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Tetrahydrobiopterin compounds modulate intracellular signaling and reactive oxygen species levels in an *in vitro* model of ischemia-reperfusion injury

Abstract: Tetrahydrobiopterin (BH_4) and 4-amino-tetrahydrobiopterin (ABH_4) prevent acute rejection after solid organ transplantation. Moreover, BH_4 also attenuates ischemia-reperfusion injury (IRI). The mechanisms underlying these protective effects are poorly defined. Activation of intracellular signaling proteins, including the mitogen-activated protein kinases (MAPKs) ERK, p38, and JNK, and the excessive production of mitochondrial reactive oxygen species (ROS) are observed mainly during early reperfusion. While the role of ROS in the initiation and progression of IRI is well understood, the contribution of individual signaling pathways is less clear. Here, we tested the potential effects of BH_4 and ABH_4 on MAPK activity and mitochondrial ROS levels. During hypoxia and reoxygenation (H/R), all three MAPKs were activated during early reoxygenation in cardiomyocytes and endothelial cells. p38 and JNK activation were further augmented by BH_4 and ABH_4, whereas ERK activation was not affected. Pretreatment with BH_4 and ABH_4 reduced the basal mitochondrial ROS levels as well as the H/R-induced increase in ROS. Prolonged incubation with ABH_4, however, showed pro-apoptotic effects in cardiomyocytes. These data suggest that a protective effect of BH_4 and ABH_4 pretreatment may be attributed mainly to their antioxidant capacity. The effects on intracellular signaling are complex and warrant further investigations.

Keywords: 4-amino-tetrahydrobiopterin (ABH_4); ischemia-reperfusion injury; mitogen-activated protein kinase (MAPK) signaling; reactive oxygen species (ROS); tetrahydrobiopterin (BH_4).

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Introduction

Ischemia-reperfusion (I/R) injury (IRI), defined as cellular damage following restoration of oxygen and nutrient supply to previously viable ischemic tissue [1], constitutes a serious problem during organ transplantation and represents a major reason for early graft dysfunction [2]. Although reperfusion is essential to avoid irreversible tissue damage, on its own, it triggers a series of pathophysiological changes further aggravating tissue injury initiated by ischemia [3]. The resulting IRI is a highly complex and multifaceted process, which, apart from intracellular damage, also involves extracellular inflammatory responses with crosstalk between these pathologic processes [2]. The activation of intracellular signaling pathways and the production of excessive mitochondrial reactive oxygen species (ROS) are critical for the initiation and progression of IRI. ROS induce damage through oxidation of biomolecules and may further activate intracellular signaling pathways promoting the overall injurious process [4, 5]. ROS may also importantly enhance IRI through inflammasome activation following cellular stress [6, 7]. Among other intracellular signaling proteins, mitogen-activated protein kinases (MAPKs) are activated, contributing to the structural and functional

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changes seen during IRI [8]. The activation of the MAP kinases ERK, p38, and JNK is most pronounced during early reperfusion and may even precede an excessive increase in ROS, making them potential targets for therapeutic intervention [9].

The pteridines tetrahydrobiopterin (BH_4) and 4-amino-tetrahydrobiopterin (ABH_4) demonstrated beneficial effects in several animal models of organ transplantation. While only BH_4 could attenuate IRI after solid-organ transplantation [10–13], both pteridines improved organ survival and performance by preventing acute rejection [14–16]. BH_4 is an essential cofactor of several enzyme systems, including the three nitric oxide synthase (NOS) isoenzymes [17]. Oxidative stress, as it occurs during IRI, causes depletion of intracellular BH_4 levels, which leads to NOS uncoupling, and subsequently to increased ROS production, enhanced vascular endothelial dysfunction, and overall increased tissue damage [18]. Exogenous administration of BH_4 prevents NOS uncoupling and may be a strategy to minimize IRI [19].

ABH_4 is a structural analogue of BH_4 that acts as a reversible inhibitor of all three NOS isoenzymes, with highest selectivity for the inducible NOS (iNOS) [20]. Excessive nitric oxide (NO) production by iNOS is involved in the pathophysiology of many inflammatory processes as well as in reperfusion injury, triggering increased production of inflammatory cytokines and ROS [10]. Inhibition of iNOS, however, did not account for the aforementioned protective effects of ABH_4 seen during acute rejection, as these occurred independently of the iNOS-inhibitory effect of ABH_4 [14]. Additionally, ABH_4 exhibited immunosuppressive effects, which did not relate to the iNOS-inhibiting properties of the compound either [21].

The poorly defined cellular and molecular mechanisms of pteridine action in ameliorating IRI prompted us to investigate the effects of BH_4 and ABH_4 on three parameters shaping the cellular and organismic response to I/R: intracellular MAPK signaling, ROS production, and cell death. During ischemia and early reperfusion in particular, changes in oxygen and nutrient concentrations serve as important triggers for events that are critical for further progression to full blown IRI. These alterations in the cellular environment can be mimicked in hypoxia/reoxygenation (H/R) experiments *in vitro*. This model is thus particularly suitable to obtain a fast overview of possible links between I/R-associated changes in these intracellular processes and the observed protective effects of the pteridine compounds.

Materials and methods

Cell culture

HL-1 cardiomyocytes and human umbilical vein endothelial cells (HUVECs) were used to study pteridine effects. The cardiac muscle cell line HL-1-deriving from AT-1 mouse atrial cardiomycoytes retains a differentiated adult cardiac phenotype and has therefore been extensively used to investigate cardiac biology *in vitro* [22, 23]. HL-1 cells were grown in gelatin/fibronectin-coated flasks and maintained in Claycomb cell culture medium (SAFC Biosciences, St. Louis, MO, USA) supplemented with 10% FBS (PAA, Pasching, Austria), 100 µM norepinephrine (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Gibco Invitrogen, Grand Island, NY, USA), 100 U/mL penicillin (PAA), and 100 µg/mL streptomycin (PAA) as described previously [23]. HUVECs are isolated endothelial cells from human umbilical veins commonly used for *in vitro* studies of vascular biology [24, 25]. HUVECs were grown in gelatin-coated flasks and cultivated in endothelial basal medium (EBM) supplemented with 5% FBS, recombinant human epidermal growth factor, bovine brain extract, hydrocortisone, and gentamycin/amphotericin B (EGM® MV BulletKit®; Lonza, Basel, Switzerland).

All cells were cultivated in T75 flasks under sterile growth conditions at 37°C in an atmosphere of 5% CO_2 and 95% air at a relative humidity of 95%. Cells were passaged at a split ratio of 1:3 upon reaching approximately 80% confluence by trypsinization and resuspension.

Vials with solid BH_4 and ABH_4 aliquots (Schircks Laboratories, Jona, Switzerland) were stored at -20°C. Stock solutions (10 mM) of BH_4 and ABH_4 were prepared shortly before use by dissolving 1–3 mg of BH_4 and ABH_4 in an appropriate amount of supplement-free cell culture medium.

Hypoxia/Reoxygenation studies

To mimic I/R *in vitro*, a previously described and well-established model of H/R was used [9]. For H/R experiments, cells were incubated in serum-deprived starvation medium (containing 0.05% FBS), placed in a Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA, USA), and exposed to 0.5% O_2 (monitored by a microPac oxygen probe; Dräger, Lübeck, Germany) at 37°C for 45 min to 1 h. For reoxygenation, a normoxic environment was reestablished and cells were kept in serum-rich maintenance medium.

Protein analysis

At the indicated time points, cells were harvested and lysates were prepared using ice-cold NP-40 lysis buffer (25 mM TRIZMA base, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 25 mM β-glycerophosphate, 10% glycerin, 0.75% NP-40, 25 mM NaF) containing 1:100 Protease Inhibitor Cocktail Set I (Calbiochem, Darmstadt, Germany) and 1 mM sodium orthovanadate. The resulting suspension was centrifuged at 13,200 rpm for 10 min at 4°C. The supernatant lysate was supplemented with 6× Laemmli buffer (120 mM Tris-HCl pH 6.8, 12% SDS, 12 mM EDTA pH 8.0, 20% glycerin, 0.1% bromphenol blue, 4.75%

2-mercaptoethanol), heated at 95°C for 5 min, and used for further analysis. Protein concentration in cell lysates was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). To ensure the loading of equal concentrations for protein separation, lysates were diluted with appropriate amounts of lysis buffer and Laemmli buffer. Protein separation and Western immunoblotting were performed as previously described [26]. Activation of MAPKs was assessed by probing blots with the following phosphorylation-specific antibodies: phospho-ERK (sc-16982-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p38 (9211S; Cell Signaling Technology, Danvers, MA, USA), or phospho-JNK (AF-1205; R&D Systems, Minneapolis, MN, USA). The total ERK, p38, and JNK protein levels were detected using the antibodies sc-94, sc-7972, and sc-571 (all Santa Cruz Biotechnology), respectively. Relative densities of protein bands were analyzed using the gel analyzer option of the ImageJ software (Scion Corporation, Frederick, MD, USA). Relative kinase activation levels were determined by normalizing phospho-MAPK signals to total MAPK levels. Probing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (AM4300; Ambion, Austin, TX, USA) was used as a loading control.

Fluorescence imaging of mitochondrial ROS

For mitochondrial ROS measurements, 60×10^3 to 70×10^3 cells were seeded on 8-well Lab-Tek Chambered Coverglass (Nalge Nunc, Rochester, NY, USA) and exposed to treatment. ROS were detected by loading cells with 0.2 µM MitoTracker Red CM-H_2XRos (Invitrogen Molecular Probes, Eugene, OR, USA). Hydrogen peroxide (1.5 mM) was used as a positive control. After 30 min of incubation, cells were supplied with fresh growth medium and analyzed by fluorescence microscopy using an Olympus IX-70 inverse microscope. For H/R experiments, the fluorescent probe was added to the starvation medium before exposing cells to 45 min of hypoxia. Digital images were acquired using Kappa ImageBase software (Kappa opto-electronics GmbH, Gleichen, Germany). Quantitative measurements of fluorescent signals were performed using inverted gray-scale images and Scion Image for Windows software (Scion Corporation). Mean density measurements of 80–120 cells/well were averaged.

Flow cytometry

Apoptosis of HL-1 cells following pteridine pretreatment was assessed after annexin V-FITC/propidium iodide (PI) staining. Stained cells were examined by FACS using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The percentage of viable cells was determined by analysis of the dot plots using BD CellQuest software.

Statistical analysis

All data are presented as mean±standard deviation (SD). Statistical analysis was performed in GraphPad Prism (GraphPad, San Diego, CA, USA) using one-way analysis of variance followed by Newman-Keuls test for multiple comparisons (mean comparison of all pairs of columns) or Dunnett's test (mean comparison to a single control column). The level of significance was defined as $p<0.05$.

Results

H/R alter MAPK signaling

Hypoxia and especially the transition from hypoxia to reoxygenation are marked by a strong activation of the MAP kinases ERK, p38, and JNK, as published previously [9]. We first confirmed these findings in the cell models used in our experiments – HL-1 cardiomyocytes and HUVECs – since cardiomyocytes and endothelial cells are critical targets for IRI [27]. Both cell lines were subjected to 45 min of hypoxia and different reoxygenation times ranging from 10 min to 24 h. MAPK activity was assessed with the help of phosphorylation-specific antibodies as described in Materials and methods. In HL-1 cells, hypoxia by itself only increased the activity of p38 (2.5-fold increase vs. control, $p<0.05$), but not that of ERK and JNK, whereas in HUVECs, the activity of all three MAPKs was slightly, although not significantly, elevated (ERK: 1.7-fold vs. control; p38: 2.8-fold vs. control; JNK: 2.2-fold vs. control) (Figure 1). The strongest MAPK activation occurred 10 min after reoxygenation. ERK, p38, and JNK phosphorylation was strongly increased in both HL-1 cells (ERK: 3.6-fold, $p<0.05$; p38: 3.6-fold, $p<0.001$; JNK: 4.8-fold, not significant) and HUVECs (ERK: 3.1-fold, $p<0.05$; p38: 5.3-fold, $p<0.05$; JNK: 4.1-fold, not significant). After 24 h of reoxygenation, the level of MAPK activity was comparable to the control group again.

BH_4 and ABH_4 pretreatment modulates H/R-induced MAPK activation

To investigate the effects of the pteridines BH_4 and ABH_4 on MAPK signaling, HL-1 cells and HUVECs were pretreated with BH_4 or ABH_4 for 5 h with concentrations ranging from 0.5 to 1.5 mM. Subsequently, the pteridine-containing cell culture medium was removed and replaced by starvation medium before exposing cells to 1 h of hypoxia followed by 10 min of reoxygenation, the time point when maximum MAPK activation had been observed (Figure 1). Activation of all three MAPKs was found in untreated cells undergoing H/R as compared with controls maintained under normoxic conditions. In the case of ERK, this H/R-induced activation was largely unaffected by BH_4 and ABH_4 in both cell lines (Figure 2C and D). Only in HUVECs, the highest concentration of ABH_4 (1.5 mM) significantly reduced ERK activation to 31% of normoxic control levels ($p<0.001$ vs. other concentrations of ABH_4 and untreated H/R control). H/R-induced p38 and JNK activation, in contrast, were

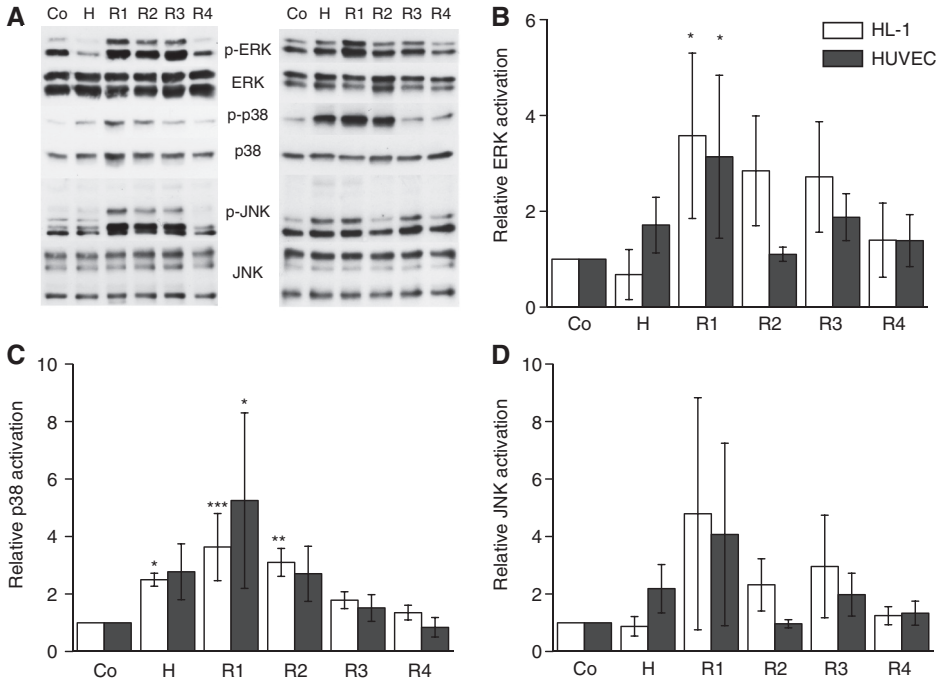

Figure 1 Alterations in MAPK activity during H/R in HL-1 cells and HUVECs. Cells were exposed to 45 min of hypoxia followed by reoxygenation. Immediately after hypoxia (H) and after 10 min (R1), 2 h (R2), 6 h (R3), and 24 h (R4) of reoxygenation, cells were harvested and MAPK activity was analyzed by Western blotting. Representative immunoblots are shown (A). (B–D) Summary bar graphs of ERK (B), p38 (C), and JNK (D) activation during H/R. Relative activation levels were calculated by normalizing phospho-MAPK signals to total MAPK levels; controls (Co) were set to 1.0. Data are expressed as mean±SD (n=3, *p<0.05, **p<0.01, ***p<0.001 vs. control).

further increased by BH_4 and ABH_4 pretreatment in both cell types in a concentration-dependent way (Figure 2E–H). The highest concentration (1.5 mM) caused p38 activity to increase 14.7-fold in the case of BH_4 and 11-fold in the case of ABH_4 in HL-1 cells and 38-fold (BH_4) and 35.5-fold (ABH_4) in HUVECs, respectively. pJNK levels in HL-1 cells were elevated 15.4-fold ($p<0.01$ vs. untreated H/R-control) by 1.5 mM of BH_4 and 5.5-fold by the same concentration of ABH_4. In HUVECs, BH_4 treatment caused a 5-fold ($p<0.001$ vs. untreated H/R control) and ABH_4 a 4.8-fold ($p<0.01$ vs. untreated H/R control) increase in JNK activity at 1.5 mM.

Basal mitochondrial ROS levels are reduced by BH_4 and ABH_4 pretreatment

To investigate whether mitochondrial ROS represent potential targets for BH_4 and ABH_4 action, HL-1 cells and HUVECs were incubated with pteridine concentrations ranging from 0.5 to 1 mM for 30 min or 5 h under normoxic conditions. Incubation with either pteridine resulted in a significant decrease of basal mitochondrial ROS levels (Figure 3). In HL-1 cells, BH_4 at a concentration of 0.5 mM caused ROS levels to drop to 65% of the control level ($p<0.05$) after 30 min and to 82.2% (not significant) after 5 h. BH_4 with a concentration of 1 mM reduced ROS to 78.6% and 77.9% (both $p<0.05$), respectively. ABH_4 showed similar results with a decline in ROS levels to 74.8% after 30 min and 73.6% after 5 h (both $p<0.05$) in the 0.5-mM group and to 69% ($p<0.05$) and 87.8% (not significant) in the 1-mM group, respectively. The findings obtained in HUVECs were comparable to these observations. BH_4 with a concentration of 0.5 mM decreased ROS levels to 69.3% (30 min, $p<0.05$) and 72.9% (5 h, not significant) of control levels, whereas a concentration of 1 mM caused a decrease to 83.2% and 79.9% (both not significant). In ABH_4-treated HUVECs, ROS levels dropped to 66.8% and 67.7% (both $p<0.05$) after pretreating with 0.5 mM and to 64.9% and 61.7% (both $p<0.05$) after pretreatment with 1 mM. In both investigated cell types, there was no significant difference between the 30-min and the 5-h groups, between BH_4 and ABH_4 at either concentration, and between different concentrations of the same compound.

BH_4 and ABH_4 pretreatment attenuates H/R-induced ROS formation

Apart from MAPK activation, the transition from hypoxia to reoxygenation is also marked by a significant increase

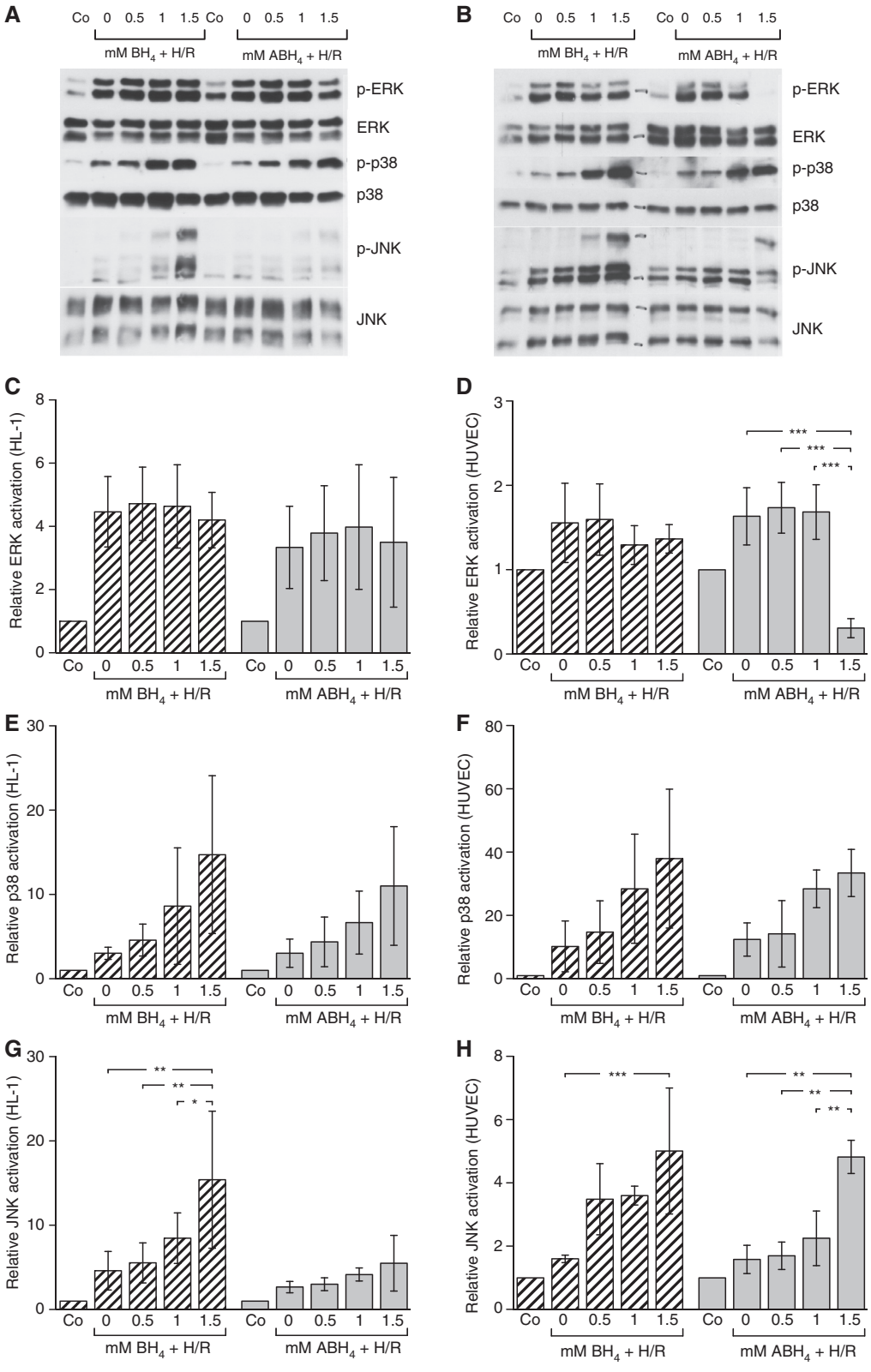

Figure 2 Modulation of H/R-induced MAPK activation by BH_4 and ABH_4 in HL-1 cells and HUVECs. Cells were pretreated with 0.5, 1, or 1.5 mM of BH_4 or ABH_4 for 5 h or left untreated (0 mM BH_4/ABH_4) before exposing them to 1 h of hypoxia followed by 10 min of reoxygenation. (A and B) Representative immunoblots for HL-1 cells (A) and HUVECs (B). (C–H) Summary bar graphs of BH_4 and ABH_4 effects on ERK, p38, and JNK signaling in HL-1 cells (C, E, G) and HUVECs (D, F, H) during H/R. Relative activation levels were calculated by normalizing phospho-MAPK signals to total MAPK levels; untreated normoxic controls (Co) were set to 1.0. Data are expressed as mean±SD (n=3, *p<0.05, **p<0.01, ***p<0.001).

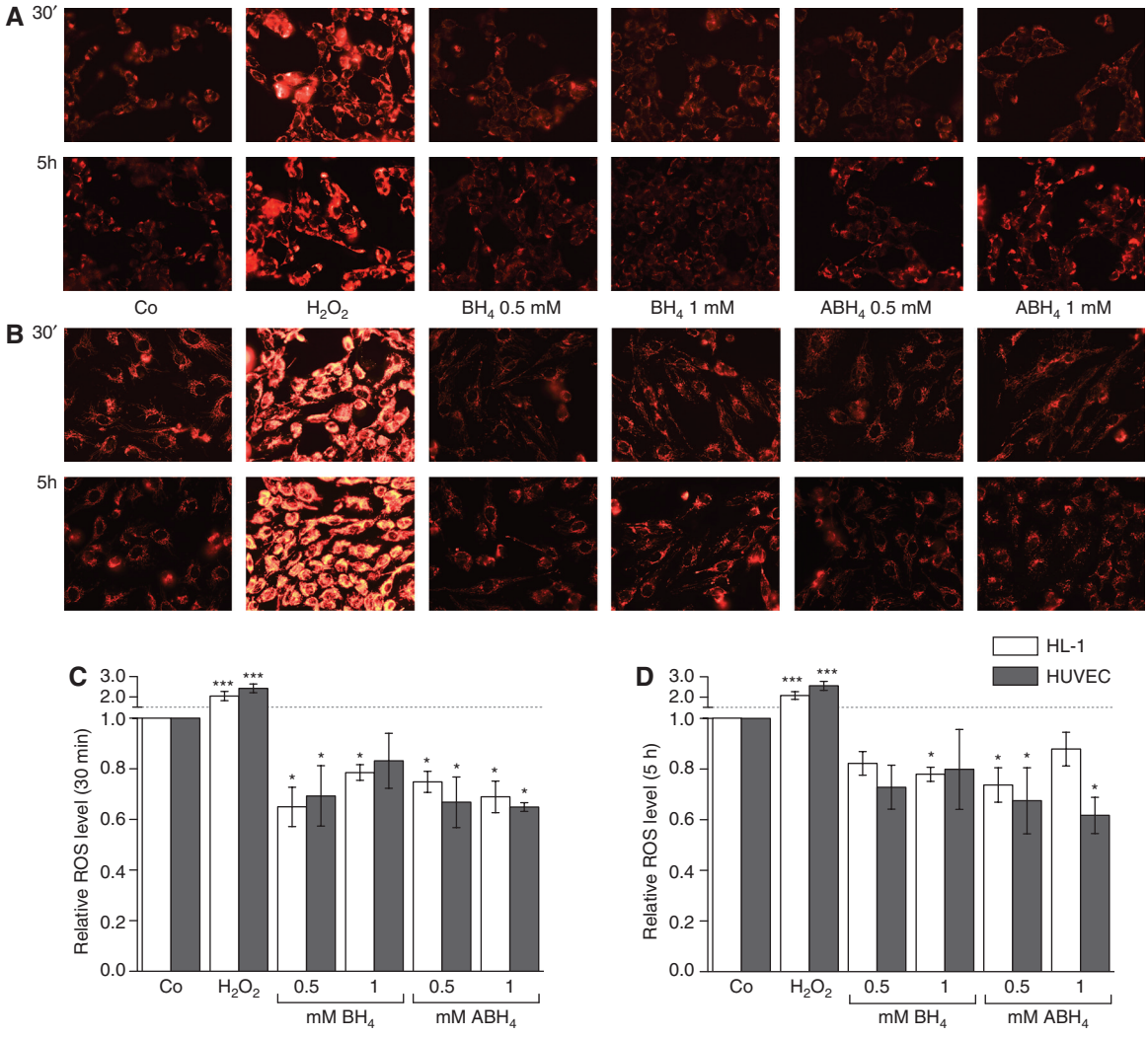

Figure 3 Effects of BH_4 and ABH_4 treatment on basal mitochondrial ROS levels. HL-1 cells and HUVECs were treated with 0.5 mM or 1 mM of BH_4 or ABH_4 for 30 min or 5 h. Mitochondrial ROS levels were assessed by fluorescence microscopy during early reoxygenation (≤5 min). Representative fluorescence images are shown for HL-1 cells (A) and HUVECs (B). (C and E) Summary bar graphs of BH_4 and ABH_4 effects on basal ROS levels in HL-1 cells and HUVECs after 30 min (C) and 5 h (D). H_2O_2 was used as a positive control. Fluorescence signal of controls (Co) was set to 1.0. Data are expressed as mean±SD (n=3, *p<0.05, ***p<0.001 vs. control).

in mitochondrial ROS levels [9]. Excessive mitochondrial ROS production is a major early contributor to the development of IRI. Attenuating this rise in ROS production might therefore be a strategy for limiting I/R-associated cell and organ injury. To investigate the effects of BH_4 and ABH_4, HL-1 cells and HUVECs were pretreated at concentrations ranging from 0.5 to 1 mM for 5 h before exposing them to 1 h of hypoxia followed by a short period of reoxygenation (≤5 min). As expected, untreated cells undergoing H/R showed significantly elevated mitochondrial ROS levels comparable with cells treated with hydrogen peroxide, which served as positive control (Figure 4). In HL-1 cells, a 2.4-fold increase ($p<0.01$ vs. normoxic control) was observed. In HUVECs, ROS levels were elevated 1.8-fold ($p<0.01$ vs. normoxic control) after H/R. In HL-1 cells treated with 0.5 or 1 mM of BH_4, ROS levels were reduced by 34% (1.6-fold increase vs. normoxic control) and 35% (1.7-fold, both $p<0.05$ vs. untreated H/R), respectively. ABH_4 with a concentration of 0.5 mM caused a 42% reduction (1.4-fold), whereas a concentration of 1 mM lowered ROS levels by 36% (1.5-fold, both $p<0.05$ vs. untreated H/R). In HUVECs, H/R-induced ROS were decreased by 29% (1.3-fold) in the case of 0.5 mM of BH_4, by 35% (1.2-fold) in the case of 1 mM of BH_4, by 34% (1.2-fold) in the case of 0.5 mM of ABH_4, and by 41% (1.1-fold) in the case of 1 mM of ABH_4 (all $p<0.01$ vs. untreated H/R). There was no significant difference between BH_4- and ABH_4-treated H/R groups and normoxic control, between BH_4 and ABH_4

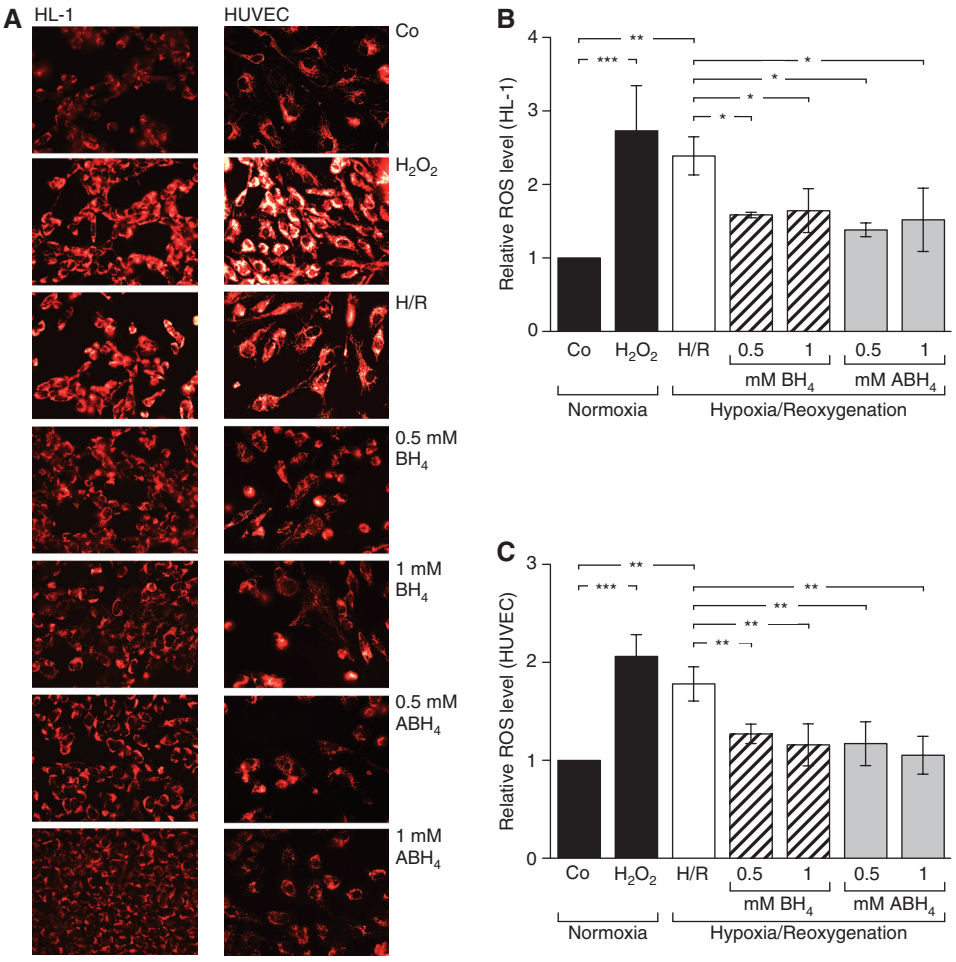

Figure 4 Effects of BH_4 and ABH_4 treatment on H/R-induced ROS production. HL-1 cells and HUVECs were pretreated with 0.5 or 1 mM of BH_4 or ABH_4 for 5 h, followed by 45 min of H/R. Mitochondrial ROS levels were assessed by fluorescence microscopy. Representative fluorescence images are shown (A). (B and C) Summary bar graphs of BH_4 and ABH_4 effects on ROS during H/R in HL-1 cells (B) and HUVECs (C). H_2O_2 was used as a positive control. Fluorescence signal of controls (Co) was set to 1.0. Data are expressed as mean±SD (n=3, *p<0.05, **p<0.01, ***p<0.001).

in either concentration and between different concentrations of the same compound.

Prolonged ABH_4 treatment causes apoptosis

To investigate the potential cytotoxic effects of BH_4 and ABH_4 in the concentrations used, HL-1 cells were incubated with both compounds for 24 h before assessing cell viability. Cells treated with ABH_4 at high concentrations showed significantly reduced cell viability at concentrations above 0.5 mM (Figure 5): 1 mM of ABH_4 reduced the percentage of viable cells to 54.3% ($p<0.01$ vs. control) and 1.5 mM caused a decrease to 28.6% ($p<0.001$ vs. control). BH_4 treatment did not significantly increase apoptosis in HL-1 cells. Concentrations of 0.5 and 1 mM showed viabilities comparable to untreated control cells (89.3% and 86.9% respectively). Only 1.5 mM of BH_4 caused a minor, although not significant, decrease in cell viability (67.9%).

Discussion

IRI critically determines the outcome after solid organ transplantation, causing delayed graft function and increased rates of acute graft failure, which may result in retransplantation or even patient death [28]. While the role of ROS in the pathology of IRI is well documented, the use of common antioxidants yielded no clinical benefit. Also, the activation of intracellular signaling pathways is

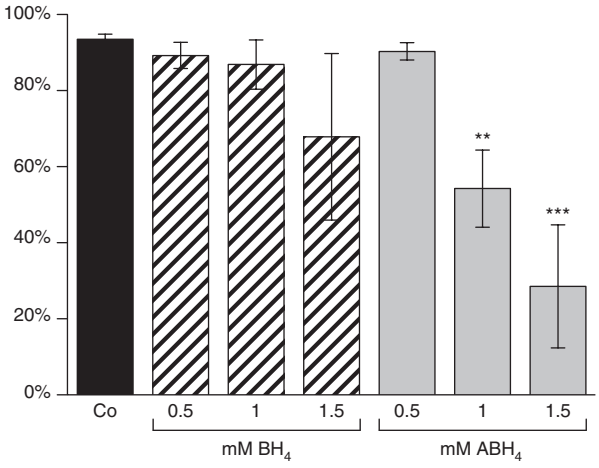

Figure 5 Effects of BH_4 and ABH_4 treatment on cell survival in HL-1 cells. HL-1 cells were treated with 0.5, 1, or 1.5 mM of BH_4 or ABH_4 for 24 h. Cell survival and apoptosis were measured by annexin V/PI staining. The percentage of viable cells is shown. Data are expressed as mean±SD (n=3, **p<0.01, ***p<0.001 vs. control).

observed during IRI, but the emerging picture is complex and individual signaling proteins may have both beneficial (anti-apoptotic) and adverse (pro-inflammatory) effects as demonstrated for IKK [29, 30]. Therefore, novel strategies to prevent IRI are necessary. The pteridines BH_4 and ABH_4 demonstrated protective effects during IRI and allograft rejection in several animal models [10–16]. Still, no conclusive picture has emerged so far of the mechanisms underlying these protective effects. The present study therefore aimed at relating these observations to molecular and cellular processes that typically occur during IRI.

Excessive production of ROS is a well-understood feature of IRI and a key initiating event leading to tissue damage. The transition from ischemia/hypoxia to reperfusion/reoxygenation in particular shows a marked increase in mitochondrial ROS levels [9]. Our data demonstrate that pretreatment of cardiomyocytes and endothelial cells with BH_4 and ABH_4 significantly reduces basal mitochondrial ROS levels and attenuates H/R-induced ROS production. Whether these antioxidant effects are the result of reduced ROS production or the consequence of radical scavenging by BH_4 and ABH_4 remains to be determined. Several considerations, however, support the theory of ROS scavenging: (i) BH_4 is one of the most potent naturally occurring reducing agents and may be oxidized in conditions of oxidative stress [31]; (ii) the same most likely applies to ABH_4, which exhibits chemical redox properties comparable to BH_4 [20]; (iii) the strikingly similar effects of BH_4 and ABH_4 suggest that the antioxidant effect is more likely due to the specific chemical nature of the compounds, since the chemical characteristics of both are very similar, whereas cellular functions differ considerably (cofactor function vs. enzyme inhibition) [20].

The involvement of signaling pathways in the onset and progression of IRI has also been well established [2]. Among these, MAPKs are of crucial importance [8]. Sucher et al. [9] could previously show a strong activation of ERK, p38, and JNK during early reoxygenation/reperfusion *in vitro* and *in vivo*. In the present study, we were able to confirm this MAPK activation pattern in cardiomyocytes and could further show that endothelial cells, which are major contributors to the pathogenesis of IRI, respond in the same way when exposed to H/R. These findings support the concept of MAPK activation as a universal signaling response to H/R.

Pretreatment with BH_4 and ABH_4 could further modulate these alterations in MAPK activities: ERK activation remained unaffected by pteridine pretreatment, whereas the stress kinases p38 and JNK were further activated by both compounds in a concentration-dependent way. These findings are striking and in contrast to the commonly proposed functions of these kinases. While the ERK pathway is well established as a protective "survival" cascade [32–34], the stress kinases p38 and JNK are thought to respond primarily to cellular stress as components of "death" signaling [8]. This is in line with our observations with 1.5 mM of ABH_4 in HUVECs, where we observed decreased ERK activity, increased p38 and JNK levels, and apoptosis. However, mitogenic and even survival functions of these kinases have also been described [35]. In the setting of IRI, the protective effects of ERK were confirmed [36], whereas increasing evidence supports the role of stress kinase signaling as pro-death pathways. Inhibition of p38 and/or JNK activation could therefore ameliorate tissue damage in various models of IRI [37–45]. As for BH_4, the ability to induce cell death by activating MAPK signaling pathways has previously been reported in neuronal cells. JNK activation, but not ERK and p38, was shown to mediate BH_4-induced dopaminergic cell death [46]. A subsequent study could also confirm the involvement of ERK and p38 in this process [47]. These findings are in contrast to the observed protective effects of BH_4 on IRI. The role of signaling pathways in IRI is in general very complex and their contributions to I/R-associated damage, either beneficial or disadvantageous, vary significantly and may even be ambiguous. For instance, inhibiting NF-κB by ablating IKK efficiently prevented a systemic inflammatory response during intestinal I/R but also caused severe apoptotic damage

to the reperfused mucosa due to a lack of NF-κB survival activity [29].

MAPK signaling pathways have been implicated in the control of mitochondrial ROS levels. Sucher et al. [9] could show that alterations in MAPK signaling precede changes in mitochondrial ROS and calcium levels. Inhibiting p38 and MEK significantly reduced these mitochondrial changes and, in the case of p38, could also prevent H/R-induced cell death. Signaling *via* the p38 pathway, however, also showed protective effects. Overexpression of MKK6, an upstream activator of p38, exhibited cardioprotective effects most likely due to reduced mitochondrial ROS generation [48, 49]. The involvement of the Raf-MEK-ERK pathway in controlling mitochondrial ROS production has also been reported [34]. Although not directly tested, the presented data seem not to confirm the mentioned link between MAPK activation and increased ROS levels. Despite increased stress kinase signaling, antioxidant effects were observed following BH_4 and ABH_4 pretreatment, suggesting that other potentially more complex mechanisms of action account for the reduced ROS levels.

Apart from their protective effects during IRI, BH_4 and particularly ABH_4 have demonstrated immunosuppressive and pro-apoptotic properties in cells of the immune system. ABH_4 could effectively induce apoptosis in T cells as well as in murine macrophages [21, 50]. Our data demonstrate that high concentrations of ABH_4 present for longer periods of time cause a significant amount of cell death in HL-1 cardiomyocytes. These findings confirm a pro-apoptotic capacity of ABH_4, which was previously observed to a similar extent in promyleoid 32D cells (unpublished data).

In conclusion, both BH_4 and ABH_4 pretreatment results in significant antioxidant capacities, whereas prolonged treatment, in particular with ABH_4, causes cell death. Furthermore, pteridine supplementation modulates intracellular signaling, which is known to be an integral part in the initiation and progression of IRI. Assessing how these changes are related to the protective and immunosuppressive effects of pteridines warrants further investigations. Given the positive results of pteridine supplementation in numerous animal studies of organ transplantation (see above), application of these compounds in the clinical setting may become practical. The results of our study provide insight into potential mechanisms of pteridine action during IRI and might further promote the introduction of pteridines as a treatment option in the clinical setting of transplantation.

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