Scientific paper

Inhibition of Biliverdin Reductase Diminished the Protective Activity of Bilirubin and Biliverdin Against Oxidative Stress-Induced Injury in Human Vascular Endothelium

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Abstract

Endothelial dysfunction is an important risk factor for the development of cardiovascular diseases, and its condition is further aggravated by oxidative stress. Biliverdin (BV) and bilirubin (BR) are potent antioxidants that protect endothelial cells, with biliverdin reductase (BVR) converting BV to BR to maintain redox balance. This study explored BVR's role in mediating these protective effects under normoxic and hypoxia-reoxygenation conditions. Pharmacological inhibition of BVR reduced the protective effects of BV and BR, as evidenced by the decreased cell viability, cellular antioxidant activity, and intracellular bilirubin levels. Activation of ERK1/2 reduced BVR's protective function, while its inhibition enhanced it. Additionally, disruption of the BVR-ERK interaction further modulated these effects, highlighting BVR-ERK1/2 interaction sites as potential therapeutic targets for oxidative stress-induced endothelial dysfunction.

Keywords: Antioxidant, bilirubin, biliverdin, biliverdin reductase, human endothelium, oxidative stress

1. Introduction

Endothelial dysfunction, characterized by impaired vasodilation, increased oxidative stress, and inflammation, is a critical factor in the development of cardiovascular disorders. These conditions lead to pathologies such as atherosclerosis, hypertension, and myocardial infarction.[1] Importantly, hypoxia-reoxygenation (H/R) injury significantly contributes to endothelial dysfunction, particularly in ischemic heart disease and stroke.[2] During ischemia, reduced oxygen supply leads to the accumulation of metabolic byproducts and reactive oxygen species (ROS) after reperfusion, which are central to the pathophysiology of endothelial dysfunction.[1] These highly reactive molecules cause oxidative damage to lipids, proteins, and DNA, leading to endothelial damage and thereby exacerbating cardio-vascular disease. The key pathophysiological mechanism involves nitric oxide (NO) inactivation due to the superoxide anion reaction with NO to form peroxynitrite (ONOO−), which reduces NO availability, impairs vasodilation, and increases vascular tone.[3] Additionally, oxidative stress activates the NF-κB pathway, promoting pro-inflammatory cytokines, adhesion molecules, and chemokines, leading to leukocyte adhesion and vascular inflammation. Excessive ROS can also induce endothelial cell apoptosis and senescence, thus reducing the regenerative capacity and compromising vascular integrity. In addition, ROS-mediated lipid peroxidation damages endothelial cell membranes and lipoproteins, thereby contributing to advanced atherogenesis[1]. Consequently, mechanisms that mitigate ROS-induced damage are of significant interest for vascular health.[4]

In this regard, endogenous bile pigments such as biliverdin (BV) and bilirubin (BR) have gained attention because of their inherent antioxidant properties.[5,6] Several studies have demonstrated an inverse correlation between bilirubin levels and the incidence of cardiovascular diseases, suggesting that higher bilirubin levels are associated with a reduced risk of conditions such as atherosclerosis

and coronary artery disease.[7] The metabolic pathway involving the conversion of heme to biliverdin and subsequently to bilirubin is facilitated by the enzymes heme oxygenase-1 (HO-1) and biliverdin reductase (BVR), respectively.[8] The addition of both BV and BR to endothelial cells can effectively neutralize ROS, thereby protecting the human endothelium from oxidative damage.[9] Bilirubin, a potent antioxidant, reacts with ROS and is oxidized to biliverdin.[10] BVR converts biliverdin back into bilirubin, thereby establishing an important cyclic antioxidant system.[11,12]

Recent research has elucidated the multifaceted role of BVR, highlighting its function not only as an antioxidant enzyme, but also as a dual-specificity kinase (Ser/Thr/Tyr) that regulates the transcription of inflammatory mediators.[13,14] Furthermore, there is a significant interplay between BVR and the extracellular signal-regulated kinase (ERK) pathway. BVR contains specific motifs known as C-box (cysteine-rich domain) and D-box (docking domain), through which it binds to ERK. In this way, BVR acts as a scaffold for ERK1/2 and modulates its cellular localization and activity within the cell.[15] This influences various cellular processes, including cell survival, proliferation, and inflammation.[16,17] The interaction of BVR with ERK1/2 involves phosphorylation of BVR, which subsequently facilitates the phosphorylation and activation of ERK1/2. Once activated, ERK1/2 translocates to the nucleus, where it modulates the activity of transcription factors such as NF-κB and AP1, and thus influences the expression of genes involved in cell proliferation, differentiation, apoptosis, and cell survival.[10] Additionally, BVR influences glucose metabolism and insulin signaling, contributing to metabolic homeostasis.[18]

Importantly, the knockout of BVR in human aortic endothelial cells leads to increased oxidative stress and endothelial-to-mesenchymal transition, which is characterized by the loss of endothelial markers.[19] Similarly, another study indicated that silencing BVR in human endothelial cells results in increased levels of reactive oxygen and nitrogen species (RONS), and bilirubin, but not biliverdin, increases the expression of the protective protein GTP cyclohydrolase in cells.[20] This indicates that BVR and bilirubin have both direct and indirect antioxidant properties that are important for the protection of endothelial cells.[20] Interestingly, direct intrathecal application of BVR ameliorated the clinical and pathological signs of experimental autoimmune encephalomyelitis more efficiently than traditional antioxidant enzymes, further suggesting a crucial protective role for BVR against cellular oxidative stress.[21]

In this study, we aim to demonstrate that pharmacological inhibition of BVR can directly limit the conversion of biliverdin to bilirubin, leading to decreased bilirubin levels and consequently diminishing its antioxidant protective capacity. Reduced bilirubin levels make endothelial cells more vulnerable to oxidative damage. Our primary goal is to delineate the impact of BVR on the protective activity of biliverdin (BV) and bilirubin (BR) on endothelial function under conditions of increased oxidative stress, both in normoxia and following hypoxia-reoxygenation injury. In addition, our study explored the complex role of BVR beyond its classical enzymatic functions. Recent evidence suggests that BVR may also act as a modulator of the ERK pathway, which is crucial for cellular response to oxidative stress. To specifically explore this modulation, we aim to inhibit the interaction between BVR and ERK by employing antagonists targeting the C-box and D-box binding sites. This approach aims to disrupt BVR's non-classical signaling functions, providing new insights into the dual contributions of its enzymatic activity and signaling pathways under oxidative stress conditions. Including normoxia with induced oxidative stress and hypoxia-reoxygenation (H-R) conditions allowed us to evaluate BVR's function under challenging stress conditions and its critical role during recovery. Under basal conditions, the BVR cycle of converting bilirubin back to biliverdin shows minimal changes upon inhibition, as the demand for antioxidant recovery is low. However, under conditions simulating cardiovascular pathologies where oxidative stress is elevated, efficient recovery of bilirubin through BVR is essential. This study aims to elucidate the role of BVR under these specific conditions.

2. Materials And Methods

2. 1. Chemicals

Bilirubin, biliverdin, apomorphine, chlorpromazine, U-46619, PD-98059, $CaCl_2$, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), methanol (all Sigma Aldrich, Germany); fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Hanks' balanced salt solution (HBSS), HEPES Buffered Saline Solution (BSS), L-glutamine, penicillin-streptomycin solution, and dimethyl sulfoxide (DMSO) (all Merck, Germany); peptide FGFPAFSG, peptide KKRILHCLGLA (both purchased from Biocat GmbH, Heidelberg, Germany).

2. 2. Human Endothelial Cell Culture EA.hy926

All experiments were conducted using the human endothelial cell line EA.hy926. Cells were cultured in DMEM supplemented with 10% heatinactivated fetal bovine serum (FBS), 1 mM L-glutamine, and 1 mM penicillin-streptomycin solution. Cells were maintained at 37 °C in a humidified incubator with 95% air and 5% CO_2. The culture medium was changed every 2-3 days, and the cells were trypsinized using a 0.25% trypsin-EDTA solution for subculturing when confluence reached approximately 70–80%. The cells were then seeded in 96-well plates at a seeding concentration of 1×10^4 cells/well.

2. 3. Experimental Design

In Figure 1, we present a general overview of our experimental design, consisting of four experimental phases, namely i. normoxia (24 h) or hypoxia (15 h) – reoxygenation (9 h); ii. pharmacological protocols that influence BVR activity (incubation with inhibitors, antagonists, and activators); iii. condition of increased oxidative stress (1h incubation with peroxyl-radical initiator); iv. functional cell assays (cell viability, cellular antioxidant assays, cell lysis for HPLC-MS measurements). In general, we aimed to perform three independent biological experiments for each experimental group and cell assay, each consisting of six technical replicates.

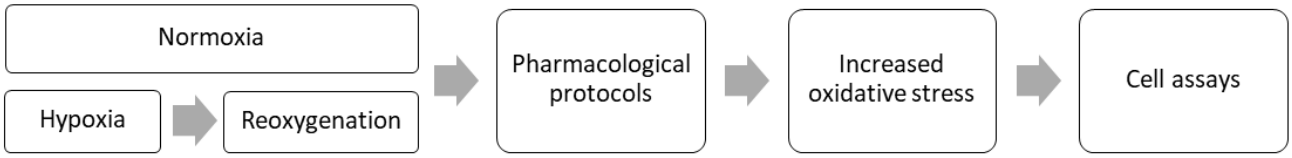

Figure 1. Schematic overview of experimental workflow. In the first phase, cells were introduced to either normoxia or the hypoxia-reoxygenation protocol for 24h, followed by various pharmacological treatments for 1-2h. All the cells were exposed to increased oxidative stress conditions with peroxyl radical initiation. Finally, various cell assays (cell viability, cellular antioxidant assay, and cell lysis for HPLC-MS analysis) were performed.

2. 4. Phase 1. Normoxia and Hypoxia-Reoxygenation

To simulate hypoxia-reoxygenation (H/R) injury, endothelial cells were incubated in a hypoxic chamber with 1% O_2 and 5% CO_2 at 37 °C for 15 h. This was followed by reoxygenation under normoxic conditions (95% air and 5% CO_2) at 37 °C for 9 h, to mimic the pathological conditions of hypoxia-reoxygenation. In parallel, endothelial cells were incubated under normoxic conditions (95% air and 5% CO_2) for 24 h.

2. 5. Phase 2. Pharmacological Protocols Influencing BVR Activity

Inhibition of the Biliverdin Reductase (BVR)

To inhibit BVR, endothelial cells were incubated with 10 μM apomorphine (AP) for 1 h, followed by addition of bilirubin or biliverdin (10 nM and 100 nM) for 30 min. To exclude the role of dopamine receptor involvement in our observed phenomena with apomorphine (apomorphine is also a dopamine agonist), we performed another experimental series with pre-incubation with 10 μM chlorpromazine (CPZ), a non-selective dopamine receptor antagonist that blocks subtypes D_1, D_2, D_3, and D_4 receptors), for 60 min, followed by apomorphine addition at 10 μM for 60 min, followed by the addition of bilirubin and biliverdin (10 nM and 100 nM) for 30 min.

Activation and inhibition of extracellular signal-regulated kinases (ERKs)

To activate ERK1/2, endothelial cells were incubated for 30 min with U-46619 at 1 μM. The cells were then incubated with bilirubin (100 nM) for 30 min. To further investigate the involvement of ERK1/2 in the protective activity of bilirubin, we inhibited ERK1/2 activity in endothelial cells with previously activated ERK1/2 enzymes (using U-46619) by incubating for 30 min with PD98059 at 20 μM, followed by incubation with bilirubin (100 nM) for 30 min.

Inhibition of BVR – ERK interaction using C-BOX and D-BOX inhibitors

The peptides FGFPAFSG (C-Box inhibitor) and KKRIHCLGLLENGTH (D-Box inhibitor) were introduced into endothelial cells using a calcium phosphate transient transfection protocol. FGFPAFSG and KKRILHCLGLA peptides (15 μL at 250 mg/mL dissolved in PBS) were suspended in 50 μL of 2.5 M $CaCl_2$ buffer. Each peptide solution was prepared separately. Then, 500 μL of 2x HEPES Buffered Saline Solution (BSS) was placed in a 15 mL conical tube. The C-box inhibitor/$CaCl_2$ solution and D-box inhibitor/$CaCl_2$ were added dropwise to this tube while stirring. The precipitate was allowed to form at room temperature for 20 min and then spread evenly over the cells along with their medium. The cells were gently shaken to ensure even distribution and incubated at 37 °C with 95% air and 5% CO_2 atmosphere for 16 h. The medium was then removed, the cells were washed twice with PBS, and fresh complete medium was added. Subsequently, the cells were incubated with bilirubin and biliverdin at concentrations of 10 and 100 nM for 30 min.

Incubation with bilirubin and biliverdin

All pharmacological pretreatments ended with incubation with either bilirubin or biliverdin (10 nM and 100 nM) for 30 min. Bilirubin and biliverdin were prepared freshly just prior to the experiment in the dark conditions in the 5 mM DMSO stock solution, which was further diluted into the non-complete DMEM solution for the cell experiments.

2. 6. Phase 3. Increased Oxidative Stress

To simulate increased oxidative stress conditions, endothelial cells in all experimental groups were exposed to the peroxyl-radical initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (ABAP) at 15 μM for 1 h before functional cell assays. ABAP was freshly prepared just

prior to experimental treatment. Endothelial cells in the control group were not exposed to oxidative stress, but continued incubation under normoxic conditions at 37 °C with 95% air and 5% CO_2 atmosphere for 1 h.

2. 7. Phase 4. Functional cell Assays

Cell viability assays

Cell viability was assessed using resazurin (Alamar Blue® reagent; Thermo Fisher, USA) at 37 °C for 3 h. The incubation time was optimized to maximize the signal-to-noise ratio without reaching saturation in preliminary experiments on Ea.hy926 cells. The fluorescence signal was recorded using a fluorescence reader (BioTek Synergy H1 multimode plate reader, Agilent, USA) in the filter detection mode with excitation at 530–570 nm and emission at 580–620 nm. Briefly, resazurin is a blue, non-fluorescent dye that serves as a redox indicator. In viable cells, metabolically active enzymes reduce resazurin to resorufin, a pink highly fluorescent compound. The conversion was directly proportional to the number of viable cells, allowing quantification of cell viability. Cell viability was calculated by comparing the fluorescence intensity of the treated samples with that of the untreated controls (100% viability). The following equation was used:

$$Cell\ viability(\%) = \left(\frac{\text{Fluorescence of treated samples}}{\text{Fluorescence of control samples}}\right) \times 100 \quad (1)$$

Equation 1. Cell viability assessment using resazurin reduction assay with fluorescence readings.

Cellular antioxidant assays

To assess the antioxidant capacity of endothelial cells EA.hy926, we performed a cellular antioxidant assay using the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) and the peroxyl radical-generating reagent 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP), as previously described [9]. Endothelial cells were washed twice with PBS, and then incubated for 30 min with non-complete albumin-free DMEM, supplemented with 1 mM L-glutamine and 50 µM DCFH-DA. This step allows DCFH-DA to permeate cells and be hydrolyzed to non-fluorescent DCFH, which is then oxidized to fluorescent DCF in the presence of reactive oxygen species (ROS). After incubation, the DCFH-DA solution was removed, and cells were washed twice with PBS to eliminate excess probe. The cells were then treated with the peroxyl radical-generating reagent ABAP at a concentration of 5 mM in 100 µL Hank's buffered saline solution (HBSS). The blank wells were filled with HBSS solution without ABAP, serving as a baseline control for the assay. Fluorescence emission was measured every 5 minutes for one hour at 538 nm with excitation at 485 nm using a fluorescence reader (BioTek Synergy H1 multimode plate reader, Agilent, USA). The cellular antioxidant activity (CAA) was calculated as a percentage of the control value using the following equation:

$$\text{CAA units (\%)} = 100 - \left[\left(\frac{\int \text{SA}\, dt}{\int \text{CA}\, dt}\right) \times 100\right] \quad (2)$$

Equation 2. Calculation of cellular antioxidant activity (CAA) of endothelial cells in the experimental group. Where **∫ SA dt** represents the integrated area under the fluorescence readings of the sample with the blank subtracted, measured over time, while **∫ CA dt** stands for the integrated area under the control fluorescence curve (with blank subtracted) over time.

This approach allows the quantification of antioxidant activity within cells by comparing the fluorescence intensity of treated samples against untreated controls. A higher percentage (expressed as CAA units in %) indicates a greater antioxidant capacity, reflecting the improved ability of cells to neutralize oxidative stress induced by ABAP.

Measurement of intracellular bilirubin and biliverdin levels in endothelial cells using HPLC-MS

For this set of experiments, human endothelial cells (EA.hy926) were cultured in 75 cm^2 cell culture flasks to provide sufficient material for bilirubin and biliverdin measurements. These larger culture flasks, as opposed to 96-well plates, were necessary to ensure adequate cell quantities for reliable HPLC-MS analysis. Following the completion of pharmacological treatments, as previously described, the cells were washed twice with PBS to remove residual media, dead cells, and other extracellular components. A 1:1 solution of DMSO and methanol was used to lyse cells. This solvent mixture is effective for the extraction of bilirubin and biliverdin, while preserving their stability. The flasks were gently scraped using Nunc™ Cell Scrapers (Thermo Fisher Scientific, USA) to mechanically harvest the adherent endothelial cells. Mechanical scraping was continued until all endothelial cells were completely detached and collected from the flask surface. All cell lysate samples were transferred into Eppendorf vials, which were then covered with aluminium foil to protect the samples from light exposure that could degrade bilirubin and biliverdin. The samples were stored at –80 **°C** until further analysis.

The intracellular levels of bilirubin and biliverdin were quantified using a validated high-performance liquid chromatography-mass spectrometry (HPLC-MS) method, as described previously[22,23]. We utilized a reverse-phase ultra-high-performance liquid chromatography (UHPLC) system coupled with a mass spectrometer equipped with an electrospray ionization (ESI) source operating in the positive ion mode. The separation was achieved on a Kinetex C18 EVO column (100 × 2.1 mm i.d., 1.7 µm) with a

guard column, employing a gradient elution with 5 mM ammonium formate at pH 3 as solvent A and a mixture of acetonitrile, water, and ammonium formate as solvent B. The flow rate was set at 0.8 mL/min, and the column temperature was maintained at 35°C. Mass spectrometry detection was performed in selected reaction monitoring (SRM) mode, allowing precise quantification of bilirubin and biliverdin. The transitions monitored were m/z 583.2 → 297.2 for biliverdin and m/z 585.2 → 299.0 for bilirubin, ensuring high sensitivity and selectivity. This method has linearity over the concentration range of 0.5–100 nM, with limits of detection and quantitation at 0.1 nM and 0.5 nM, respectively [22].

To ensure accurate and comparable results across samples, the total cellular protein content in each sample was determined using Bradford assay. Bilirubin and biliverdin concentrations were normalized to the total cellular protein content in each sample. This normalization accounts for any variability in cell number or size, allowing for the expression of bilirubin and biliverdin cellular levels in nanomoles per microgram of total protein (nmol/μg).

2. 8. Statistical Analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical differences between groups were evaluated using one-way ANOVA followed by Bonferroni's post-hoc test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3. 1. Inhibition of Biliverdin Reductase Diminished the Protective Activity of Bilirubin and Biliverdin After Increased Oxidative Stress

To investigate the role of biliverdin reductase (BVR) in protecting activity of bilirubin and biliverdin on the endothelium against oxidative stress, we conducted experiments using human endothelial cells (EA.hy926) exposed to varying conditions of oxidative stress and treatments with bilirubin, biliverdin, and the BVR inhibitor apomorphine.

3. 1. 1. Impact of BVR inhibition on cell viability of human endothelial cells

Oxidative stress induced by the peroxyl radical-generating reagent ABAP reduced endothelial cell viability, whereas bilirubin significantly increased cell viability. This increase in cell viability was concentration-dependent, with 100 nM bilirubin providing greater protection than 10 nM, as shown in Figure 2A. Apomorphine, used as a BVR inhibitor, did not decrease cell viability *per se* in control experiments without oxidative stress, indicating that it does not possess inherent cytotoxic effects under normoxic conditions. However, pre-incubation with apomorphine significantly decreased the protective effect of bilirubin, resulting in reduced cell viability compared to the bilirubin-treated groups without apomorphine ($p < 0.05$). This suggests that inhibition of BVR impairs the ability of bilirubin to protect endothelial cells from oxidative damage.

The effects of biliverdin treatment were examined in parallel experiments under normoxic conditions, as shown in Figure 2B. Similar to bilirubin, biliverdin treatment also enhanced cell viability after oxidative stress. The protective effect of biliverdin was concentration-dependent, with 100 nM showing more pronounced protection than 10 nM ($p < 0.05$). Pre-treatment with apomorphine significantly reduced the protective effect of biliverdin, as evidenced by decreased cell viability compared to the biliverdin-treated groups without apomorphine ($p < 0.05$), indicating that BVR inhibition also diminishes the protective capabilities of biliverdin.

To simulate more severe stress conditions, endothelial cells were subjected to hypoxia (15 h), followed by reoxygenation (9 h) before bilirubin treatment, as shown in Figure 2C. The injury induced by the pre-treatment protocol of hypoxia-reoxygenation was significantly greater than that under normoxic conditions, as evidenced by lower baseline cell viability in the oxidative stress-only group (group ABAP). Despite increased oxidative stress, bilirubin at both 10 nM and 100 nM concentrations maintained its protective role, enhancing cell viability compared to untreated controls ($p < 0.05$). Consistent with previous findings, apomorphine pre-treatment significantly reduced the protective effect of bilirubin, leading to decreased cell viability compared to that in the bilirubin-only treated groups ($p < 0.05$). Similar experiments were conducted with biliverdin treatment following hypoxia-reoxygenation injury (Figure 2D). Biliverdin demonstrated protective properties, increasing cell viability at 10 nM and 100 nM in a concentration-dependent manner under severe oxidative stress conditions. Pre-incubation with apomorphine diminished the protective effects of biliverdin, as shown by reduced cell viability in comparison to biliverdin-treated groups without apomorphine ($p < 0.05$).

3. 1. 2. Impact of BVR Inhibition on Cellular Antioxidant Activity (CAA) in Human Endothelial Cells

The effect of biliverdin reductase (BVR) inhibition on cellular antioxidant activity (CAA) in human endothelial cells was systematically examined under normoxic and hypoxia-reoxygenation conditions. These findings are depicted in Figure 3, with panels A and B corresponding to normoxic conditions and panels C and D to hypoxia-reoxygenation conditions. We observed that both bilirubin and

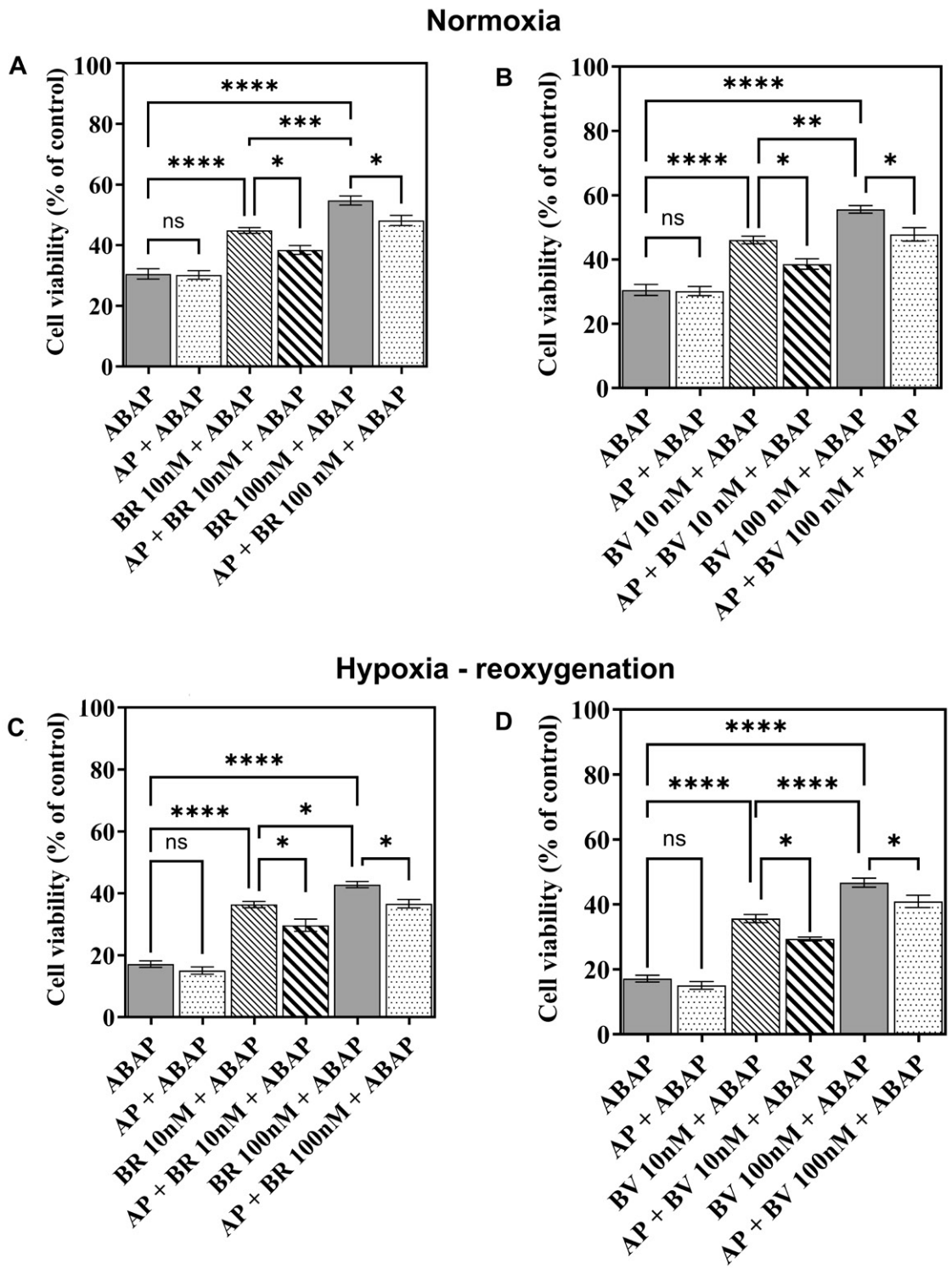

Figure 2. Assessment of cell viability of human endothelial cells EA.hy926 under various treatment conditions. This figure presents the impact of biliverdin reductase (BVR) inhibition on the protective effects of bilirubin and biliverdin against oxidative stress in EA.hy926 cells. Experimental groups included: (1) ABAP, where cells were subjected only to oxidative stress induced by 15 μM ABAP; (2) AP + ABAP, inhibition of BVR with 10 μM apomorphine (AP) prior to oxidative stress; (3) BR + ABAP, pre-treatment with bilirubin (10 nM or 100 nM) prior to oxidative stress; (4) AP + BR + ABAP, inhibition of BVR activity with 10 μM AP, followed by bilirubin (10 nM or 100 nM) pre-treatment prior to oxidative stress; (5) BV + ABAP, pre-treatment with biliverdin (10 nM or 100 nM) prior to oxidative stress; and (6) AP + BR + ABAP, inhibition of BVR activity with 10 μM AP, followed by biliverdin (10 nM or 100 nM) pre-treatment before oxidative stress. Cell viability was quantified and expressed as a percentage of control group viability, which was set as 100%. Data are presented as mean ± SEM. Each data point represents the mean of three independent experiments, each consisting of six technical replicates. Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test to determine significance (*$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

biliverdin exhibited slightly higher antioxidant activities under normoxic conditions than under hypoxic conditions.

Under normoxic conditions, the inhibition of BVR by apomorphine significantly reduced the CAA of both bilirubin and biliverdin. Specifically, Figure 3A shows that the antioxidant activity of bilirubin notably diminished when BVR activity was inhibited. A similar reduction was observed for biliverdin (Figure 3B), indicating that the antioxidative capacity of both bile pigments is critically dependent on BVR activity.

When the experiment was extended to hypoxia-reoxygenation conditions, a comparable pattern was observed. As shown in Figure 3C, the CAA of bilirubin was

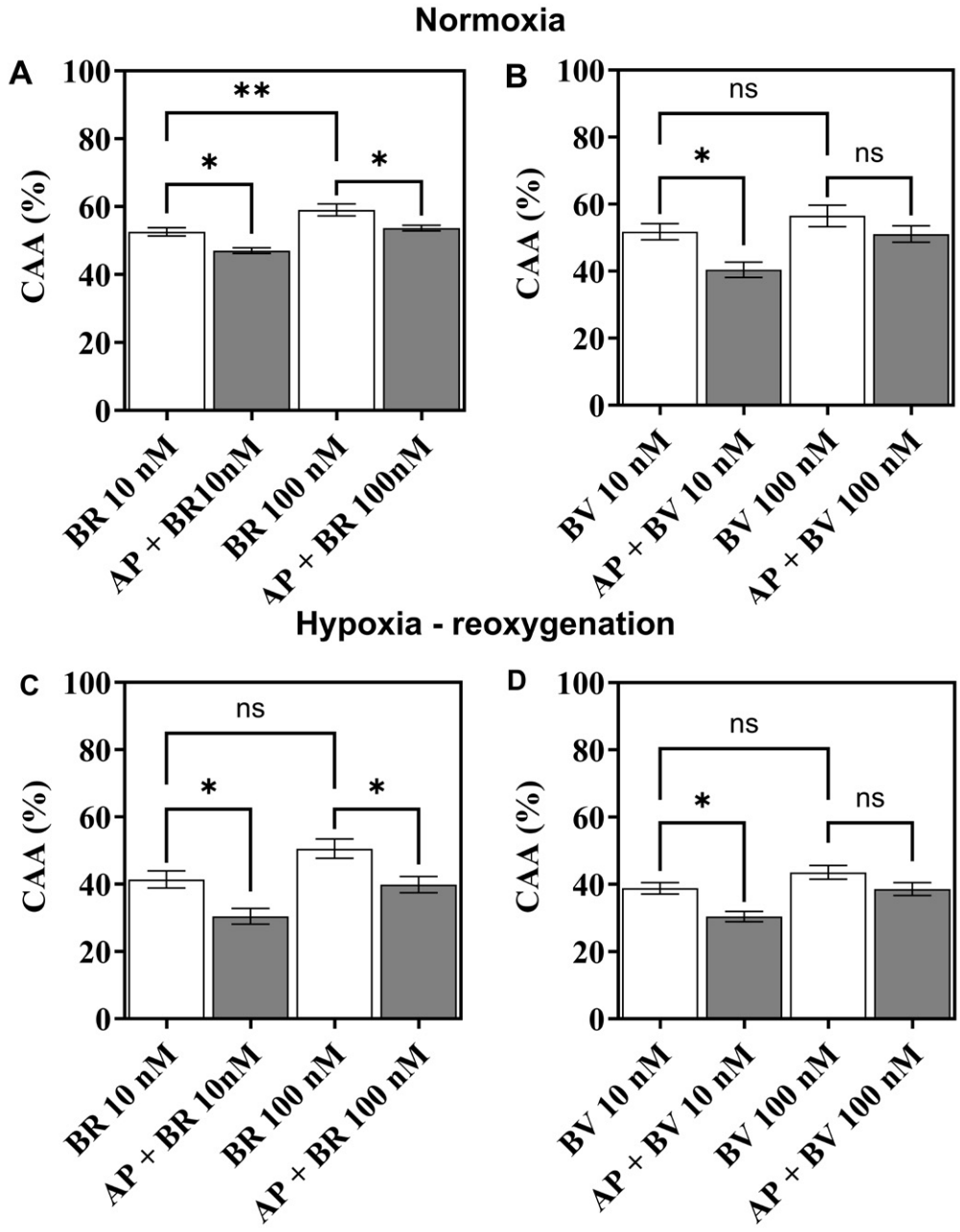

Figure 3. Assessment of cellular antioxidant activity in human endothelial cells EA.hy926 under various treatment conditions. This figure presents the effects of biliverdin reductase (BVR) inhibition on the cellular antioxidant activities of bilirubin and biliverdin in human endothelial cells EA.hy926. Experimental groups included: (1) BR, cells treated with bilirubin (10 nM or 100nM); (2) AP + BR, cells pre-incubated with 10 μM apomorphine (BVR inhibitor) followed by treatment with 10 nM or 100 nM bilirubin; and (3) AP + BV, cells were pre-incubated with 10 μM apomorphine, followed by treatment with 10 nM or 100 nM biliverdin. Following these treatments, the cellular antioxidant activity (CAA) was measured. The results are presented as the mean ± SEM. Each data point represents the average of three independent experiments, each consisting of six technical replicates. Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test to determine significance. Labels indicate: ns, non-significant, *$p \leq 0.05$, **$p \leq 0.01$.

reduced following BVR inhibition, mirroring findings under normoxic conditions. Similarly, Figure 3D shows a reduction in the antioxidant activity of biliverdin when BVR was inhibited during hypoxia-reoxygenation. An interesting exception was observed when 100 nM biliverdin was used in conjunction with apomorphine under hypoxia-reoxygenation conditions. In this case, the effect of BVR inhibition on CAA was not statistically significant, suggesting a potential differential response of biliverdin at this concentration.

3. 1. 3. Effects of BVR Inhibition on Cellular Bilirubin and Biliverdin Levels in Human Endothelial Cells

The impact of increased oxidative stress induced by ABAP, both under normoxic conditions and following hypoxia-reoxygenation, as well as the inhibition of biliverdin reductase (BVR), on cellular bilirubin and biliverdin levels in EA.hy926 cells is presented in Figure 4.

Under normoxic conditions, ABAP exposure significantly decreased cellular bilirubin levels (Figure 4A), with a similar reduction observed in biliverdin levels (Figure 4B). When bilirubin was added (100 nM), cellular bilirubin levels increased with minimal changes in biliverdin levels. In contrast, addition of biliverdin (100 nM) elevated both biliverdin and bilirubin levels. BVR inhibition by apomorphine slightly decreased cellular bilirubin levels when bilirubin was added, whereas biliverdin levels remained unchanged. In the presence of biliverdin and BVR inhibition, bilirubin levels decreased; however, biliverdin levels were unaffected.

Following hypoxia-reoxygenation, ABAP further decreased bilirubin levels, showing a threefold reduction

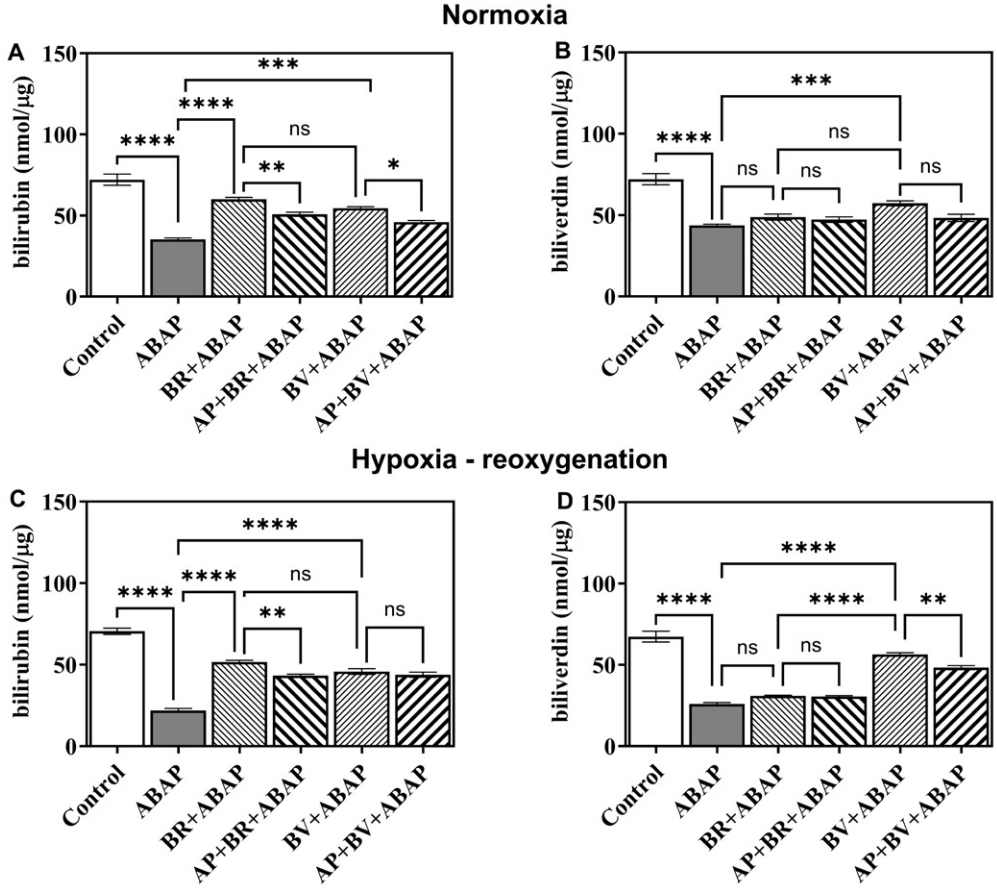

Figure 4. Measurement of bilirubin and biliverdin levels in human endothelial cells EA.hy926 using HPLC-MS. The experiments were performed using normoxic (panels A and B) and hypoxia-reoxygenation protocols (panels C and D). Results are presented as cellular bilirubin (panels A and C) or cellular biliverdin (panels B and D) levels normalized to the total quantity of cellular proteins (nmol/µg). Experimental groups included: (1) control group, in which cells were not exposed to any experimental protocol; (2) ABAP, oxidative stress induced by 15 µM ABAP; (3) BR + ABAP, cells treated with 100 nM bilirubin (BR) prior to oxidative stress (15 µM ABAP); (4) AP+BR+ABAP, cells pre-incubated with 10 µM apomorphine (AP), then pre-treated with 100 nM bilirubin (BR) before oxidative stress (15 µM ABAP); (5) BV + ABAP, cells treated with 100 nM biliverdin (BV) before oxidative stress (15 µM ABAP); and (6) AP+BR+ABAP, cells pre-incubated with 10 µM apomorphine (AP), then pre-treated with 100 nM biliverdin (BV) before oxidative stress (15 µM ABAP). Results are expressed as mean ± SEM. Each data point represents the mean of three independent experiments, each consisting of six technical replicates. Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test to determine significance. Labels indicate: ns, non-significant, *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

compared to controls (Figure 4C) and similarly reduced biliverdin levels (Figure 4D). Pre-incubation with 100 nM bilirubin significantly increased cellular bilirubin levels with a marginal increase in biliverdin levels. Conversely, biliverdin incubation raised bilirubin levels more than biliverdin itself. BVR inhibition caused a significant reduction in bilirubin levels after the addition of either bilirubin or biliverdin, with a minimal impact on biliverdin levels.

3. 2. Primary Mechanism of Apomorphine Involvement in Diminished Bilirubin and Biliverdin Protective Activity is Via Inhibition of BVR and Not Via Agonistic Activity on Dopamine Receptors in Endothelium

To assess whether the effects of apomorphine, a dopamine receptor agonist, on the protective activities of bilirubin and biliverdin were mediated by dopamine recep-

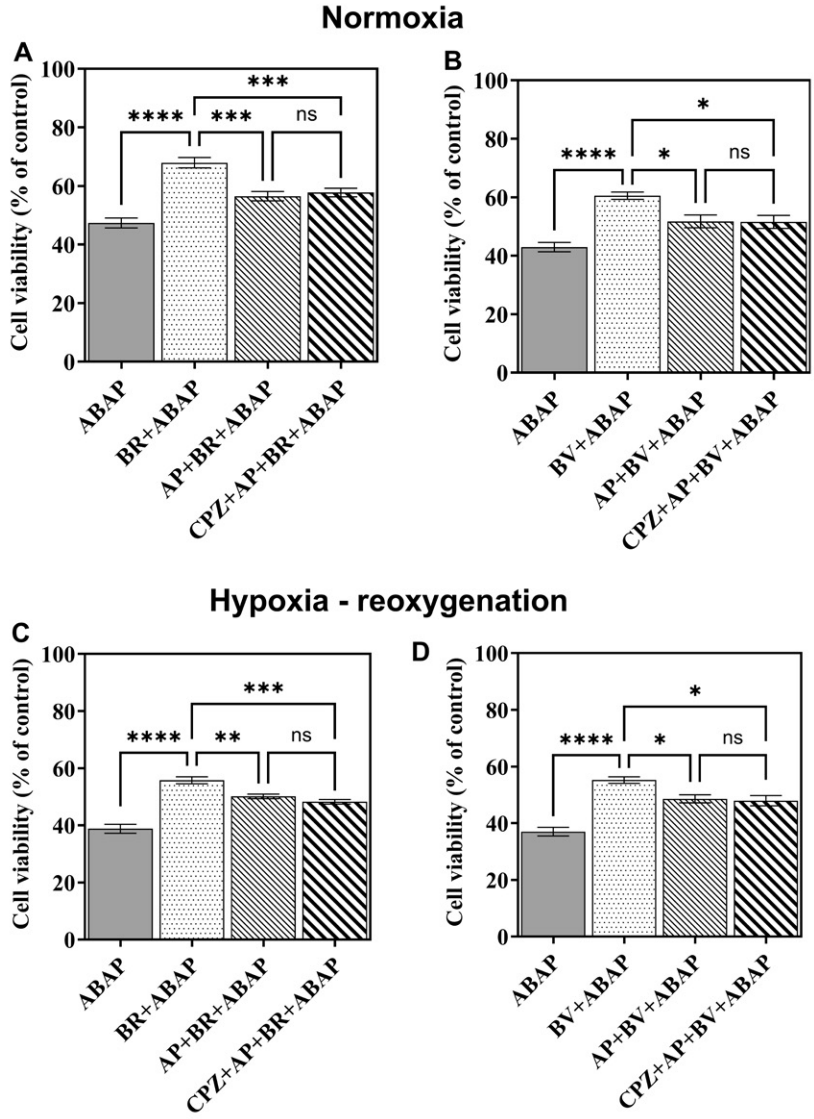

Figure 5. Evaluation of dopamine receptor involvement in the modulation of bilirubin and biliverdin protective activities by apomorphine in human endothelial cells EA.hy926. The results presented in this figure show that dopamine receptors, blocked by the dopamine receptor antagonist chlorpromazine, do not influence the effects of apomorphine on the protective activities of bilirubin and biliverdin under oxidative stress conditions. Experiments were performed under normoxic (panels A and B) and hypoxia-reoxygenation protocols (panels C and D). Experimental groups included: (1) ABAP – oxidative stress induced by 15 μM ABAP; (2) BR + ABAP, cells treated with 100 nM bilirubin (BR) before oxidative stress (15 μM ABAP); (3) AP+BR+ABAP, cells pre-incubated with 10 μM apomorphine (AP), a BVR inhibitor, followed by treatment with 100 nM bilirubin (BR) before oxidative stress (15 μM ABAP); (4) CPZ+AP+BR+ABAP, cells were pre-treated with 10 μM chlorpromazine (CPZ), followed by 10 μM apomorphine (AP), then cells were treated with 100 nM bilirubin (BR) before oxidative stress (15 μM ABAP). Similar experiments were performed using 100 nM biliverdin (BV), as shown in panels B and D. Data are presented as mean ± SEM. Each data point represents the mean of three independent experiments, each consisting of six technical replicates. Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test to determine statistical significance. Labels indicate: ns, non-significant, *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

tors, we conducted additional experiments using chlorpromazine, a non-selective dopamine receptor antagonist (Figure 5). Pre-incubation with chlorpromazine did not alter the observed effects of apomorphine on cell viability in either the bilirubin (Figure 5A) or biliverdin (Figure 5B) treatment groups under normoxic and hypoxia-reoxygenation conditions. The reduction in protective activity observed with apomorphine was consistent, regardless of chlorpromazine pre-treatment, indicating that dopamine receptors were not involved in mediating the

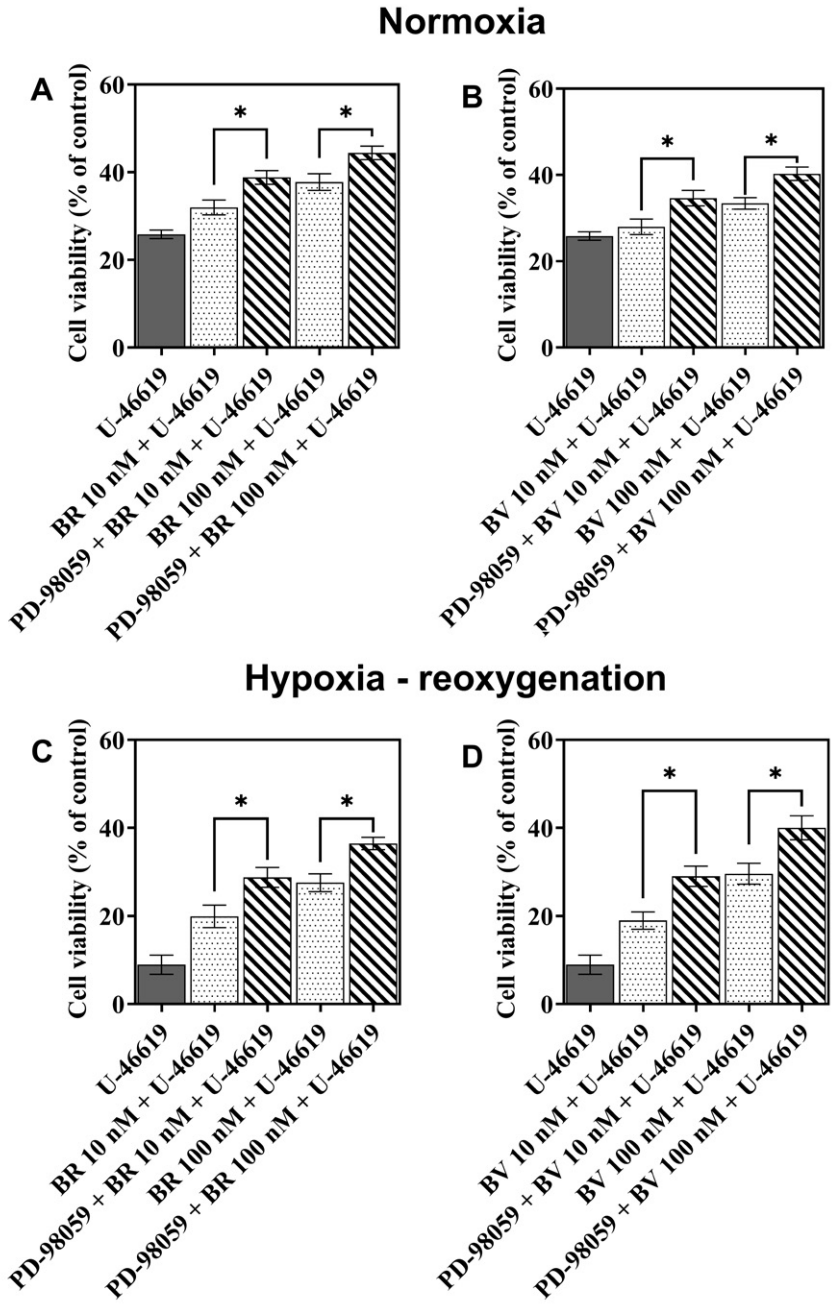

Figure 6. Assessment of ERK1/2 kinase modulation on the protective activity of bilirubin and biliverdin against oxidative stress in human endothelial cells EA.hy926. Experiments were conducted under normoxia (panels A and B) and hypoxia-reoxygenation conditions (panels C and D). The experimental setup involved the initial activation of ERK1/2 kinases using pretreatment with 1 μM U46619, followed by different treatments, and subsequent cell viability assay. Experimental groups included: (1) U-46619 alone, for baseline activation of ERK1/2 kinases; (2) BR + U-46619, where cells were treated with bilirubin (BR) (10 nM or 100 nM) after initial ERK1/2 activation (1 μM U-46619); (3) PD-98059 + BR + U46619, inhibition of ERK kinases by 20 μM PD-98059 after U-46619 pre-treatment, then followed by bilirubin (10 nM or 100 nM). Similar experiments were performed with 10 and 100 nM biliverdin (BV) under normoxia (panel B) and hypoxia-reoxygenation (panel D). Data are presented as mean ± SEM. Each data point represents the mean of three independent experiments, each consisting of six technical replicates. Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test to determine significance. Labels indicate: ns, non-significant, *p ≤ 0.05.

effects of apomorphine. These findings confirm that the diminished protective effects of both bilirubin and biliverdin in the presence of apomorphine are due to the inhibition of BVR as a primary pharmacological mechanism rather than any agonistic activity of apomorphine on dopamine receptors located on the endothelial cell surface. In addition, the effect of apomorphine was evaluated following hypoxia-reoxygenation injury (Figure 5C for bilirubin and Figure 5D for biliverdin). In both scenarios, the presence of chlorpromazine did not influence the effect of

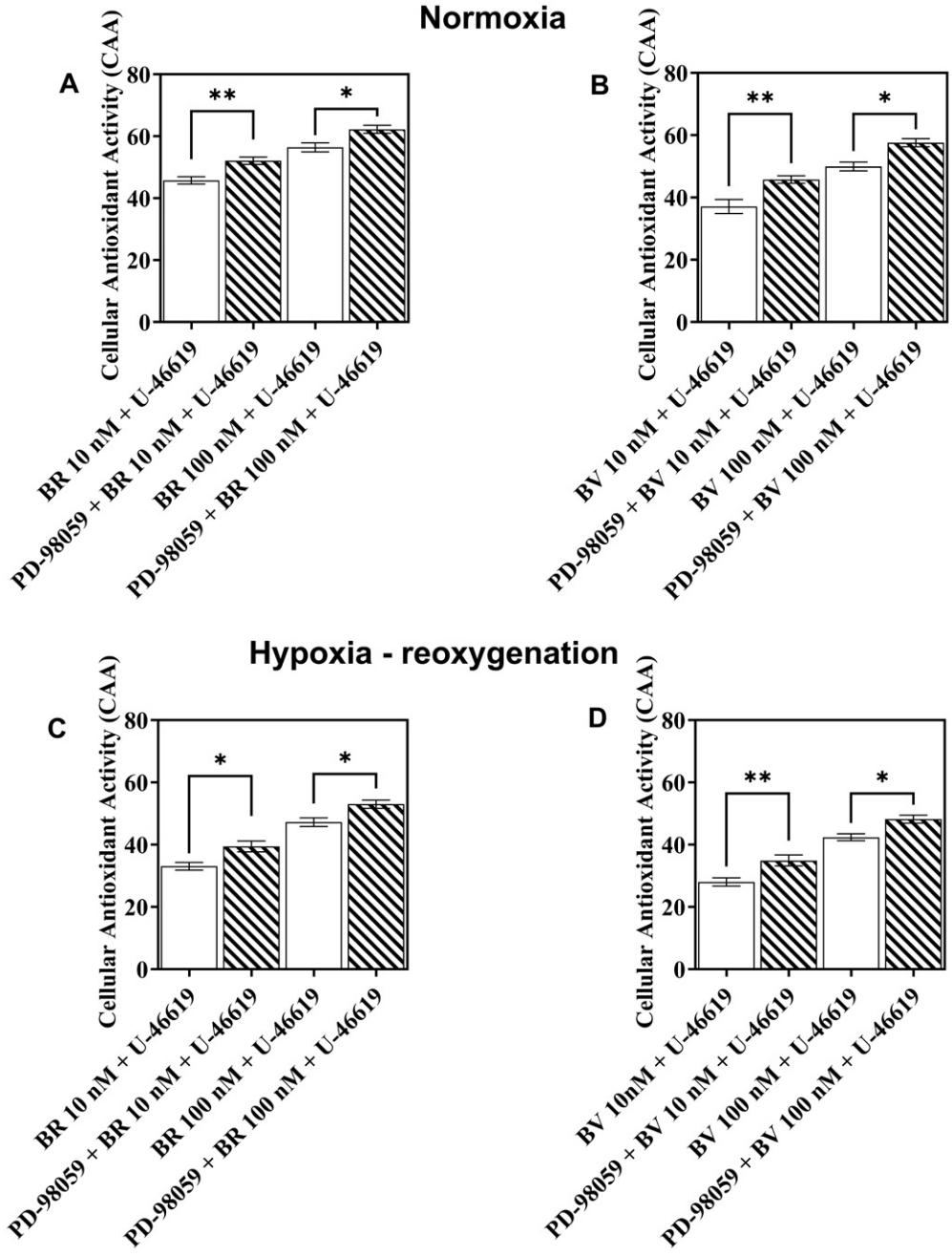

Figure 7. Evaluation of ERK1/2 kinase modulation on the cellular antioxidant activity (CAA) of bilirubin and biliverdin in human endothelial cells EA.hy926. Experiments were conducted under normoxia (panels A and B) and hypoxia-reoxygenation conditions (panels C and D). The experimental protocol involved the initial activation of ERK1/2 (by 1 μM U-46619 pre-treatment), followed by different treatments, and a final CAA assay to measure the cellular antioxidant activity of bilirubin and biliverdin. Experimental groups included: (1) BR + U46619, where cells were treated with bilirubin (10 nM or 100 nM) after ERK1/2 activation by 1 μM U-46619; (2) PD-98059 + BR + U46619, inhibition of ERK kinases by 20 μM PD-98059 after 1 μM U-46619 pre-treatment, then followed by bilirubin (10 nM or 100 nM). Similar experiments were performed with 10 and 100 nM biliverdin (BV) under normoxia (panel B) and hypoxia-reoxygenation (panel D). Data are presented as mean ± SEM. Each data point represents the average of three independent experiments, each consisting of six technical replicates. Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test to determine significance. Labels indicate: *$p \leq 0.05$, **$p \leq 0.01$.

apomorphine, further supporting the role of BVR inhibition as the primary mechanism of apomorphine.

3. 3. Activation or Inhibition of ERK1/2 Kinases Influences Bilirubin and Biliverdin Protective Activity

The effects of ERK1/2 kinase modulation on the protective activity of bilirubin and biliverdin on endothelial cell viability are shown in Figure 6. Activation of ERK1/2 by U-46619 under normoxic conditions before oxidative stress injury significantly reduced cell viability. However, both bilirubin (Figure 6A) and biliverdin (Figure 6B) improved cell viability in a concentration-dependent manner, that is 100 nM provided greater protection than 10 nM did. Importantly, inhibition of pre-activated ERK1/2 with PD-98059 enhanced the protective effects of both bilirubin and biliverdin, as evi-

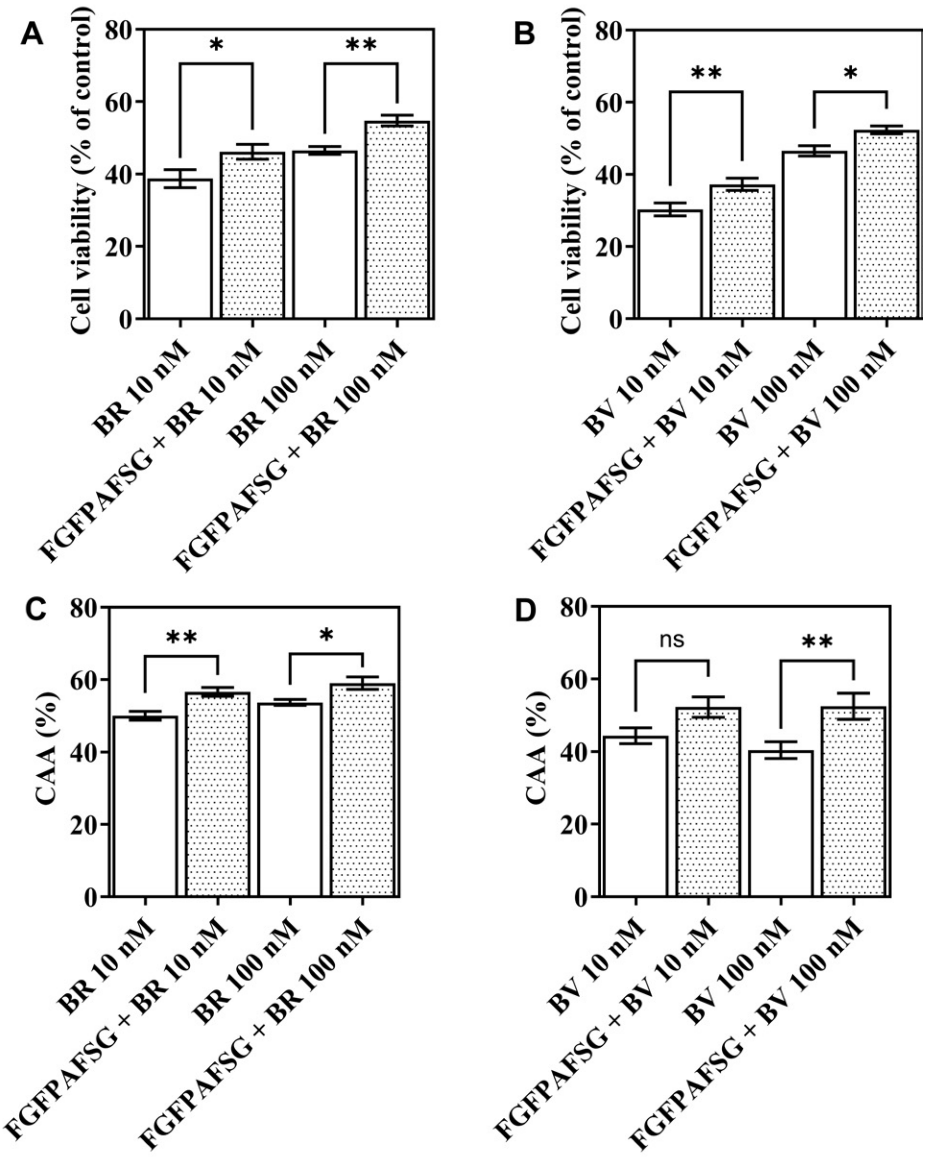

Figure 8. Assessment of the inhibition of BVR interaction with ERK1/2 kinases by C-Box inhibitor (peptide FGFPAFSG) on cell viability and cellular antioxidant activity in human endothelial cells EA.hy926. This figure depicts the effects of inhibiting BVR's interaction with ERK1/2 kinases using a CBox inhibitor, peptide FGFPAFSG, under normoxic conditions. Panels A and B show the effects on cell viability, and panels C and D show effects on cellular antioxidant activity with bilirubin and biliverdin, respectively. Experimental groups included: (1) BR, bilirubin (BR) treatment alone (10 nM and 100 nM) followed by ABAP-induced oxidative stress; (2) BV, biliverdin (BV) treatment alone (10 nM and 100 nM) followed by ABAP-induced oxidative stress; (3) FGFPAFSG + BR, pre-treatment with C-Box inhibitor, followed by bilirubin (BR) incubation (10 nM and 100 nM), and then followed by ABAP-induced oxidative stress; (4) FGFPAFSG + BV, pre-treatment with C-Box inhibitor, followed by biliverdin (BV) incubation (10 nM and 100 nM), and then followed by ABAP-induced oxidative stress. Data are presented as mean ± SEM. Each data point represents the mean of three independent experiments, each consisting of six technical replicates. Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test to determine significance. Labels indicate: ns, non-significant, *$p \leq 0.05$, **$p \leq 0.01$.

denced by the increased cell viability (Figure 6A and 6B).

Under hypoxia-reoxygenation conditions (Figures 6C and 6D), the negative impact of U-46619 on cell viability was even more pronounced, showing a 2.5-fold greater reduction in viability compared to normoxic conditions. In contrast, the inhibition of ERK1/2 by PD-98059 markedly increased the protective efficacy of bilirubin (Figure 6C) and biliverdin (Figure 6D), resulting in significantly higher cell viability.

Furthermore, inhibition of ERK1/2 with PD-98059 significantly increased the cellular antioxidant activity of both bilirubin (Figure 7A) and biliverdin (Figure 7B) under normoxic conditions. Under hypoxia-reoxygenation conditions, the inhibition of ERK1/2 with PD-98059 also resulted in a marked increase in the antioxidant activity of both bilirubin (Figure 7C) and biliverdin (Figure 7D).

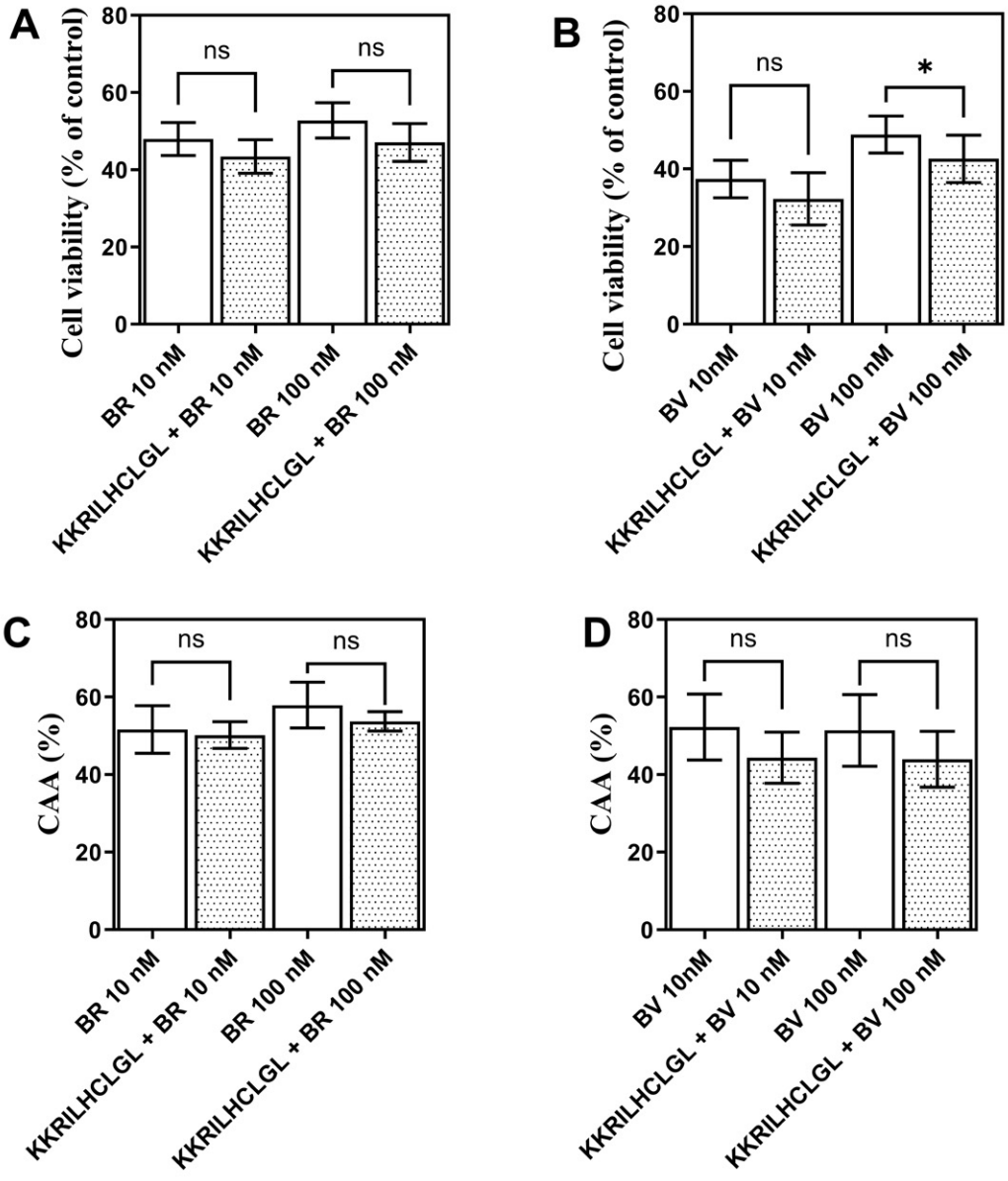

Figure 9. Assessment of the inhibition of BVR interaction with ERK1/2 kinases by D-Box inhibitor KKRILHCLGLA (D-Box inhibitor) on cell viability and cellular antioxidant activity in human endothelial cells EA.hy926. This figure depicts the effect of inhibiting the interaction between BVR and ERK1/2 kinases using the D-Box inhibitor KRILHCLGLA under normoxic conditions. Panels A and B show the effects on cell viability, and panels C and D show effects on cellular antioxidant activity with bilirubin and biliverdin, respectively. Experimental groups included: (1) BR, bilirubin (BR) treatment alone (10 nM and 100 nM) followed by ABAP-induced oxidative stress; (2) BV, biliverdin (BV) treatment alone (10 nM and 100 nM) followed by ABAP-induced oxidative stress; (3) KKRILHCLGL + BR, pre-treatment with D-Box inhibitor, followed by bilirubin (BR) incubation (10 nM and 100 nM) and then followed by ABAP-induced oxidative stress; (4) KKRILHCLG + BV, pre-treatment with D-Box inhibitor, followed by biliverdin (BV) incubation (10 nM and 100 nM), and then followed by ABAP-induced oxidative stress. Data are presented as mean ± SEM. Each data point represents the average of three independent experiments, each consisting of six technical replicates. Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test. Labels indicate: ns, non-significant, *$p \leq 0.05$.

3. 4. Inhibition of BVR Interaction with ERK1/2 Kinases Enhances Bilirubin and Biliverdin Protective Activity

To study the influence of disruption of the interaction between biliverdin reductase (BVR) and ERK1/2 kinases on the protective activity of bilirubin and biliverdin, we used C-Box and D-Box inhibitors under normoxic conditions.

Inhibition of the BVR-ERK interaction via a C-Box inhibitor (FGFPAFSG peptide) resulted in a modest enhancement of the protective effects of bilirubin and biliverdin. Specifically, the C-Box inhibitor slightly increased cell viability in the presence of bilirubin (Figure 8A) and biliverdin (Figure 8B). Similarly, a minor elevation in cellular antioxidant activity was observed when the C-Box inhibitor was applied, as shown in Figure 8C for bilirubin and Figure 8D for biliverdin. Although these effects were statistically significant, they were relatively small, indicating a subtle contribution of the C-Box interaction in modulating the cytoprotective functions of these bile pigments.

Conversely, the D-Box inhibitor (KRILHCLGLA peptide) did not significantly affect the protective activity of bilirubin or biliverdin, as demonstrated by the lack of change in both cell viability (Figure 9A for bilirubin, Figure 9B for biliverdin) and cellular antioxidant activity (Figure 9C for bilirubin, Figure 9D for biliverdin). These findings suggest that the D-Box interaction between BVR and ERK1/2 kinases does not play a major role in the modulation of the protective mechanisms of bilirubin and biliverdin under the conditions tested.

4. Discussion

Our study provides compelling evidence for the critical role of biliverdin reductase (BVR) in mediating the protective effects of bilirubin and biliverdin against oxidative stress-induced injury in human vascular endothelial cells. Our findings demonstrate that the inhibition of BVR, particularly through the pharmacological agent apomorphine, significantly diminishes the cytoprotective and antioxidant activities of these bile pigments. Moreover, we elucidated the intricate interplay between BVR and the extracellular signal-regulated kinase (ERK1/2) pathway, highlighting how ERK1/2 activation modulates the efficacy of BVR-mediated protection.

Role of biliverdin reductase in endothelial protection conferred by bilirubin and biliverdin

Biliverdin reductase (BVR) is a critical enzyme in the heme catabolic pathway that catalyzes the reduction of biliverdin to bilirubin. This conversion is not merely a metabolic step; it represents a key aspect of the cellular antioxidant defence system.[24] Bilirubin, a product of BVR activity, is a potent scavenger of reactive oxygen species (ROS), providing robust protection against oxidative damage in various cell types, including endothelial cells.[9] Bilirubin is oxidized to biliverdin, and BVR converts biliverdin back to bilirubin.[6] In case of high and intense oxidative stress, some bilirubin is lost and converted to bilirubin oxidizing products (BOXes).[25] Given that BOXes are vasoactive and capable of inducing significant vasoconstriction while further exacerbating oxidative stress,[26] it is crucial that biliverdin is efficiently recycled back into bilirubin under conditions of increased oxidative stress. Thus, BVR's contribution to the antioxidant defence is to ensure the availability of bilirubin, which can neutralize ROS and thus protect cellular components from oxidative damage. This cyclic mechanism is crucial for sustaining redox homeostasis within endothelial cells, particularly under conditions of increased oxidative stress, such as those encountered during ischemia-reperfusion injury.

Our results demonstrated that inhibition of BVR using apomorphine led to a significant reduction in both cell viability and cellular antioxidant activity. Recently, studies have begun to identify potential pharmacological tools for inhibiting BVR. For instance, screening of 1,280 FDA-approved compounds *in vitro* led to the identification of 26 compounds as BVR inhibitors.[27] Similarly, another study identified 20 inhibitors from a screening of 1,496 compounds.[28] To rule out the dopamine agonist activity of apomorphine, we pre-incubated samples with the non-selective dopamine receptor antagonist chlorpromazine. Our results showed that chlorpromazine, which effectively blocked dopamine receptors, did not reduce apomorphine activity. This suggests that the observed reduction in bilirubin and biliverdin's protective effects is attributable to apomorphine's role as a BVR inhibitor rather than its agonist activity on dopamine receptors. Our findings underscore the essential role of BVR in maintaining endothelial cell integrity under oxidative stress conditions. The observed reduction in cell viability following BVR inhibition was due to the decreased availability of bilirubin, impairing the cell's capacity to neutralize ROS-induced damage. Our experiments confirmed that intracellular bilirubin levels in endothelial cells were significantly reduced when BVR was inhibited with apomorphine compared to the control groups. Notably, we observed this reduction only under oxidative stress conditions, in both normoxic and hypoxia-reoxygenation pretreatments. However, under normal, non-stressed conditions (normoxia and without induced oxidative stress with ABAP), apomorphine incubation did not lead to significant changes in intracellular bilirubin or biliverdin levels. We also observed that the addition of biliverdin led to an increase in intracellular bilirubin levels, which could be explained by the rapid conversion of biliverdin to bilirubin by BVR. The data on intracellular bilirubin levels were consistent with the

findings from cell viability and cellular antioxidant activity (CAA) assays; higher intracellular bilirubin levels were associated with improved cell viability after oxidative stress and with improved intracellular antioxidant properties.

Our experimental findings align with previous research highlighting the protective role of BVR in various cellular contexts. For instance, BVR deficiency in human endothelial cells has been shown to increase oxidative stress and promote endothelial-to-mesenchymal transition, a process associated with vascular pathology.[19] Similarly, in human endothelial cells (HUVECs) with silenced BVR expression, there was a notable rise in reactive oxygen and nitrogen species. Furthermore, a study on cyanobacteria demonstrated that inactivation of BVR resulted in higher ROS levels compared to cells with intact BVR activity.[29] From a pharmacological point of view, one study showed that atorvastatin significantly upregulated BVR expression and increased BVR protein levels in the parietal cortex.[30] This suggests that some protective drugs that increase the cellular bioavailability of bilirubin might act as inducers of BVR. However, the research field of increasing BVR levels in the endothelium has not yet been fully addressed. In conclusion, our study, along with several others, emphasizes the protective role of BVR in preventing oxidative damage and preserving endothelial function.

Impact of ERK1/2 Modulation on BVR Activity

The interaction between biliverdin reductase (BVR) and extracellular signal-regulated kinase 1/2 (ERK1/2) represents a significant intersection of cellular signaling pathways that govern both the oxidative stress response and cell survival mechanisms. In addition to its well-characterized role in the conversion of biliverdin to bilirubin, BVR also functions as a scaffold protein for ERK1/2 kinase and acts as a nuclear transporter of ERK,[31] thereby influencing its activity and downstream signaling cascades. This dual role of BVR places it at a critical juncture, where metabolic and signaling pathways converge, impacting the overall cellular response to oxidative stress. Interestingly, several cellular stressor factors, including mitogens, cytokines, free radicals, and insulin, that activate MAPK also activate BVR.[16]

Our study elucidated the modulatory effects of ERK1/2 on BVR activity and its subsequent impact on the protective functions of bilirubin and biliverdin in endothelial cells. Specifically, activation of the ERK1/2 signaling pathway by the pharmacological agent U-46619 significantly impaired the cytoprotective effects of both bilirubin and biliverdin, as evidenced by reduced cell viability and antioxidant activity. We used U-46619, a thromboxane A2 mimic, to activate the ERK1/2 pathway. It acts primarily through the thromboxane receptor, leading to the activation of G-proteins, which subsequently activate the MAPK/ERK pathway. These results were further emphasized under hypoxia-reoxygenation pre-treatment. We speculate that ERK1/2 activation compromises the ability of BVR to maintain adequate bilirubin levels, thereby diminishing its antioxidative capacity under stress conditions. The mechanistic basis for this impairment involves phosphorylation of BVR, which is facilitated by its interaction with ERK1/2. BVR contains specific docking sites, such as the C-box and D-box motifs, that enable its binding to ERK1/2.[16] Once bound, ERK1/2 can phosphorylate BVR,[16] potentially altering its enzymatic activity and its ability to convert biliverdin to bilirubin. We can hypothesize further that this phosphorylation event may also influence the subcellular localization of BVR, thereby impacting its accessibility to biliverdin and its role in the cellular antioxidant defence system. Furthermore, the role of phosphorylation in BVR's function extends beyond simple enzymatic modulation. As a scaffold protein, BVR facilitates the assembly of signaling complexes that include ERK1/2 and other kinases, thereby influencing the broader cellular response to stress.[16] The phosphorylation of BVR by ERK1/2 may disrupt these scaffolding functions, leading to altered signaling outcomes that compromise cell survival pathways. This disruption can manifest as reduced activation of protective pathways, such as those mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or activator protein 1 (AP-1), both of which are influenced by ERK1/2 signaling and are critical for cellular survival in the face of oxidative challenges.[32] Similarly, BVR has been identified to form several other protein-protein interactions, such as with protein kinase C,[33] thereby suggesting that physical interaction between BVR and proteins of the signaling network is important for its mechanism of action.

Interestingly, our findings also demonstrated that inhibition of ERK1/2 activity enhances the protective effects of bilirubin and biliverdin. We achieved this inhibition using PD-98059, a specific inhibitor of MEK1 and MEK2, the upstream kinases in the MAPK/ERK pathway.[34] This protective enhancement is particularly evident in the context of oxidative stress, where the inhibition of ERK1/2 not only restores but also amplifies the antioxidant activity and cytoprotective functions of both bilirubin and biliverdin. The results of this experimental series further indicate that ERK1/2 activation serves as a negative regulator of BVR-mediated protection and that blocking this pathway can potentiate the antioxidative effects of bilirubin and biliverdin. In another study on arterial smooth muscle cells, the protective role of BVR under hypoxia was demonstrated, as well as the involvement of the ERK1/2 pathway.[35] In conclusion, modulation of BVR activity by ERK1/2 represents a critical control point in the cellular response to oxidative stress. Although ERK1/2 activation can impair the protective effects of bilirubin and biliverdin, its inhibition can enhance these effects, highlighting the potential of targeting

this pathway for therapeutic interventions aimed at preserving endothelial function in oxidative stress-related diseases.

Effects of C-Box and D-Box Inhibitors on BVR-ERK Interaction

The interaction between biliverdin reductase (BVR) and ERK1/2 kinases is mediated by specific motifs within the BVR, known as the C-box and D-box.[16] These motifs facilitate the binding of BVR to ERK1/2, influencing downstream signaling events, and consequently, the cellular response to oxidative stress. In our study, we explored the effects of inhibiting these specific interactions using peptides that target the C-box and D-box motifs, thereby disrupting the BVR-ERK1/2 interaction. The differential effects of these inhibitors provide insights into the specific roles of these binding interactions in modulating the protective activities of bilirubin and biliverdin.

Modest Enhancement of Bilirubin Protective Activity with C-Box Inhibition

The inhibition of the C-box interaction using the FGFPAFSG peptide yielded a modest yet statistically significant enhancement of the protective activities of both bilirubin and biliverdin. We observed this enhancement in terms of increased cell viability and cellular antioxidant activity. The C-box motif within the BVR is also known as the high-affinity ERK binding site, and thus plays a crucial role in the docking and subsequent phosphorylation of BVR by ERK1/2.[36] Interestingly, the C-terminal part of BVR is critical for its catalytic activity, although it lies in a disordered region of the BVR molecule,[37] suggesting that various regions of BVR influence its catalytic function. By inhibiting this interaction, it is likely that the phosphorylation of BVR is reduced, thereby maintaining its enzymatic activity in converting biliverdin to bilirubin. This, in turn, may enhance the availability of bilirubin, allowing for more effective scavenging of reactive oxygen species (ROS) and greater protection of endothelial cells from oxidative damage.

The modest nature of the enhancement observed with C-box inhibition suggests that while this interaction is important, it may not be the sole determinant of BVR's protective function. The partial relief of the inhibitory effects of ERK1/2 on BVR through C-box inhibition indicates that other factors or interactions within the cell may also contribute to the overall regulation of BVR activity. Nonetheless, our data support the notion that disrupting the C-box-mediated interaction between BVR and ERK1/2 can positively influence the antioxidative capacity of bilirubin and biliverdin, albeit to a limited extent.

Lack of Significant Effects on Bilirubin Protective Activity with D-Box Inhibition

In contrast to the effects observed with C-box inhibition, disruption of the D-box interaction using the KRILHCLGLA peptide did not yield significant changes in the protective activities of bilirubin and biliverdin. Both cell viability and cellular antioxidant activity remained unaffected by inhibition of the D-box motif. This lack of an effect suggests that the D-box-mediated interaction between BVR and ERK1/2 may not play a critical role in modulating the enzymatic activity of BVR or its ability to protect against oxidative stress.

Indeed, the D-box motif is considered a low-affinity binding site for several kinases, including ERK, and substrates in the MAPK signaling cascade, and is generally involved in stabilizing protein-protein interactions rather than directly influencing the catalytic activity of enzymes.[36] In the context of BVR, it is possible that the D-box interaction with ERK1/2 primarily facilitates the spatial organization of these proteins within the cell, without significantly altering the enzymatic function of BVR. Therefore, inhibition of this interaction may not affect the protective roles of bilirubin and biliverdin.

The differential effects observed with C-box and D-box inhibition highlight the distinct roles that these motifs play in BVR-ERK1/2 interaction. C-box is more directly involved in modulating BVR activity and, consequently, the protective effects of bilirubin and biliverdin. In contrast, the D-box may play a more structural or organizational role, which does not significantly affect the antioxidant functions of these bile pigments. These findings suggest that targeted modulation of the C-box interaction could be a potential strategy for enhancing the protective effects of BVR in endothelial cells under oxidative stress. However, the modest enhancement observed also indicates that this approach alone may not be sufficient to fully restore or amplify the antioxidative capacity of BVR. Future studies should explore combinatory approaches that target multiple aspects of the BVR-ERK1/2 interaction or other related signaling pathways to achieve more robust protective effects.

Study limitations

Our study has several limitations that should be acknowledged. First, the experiments were conducted *in vitro* using human endothelial cells, which, while informative, do not fully replicate the complex *in vivo* environment of human vasculature. Second, the study primarily focused on the interaction between BVR and ERK1/2, potentially overlooking other signaling pathways that may also interact with BVR and influence its protective functions. Additionally, the use of pharmacological inhibitors, while effective in elucidating mechanisms, may have off-target effects that could confound our results. These limitations suggest the need for further research to validate our findings *in vivo* and to explore other pathways that might interact with BVR. More comprehensive studies are needed to fully understand the long-term effects of modulating BVR activity in the context of endothelial function.

5. Conclusions

In conclusion, this study highlights the critical role of biliverdin reductase (BVR) in protecting endothelial cells from oxidative stress. In Figure 10, we schematically present the interplay of BVR with ERK, the role of BVR in increased oxidative stress conditions, as well as all key approaches that we experimentally addressed in our study. Collectively, our results reinforce the importance of BVR as a key endothelial regulator of endogenous antioxidant activity of bilirubin. Building on the findings of this study, further research should investigate the interactions between BVR and other signaling pathways beyond ERK1/2, as this could reveal additional mechanisms through which BVR confers its protective effects. Finally, the development of specific BVR modulators that can selectively enhance or inhibit its activity could pave the way for novel therapeutic strategies aimed at preventing or treating endothelial dysfunction and related cardiovascular diseases. In addition, the development of novel small-molecule C-box inhibitors may lead to novel pharmacological strategies for treating endothelial dysfunction in both normoxia and after ischemia-reperfusion injury. However, the complexity of BVR interactions with several intracellular proteins and their versatile functions require cautious and well-targeted approaches for future clinical applications.

Conflicts of Interest

The authors declare no conflicts of interest. The funding sources were not involved in the study design, collection, analyses, interpretation of data, writing of the manuscript, or decision to publish the results.

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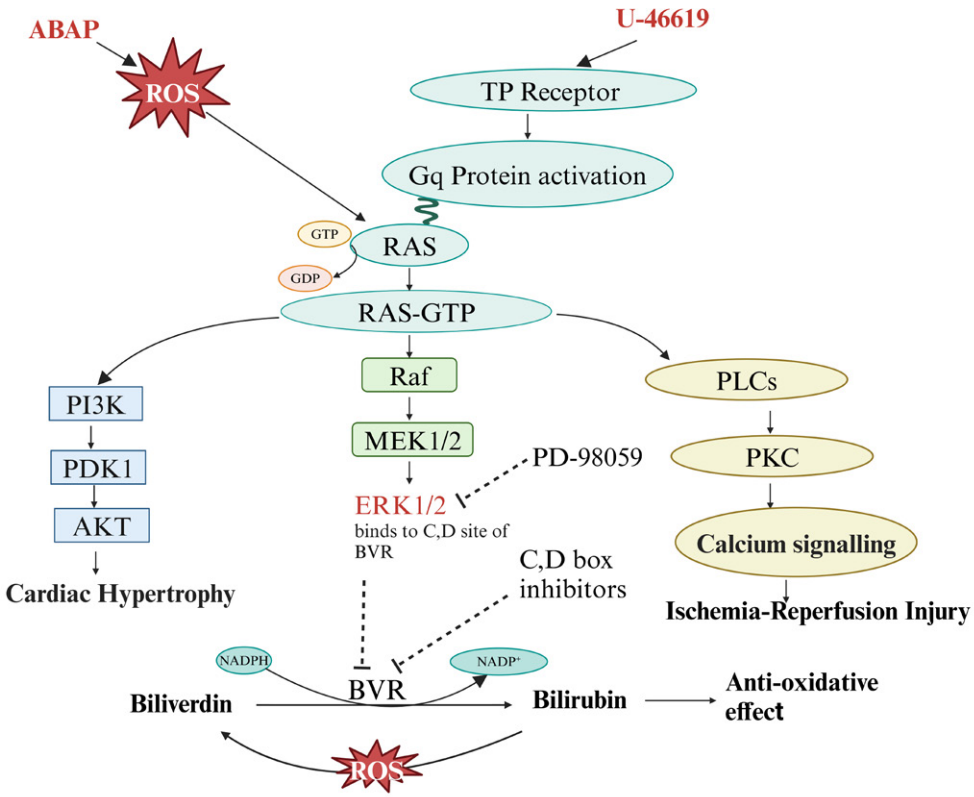

Figure 10. Schematic representation of the interplay between oxidative stress, ERK1/2 signaling, and biliverdin reductase (BVR) in endothelial cells. This figure illustrates the complex interactions between reactive oxygen species (ROS) generated by 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP) and the activation of thromboxane A2 (TXA2) receptors by U-46619, leading to activation of the Rat Sarcoma (RAS) protein. Activated RAS initiates several downstream pathways, including phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and rapidly accelerated fibrosarcoma/mitogen-activated protein kinase/extracellular signal-regulated kinase (Raf/MEK/ERK) signaling cascades. Additionally, phospholipase C/protein kinase C (PLC/PKC) - mediated calcium signaling is implicated in ischemia-reperfusion injury. Bilirubin, produced via the BVR pathway, provides important antioxidative protection, however its activity is modulated by the activity of ERK1/2. This hypothesis was experimentally tested in our study by the inhibition of BVR activity, by activation and inhibition of ERK1/2 kinase activity, and by using C-box and D-box inhibitors that interfere with ERK1/2 binding to BVR. The figure was created using graphical elements from BioRender.com.

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Povzetek

Endotelijska disfunkcija je pomemben dejavnik tveganja za razvoj srčno-žilnih bolezni, njeno stanje pa dodatno poslabša oksidativni stres. Biliverdin (BV) in bilirubin (BR) sta močna antioksidanta, ki ščitita endotelijske celice, pri čemer encim biliverdin reduktaza (BVR) pretvarja BV v BR za vzdrževanje redoks ravnovesja. Naša raziskava je preučevala vlogo BVR pri posredovanju teh zaščitnih učinkov v normoksičnih pogojih in pogojih hipoksije-reoksigenacije. Farmakološka inhibicija BVR je zmanjšala zaščitne učinke BV in BR, kar se kaže v zmanjšani viabilnosti celic, znotrajcelični antioksidativni sposobnosti in nižjih ravneh znotrajceličnega bilirubina. Aktivacija ERK1/2 je zmanjšala zaščitno funkcijo BVR, medtem ko jo je njena inhibicija povečala. Poleg tega je zaviranje interakcije med BVR in ERK dodatno vplivalo na delovanje BV in BR, kar kaže, da bi lahko vezavna mesta interakcije BVR-ERK1/2 predstavljala potencialne terapevtske tarče za zdravljenje endotelijske disfunkcije, povzročene z oksidativnim stresom.