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Purinergic Receptor Antagonism Prevents Cold Preservation-Induced Cell Death Independent of Cellular ATP Levels

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Abstract

Background—Purinergic (P2Y) receptors play an important role in intracellular Ca^{2+} regulation in hepatocytes. Prevention of mitochondrial Ca^{2+} (mCa^{2+}) overload during ischemic conditions prevents cellular cell death during the early reperfusion period. P2Y-antagonists are cytoprotective in other settings. We studied the effect of P2Y receptor antagonism on mitochondrial associated cell death during the period of cold storage.

Methods—HepG2 cells were stored in UW with or without 300 μM reactive blue 2 (RB2) or 10μM ruthenium red (RR) under either normoxic-hypothermic (NH) or hypoxic-hypothermic (HH) conditions. Cytoplasmic cytochrome c levels were studied by transfection of cytochrome c-GFP. Immunofluorescence determined the intracellular, spatio-temporal distribution of Bax, and TUNEL staining was used to evaluate cell death. Intracellular compartmental ATP levels were assayed by transfecting with luciferase vectors specific for cytoplasm (PcDNA3-luciferase-LL/V) and mitochondria (PcDNA3-COX8-luciferase).

Results—Bax translocation to the mitochondria occurred immediately following cold storage and was followed by cytochrome c-GFP redistribution to the cytosol during rewarming. RB2 treatment significantly attenuated Bax translocation, cytochrome c-GFP redistribution, and cell death following both storage conditions. Both RR and RB2 provided cytoprotection *despite* ongoing cytoplasmic ATP consumption during cold ischemia.

Conclusion—These data indicate that the cytoprotective effects of mCa^{2+} uptake inhibition and P2Y receptor antagonism are independent of cytoplasmic ATP levels during cold ischemia.

Introduction

Purines and pyrimidines are important signaling molecules that affect a diverse array of cellular processes such as proliferation, differentiation, and cell death by interacting with purinergic receptors[1–3]. These receptors include the ligand gated channel P2X receptors and the G-protein coupled P2Y receptors[1]. More specifically, the mammalian P2Y receptors are coupled

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to the hydrolysis of phosphatidylinositol 4,5-bisphosphate and subsequent inositol 1,4,5-triphosphate-mediated release of intracellular Ca^{2+}[1,4]. Purinergic receptors play important roles in hepatocyte intracellular Ca^{2+} signaling, such as the response to osmotic shifts and cellular swelling[5–9]. Intracellular Ca^{2+} regulation is also the proposed mechanism underlying the P2 receptor mediation of glycogen metabolism and cellular proliferation, in hepatocytes[10].

Altered intracellular and mitochondrial Ca^{2+} (mCa^{2+}) regulation during periods of ischemia and reperfusion contribute to primary hepatocyte injury. Cold ischemia is known to induce an increase in cytosolic Ca^{2+} concentration via both release from intracellular stores and increased cellular uptake[11–13]. Depletion of cellular ATP during periods of ischemia acts synergistically with the elevated cytoplasmic Ca^{2+} concentration to cause activation of the mCa^{2+} uniporter[14–19]. Mitochondria were thought initially to serve as simple calcium buffers during conditions causing increased cytosolic calcium, such as ischemia. However, it is now known that mCa^{2+} uptake during these conditions serves to stimulate mitochondrial ATP production as part of a still poorly defined feedback mechanism[20]. Recently described P2Y receptors in the mitochondrial membrane, which play a role in the regulation of mCa^{2+} uptake, may play a role in this feedback mechanism[6,20,21]. Despite the initial increase of ATP production, as ischemia persists, electron transport is stopped and the continued mitochondrial accumulation of calcium compromises the mitochondrial membrane potential ($\Delta\Psi$). In an attempt to maintain$\Delta\Psi$, F0F1 synthase reverses its activity, and the mitochondria becomes an ATP consumer via reversal of the adenine nucleotide transporter (ANT)[22]. This preserves $\Delta\Psi$ for the short term, but also allows ongoing uptake of calcium and further depletion of cellular ATP. Ultimately, mCa^{2+} overload initiates mitochondrial associated apoptosis and cell death.

While little is known about the role of hepatocyte P2Y receptors in cold ischemia and reperfusion injury, available data suggests that adenosine nucleotides can act directly on P2Y receptors to induce cell death[23–25]. Stimulation of P2 receptors by ATP has been implicated in many models of apoptosis and neurotoxicity[3,23,26–28], and P2Y antagonists have been demonstrated to provide neuroprotection during ischemia[29–31]. The P2Y antagonists Suramin and RB2 have been shown to provide neuroprotection following exposure to dequalinium by preserving $\Delta\Psi$[32].

We have recently demonstrated that direct antagonism of the mCa^{2+} uniporter prevents a Bax-dependent apoptosis early during the reperfusion period[33]. Because of the ischemic neuroprotection afforded by P2Y antagonism in some models and the close relationship between purinergic receptor activation and intracellular calcium regulation, we investigated the role of reactive blue 2 (RB2), a non-competitive P2 inhibitor which does not discriminate between P2Y receptor subtypes[1], in hepatocyte cold ischemia and reperfusion injury. We report that addition of RB2 to the cold storage solution attenuates Bax-dependent early reperfusion cell death, similar to that observed when the mCa^{2+} calcium uptake is inhibited by the well established mCa^{2+} uniporter inhibitor, ruthenium red (RR). In addition, both RB2 and RR provide their cytoprotection *despite* ongoing cellular ATP consumption.

Materials and Methods

Cell culture and cold ischemic conditions

The human HepG2 hepatoblastoma cell line (HepG2, ATCC, Rockville, MD, Catalog No. HB-8065) was chosen because of its stability and predictable growth as well as retained characteristics of primary hepatocytes[34–37]. Cells were grown at 37°C, 5% CO_2 in MEM (ATCC, Manassas, VA), with 10% fetal bovine serum (Cascade Biologicals, Winchester, MA), and 1% penicillin/streptomycin (Gibco, Grand Island, NY) to 80% confluence. The media was replaced with Belzer solution (UW), and cells were incubated at 4°C in either normoxic or

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hypoxic conditions for 6 hours. Hypoxia was achieved by placing the cells in an airtight incubator (Forma Scientifica, Marietta, OH) which was flushed with 5% CO_2 and 95% N_2 until the oxygen content in the container reached < 0.1% as verified using a dissolved O_2 meter (Model 4000, VWR Scientific Products, Suwannee, GA). To render the storage solution hypoxic before experiments were carried out, UW was pre-incubated in the hypoxic chamber in an open sterile container for 8 hr before experiments were carried out. This resulted in a final O_2 concentration of < 0.1% as measured with the dissolved O_2 meter. Reactive Blue 2 (RB2) (Sigma, St Louis, MO; 300 μM) or Ruthenium Red (RR) (Sigma, 30 μM) was added to the UW solution of selected samples before storage of cells.

For experiments requiring “reperfusion”, the UW was replaced with warm, oxygenated MEM following storage, and the cells were incubated at 37°C to simulate reperfusion.

TUNEL staining, Immunofluorescence, and fluorescence microscopy

TUNEL (TdT-mediated dUTP Nick-End Labeling) staining was performed after 180 minutes of reperfusion as described previously[33]. The percentage of TUNEL positive cells per high powered field (HPF) were recorded. Four HPF for each of 3 experiments are reported.

Immunofluorescence for Bax was performed immediately following cold storage (no reperfusion) using the methods previously described[33]. The percentage of cells demonstrating Bax translocation per high powered field (HPF) were recorded. Four HPF for each of 3 experiments are reported.

To obtain a qualitative measure of mitochondrial cytochrome c release as a marker of apoptosis, HepG2 cells were transiently transfected with cytochrome c-GFP (gift from Dr. Nieminen) as described by Nieminen [38]. The transfected cells were grown, fixed, and studied as previously described[33].

Western analysis

Western analysis was carried out using methods previously described[33]. The antibodies used were as follows: anti P44/42 MAPK and anti-phosphorylated P44/42 MAPK antibodies (1:1000, Cell Signaling Technology).

Determination of Intracellular ATP

Luciferase vectors, PcDNA3-luciferase-LL/V (cytoplasmic luciferase) and PcDNA3-COX8-luciferase (mitochondrial luciferase) were a gift of Dr. Manfredi[39]. HepG2 cells were transfected with the selected PcDNA containing the engineered luciferase gene using FuGENE6 (Roche Applied Science, Indianapolis, IN) as described by the manufacturer. Cells were grown for 48 hours and then subjected to the storage conditions described above [with or without RR (30μM) or RB2 (300 μM)]. Following storage, cells were counted using the trypan blue exclusion method to insure no difference in the rate of cell death during the storage period. 2×10^5 cells were resuspended in tricine buffer with 10 μL beetle luciferin. Luminescence was measured (BioOrbit Model 1251) for 30 seconds and peak values were used for analysis (N=6 for each group)[40].

Untreated (MEM 37°C storage) cells served as a control. Standard ATP curves were performed in the presence of varying concentrations of RR and RB2 to control for interference from these compounds. Six experiments are reported for each condition.

Statistical Analysis

Statistical significance was determined using a two-tailed-homoscedastic student t-test. A p value of ≤ 0.05 was considered significant.

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Results

Reactive Blue 2 attenuates cell death following hypothermic storage

Following 6 hours of normoxic-hypothermic (NH) or hypoxic-hypothermic (HH) storage and 180 min of rewarming, TUNEL staining was performed. Figure 1 summarizes the results of these experiments. NH stored HepG2 cells demonstrate approximately 27.5% TUNEL positive nuclei per high power field after 180 min of rewarming. The percentage of apoptotic cells increases significantly to 45% (p=0.034) when cells are rewarmed for 180 min following HH storage. Addition of RB2 to the UW storage solution decreases the rate of cellular cell death following NH storage to 2.5% (p=0.001), while addition of RB2 to HH stored cells decreased cell death rates to 21% (p=0.001). These results are similar to those found when ruthenium red (RR) is added to the storage solution[33].

RB2 attenuation of cell death is not mediated via a ERK 1, 2-mechanism

P2 agonists have been demonstrated to promote apoptosis via pathways resulting in activation of ERK 1, 2, and P2 antagonists have been demonstrated to prevent this activation[41–43]. We studied the expression levels and phosphorylation states of ERK 1, 2 following our storage conditions by Western analysis. Figure 2 demonstrates that neither ERK 1, 2 expression levels nor phosphorylation states were altered by our experimental conditions, indicating that any effect of RB2 is independent of this pathway.

Reactive Blue 2 attenuates hypothermic storage-induced Bax translocation from cytosol to mitochondria

We have previously demonstrated that Bax translocation to the mitochondria plays a major role in early reperfusion apoptosis following hypothermic storage in HepG2 cells[33]. Inhibition of mCa^{2+} uptake by RR attenuated this occurrence. During the current experiments, RB2 was added to the storage solution. Figure 3 demonstrates representative examples of Bax immunofluorescence following hypothermic storage with and without RB2 treatment. As shown, the Bax staining pattern was diffuse in untreated cells indicating a equal distribution throughout the cytoplasm. Following cold storage (NH or HH), the Bax staining pattern became punctate, indicating translocation to mitochondria. When RB2 is added to the UW during storage, attenuation of the punctate Bax staining pattern was observed. When quantified (Figure 3), punctate Bax staining occurred in approximately 35% of cells per high power field following NH storage and increased significantly to 45% following HH storage. Addition of RB2 to the UW storage solution significantly decreased the rate of punctate Bax staining to 12% (p=0.001) following NH storage and 22% (p=0.009) following HH storage.

RB2 treatment prevents cytochrome c release following Bax translocation

Cytochrome c release from the mitochondria is an important downstream occurrence following Bax translocation, indicating that the cell is committed to apoptosis. To insure that Bax translocation in our model signified commitment of the cell to apoptosis, we qualitatively studied cytochrome c release. In order to visualize the spatio temporal intracellular distribution of cytochrome c during our study, HepG2 cells were transfected with GFP tagged cytochrome c and subjected to the storage and rewarming conditions. Figure 4 demonstrates representative photomicrographs. In untreated cells, cytochrome c-GFP is visualized in punctate grouping in the cytoplasm, indicating association with mitochondria. Cold storage in UW (both NH and HH) alone (without rewarming) resulted in very little diffusion of the GFP. In contrast, the GFP staining pattern is very diffuse following 6 hr of cold storage and 3hr of reperfusion. Addition of RB2 to UW attenuates this occurrence. These studies indicate that the Bax translocation precedes cytochrome c release in our model of cold storage and reperfusion.

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Similar to the observation when cells were treated with RR, RB2 treatment of cells during cold storage attenuates cell death of cells during reperfusion[33].

Hypothermic storage depletes cytosolic but not mitochondrial ATP concentrations

HepG2 cells were transfected with a PcDNA3-luciferase-LL/V (LL/V) cDNA construct to report cytosolic ATP levels following cold storage. Following storage, the stored transfected cells were counted using the trypan blue exclusion method. There was no difference in the number of necrotic cells immediately following NH storage when cells subjected to cold storage in UW alone were compared to cells stored in UW with RB2 (15% vs 20%; $p = 0.06$) or RR (15% vs 18%; $p = 0.30$). There was also no difference in cell death immediately following HH storage (storage alone, 15%; RB2, 20%, $p = 0.13$; RR, 15%, $p = 0.89$). In addition, there was no difference in cell death during storage when the UW alone groups (NH and HH) were compared with each other (15% vs 15%; $p = 0.31$). Therefore differences detected in ATP levels between groups are not due to differences in cell death during the storage period. As an additional quality assurance measure, standardized ATP curves were performed in the presence of varying concentrations of RR and RB2. Neither RR nor RB2 caused absorbance of light which interfered with the luminescence assay.

For each group, 2×10^5 total cells of each group were resuspended in tricine buffer with 10 μL beetle luciferin. Luminescence was then measured. As demonstrated in Figure 5, cytosolic ATP levels were significantly reduced by both NH and HH storage conditions. When compared to untreated controls, NH storage reduced cytoplasmic ATP levels by 46% ($p = 0.0002$) and HH storage reduced cytoplasmic ATP levels by 61% ($p=0.0005$). In addition, HH storage resulted in a further decrease in cytoplasmic ATP levels when compared to NH ($p=0.05$).

When the cells were transfected with PcDNA3-COX8-luciferase (COX-8), NH and HH had slightly lower mitochondrial ATP levels than untreated controls. However, there was no statistically significant difference. This is also illustrated in Figure 5.

RB2 and RR treatment results in further cytoplasmic ATP depletion during both NH and HH storage

When compared to cells undergoing NH or HH storage alone, the addition of RB2 to the storage solution resulted in lower cytoplasmic ATP levels following the same period of storage (Figure 6). Cells undergoing NH storage with RB2 had 28% less cytosolic ATP than did cells undergoing NH storage alone ($p=0.003$). Similarly, cells undergoing HH storage with RB2 had 25% less cytosolic ATP than cells undergoing HH storage alone ($p=0.005$). In a similar manner, NH and HH storage with RR resulted in lower cytoplasmic ATP levels (16%, $p=0.028$; 17%, $p=0.032$) than cells subjected to cold storage alone (Figure 6). There was no significant difference in the cytoplasmic ATP levels between the RR and RB2 groups following either storage method.

Neither addition of RB2 nor RR to the cold storage solution had a significant effect on mitochondrial ATP levels.

Discussion

The experiments presented in this manuscript were designed to determine if P2 receptor antagonism had a cytoprotective role in cold ischemia and reperfusion and to elucidate potential mechanisms of these cytoprotective effects. Our results show that RB2, when added to the cold storage solution, attenuates a Bax-dependent cell death during the early rewarming period following cold storage. We have previously described that inhibition of mCa^{2+} uptake during cold ischemic storage results in cytoprotection via the same mechanism[33]. The cytoprotection

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afforded by RB2 in our model occurs in a similar time frame and to an almost identical extent as that provided by RR[33].

Neither RR nor RB2 had significant effects on *mitochondrial* ATP levels during cold storage when compared to untreated cells undergoing cold storage alone. We expected that by preventing mCa^{2+} uptake during the cold storage period RR would decrease the calcium driven stimulus for the mitochondria to increase ATP production, resulting in a more rapid depletion of the existing cytosolic ATP. As predicted, inhibition of mCa^{+2} uptake with RR resulted in decreased level of cytosolic ATP compared to cells undergoing storage alone. We observed an identical effect from purinergic inhibition with RB2 during the period of cold storage. The decreased cytoplasmic ATP levels along with the unchanged mitochondrial ATP levels suggests that cells stored with RR or RB2 have higher rates of total cellular ATP consumption than do cells subjected to cold storage alone. In cells subjected to cold storage in UW alone, the rate of cell death was higher than that of cells cold stored in UW with RR or RB2. Therefore, as cells die, the total ATP consumption of the population of cells studied decreases resulting in higher detectable ATP levels. For cells protected from apoptosis, hydrolysis of mitochondrial ATP is required at significant levels in order to maintain important cellular processes, such as preservation of $\Delta\Psi$. The ATP is supplied to the mitochondria at the expense of cytoplasmic ATP stores via reversal of the ANT[22]. Our observation suggests that more energy in the form of ATP is required to sustain cellular processes during ischemia than to undergo cell death.

A potential explanation for the similar results observed with RR and RB2 may be the recently documented presence of P2Y receptors in the mitochondrial membrane of hepatocytes[6,21]. Similar to the P2 receptors in the cell membrane, the mitochondrial receptor analogs play a role in the regulation of mCa^{2+} uptake, potentially via the hydrolysis of phosphatidylinositol 4,5-bisphosphate[6,44]. The existence of these receptors suggests a mechanism by which ATP and/or ADP may participate in an ATP feedback mechanism by regulating mCa^{2+} uptake[6,20,21]. If these P2Y-like receptors play a major role in the regulation of mCa^{2+} uptake, P2Y antagonists should convey a cytoprotection similar to that which has been demonstrated with RR. Together these data suggest, but do not prove, that in our cold ischemic model, RB2 is conveying cytoprotection in a manner similar to RR -- inhibition of mCa^{2+} uptake. To further strengthen this hypothesis, the failure of our model to stimulate ERK 1, 2 activation rules out suppression of ERK 1,2 activation as the mechanism of cytoprotection.

Recently, a non-purinergic mechanism of RB2 has been reported. RB2 has been observed to inhibit phosphatidylinositol 3-kinase (PI 3-K) in rat glioma cells [45]. The PI 3-K/AKT pathway is crucial to many aspects of cell growth and survival. Inhibition of PI 3-K leads to an increase of apoptosis, and this has been exploited as a potential target for cancer therapeutics [46,47]. Thus, if RB2 were affecting our cells via this mechanism, we would have expected an increase in cell death in RB2 treated cells rather than the decrease which was actually observed.

A final consideration regarding our observations is the argument that the findings of the TUNEL staining and ATP measurements presented in this manuscript can be explained by an increased rate of necrotic cell death during cold storage in the presence of RR or RB2. However, the trypan blue exclusion method was used to count cells for the ATP studies. This counting method revealed that neither RR nor RB2 storage resulted in significantly greater necrotic cell death than did storage alone. In our model of cold ischemic storage, Bax translocation to the mitochondria occurs during cold storage resulting in cytochrome c release and cellular apoptosis during the reperfusion period. Bax translocation and cellular apoptosis occurred at similar rates suggesting that the cell death noted in our study is Bax translocation dependent. The cell death induced in this study is attenuated at similar rates by RR and RB2. In addition, the cytoprotection afforded by RR and RB2 occurs *despite* cytosolic ATP consumption beyond that of untreated control cells.

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In summary, the data presented here demonstrate that neither purinergic antagonism nor inhibition of mCa^{2+} uptake during the cold storage period maintains cytoplasmic ATP levels. While it is clear that cold storage with RR or RB2 results in an increase in total cellular utilization of ATP during the storage period, at this point it remains unclear if inhibition of mCa^{2+} uptake allows the cell to use ATP more efficiently or if mitochondrial calcium uptake itself is a specific trigger for cell death independent of ATP levels.

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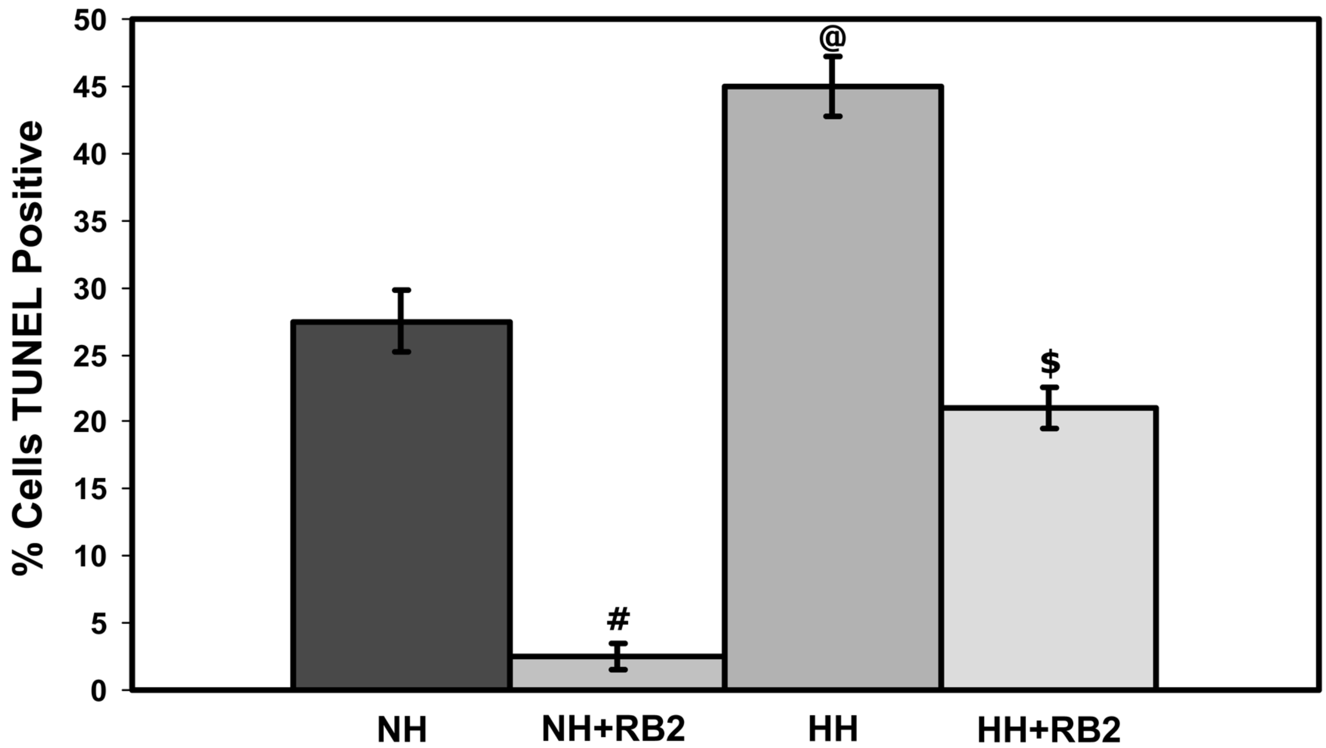

Figure 1.

TUNEL results following hypothermic storage with RB2 reported as percentage of cells TUNEL positive per high power field. Following NH storage, 27.5% of cells are TUNEL positive after 180 min of rewarming. Following HH storage, the percentage of apoptotic cells increases significantly to 45% (@; p=0.034). Addition of RB2 to the UW storage solution decreases the rate of cellular apoptosis to 2.5% (#, p=0.001) and 21% ($, p=0.001) following NH and HH storage respectively.

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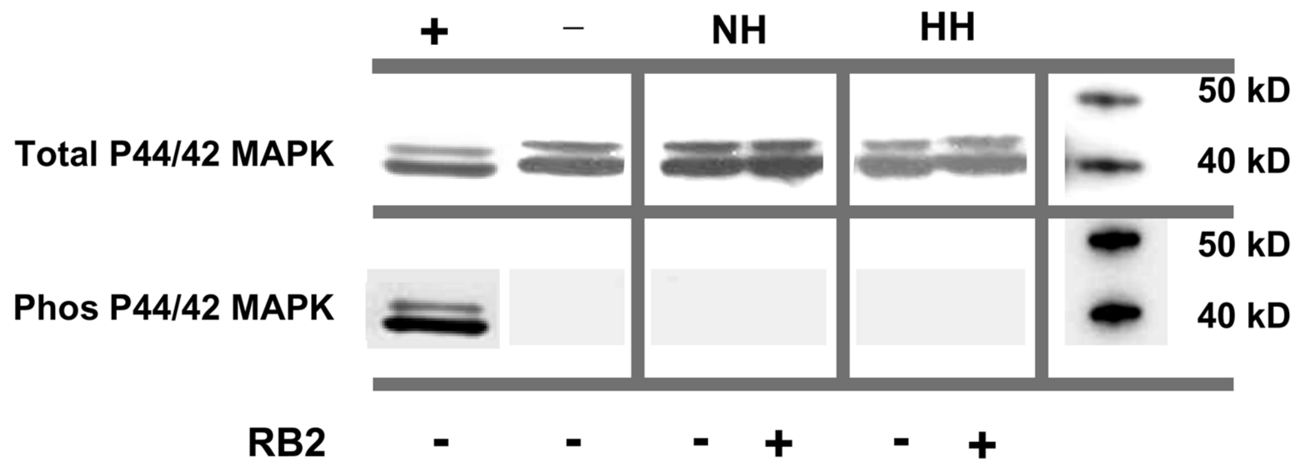

Figure 2.
Western analysis demostrating ERK 1,2 epression levels and ERK 1,2 posphorylation states. Neither ERK 1, 2 expression levels nor phosphorylation states were altered by our experimental conditions. Positive and negative controls are shown.

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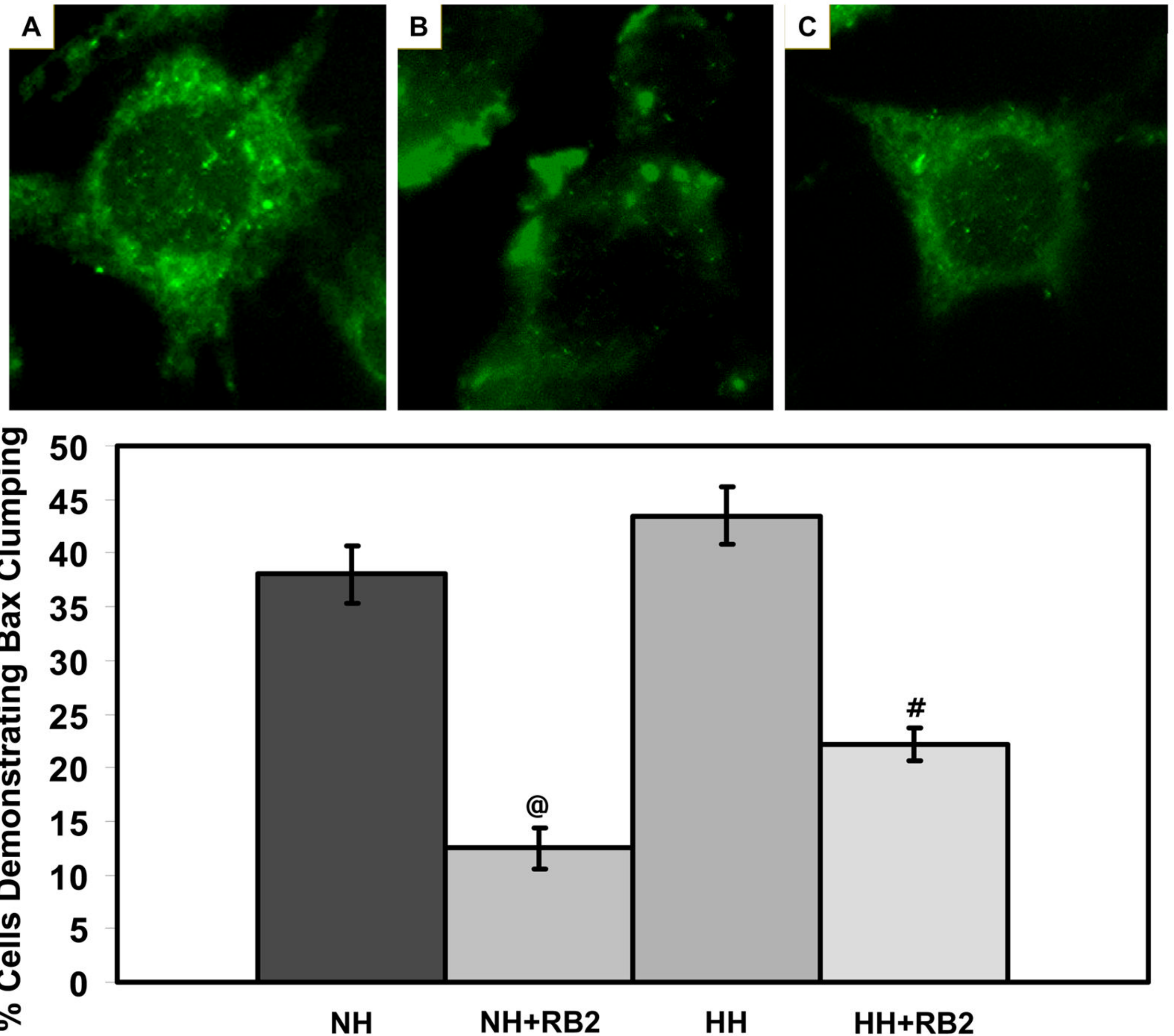

Figure 3.
Representative examples of Bax immunofluorescence following hypothermic storage with and without RB2 treatment. (A) In the untreated cell, Bax staining occurs diffusely throughout the cytoplasm. (B) Following cold storage, Bax translocation to the mitochondria results in a punctate pattern of immunofluorescence staining. This photo is representative of the similar patterns were observed for both NH and HH storage. (C) Cold storage (NH or HH) in UW containing RB2 attenuates this mitochondrial translocation and preserves the diffuse cytoplasmic staining pattern. The graft reports percentage of cells per high power field demonstrating punctate Bax staining. Following NH storage, approximately 35% of cells demonstrate punctate staining. This increased significantly to 45% following HH storage. Addition of RB2 to the UW storage solution significantly decreased the rate of punctate Bax staining to 12% (@, p=0.001) following NH storage and 22% (#, p=0.009) following HH storage.

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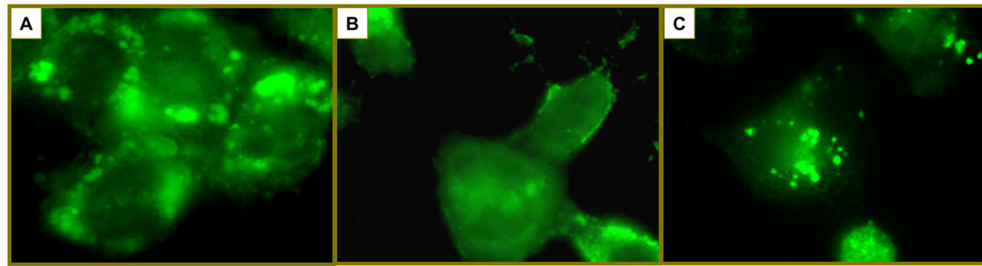

Figure 4.

Representative photomicrographs showing cytochrome c-GFP distribution. A: In untreated cells, cytochrome c-GFP is visualized in punctate groupings in the cytoplasm, indicating association with mitochondria. B: Following 6 hr of cold storage and 3hr of reperfusion, GFP is widely distributed in the cytoplasm. C: RB2 treatment attenuates this occurrence.

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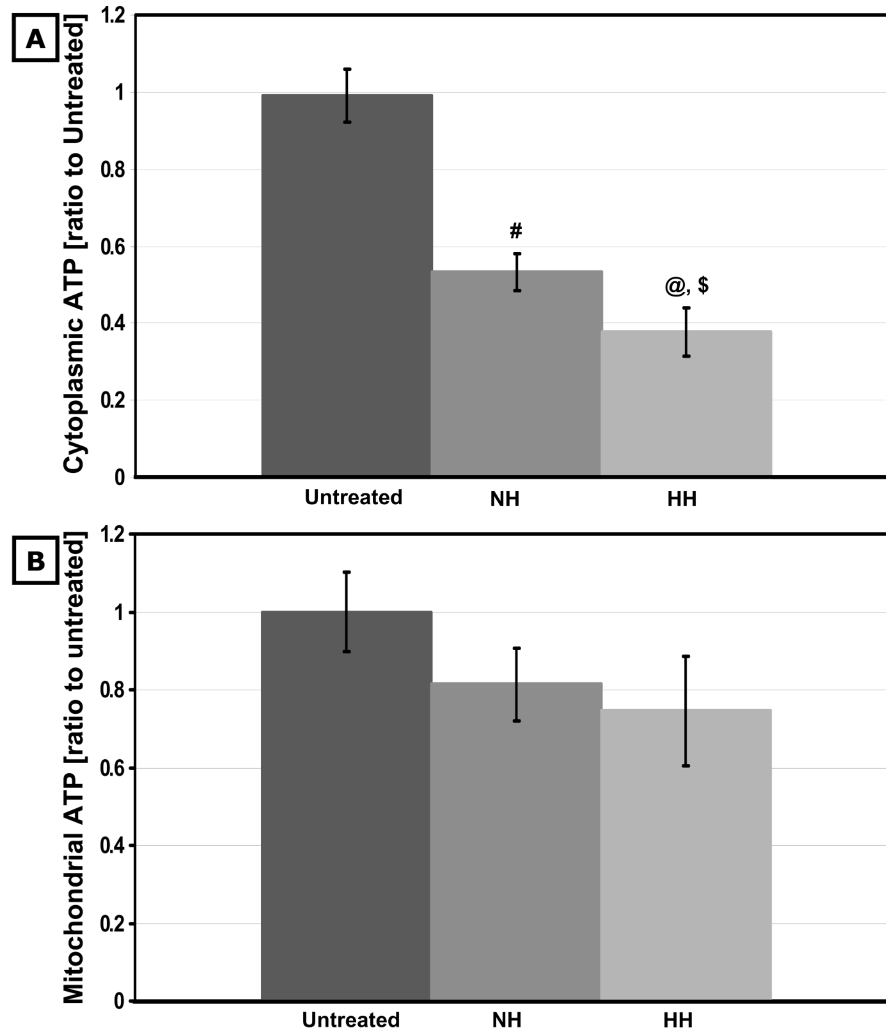

Figure 5.

Cellular compartmental ATP levels following hypothermic storage. (A) When compared to untreated controls, NH storage reduced cytoplasmic ATP levels by 46% (#, p = 0.0002) and HH storage reduced cytoplasmic ATP levels by 61% (@, p=0.0005). In addition, HH storage resulted in a further decrease in cytoplasmic ATP levels when compared to NH ($, p=0.05). (B) Mitochondrial ATP levels were slightly lower following NH and HH storage. However, there was no statistically significant difference compared to untreated controls.

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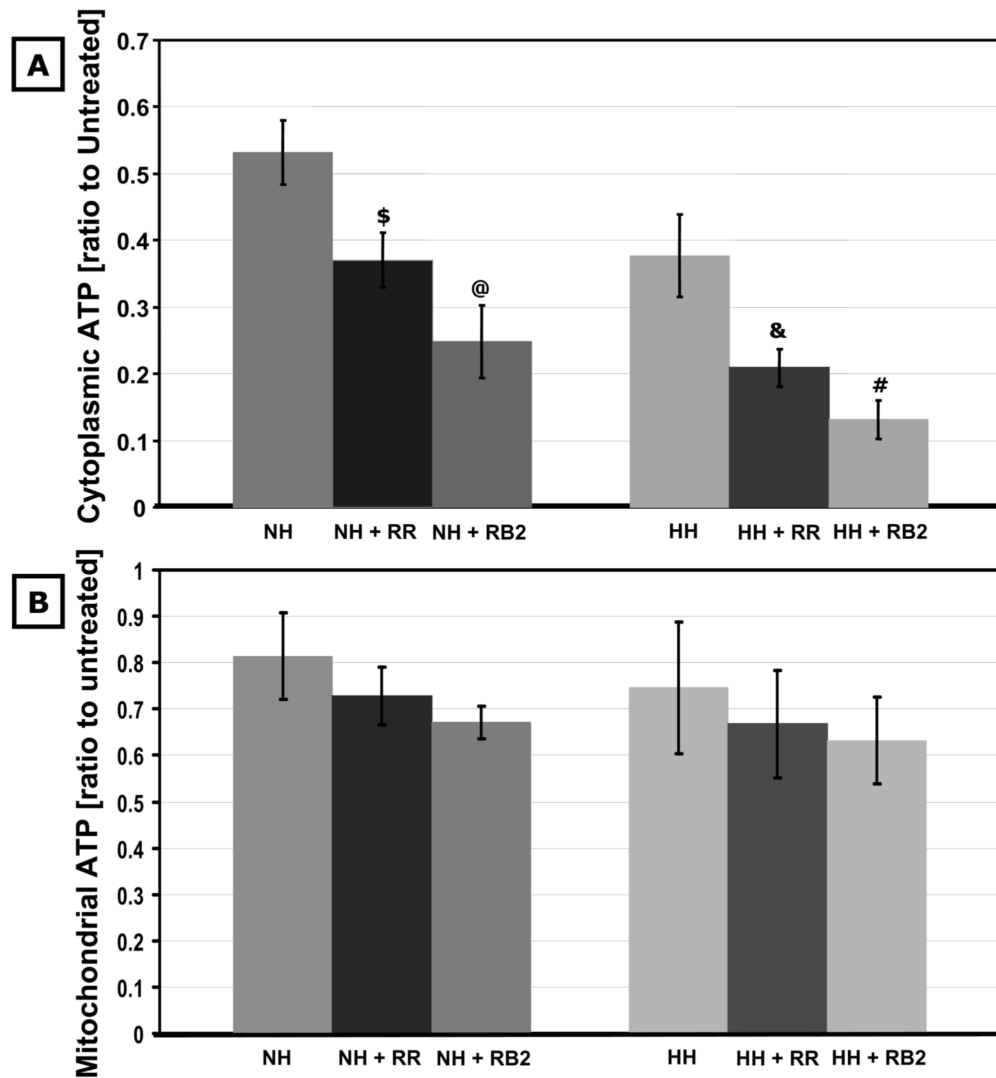

Figure 6.

Storage with RB2 or RR results in further decreases in cytoplasmic ATP levels. (A) Addition of RB2 to the storage solution results in 28% less cytosolic ATP when compared to cells undergoing NH storage alone (@, p=0.003). Similarly, cells undergoing HH storage with RB2 had 25% less cytosolic ATP than cells undergoing HH storage alone (#, p=0.005). NH and HH storage with RR also resulted in lower cytoplasmic ATP levels [($, 16%, p=0.028);(&, 17%, p=0.032)] than cells in stored without RR treatment. (B) Neither addition of RB2 nor RR to the cold storage solution had a significant effect on mitochondrial ATP levels.