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HDAC1 localizes to the mitochondria of cardiac myocytes and contributes to early cardiac reperfusion injury

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ABSTRACT

Rationale: Recent evidence indicates that histone deacetylase enzymes (HDACs) contribute to ischemia reperfusion (I/R) injury, and pan-HDAC inhibitors have been shown to be cardioprotective when administered either before an ischemic insult or during reperfusion. We have shown previously that selective inhibition of class I HDACs provides superior cardioprotection when compared to pan-HDAC inhibition in a pretreatment model, but selective class I HDAC inhibition has not been tested during reperfusion, and specific targets of class I HDACs in I/R injury have not been identified.

Objective: We hypothesized that selective inhibition of class I HDACs with the drug MS-275 (entinostat) during reperfusion would improve recovery from I/R injury in the first hour of reperfusion.

Methods and results: Hearts from male Sprague-Dawley rats were subjected to *ex vivo* I/R injury ± MS-275 class I HDAC inhibition during reperfusion alone. MS-275 significantly attenuated I/R injury, as indicated by improved LV function and tissue viability at the end of reperfusion. Unexpectedly, we observed that HDAC1 is present in the mitochondria of cardiac myocytes, but not fibroblasts or endothelial cells. We then designed mitochondria-restricted and mitochondria-excluded HDAC inhibitors, and tested both in our *ex vivo* I/R model. The selective inhibition of mitochondrial HDAC1 attenuated I/R injury to the same extent as MS-275, whereas the mitochondrial-excluded inhibitor did not. Further assays demonstrated that these effects are attributable to a decrease in SDHA activity and subsequent metabolic ROS production in reperfusion.

Conclusions: We demonstrate for the first time that HDAC1 is present within the mitochondria of cardiac myocytes, and mitochondrial HDAC1 contributes significantly to I/R injury within the first hour of reperfusion.

1. Introduction

Lysine acetylation is a reversible post-translational protein modification that occurs on a multitude of proteins and regulates vital cellular processes including metabolism [1–3], cell cycle regulation [4,5], chromatin remodeling [6,7], nuclear transport [4,8–10], and autophagy [11–14], among others. It was initially proposed [15], and later confirmed that acetylation of histone tails regulates gene expression by loosening chromatin compaction and allowing transcription factors to bind DNA [16]. Eventually, non-histone protein acetylation was also discovered, starting with p53 [17] and expanding to non-histone proteins throughout the cell [18]. As the field has expanded, it has come to be appreciated that the acetylation state of non-histone proteins and enzymes can dramatically affect characteristics of the acetylated proteins, including protein stability [19,20], enzymatic activity [21,22], DNA binding [23], and intracellular localization [24]. These changes in protein characteristics can have a profound effect on cellular processes, including those determining the fate of cells subjected to injury.

Lysine acetylation occurs *via* the addition of an acetyl group to the Ɛ-amino moiety of lysine residues in a reaction catalyzed by histone acetyltransferases (HATs). The removal of acetyl groups, termed

Abbreviations: HAT, histone acetyltransferase enzyme; HDAC, histone deacetylase enzyme; I/R, ischemia reperfusion injury; dP/dt_{max}, rate of LV pressure generation; $-dP/dt_{max}$, rate of LV pressure relaxation; TTC, 2,3,5-triphenyltetrazolium chloride; mPTP, mitochondrial permeability transition pore; sI/R, simulated ischemia reperfusion injury *in vitro*; OCR, oxygen consumption rate; AMI, acute myocardial infarction; PCI, primary percutaneous coronary intervention

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deacetylation, is achieved by histone deacetylases (HDACs). HDACs consist of 4 classes, delineated based on their similarity to histone deacetylase enzymes in yeast. HDACs 1, 2, 3, and 8 comprise the class I HDACs. Class II HDACs are subgrouped into class IIa (HDACs 4, 5, 7, and 9), and class IIb HDACs (HDACs 6 and 10). Class III HDACs are the sirtuin family, differentiated from the other classes because they use NAD^+ as a cofactor. HDAC11 is the sole known class IV HDAC. In the last two decades, the function of HDACs and their promise as a treatment target in cardiovascular disease has become a topic of great interest in cardiovascular research [25,26]. Accordingly, the pharmacological inhibition of HDAC activity has been shown to be beneficial in animal models of multiple cardiac pathologies, though these discoveries have yet to be translated to the clinic.

Ischemic heart disease is the leading cause of death worldwide and the majority of these deaths are due to acute myocardial infarction [27]. Treatment for AMI is reperfusion *via* primary percutaneous coronary intervention (PCI), or coronary angioplasty [28]. Although PCI and angioplasty limit ischemic injury, reperfusion itself injures the heart by increasing ROS. We and others have shown that HDAC inhibition preceding ischemia reperfusion (I/R) injury in the heart can preserve left ventricular function and myocardial survival [29–31]. Importantly, we demonstrated that the selective inhibition of class I HDACs was more effective than pan-HDAC inhibition in preserving myocardial viability and function in the setting of I/R injury. Recently, pan-HDAC inhibition solely during the reperfusion phase of I/R injury was shown to preserve myocardial function in multiple animal models, although the exact mechanisms behind this cardioprotection have yet to be fully elucidated [32,33].

Here, we utilized a Langendorff isolated heart model to test the hypothesis that selective pharmacological class I HDAC inhibition during the reperfusion phase alone would confer cardioprotection from I/R injury. In doing so, we discovered that HDAC1 localizes to the mitochondria of cardiac myocytes and modifies oxidative metabolism, contributing to ROS production and mitochondrial injury in the reperfusion phase of I/R injury.

2. Methods

2.1. Langendorff heart isolation

Rats weighing 300–400 g (Harlan, Frederick MD) were cared for in accordance with the National Institutes of Health (NIH) guidelines and those of the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina. Rats were anesthetized with ketamine/xylazine (85/15 mg/kg) *via* intraperitoneal injection. Following confirmation of anesthesia, rats were tracheotomized with a 16 g catheter and ventilated with 8 mL/kg/min room air at a rate of 70 strokes/min with a rodent ventilator. 1000 U/kg heparin was administered into the jugular vein and allowed to circulate for 30 s prior to thoracotomy. Midsternal thoracotomy was performed to expose the heart, followed by *in situ* cannulation of the aorta. The hearts were removed and immediately attached to a non-recirculating Langendorff constant pressure perfusion apparatus. Hearts were perfused with oxygenated (95% O_2 + 5% CO_2) modified Krebs Henseleit buffer (in mM: 112 NaCl, 5 KCl, 1.2 $MgSO_4$, 1 K_2HPO_4, 1.25 $CaCl_2$, 25 $NaHCO_3$, 11 D-glucose, 0.2 octanoic acid, pH = 7.4) maintained at 37.4 °C through the use of custom crafted water-jacketed glassware.

2.2. Left ventricular functional assessment

A saline filled balloon attached to a pressure transducer was inserted into the left ventricle and set to a minimum diastolic pressure of 10 mm Hg to allow for real time monitoring of left ventricular function. The pressure transducer was attached to a PowerLab 8/30 analog to digital converter and to a computer running LabChart Pro software (ADInstruments, Colorado Springs CO). This allowed the direct measurement of heart rate, systolic pressure, diastolic pressure, dP/dt_{max} and $-dP/dt_{max}$. Developed pressure was calculated as the difference between maximum systolic and minimum diastolic pressures. Rate pressure product was calculated as the product of the heart rate and the developed pressure. Coronary flow was continuously measured *via* an inline flow meter (Transonic Systems, Ithica NY).

2.3. Ischemia reperfusion injury and HDAC inhibition

Global ischemia was achieved by complete cessation of buffer flow for 30 min, followed by 60 min of reperfusion. Administration of 10 nM MS-275, 10 nM LL-66, or 10 nM LL-224 at reperfusion was achieved by dissolving MS-275, LL-66, or LL-224 in the perfusion buffer during the ischemic period. At the termination of reperfusion, hearts were briefly placed at −80 °C until firm but not frozen, then sliced into 2 mm sections. Slices were then either used for infarct staining or flash frozen in liquid nitrogen and stored at −80 °C.

2.4. Infarct staining

Triphenyl Tetrazolium Chloride (TTC) was used to stain for area of infarction at the termination of the I/R experiments, using the method of Ferrera et al. [34]. One slice from each heart was incubated in 1% TTC at 37.4 °C for 20 min, then fixed in 10% formalin solution overnight. Slices were photographed and analyzed for infarct area using imageJ software. Infarct size was reported as a percent of total left ventricular area.

2.5. Mitochondrial isolation and proteinase K digestion

Mitochondria were isolated from rat ventricular cardiac tissue by differential centrifugation, as described previously [35]. Briefly, hearts were excised from rats, the atria were removed, and the ventricles were minced in ice-cold mitochondrial isolation medium (in mM: 220 mannitol, 75 sucrose, 5 MOPS, 0.5 EGTA, 2 taurine, pH 7.25). The minced tissue was then homogenized by polytron. Trypsin was added to the homogenate (1 mg/100 mg wet tissue) for 5 min to digest contractile proteins, and 0.2% BSA was added to stop the digestion. Homogenates were then centrifuged at 600*g* for 10 min at 4 °C. The supernatant was removed and re-centrifuged at 600*g* for 10 min at 4 °C. The supernatant was again removed, and centrifuged at 5500*g* for 15 min. The pellet was then re-suspended in buffer B (in mM: 137 KCl, 2 KH_2PO_4, 2.5 $MgCl_2$, 20 HEPES, 0.5 EGTA). This procedure was also used for isolation of skeletal muscle mitochondria. For isolation of liver mitochondria, the trypsin digestion was omitted. For western blotting, antibodies against HDAC1 (Santa Cruz sc-6298, Abcam 53091), HDAC2 (Santa Cruz sc-7899), HDAC3 (Santa Cruz sc-11417), HDAC8 (Santa Cruz sc-11,405), α/β-tubulin (Cell Signaling 2148) and histone H3 (Cell Signaling 9715) were used. Proteinase K digestion was performed according to standard protocols [36]. Isolated mitochondria (100 μg per sample) were re-suspended in buffer B or buffer B containing 2% triton-X100. Mitochondria were then incubated in the presence of 50 μg/mL proteinase K (Qiagen, Germantown, MD) for 0, 20, 40 or 60 min at 37 °C. Following digestion, samples were immediately boiled in SDS and processed for western blotting with HDAC1, HDAC2, and ACAA2 (Santa Cruz sc-100847).

2.6. Myocyte isolation and immunofluorescence staining

Rats were anesthetized using 5% isoflurane, subjected to midsternal thoracotomy and removal of the heart. The hearts were then cannulated and attached to a perfusion apparatus, flushed with 37 °C perfusate (in mM: 113 NaCl, 4.7 KCl, 0.6 KH2PO4, 0.6 Na2HPO4, 1.2 MgSO4, 12 NaHCO3, 10 KHCO3, 10 HEPES, 0.1 adenosine, 0.1 gadolinium chloride, 6.4 sodium pyruvate, 30 taurine, 10 2,3-butanedione monoxime (BDM)) and perfused with recirculating digestion medium