HDAC2 is required by the physiological concentration of glucocorticoid to inhibit inflammation in cardiac fibroblasts

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ABSTRACT

We previously suggested that endogenous glucocorticoids (GCs) may inhibit myocardial inflammation induced by lipopolysaccharide (LPS) *in vivo*. However, the possible cellular and molecular mechanisms were poorly understood. In this study, we investigated the role of physiological concentration of GCs in inflammation induced by LPS in cardiac fibroblasts and explored the possible mechanisms. The results showed that hydrocortisone at the dose of 127ng/ml (equivalent to endogenous basal level of glucocorticoids) inhibited LPS (100ng/ml)-induced productions of TNF- α and IL-1 β in cardiac fibroblasts. Xanthine oxidase/xanthine (XO/X) system impaired the anti-inflammatory action of GCs through downregulating HDAC2 activity and expression. Knockdown of HDAC2 restrained the anti-inflammatory effects of physiological level of hydrocortisone, and blunted the ability of XO/X system to down-regulate the inhibitory action of physiological level of hydrocortisone on cytokines. These results suggested that HDAC2 was required by the physiological concentration of glucocorticoid to inhibit inflammatory response. The dysfunction of HDAC2 induced by oxidative stress might be account for GC resistance and chronic inflammatory disorders during the cardiac diseases.

Key words: Fibroblasts, Glucocorticoid, Histone deacetylase, HDAC2, Inflammation, Resistance

Introduction

Chronic inflammation is the key component in cardiac remodeling and heart failure pathology (Anker and von Haehling 2004; Candia et al. 2007; Deten et al. 2002; Genth-Zotz et al. 2004). Thus, resolving inflammation not only helps to attenuate the ventricular remodeling but also promises to delay potential heart failure events. Despite recent advances in interventional strategies targeting inflammation of cardiovascular disease (CVD), but the simply anti-inflammatory treatment always failed in clinical practice, some even resulted in adverse outcomes that were realized in heart failure trials (Chung et al. 2003; Kleinbongard et al. 2010; Mann et al. 2004). Therefore, the development of new therapeutically promising strategies that support the activation of endogenous inhibitory pathways to suppress inflammation and restore inflamed tissue to the prior physiological function and normal homeostasis is hallmarks for the treatment of cardiac diseases.

Glucocorticoids (GCs) are vital stress hormones and key neuroendocrine regulators that regulate a wide array of physiological functions. Also, exogenous pharmacological doses of GCs are the most effective anti-inflammatory agents for the treatment of severe inflammatory diseases such as asthma and rheumatoid arthritis. But the role of endogenous GCs in regulation of inflammatory response is not yet well defined. We recently reported that endogenous GCs could act as an important anti-inflammatory factor to regulate cardiac myocardial inflammatory response *in vivo*, and down-regulation of endogenous GCs function might contribute to myocardial inflammatory disorders (Zhang et al. 2012). However, its exact molecular mechanisms

and the possible cellular target in the heart remain unclear.

Traditionally, GCs act by binding to cytosolic glucocorticoids receptor (GR), which upon binding becomes activated and rapidly translocates to the nucleus. Within the nucleus, the GC-GR complex can bind to specific glucocorticoid response elements on DNA to enhance transcription (transactivation) of anti-inflammatory genes and/or to inhibit transcription (transrepression) of pro-inflammatory genes. Growing evidence shows that histone acetylase (HAT) and histone deacetylase (HDAC) play critical roles in modulating a variety of genes transcription (Adcock et al. 2004; Barnes 2011; Baschant and Tuckermann 2010). There is compelling evidence that increased gene transcription is associated with an increase in histone acetylation, whereas hypoacetylation is correlated with reduced transcription or gene silencing (Govindan 2010; Rahman et al. 2004). GC-GR complex are capable of recruiting corepressor proteins that have HDAC activity to deacetylate core histone proteins bound to DNA, therefore, switch off inflammatory gene transcription.

Recent studies have reported that oxidative stress reduces HDAC activity of lung parenchyma in chronic obstructive pulmonary disease (COPD), and this decrease may account for amplified inflammation and resistance to the anti-inflammatory effects of corticosteroids in COPD. On the other hand, increasing HDAC activity may be useful in attenuating inflammation and reversing GCs resistance (Adcock and Ito 2005; Barnes 2010; Barnes and Adcock 2009; Freishtat et al. 2010; Marwick and Chung 2010). These findings indicate the decreased activity of HDAC mediated by oxidative stress may down-regulate the anti-inflammatory function of endogenous

GCs, which may contribute to the chronic inflammatory disorders.

Cardiac fibroblasts are composed of the most interstitial components in the myocardium, and play a critical role in tissue repair after heart infarction. Experimental studies also have suggested that fibroblasts possess potent immunomodulatory and inflammatory properties, and can produce cytokines and chemokines when becoming activated, which may stimulate over-proliferation of fibroblasts, therefore contributes to the cardiac fibrosis and heart remodeling (Camelliti et al. 2004; Goldsmith et al. 2004; Haudek et al. 2010). Our previous study showed that physiological concentration of GCs could inhibit pro-inflammatory cytokine-stimulated proliferation of cardiac fibroblasts (He et al. 2011). In this study, we further investigated the effect of physiological concentration of GCs on inflammation induced by LPS in cardiac fibroblasts. Also, we used xanthine oxidase/xanthine (XO/X) system to induce reactive oxygen species (ROS) and explored the possible mechanisms by which oxidative stress down-regulates the anti-inflammatory function of endogenous GCs and promotes inflammation.

Materials and methods

Reagents

Xanthine, Xanthine oxidase, Allopurinol and LPS (*Escherichia coli*, 0111: B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrocortisone was purchased from Merck Chemicals, Germany. Lipofectamine 2000 or RNAi against HDAC2 was purchased from or synthesized by Life technology.

Animals and treatments

Male Sprague-Dawley rats (200~250g body BW) were purchased from the experimental animal center of Guangdong Province (Guangzhou, China). Rats received a standard diet and water ad libitum and were treated according to National Institute of Health Guidelines on the Care and Use of Experimental Animals. An i.p. injection of 1mg/kg LPS (*Escherichia coli*, 0111: B4, Sigma; St. Louis, MO) was used to induce inflammatory response.

Histologic analysis

After treatment with LPS for 1h, the rat hearts were removed and longitudinally cut into blocks. After fixed in 4% PB-buffered paraformaldehyde followed by 10-30% graded sucrose solutions, the tissue blocks were embedded in Tissue-Tek O.C.T compound (Sakura Finetek USA, Inc), flash frozen in liquid nitrogen, and then sectioned on cryostats. Sections were conducted immunostaining with primary antibody against IL-1 β (Millipore Inc., U.S.A.) and appropriate second antibody. The staining was detected by 3'3'diaminobenzidine and the IL-1 β -positive cells were observed and imaged under microscope.

Isolation and culture of cardiac fibroblasts of adult rats

Cardiac fibroblasts of adult rats were prepared according to the previously described methods (Villarreal et al. 1993). In brief, rats were anaesthetized and hearts were removed under sterile conditions. After removing atria and vessels, ventricular tissue was digested repeatedly by 0.1% collagenase (type II, Gibco AG) plus 0.125% trypsin solution under constant shaking at 37°C for 10min. The cell pellet was

collected by centrifuging and suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). To remove non-fibroblast cells, cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ and 95% air for 2h, and supernatant was aspirated. The adhering cells, mainly fibroblasts, were cultured in DMEM at 37°C with 5% CO₂ and passaged by trypsin when confluent. Cardiac fibroblasts at passage 2~3 were used for the following experiments.

Cytokines assay

After treatment with different drugs for indicated time, the cells were lysed with cell lysis buffer (Cell Signaling Technology, Beverly, MA). The protein was extracted and protein content was quantified by the BCA protein assay (Pierce). The amount of TNF- α and IL-1 β in supernatants was measured using ELISA kits for TNF- α and IL-1 β (eBioScience, San Diego, CA, USA) following the manufacturer's instructions.

HDAC activity assay

Nuclear proteins were extracted from cultured cardiac fibroblasts of adult rat using the Nuclear Extraction Kit (Millipore international Co. U.S.A.). HDAC activity was assessed with commercially available Fluorimetric HDAC Activity Assay Kit (BioVision, Mountain View, CA) following the detailed instructions provided by the manufacturer. The results were expressed as the Relative Fluorescence Units *per* mg of protein.

Western blotting analysis

After treatment, nuclear proteins extracted from cardiac fibroblasts were separated by SDS-PAGE and transferred to PVDF membranes (Roche Molecular

Biochemicals, Mannheim, Germany). The membranes were blocked and detected with anti-HDAC2 antibody (Millipore Inc., U.S.A.), anti-LaminB1 antibody (Bioworld Inc., U.S.A.) respectively. The density of target bands was accurately determined by the computer-aided Quantity One analysis system.

Reactive oxygen species assay

The ROS-specific fluorescent probe, 2',7'-dichlorofluorescein diacetate (H₂DCF-DA, Molecular Probes, Inc.), was used to assess the levels of intracellular reactive oxygen species (ROS). The intensity of the fluorescence is in proportion to the levels of intracellular reactive oxygen species. Briefly, after exposing cells to the XO/X system (1.5u/L/0.5mmol/L) for 0, 6, 8 hours respectively, cardiac fibroblasts were incubated with H₂DCF-DA (5μM/L) in HBSS under 37 °C for 30 minutes. Fluorescent signals were obtained with a fluorescence conversion microscope (Nikon TE300-ECI) and analyzed by the image processing and analysis system. Five to eight randomly selected fields in each well were used to assess the levels of ROS.

Immunofluorescent staining

After treatment with LPS for 6h in the presence and absence of physiological level hydrocortisone, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% TritonX-100 and blocked with PBS containing 2% goat serum. Immunofluorescent staining was performed using primary antibody against mouse anti-vimentin, anti-HDAC2, anti-TNF-α, anti-IL-1β (Millipore Inc., U.S.A.) and second antibody conjugated to Alex-488 (green) or Cy3 (red). Nuclei were co-stained

with DAPI (blue) (Roche Molecular Biochemicals, Mannheim, and Germany). Cells were observed and imaged by the fluorescence microscope.

HDAC2 knockdown

RNAi against HDAC2 was synthesized (Life Technology, USA) and transfected into cardiac fibroblasts using lipofectamine 2000 reagent (Life Technology, USA). Scramble nucleotide sequence served as the non-silencing control and Block-iTTM fluorescent oligo was used to detect the efficiency of transfection. After fibroblasts were transfected for 24h-72h, specificity and degree of knockdown were confirmed by western blotting.

Statistical analysis

All values were expressed as mean $\pm$ standard deviation. Data were subjected to analysis by ANOVA using SPSS 17.0 software (SPSS Science, Chicago, IL, USA). A value of $P < 0.05$ was taken as statistically significant.

Results

Cardiac fibroblasts could be the contributor to the source of pro-inflammatory cytokines induced by LPS in rat hearts

To investigate whether cardiac fibroblasts could contribute to the inflammatory response induced by LPS in the heart, rats received an i.p. injection of LPS (1mg/kg) and the expression of IL-1 β was detected by immunohistochemistry in the heart tissue sections. The results showed that clustered or scattered inflammatory lymphocytes or polymorphonuclear leucocytes were histologically identified in LPS-injected heart tissue but not in that of control. Immunostaining showed an obvious accumulation of

IL-1 β -positive cardiac fibroblasts in the connective tissue of the myocardium including the heart valve, myocardium, perivascular tissue and epicardium of the myocardium, while few IL-1 β -positive staining was observed in cardiac myocytes (Fig. 1A-D).

LPS induced pro-inflammatory cytokine production in cultured cardiac fibroblasts of adult rats.

To induce inflammatory response, cardiac fibroblasts of adult rats were incubated with LPS (100ng/ml) at indicated time points or using different concentrations (1-10000ng/ml) for 6h. The results showed that LPS stimulated TNF- α , and IL-1 β production in a time-dependent (Fig. 2B-C) and concentration-dependent manner (Fig. 2D-E). The maximal release was reached at 4h and 6h respectively and remained higher levels until 24h.

Physiological levels of hydrocortisone inhibited LPS-stimulated cytokines production in cardiac fibroblasts of adult rats.

As shown in figure3, LPS (100ng/ml) treatment induced the production of TNF- α and IL-1 β in cultured cardiac fibroblasts. Pretreatment with physiological level of hydrocortisone (determined previously that serum basal corticosterone level in normal SD rats was around 127 ng/ml) (Zhang et al.2012) had little effects on the basal levels of TNF- α and IL-1 β , but significantly inhibited LPS-induced the production of TNF- α and IL-1 β . (Fig 3A-B).

Oxidative-stress impaired the inhibitory effect of physiological level of hydrocortisone on LPS-induced cytokines production in cardiac fibroblasts of

adult rats.

To investigate whether oxidative-stress injury participated in GCs resistance, we examined the effect of XO/X system and antioxidant on the production of TNF- α and IL-1 β . The results showed that exposure of cells to both LPS and XO/X system evoked robust oxidative-stress response as evidenced by increased fluorescence intensity of ROS-sensitive fluorescent probe H2DCFDA in a time-dependent manner (**Fig. 4A**). Pretreatment of cells with XO/X system for 24h had little effects on the basal levels of TNF- α and IL-1 β , but caused significantly increases of LPS-induced production of TNF- α and IL-1 β , and down-regulation of the inhibitory effect of physiological concentration of hydrocortisone on the production of those cytokines. However, when allopurinol, a xanthine oxidase inhibitor, was added before XO/X system treatment, the ability of XO/X system to down-regulate the inhibitory effects of physiological level of hydrocortisone on cytokines was abolished (**Fig. 4B-C**).

XO/X system reduced HDAC activity and HDAC2 expression in cultured cardiac fibroblasts of adult rats. While, Antioxidant allopurinol restored HDAC activity and HDAC2 expression induced by XO/X system.

We and others had found that HDAC was involved in the anti-inflammatory action of GCs, therefore, we tested whether the inhibitory effects of XO/X system on anti-inflammatory action of physiological level of hydrocortisone were also associated with the down-regulation of HDAC functions. As shown in figure 5A-B, exposure of cardiac fibroblasts to XO/X system dose-dependently decreased HDAC activity. After 24 hours of treatment with 1.5/0.5 XO/X system, the HDAC activity was decreased to

76.7%. In contrast, physiological level of hydrocortisone caused markedly increase of HDAC activity in basal or in response to LPS challenge, but such effects were abrogated in the presence of XO/X system. Similarly, pretreatment with allopurinol blunted the ability of XO/X system to down-regulate HDAC activity.

Immunofluorescent staining and western blotting analysis showed that exposure of cardiac fibroblasts to XO/X system decreased expression of HDAC2 in a dose-dependent manner. In contrast, physiological level of hydrocortisone markedly up-regulated HDAC2 expression either in basal or in response to LPS challenge. Likewise, such effects of physiological level of hydrocortisone on expression of HDAC2 were significantly attenuated by XO/X system, but not affected in the presence of allopurinol (Fig. 5C-E).

HDAC2 was required by GCs to restore the inhibitory effect of physiological level hydrocortisone on LPS-induced inflammatory response in cardiac fibroblasts of adult rats.

The involvement of HDAC2 in the anti-inflammatory action of GCs was assessed by use of specific RNAi against HDAC2. As shown in figure 6A-B, targeted RNAi against HDAC2 achieved significant knockdown of HDAC2 protein to 15.7 %, and the activity of HDAC to 28.7 %, whereas, scramble sequence had no effect on the expression of HDAC2. LPS treatment induced the production of TNF- α and IL-1 β in HDAC2 RNAi-transfected cardiac fibroblasts. However, after HDAC2 knockdown, the ability of physiological level of hydrocortisone to prevent LPS-induced increases of TNF- α and IL-1 β was markedly attenuated (fig. 6C-D). Also, the ability of XO/X system to down-regulate the inhibitory effects of physiological level of

hydrocortisone on cytokines was significantly blunted (Fig. 6E-F).

4. Discussion

In the present study, we showed that cardiac fibroblasts of adult rats could be the target cells of LPS to produced pro-inflammatory cytokines of TNF- α and IL-1 β . Physiological concentration of hydrocortisone was capable of inhibiting LPS-induced the productions of TNF- α and IL-1 β . However, this inhibitory effect was weakened by oxidative-stress *via* reducing HDAC activity and HDAC2 expression in cultured cardiac fibroblasts of adult rats. Allopurinol, an antioxidant and xanthine oxidase inhibitor could restore the inhibitory effect of physiological concentration of hydrocortisone on those cytokines induced by LPS. Furthermore, we found that knockdown of HDAC2 significantly impaired the inhibitory effects of physiological level of hydrocortisone on cytokines, and prevented XO/X system from down-regulating anti-inflammatory action of physiological level of hydrocortisone, suggesting that HDAC2 was required by the physiological concentration of glucocorticoid to inhibits inflammatory responses induced by LPS in cardiac fibroblasts of adult rats.

Accumulating evidence showed that fibroblasts could become pro-inflammatory, and were able to produce a broad spectrum of pro-inflammatory cytokines and chemokines under stimulus of hypoxia, mechanical stress or endotoxin (Goldsmith et al. 2004; Haudek et al. 2010) . The action of those cytokines or chemokines on the cardiovascular system was involved in stimulating cell division,

proliferation and differentiation (Turner et al. 2009), etc. Indeed, we found that IL-1 β was expressed in the cytoplasm of cardiac fibroblasts in cardiac connective tissue after LPS injection, and TNF- α and IL-1 β could be induced by LPS in a time and dose-dependent manner in cardiac fibroblasts of adult rats, indicating that cardiac fibroblasts could be the contributor to the source of pro-inflammatory cytokines during the development of cardiac diseases. Furthermore, we observed that hydrocortisone used at the dosage equivalent to endogenous basal level of glucocorticoids could inhibit LPS-induced the productions of TNF- α and IL-1 β in cardiac fibroblasts of adult rats, confirming our results *in vivo* that endogenous, physiological levels of GCs are able to exert an anti-inflammatory effect on myocardial inflammatory response. We had reported previously that physiological level of hydrocortisone also attenuated proliferation of cardiac fibroblasts stimulated by TNF- α and IL-1 β . Combined together, these evidences suggested that inhibition of myocardial inflammation by endogenous, physiological levels of GCs might be helpful to prevent cardiac fibroblasts from the inflammation-induced hype-proliferation and following cardiac fibrosis and cardiac remodeling.

Different mechanisms are involved in the anti-inflammatory action of GCs and HDAC-mediated reversal of the hyperacetylation of core histones and transrepression on proinflammatory transcriptional factors such as NF- κ B and AP1 have been suggested to be critical for GC-GR to suppress proinflammatory gene expression (Barnes 2011; Baschant and Tuckermann 2010; Rhen and Cidlowski 2005). As such, dysregulation of HDAC can interfere with anti-inflammatory function of GC-GR, and

lead to the inflammatory outcomes. Barnes and his colleagues (Adcock and Ito 2005; Barnes 2010; Barnes and Adcock, 2009) have reported that smoking induces oxidative stress and insensitivity to exogenous GCs treatment in asthma, possible through reduced HDAC activity in their lung parenchyma. Investigations from others also demonstrate enhanced oxidative stress and inflammatory response, but deficient HDAC and GC resistance in COPD patients and patients with severe asthma. Methyl-xanthine theophylline or polyphenol curcumin treatment may restore HDAC activity, thereby ameliorate inflammatory state and reverse GC resistance (Ito et al. 2002). Consistent with these results, we showed an evil circle between oxidative stress and the inflammation. That is oxidative stress could be induced by LPS, and vigorous oxidative stress elicited by XO/X system was followed by more vigorous inflammatory response induced by LPS in cardiac fibroblasts. Notably, enhanced oxidative stress down-regulated the inhibitory effect of physiological concentration of hydrocortisone on inflammation induced by LPS and dose-dependently reduced HDAC activity in cardiac fibroblasts of adult rats, while, antioxidant allopurinol retained inhibitory effect of physiological concentration of hydrocortisone on inflammation and HDAC activity.

HDAC2, one of members of class I HDACs, was found to be anti-inflammatory and mainly localized to the nucleus (De Ruijter et al. 2003; Ito et al. 2006). Our *in vivo* experiments had revealed that HDAC2 were selectively recruited to GC-GR complex following the LPS challenge, indicating that HDAC2 may functionally be involved in the anti-inflammatory action of endogenous GCs (Zhang et al. 2012).

Here, we found that HDAC2 was down-regulated by XO/X system at protein level. Physiological level of hydrocortisone was able to up-regulate HDAC2 expression in response to the LPS challenge, but this effect was abolished by XO/X system, reversed by allopurinol. Specifically, HDAC2 knockdown markedly attenuated anti-inflammatory effects of physiological level of hydrocortisone, and blunted the ability of XO/X system to down-regulate the inhibitory action of physiological level of hydrocortisone on cytokines induced by LPS. Results from Ito lab (Ito et al. 2002; Ito et al. 2006; Ito et al. 2005) also showed that corticosteroids suppressed inflammatory genes in asthma by recruiting HDAC2 to the NF- κ B -activated inflammatory gene complex. Oxidative stress induced the GC resistance or dysfunction by decreasing the activity of HDAC2 possible through nitrifying tyrosine residues within HDAC2, therefore interfering with its enzymatic efficiency and making them more susceptible to protein degradation via the proteasome. Conversely, over-expression of HDAC2 in insensitive alveolar macrophages from patients with COPD was able to restore GC sensitivity (Marwick et al. 2010; Moodie et al. 2004; Rajendrasozhan et al. 2008). These data together provided evidence that anti-inflammatory action of endogenous, physiological level of GCs significantly related to HDAC2 functions. In the way of reducing the activity and expression of HDAC2, oxidative stress may down-regulate the actions of GCs on inflammation, thereby contribute to the inflammatory response and GC resistance. Actually, that may be the cases for cardiovascular diseases and CHF, in which both of increased oxidative stress and chronic inflammation were increasingly recognized as important

pathogenic mechanisms (Chen et al. 2004; Gioda et al. 2010; Grieve and Shah 2003; Xu et al. 2011). Thus, the simply anti-inflammatory treatment with exogenous glucocorticoids for those patients with CHF always failed to delay the potential heart failure events in the clinic practice. Instead, chronic administration of exogenous glucocorticoids usually caused multiple adverse outcomes such as hyperglycemia, obesity, and even cardiac fibrosis under the conditions of oxidative stress, indicating that the impairment of anti-inflammatory mechanism of GCs or GC resistance induced by oxidative stress may participate in the pathological processes of CHF. Therefore, we supposed that inhibition of oxidative stress and amelioration of endogenous GC resistance might be a new therapeutically promising strategy to eventually resolve inflammation and delay the progression of heart failure.

Disclosure

No conflicts of interest, financial or otherwise, are declared by the authors.

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Legends

Figure 1. LPS induced the secretion of IL-1 β in rat cardiac fibroblasts. An i.p. injection of 1mg/kg LPS was used to induce myocardial inflammatory response. Hearts of rats were dissected at 1h after LPS injection and immunohistochemical staining was performed as described in the text. Brown madder (DAB) indicates localization of IL-1 β . Blue (hematoxylin) indicates localization of nucleus. Red arrows point to inflammatory cells infiltration. Black arrows point to IL-1 β -positive cardiac fibroblasts. (Figures are the representative of 1 of 8 sections from individual rat. Original magnification $\times$ 400).

Figure 2. LPS time and concentration-dependently induced the production of TNF- α and IL-1 β in cultured cardiac fibroblasts. (A) Cardiac fibroblasts were identified by vimentin (B) and (C) the levels of TNF- α and IL-1 β in adult rat cardiac fibroblasts after treatment with LPS (100ng/ml) for different time points. (D) and (E) the levels of TNF- α and IL-1 β in adult rat cardiac fibroblasts after treatment with LPS at different concentrations for 6h. All data shown represent the mean $\pm$ SD of five independently experiments.

Figure 3. The physiological level of hydrocortisone inhibited the production of TNF- α and IL-1 β induced by LPS in cultured cardiac fibroblasts. (A) and (B) the levels of TNF- α and IL-1 β in adult rat cardiac fibroblasts pretreated with hydrocortisone (127ng/ml) for 18 hours before LPS treatment. **, $P < 0.01$ vs. control group or LPS group. All data shown represent the mean $\pm$ SD of the five independently experiments.

Figure 4. XO/X system impaired the inhibitory effect of physiological level of hydrocortisone on LPS-induced cytokines production in cardiac fibroblasts of adult rats. (A) H₂DCFDA staining was performed to determine the levels of ROS in cultured cardiac fibroblasts treated with LPS for 6h or 0.5mmol/L xanthine/1.5u/L xanthine oxidase for 0h, 6h, or 8h. Fluorescent intensity was measured by fluorescence microscopy. Original magnification was 100×. (B) and (C) the levels of TNF- α and IL-1 β in adult rat cardiac fibroblasts pretreated with hydrocortisone (127ng/ml) for 18 hours before LPS treatment in the presence of XO/X (1.5u/L/0.5mM/L). *, $P < 0.05$ and **, $P < 0.01$ vs. control group or as indicated. All data shown represent the mean $\pm$ SD of five independent experiments.

Figure 5. XO/X system reduced HDAC activity and HDAC2 expression in a concentration-dependent manner, which was partially reversed by antioxidant allopurinol. Cardiac fibroblasts were incubated with different doses of XO/X or XO/X (1-2u/L/0.5mM/L) for 24h in the presence or absence of hydrocortisone (127ng/ml) and/or allopurinol before LPS treatment. Six hours later, the cells were harvested and nuclear proteins were extracted. (A) and (B) The HDAC activity was measured using Fluorimetric HDAC Activity Assay Kit. (C) Cells were treated with XO/X (1.5u/L/0.5mmol/L) or hydrocortisone(127ng/ml) for 24h, then immunostaining was conducted with antibody against HDAC2 (Cy3 red). Nuclei were co-stained with DAPI (blue). Original magnification was 400×. (D) and (E) The expression of HDAC2 were detected by western blotting analysis. LaminB1 or Histone H3 was used as loading control. *, $P < 0.05$ and **, $P < 0.01$ vs. control group

or as indicated. All data represent mean $\pm$ SD of three independent experiments.

Figure 6. HDAC2 was required by GCs to restore the inhibitory effect of physiological level hydrocortisone on LPS-induced inflammatory response in cardiac fibroblasts of adult rats

(A) and (B) HDAC2 knockdown reduced HDAC2 expression and HDAC activity. Scrambled sequences served as non-silencing controls in all experiments involving RNAi knockdown of HDAC2. $n=4$, **, $P<0.01$ vs. control group. **(C) and (D)** immunofluorescent staining showed the levels of TNF- α and IL-1 β in adult rat cardiac fibroblasts pretreated with hydrocortisone (127ng/ml) for 18 hours before LPS treatment in the presence of HDAC2 siRNA. **(D) and (E)** HDAC2 knockdown weakened the anti-inflammatory effects of physiological level hydrocortisone. $n=4$, **, $P<0.01$ vs. control group.

Figure 1.

81x36mm (300 x 300 DPI)

Draft

Figure 2.

274x340mm (300 x 300 DPI)

Figure 3.

273x109mm (300 x 300 DPI)

A

Figure 4.

222x225mm (300 x 300 DPI)

Figure 5.

284x451mm (300 x 300 DPI)

Figure 6.

283x364mm (300 x 300 DPI)