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Cardioprotection by modulation of mitochondrial respiration during ischemia–reperfusion: Role of apoptosis-inducing factor

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ABSTRACT

The transient, reversible blockade of electron transport (BET) during ischemia or at the onset of reperfusion protects mitochondria and decreases cardiac injury. Apoptosis inducing factor (AIF) is located within the mitochondrial intermembrane space. A release of AIF from mitochondria into cytosol and nucleus triggers caspase-independent cell death. We asked if BET prevents the loss of AIF from mitochondria as a mechanism of protection in the buffer perfused heart. BET during ischemia with amobarbital, a rapidly reversible inhibitor of mitochondrial complex I, attenuated a release of AIF from mitochondria into cytosol, in turn decreasing the formation of cleaved and activated PARP-1. These results suggest that BET-mediated protection may occur through prevention of the loss of AIF from mitochondria during ischemia–reperfusion. In order to further clarify the role of mitochondrial AIF in BET-mediated protection, Harlequin (Hq) mice, a genetic model with mitochondrial AIF deficiency, were used to test whether BET could still decrease cell injury in Hq mouse hearts during reperfusion. BET during ischemia protected Hq mouse hearts against ischemia–reperfusion injury and improved mitochondrial function in these hearts during reperfusion. Thus, cardiac injury can still be decreased in the presence of down-regulated mitochondrial AIF content. Taken together, BET during ischemia protects both hearts with normal mitochondrial AIF content and hearts with mitochondrial AIF deficiency. Although preservation of mitochondrial AIF content plays a key role in reducing cell injury during reperfusion, the protection derived from the BET is not fully dependent on AIF-driven mechanisms.

1. Introduction

Cardiac ischemia damages the mitochondrial electron transport chain (ETC) leading to greater myocardial injury following ischemia–reperfusion (IR) [1–3]. The transient blockade of electron transport (BET) at complex I by the reversible inhibitor amobarbital protects mitochondria against IR damage and decreases cardiac injury in buffer perfused hearts [3,4]. BET has been shown to improve oxidative phosphorylation [3,5], maintain the bcl-2 content [5], and decrease the opening of the mitochondrial permeability transition pore (MPTP) [5] in mitochondria following ischemia–reperfusion that leads to decreased release of cytochrome *c* from mitochondria during IR.

Apoptosis inducing factor (AIF) is a nuclear encoded flavoprotein that is located within the mitochondrial intermembrane space and is attached to the inner mitochondrial membrane [6–9]. AIF exhibits a pro-survival role within the mitochondrial intermembrane space via its potential antioxidant properties. In contrast, the release of AIF from mitochondria into the cytosol followed by translocation into the nucleus increases cell death by inducing chromatin condensation and DNA fragmentation in a caspase-independent manner [7,9,10]. A lower expression of mitochondrial AIF in Harlequin (Hq) mice increases myocardial injury following IR *in vivo* [11], indicating that mitochondrial AIF deficiency augments myocardial injury. In contrast, preservation of mitochondrial AIF content by administering a calpain inhibitor decreases cardiac injury during reperfusion *in vitro*, suggesting that preservation of mitochondrial AIF content is protective [12]. We asked if BET during ischemia could preserve mitochondrial AIF content during IR. The release of cytochrome *c* from mitochondria into cytosol triggers caspase-dependent programmed cell death [13], whereas relocation of AIF from mitochondria into cytosol activates caspase-independent death mechanisms [14]. We propose that BET will prevent the activation of both caspase-dependent and independent cell death by preventing the release of cytochrome *c* and AIF from mitochondria into cytosol.

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In order to further address the role of mitochondrial AIF content in BET-mediated protection during IR, Hq mice with mitochondrial AIF deficiency were used in the present study. If the cardioprotective mechanism of BET is largely due to attenuation of IR-induced AIF release from mitochondria, then Hq mice should derive no additional protection from treatment with amobarbital immediately before ischemia. Surprisingly, administration of amobarbital before ischemia still protected mitochondria and decreased cardiac injury in buffer perfused Hq mouse hearts following IR, indicating that the BET-mediated protection is not solely through preservation of mitochondrial AIF content nor attenuation of mitochondrial AIF release.

2. Methods

2.1. Animal models and isolated, perfused heart preparation

The experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of Virginia Commonwealth University and the McGuire Department of Veterans Affairs Medical Center.

Male rats (Fischer 344, 6 mo. age) were obtained from Harlan (Indianapolis, IN) [3]. Male Hq mice (B6CBACaA$^{w\text{-}j}$/A-Pdc8^{Hq}/J) [11] were obtained from Jackson Laboratories (Bar Harbor, Maine) and maintained by breeding. Rats or mice were anesthetized with pentobarbital sodium (100 mg/kg i.p.) and anti-coagulated with heparin (1000 IU/kg i.p.) [15]. Hearts were excised and retrograde perfused via the aorta in the Langendorff mode [3,12]. In untreated hearts, the heart (mouse or rat) was buffer-perfused for 15 min followed by 25 min global ischemia at 37 °C and 30 min of reperfusion. In BET-treated hearts, hearts followed the same perfusion protocol except that amobarbital [15] was perfused in identical Krebs–Henseleit buffer for 1 min before ischemia [3]. The LDH (lactate dehydrogenase) content was determined in freshly collected coronary effluent samples during 30 min reperfusion as an index of cardiac injury [3,12].

2.2. Isolation and study of cardiac mitochondria

Subsarcolemmal mitochondria (SSM) were isolated from buffer perfused rat hearts [15,16], and a mixed population of mitochondria were isolated from single mouse heart [17]. Oxidative phosphorylation in freshly isolated mitochondria was measured using a Clark-type oxygen electrode at 30 °C [16]. Glutamate (20 mM) + malate (10 mM) (complex I substrate), succinate (20 mM) plus 7.5 uM rotenone (complex II substrate) and TMPD (N,N,N′,N′ tetramethyl p-phenylenediamine, 1 mM)-ascorbate (10 mM, complex IV substrate via cytochrome *c*) +7.5 uM rotenone were used as electron donors to specific sites in the ETC. The net release of H_2O_2 from isolated mitochondria was measured using the oxidation of the fluorogenic indicator amplex red in the presence of horseradish peroxidase [18]. The maximal production of H_2O_2 from complex I was measured when glutamate + malate was used as the complex I substrate in the presence of rotenone. Antimycin A inhibition was used to generate maximal H_2O_2 release from complex III when succinate + rotenone was the substrate [18].

2.3. Western blotting

Proteins were first separated using 4–15% gradient Bis-Tris gels and then transferred to PVDF membranes (Millipore, Billerica, MA) using semi-dry transfer (Bio-Rad, Hercules, CA) [12]. Anti-cytochrome *c* antibody was purchased from Invitrogen (Grand Island, NY) and anti-tubulin from Sigma (St. Louis, MO). Other primary antibodies were purchased from Cell Signaling (Danvers, MA).

2.4. Statistical analysis

Data were expressed as the mean ± standard error of the mean. Differences between two groups were compared by unpaired student *t*-test, and differences between three groups were compared by one-way ANOVA. The Student-Neuman–Keuls test was used to test the significance of multiple comparisons among groups (*Sigmastat 3.5*, Gothenburg, Sweden). A difference of $p < 0.05$ was considered significant.

3. Results

3.1. BET during ischemia by amobarbital treatment preserved the mitochondrial AIF content and inhibited the activation of PARP-1 in cytosol in rat hearts following IR

Amobarbital (2 mM) [15] given before ischemia decreased LDH content in coronary effluent [mean ± SEM: untreated hearts following IR 620 ± 65 (mU/mg/min), $n = 8$; Amobarbital + IR, 130 ± 40*, $n = 5$; *$p < 0.05$ vs. untreated hearts] and improved cardiac function [mean ± SEM: untreated hearts 55 ± 7 (mmHg), $n = 8$; Amobarbital + IR, 109 ± 15*, $n = 5$. *$p < 0.05$ vs. untreated hearts] during reperfusion, supporting that BET during ischemia reduced cardiac injury in buffer perfused rat hearts [3].

The mitochondrial AIF content was dramatically decreased in mitochondria from rat hearts following IR compared to time control hearts, whereas BET preserved mitochondrial AIF content (Fig. 1, Panel A). The t-AIF content (truncated AIF) within mitochondria was also decreased following IR. BET during ischemia prevented the loss of t-AIF from mitochondria (Fig. 1, Panel A).

IR led to a decrease in the content of full length of PARP-1 in cytosol and an increase in the content of cleaved PARP-1 in cytosol compared to time controls (Fig. 1, Panel B), indicating that IR activates PARP-1. BET, in contrast, preserved the content of full length PARP-1 and decreased the formation of truncated PARP-1 during IR (Fig. 1, Panel B), providing evidence that BET during ischemia prevents PARP activation during IR.

3.2. BET during ischemia decreased the release of cytochrome c from mitochondria and prevented the activation of caspase 3 in rat heats following IR

The content of cytochrome *c* increased in the cytosol following IR compared to time control hearts (Fig. 3, Panels A & B), in line with the previously observed decrease in cytochrome *c* content within mitochondria [3,15]. Although the content of full length caspase 3 was not decreased following IR (data not shown), the content of cleaved caspase 3 present in cytosol was significantly increased (Fig. 2, Panels A & B). BET during ischemia dramatically decreased the formation of the cleaved caspase 3 in rat heart cytosol following IR (Fig. 2, Panels A & B). The decrease in cytochrome *c* content in cytosol and the reduced formation of cleaved caspase 3 suggest that BET decreases activation of the intrinsic pathway caspase-dependent programmed cell death pathway in buffer perfused rat hearts following IR.

2.3. Ischemia alone did not decrease the contents of AIF and t-AIF within rat heart mitochondria

Ischemia did not lead to decreased contents of AIF and t-AIF in rat SSM compared to time control (Fig. 3). These results suggest that a release of AIF or t-AIF from mitochondria occurs during

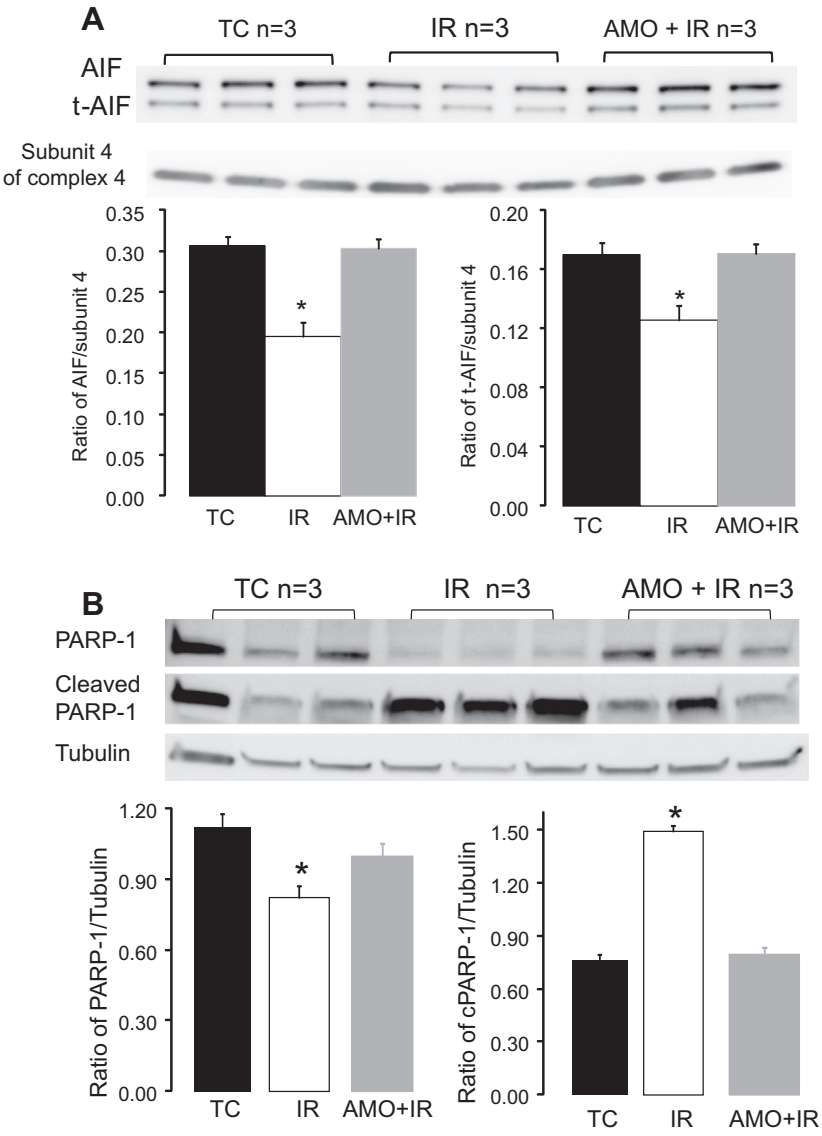

Fig. 1. Blockade of electron transport preserved the AIF content within mitochondria and decreased the formation of cleaved PARP-1 following ischemia-reperfusion (IR). *Panel A (mitochondria):* IR decreased the contents of AIF and t-AIF in rat heart mitochondria following IR compared to time control (TC), whereas blockade of electron transport before ischemia with amobarbital (AMO) maintained AIF within mitochondria. Subunit 4 of cytochrome oxidase was used as a mitochondrial protein loading control. *Panel B (cytosol):* IR led to a decrease in full length PARP-1 and increased the formation of cleaved PARP-1 compared to the time control group, indicating that IR activated PARP-1 in the cytosol. Blockade of electron transport before ischemia with amobarbital (AMO) preserved the content of full length PARP-1 and decreased the formation of the cleaved PARP-1 measured following reperfusion, indicating that blockade of electron transport only during ischemia prevented PARP-1 activation during IR. Tubulin was used as a cytosolic protein loading control. Data are expressed as mean ± SEM; $^{*}p < 0.05$ vs. TC and IR, p = NS TC vs. AMO, $n = 3$ in each group.

reperfusion rather than during ischemia. Ischemia markedly decreased cytochrome *c* content in SSM compared to time control (Fig. 3), consistent with previous studies that showed cardiac ischemia led to mitochondrial cytochrome *c* loss in buffer perfused rat hearts.

3.4. BET during ischemia by amobarbital treatment decreased myocardial injury in Hq mice during IR

Although amobarbital (2 mM) was an optimal dose to protect rat hearts during IR [3], amobarbital (1 mM) provided the best protection in buffer perfused mouse hearts during IR based upon preliminary experiments (data not shown). Amobarbital (1 mM) given before ischemia improved cardiac function and decreased myocardial injury in C57BL/6 mouse hearts during IR (Supplemental Table 1). Therefore, the same dose of amobarbital was used to test if BET during ischemia could protect Hq mouse hearts during IR. BET before ischemia significantly improved left ventricular developed pressure (LVDP) in Hq mouse hearts during reperfusion compared to untreated hearts (Fig. 4A). The LDH release into coronary effluent during the 30 min reperfusion period was markedly decreased in amobarbital-treated hearts compared to untreated hearts (Fig. 4B), supporting that BET during ischemia decreased cardiac injury in the Hq mouse heart during reperfusion.

2.5. BET during ischemia protected Hq mouse heart mitochondria during IR

IR decreased the maximal ADP (2 mM) stimulated rate of oxidative phosphorylation in Hq mouse heart mitochondria following IR compared to non-ischemic time control perfused hearts when glu-

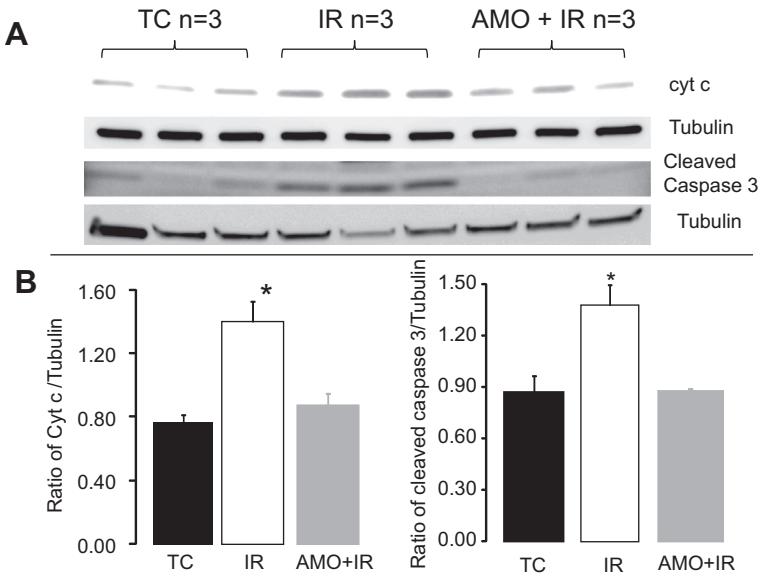

Fig. 2. Blockade of electron transport during ischemia decreased the release of cytochrome c into cytosol with less formation of cleaved caspase 3 in the rat heart following ischemia-reperfusion (IR). *Panel A:* IR led to cytochrome *c* release from mitochondria into cytosol as shown by the increased cytochrome *c* content compared to time control (TC). The cytochrome *c* content in cytosol was decreased in amobarbital (AMO) treated hearts, indicating less cytochrome *c* release from mitochondria. Greater cytochrome *c* release during IR increased the formation of the cleaved caspase 3 in cytosol, whereas the content of cleaved caspase 3 was decreased in AMO-treated hearts. Tubulin was used as a cytosolic protein loading control. *Panel B:* quantification of the cytochrome *c* content and the cleaved caspase 3 in cytosol. Data are expressed as mean ± SEM; $^{*}p < 0.05$ vs. TC and IR, p = NS TC vs. AMO, $n = 3$ in each group.

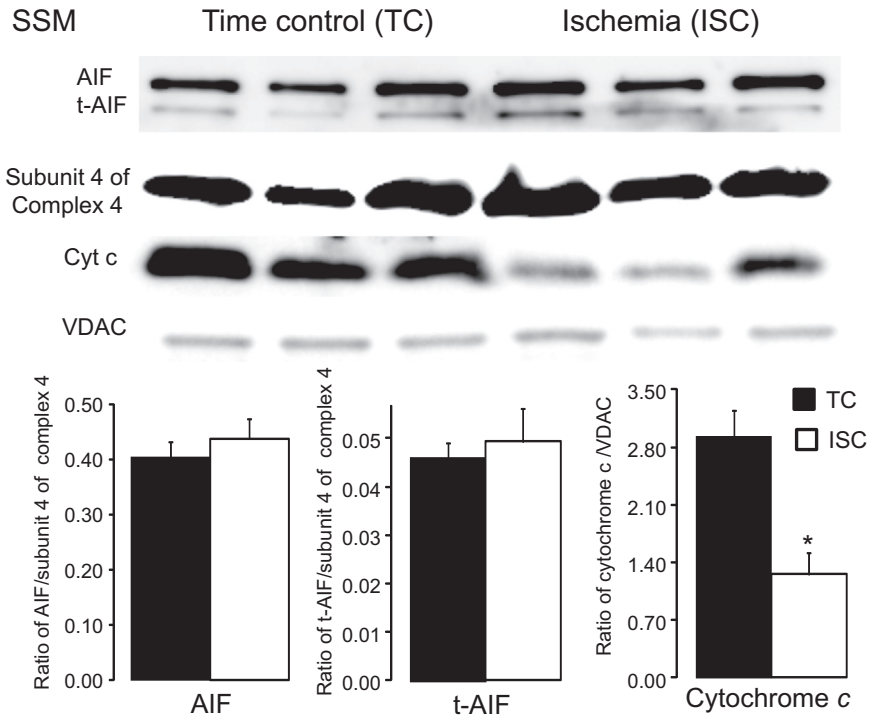

Fig. 3. Ischemia led to decreased cytochrome c content without alteration of AIF and t-AIF contents in rat heart mitochondria. Ischemia alone did not lead to decreased contents of AIF and t-AIF in rat SSM compared to time control (Fig. 3). However, ischemia markedly decreased cytochrome *c* content in SSM compared to time control (Fig. 3), consistent with previous studies that showed cardiac ischemia led to mitochondrial cytochrome *c* loss in buffer perfused rat hearts. Subunit 4 of complex IV was used as a mitochondrial protein loading control for AIF and t-AIF. VDAC (voltage dependent anion channel) was used as a loading control for cytochrome *c*. Mean ± SEM: $^{*}p < 0.05$ vs. time control, $n = 3$ in each group.

tamate + malate was used as the complex I substrate (Fig. 4C). The rate of oxidative phosphorylation was not altered in Hq mouse heart mitochondria following IR compared to time controls when succinate + rotenone (complex II, Fig. 4D) or TMPD-ascorbate were used as substrates [mean ± SEM: Time control, 1059 ± 64; IR, 1005 ± 76; amobarbital + IR, 1165 ± 24 (nAO/min/mg). p = NS, n = 4–5 in each group]. Thus, IR resulted in a defect in the proximal ETC segment in Hq mouse heart mitochondria. Compared to untreated hearts after IR, BET during ischemia significantly improved the rate of oxidative phosphorylation measured using glutamate + malate as a complex I substrate (Fig. 3A), suggesting that BET protected the proximal ETC against IR-mediated damage.

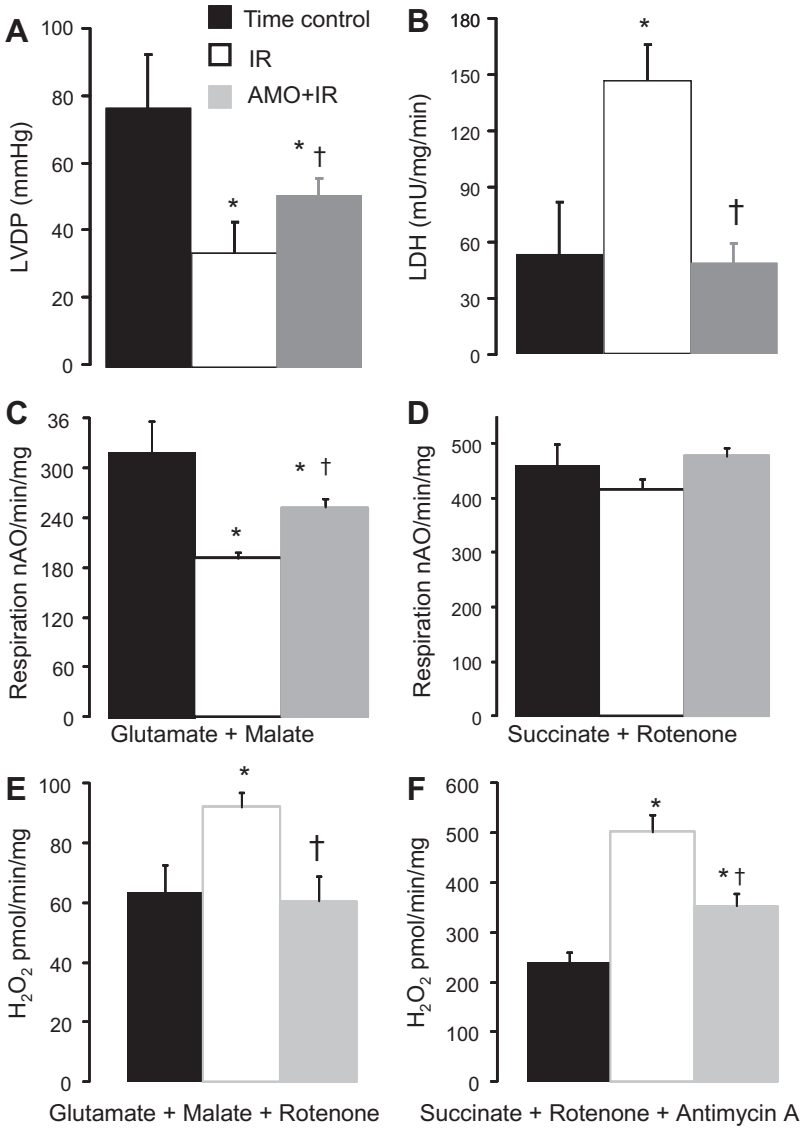

Fig. 4. Blockade of electron transport decreased cardiac injury and improved mitochondrial function in the Harlequin mouse heart following ischemia-reperfusion (IR). *Panels A & B:* Amobarbital (1 mM) treatment before ischemia markedly improved left ventricular developed pressure (LVDP) during reperfusion compared to the untreated hearts (Panel A). Amobarbital given before ischemia significantly decreased the LDH release from the Harlequin mouse heart compared to untreated IR, supporting that blockade of electron transport during ischemia protected the Harlequin heart during reperfusion. Data are expressed as mean ± SEM; $^{*}p < 0.05$ vs. Time control, $^{\dagger}p < 0.05$ vs. ISC-REP, $n = 5–8$ in each group. *Panels C & D:* Compared to the untreated hearts, amobarbital (AMO) treatment immediately before ischemia improved the maximal rate of oxidative phosphorylation in mitochondria following IR using glutamate + malate (Panel C) as substrate. In contrast, in the Harlequin mouse heart the rate of respiration with succinate (+rotenone) as substrate was unaltered by IR (Panel D). *Panels E & F:* The maximal release of H_2O_2 from mitochondria was increased in Harlequin mouse heart mitochondria following IR compared to control hearts when glutamate + malate (Panel E) or succinate (+rotenone) were used as substrates (Panel F). Blockade of electron transport decreased the maximal H_2O_2 generation using both substrates (Panels E, F). Mean ± SEM; $^{*}p < 0.05$ vs. time control, $^{\dagger}p < 0.05$ vs. ISC-REP, $n = 4–5$ in each group.

In wild type mice, IR led to decreased t-AIF content in mitochondria accompanied by increased t-AIF content in cytosol compared to time control (Supplemental Fig. 1). AIF content was not altered in wild type following IR vs. time control. In time control hearts, AIF content was significantly decreased in Hq mouse heart mitochondria compared to wild type. IR did not led to a further decrease of AIF from mitochondria in Hq mouse heart mitochondria vs. time control. The t-AIF was not detected in Hq mouse heart mitochondria, but IR did lead to slightly increased t-AIF content in cytosol in Hq mouse heart compared to time control (Supplemental Fig. 1) Cytosolic t-AIF content in Hq mouse heart following IR was much lower than that in wild type mouse heart following IR (Supplemental Fig. 1).

IR increased the maximal capacity for H_2O_2 production and release from Hq mitochondria measured using complex I substrates (Fig. 4E). The maximal production and net release of H_2O_2 was assessed using glutamate + malate as substrate in the presence of rotenone, an irreversible inhibitor of complex I [19]. Amobarbital treatment immediately before ischemia also decreased the release of H_2O_2 compared to mitochondria from untreated IR hearts (Fig. 3E). Using succinate + rotenone as substrate in the presence of antimycin A, a complex III Q_i center inhibitor, to generate maximal net H_2O_2 release dependent upon complex III [18], release was also substantially decreased in mitochondria isolated from amobarbital-treated hearts following IR (Fig. 3F).

4. Discussion

Blockade of electron transport using amobarbital before ischemia decreases myocardial infarct size by protecting mitochondrial

function and decreasing ROS production [3]. Here we show that BET prevents the release of AIF from mitochondria and leads to decreased activation of PARP-1, the latter known to induce caspase-independent cell death [14,20]. In addition, a decrease in the release of cytochrome *c* from mitochondria into cytosol reduces the formation of cleaved caspase 3 in cytosol, supporting that BET during ischemia decreases caspase-dependent cell death during reperfusion. Thus, BET during ischemia not only protects mitochondrial function [2,3,15], it also favors the mitochondrial retention of key peptide mediators of cell death, AIF and cytochrome *c*, leading to attenuation of both caspase-dependent and caspase-independent death pathways during IR. Blockade of electron transport during ischemia prevents the damage to mitochondria [2,15] that results in decreased mitochondrial-driven cardiomyocyte death during reperfusion [2,3], likely mediated at least in part via the attenuation of mitochondrial-driven cell death pathways as shown in the present study.

Most AIF is anchored on the mitochondrial inner membrane [7,8,20]. Activation of mitochondrial u-calpain cleaves the AIF to t-AIF that is then delocalized into the intermembrane space. Next, t-AIF is then released from mitochondria into cytosol when the permeability of outer mitochondrial membrane is increased [7,8,12]. Ischemia leads to a release of cytochrome *c* from rat heart mitochondria, but the contents of AIF and t-AIF are not decreased. The decreased AIF and t-AIF contents in rat heart mitochondria following IR suggests that reperfusion leads to the loss of AIF and t-AIF from mitochondria. Calpain is activated during reperfusion rather than during ischemia [21]. The loss of AIF and t-AIF from mitochondria that occurs during reperfusion but not during ischemia suggests that activation of mitochondrial calpain contributes to the release of AIF and t-AIF from mitochondria into cytosol [12]. Amobarbital given before ischemia preserves the mitochondrial AIF content, indicating that BET during ischemia may prevent the mitochondrial u-calpain activation. The permeability of the outer mitochondrial membrane is increased in buffer perfused hearts following IR [3,16], whereas amobarbital treatment before ischemia leads to decreased permeation of outer mitochondrial membrane during reperfusion, at least in part by preventing MPTP opening [5]. The preserved contents of AIF and cytochrome *c* in mitochondria isolated from amobarbital-treated hearts support that BET during ischemia maintains the integrity of the outer mitochondrial membrane [5].

AIF relocates to the nucleus once it is released from mitochondria into cytosol [12,22]. Relocation of AIF results in chromatin condensation and DNA fragmentation that induces caspase-independent apoptosis [22]. The DNA fragmentation induced by AIF relocation to the nucleus can activate PARP-1 (a nuclear DNA repair enzyme), which ligates NAD^+ onto poly(ADP-ribose) (PAR), and increases the formation of the cleaved PARP-1 [7,23]. Amobarbital treatment preserves the mitochondrial AIF content in association with less cleaved PARP-1 formation during reperfusion, suggesting that BET can reduce caspase-independent cell death by preventing the loss of AIF from rat heart mitochondria during reperfusion.

In Harlequin mouse heart mitochondria, AIF content is significantly decreased compared to wild type heart mitochondria [11,24] (Supplemental Fig. 1). Cardiac injury is increased in Hq mouse hearts following *in vivo* IR [11], supporting that mitochondrial AIF content is critical to cardiac protection during IR. In the present study, amobarbital treatment leads to decreased cardiac injury in buffer perfused Harlequin mouse hearts. Oxidative phosphorylation is also improved in amobarbital-treated Hq mouse heart mitochondria following IR. The protection of the ETC by amobarbital results in a decrease in the net release of H_2O_2 from Hq mouse heart mitochondria. Thus, BET during ischemia decreases myocardial injury by protecting mitochondria and reducing oxidative stress during reperfusion even in Hq mouse hearts with reduced mitochondrial AIF content. ETC damage from ischemia is positioned upstream of cytochrome *c* and AIF release during IR. Taken together, BET during ischemia protects hearts independent of mitochondrial AIF content and the extent of mitochondrial AIF release. BET-mediated protection is not solely dependent on the attenuation of mitochondrial AIF release and the prevention of activation of caspase-independent cell death programs. These results highlight that prevention of a release of cytochrome *c* from mitochondria and subsequent activation of caspase cascades plays a critical role in BET-mediated cardioprotection.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.033.

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