Biochemical and Biophysical Research Communications 435 (2013) 627–633

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 6 May 2013
Available online 16 May 2013

Keywords:
Electron transport chain
Amobarbital
PARP-1
Caspase 3
Apoptosis
Harlequin mice

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.

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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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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.05.033

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

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