



Inhibition of p300-HAT results in a reduced histone acetylation and down-regulation of gene expression in cardiac myocytes

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ABSTRACT

Aims: Histone acetylation plays an important role in cardiogenesis, but the underlying mechanism is unclear. In this study, we investigated the relationship between histone hypo-acetylation and the expression of cardiac-specific genes to explore the underlying mechanisms.

Main methods: Cardiac-specific genes that physically interacted with p300 protein in mouse hearts were analyzed using chromatin immunoprecipitation (ChIP) assays. The cultured mouse neonatal cardiac myocytes were treated with curcumin with different concentrations and durations. The changes of histone acetyltransferase (HAT) activities, histone acetylation, cardiac-specific genes expression, and structure of chromatin were assessed by ELISA, Western blotting, quantitative RT-PCR, and ChIP assays, respectively.

Key findings: Results from the ChIP assay showed that GATA4, Nkx2.5, and Mef2c physically interacted with p300 protein. After treatment with 30 μ M curcumin for 24 h, the HAT activities of cardiac myocytes were inhibited significantly. And the acetylation of whole histone H3 was reduced by 0.3983-fold compared to control groups ($P < 0.05$). Accordingly, the expression of cardiac-specific genes, GATA4, Nkx2.5, and Mef2c, were significantly down-regulated. Acetylation of histone H3 bound with promoter regions of these genes was significantly reduced.

Significance: p300 interacts with cardiac-specific genes, GATA4, Nkx2.5 and Mef2c, and inhibition of p300-HAT by curcumin down-regulates their expression through the inhibition of histone H3 acetylation in the promoter regions. This finding indicates that p300-HAT mediated histone H3 acetylation plays an important role in the regulation of cardiac gene expression, which is a novel mechanism of epigenetic regulation in the heart during the development and in case of some congenital heart diseases.

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Introduction

Modification of histone acetylation is an important mechanism in the regulation of gene expression (Allfrey et al., 1964). Acetylation of amino-terminal lysine residues of histone tails loosens the chromatin structure thereby to facilitate the accessibility of transcriptional factors to DNA templates and to promote the gene expression. Deacetylation of histone, on the other hand, renders the chromatin structure more dense, which represses or silences gene transcription (Clayton et al., 2006; Backs and Olson, 2006). Modification of histone acetylation is a reversible dynamic process mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Based on the protein homology, substrate specificity, and functional

consequences, nuclear HATs could be broadly classified into different subgroups, in which p300 is an important subtype (Roth et al., 2001).

Recently several studies demonstrated that p300 played an important role in embryonic development. Yao et al. (1998) reported that an appropriate expression of p300 was essential for mouse embryonic development. p300 homozygous knockout mice died during gestation period between days E9 to E11.5, with obvious defects in neurulation, cell proliferation, and perturbed heart development. p300 heterozygotes also manifested considerable embryonic lethality. Shikama et al. (2003) reported similar findings that p300 (−/−) mice and p300 (+/−) led to embryonic or neonatal lethality with impaired formation of cardiovascular system, lungs, and small intestine.

We have demonstrated previously that hyper-acetylation of histone enhanced the differentiation of mesenchymal stem cells (MSCs) to cardiac myocytes with an increased expression of cardiac-specific genes and proteins (Feng et al., 2009). In another in vivo study, we found that several different HATs subtypes were related to

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mouse embryonic heart development, while their roles were different. p300 had a higher expression while others had no expression or low expression in crescent-shaped cardiogenic plate, indicating that p300 played an essential role during the heart development, especially for cardiac progenitor cell inducing and migrating (Chen et al., 2009). All these studies indicate that p300 plays a critical role in physiological growth and differentiation of cardiac myocytes during heart development, however, the mechanism remains unclear. Thus, it is imperative that we clarify the regulation role of histone acetylation mediated by p300 on cardiac-specific genes and to explore the mechanism of this regulation.

Curcumin is a natural polyphenolic compound abundant in the rhizome of *Curcuma longa*. Recently many studies show that curcumin is a specific inhibitor of p300-HAT (Miquel et al., 2002; Mai et al., 2006; Morimoto et al., 2008). Curcumin could inhibit HAT activity and block histone H3 acetylation in vitro, but do not affect the cells that express a dominant negative version of p300 (Balasubramanyam et al., 2004; Kang et al., 2005; Marcu et al., 2006). These findings provide an evidence that p300 is a specific molecular target of curcumin. So in the present study we chose curcumin to inhibit the HAT activity in cardiac myocytes and to explore the mechanism of cardiac-specific genes expression regulated by histone acetylation and p300.

Materials and methods

Chromatin immunoprecipitation (ChIP) assay in cardiac tissues

Healthy and adult Kunming (KM) mice (weight 28–32 g) were purchased from the Experimental Animal Center in Chongqing Medical University (Chongqing, China). All procedures on experimental animals were approved by the Animal Care and Use Committee at the Chongqing Medical University. Mice were mated at 5:00 pm and females were examined for a vaginal plug the following morning. At noon time, if a vaginal plug was observed, it was considered as embryo day 0.5 (E0.5). On days E10.5 and E16.5, female mice were killed by cervical dislocation and the embryonic hearts were collected. Hearts from 1- to 2-day-old neonatal mice were also collected. After homogenization of the heart tissues, formaldehyde was added into the samples to cross link proteins and DNA. ChIP experiments were performed using a ChIP assay kit (Millipore, Massachusetts, USA). After cross-linking, the DNA was cut into small fragments by sonication. The condition of sonication to shear DNA was 10 s each time with an interval of 30 s to cooling down and then repeated 70 times. Then protein–DNA complex was recruited and precipitated by monoclonal anti-p300 antibody (ChIP grade, Abcam, Cambridge, England) and then DNA was extracted using phenol/chloroform. The experiment contains both positive control group (precipitated by anti-RNA polymerase II antibody) and negative control group (precipitated by normal mouse IgG). To identify the heart development-related genes, which may interact with p300 protein and be regulated by p300, specific primers were designed for recognizing the promoters of GATA4, Nkx2.5 and Mef2c in PCR assays. To confirm the specific interaction between p300 and genes, we designed a pair of primers targeting the AT-rich (ATr) motif of α -mouse skeletal actin promoter (SA ATr site) as a positive control. For the negative control, we designed a pair of primers targeting the serum response element (SRE) binding site of mouse α -skeletal actin promoter (SA SRE site) which is reported not to bind with p300 (Slepek et al., 2001). The sequences of specific primers were as follows: GATA4, forward: 5'-CACTGACGCC-GACTCCAACTAA-3'; reverse: 5'-CGACTGGGTCCAATCAAAGG-3'. Nkx2.5, forward: 5'-CTTCTGGCTTCAATCCATCCTCA 3'; reverse: 5'-CGGGCAGTTCTGCGTCACCTA-3'. Mef2c, forward: 5'-CACGCATCT-CACCGCTTGACG-3'; reverse: 5'-CACCAGTGCCTTCTGCTTCTCC-3'. SA ATr site, forward: 5'-CCATCCCAGCTTCCATAAG-3'; reverse: 5'-CTGCTTCCCATGCACCTT-3'. SA SRE site, forward: 5'-GAAGGAAGG-GACTCTAGTGCC-3'; reverse: 5'-CTGTCCCCTTGACAGGTT-3'.

The annealing temperature was 60 °C for GATA4 and Nkx2.5, 68 °C for Mef2c, and 57 °C for ATr and SRE sites of α -skeletal actin promoter. Products of PCR were detected by 2% gel electrophoresis.

Culture of neonatal mouse cardiac myocytes

Primary cultures of cardiac myocytes were prepared as described previously (Sreejit et al., 2008). Cardiac myocytes were collected from the hearts of 1- to 2-day-old Balb/C mice (Experimental Animal Center of Chongqing Medical University). After being purified by differential velocity adherent, the suspension cells were seeded onto 6-well culture plates at a density of 1×10^6 cells/well coated with L-polylysine (Sigma, Santa Clara, California, USA) and cultured in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS), BrdU (final concentration 0.1 mM) and penicillin/streptomycin (final concentration 100 μ g/ml). After 48 h the medium was replenished for the first time. After 72 h, the cells were randomly divided into three groups, the curcumin group (treated with curcumin), the DMSO control group (treated with DMSO) and the untreated control group (treated with nothing). Curcumin (Sigma, Santa Clara, California, USA) was dissolved in DMSO to make a 50 μ M stock solution and stored in -20 °C. When cardiac myocytes were in good state, curcumin was added into the medium at the final concentration of 5 μ M, 10 μ M, 20 μ M, 30 μ M, 40 μ M and 50 μ M respectively. The treatment time of curcumin with the cells varied from 6 h, 12 h, 24 h, 36 h to 48 h. The cell viability was monitored by counting the cell number and observing the cell contraction and cellular morphology.

HAT activity assay

After treatment of the cells with different concentrations of curcumin (5 μ M, 10 μ M, 20 μ M, 30 μ M, 40 μ M, and 50 μ M) and different durations (6 h, 12 h, 24 h, 36 h, and 48 h), the cultured cardiac myocytes were collected and nuclear proteins were extracted using Nuclear Extract Kit (Active Motif, Carlsbad, California, USA) following the manufacturer's instructions. The HAT activities of the nuclear protein extraction were determined with an indirect ELISA assay using a HAT assay kit (Millipore, Massachusetts, USA). The ELISA plates were pre-coated with 100 μ l of 1 μ g/ml reconstituted histone H3 and incubated overnight at 4 °C. Nuclear extraction (20 μ g) was incubated with HAT reaction cocktail at room temperature for 15 min. Then the HAT assays were performed according to the manufacturer's instructions. The ELISA plates were read by an enzyme micro-plate reader (Thermo Scientific, New York, USA) at the wavelength of 450 nm and 570 nm. Well to well variations were removed by using the 570 nm values subtracted from the 450 nm values. Each assay group was determined in triplicate samples.

Western blotting

After treatment of the cells with curcumin at an optimal concentration and duration, the cultured cardiac myocytes were collected and nuclear proteins were extracted. Nuclear proteins were separated and electrophoresed on 15% Bis-Tris polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, USA). After incubation with 5% nonfat milk for 1 h, the blots were probed with rabbit polyclonal antibodies against acetylated groups of histone H3 (Abcam, Cambridge, England, 1:100 dilution) or mouse monoclonal antibody against β -actin (Abcam, Cambridge, England, 1:5000 dilution) in tris buffer solution (TBS) plus 5% nonfat milk at 4 °C overnight. HRP conjugated goat anti-rabbit and anti-mouse antibodies (Santa Cruz Biotechnology, California, USA) were used as the secondary antibody respectively. The immunoreactive protein bands were detected with an Enhanced

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