GR mRNA levels were all upregulated in control palates on GD12. The increases in AhR and ARNT mRNA were detected by 12 h, while GR upregulation occurred in the second part of the 24-h period. Gestation days 12 through 13 comprise a period of vertical outgrowth of the palatal shelves, which are on either side of the tongue, followed by the elevation of the shelves to a horizontal position over the tongue (Zimmerman, 1997). In contrast to our findings of a statistically significant increase in GR mRNA at this developmental stage, Lau et al., (1993) reported a 30% decrease in GR mRNA in the B10.A mouse embryo immediately after elevation of the palatal shelves. In that study, mRNA of constitutively expressed genes was compared to GR mRNA in a semiquantitative PCR analytical method. Although there are substantial differences between the methods used to quantify the mRNA, the different outcomes of these studies may reflect strain variation. The increase in GR and AhR mRNAs on GD12 was not affected by exposure to TCDD or HC + TCDD. However, in HC-exposed palates, although GR was upregulated in the initial 24 h, the increase in AhR occurring between 4 and 12 h was not statistically significant (p = 0.067, n = 5). Although it is possible that HC attenuated the increase in AhR, a larger sample would be needed to show the expected upregulation. The upregulation of AhR, ARNT and GR during this period may be coincidental; however, it cannot be ruled out that these genes have a role in the initial growth and elevation of the shelves.

The relative number of copies of each mRNA was found to be quite different for AhR, GR and ARNT and relative abundance of mRNA was AhR > ARNT > GR. The differences between the gene expression levels were 25.8:4.3:1 (based on an average value for control tissues). Higher levels of AhR relative to ARNT were reported for female Sprague-Dawley rats (Pollenz et al., 1998) as well as in several mammalian cell culture lines derived from both hepatic and non-hepatic sources using Western blot analysis.
for protein (Holmes and Pollenz, 1997). In the Holmes and Pollenz study, the ratio of AhR to ARNT ranged from 0.3 in H411E cells to 10 in Hepa-1 cells. The C57BL/6N embryonic craniofacial cells appear to express 6 times more AhR than ARNT. ARNT dimerizes with other bHLH proteins and regulates multiple gene pathways through these complexes (Rowlands and Gustaffsson, 1997). ARNT heterodimerizes with hypoxia induction factor-1α (HIF1α) to directly up-regulate genes in the hypoxic-response pathway and this constitutes an important mechanism for responding to anemia, wound healing, and high altitude, and may be important for normal placental and embryonic development (Wenger and Gassmann, 1997, review). The transgenic knockout of ARNT is lethal in utero and the lethality is linked to angiogenesis in the yolk sac (Maltepe knockout of ARNT is lethal in utero and the lethality is (Wenger and Gassmann, 1997, review). The transgenic knockout of ARNT is lethal in utero and the lethality is linked to angiogenesis in the yolk sac (Maltepe et al., 1997) and placenta (Kozak et al., 1997), processes that may well be linked to hypoxic responses during development. Since no homozygous knockout embryos survive beyond GD11, it is not possible to determine if the embryos form a normal secondary palate. The equilibrium between the bHLH proteins (including ARNT) has been proposed as a factor in regulating biological responses. In particular, it was proposed that recruitment of ARNT to complex with AhR may affect the formation of dimers with its other partners (Holmes and Pollenz, 1997).

Maintaining the relative levels of expression of AhR and ARNT may not only be critical to normal embryonic development, but disruption of the balance of these factors could be important in the response to TCDD. The decreased expression of ARNT in TCDD-exposed palates at 24 h and the prolonged deficiencies in ARNT mRNA in the HC and HC + TCDD tissues may have physiological importance. The most prominent response detected in this study was the downregulation of ARNT, and the developmental timing and duration of the effect correlated with differential outcomes in palatogenesis. The etiology of clefting after a single dose of TCDD on GD12 involved failure of opposing palates to fuse, and these palates had a pronounced decrease in ARNT of relatively short duration. Prolonged deficits in ARNT mRNA correlated with clefting due to formation of small palatal shelves and was observed after both treatments that produce identical depression in the mRNA.

In summary, the developing secondary palate AhR, ARNT, and GR mRNAs were upregulated during early outgrowth and elevation of the palatal shelves. The expression of AhR and ARNT correlated with that of GR. GR was expressed at lower levels than either AhR or ARNT and ARNT mRNA was generally 5–6-fold lower than that of AhR. The most pronounced effects of treatment were reduction in ARNT mRNA. Maintaining the appropriate relative expression of these genes may be important in regulation of development, and decreasing the availability of ARNT may be a significant factor in response to TCDD, HC and HC + TCDD.

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REFERENCES


