In mammalian cells, heme is degraded by heme oxygenase to biliverdin, which is then reduced to bilirubin by biliverdin reductase (BVR). Both bile pigments have reducing properties, and bilirubin is now generally considered to be a potent antioxidant, yet it remains unclear how it protects cells against oxidative damage. A presently popular explanation for the antioxidant function of bilirubin is a redox cycle in which bilirubin is oxidized to biliverdin and then recycled by BVR. Here, we reexamined this putative BVR-mediated redox cycle. We observed that lipid peroxidation-mediated oxidation of bilirubin in chloroform, a model of cell membrane-bound bilirubin, did not yield biliverdin, a prerequisite for the putative redox cycle. Similarly, transformation of human BVR into hmx1 (heme oxygenase) mutant yeast did not provide protection against H2O2 toxicity above that seen in hmx1 mutant yeast expressing human heme oxygenase-1. Together, these results argue against the BVR-mediated redox cycle playing a general or important role as cellular antioxidant defense mechanism.

Biliverdin reductase (BVR)\(^3\) forms part of the major pathway for the disposition of cellular heme in mammalian cells. This pathway is initiated by heme oxygenase, which converts heme to carbon monoxide, iron, and biliverdin, which in turn is reduced to bilirubin by BVR at the expense of NADPH. Because of its intramolecular hydrogen bonding, the bilirubin produced is sparingly soluble in water at physiological pH and ionic strength (1). Hence, bilirubin is usually tightly bound to albumin in order to be transported within the blood circulation (2), from which it is removed mainly through uptake by hepatocytes. Once bilirubin is transferred across the cell membrane of hepatocytes, it binds glutathione S-transferases before being transformed to water-soluble derivatives by conjugation of one or both of its propionyl groups before its excretion into bile and then the intestine (3).

Under physiological conditions, plasma bilirubin concentrations in humans range from ~5 to 20 \(\mu\)M, practically all of which is unconjugated pigment bound to albumin (1). Abnormally high plasma concentrations are associated with the risk of developing neurologic dysfunction due to preferential deposition of bilirubin in brain and its toxic effects on cell functions. In fact for many years, biliverdin and bilirubin were generally regarded as waste products of heme metabolism in higher animals, although earlier work suggested that these bile pigments might play a role as natural antioxidants, since small quantities of the pigment stabilize vitamin A and \(\beta\)-carotene during intestinal uptake, and animals with low plasma bilirubin showed early signs of vitamin E deficiency (4, 5).

In a series of in vitro studies, Stocker et al. (6–8) demonstrated that unconjugated bilirubin, at micromolar concentrations, efficiently scavenged peroxyl radicals in homogenous solution or multimolecular liposomes. At physiologically relevant oxygen tension, bilirubin surpassed peroxyl radicals in homogenous solution or multimolecular liposomes (8), and it is thought to protect plasma proteins and lipids from many but not all oxidants (9). However, it is less clear whether this antioxidant activity extends to in vivo situations or protection of cells from oxidative stress. Although produced in essentially all cells, the normal range of cellular bilirubin concentrations is unknown. However, it is probably in the low nanomolar range, well below that of established cellular antioxidants, such as glutathione and ascorbate, arguing against bilirubin being an important cellular antioxidant. Nonetheless, in vitro studies with rat neuronal cultures showed that the presence of 10 \(\mu\)M bilirubin in the culture medium protected cells against 10,000-fold higher concentrations of hydrogen peroxide (10). Later, Barañano et al. (11) confirmed such observations in HeLa cells and demonstrated that BVR depletion increased reactive oxygen species (ROS) and cell
Biliverdin Reductase and Cellular Antioxidant Defense

dearth. This led to the following proposal of the BVR-amplified redox cycle. While acting as an antioxidant, bilirubin is oxidized to biliverdin that is then reduced back to bilirubin by the ubiquitous and abundant BVR.

An important underlying assumption of this amplification cycle is that ROS-mediated bilirubin oxidation in cells is specific and yields substantial if not stoichiometric amounts of biliverdin. Inconsistent with this assumption, however, earlier studies showed that high yields of biliverdin formation are limited to certain oxidants (i.e., peroxyl radicals) and albumin-bound bilirubin. In cells, bilirubin is probably present in membranes, bound to proteins other than albumin, or present in conjugated form. Therefore, we reexamined the putative redox amplification cycle. Our results show that reaction of these forms of bilirubin with 1e- or 2e-oxidants at best generates modest amounts of biliverdin. Furthermore, overexpression of BVR does not protect mammalian or yeast cells from hydrogen peroxide-mediated damage, thereby casting doubt on the importance of the putative BVR redox cycle for cellular antioxidant protection.

EXPERIMENTAL PROCEDURES

Materials—HPLC grade methanol and glacial acetic acid were obtained from Scharlau (Barcelona, Spain). 2,2'-Azobis(2-amidinopropane)hydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) were obtained from Wako Pure Chemical Industries (Japan). Biliverdin hydrochloride, bilirubin ditaurate, and bilirubin 1Xα were purchased from Frontier Scientific (Logan, UT). Equine serum albumin was obtained from U.S. Biological (Swampscott, MA). Penicillin/streptomycin, trypsin, Opti-MEM, l-glutamine, and 5(6)-carboxy-2',7'-dichlorofluorescein diacetate were purchased from Invitrogen. Polyclonal rabbit anti-biliverdin reductase antibody was obtained from Stressgen Bioreagents (Ann Arbor, MI), and goat anti-rabbit IgG conjugated with horseradish peroxidase was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FuGENE® HD transfection reagent and protease inhibitor mixture tablet were obtained from Roche Applied Science. Nitrocellulose membranes were purchased from Amersham Biosciences. Solutions of NaCl (0.9%) were obtained from Baxter (Munich, Germany), NaOH pellets were from Merck, and phosphate-buffered saline tablets (Dulbecco A) were from Oxoid (Cambridge, UK). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from SAFC Biosciences, and fetal bovine serum (FBS) was from JRH Biosciences. All other reagents and chemicals were obtained from Sigma unless otherwise stated. The concentration of commercial H₂O₂ (30%; British Drug House, UK) was determined using ε₂₄₀nm = 43.6 M⁻¹ cm⁻¹ and then diluted appropriately to the concentrations indicated throughout.

Oxidation of Protein-bound Bilirubin—Bilirubin dissolved in 50 mM NaOH was added to either equine glutathione S-transferase (GST) or essentially fatty acid-free human serum albumin in 10 mM phosphate-buffered saline (PBS; pH 7.3). For human serum albumin, purified linoleic acid, dissolved in methanol, was added as an aqueous dispersion, and the solution was stirred gently at 4°C until it became clear, as described previously (7). An aliquot of cold AAPH (500 mM) was then added. The final concentrations of reactants were as follows: 50 μM GST or 500 μM human serum albumin containing 2 mM linoleic acid, 20 μM bilirubin, and 50 mM AAPH. The oxidation was initiated by placing the reaction tube in a water bath set at 37°C. For some oxidation by H₂O₂, we used glucose in the presence of glucose oxidase to generate H₂O₂ at a constant rate. Briefly, 1.5 units/ml glucose oxidase from Aspergillus niger (low catalase activity) dissolved in PBS and containing 50 μM diethylenetriamine pentaacetic acid (DTPA) was added to GST (final concentration 50 μM) or a clear solution of bilirubin bound to human serum albumin containing purified linoleic acid and prepared as described above. Oxidation was initiated by adding 5 mM D-glucose and placing the reaction tube at 37°C. Aliquots (100 μl) of the reaction mixture were removed at the time points indicated, and the pigments were extracted with 400 μl of ice-cold methanol. After centrifugation at 13,600 × g for 5 min, 100 μl of the supernatant was subjected to HPLC analysis.

Oxidation of Non-protein-bound Bilirubin—Cold AAPH (50 mM final concentration) was added to bilirubin (20 μM) dissolved in DMSO, and oxidation was carried out under air and at 37°C. Alternatively, AMVN (50 mM) was added to bilirubin (100 μM) dissolved in chloroform, and the oxidation again started at 37°C. For oxidation of conjugated bilirubin, AAPH (50 mM) or 5 mM D-glucose and 1.5 units/ml glucose oxidase in 50 μM DTPA was added to bilirubin ditaurate (100 μM) dissolved in PBS. Oxidation was initiated by placing the reaction tubes at 37°C. At the time points indicated, aliquots (100 μl) of the reaction mixture were removed and subjected to HPLC.

HPLC Determination of Bile Pigments—Separation of biliverdin and bilirubin was achieved using an analytical LC-18 column, 25 cm × 4.6 mm (Supelco, Bellefonte, PA), eluted with 0.1 M di-n-octylamine acetate in methanol and water (96:4, v/v) at 1 ml/min, as described previously (12). Under these conditions, biliverdin and bilirubin eluted at 5 and 12 min, respectively. Biliverdin was detected at 380 or 665 nm, whereas bilirubin was detected at 460 nm. Bile pigments were quantified by their peak area comparison with standard curves constructed from authentic standards. For the separation of bilirubin and biliverdin conjugates, 0.1 M di-n-octylamine acetate in 80% methanol/20% water (v/v) was used as the mobile phase eluted at 1 ml/min. The retention time of bilirubin ditaurate was ~22 min, and that of its putative biliverdin conjugate ~15 min. Bilirubin ditaurate and biliverdin ditaurate were detected at 460 and 665 nm, respectively. To quantify biliverdin ditaurate, non-conjugated biliverdin was used as standard due to the unavailability of biliverdin ditaurate.

Cell Culture Studies—HeLa cells were cultured in low glucose DMEM, supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 95% air, 5% CO₂ at 37°C, and the medium was replaced every 3–4 days. At 90% confluence, cells were disrupted with 0.15% trypsin and 1 mM EDTA for 5 min, washed, and plated in new culture plates and flasks for experiments.

Construction of BVR Plasmid—Total RNA was extracted from HepG2 cells by TRIzol reagent (Invitrogen) and converted
to cDNA with a Superscript first strand synthesis kit (Invitro-
gen). BVRA was then amplified by PCR with AmpliTaq (Applied Biosystems, Foster City, CA), using the primer pair 5′-TTAT-
AGGATGCGATGCAATGAGCCCG-3′ and 5′-CAG-
AAAGATCTCGAGAATGCTACATCA-3′. The ther-
mocycle used was as follows: 1 cycle of 94 °C for 3 min; 40 cycles
of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; 1 cycle
of 72 °C for 5 min. The PCR product was resolved on a 1.5% agar-
ose gel and purified using a gel purification kit obtained from
Qiagen (Valencia, CA). The purified cDNA was then cloned
into pGEM-T easy vector (Promega, Madison, WI) and trans-
fomed into TOP10 chemically competent cells (Invitrogen).
The pGEM-T easy-BVR construct was propagated, purified
with the QIAPREP spin miniprep kit (Qiagen), and digested
with BamHI (Fermentas, Glen Burnie, MD) to excise the BVR
cDNA. pcDNA3 vector (Invitrogen) was also digested with
BamHI and treated with calf intestinal alkaline phosphatase
(Invitrogen). Both the BVR cDNA and pcDNA3 vector were
resolved and purified from a 1% agarose gel and then ligated
with Ligation (DNA sense sequences 5′-GACGAGAGCATGTT-
GAACCTCTTG-3′, 5′-TCCTCAGCCTCCTGAACCTG-3′,
and 5′-AGAGGTGAGGTGGCCTATATC-3′) using Lipo-
fectamine 2000 (Invitrogen), according to the manufacturer’s
instructions. Cells were then incubated for 48 h in DMEM sup-
plemented with 10% FBS before being subjected to assays. As a
control, cells were transfected with 60 pmol scrambled siRNA
(medium negative control stealth RNAi; Invitrogen; catalogue
number 12935-300). The scrambled siRNA was not a match to
any sequence in the human BLAST database.

**Determination of Cell Viability**—Cell viability was deter-
mined by the trypsin blue exclusion method or the 3-(4,5-di-
methylthiazl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)
assay. Cells were seeded at 3 × 10^5, 1 × 10^5, and 7.5 × 10^4
cells/well into a 6-well, 12-well, and 96-well plate, respectively,
for cell viability measurement by the trypsin blue exclusion
method. Two vials of cells were collected for each time point,
one containing the spent medium with floating dead cells and
the other containing trypsized live cells still attached to the
plate after medium collection and one wash with PBS. The cell
suspension was centrifuged at 720 × g for 5 min, and cells were
resuspended in an adequate volume of culture medium. Ali-
quots of cell suspension (10 μl) were then stained with 10 μl of
0.4% trypsin blue solution. Approximately 100 cells were
counted, and cell viability was calculated as follows: percentage
of cell viability = ((total cells – dead cells)/total cells) × 100.

For the MTT assay, cells in DMEM plus 10% FBS were seeded
in 24-well plates and treated as described. At the time points
indicated, the medium was removed, and the cells were washed
with PBS. Fresh medium (200 μl) and MTT (1 mM) were then
added, and the cells were incubated at 37 °C for 1 h in a humid-
ified atmosphere of 95% air, 5% CO2 before medium was
removed. The dark blue crystals in the cells were then dissolved
by adding 150 μl of DMSO, followed by incubation at 37 °C for
10 min. Finally, 20 μl of the resulting mixture were diluted in
100 μl of DMSO, and its absorbance at 570 nm was determined
using a plate reader. Cell viability was calculated as percentage
of cell viability = (A_570 nm of treated cells/A_570 nm of untreated
cells) × 100.

**Yeast Cell Sensitivity to H2O2**—Cells were grown to exponen-
tial phase (A_600 = 1) in synthetic-defined medium at 30 °C and
then treated for 1 h with H2O2 (0–20 μM). Aliquots of cells
were removed, diluted in fresh YEPD medium, and plated in
triplicate on YEPD plates, and the number of viable colonies
was counted after 3 days of growth.

**Measurement of Cellular BVR Activity**—BVR activity was
determined by the formation of bilirubin using ε_540 nm = 53.0
mm⁻¹ cm⁻¹ (12). Briefly, cells lysed with 200 μl of PBS plus
0.1% Triton X-100 were scraped and collected into an Eppen-
dorf tube and centrifuged at 13,000 × g for 5 min at 4 °C. An
aliquot of the resulting supernatant corresponding to 200 μg of
protein (determined by the bicinchoninic acid assay) was then
Biliverdin Reductase and Cellular Antioxidant Defense

added to an NADPH-generating system in a plastic cuvette at the following final concentrations: 200 μg of cellular protein, 5–50 μM biliverdin added as DMSO solution, 2 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, 1 mM NADPH in Buffer A (PBS containing 250 mM D-glucose, 20 mM Tris, and one protease inhibitor mixture tablet, pH 7.3). The final volume of the reaction mixture was 1 ml. The reaction was monitored in a spectrophotometer (Beckman DU 800) for 120 min at 37 °C. The bilirubin generated was detected by reading absorbance at 450 nm and quantified using ε 450 nm = 53.0 M cm⁻¹.

Measurement of ROS Levels—ROS levels were determined using 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate, as described previously (13) with modifications. HeLa cells in 24-well plates were transfected for 18–24 h with empty vector or human BVR, as described above. On the day of the experiments, cells were washed with PBS and then incubated for 30 min at 37 °C with dichlorofluorescein diacetate (100 μM, dissolved in DMSO) in DMEM plus 1% FBS. Following incubation, cells were washed with washing buffer and treated with the indicated concentration of H₂O₂ in Krebs. Fluorescence of the cells in each well was then measured every 5 min for 30 min in a fluorescence plate reader maintained at 37 °C. Excitation was set at 485 nm, and emission was detected at 538 nm. ROS levels in H₂O₂-treated cells were determined as the change in fluorescence over 30 min compared with untreated cells.

Western Blot Analysis—Mammalian cells were lysed in PBS plus 0.1% Triton X-100 supplemented with protease inhibitors. Yeast cell lysates were prepared by breaking cells in 50 mM KPB, 100 mM NaCl, 1 mM EDTA supplemented with protease inhibitors on a vortex in the presence of acid-washed glass beads for 45 s and then placed on ice for 30 s. Western blotting analysis was performed on lysates by loading equal amounts of protein (8–15 μg) onto 10% polyacrylamide gels (NU-PAGE, Invitrogen) using SeeBlue® Plus2 (Invitrogen) as molecular mass standards. Electrophoresis was performed at 200 V for 90 min using a MiniProtean II electrophoresis system (Invitrogen XCell Surelock system), and separated proteins were transferred onto nitrocellulose membranes (Amersham Biosciences) at 30 V for 90 min using a miniblot module (NOVEX, San Diego, CA). The blotted membranes were blocked in 5% (w/v) skim milk powder with 0.1% (v/v) Tween 20 in Tris-buffered saline for 3–4 h at room temperature. Blocked membranes were probed overnight at 4 °C with the following primary antibodies at 1:2000 dilutions in 5% (w/v) skim milk and Tween 20-containing Tris-buffered saline: rabbit polyclonal antibody against human BVR (StressGen); rabbit polyclonal antibody against human HO-1 (StressGen); mouse monoclonal antibody against human α-tubulin (Sigma). Membranes were then probed for 1 h at room temperature with sheep anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated IgG (Bio-Rad) (1:5,000 dilution) in 1% skim milk and 0.1% Tween 20 in Tris-buffered saline. Proteins were detected by ECL according to the manufacturer’s instructions (Amersham Biosciences). For yeast cell lysates, HO-1 and BVR were detected essentially the same as above, but primary and secondary antibodies were diluted in 3% bovine serum albumin and Tween 20-containing Tris-buffered saline.

Reverse Transcription-PCR of Yeast cDNA for BVR and HO-1—Yeast cells were grown to exponential phase (A₆₀₀ = 1), cells were broken in TRizol reagent (Invitrogen) on a vortex in the presence of acid-washed glass beads for 45 s followed by placement on ice for 30 s; and the RNA was then extracted according to the manufacturer’s instructions. cDNA was synthesized using the SuperScript III first strand synthesis system for reverse transcription-PCR.

Statistical Analysis—Statistical differences between treatments and controls for BVR activity were examined using the Wilcoxon-Mann-Whitney rank sum test. Where appropriate, data were analyzed using a two-factor repeated measures analysis of variance with post hoc Bonferroni test. Significance was accepted at p < 0.05.

RESULTS

Oxidation of Bilirubin by Peroxyl Radicals or H₂O₂ Yields Modest Amounts of Biliverdin—An important underlying assumption of the BVR-catalyzed oxidation-reduction cycle proposed by Baranano et al. (11) is that oxidation of bilirubin is specific and yields substantial if not stoichiometric amounts of biliverdin. Since bilirubin is lipophilic and essentially insoluble in water, the pigment associates with either proteins or lipids. We therefore examined the yield of biliverdin formation using bilirubin bound to albumin or GST and aqueous peroxyl radicals derived from thermolabile AAPH as the oxidant. Albumin and GST are extra- and intracellular binding proteins of bilirubin, respectively (14), whereas peroxyl radicals were used as a
model for hydroperoxyl radicals (i.e. the protonated superoxide radical). Albumin-bound bilirubin was oxidized rapidly by peroxyl radicals (Fig. 1A), as reported earlier (7). Under the conditions used, peroxyl radicals were produced at \( \sim 3 \mu\text{mol/min} \), and bilirubin oxidation was complete in 20 min. This time point coincided with maximal accumulation of biliverdin to \( \sim 30\% \) of the initial bilirubin concentration (Fig. 1A). Subsequently, biliverdin became oxidized, as indicated by the time-dependent decrease in the concentration of that pigment (Fig. 1A). Peroxyl radicals also effectively oxidized GST-bound bilirubin, with complete consumption observed within 15 min. In contrast to the situation with albumin-bound bilirubin, there was only a modest accumulation of biliverdin, corresponding to \( <10\% \) of the initial bilirubin concentration (Fig. 1B). Similarly, peroxyl radicals effectively oxidized bilirubin ditaurate, a model of the water-soluble, conjugated bilirubin, but the corresponding biliverdin formed was a minor oxidation product only (Fig. 1C). We next investigated the oxidation of bilirubin by \( \text{H}_2\text{O}_2 \), since 10 \( \mu\text{M} \) bilirubin has been shown to protect cells from damage caused by a 10,000-fold molar excess of \( \text{H}_2\text{O}_2 \) (10, 11). In sharp contrast to peroxyl radicals, albumin-bound bilirubin was essentially resistant to \( \text{H}_2\text{O}_2 \), a non-radical oxidant that was generated at constant rate using glucose oxidase plus glucose, with negligible biliverdin being detected (Fig. 1D). We chose conditions such that the rate of \( \text{H}_2\text{O}_2 \) generation was 5 \( \mu\text{mol/min} \) (i.e. similar to the rate at which we employed peroxyl radicals that readily oxidized bilirubin). Interestingly, \( \text{H}_2\text{O}_2 \)-oxidized bilirubin bound to GST, albeit at a slower rate compared with peroxyl radicals and with biliverdin accumulation corresponding to \( \sim 25\% \) of the bilirubin present initially (Fig. 1E). Oxidation of bilirubin ditaurate by \( \text{H}_2\text{O}_2 \) was ineffective (Fig. 1F) and failed to produce substantive amounts of the biliverdin conjugate. These results suggest that the oxidation of bilirubin to biliverdin by peroxyl radicals or \( \text{H}_2\text{O}_2 \) is modest and depends on the nature of the protein to which bilirubin binds.

We next examined whether the conformation of bilirubin affected the extent of its oxidation to biliverdin. As indicated, bilirubin is scarcely soluble in water (1) due to intramolecular hydrogen bonds that give the pigment its “ridge-tile” conformation (15). These hydrogen bonds are present when bilirubin associates with cell membranes or is dissolved in non-polar solvents like chloroform but are weakened when the pigment is bound to albumin or dissolved in polar solvents like DMSO (16). Peroxyl radicals effectively oxidized bilirubin, irrespective of whether chloroform or DMSO was used as the solvent (Fig. 2). However, there was a significant difference in the extent of biliverdin formation, with negligible “yield” in chloroform (Fig. 2A) and \( \sim 16\% \) yield in DMSO (Fig. 2B). These results suggest that relaxation of intramolecular hydrogen bonds facilitates biliverdin formation during peroxyl radical-mediated oxidation of bilirubin and that membrane-bound bilirubin is less likely to produce biliverdin under these oxidizing conditions.

Overexpression of BVR Does Not Affect Bilirubin Oxidation or Protect Cells against \( \text{H}_2\text{O}_2 \)-mediated Death—We next investigated whether overexpression of BVR protected cells from oxidants. Initially, we added bilirubin to lysates prepared from HeLa cells without and with BVR overexpression and then exposed these lysates to peroxyl radicals or \( \text{H}_2\text{O}_2 \). As expected, BVR overexpression significantly increased BVR protein and activity (Fig. 3A). In lysates prepared from control (vector-transfected) cells supplemented with exogenous bilirubin, exposure to peroxyl radicals resulted in rapid oxidation of the pigment (filled circles in Fig. 3B). This was associated with significant, albeit small, accumulation of biliverdin, corresponding to \( \sim 5\% \) of the initial bilirubin. If BVR acted as a cytoprotectant via the proposed redox cycle, one would expect its overexpression to slow down the consumption of bilirubin by oxidants. Contrary to this expectation, neither the rate of peroxyl radical-mediated consumption of bilirubin nor biliverdin accumulation was affected by BVR overexpression (compare filled with empty symbols in Fig. 3B). When \( \text{H}_2\text{O}_2 \) was used as the oxidant, bilirubin oxidation was barely detectable without biliverdin accumulation, and both processes were unaffected by BVR overexpression (Fig. 3C). These results indicate that in HeLa cells, the operation of the proposed redox cycle, if occurring, is limited by the low yield of biliverdin formation during oxida-
H2O2 was then added as a bolus to the cells in DMEM plus 10% FBS. At the respective pigment for 1 h in DMEM plus 1% FBS and then washed with PBS. H2O2 was then added as a bolus to the cells in DMEM plus 10% FBS. At the indicated time points, cell viability was determined by trypan blue exclusion and expressed as the percentage of untreated cells at the zero time point. Data represent mean ± S.E. of three independent experiments, each done in triplicate.

If BVR protected cells against oxidative stress, one would expect its overexpression to protect against ROS-mediated cell death. Challenge of HeLa cells with H2O2 resulted in a time-dependent loss of viability, as assessed by trypan blue exclusion (Fig. 4A). Overexpression of BVR failed to protect cells against H2O2-mediated death (Fig. 4A). To examine whether this apparent lack of protection was due to the absence of substantial bile pigments for the redox cycle to operate, we preloaded cells with biliverdin or bilirubin by preincubation with either pigment prior to H2O2 treatment. However, such loading of cells with the pigments also failed to significantly affect H2O2-mediated loss of cell viability (Fig. 4, B and C). Control experiments confirmed that preincubation of cells with biliverdin or bilirubin alone did not significantly affect cell viability (data not shown).

Previous work by Barañano et al. (11) showed that knockdown of BVR led to increased cellular levels of ROS. We therefore examined whether BVR overexpression would decrease cellular ROS, as assessed by dichlorofluorescein diacetate fluorescence. As expected, treatment of control HeLa cells with increasing H2O2 for 30 min caused an increase in fluorescence, indicative of increased cellular levels of ROS (Fig. 5). However, BVR overexpression did not significantly affect fluorescence following H2O2 treatment (Fig. 5), suggesting that increased BVR does not provide cells with additional protection against H2O2-mediated ROS formation.

We next decreased BVR expression in HeLa cells and compared the response of these cells to H2O2 with that of corresponding control cells, transfected with scrambled siRNA. Transfection of cells with three different siRNAs specific to the human BVR gene decreased the expression of BVR protein and BVR activity by ~70% (Fig. 6A), yet this did not exacerbate cell death caused by H2O2, as assessed by the MTT assay (Fig. 6B). Similarly, preloading siRNA-treated cells with biliverdin or bilirubin did not affect H2O2-mediated loss in cell viability (Fig. 6C).

Expression of Human BVR Does Not Increase the Resistance of Yeast against H2O2 Toxicity—It is conceivable that endogenous BVR may confound any potential effect of the approaches used above to modulate BVR activity in HeLa cells, since in most mammalian cells heme degradation by heme oxygenase is tightly coupled with BVR activity (17). In contrast, yeast cells do not contain BVR activity and only possess a single heme oxygenase, encoded for by HMX1 (18, 19). Therefore, we transformed a null hmx1 mutant yeast strain with human HO-1 (heme oxygenase-1) alone (Fig. 7, A and B, lane 1) or together with human BVR (Fig. 7, A and B, lane 2) and then assessed survival in response to H2O2. As expected, and similar to the situation in mammalian cells, overexpression of human HO-1 alone increased survival of the hmx1 null mutant yeast (Fig. 7C). However, when HO-1 and BVR were co-expressed, there was no further increase in survival to that seen with HO-1 overexpression alone (Fig. 7D). Therefore, even in the absence of endogenous BVR, overexpression of BVR does not increase protection against oxidant treatment beyond that provided by heme oxygenase.
Biliverdin Reductase and Cellular Antioxidant Defense

FIGURE 7. Overexpression of human HO-1 but not human BVR rescues the sensitivity of hmx1 mutant yeast to oxidant challenge. Null hmx1 mutant strains were transformed with pESC-LEU-HO-1 and pESC-LEU-HO-1-BVRA. Expression of HO-1 and BVR was induced with the addition of galactose to the growth medium. Cells extracts were prepared as described under “Experimental Procedures” for cDNA synthesis and reverse transcription-PCR (A) and Western blotting (B). In A, the presence of both the HO-1 (row 1) and BVR (row 2) transcript was verified by agarose gel electrophoresis and by sequencing (note that the presence of a band (in row 2) in the hmx1 transformed with pESC-LEU-HO-1 was investigated by sequencing and was confirmed to be a nonspecific sequence (data not shown)). Data are representative of three independent experiments. B, Western blot showing expression of human HO-1 and BVR protein at 32 and 39 kDa, respectively, in yeast cells transformed with pESC-LEU-HO-1 and pESC-LEU-HO-1-BVRA and cultured in the presence of galactose. Null hmx1 mutant yeast cells were transformed with galactose-inducible multicopy plasmids containing human HO-1 (C) or human HO-1 and human BVR (D). Cells were grown to exponential phase in medium containing raffinose (1) or galactose (2) to respectively prevent or induce expression of the gene(s) contained in the multicopy plasmid and treated for 1 h with H2O2 at the concentration indicated. Survival was determined as described under “Experimental Procedures,” and results are expressed relative to non-treated control cultures. Data represent the mean ± S.E. of three independent experiments performed in triplicate.

DISCUSSION

Over the last decade, evidence has emerged, suggesting that BVR possesses important properties in addition to its well established function in heme metabolism. These non-metabolic activities include modulation of cell signaling and kinase activity (20). In addition, BVR has been proposed to play an important role in protecting cells against H2O2-mediated oxidative stress via a redox amplification cycle, involving NADPH-dependent “recycling” of bilirubin subsequent to its oxidation to biliverdin (21). Using a combination of in vitro and cellular studies with varying oxidants and forms of bilirubin, as well as modulation of BVR activity, our studies question the importance of this putative antioxidant redox cycle and hence the role of BVR as a biologically relevant antioxidant.

A central underlying assumption of the redox amplification cycle is that cellular oxidation of bilirubin is specific, yielding considerable if not stoichiometric amounts of biliverdin. Contrary to this assumption, however, biliverdin at best was formed only to a modest extent under the various conditions, including those that caused complete oxidation of bilirubin. Foremost, H2O2 generated by glucose/glucose oxidase was inefficient in oxidizing bilirubin to biliverdin, irrespective of whether the pigment was bound to isolated proteins, added to cell lysates, or presented separately in a water-soluble, conjugated form.

In the model of extracellular (albumin-bound) bilirubin, oxidative conversion to biliverdin was clearly more substantial with peroxyl radicals than H2O2. Similarly, oxidation of GST-bound bilirubin, a model for cell protein-bound pigment, yielded only modest amounts of biliverdin; however, this accumulation was more significant in the presence of H2O2. In contrast, oxidation of the water-soluble bilirubin conjugate by 1e- or 2e-oxidants resulted in minimal biliverdin conjugate, suggesting that protein binding may install a certain strain and conformation that renders the pigment more susceptible to specific oxidation by various oxidants. This notion is supported by the lack of biliverdin accumulation during peroxyl radical-mediated oxidation of bilirubin dissolved in chloroform (Fig. 2A). Together, these findings suggest that disruption of intramolecular hydrogen bonds facilitates oxidant-mediated biliverdin formation. It highlights that membrane-bound bilirubin is unlikely to produce biliverdin upon peroxyl radical oxidation. Also, our model of cellular bilirubin oxidation by peroxyl radicals or H2O2 in the presence of lysates overexpressing BVR suggested that biliverdin formation is the limiting factor in any putative redox cycle.

Bilirubin is now considered an important antioxidant, especially in blood plasma, as demonstrated by many chemical and biochemical in vitro studies. However, these studies have established clearly that not all reactive species effectively oxidize bilirubin and that, when oxidation occurs, it only generates modest levels of biliverdin. For example, superoxide anion radical generated by polymorphonuclear leukocytes does not react with conjugated bilirubin (22), a result comparable with our H2O2-mediated oxidation of conjugated bilirubin (Fig. 1F). However, oxidation of free bilirubin by peroxyl (8) and α-tocopheroxy (23) radicals does occur, but it yields predominantly nonspecific oxidation products. Similarly, early work on the photooxidation of bilirubin, which involves singlet oxygen, indicated that biliverdin was not a major oxidation product (24, 25), and Reed et al. (26) reported biliverdin to account for only about 15% of bilirubin oxidized by microsomes. Our inability to detect biliverdin in some of the reactions probably was not limited by the analytical method used, since our results are also consistent with a recent study where the oxidation of bilirubin was assessed by mass spectrometry (27). That study clearly showed that biliverdin accounted for only 14% of the initial bilirubin, oxidized with H2O2 in the presence of Fe-EDTA or with uncoupled cytochrome P450 (27). The majority of the accumulated products (>40%) in this instance were putative dipyrrole compounds (m/z 299–315), smaller than biliverdin. Furthermore, oxidation of conjugated bilirubin by strong oxidants, such as hypochlorite or peroxynitrite, in aqueous buffer only yields negligible amounts of biliverdin (22, 28). Contrastingly, albumin-bound bilirubin does not react efficiently with hypochlorite, an effect attributed to the reactive amino acids of albumin out-competing bilirubin for this oxidant (29).

Binding of bilirubin to the primary binding site of albumin is thought to involve ion pairing, hydrogen bonding, and π-inter-
actions, thereby fixing the two planar dipyrrrole rings of the pigment in an out-of-plane position (2) and disrupting its intramolecular hydrogen bonds (30). This appears to confer increased reactivity of bilirubin to certain oxidants. In fact, the rate constant for the reaction of albumin-bound bilirubin with peroxyl radicals is ~30 times higher than the rate with unconjugated pigment (8). Conversely, albumin-bound bilirubin acts as an antioxidant against treatment of human plasma by peroxynitrite, but in this case the rate of oxidation is much slower, and interestingly, biliverdin was the major oxidation product detected (28). These studies indicate that biliverdin may be a significant oxidation product of protein-bound bilirubin and highlight the potential antioxidant capacity of biliverdin in vitro. However, support for an in vivo antioxidant activity of bilirubin is mainly indirect. For example, total serum bilirubin inversely relates to cardiovascular disease (31), and subjects with Gilbert’s syndrome, who have elevated serum bilirubin due to defective bilirubin conjugation, have decreased incidence of ischemic heart disease (32). Also, hyperbilirubinemic Gunn rats show decreased plasma lipid peroxidation after exposure to >95% oxygen (33). These studies indicate that bilirubin might act as an extracellular antioxidant, but whether cellular bilirubin reacts with oxidants to generate biliverdin for BVR is not clear.

BVR was described first in 1965 by Singleton and Laster (34) as the catalytic enzyme responsible for the 2e-reduction of biliverdin IXα to bilirubin, and for many years this was its only ascribed function. Recently, however, a role for the enzyme in cell signaling (35) and protection against oxidative stress has been reported (21). The latter role is thought to be based on the BVR-catalyzed redox cycle and supported by the increase in H₂O₂-induced cell death following knockdown of BVR by RNA interference (11, 36). In those studies, H₂O₂-mediated cell death was exacerbated by ~25% with 80–90% BVR knockdown, whereas we observed no effect on H₂O₂-mediated cell death with variations in BVR activity ranging from 30 to 70% of that seen in control cells. Also, BVR overexpression failed to decrease the levels of ROS in H₂O₂-treated cells. Together, our studies do not support the notion that BVR acts as a significant cellular antioxidant via the putative redox cycle. Others have reported depletion of BVR to increase cellular DCF fluorescence (11, 36). However, whether increased DCF fluorescence truly indicates increased ROS remains to be established. This is because H₂O₂ treatment causes apoptosis of cells, and cytochrome c released during apoptosis is well known to give rise to increased DCF fluorescence (37, 38). Therefore, the reported increased DCF fluorescence (11, 36) might not reflect increased ROS but instead apoptosis-associated release of cytochrome c.

The lack of protection seen with either BVR overexpression or BVR knockdown could be interpreted as cells requiring only a low level of BVR for the redox cycle of biliverdin to bilirubin to function optimally. Inconsistent with this interpretation, however, we observed that overexpression of human BVR in yeast cells that do not normally contain endogenous BVR did not alter the H₂O₂ sensitivity.

Our studies do not exclude the possibility that BVR protects cells independent of the proposed redox cycle. This is underlined by a recent study where depletion of BVR attenuated arsenite-mediated apoptosis compared with control cells and, interestingly, HO-1-depleted cells (39). The authors suggested that BVR offers protection against arsenite independent of bile pigments. Indeed, there is increasing evidence that BVR has multiple functions, including a role in cell signaling (40) and as a transcriptional factor (41, 42). It is conceivable that disruption of signaling pathways could trigger apoptosis. It is also worth recalling that regeneration of bilirubin following its reaction with radicals does not necessarily require BVR. For example, during oxygen-dependent free radical reactions in water, bilirubin is regenerated via isomerization, without involvement of BVR or intermediacy of biliverdin (43). However, even if cellular recycling of bilirubin from biliverdin were to take place, it would be expected to occur only secondarily to the action of ascorbate (44) and ubiquinol-10 (23). These first line antioxidants are abundant and ubiquitous (45), effectively compete with bilirubin for radicals (23), and are recycled (46, 47). Therefore, the relative importance of bilirubin as a cellular antioxidant in vivo would be expected to be limited, unless the mentioned first line antioxidants become depleted. Such a situation occurs to at least some extent in cultured cells that are deficient in ascorbate unless the culture medium is supplemented with vitamin C.

In a recent study, Seldak et al. (48) reported knockdown of BVR in human cell lines to increase H₂O₂-mediated lipid peroxidation, supporting the notion that bilirubin acts as an endogenous antioxidant for cellular lipids. Indeed, it is now well established that bilirubin can effectively protect lipids from oxidation (8). However, our present study questions whether, and if so to what extent, this translates into cellular protection against H₂O₂-mediated cell death, as implied by the original bilirubin/biliverdin redox cycle (11). In this context, we note that Seldak et al. reported knockdown of BVR to increase lipid peroxidation in unchallenged cells without observing a significant change in cell death. Conversely, in H₂O₂-exposed cells, knockdown of BVR increased cell death without significantly increasing lipid peroxidation (compare Figs. 3 and 4 in (48)). We interpret these data as further evidence for a dissociation of cellular BVR levels and H₂O₂-induced cell death.

In summary, our studies show that bilirubin oxidation does not generally generate biliverdin specifically and that BVR is not an important cellular protector via the antioxidant redox cycle proposed by Snyder and co-workers (11). However, our studies do not exclude the possibility that BVR has an antiapoptotic activity. Future studies addressing this possibility are clearly warranted.

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Biliverdin Reductase and Cellular Antioxidant Defense

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