Chapter 2

Aspirin pretreatment reduced heat stress injury to chicken myocardial cells

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Abstract:

Heat stress injury causes considerable loss in breeding industry annually. In this research, we tested whether heat stress-induced myocardial cell damage can be prevented by aspirin (ASA) pre-treatments and if this is associated with an aspirin-induced up-regulation of heat shock proteins (Hsps). Primary cultured chicken myocardial cells and SPF chickens were used to build in vitro and in vivo stress models. Cytopathological lesions as well as myocardial cell damage-related enzymes, such as creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in cultural media or blood, were used to monitor myocardial cell injury. All myocardial cell injury indicators revealed aspirin pre-treatments reduced heat stress injury both in vitro and in vivo. However, aspirin’s protective function against heat stress was suppressed when the induction of heat shock proteins was inhibited. These results indicated the potential value of aspirin pre-treatments as an anti-heat stress solution and it can be related to the induction of heat shock proteins.

Key words: Heat stress, Aspirin, Heat shock proteins, Chicken, Myocardial cell
Introduction

The breeding industry has been suffering from great economic losses due to the seasonal climate in many regions (1, 2). Even in a developed country as United States, it was estimated that $2.4 billion loss is due to non-protection from heat exposure ($897 million, $369 million, $299 million and $128 million for dairy, beef, swine and poultry industries, respectively) (2). High temperature exposure leads to dysfunction in multiple organs, including the heart, one of the most important organs in multicellular organisms (3). Such myocardial injury is believed to be responsible for sudden death induced by heat stress (4, 5). Even in animals that survived from heat stress, acute intracorporal damage would affect the breeding industries. In fact, due to feather cover, lack of perspiratory glands and fast growth in modern commercial breeding facilities, poultry species, such as broiler chickens are more defenseless to high temperatures than mammals (2, 6, 7). Thus, developing a feasible solution to protect animals, especially poultry species such as chickens, from heat stress is an important aim.

To survive from HS, organisms from fungi to mammals have evolved endogenous anti-HS injury systems (8). Across both organs and species, these systems include heat shock proteins (HSPs) (8, 9). Previous studies have asserted that some HSPs possess potent stress resistance activities, particularly HspB1 (10, 11), HspD1 (12), HspA1A (13, 14), and HspC1 (15). A number of researchers believe that heat stress damage can be largely diminished if the Hsps are appropriately induced (8, 9). Under natural circumstances, many stimuli such as oxidation (16, 17), ultraviolet radiation (18), and hyperthermia are capable of inducing Hsps. Scientists have attempted to develop various strategies to induce Hsps in stressed animals via physical, chemical, and biological stimulation; however, most of these attempts were either too costly or produced limited benefits, and some even resulted in higher mortality rates than HS itself (19-21). Acetyl salicylic acid (ASA), also known as aspirin, is widely used for its broadly therapeutic anti-inflammatory and antipyretic properties. ASA has heart protective functions that were initially considered to act through platelet interactions (22). Although non-aspirin nonsteroidal anti-inflammatory drugs (NSAIDs) have anti-inflammatory and antiplatelet properties similar to aspirin, few of them induce the same myocardial protective effects against acute myocardial infarction (AMI) (23). Therefore, it is possible that ASA confers heart protection by another mechanism. ASA induces different HSP family members in different species (24-26), including HspB1 (27). Because Hsps are widely believed to protect against cell injury caused by different stress factors, it is reasonable to consider that ASA may protect against myocardial injury from heat stress by inducing one of the Hsps. In fact, previous studies have suggested that ASA helps to reduce stress injury in a manner closely related to the heat shock response (26-28).

This research is aimed to test if aspirin can prevent heat stress induced myocardial cell injury in vitro and in vivo stress models. To study the connection between aspirin-induced heat stress resistance
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and HSPs induction, we used BAPTA-AM as a Ca\(^{2+}\)-chelating agent, a treatment that was recently shown to inhibit some HSPs (29-33). Our data show that aspirin pretreatment indeed has cytoprotective effect against heat stress injury and suggest that induction of Hsps can be important to this protective effect.

**Results**

**Aspirin (ASA) pretreatment prevented heat stress injury in primary cultured chicken myocardial cells**

To evaluate the heat stress injury in primary cultured myocardial cells with or without ASA pretreatments, CK-MB, LDH and cytopathological observation were used as indicators. Upon exposure to heat stress, the permeability of the myocardial cell membrane increases and leads to the extracellular release of a series of enzymes, including CK-MB and LDH. These enzymes are generally used as cardiomyocyte damage-related enzymes (34-37), especially CK-MB. Heat shock resulted increases in CK-MB and LDH in the supernatant of myocardial cells (Figure 1, A and B). Cell pre-treated with aspirin (ASA) displayed lower level of CK-MB and LDH at most of the time points. This indicated ASA pretreatments reduced heat stress injury on primary cultured chicken’s myocardial cells (Figure 1, A and B).

Additionally, cytopathological analysis of the primary cultured chicken myocardial cells after heat treatment revealed acute vacuolar degeneration in the cytoplasm of myocardial cells immediately upon exposure to heat shock. This vacuolar degeneration, accompanied by granular degeneration, developed as the period of heat stress was prolonged and finally led to karyopyknosis and inadherence, after 15 hours of heat exposure (Figure 1C). In the ASA-pretreated group, vacuolar and granular degeneration could be observed in the cytoplasm of the myocardial cells after 1 h of heat stress exposure, but the degeneration was reduced compared to that in the HS group and no major pathological lesions were observed in the myocardial cells of the ASA-HS group after 15 hours of heat treatment. Without heat stress involved, no obvious pathological change was observed in the ASA group from the beginning to the end of the experiment (Figure 1, C).

Together, these results indicated ASA pretreatments reduced heat stress injury in chicken myocardial cells *in vitro*.

**Inhibition of Aspirin-Induced HS Resistance by BAPTA-AM**

Aspirin has been suggested to induce the heat shock response, leading to the up-regulation of Heat Shock Proteins. Indeed, the induction of several HSPs was enhanced by non-toxic ASA pre-treatments (1 mg/ml) compared to heat shock alone (Figure 2A, compare circles and squares). This suggested that the induction of these HSPs may be responsible for the ASA-induced heat stress resistance and Hsps. To
test this, we employed the heat shock response inhibitor, BAPTA-AM, (29-33) at non-toxic conditions BAPTA-AM (18 μM). Whilst BAPTA-AM alone did not significantly alter HSP levels (Figure 2 A, diamonds), it clearly reduced the ASA-induced elevation in the expression of all HSP tested (Figure 2 A, triangles).

Next, we asked whether BAPTA-AM also reversed the protection of ASA against heat induced damage by measuring again the release of CK-MB and LDH in the supernatants of primary cultured myocardial cells and the cytopathological observations as before. Treatment of cells with BAPTA-AM did not increase heat-induced damage to the cardiomyocytes (Figure 2B, C). When combining BAPTA-AM with ASA treatment, the protective action of ASA against heat damage was completely suppressed reflected by both increased as CK-MB and LDH release (Figure 2B) as well as stronger histological damage (Figure 2C).

**ASA pretreatment prevented heat stress injury in chicken myocardial cells in vivo**

Having established that ASA could prevent agains heat damage in vitro, we next tested if ASA pretreatment could also reduce heat-induced injury in animals. Hereto, the levels of the same cardiomyocyte damage-related enzymes, CK-MB and LDH, were analyzed in the serum of chickens and
Aspirin pretreatment reduced heat stress injury to chicken myocardial cells showed substantive increases after exposure to various periods of heat (Figure 3, A B). Indeed, also in vivo application of ASA conferred a substantial protection against heat-induced cardiac injury (Figure 3 A and B).

**Figure 2. ASA induced heat stress resistance can be suppressed by blocking Hsp induction.**

A, The expression of HSPs (HspB1, HspD1, HspA1A, and HspD1) in different groups, cells in HS group were exposed to heat stress from 0 h; cells in A group were treated with ASA 2 hours before heat stress (-2 h); cells in B group were treated with BAPTA-AM 2 hours before heat stress (-2 h); cells in A+B group were treated with both of ASA and BAPTA-AM 2 hours before heat stress. HSPs expressions at 0, 2, and 5 h were compared with the baseline level (-2 h), ** P < 0.01; * P < 0.05. B, Cytopathological image of cells exposed to heat stress without any pretreatments. C, Cytopathological image of cells exposed to heat stress with ASA pre-treatments 2 hours in advance. D and E, Variation of CKMB and LDH in HS group and A group. F, Cells in A+B group which is treated with ASA and BAPTA-AM 2 hours in advance displayed serious cytopathological damage after heat stress exposure. G and H, Level of CKMB and LDH
in A+B group also indicated cells were injured after heat stress exposure.

**Figure 3. ASA pre-treatment reduces heat stress injury in chicken’s myocardial cell in vivo.**

Chickens in ASA-HS group were administrated with ASA orally 2 h in advance (-2 h). Then cells in both groups were exposed to heat stress. A and B, variation of CKMB and LDH in serum. ** P < 0.01; * P < 0.05. C, Representative histopathological images of chicken myocardial cells following heat exposure. Hematoxylin and eosin staining. Scale bar = 10 μm. HS group: By 5 hours, acute degeneration in numerous vacuoles (↑) was observed in the myocardial fibers. Some nuclei within myocardial cells appeared shrunken and dull-stained, characteristic of karyopyknosis (→). At later time points, several nuclei displayed the characteristics of karyopyknosis (→) suggesting that acute injury remained in myocardial cells by 15 hours. ASA-HS group: By 5 hours there was no significant injury except for acute degeneration observed by myocardial cells morphology at later time points.

In line, the histopathological damage revealed after heat shock was dramatically reduced in ASA pretreated animals (Figure 3C). In the heat only group, the myocardial cells became swollen in shape after 1 hour heat exposure and some nuclei appeared shrunken and dull-stained, characteristic of karyopyknosis 5 hours after heating and vacuolar degeneration was observed among the myocardial after 15 hours. Chickens that were given ASA prior to heat exposure also displayed swollen myocardial cells with vacuolar degeneration after 1 hour of heat stress treatment, but except for the vacuolar degeneration at 1 hour, there was no significant injury in myocardial cells at later time points in this group.

**Discussion**

Here, we show that Aspirin (ASA) pretreatment reduces heat-induced myocardial cell damage both in vivo and in vitro. Our cellular data furthermore reveal that the ASA-related increased expressions of HSPs was key to its protective effects as the ablation of this response by BAPTA-AM also negated the damage protection of the ASA treatment. Resistance to HS injury has been often related to elevated HSP expression (8, 38). In the heart, especially small HSP have been suggested to been crucial for a proper maintenance of cardiac function (11, 38). Indeed, of the HSP tested here, especially the expression of
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HspB1 was elevated most (i.e. by over one order of magnitude compared to the baseline level). In fact, the overexpression of HspB1 has been reported to attenuate cardiac dysfunction in transgenic mice (11).

The finding of BAPTA-AM as a potent inhibitor of ASA-induced HSP expression and heat stress is striking. BAPTA-AM is known as an intracellular Ca\(^{2+}\) chelating agent (39, 40). This leads to a novel but reasonable hypothesis that intracellular Ca\(^{2+}\) might be involved in ASA-induced upregulation of Hsps. Although the complex relations between ASA and intracellular Ca\(^{2+}\) remain unclear (41-43), some studies have indicated that ASA acts at sites beyond the adenylate cyclase/cAMP system and before the proton pump (44). And in fact, many biological effects may be related to such ASA-induced increases in Ca\(^{2+}\) (45, 46). Intracellular free Ca\(^{2+}\) increased by heat stress could increase the complex formation between heat shock transcriptional factor (HSF) and heat shock elements HSE, hereby activating the heat shock response (47, 48). Besides activating the heat shock response, increased Ca\(^{2+}\) levels have also been reported to activate kinase or phosphatase that can lead to the phosphorylation of a series of proteins including HspB1 (49). Phosphorylated HspB1 will de-oligomerize into dimers which is the activated form of HspB1. This could be an alternative and non-exclusive contributor to the protective effect evoked by the ASA pretreatment (50-52). However, this require further research.

Materials and Methods

**Cell stress model**

Primary cultured chicken myocardial cells were provided by Shanghai Fu Meng Biological Technology Ltd (Shanghai, China). Cells were cultured (37°C and 5% CO\(_2\)) on the petri dishes for 72 h until the fusion rate was higher than 90%. The cell culture medium contained 20% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 IU penicillin and streptomycin (HyClone, Logan, UT, USA) in Dulbecco’s modified Eagle’s medium (Thermo Scientific, Waltham, MA, USA). Heat stress were given by water bath (40 ± 1°C). After treatments, at each time point, the supernate of the cell culture media from each group was collected, and myocardial cells were harvested. Samples for ELISA and enzymes detections were frozen in liquid nitrogen. The working concentrations of aspirin (1 mg/ml) and BAPTA-AM (18 μM) were measured by MTT assay in a preliminary experiment (data not shown). Aspirin powder (>98% purity) and BAPTA-AM powder (>95% purity) were purchased from Sigma, St. Louis, MO, USA. Additional cells were also cultured on poly-L-lysine (PLL) treated glass coverslips (Sigma, USA) for pathological analyses.

**Animal stress model**

One-day-old specific pathogen free (SPF) chickens were purchased from Qian Yuan Hao Biotechnology Company, Nanjing, China and raised in an air chamber (GJ-1, Suzhou Fengshi Laboratory
Animal Equipment Co. Ltd, China) at constant temperature (25 ± 1 ℃). The entire chicken population was vaccinated against Newcastle disease (ND) and infectious bursal disease (IBD) on the 7th and the 14th days, respectively. The birds were given 30 days to acclimate to their new housing and to recover from environmental stress. Heat stressed were given by rapidly and gently shifting the animals 25 ± 1 ℃ to a preheated air chamber 40 ± 1 ℃ with 60% ~ 70% humidity. During the experiment, birds were allowed free access to food and water ad libitum during heat stress exposure. After collecting 10ml blood samples, 10 chickens stood for 10 biological repeats were sacrificed humanely by decapitation at each time points all the groups. Heart samples were collected. Each heart specimen was cut into two pieces from the medial axis, one half was fixed in 10% formalin for morphological studies while another one was frozen in liquid nitrogen for ELISA and enzymes detections. The heat-stress experiment adhered to the guidelines of the regional Animal Ethics Committee and was approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

**Experimental process**

Primary cultured cells or animals were divided into three groups: HS group (heat stress challenge), ASA-HS group (pretreated with ASA before heat stress) and ASA group (treated with ASA alone). Except for cells or animals in the HS group, those in the ASA-HS group and the ASA group were administered ASA 2 h before the heat stress phase. This time point was marked as -2 h. As soon as the heat stress phase began, myocardial cells in the ASA group were maintained under normal conditions and served as one of the control groups (non-heated cells); meanwhile, the other two groups, the HS group and the ASA-HS group, were exposed to heat stress for different time span (0, 1, 2, 3, 5, 7, 10, 15 and 24 h).

**Detection of enzymes associate with heart damage**

Supernatants of cultured cells or serum (1.5 mL) was collected from chickens after exposure to the treatments described above. The LDH and CK-MB enzyme activities were measured according to the manufacturer’s instructions (Nanjing Jiancheng Biochemical Reagent Co. Ltd., Nanjing, China) with a clinical biochemical indicator auto-analyzer (Vital Scientific NV, The Netherlands). Each sample was analyzed five consecutive times.

**Pathological observation**

For cytopathological tests, myocardial cells cultured on PLL-pre-treated coverslips in vitro were fixed in paraformaldehyde overnight and were washed with PBS (pH 7.4) twice before staining. Heart tissue samples were obtained and preserved in 10% formalin. The samples were embedded in paraffin
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and then cut into 5-µm-thick serial sections. All sections were stained with hematoxylin and eosin (H&E), and the images were obtained using a light microscope.

**ELISA Assays**

Total proteins were extracted using RIPA lysis buffer (WB-0071, Dingguo Changsheng Biotechnology Co., Ltd., Nanjing, China). Concentrations of total proteins were measured using a Micro BCA assay kit (BCA01, Dingguo Changsheng Biotechnology Co., Ltd., Nanjing, China) and samples were diluted with lysis buffer to normalize the protein loading amounts. The concentrations of four HSPs (HspB1, HspD1, HspA1A, and HspC1) were measured by ELISA kits according to manufacturer’s instructions (MBS700383 for HspB1, MBS737544 for Hsp60, MBS704670 for Hsp90, and MBS740753 for Hsp70, MyBiosource, USA).
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