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gp-91 mediates histone deacetylase inhibition-induced cardioprotection

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ABSTRACT

We have recently shown that the inhibition of histone deacetylases (HDAC) protects the heart against ischemia and reperfusion (I/R) injury. The mechanism by which HDAC inhibition induces cardioprotection remains unknown. We sought to investigate whether the genetic disruption of gp-91, a subunit of NADPH-oxidase, would mitigate cardioprotection of HDAC inhibition. Wild-type and gp-91$^{-/-}$ mice were treated with a potent inhibitor of HDACs, trichostatin A (TSA, 0.1 mg/kg, i.p.). Twenty-four hours later, the perfused hearts were subjected to 30 min of ischemia and 30 min of reperfusion. HDAC inhibition in wild-type mice produced marked improvements in ventricular functional recovery and the reduction of infarct size. TSA-induced cardioprotection was eliminated with genetic deletion of gp91. Notably, Western blot and immunostaining displayed a significant increase in gp-91 in myocardium following HDAC inhibition, which resulted in a mildly subsequent increase in the production of reactive oxygen species (ROS). The pre-treatment of H9c2 cardiomyoblasts with TSA (50 nmol/l) decreased cell necrosis and increased viability in response to simulated ischemia (SI), which was abrogated by the transfection of cells with gp-91 siRNA, but not by scrambled siRNA. Furthermore, treatment of PLB-985 gp91$^{+/+}$ cells with TSA increased the resistance to SI, which also diminished with genetic disruption of gp91 in gp91^{phox}-deficient PLB-985 cells. TSA treatment inhibited the increased active caspase-3 in H9c2 cardiomyoblasts and PLB-985 gp91$^{+/+}$ cells exposed to SI, which were prevented by knockdown of gp-91 by siRNA. These results suggest that a cascade consisting of gp-91 and HDAC inhibition plays an essential role in orchestrating the cardioprotective effect.

1. Introduction

Histone acetyltransferases (HAT) and histone deacetylases (HDAC) have recently garnered the attention because they have emerged as an important mechanism in the regulation of a variety of cellular responses. Histone acetylation is mediated by histone acetyltransferase, which results in the modification of the structure of chromatin leading to nucleosomal relaxation and altered transcriptional activation. In contrast, the reverse reaction is mediated by histone deacetylase which induces deacetylation, chromatin condensation, and transcriptional repression [1–5]. The acetylation status of the histone tails is determined by the interplay between HATs and HDACs. The opposing actions of HAT and HDACs allow gene expression to be exquisitely regulated through chromatin remodeling.

Since the identification of HDAC 1 (named HD 1) [6], over a dozen of HDACs have been described in mammals [7]. These HDACs can be categorized into three distinct classes. Class I HDACs consist of HDACs 1, 2, 3, and 8, which are ubiquitously expressed and predominantly located in nuclei. Class II HDACs include HDACs 4, 5, 7 and 9. In contrast to class I, class II HDACs exhibit a tissue specific pattern of expression. HDAC 4 and HDAC 5 are found highly expressed in the heart, brain, and skeletal muscle and shuttle between the nucleus and cytoplasm [8–11]. We have recently demonstrated that the inhibition of histone deacetylase with a selective inhibitor, trichostatin A, showed cardioprotective effects against I/R injury [12]. This is consistent with the observations that the inhibition of HDAC in myocytes silences the fetal gene activation, blocks cardiac hypertrophy and prevents cardiac remodeling [13–15]. Furthermore, HDAC inhibition has previously been shown to markedly decrease infarct size and reduce ischemia-induced neurological deficit scores in focal cerebral ischemia model of rats [16].

ROS have a central role in diverse physiological and pathological processes. When produced in large amounts by professional immune cells such as neutrophil granulocytes, ROS have antimicrobial activity serving in the first line of host defense [17]. However, ROS produced at low levels by non-immune cell have been implicated in growth factor signaling, mitogenic response, apoptosis, and oxygen sensing [18,19]. Our study has suggested that a mild generation of ROS protected the heart against myocardial necrosis [20]. A plethora of evidence has well indicated that endothelial cells and vascular smooth muscle cells

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express an ROS generating, NADH/NADPH-dependent oxidase containing gp-91, a NADPH-oxidase subunit, which is a membrane-bound component of NADPH-oxidase [21–23]. In addition to these membrane-bound complexes, several other cytosolic proteins, including p67, p47, p40 and rac, translocate to the membrane and associate with the gp-91 subunits and $p22^{phox}$ to assemble an enzyme complex that facilitates electron transfer from NADPH to molecular oxygen, leading to the generation of superoxides [24]. Even though the investigations have supported that ROS generate a preconditioning effect to confer protection, the role of gp-91 attributable to this event remains unknown. Particularly, the inhibition of HDACs has been shown to result in the generation of ROS in tumor cell lines [25]. The beneficial effects of mild ROS in preconditioned hearts were well addressed and HDAC inhibition has been shown to effectively trigger a generation of ROS. However, it also leaves the question open as to whether HDAC inhibition co-ordinates with gp-91 to transduce a signaling pathway to protect the heart against I/R injury. In this study, we investigated: 1) whether the cardioprotective effects induced with HDAC inhibitor, TSA, could be diminished with the targeted deletion of gp-91; 2) whether HDAC inhibition would enhance gp-91 of NADPH-oxidase and ROS production in myocardium; 3) whether genetic suppression of gp-91 with siRNA would eliminate the resistance of H9c2 cardiomyoblast cells in response to simulated ischemia in the presence of HDAC inhibition; 4) whether the protective effects of HDAC inhibition were absent in the $gp91^{phox}$-deficient PLB-985 cells but present in wild-type cells; and 5) whether disruption of gp-91 would mitigate the anti-apoptotic effect of HDAC inhibition in the *in vitro* cultured cells exposed to simulated ischemia. To the best of our knowledge, this is the first study to provide new insight into our understanding of novel mechanisms of ischemic injury and developing therapeutic strategies for heart disease.

2. Materials and methods

2.1. Animals

Adult male C57/BL6 wild-type and $gp91^{-/-}$ mice were supplied by Jackson laboratories (Bar Harbor, Maine). All animal experiments were conducted in accordance with the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health. The experimental protocol was approved and carried out in accordance with the guidelines adhered to the Institutional Animal Care and Use Committee.

2.2. Chemical supplies and antibodies

Trichostatin A was obtained from Calbiochem (San Diego, CA). N-(2-mercaptopropionyl)-gel electrophoresis supplies were obtained from Bio-Rad Laboratories (Hercules, CA). The perfusion chemicals were purchased from Sigma (St. Louis, MO). Gp-91 polyclonal rabbit antibody, β-actin, gp-91 siRNA and negative control scrambled siRNA were purchased from Santa Cruz Biotechnology Inc. Mouse anti-sarcomeric actinin antibody was obtained from Sigma (St. Louis, MO). Anti-rabbit horseradish peroxidase-conjugated secondary antibody was purchased from Amersham (Piscataway, NJ).

2.3. Langendorff isolated heart perfusion

The methodology of Langendorff's isolated perfused heart preparation has been described previously in detail [26–29]. Briefly, mice were anesthetized with an intraperitoneal injection (i.p.) of pentobarbital sodium (120 mg/kg). The hearts were rapidly excised and arrested in ice-cold Krebs–Henseleit buffer. They were then cannulated via the ascending aorta for retrograde perfusion by the Langendorff method using Krebs–Henseleit buffer containing (in mM) 110 NaCl, 4.7 KCl, 1.2 $MgSO_4$ $7H_2O$, 2.5 $CaCl_2$ $2H_2O$, 11 glucose, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 0.5 EDTA. The buffer, aerated with 95% O_2:5% CO_2 to give a pH of 7.4 at 37 °C, was perfused at a constant pressure of 55 mmHg. A water-filled latex balloon, attached to the tip of polyethylene tubing, was then inflated sufficiently to provide a left ventricular end-diastolic pressure (LVEDP) of 10 mmHg. Myocardial function was measured including left ventricular developed pressure (LVDP), LVEDP, RPP, heart rate and coronary flow. LVDP was calculated by subtracting LVEDP from the peak systolic pressure. Rate pressure product (RPP), an index of cardiac work, was calculated by multiplying LVDP with heart rate.

2.4. Measurement of myocardial infarction

The infarction size was measured with a modification as previously described [26–29]. At the end of reperfusion, hearts were perfused with 10% triphenyltetrazolium chloride (TTC), and then removed from the Langendorff perfusion apparatus. The frozen hearts were then cut from apex to base into transverse slices. After staining, 10% TTC buffer was replaced, and then the slices were fixed in formaldehyde for measurement of the infarcted areas using computer morphometry NIH image software (Image *J* 1. 36, NIH). The infarct size was calculated and presented as the percentage of risk area, defined as the sum of total ventricular area minus cavities.

2.5. Experimental protocol 1

Mice were randomized into three experimental groups that underwent the following treatments, as shown in Fig. 1: 1) Vehicle group: wild-type mice receiving an i.p. injection of 0.1 ml vehicle (DMSO); 2) TSA + wild-type group: wild-type animals were treated the same as group 1 except that TSA (TSA, 0.1 mg/kg, i.p.) was given to wild-type animals; 3) TSA + $gp\text{-}91^{-/-}$ group: the same as group 2 except that mice with disruption of gp-91 were administered with TSA. Twenty-four hours later, the hearts were subjected to 30 min of stabilization and 30 min of ischemia followed by 30 min of reperfusion.

Another subset of animals without sustained ischemia and reperfusion was treated with or without TSA solely for the purpose of measuring the gp-91 protein. Animals were treated with TSA for 30 min, and heart tissues were collected. Cardiac lysates were extracted as previously described [37]. Briefly, the hearts were frozen in liquid nitrogen, ground and suspended in 1 ml of lysis buffer containing 50 mM Tris HCl (pH 7.4), 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, 150 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 5 mM EGTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 5 μg/ml pepstatin A. Mixtures were homogenized and microcentrifuged at 16,000 × *g* for 20 min. The protein content of the supernatant was determined using the detergent compatible-protein assay (Bio-Rad). In addition, frozen hearts following treatments were used for the detection of ROS in cardiac sections.

2.6. Western blot analysis

The proteins were detected using polycolonal anti-gp-91, active caspase-3 from CalBiochem (San Diego, CA) and β-actin antibodies. For immunoblotting, proteins (50 μg/lane) were separated by 6% SDS-PAGE (gp-91) and 10% SDS-PAGE (active caspase-3). Proteins were then transferred onto a nitrocellulose membrane for 2 h at 100 V. The membrane was blocked with 5% non-fat dry milk in 1× Tris-buffered saline containing 0.5% Tween 20 for 1 h. The blots were incubated with respective primary antibodies (1:1000 dilution) for 2 h and visualized by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000 dilution) for 1 h. The immunoblots were developed with the ECL Chemiluminescence Detection Reagent (Amersham Pharmacia Biotech). The densitometric results were normalized to the control group and expressed as percentages of control values.