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Diverse Chemicals Including Aryl Hydrocarbon Receptor Ligands Modulate Transcriptional Activity of the 3'Immunoglobulin Heavy Chain Regulatory Region

Rebecca A. Henseler, Eric J. Romer, and Courtney E.W. Sulentic

Department of Pharmacology & Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, OH 45435, USA

Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a known disruptor of B-cell differentiation and a ligand for the aryl hydrocarbon receptor (AhR), induces binding of the AhR to dioxin responsive elements (DRE) in sensitive genes. The Ig heavy chain (IgH) gene is a sensitive target of TCDD and may be transcriptionally inhibited by TCDD through inhibition of the 3'IgH transcriptional regulatory region (3'IgHRR). While the 3'IgHRR contains binding sites for several transcription factors, two DRE motifs were also identified which may be responsible for TCDD-induced inhibition of 3'IgHRR activation and may implicate the AhR as an important regulator of IgH expression. The objectives of the present study were to determine if 3'IgHRR modulation is limited to TCDD or if structurally diverse chemicals (AhR ligands and non-AhR ligands) from environmental, industrial, dietary or pharmaceutical origin are also capable of modulating the 3'IgHRR and to verify a correlation between effects on a stable 3'IgHRR reporter and the endogenous IgH protein. Utilizing a CH12.LX mouse B-cell line that stably expresses a 3'IgHRR-regulated transgene, we identified an inhibition of both 3'IgHRR activation and IgH protein expression by the non-dioxin AhR activators indolo(3,2-b) carbazole, primaquine, carbaryl, and omeprazole which followed a rank order potency for AhR activation supporting a role of the AhR in the transcriptional regulation of the 3'IgHRR and IgH expression. However, modulation of the 3'IgHRR and IgH expression was not limited to AhR activators or to suppressive effects. Hydrogen peroxide and terbutaline had an activating effect and benzyl isothiocyanate was inhibitory. These chemicals are not known to influence the AhR signaling pathway but have been previously shown to modulate humoral immunity and/or transcription factors that regulate the 3'IgHRR. Taken together these results implicate the 3'IgHRR as a sensitive immunological target and are the first to identify altered 3'IgHRR activation by a diverse range of chemicals.

Keywords

3'IgHRR; immunoglobulin; aryl hydrocarbon receptor; B-cell line; in vitro

Corresponding author: Dr. Courtney E.W. Sulentic, Dept. of Pharmacology & Toxicology, 206 Health Sciences Bldg, Boonshoft School of Medicine, Wright State University, Ohio 45435, phone: 937-775-3583, fax: 937-775-7221, e-mail: E-mail: courtney.sulentic@wright.edu.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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INTRODUCTION

2,3,7,8-Tetracholordibenzo-*p*-dioxin (TCDD) is a potent and persistent environmental toxicant, which produces a variety of biological effects in animal and cellular models (Birnbaum and Tuomisto, 2000). Of these effects, immune suppression including inhibition of B-lymphocyte differentiation into antibody secreting cells is one of the most sensitive consequences of TCDD exposure (Kerkvliet, 2002). Inhibition of Ig expression and secretion in B lymphocytes has been well documented with several studies supporting the involvement of the aryl hydrocarbon receptor (AhR) signaling pathway; however, the specific mechanism remains unclear (Holsapple et al., 1991; Sulentic et al., 1998; Sulentic et al., 2000; Vorderstrasse et al., 2001).

The AhR and its dimerization partner AhR nuclear translocator (ARNT) are believed to regulate transcription by binding dioxin responsive elements (DREs) in regulatory regions of dioxin-sensitive genes (for review, see Okey, 2007). The CH12.LX B-cell line has provided a useful model in studying the effects of TCDD on Ig expression in B cells and has lead to the identification of a novel transcriptional target of TCDD: the Ig heavy chain (IgH) locus (Sulentic et al., 2000; Sulentic et al., 2004b). Regulation of the murine IgH locus is governed through a complex interaction of several regulatory elements. One such element, the 3'IgH regulatory region (3'IgHRR), appears to mediate processes late in B-cell differentiation such as up-regulation of IgH expression as well as class switch recombination (Manis et al., 1998; Pinaud et al., 2001). The 3'IgHRR is a large, approximately 40 kb region that lies downstream of the IgH constant regions. The 3'IgHRR is most often associated with four enhancer domains (hs3A; hs1,2; hs3B; and hs4) which contain DNA binding sites for several transcription factors that appear to be important regulators of enhancer activity (reviewed by Khamlichi et al., 2000). The AhR may also contribute to the regulation of the 3'IgHRR since at least two functional DRE sites (i.e., TCDD-induced AhR and ARNT binding) have been identified within the hs1,2 and hs4 enhancers (Sulentic et al., 2000). Correspondingly, in the CH12.LX cell line TCDD profoundly inhibited LPS activation of a luciferase reporter construct regulated by the 3'IgHRR (Sulentic et al., 2004b), correlating with the significant inhibition of LPS-induced IgH gene expression and antibody secretion by TCDD and other polychlorinated dibenzo-*p*-dioxin congeners that followed a structure activity relationship for AhR-mediated effects (Sulentic et al., 1998; Sulentic et al., 2000). Interestingly, the incidence and/or severity of the human diseases, IgA nephropathy and Celiac disease, have been associated with altered regulation of the 3'IgHRR (Aupetit et al., 2000; Frezza et al., 2004). Therefore, chemical-induced modulation of the 3'IgHRR likely has significant implications regarding the potential for altered Ig regulation and impaired immunity.

The most widely studied exogenous AhR ligands are the highly persistent halogenated aromatic hydrocarbons (HAHs), of which TCDD is considered the prototype, and the less persistent polycyclic aromatic hydrocarbons (PAHs). However, it is becoming increasingly evident that exogenous activators of the AhR signaling pathway are not limited to HAHs or PAHs but also comprise a diverse array of chemicals including those of dietary or therapeutic origin; and studies have also identified indoles, tetrapyroles, and arachidonic acid metabolites as potential endogenous, albeit low affinity, ligands for the AhR (reviewed in (Denison and Nagy, 2003; Nguyen and Bradfield, 2008)). Therefore an objective of the present study was to determine if the suppression of 3'IgHRR transcriptional activity and Ig expression was dioxin-specific or also sensitive to non-dioxin AhR ligands. However, the 3'IgHRR is clearly regulated by many transcription factors and signaling pathways and consequently may be a sensitive toxicological and clinical target of a broader range of chemicals. Indeed, an additional objective of this study was to validate the utility of our 3'IgHRR model in identifying chemicals that can alter 3'IgHRR activity. Therefore, we expanded our evaluation to chemicals not known to modulate the AhR

signaling pathway but shown to modulate the antibody response or signaling pathways involved in 3'IgHRR activity.

Using the well-characterized CH12.LX cell line and both transiently and stably expressed 3'IgHRR reporters, we found that modulation of 3'IgHRR activity was not limited to the AhR ligand TCDD but was sensitive to diverse AhR activators as well as chemicals that influence non-AhR signaling pathways implicated in 3'IgHRR regulation. Additionally, effects on 3'IgHRR activity correlated well with effects on endogenous IgH protein expression. Collectively, these results support the hypothesis that the 3'IgHRR is a sensitive immunological target of a diverse range of chemicals and that altered 3'IgHRR activity will alter IgH expression and antibody-mediated immune function. Furthermore, these results support a role of the AhR in 3'IgHRR transcriptional activity but also underscore the complexity of 3'IgHRR regulation and the diversity of chemicals that may alter 3'IgHRR activity.

MATERIALS AND METHODS

Chemicals and Reagents

TCDD, 1-monochlorodibenzo-*p*-dioxin (MCDD), and 2,2',3,5',6-pentachlorobiphenyl (PCB95) in 100% dimethyl sulfoxide (DMSO) were purchased from AccuStandard Inc. (New Haven, CT). Hydrogen peroxide solution (30 wt. % in water) and the neat form of carbaryl, primaquine, omeprazole, terbutaline, and carbachol were purchased from Sigma Aldrich (Milwaukee, WI). The certificates of product analysis stated the purity of TCDD, MCDD, PCB95, primaquine, carbaryl, omeprazole, terbutaline, and carbachol to be 99.1%, 100%, 100%, 98%, 98.9%, 100%, 99% and 99%, respectively. Indolo(3,2,b)carbazole (ICZ) was generously provided by Dr. Leonard F. Bjeldanes (University of California, Berkeley, CA). ICZ, carbaryl, primaquine, and omeprazole were dissolved in DMSO. Terbutaline and carbachol were dissolved in water. Benzyl isothiocyanate (BITC) dissolved in a 50% DMSO phosphate buffered saline (PBS) solution was generously provided by Dr. Sanjay Srivastava (Texas Tech University) and Dr. Thomas Brown (Wright State University). See Table 1 for a list of the chemicals tested and a summary of their purity, vehicle, final vehicle concentration, and range of concentrations tested. Lipopolysaccharide (LPS, *Escherichia coli*) and DMSO were purchased from Sigma Aldrich.

Cell Lines

The CH12.LX B cell line derived from the murine CH12 B cell lymphoma (Arnold et al., 1983), which arose in B10.H-2^aH-4^bp/Wts mice (B10.A × B10.129), has been previously characterized by Bishop and Haughton (Bishop and Haughton, 1986) and was a generous gift from Dr. Geoffrey Haughton (University of North Carolina, Chapel Hill, NC). Since its initial characterization, the CH12.LX cell line has been extensively utilized to study a variety of cellular processes specific to B cells and has provided a useful model in studying the effects of TCDD on B-cell differentiation. Employing the γ 2b mini-locus model which was developed and generously provided by Dr. Laurel Eckhardt (Hunter College, The University of New York City, NY) (Shi and Eckhardt, 2001), we generated the CH12. γ 2b-3'IgH cell line. The CH12. γ 2b-3'IgH cell line is a subclone isolated from CH12.LX cells that were under antibiotic selection to induce the stable expression of a transgene (γ 2b Ig heavy chain gene) regulated by the 3'IgHRR. Inducible expression of the γ 2b transgene in the CH12. γ 2b-3'IgH was confirmed by Real-Time RT-PCR and Enzyme-Linked Immunosorbent Assay (ELISA) analysis (data not shown and Fig. 2). Analysis of the CH12. γ 2b-3'IgH cells by flow cytometry and ELISA identified them as IgA expressing B cells and genomic analysis identified insertion of one copy of the γ 2b transgene (Fig. 5 and 7 and data not shown). The CH12. γ 2b-3'IgH cells do not endogenously express γ 2b IgH (major component of an IgG2b antibody) or secrete IgG2b (data not shown). Cells were grown in RPMI 1640 media (MediaTech, Herndon, VA) supplemented

with 10% bovine calf serum (Hyclone, Logan, UT), 13.5mM HEPES, 23.8 mM sodium bicarbonate, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 50 µM β-mercaptoethanol. Cells were maintained at 37°C in an atmosphere of 5% CO₂.

Transient Transfection

Luciferase reporter plasmids were generously provided by Dr. Robert Roeder (Rockefeller University, New York, NY) and included the 5-kb reporter with a variable heavy chain (V_H) promoter upstream of the luciferase gene which served as a promoter alone control and the 12.4-kb reporter with the upstream V_H promoter and the 3'IgHRR downstream of the luciferase gene. Plasmids were constructed using a pGL3 basic luciferase reporter construct (Promega, Madison, WI) as described previously (Ong et al., 1998).

Transient transfections were performed as follows. CH12.LX cells (2.2×10^7) were resuspended in 440 µl of culture media with 22 µg of plasmid (3'IgHRR or V_H promoter alone) and 200 µl transferred into two 2-mm gap electroporation cuvettes (Molecular BioProducts, San Diego, CA). Cells were electroporated using an electro cell manipulator (ECM 630; BTX, San Diego, CA) with the voltage at 250 V, the capacitance at 150 µF, and the resistance at 75 ohms. For each plasmid, multiple transfections were pooled then cells aliquoted into 12-well plates at 2.0×10^5 cells/ml.

Luciferase Assay

Immediately after transfection, CH12.LX cells, in the absence or presence of LPS stimulation, were treated for 48 hr with TCDD, ICZ, carbaryl, primaquine, omeprazole, terbutaline, carbachol, hydrogen peroxide, or BITC or the appropriate vehicle control (see Table 1). Between chemicals the DMSO vehicle concentrations varied according to the effective concentration range of the chemical and the stock concentration. Based on initial studies, 48 hr is optimum for LPS to maximally induce the 3'IgHRR luciferase reporter; therefore, this time point was selected to evaluate the effects of the test chemicals on LPS-induced 3'IgHRR activation. Notably, a similar profile of TCDD-induced inhibition of 3'IgHRR activation occurs following a 24-hr incubation period but the activation by LPS is not as pronounced or dependable which may relate to the relatively low transfection efficiency of the large 12.4 kb 3'IgHRR plasmid (unpublished observations and (Sulentic et al., 2004b). After the 48-hr incubation period, cells were washed with 1x PBS and then lysed with a 1x reporter lysis buffer (Promega). Samples were immediately frozen at -80°C. Treatments were either in triplicate or quadruplicate. To measure luciferase enzyme activity, samples were thawed at room temperature and then 20 µl of sample lysate was mixed with 100 µl of luciferase assay reagent (Promega) using an autoinjector. Luciferase activity or luminescence was measured with a luminometer (Berthold detection systems; Oak Ridge, TN) and represented as relative light units or percent effect with the LPS control set to 100% effect.

Protein Isolation for γ2b and IgA Analysis

CH12.LX cells or CH12.γ2b-3'IgH cells, in the absence or presence of LPS stimulation, were treated with TCDD, ICZ, carbaryl, primaquine, omeprazole, terbutaline, carbachol, hydrogen peroxide, or BITC or the appropriate vehicle control (see Table 1) then plated into 12-well plates. For γ2b protein isolation, CH12.γ2b-3'IgH cells were plated at a concentration of 2.5×10^4 cells/well and incubated for 48 hr. For IgA protein isolation, CH12.LX cells were plated at a concentration 2.0×10^5 cells/ml and incubated for 24 to 48 hr. Following the incubation period, cells were centrifuged at 3000 rpm, lysed with mild lysis buffer (1% NP-40, 150 mM NaCl, 10 mM NaPO₄, 2 mM EDTA) containing freshly added protease inhibitors (Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics, Indianapolis, IN). Lysed cells were centrifuged at 14,000 rpm then supernatants were collected and stored at -80 °C

until analysis. To measure $\gamma 2b$ or IgA, cell lysates were thawed on ice and protein concentrations were determined by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Samples were then diluted to the lowest sample concentration and 1 or 2 μg of total protein was analyzed for $\gamma 2b$ or IgA by ELISA.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cell lysates were analyzed for IgA or $\gamma 2b$ by sandwich ELISA as described previously (Sulentic et al., 2000). Colorimetric detection was performed every minute over a 1-h period using a Spectramax plus 384 automated microplate reader with a 405-nm filter (Molecular Devices, Sunnyvale, CA). The SOFTmax PRO analysis software (Molecular Devices) calculated the concentration of IgA or $\gamma 2b$ in each sample from a standard curve generated from the kinetic rate of absorption for known IgA or $\gamma 2b$ concentrations, respectively. Results are represented as the mean ng IgA or $\gamma 2b$ per 2 μg of total protein $\pm$ S.E. ($n=3$ to 4) or the mean percent effect $\pm$ S.E. ($n=3$ to 4) with the LPS control set to 100% effect.

Statistical Analysis of Data

Mean $\pm$ S.E. ($n=3$ to 4) was determined for each treatment group of a given experiment. For figures comparing the concentration response of multiple chemicals, the data was normalized to background and transformed to percent effect with the LPS control set to 100% effect. A statistical difference in the untransformed data (i.e. prior to transformation to percent effect) between treatment groups and the vehicle controls was determined by Dunnett's two-tailed t test. For IC_{50} generation, a complete concentration-response curve for each chemical was determined. Concentration-response curves were fit by a four-parameter logistic concentration-response equation given as $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogIC}_{50} - X) * \text{Hill Slope}})$. X is the logarithm of concentration. Y is the response. The derived IC_{50} (concentration generating half-maximal response) and 95% confidence interval for each AhR activator were expressed as a molar (M) concentration and were generated from the best-fit value for all data sets ($n=3$ to 4) (Fig. 4).

RESULTS

AhR ligands inhibit LPS-induced 3'IgHRR activation in CH12.LX B cells

Though we have previously identified AhR binding within the 3'IgHRR and AhR-dependent transcriptional regulation of the hs4 enhancer, the role of the AhR in mediating TCDD-induced inhibition of the 3'IgHRR is unknown. Further, TCDD represents a large class of chemicals, those known and those yet to be discovered, that activate the AhR signaling pathway and therefore may modulate 3'IgHRR activity. Notably, the effect of chemicals in general on 3'IgHRR activity has been largely unexplored. Therefore, to determine if the effect of TCDD on 3'IgHRR activity is truly representative of all AhR activators including non-dioxin AhR ligands, we examined the effect of four structurally diverse, non-dioxin chemicals (Table 1) on a luciferase reporter regulated by the 3'IgHRR that was transiently transfected into CH12.LX B cells. As previously demonstrated, the polyclonal B-cell activator and toll-like receptor-4 (TLR-4) ligand LPS significantly activated the 3'IgHRR (Fig. 1) with optimum induction occurring at 48 hr of stimulation (unpublished observations). Similar to the effect seen with TCDD (Fig. 1, inset and (Sulentic et al., 2004b), we identified a concentration-dependent inhibition, at concentrations that did not affect cell viability (data not shown), of LPS-induced 3'IgHRR activity by the following activators of the AhR signaling pathway: the dietary metabolite ICZ; the antimalarial drug primaquine; the pesticide carbaryl; and the proton pump inhibitor omeprazole (Prilosec) (Fig. 1, A–D). Each of these chemicals has been shown to either bind the AhR and/or induce AhR/DRE binding and Cyp1a1 activation and previous reports have determined their potency in relation to TCDD (Chen et al., 1995; Werlinder et al., 2001; Backlund and Ingelman-Sundberg, 2004; Bohonowych and Denison, 2007) suggesting a

rank order potency of TCDD > ICZ > carbaryl $\geq$ primaquine > omeprazole. Indeed μM concentrations of carbaryl, primaquine, and omeprazole were required to significantly inhibit LPS-induced 3'IgHRR activation; whereas nM concentrations of ICZ and TCDD resulted in inhibition. Additionally, the V_H promoter control which showed little activity alone was activated by LPS but to a lesser degree than the 3'IgHRR and was not modulated by ICZ, primaquine, carbaryl, or omeprazole at concentrations that had produced a marked inhibition of LPS-induced 3'IgHRR activation suggesting that these chemicals target the 3'IgHRR versus the V_H promoter (data not shown). Furthermore, as expected these AhR activators had no effect on 3'IgHRR in the absence of LPS stimulation (data not shown).

To more closely approximate the situation on the IgH chromosome and to avoid the many limitations of transient transfection experiments, we generated an experimental model (CH12. γ 2b-3'IgH) with the CH12.LX cells that stably expresses a γ 2b heavy chain transgene under the regulation of the 3'IgHRR (Shi and Eckhardt, 2001). Analysis of the CH12. γ 2b-3'IgH cells by flow cytometry and ELISA analysis identified them as IgA expressing B cells and genomic analysis suggested the insertion of one copy of the γ 2b transgene (data not shown). As determined by Real-Time RT-PCR and ELISA analysis, the CH12. γ 2b-3'IgH cells do not endogenously express γ 2b IgH (major component of an IgG2b antibody) or secrete IgG2b (data not shown). Similar to our 3'IgHRR transient reporter results, TCDD, ICZ, carbaryl, primaquine, and omeprazole inhibit LPS-induced γ 2b transgene expression in a concentration-dependent manner (Fig. 2 and Fig. 3A–D) which followed a rank order potency for AhR activation, i.e. TCDD > ICZ > primaquine > carbaryl = omeprazole (Fig. 4). Additionally, the dioxin congener MCDD and the non-coplanar PCB95 congener, which have no affinity for the AhR, had no effect on γ 2b expression (Fig. 2A) even at concentrations as high as 300 nM (data not shown), supporting a role of the AhR in the above rank order potency and verifying the specificity of this response. Furthermore, since the 3'IgHRR appears to play a significant role in IgH expression (Manis et al., 1998; Pinaud et al., 2001; Shi and Eckhardt, 2001), we verified that the effects on the γ 2b transgene reflected the effects on endogenous IgA protein expression. Indeed, TCDD, ICZ, primaquine, carbaryl, and omeprazole significantly inhibited IgA protein expression in the parental CH12.LX cells (data not shown) and in the CH12. γ 2b-3'IgH cells (Fig. 5). MCDD, similar to previous results (Sulentic et al., 2000), and PCB95 had no effect on IgA protein expression (data not shown). Although TCDD has long been known to inhibit Ig expression and secretion in B cells, the specific transcriptional target(s) has been less clear. These results along with previous studies (Sulentic et al., 1998; Sulentic et al., 2000; Sulentic et al., 2004a; Sulentic et al., 2004b) implicate an inhibitory mechanism involving the AhR and targeting the transcriptional activity of the 3'IgHRR.

Modulation of LPS-induced 3'IgHRR activation by non-AhR ligands in CH12.LX B cells

The above results are the first to support an inhibition of the 3'IgHRR by structurally diverse, non-dioxin AhR ligands; however, the 3'IgHRR is known to be regulated by many transcription factors and signaling pathways including but not limited to NF- κ B, Oct, and OCA-B (Michaelson et al., 1996; Khamlichi et al., 2000; Podojil et al., 2004). Consequently, it is likely that the 3'IgHRR can also be modulated by a diverse range of chemicals in addition to ligands for the AhR as identified above. As mentioned earlier the effect of chemicals in general on 3'IgHRR activity has not been previously explored and if 3'IgHRR activity can be altered by a wide array of chemicals through differing mechanisms, the 3'IgHRR may be a highly relevant toxicological or pharmacological target due to its proposed role in Ig expression and B-cell function. Therefore, we expanded the evaluation of our model to include chemicals not known to modulate the AhR signaling pathway but shown to modulate the antibody response or signaling pathways involved in 3'IgHRR activity. We initially tested hydrogen peroxide, terbutaline, and carbachol which are known to influence transcription factors that regulate the 3'IgHRR and/or to alter the antibody forming response. Hydrogen peroxide is a well known

activator of the NF- κ B signaling pathway (reviewed in Gloire et al., 2006), which is an important pathway in B-cell activation. Terbutaline is an agonist for the β_2 -adrenergic receptor and has been previously shown to induce OCA-B binding to the 3'IgHRR and to increase serum IgG1 levels in mice as well as IgY1 transcripts in primary mouse splenic B cells following in vivo administration of terbutaline (Podojil et al., 2004). Carbachol is a cholinergic agonist for the muscarinic and nicotinic receptors and has been previously shown to enhance the T cell-dependent antibody-forming cell response in mouse splenocytes (Pruett et al., 1992). Both hydrogen peroxide and terbutaline enhanced LPS-induced expression of the γ 2b transgene (Fig. 6A and B) and 3'IgHRR luciferase reporter (data not shown); whereas, carbachol had no effect on γ 2b expression (Fig. 6C) or 3'IgHRR luciferase activity (data not shown). The effect of terbutaline was concentration-dependent as opposed to hydrogen peroxide which consistently demonstrated a spike in activation that resolved with increasing concentration (Fig. 6A and B). Additionally, terbutaline and carbachol did not affect V_H promoter activity in LPS-stimulated cells nor did they affect basal activity of the V_H promoter or the 3'IgHRR in the absence of LPS stimulation (data not shown). Furthermore, we again verified that the effects on the γ 2b transgene reflected the effects on endogenous IgA protein expression. Correspondingly, terbutaline significantly enhanced LPS-induced IgA protein expression in the CH12. γ 2b-3'IgH cells; carbachol had no effect (Fig. 7). However, for hydrogen peroxide there was some variability in inducing the enhancement of IgA expression which may relate to the unstable nature of ROS and/or the antioxidant defense capacity of the CH12.LX B cells (Fig. 7).

We also evaluated the isothiocyanate BITC which has been shown to alter NF- κ B signaling and the production of ROS (Srivastava and Singh, 2004; Jakubikova et al., 2006) and to induce anti-carcinogenic effects in animal models (reviewed in (Hecht, 2000)). Isothiocyanates are highly bioavailable dietary metabolites that are released during the normal consumption of cruciferous vegetables (Hecht, 2000). BITC modulated 3'IgHRR activity by producing a concentration-dependent inhibition of LPS-induced expression of the γ 2b transgene (Fig. 6D) and of the 3'IgHRR luciferase reporter (data not shown). Consistent with the 3'IgHRR reporters, BITC also inhibited endogenous IgA protein expression in LPS-stimulated cells (Fig. 7).

These results support our hypothesis that the 3'IgHRR is sensitive to a wide range of chemicals (a question previously unexplored) which may have toxicological significance. This broad modulation is not entirely surprising given the number of transcription factors and signaling pathways thought to be involved in 3'IgHRR activity. Although the exact mechanism for the effects of the above chemicals on 3'IgHRR activity is unknown each chemical was selected based on previous reports demonstrating either an inhibition of Ig expression or modulation of a signaling pathway implicated in 3'IgHRR regulation; therefore these chemicals were considered likely to modulate 3'IgHRR activity. Furthermore, this study is the first to evaluate the effect of several diverse chemicals on 3'IgHRR activity and to compare these results with effects on endogenous IgH expression. Results support a direct correlation between 3'IgHRR activity and IgH expression and underscore the sensitivity of the 3'IgHRR to chemical-induced modulation.

DISCUSSION

The 3'IgHRR appears to mediate processes late in B-cell differentiation such as up-regulation of IgH expression as well as class switch recombination; both are critical processes in immunoglobulin or antibody production (Manis et al., 1998; Pinaud et al., 2001). Our past studies have identified the 3'IgHRR as a sensitive target of TCDD and have supported a role of the AhR signaling pathway in TCDD-induced inhibition of 3'IgHRR activation (Sulentic et al., 2000; Sulentic et al., 2004b). Therefore, a major focus of the present studies was to

determine if inhibition of 3'IgHRR activation applied to a diverse range of non-dioxin AhR activators. To this end, we chose to evaluate the following structurally diverse, non-dioxin AhR activators: the dietary metabolite ICZ; the antimalarial drug primaquine; the pesticide carbaryl; and the proton pump inhibitor omeprazole.

In the current studies, we utilized the CH12.LX B-cell line that has been extensively utilized to study a variety of cellular processes specific to B cells and has been well-characterized in regards to TCDD-induced inhibition of B-cell function and to the AhR signaling pathway (Sulentic et al., 2004b). Using a transient luciferase reporter and a stably integrated γ 2b reporter, both under the regulation of the 3'IgHRR, we identified an inhibition of LPS-induced 3'IgHRR activity that followed a rank order potency for AhR activation. These results suggest that the AhR is a significant regulatory element in the 3'IgHRR and that the 3'IgHRR is a significant target of chemicals that modulate the AhR signaling pathway. Indeed, we have previously characterized two DRE-like motifs within the hs1,2 and hs4 enhancers of the 3'IgHRR and identified AhR/ARNT binding to these motifs (Sulentic et al., 2000). Interestingly, the DRE motif is in close proximity to or overlapping a κ B motif in the hs1,2 and hs4 enhancers, respectively; and mutational analysis identified a dependence on protein binding to both the DRE and κ B motifs in the transcriptional effect of TCDD on hs4 enhancer activity in LPS-activated CH12.LX cells (Sulentic et al., 2000; Sulentic et al., 2004a). A direct association between the AhR and the NF- κ B/Rel subunits RelA and RelB has been previously demonstrated in other cellular models (Tian et al., 1999; Kim et al., 2000; Vogel et al., 2007). Therefore it is possible that the AhR modulates 3'IgHRR transcriptional activity directly through DRE binding and/or indirectly by associating with specific NF- κ B/Rel proteins and influencing the composition of NF- κ B/Rel dimers binding to the κ B site. In support of this possibility, κ B binding within the 3'IgHRR has been shown to play either an inhibitory or activating role in the transcriptional activity of the hs1,2 enhancer which was dependent on the B-cell activation state (Michaelson et al., 1996) and is perhaps mediated by binding of activating versus inhibitory NF- κ B/Rel protein dimers as seen in the germline γ 1 promoter (Lin et al., 1998). Therefore, ongoing studies are focused on testing the hypothesis that the inhibitory effect of TCDD on 3'IgHRR activation is mediated through an AhR-dependent shift in the NF- κ B/Rel protein complexes binding to κ B motifs within the hs1,2 and hs4 enhancers. Lastly, if the 3'IgHRR is a significant transcriptional regulator of the IgH gene then altered 3'IgHRR activity should directly influence IgH transcription leading to altered Ig protein levels. Correspondingly, the AhR activators used in this study inhibited endogenous IgH protein expression in a similar manner to the 3'IgHRR further supporting a critical role of the 3'IgHRR in IgH expression. Moreover these results extend our previous studies in which we demonstrated an AhR structure activity relationship for the inhibition of IgH mRNA expression and Ig secretion by chlorinated dibenzo-p-dioxin congeners (Sulentic et al., 2000). In fact for these endpoints the generated IC₅₀'s for TCDD-induced inhibition correlated well with the IC₅₀ generated here for TCDD-induced inhibition of 3'IgHRR activity.

A second objective of these studies was to validate the use of the CH12. γ 2b-3'IgH cell line that stably expresses the γ 2b-3'IgHRR reporter as an appropriate model to evaluate chemical-induced modulation of 3'IgHRR activity. For the AhR activators, there is a general concordance between the transient luciferase and stable 3'IgHRR reporters suggesting that both are sensitive indicators of 3'IgHRR modulation. Additionally, the IC₅₀'s generated for a particular AhR activator using either the transient or stable reporter did not substantially differ and fell within or just outside the best fit 95% confidence interval for either reporter. Significantly, modulation of the 3'IgHRR in our reporters correlated with the effect of these chemicals on endogenous Ig protein expression. This correlation between effects on the transient and stable 3'IgHRR reporters and endogenous Ig protein expression also occurred with non-AhR activators. This is particularly important since the 3'IgHRR has been shown to be regulated by multiple transcription factors including but not limited to NF- κ B, OCA-B, and Oct-2. Thus, it is likely

that the 3'IgHRR can also be modulated by a diverse array of chemicals in addition to AhR agonists and the ability to alter 3'IgHRR activity through differing mechanisms suggests that the 3'IgHRR may be a highly relevant toxicological or pharmacological target due to its role in Ig expression and B-cell function. Therefore, we expanded the evaluation of our model to include chemicals (terbutaline, hydrogen peroxide, carbachol, and BITC) which have been shown to influence these transcription factors and/or to alter B-cell differentiation into antibody-secreting cells.

In vivo administration of terbutaline has been previously shown to increase serum IgG1 levels and mature Ig γ 1 transcripts in mice treated with the B-cell stimulants IL-4 and antibodies against the B-cell surface signaling receptors, CD40 and CD86 (Podojil et al., 2004). This effect appears to be mediated by the activation of β_2 -adrenergic receptors and CD86 resulting in an increase in the expression of OCA-B and Oct-2, respectively, and increased binding of the OCA-B/Oct-2 dimer to octomer motifs within both the hs1,2 and hs4 enhancers of the 3' IgHRR; however, 3'IgHRR activity was not evaluated (Podojil et al., 2004). Here, we demonstrate a terbutaline-induced enhancement of 3'IgHRR activity (transient and stable reporters) and of IgH protein expression in CH12.LX B-cells stimulated with the TLR4 ligand LPS. Taken together terbutaline enhanced 3'IgHRR activity and Ig expression in B cells activated with stimuli (LPS versus anti-CD86, anti-CD40 and IL-4) that activate different surface receptors and signaling pathways.

Low concentrations of hydrogen peroxide also enhanced LPS-induced 3'IgHRR activity (transient and stable reporters) and IgH protein expression though the effect on IgH tended to be more variable in its magnitude and significance which may reflect the unstable nature of ROS and/or the antioxidant defense capacity of CH12.LX cells. Notably, hydrogen peroxide and other ROS have been shown to induce NF- κ B/Rel activation (reviewed by Gloire et al., 2006) which may, at least in part, mediate hydrogen peroxide's effects on 3'IgHRR activity due to the importance of the NF- κ B signaling pathway in B-cell activation and 3'IgHRR activity. Additionally, several reports support a physiological role of ROS in lymphocyte activation. Specifically, hydrogen peroxide increased phosphorylation and Ca²⁺ mobilization in B cells that was similar to antigen-induced receptor activation (reviewed in Verweij and Gringhuis, 2002). Clearly the concentration and duration of the redox signal will be an important factor in the ultimate effect on cell function and it has been proposed that chronic oxidative stress will result in cellular damage while acute oxidative stress will induce activation signals akin to receptor activation by antigen (Verweij and Gringhuis, 2002).

We also evaluated the effect of the muscarinic and nicotinic agonist carbachol. Previous studies with carbachol demonstrated an increase in the IgM response to the T-cell dependent antigen sheep red blood cell, which requires signals from T cells and macrophages to induce Ig expression and antibody secretion in B cells (Pruett et al., 1992). The cellular target and molecular mechanism for this effect is unknown and carbachol did not alter LPS-induced 3' IgHRR activation or endogenous IgH protein expression in our model. Although there could be several reasons for this including a lack of functional cholinergic receptors, perhaps the most likely conclusion is that carbachol does not directly target B cells but instead targets T-cell and/or macrophage functions. The CH12. γ 2b-3'IgH cellular model is limited to chemicals that directly target B-cell function as opposed to indirect effects mediated by altered T-cell and/or macrophage function.

Isothiocyanates including BITC are highly bioavailable dietary metabolites released during the normal consumption of cruciferous vegetables and appear to have anti-carcinogenic properties that may be related to their modulation of NF- κ B/Rel activity, ROS production, and/or Phase I and II metabolic enzyme activity (Hecht, 2000; Srivastava and Singh, 2004; Jakubikova et al., 2006). While the effects of BITC on Ig expression and secretion have not been previously

evaluated, due to its previously reported effects on NF- κ B/Rel activity and ROS production and its high bioavailability, BITC seemed a good candidate to alter 3'IgHRR activity. Indeed, BITC inhibited both the 3'IgHRR transient and stable reporters as well as endogenous Ig expression. Although the exact mechanism for this effect is unclear and may be mediated through modulation of NF- κ B/Rel activity, our model has identified BITC as a direct modulator of 3'IgHRR activity and Ig expression and due to its reported bioavailability during normal consumption of fruits and vegetables, BITC may alter B-cell function in humans.

Clearly, the 3'IgHRR and IgH expression in our cellular model is sensitive to modulation by exogenous chemicals which is a novel observation and may have important implications to human health particularly considering the multiple signaling pathways and transcription factors that appear to regulate 3'IgHRR activity and the diversity of chemicals, known and yet to be identified, that alter these pathways. In assessing the potential risk to human health several biokinetic factors need to be considered including bioavailability and distribution, effective concentrations, and half-life which will determine if target cells (i.e. B cells in the lymph nodes, spleen, and gastrointestinal tract) will be exposed to sufficient concentrations to mediate an effect. There is also the question of whether a synergistic, additive, or antagonistic effect will result from the simultaneous exposure to multiple chemicals in the environment, diet, and therapeutics that modulate the same or different signaling pathways involved in 3'IgHRR activity (i.e. NF- κ B versus AhR versus β -adrenergic receptors, etc).

Interestingly, non-persistent AhR activators demonstrate AhR-induced Cyp1a1 induction without dioxin-like toxicities which are thought to be mediated by the sustained activation of the AhR (reviewed in Okey, 2007). Whether weak and/or non-persistent AhR activators could sufficiently modulate Ig expression and B-cell function in vivo and the relative risk to human immunocompetence is uncertain. Hu and coworkers identified six marketed drugs including omeprazole that are weak, nonpersistent AhR ligands and noted that there have been no reports of dioxin-like toxicities in rats and no evidence of immunosuppression in exposed humans (Hu et al., 2007). However, we propose that non-persistent AhR activators (and other modulators of the 3'IgHRR) directly target IgH transcription much like Cyp1a1 and will likely produce subtle effects on B-cell function and immunocompetence which may not have been directly evaluated or may be difficult to even identify in humans. Furthermore, the etiology for many immune ailments from increased infections to autoimmunity is not clearly defined and probably depends on both environmental factors and genetics.

It is intriguing that the 3'IgHRR has been associated to date with several human immune-related disorders including Celiac disease, IgA nephropathy, Burkitt's lymphoma, and more recently systemic sclerosis, plaque psoriasis, psoriatic arthritis, and dermatitis herpetiformis (Aupetit et al., 2000; Frezza et al., 2004; Wang and Boxer, 2005; Frezza et al., 2007; Cianci et al., 2008). Interestingly, disease incidence and/or severity of these diseases (except Burkitt's lymphoma) appears to correlate with a polymorphism within the hs1,2 enhancer of the 3'IgHRR. This polymorphism is characterized by varying repeats of a 38 to 44 bp invariant sequence containing potential binding sites for NF- κ B, AP-1, Sp-1, and NF-1 (Denizot et al., 2001); we have also identified a DRE-like site that is fairly well conserved with the functional DRE we have previously evaluated in the mouse hs1,2 and like the mouse hs1,2 the DRE-like site is in close proximity to a NF- κ B binding site (Sulentic et al., 2000). It is tempting to speculate that altered 3'IgHRR activity by chemicals could modulate the occurrence and severity of these diseases and that the hs1,2 polymorphism may confer an increased sensitivity to AhR ligands and other modulators of the 3'IgHRR. The AhR offers an appealing avenue to explore for understanding mechanisms of altered immunocompetence and future therapeutic interventions.

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Nonstandard abbreviations

TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
AhR	aryl hydrocarbon receptor
DRE	dioxin responsive element
IgH	immunoglobulin heavy chain
3'IgHRR	3'IgH regulatory region
ARNT	AhR nuclear translocator
HAHs	halogenated aromatic hydrocarbons
PAHs	polycyclic aromatic hydrocarbons
DMSO	dimethyl sulfoxide
ICZ	indolo(3,2, <i>b</i>)carbazole
BITC	benzyl isothiocyanate
PBS	phosphate buffered saline
LPS	lipopolysaccharide
RT-PCR	reverse transcription-polymerase chain reaction
ELISA	

	enzyme-linked immunosorbent assay
V_H	variable heavy chain
IC₅₀	50% inhibitory concentration
NF-κB	nuclear factor-κB
Oct	octamer-binding factor
OCA-B	Oct-1-associated coactivator
AP-1	activator protein 1
NF-1	neurofibromatosis type 1

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Figure 1. AhR activators inhibit LPS-induced 3'IgHRR luciferase reporter activity

CH12.LX cells were transiently transfected with the 3'IgHRR reporter plasmid then treated for 48 hr with vehicle (0.01% DMSO for ICZ and primaquine; 0.019% for TCDD; or 0.1% DMSO for carbaryl and omeprazole); or varying concentrations of indolo(3,2,b)carbazole (ICZ) (A), primaquine (B), carbaryl (C), or omeprazole (D); or 30nM TCDD (inset figure) in the presence of LPS (3 μg/ml) stimulation. Luciferase enzyme activity (mean ± SE, n=3) was measured in relative light units (RLU) then normalized to background and transformed to percent effect with the LPS control set to 100% as shown on the y-axis. NA denotes the unstimulated naïve control. Significance was determined by a 1-way ANOVA followed by a Dunnett's post-hoc test. A "*" or "**" denotes significance from the DMSO control at $p < 0.05$ or $p < 0.01$, respectively. A "††" denotes significance of the NA control from the LPS control at $p < 0.01$, respectively.

Figure 2. Concentration-dependent inhibition of the 3'IgHRR-regulated $\gamma 2b$ transgene by TCDD but not by MCDD or PCB95

Stably transfected CH12. $\gamma 2b$ -3'IgH cells were treated for 48 hr, in the presence of LPS (3 μ g/ml) stimulation, with vehicle (0.019% DMSO) or A) 30 nM concentrations of MCDD, PCB95, or TCDD; or B) varying concentrations of TCDD. In contrast to the AhR high affinity ligand TCDD, the MCDD congener and PCB95 have no affinity for the AhR. $\gamma 2b$ protein expression (mean $\pm$ SE, $n=3$) in the cell lysate was determined by sandwich ELISA and standardized to ng $\gamma 2b$ per 1 or 2 μ g of total protein as shown on the y-axis. NA denotes the unstimulated naïve control. Significance was determined by a 1-way ANOVA followed by a Dunnett's post-hoc test. A "***" denotes significance from the DMSO control at $p<0.01$. A "††" denotes significance of the NA control from the LPS control at $p<0.01$.

Figure 3. Concentration-dependent inhibition of the 3'IgHRR-regulated γ 2b transgene by non-dioxin AhR activators

Stably transfected CH12. γ 2b-3'IgH cells were treated for 48 hr with vehicle (0.01% DMSO for ICZ and primaquine and 0.1% DMSO for carbaryl and omeprazole) or varying concentrations of ICZ (A), primaquine (B), carbaryl (C), or omeprazole (D), in the presence of LPS (3 μ g/ml). γ 2b protein expression (mean $\pm$ SE, n=3) in the cell lysate was determined by sandwich ELISA and standardized to ng γ 2b/2 μ g of total protein. γ 2b protein expression was then normalized to background and transformed to percent effect with the LPS control set to 100% as shown on the y-axis. NA denotes the unstimulated naïve control. Significance was determined by a 1-way ANOVA followed by a Dunnett's post-hoc test. A "*" or "**" denotes significance from the DMSO control at $p < 0.05$ or $p < 0.01$, respectively. A "††" denotes significance of the NA control from the LPS control at $p < 0.01$.

AhR activator	IC ₅₀	95% CI
■ TCDD	4.7×10^{-10}	2.6×10^{-10} to 8.8×10^{-10}
▲ ICZ	1.2×10^{-7}	9.6×10^{-8} to 1.4×10^{-7}
▼ Primaquine	2.2×10^{-5}	1.8×10^{-5} to 2.7×10^{-5}
● Carbaryl	3.8×10^{-5}	3.1×10^{-5} to 4.6×10^{-5}
◆ Omeprazole	3.8×10^{-5}	3.3×10^{-5} to 4.5×10^{-5}

Figure 4. Inhibition of the 3'IgHRR-regulated $\gamma 2b$ transgene follows a rank order potency
Percent inhibition was calculated from 3 to 4 separate concentration-response curves for each AhR activator (one representative curve shown) and fit to a four-parameter logistic concentration-response equation to generate the average 50% inhibitory concentration (IC₅₀) and 95% confidence interval for each AhR activator. The vehicle control is 0.01% DMSO for ICZ and primaquine; 0.019% DMSO for TCDD; and 0.1% DMSO for carbaryl and omeprazole. NA denotes the unstimulated naïve control.

Figure 5. Inhibition of endogenous IgH protein expression by TCDD and non-dioxin AhR activators CH12.γ2b-3'IgH cells were treated for 24 hr with vehicle (0.01% DMSO for TCDD, ICZ and primaquine or 0.1% DMSO for carbaryl and omeprazole) or specific concentrations of TCDD (10 nM), ICZ (500nM), primaquine (PMQ; 50μM), carbaryl (CBRL; 100μM), or omeprazole (OME; 62.5μM), in the presence of LPS (3 μg/ml). Concentrations that produced a maximum inhibition of LPS-induced 3'IgHRR activity were used. IgA protein expression (mean ± SE, n=3) in the cell lysate was determined by standard ELISA and standardized to ng IgA/2μg of total protein. NA denotes the unstimulated naïve control. Significance was determined by a 1-way ANOVA followed by a Dunnett's post-hoc test. A "***" denotes significance from the DMSO control at $p < 0.01$. A "††" denotes significance of the NA control from the LPS control at $p < 0.01$.

Figure 6. Inhibition of the 3'IgHRR-regulated $\gamma 2b$ transgene by non-AhR ligands

Stably transfected CH12. $\gamma 2b$ -3'IgH cells were treated for 48 hr with varying concentrations of hydrogen peroxide (H_2O_2) (A), terbutaline (B), carbachol (C), and BITC (D) in the presence of LPS (3 $\mu g/ml$) stimulation. $\gamma 2b$ protein expression (mean $\pm$ SE, $n=3$) in the cell lysate was determined by sandwich ELISA and standardized to ng $\gamma 2b/2\mu g$ of total protein. $\gamma 2b$ protein expression was then normalized to background and transformed to percent effect with the LPS control set to 100% as shown on the y-axis. NA denotes the unstimulated naïve control. Significance was determined by a 1-way ANOVA followed by a Dunnett's post-hoc test. A "*" or "**" denotes significance from the appropriate LPS or vehicle (0.05% DMSO for BITC) control at $p<0.05$ or $p<0.01$, respectively. A "†" or "††" denotes significance of the NA control from the LPS control at $p<0.05$ or $p<0.01$, respectively.

Figure 7. Altered IgA secretion correlated with effects on 3'IgHRR activity

CH12.γ2b-3'IgH cells were treated with a specific concentration of TCDD (10nM), carbachol (CBOL; 100μM), terbutaline (TERB; 100μM), hydrogen peroxide (H₂O₂; 30μM), or BITC (5μM), or the appropriate vehicle control (VH, 0.01% DMSO for TCDD or 0.05% DMSO for BITC) in the presence of LPS (3 μg/ml) stimulation. IgA protein expression (mean ± SE, n=3) in the cell lysate was determined by sandwich ELISA and standardized to ng IgA/2μg of total protein. Three experiments (separated by the vertical lines) are graphed together and for comparative purposes. IgA protein expression was normalized to background and transformed to percent effect with the LPS control set to 100% as shown on the y-axis. NA denotes the unstimulated naïve control. Significance was determined by a 1-way ANOVA followed by a Dunnett's post-hoc test. A "*" denotes significance from the appropriate LPS or vehicle (0.01% DMSO for TCDD and 0.05% DMSO for BITC) control at $p < 0.05$. A "†" or "††" denotes significance of the NA control from the LPS control at $p < 0.05$ or $p < 0.01$, respectively.

Table 1**Test chemicals**

Non-dioxin AhR ligands include ICZ, primaquine, carbaryl, and omeprazole. Negative controls for AhR binding include 1-monochlorodibenzo-*p*-dioxin and the non-coplanar PCB95 both of which have no affinity for the AhR. Chemicals not known to modulate the AhR signaling pathway but shown to modulate the antibody response or signaling pathways involved in 3'IgHRR activity include hydrogen peroxide, terbutaline, carbachol, and BITC. DMSO vehicle concentrations varied according to the solubility and effective concentration range of the chemical. ND, not determined; NA, not applicable.

Chemical	Purity	Vehicle	Final vehicle concentration	Concentration Range Tested
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin (TCDD)	99.1%	100% DMSO	0.01% or 0.019% DMSO	0.01–30 nM
Indolo(3,2,b)carbazole ICZ	ND	100% DMSO	0.01% DMSO	0.5–500 nM
Primaquine (PMQ)	98%	100% DMSO	0.01% DMSO	6.25–100 µM
Carbaryl (CBRL)	98.9%	100% DMSO	0.1% DMSO	12.5–100 µM
Omeprazole (OME)	100%	100% DMSO	0.1% DMSO	12.5–62.5 µM
1-Monochlorodibenzo- <i>p</i> -dioxin (MCDD)	100%	100% DMSO	0.01% or 0.1%	30 and 300 nM
Polychlorinated biphenyl 95 (PCB95)	100%	100% DMSO	0.01% or 0.1%	30 and 300 nM
Hydrogen Peroxide (H ₂ O ₂)	ND	water	NA	20–50 µM
Terbutaline (TERB)	99%	water	NA	0.1–100 µM
Carbachol (CBOL)	99%	water	NA	0.01–100 µM
Benzyl Isothiocyanate (BITC)	ND	50% DMSO 1x PBS	0.05% DMSO	0.5– 5.0 µM