

Apoptosis in Murine Hepatoma Hepa 1c1c7 Wild-Type, C12, and C4 Cells Mediated by Bilirubin

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Received November 20, 2001; accepted April 18, 2002

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Elevated serum and tissue bilirubin concentrations that occur in pathological conditions such as cholestasis, jaundice, and other liver diseases are known to stimulate cytotoxic responses. In preliminary studies, we noted that bilirubin seemed to cause apoptosis in murine hepatoma Hepa 1c1c7 wild-type (WT) cells. Consequently, we investigated apoptosis caused by bilirubin in WT, mutant C12 [aryl hydrocarbon receptor (AHR)-deficient], and C4 (AHR nuclear translocator-deficient) Hepa 1c1c7 cells. Three independent measures of apoptosis were used to quantify the effects of exogenous bilirubin (0, 1, 10, 25, 50, or 100 μ M). Caspase-3 activity and cytochrome c release from mitochondria increased at 3 h post-treatment, before increased caspase-8 activity at 6 h, and nuclear condensation

by 24 h after treatment with bilirubin. No differences in whole-cell lipid peroxidation were observed between the cell types; however, intracellular reactive oxygen species (ROS) production was greater in WT cells than C12 or C4 cells 3 h after bilirubin exposure. Pretreatment of cells for 1 h with 1 or 10 μ M α -naphthoflavone, an AHR antagonist, before bilirubin exposure resulted in decreased caspase-3 activity at 6 h and nuclear condensation at 24 h in WT cells. These results indicate that bilirubin, a potential AHR ligand, causes apoptosis in murine Hepa 1c1c7 WT cells by a mechanism(s) partially involving the AHR, disruption of membrane integrity, and increased intracellular ROS production.

Bilirubin is an endogenous metabolite generated by the oxidation of heme derived from ubiquitous proteins such as cytochromes, catalases, and hemoglobin. Heme oxygenase (HO) catalyzes the rate-limiting reaction in the conversion of heme to bilirubin (Maines, 1997). There are three isoforms of the enzyme, an inducible form (HO-1) and two constitutive forms (HO-2 and HO-3), which catalyze the breakdown of heme to equimolar concentrations of carbon monoxide, iron, and biliverdin. Biliverdin is reduced rapidly to bilirubin in cells (Maines, 1997). Many stimuli are known to up-regulate HO-1, such as heat shock (Shibahara et al., 1987) and various

initiators of cellular stress responses, including heavy metals, cytokines, endotoxin, and H₂O₂ (Keyse and Tyrrell, 1989; Ryter and Tyrrell, 2000). Pathobiological conditions, including biliary obstruction, neonatal jaundice, liver diseases, and some genetic disorders, result in a marked elevation of plasma bilirubin (>300 μ M) above homeostatic levels (\sim 20 μ M) (Bhutani et al., 1999; Dennery et al., 2001).

In vivo, bilirubin has antioxidant and toxicant effects, dependent on the concentration at the tissue or cellular level. The antioxidant properties of bilirubin occur at low concentrations (\sim 0.01–10 μ M) and have been demonstrated in various systems (Stocker et al., 1987), whereas cytotoxic effects have been reported at higher concentrations (>20 μ M) in such tissues as the central nervous system (Cashore, 1990; Dennery et al., 2001). The exact mechanism(s) responsible for bilirubin-mediated toxicity remains largely unknown. Studies investigating cholestasis have indirectly shown a role for bilirubin in the induction of apoptosis in isolated hepatocytes (Miyoshi et al., 1999). More recent reports indicate that apoptosis in response to bilirubin in neuronal cells

This work was supported by Canadian Institute of Health Research grant FRN 9972 (to J.R.B.). J.M.S. is the recipient of an Ontario Graduate Scholarship. S.J.A.D. is a Kidney Foundation of Canada Research Scholar. This work has been presented previously [Seubert JM, Darmon AJ, El-Kadi AOS, D'Souza SJA, and Bend JR (2001) Apoptosis in murine hepatoma Hepa 1c1c7 WT (wild-type), C12 (AHR-deficient) and C4 (ARNT-deficient) cells triggered by bilirubin. 2nd Annual Scientific Meeting of the CIHR Oxidative Stress Consortium; 2001 May 4–6; London, ON, Canada and Seubert JM, Darmon AJ, El-Kadi AOS, D'Souza SJA and Bend JR (2001) Bilirubin induces an apoptotic response in murine hepatoma cells. Society of Toxicology 40th Annual Meeting; 2001 Mar 25–29; San Francisco, CA].

ABBREVIATIONS: HO, heme oxygenase; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear transporter; WT, wild-type hepatoma Hepa 1c1c7 murine cells; C12, AHR-deficient hepatoma Hepa 1c1c7 murine cells; C4, ARNT-deficient hepatoma Hepa 1c1c7 murine cells; ROS, reactive oxygen species; α NF, α -naphthoflavone; PI, propidium iodide; H33342, Hoescht 33342 (bisbenzamide); PBS, phosphate-buffered saline; AMC, 7-amino-4-methylcoumarin; Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; TBARS, thiobarbituric acid reactive substances.

involves NMDA receptors and a mitochondrial-dependent pathway (Grojean et al., 2000; Rodrigues et al., 2000).

Previous studies suggest that bilirubin can serve as a ligand for the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor belonging to the basic helix-loop-helix/periodicity-AHR nuclear translocator (ARNT)-simple-minded superfamily of proteins (Sinal and Bend, 1997; Phelan et al., 1998). The binding of a ligand to the AHR initiates a transformation allowing the ligand:AHR complex to translocate into the nucleus where it forms a heterodimer with the ARNT protein (Hankinson, 1995). The AHR:ARNT heterodimer has a high-affinity for specific DNA recognition sequences, known as xenobiotic responsive elements (Hankinson, 1995). In combination with earlier work, studies with AHR knockout mice and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a ligand with very high affinity for this receptor, demonstrate that many of the toxic and biologic effects after 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure are mediated via the AHR (Fernandez-Salguero et al., 1996; Mimura et al., 1997). Recent evidence has shown the AHR is also involved in a wide range of cellular responses from apoptosis to cell cycle control, indicating an interaction with multiple signaling pathways (Ma and Whitlock, 1996; Zaher et al., 1998; Near et al., 1999; Reiners and Clift, 1999; Elizondo et al., 2000; Mäkitäinen et al., 2001). Whether or not bilirubin has significant function in vivo by serving as an AHR ligand remains an unresolved issue. However, increased production of bilirubin from heme or decreased clearance under pathological conditions would enhance any AHR-mediated toxicity of bilirubin.

Consequently, we investigated the ability of bilirubin to decrease the viability of murine hepatoma Hepa 1c1c7 wild-type (WT) cells (Miller et al., 1983), C12 cells (an AHR-deficient mutant), or C4 cells (an ARNT-deficient mutant) (Israel and Whitlock, 1984). Results showed that bilirubin treatment caused concentration-dependent apoptosis in all cell types by a mechanism that involved the generation of ROS and the suppression of activated-Akt. As well, studies with the AHR antagonist α -naphthoflavone (α NF) suggest a partial role for the AHR in the bilirubin-mediated cell death. These data provide one potential explanation for cytotoxicity in pathobiological conditions characterized by elevated concentrations of bilirubin.

Experimental Procedures

Materials. Bilirubin IX was obtained from Porphyrin Products (Logan, UT). Solutions of bilirubin were prepared first by dissolving a fixed amount in 0.1 N NaOH (10% of final volume) followed by addition of an appropriate volume of PBS, pH 7.4. All experiments were protected from exposure to light. Propidium iodide (PI) and bisbenzamide (H33342) were purchased from Calbiochem-Novabiochem (La Jolla, CA). Leupeptin, pepstatin, aprotinin, phenylmethylsulfonyl fluoride, and RNase A were purchased from Roche Applied Science (Laval, PQ, Canada); proteinase K from Promega (Toronto, ON, Canada); and digitonin and paraformaldehyde from Sigma-Aldrich (Oakville, ON, Canada). Tissue culture materials were obtained from Invitrogen (Burlington, ON, Canada), and all other chemicals (reagent grade or better) were purchased from BDH (Toronto, ON, Canada) or Sigma-Aldrich.

Cell Lines and Treatment. The mouse hepatoma WT, C12, and C4 cell lines were generously provided by Dr. O. Hankinson (University of California, Los Angeles). These cells were maintained in a minimal essential medium supplemented with 10% fetal bovine serum, 20 μ M L-glutamine, 50 μ g/ml gentamicin sulfate, 100 IU/ml

penicillin, 10 μ g/ml streptomycin, and 25 ng/ml amphotericin B. The concentration of albumin in the tissue culture media was 40 μ M after the addition of 10% fetal bovine serum. Cells were grown at 37°C in a 5% CO₂ humidified environment. Cells were seeded and cultured for 24 h (until 90% confluent) and then treated with saline or 1, 10, 25, 50, or 100 μ M bilirubin for various times. For antagonist studies, cells were seeded and cultured for 24 h (90% confluent), and then a single pretreatment of 1 or 10 μ M α NF was administered for 1 h followed by treatment with 0, 10, 25 or 50 μ M bilirubin. Changes in cell morphology were examined over time using a phase-contrast microscope (Leica, Wetzlar, Germany), and cell viability was assessed by exclusion of a 0.2% solution of trypan blue.

Nuclear Morphology. Cells were cultured for 24 h on cover slips. To specifically label nuclei and assess membrane integrity, the cells were washed twice with PBS, stained with PI (12 μ g/ml PBS), a cell membrane impermeant fluorescent nuclear stain, and Hoechst 33342 (50 μ g/ml PBS), a cell permeant nuclear stain, for 10 min at 37°C. The cells were then washed repeatedly with PBS to remove the excess fluorescent stains, fixed with 3.7% paraformaldehyde in PBS, and mounted on slides (Lieberthal et al., 1998). To quantify necrotic and apoptotic cells, an inverted epifluorescence microscope DMIRB (Leica, Wetzlar, Germany) attached to an Orca I digital camera (Hamamatsu Corporation, Bridgewater, NJ) was used to visualize nuclei labeled with either stain (40 \times magnification). Stained cells were counted using OPENLAB analysis software (Improvision, Coventry, UK), and data are expressed as a percentage of the total number of cells in a field. At least three fields containing at least 500 cells were examined for each experimental condition.

Cytochrome *c* Release and Akt. The release of cytochrome *c* from mitochondria was analyzed using a modified method of Heibein et al. (1999). WT, C12, or C4 cells, plated at 1×10^6 cells/10-cm dish, were harvested after bilirubin treatment. Cells were centrifuged for 2 min at 500g, supernatant was removed, and remaining cell pellets were washed with PBS. The cell pellet was resuspended in 200 μ l of lysis buffer (75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, 200 μ g/ml digitonin, including protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 0.2 μ g/ml aprotinin, and 2.5 μ g/ml pepstatin). Cell suspensions were incubated on ice for 10 min and subsequently centrifuged at 14,000g for 10 min at 4°C. The supernatant was further centrifuged at 100,000g for 60 min at 4°C, and this supernatant was used as the cytosolic fraction. The heavy-membrane pellet produced by 14,000g fractionation was resuspended in buffer (25 mM Tris-HCl, pH 8.0, and 0.1% v/v Triton X-100). Heavy-membrane protein (25 μ g) or 50 μ g of cytosolic protein for cytochrome *c* and 20 μ g of cytosolic protein for Akt detection were resolved by denaturing electrophoresis on 15% discontinuous polyacrylamide slab gels (SDS-polyacrylamide gel electrophoresis) and electrophoretically transferred to polyvinylidene difluoride membranes (PALL Corp., Ann Arbor, MI). Protein blots were blocked for 2 h at 25°C in Tris-saline buffer (0.15 M NaCl, 3 mM KCl, and 25 mM tris(hydroxymethyl)methylamine, pH 7.4) with 5% skim milk powder. The blocking solution was removed, and blots were rinsed three times in wash buffer (Tris-saline buffer containing 0.1% Tween 20). The primary antibodies used were mouse monoclonal anti-cytochrome *c* antibody (0.25 μ g/ml; BD PharMingen, Mississauga, ON, Canada), rabbit polyclonal anti-Akt antibody (1:1000; New England BioLabs, Beverly, MA), and rabbit polyclonal anti-phospho-Akt polyclonal antibody that recognizes only phosphorylated Akt (Ser473) (1:1000; New England BioLabs). The blots were incubated for 12 h at 4°C with the primary antibody solution and then rinsed three times with wash buffer. Blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary Ab (1:2500, Promega) or horseradish peroxidase-conjugated anti-rabbit IgG secondary Ab (1:2500, Promega) for 2 h at 25°C and washed as described above. Detection of signal was performed using enhanced chemiluminescence (ECL Plus; Amersham Biosciences, Baie d'Urfé, PQ, Canada).

Caspase-8 and -3 Activities. Caspase-8 or -3 activity was determined as described previously (Reiners and Clift, 1999). In brief, WT,

C12, or C4 cells, plated at 2×10^5 cells/well in six-well plates, were grown for 24 h to 90% confluence and harvested after bilirubin treatment. Cells were centrifuged for 2 min at 500g, the supernatant was removed, and the residual pellet was washed with PBS. Cell pellets were resuspended in 200 μ l of lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaP_i, and 10 mM NaPP_i) and frozen at -70°C . Cells were thawed on ice, homogenized for 15 s, and then centrifuged at 15,000g for 15 min at 4°C . Caspase-8 and -3 activities were determined in cell lysates by monitoring the release of 7-amino-4-methylcoumarin (AMC) by proteolytic cleavage of the peptide *N*-acetyl-Ile-Glu-Thr-Asp-AMC (caspase-8) or Ac-DEVD-AMC (caspase-3) (Sigma-Aldrich) by fluorescence spectrophotometry. Reaction mixtures containing 100 μ l of cell lysate and 1.9 ml of buffer (20 mM HEPES, pH 7.4, 10% glycerol, 2 mM dithiothreitol, and 20 μ M Ac-DEVD-AMC) were incubated in the dark for 30 min at 37°C , and fluorescence was monitored at wavelengths of 380 (excitation) and 460 nm (emission). Specific activities were determined to be within the linear range of a standard curve established with AMC.

Measurement of Lipid Peroxidation. WT, C12, or C4 cells, plated at 1×10^6 cells/10-cm dish, were grown for 24 h to 90% confluence and harvested after treatment with bilirubin for 3 h. Cells were centrifuged for 2 min at 500g, the supernatant was removed, and the residual pellet was washed with PBS. Malondialdehyde formation, an indicator of the degree of lipid peroxidation, was determined in cells using the thiobarbituric acid assay (TBARS) (Ohkawa et al., 1979).

Measurement of Intracellular ROS Production. Free radical production was examined by measuring the conversion of the cell permeant probe, 2',7'-dichlorofluorescein diacetate to its fluorescent product, 2',7'-dichlorofluorescein (LeBel et al., 1992). WT, C12, or C4 cells, plated at 7.5×10^4 cells/well in 12-well plates, were grown for 24 h to 90% confluence and harvested after treatment with 1, 10, 25, 50 or 100 μ M bilirubin or PBS (control) for 3 h. Media were removed, and cells were washed with PBS and then incubated with 10 μ M 2',7'-dichlorofluorescein diacetate in Hanks' balanced salt solution for 20 min at 37°C . Cells were collected and changes in fluorescence were measured immediately in a Cary Eclipse Fluorimeter (Varian, Palo Alto, CA) (excitation and emission wavelengths of 500 and 520 nm, respectively).

Statistics. All experimental data were analyzed by the unpaired, Student's *t* test for significant differences ($P < 0.05$) between the bilirubin-treated and the corresponding control groups.

Results

Cell Viability and Nuclear Morphology. High concentrations of bilirubin are known to be cytotoxic to many different cell types (Ngai et al., 2000). Initial experiments were undertaken to determine the toxicity of bilirubin to murine hepatoma cells. Trypan blue exclusion studies demonstrated that bilirubin treatment caused significant cell death in WT cells, AHR-deficient C12 cells, and ARNT-deficient C4 cells early in the time course. By 48 h, significant decreases in cell viability in all three adherent cell types were found (data not shown). LC₅₀ values calculated from cell viability experiments at 24 h were 25, 45, and 50 μ M for WT, C12, and C4 cells, respectively (data not shown). Examination of cell morphology by phase-contrast microscopy during the initial experiments indicated that bilirubin treatment caused shrinkage, rounding, and detachment of individual cells suggestive of apoptosis (data not shown). Therefore, we proceeded to confirm our visual observations by quantifying the relative amounts of apoptosis occurring subsequent to exposure to multiple concentrations of bilirubin for various times. Three

independent measures of the apoptotic response were used for this analysis.

Fluorescence microscopy was used to assess plasma membrane integrity and nuclear morphology by staining cells with PI and H33342. Chromatin condensation and nuclear fragmentation, characteristic features of apoptosis, were present 24 h after treatment with 1 to 100 μ M bilirubin in WT, C12, or C4 cells (Fig. 1). The percentage of fragmented nuclei at 24 h increased in a concentration-dependent manner after bilirubin treatment in each cell type. However, WT cells were found to be more susceptible than C12 or C4 cells at 25 μ M bilirubin (Fig. 1), consistent with 24-h LC₅₀ values. Positive staining for PI in the nuclei of cells followed a pattern similar to H33342 observed at 24 h. Consistent with the maintenance of plasma membrane integrity during early stages of apoptosis, PI staining was not observed at lower doses of bilirubin (Fig. 1).

Cytochrome C Release. Release of cytochrome *c* from the mitochondria is an early event crucial in many signaling pathways leading to apoptosis and is directly responsible for the activation of the caspases (Susin et al., 1998). To evaluate the contribution of the mitochondrial pathway to the process of bilirubin-induced cell death, we analyzed the release of cytochrome *c* from mitochondria. Concentration-dependent cytochrome *c* release was detected in the cytosol (Fig. 2) by 3 h after bilirubin treatment in all three cell types. Initial increases in cytosolic cytochrome *c* were detected at lower concentrations of bilirubin (beginning at 1 μ M) in WT cells, whereas significant increases were not observed until higher concentrations (25 μ M) in C12 or C4 cells.

Caspase Activation. Caspase-8 is an initiator caspase, whereas caspase-3 is considered an executioner enzyme in the apoptotic pathway (Bratton et al., 2000). An *in vitro* assay was used to assess caspase-8 and caspase-3 activities in lysates derived from cells treated with various concentrations of bilirubin for 3 or 6 h. At the 3-h time point, no significant changes in caspase-8 activity were observed at any concentration of bilirubin or in any cell type (Fig. 3). On the other hand, concentration-dependent increases in caspase-3 activity occurred by 3 h in WT or C12 cells, starting

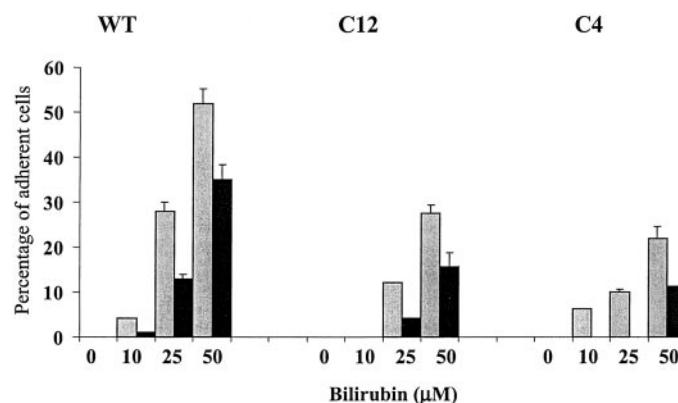


Fig. 1. Quantitative representation of bilirubin-induced changes in nuclear morphology. Hepa WT, C12, or C4 cells plated at 200,000 cells/well were grown for 24 h to 90% confluence on 18-mm cover slips in 12-well dishes and treated with 0, 1, 10, 25, 50, or 100 μ M bilirubin for 24 h. Cells were washed and stained with H33342 (50 μ g/ml) and PI (12 μ g/ml) for 10 min at 37°C and then were mounted onto slides. Nuclei were observed with a Leica epifluorescence microscope (40 \times magnification). Data represent apoptotic (□) or necrotic (■) nuclei counted from four separate fields of view. Values represent mean \pm S.D.; $n = 4$ experiments.

at 25 μM bilirubin (Fig. 4). Concentration-dependent increases in caspase-8 and -3 activities were observed in all cell types 6 h after bilirubin treatment (Figs. 3 and 4).

Lipid Peroxidation and Intracellular ROS Production. Bilirubin is known to bind and interact with lipid membranes (Brodersen, 1979). Consequently, we investigated whether bilirubin exposure would trigger an increase in lipid peroxidation in these hepatoma cell lines. Concentration-dependent increases in TBARS, an indication of elevated lipid peroxidation, occurred in each cell type after bilirubin treatment for 3 h (Fig. 5). We subsequently determined whether bilirubin generated intracellular ROS in Hepa 1c1c7 cells. A significant and concentration-dependent increase in ROS was observed in all three cell types after exposure to bilirubin for 3 h (Fig. 6). Of interest, more ROS were produced in WT cells than in C12 or C4 cells at low bilirubin concentrations (beginning at 1 μM in WT cells).

Effect of αNF on Bilirubin-Mediated Apoptosis. The preceding data indicate a selective sensitivity to bilirubin-mediated apoptosis in the different Hepa cell lines. WT cells were more susceptible than the mutant C12 or C4 cell lines, suggesting a potential involvement of an AHR signaling pathway. Therefore, experiments were conducted to evaluate the potential role of the AHR in bilirubin-induced apoptosis. Because αNF is known to act as an AHR antagonist at low concentrations (1–10 μM), preventing AHR-mediated signaling events (Merchant et al., 1990), it was used to further test this hypothesis. Cells were pretreated for 1 h with 0, 1, or 10 μM αNF , before the administration of 0, 10, 25, or 50 μM bilirubin. Significant decreases in caspase-3 activity were observed in WT cells at 6 h in cultures receiving the αNF

before bilirubin, compared with bilirubin treatments alone (Fig. 7). No significant difference in caspase-3 activity was observed in C12 or C4 cell cultures treated with bilirubin alone and αNF pretreatment at 6 h (Fig. 8). Reductions in the numbers of H333342- and PI-stained nuclei at 24 h occurred in WT cells receiving 10 μM αNF (Fig. 9) and not C12 or C4 cells (data not shown), compared with respective bilirubin treatments.

Discussion

Bilirubin has been shown to have a variety of adverse effects in most biological systems examined, but the molecular mechanisms responsible for its toxicity are incompletely understood. In vivo toxicity is observed primarily in diseases where bilirubin transiently increases above the buffering

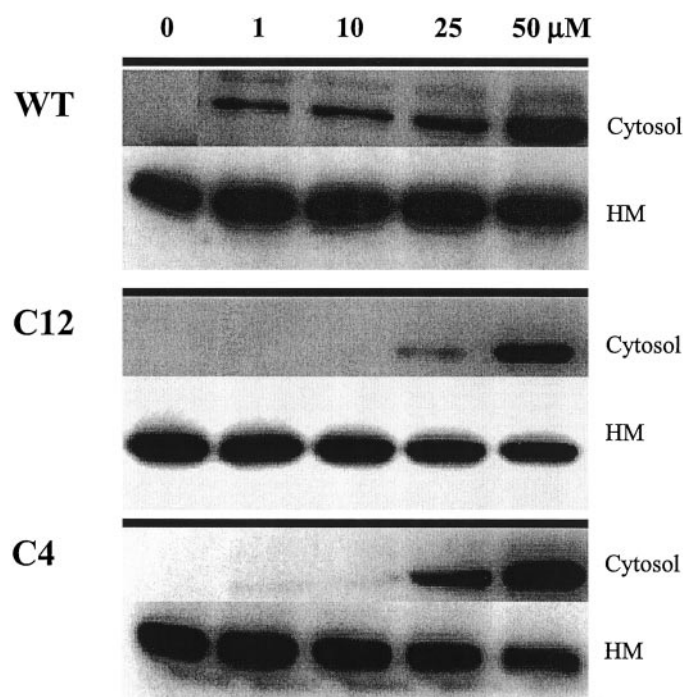


Fig. 2. Mitochondrial cytochrome *c* release in Hepa 1c1c7 cells after bilirubin treatment. WT, C12, or C4 cells plated at 200,000 cells/well in six-well plates were exposed to either PBS (control) or 1, 10, 25, 50, or 100 μM bilirubin for 3 h. Cytosolic and membrane fractions were isolated, and cytochrome *c* content was assessed by SDS-polyacrylamide gel electrophoresis followed by Western blotting. Data are from a single representative experiment which was repeated three times.

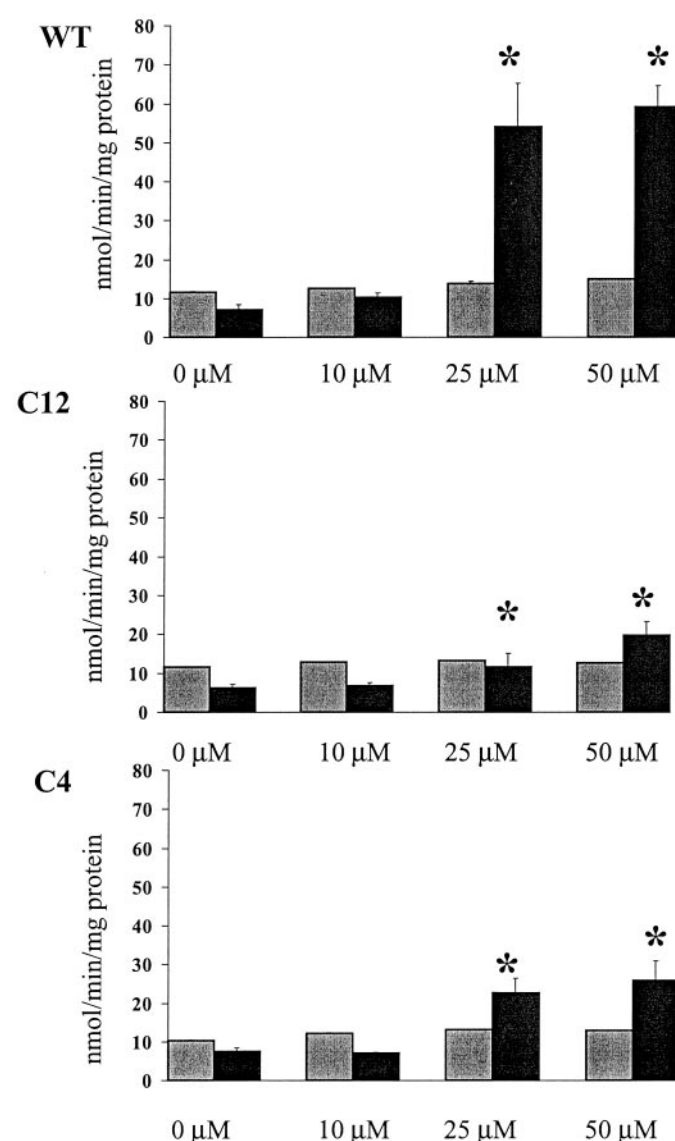


Fig. 3. Increases of caspase-8 activity by bilirubin. Hepa 1c1c7 WT, C12, or C4 cells plated at 200,000 cells/well in six-well plates were incubated for either 3 h (□) or 6 h (■) with 0, 10, 25, or 50 μM bilirubin. Cell lysates (75 μl) were incubated with *N*-acetyl-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) for 60 min at 37°C and monitored for AMC release by fluorescence. Values represent mean \pm S.D.; $n = 4$ experiments; *, $P < 0.05$ versus control values.

capacity of plasma albumin and redistributes into cells ($\sim 20 \mu\text{M}$) (Cashore, 1990; Mireles et al., 1999; Ngai et al., 2000; Dennerly et al., 2001). In the present study, bilirubin caused concentration-dependent apoptosis in the nonexcitatory mouse hepatoma Hepa 1c1c7 cell line at concentrations as low as $10 \mu\text{M}$ (Figs. 1–4). Bilirubin also caused apoptosis in AHR-deficient (C12) and ARNT-deficient (C4) mutant cells, although these cells were less sensitive to bilirubin than WT cells (Figs. 1–4). In addition, pretreatment of cells with αNF resulted in a decreased apoptotic response in WT cells and not C12 or C4 cells (Figs. 7–9). Thus, it seems that the AHR-signaling pathway may contribute to but is not required for bilirubin-mediated apoptosis in WT cells. The proposed mechanism for bilirubin-mediated apoptosis in these cell lines implicates a partial role for the AHR, in conjunction with intracellular generation of ROS (Fig. 6). The concentrations of bilirubin that cause apoptosis ($10\text{--}100 \mu\text{M}$) are similar to those that occur from overproduction of bilirubin dur-

ing some pathobiological conditions, where serum bilirubin levels greater than $300 \mu\text{M}$ are known (Bhutani et al., 1999).

A protective role for bilirubin may be inferred from the well conserved heme degradation pathway and the diverse range of inducers of HO-1. The beneficial antioxidant effects of bilirubin include scavenging of peroxy radicals and inhibition of lipid peroxidation at low concentrations ($\sim 20 \mu\text{M}$) (Stocker et al., 1987). The present findings demonstrate a role for bilirubin in initiating apoptosis, which is dependent on its elevated cellular concentration. Bilirubin-mediated apoptosis may potentially have a protective role, whereby increased local production of bilirubin after HO-1 induction provides a selective mechanism for removal of damaged or unwanted cells from tissues in a controlled process. On the other hand, circumstances resulting in the overproduction or accumulation of bilirubin may cause an abnormal apoptotic response resulting in severe toxicity.

The AHR is associated with multiple cellular functions, indicating its interaction with various signaling pathways. Analysis of cell proliferation and differentiation in AHR-defective Hepa 1c1c7 cells demonstrate the important role of the AHR in cell homeostasis and the presence of an endogenous ligand (Ma and Whitlock, 1996). AHR regulation of cell cycle involves an interaction with retinoblastoma protein

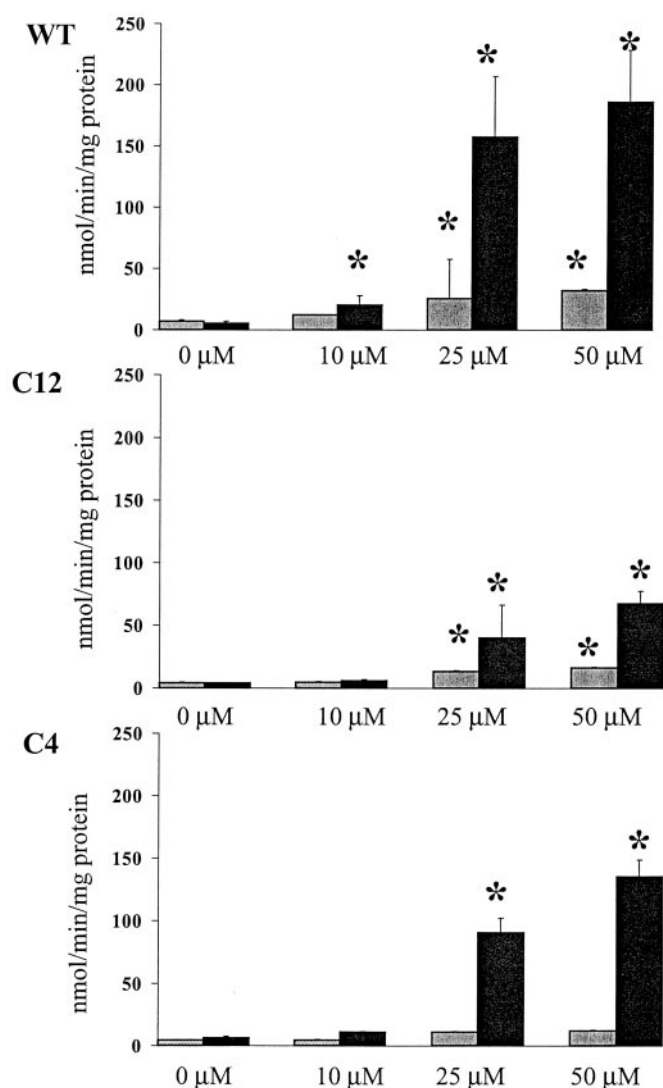


Fig. 4. Increases of caspase-3 activity by bilirubin. Hepa 1c1c7 WT, C12, or C4 cells plated at 200,000 cells/well in six-well plates were incubated for either 3 h (□) or 6 h (■) with 0, 10, 25 or $50 \mu\text{M}$ bilirubin. Cell lysates ($75 \mu\text{l}$) were incubated with Ac-DEVD-AMC for 60 min at 37°C and monitored for AMC release by fluorescence. Values represent mean \pm S.D.; $n = 4$ experiments; *, $P < 0.05$ versus control values.

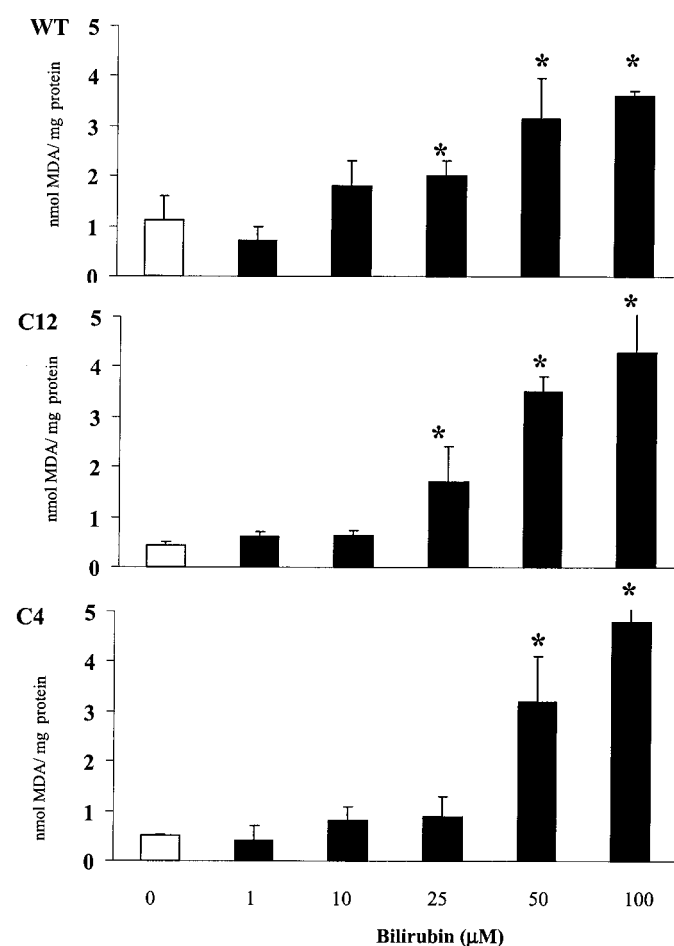


Fig. 5. Increased lipid peroxidation (TBARS) in Hepa 1c1c7 cells after incubation with bilirubin. Hepa 1c1c7 WT, C12, or C4 cells plated at 1×10^6 cells/10-mm plate were exposed to 0, 1, 10, 25, 50 or $100 \mu\text{M}$ bilirubin for 3 h. Values represent mean \pm S.D.; $n = 4$ experiments; *, $P < 0.05$ versus control values.

preventing the progression G₁ to S phase by blocking E2F-mediated transcription (Ma and Whitlock, 1996; Ge and Elferink, 1998; Elizondo et al., 2000). As the cell decides be-

tween apoptosis and continuation of cell cycle at or near the G₁/S boundary, involvement of the AHR is important in determining such an apoptotic response (Zaher et al., 1998; Reiners and Clift, 1999). A direct role for AHR in apoptosis was recently demonstrated in mouse oocytes where toxic effects after PAH exposure resulted from the induction of Bax protein by an AHR pathway (Matikainen et al., 2001). Although several exogenous AHR ligands have been reported to cause apoptosis including benzo[*a*]pyrene in Hepa 1c1c7 cells (Lei et al., 1998) and dimethylbenz[*a*]anthracene in preB cells (Near et al., 1999), some reports have indicated involvement of an endogenous ligand (Reiners and Clift, 1999; Elizondo et al., 2000). Many pathobiological conditions result in increased bilirubin levels, a significant endogenous role of

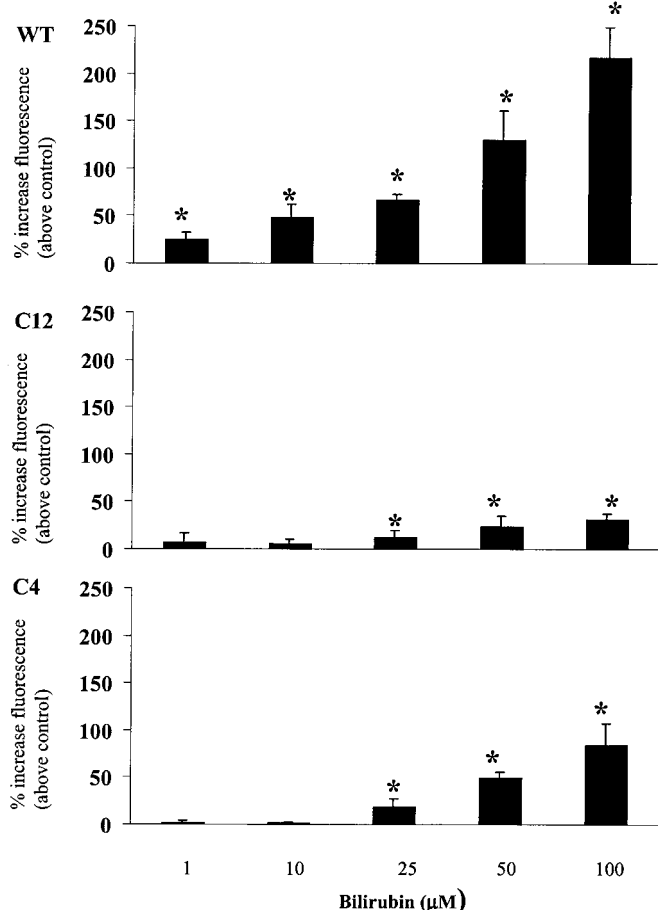


Fig. 6. Increased intracellular ROS production in Hepa 1c1c7 cells after incubation with bilirubin measured fluorometrically using 2',7'-dichlorofluorescein-diacetate as substrate. Hepa 1c1c7 WT, C12, or C4 cells plated at 1×10^6 cells/10-mm plate were exposed to 0, 1, 10, 25, 50 or 100 μ M bilirubin for 3 h. Values represent mean \pm S.D.; $n = 4$ experiments; *, $P < 0.05$ versus control values.

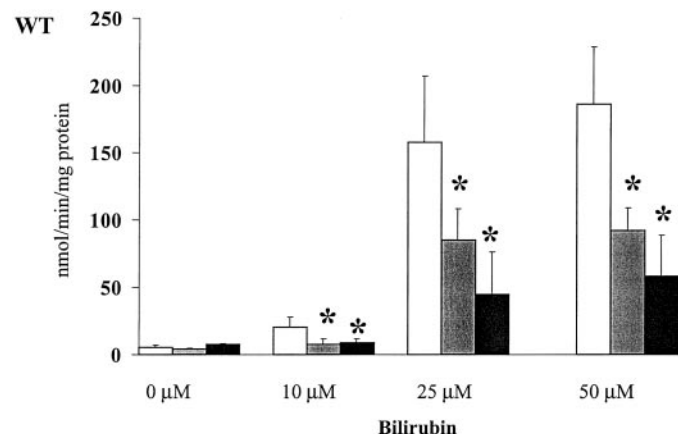


Fig. 7. Protective effect of α NF on bilirubin increased caspase-3 activity. Hepa 1c1c7 WT cells plated at 200,000 cells/well in six-well plates were preincubated for 1 h with 0 (\square), 1 (\blacksquare), or 10 (\blacksquare) μ M α NF and then for 6 h with 0, 10, 25, or 50 μ M bilirubin. Cell lysates (75 μ l) were incubated with Ac-DEVD-AMC for 60 min at 37°C and monitored for AMC release by fluorescence. Values represent mean \pm S.D.; $n = 3$ experiments; *, $P < 0.05$, α NF compared with respective bilirubin treatment.

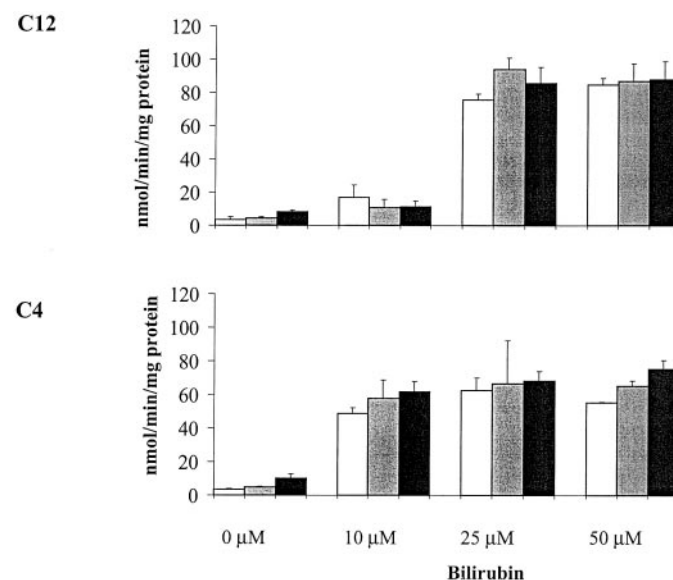


Fig. 8. Effect of α NF on bilirubin increased caspase-3 activity. Hepa 1c1c7 C12 and C4 cells plated at 200,000 cells/well in six-well plates were preincubated for 1 h with 0 (\square), 1 (\blacksquare), or 10 (\blacksquare) μ M α NF and then for 6 h with 0, 10, 25, or 50 μ M bilirubin. Cell lysates (75 μ l) were incubated with Ac-DEVD-AMC for 60 min at 37°C and monitored for AMC release by fluorescence. Values represent mean \pm S.D.; $n = 3$ experiments; *, $P < 0.05$, α NF compared with respective bilirubin treatment.

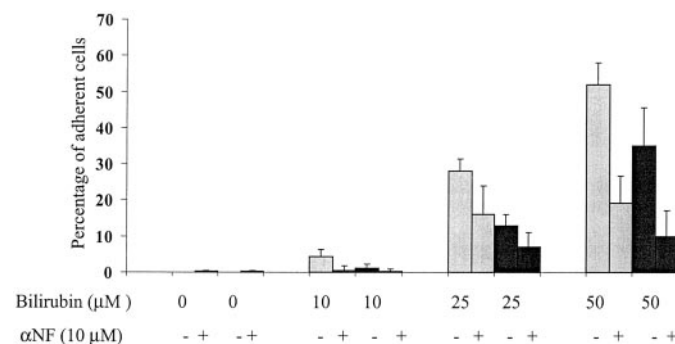


Fig. 9. Protective effect of α NF on bilirubin-mediated changes in nuclear morphology. Hepa WT cells plated at 200,000 cells/well were grown for 24 h to 90% confluence on 18-mm cover slips in 12-well dishes and pretreated with 10 μ M α NF for 1 h. Cells were subsequently treated with 0, 10, 25, or 50 μ M bilirubin for 24 h. Cells were washed and stained with 50 μ g/ml H33342 and 12 μ g/ml PI for 10 min at 37°C and then were mounted onto slides. Nuclei were observed with a Leica epifluorescence microscope (40 \times magnification). Data represent apoptotic (\square) or necrotic (\blacksquare) nuclei counted from four separate fields of view. Values represent mean \pm S.D.; $n = 4$ experiments.

which may be to affect cellular function and survival by influencing AHR-dependent or -independent responses (Sinal and Bend, 1997; Phelan et al., 1998).

The bilirubin-mediated apoptotic response that occurred in WT, C12, and C4 cells involved the early release of cytochrome *c* from the mitochondria and subsequent activation of caspase-3. Cytochrome *c* release from the mitochondria occurred in a concentration-dependent manner in all cell types treated with bilirubin at 3 h, beginning at the lower doses (1 μ M) in WT cells (Fig. 2). In this study, we observed increased caspase-3 activity (3 h) before caspase-8 activity (6 h) after bilirubin treatment (Figs. 4 and 5). Evidence exists indicating that late activation of caspase-8 may occur in a mitochondrial-dependent manner after cytochrome *c* release, downstream of caspase-3 activation (Scaffidi et al., 1998; Slee et al., 1999). In addition, caspase-3 and caspase-8 activations were impaired in embryonic stem cells derived from *CASP-9*^{-/-} mice after UV radiation, although cytochrome *c* release occurred (Hakem et al., 1998). These earlier reports are supportive of the involvement of a mitochondrial-dependent pathway in bilirubin-mediated apoptosis. The involvement of the mitochondrial-dependent pathway is consistent with recent reports indicating that bilirubin interferes with membrane permeabilization in isolated mitochondria from rat brain and liver tissues (Rodrigues et al., 2000). Not surprisingly, mitochondria were found to be involved in the apoptotic response, because they are target sites for bilirubin toxicity (Menken et al., 1966) and removal by enzymatic oxidation, at least in brain (Hansen and Allen, 1997). The magnitude of the cytochrome *c* response reflects the differences observed in cell viability between WT, C12, and C4 cells incubated with bilirubin, implying a role for the AHR in WT cells. Reduction in caspase-3 activity (Fig. 7) after α NF treatment in WT cells is consistent with the contribution of the AHR via a mitochondrial-dependent pathway. However, it is not conclusive that an AHR-mediated induction of Bax protein (Matikainen et al., 2001) occurred after bilirubin exposure.

Extracellular accumulation of bilirubin could result in binding or interaction with membrane phospholipids and sphingomyelin stimulating an apoptotic response (Nagaoka and Cowger, 1978; Notter et al., 1982; Mireles et al., 1999). The higher concentrations of bilirubin tested caused lipid peroxidation in all three cell types, indicating a disruption of membrane integrity (Fig. 5). The lack of difference in lipid peroxidation observed between WT, C12, and C4 cells presumably resulted from the interaction of the highly hydrophobic bilirubin with the outer plasma membrane on all three cell types. Ceramide, a compound generated biosynthetically or by degradation of sphingomyelin, has been reported to stimulate an AHR-dependent apoptotic response in murine Hepa 1c1c7 cells (Reiners and Clift, 1999). In contrast to ceramide, bilirubin caused apoptosis in WT, C12, and C4 cells, and this response was only partially blocked by α NF, an AHR antagonist, in WT cells. This suggests that bilirubin causes apoptosis in Hepa 1c1c7 cells by a mechanism(s) other than signaling solely by an AHR pathway.

Differences in the degree of apoptotic response observed among the Hepa 1c1c7 cell types studied demonstrated that C12 and C4 cells were more resistant to bilirubin-mediated cellular death. Observed differences in the sensitivity of WT, C12, and C4 cells to increased production of intracellular

ROS after bilirubin treatment suggest a potential role of the AHR pathway or alterations in other unknown characteristics in these cell lines. The mitochondrial electron transport components are considered a major source of ROS that is involved in apoptosis (Skulachev, 1999). Increased ROS production at 1 μ M bilirubin occurred in WT cells but not until 25 μ M in C12 or C4 cells (Fig. 7), which correlated with the concentrations for cytochrome *c* release (Fig. 2) in these cells, respectively. Although the intracellular source of the ROS production was not investigated in the present studies, these data suggest the mitochondria as a potential source. If ROS signaling plays a key role in the apoptotic response in these hepatoma cells, any alteration in the balance between the rate of ROS generation and the capacity of its antioxidant systems is important.

Stimulation of intracellular signaling pathways that activate phosphoinositide 3-kinase is known to promote the phosphorylation and subsequent activation of the serine/threonine kinase, Akt (protein kinase B), which contributes to the regulation of multiple cellular processes that function in cell survival (Kennedy et al., 1999; Rokudai et al., 2000). Bilirubin is known to inhibit protein phosphorylation catalyzed by a number of protein kinases, including protein kinase C (Sano et al., 1985; Amit and Boneh, 1993; Churn et al., 1995; Hansen et al., 1996, 1997; Hansen and Allen, 1997). Akt has a catalytic domain closely related to cAMP-dependent protein kinase and protein kinase C (Konishi et al., 1999), and our initial tests indicated inhibition of Akt phosphorylation by bilirubin. After treatment, phospho-Akt content was suppressed in a concentration-dependent manner by bilirubin at 6 h with no significant change in total Akt content in WT or C12 cells (data not shown). It is presently unclear whether bilirubin, a lipid soluble product, reached high enough concentrations within the cells to directly inhibit Akt activation. This will be at least partially dependent on whether bilirubin accumulation from extracellular sources is required and on the activity of clearance enzymes, such as of UDP-glucuronosyltransferases. However, initial observations suggest that the bilirubin-mediated apoptotic response might involve suppression of protein phosphorylation affecting key survival signals such as Akt activation.

In the present study, we report three significant observations. First, bilirubin induces an apoptotic response in murine hepatoma 1c1c7 cells; second, bilirubin generates intracellular ROS at moderate to high doses in these cells; and third, AHR has a role in the bilirubin-mediated response in WT cells. Our proposed mechanism of bilirubin-induced apoptosis in Hepa 1c1c7 cells is partially mediated by the AHR. However a more general pro-oxidant effect occurs at higher concentrations of bilirubin resulting in effects such as generation of ROS, disruption of lipid membranes and inhibition protein phosphorylation reactions, such as Akt, that cause apoptosis in C4 and C12 cells.

Acknowledgments

We thank Chris Webb for technical contributions to these experiments.

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