Research Paper

Protective and anti-fatigue effects of aspirin against heatstroke in rats

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Abstract: The purpose of this study is to determine whether aspirin can reduce interleukin-1β (IL-1β) concentration and exert protective effects against heatstroke. The heatstroke rat model was established through exposing rat to a high ambient temperature (HAT, T, 41°C, relative humidity 65%) in a simulative HAT chamber to induce heatstroke. Three parts were performed in the present experiment: (1) To determine the effects of pretreatment with aspirin against heatstroke; (2) To prove the effects of specifically reducing inducible nitric oxide synthase (iNOS) against rat heatstroke by iNOS selective prohibitor aminoguanidine (AG); (3) To determine the effects of aspirin against heatstroke and fatigue. In part 1 and 2, Sprague-Dawley rats were randomly assigned to control and aspirin groups or AG groups respectively. Mean arterial blood pressure (MAP), colonic temperature (T co), electrocardiograph (ECG) were monitored during heat exposure (HE) and blood samples were taken 0 and 60 min after HE for IL-1β assay or nitric oxide (NO) assay. In part 3, additional control and aspirin groups of conscious rats were put in a barrel with 41°C water and kept swimming until drowning over 10 s, and then intervals were recorded as survival time. The results from part 1 showed that from 0 to 50 min after HE, MAPs of control group and aspirin group were not significantly different. About 50~60 min after HE, MAPs of both groups were decreased abruptly and MAPs of control group were decreased significantly in comparison with those of aspirin group. T co of both groups was increased until to 42°C, without significant difference. Time of heatstroke onset was not significantly different, while survival time was significantly longer in aspirin group than that in control group. Plasma IL-1β concentrations in both groups were significantly increased after HE, and the concentration was significantly higher in the control group than that in aspirin group 60 min after HE. In part 3, the survival time was significantly longer in aspirin group than that in control group. In part 2, MAPs of both groups from 0 to 50 min after HE were not significantly different, whereas 55~60 min after HE, MAPs of control group were decreased significantly in comparison with those of AG group; T co of both groups was increased after HE until to 42°C, but without significant difference. The time of the heatstroke onset and survival time of AG group were significantly longer than that of control group; the plasma NO concentrations of two groups were significantly higher 60 min after HE than those 0 min after HE, and the plasma NO concentration of control group was significantly higher than that of AG group 60 min after HE. In conclusion, IL-1β may contribute to heatstroke through inducing iNOS, which attenuates the tone of peripheral blood vessel, and pretreatment with aspirin can provide preventive effects against heatstroke and reinforce the heat and fatigue endurance, which may be associated with inhibition of systemic IL-1β levels and local iNOS levels.

Key words: heatstroke; interleukin-1β; aspirin; anti-fatigue; blood pressure
Heatstroke (HE) is a medical emergency caused by prolonged exposure to high ambient temperature (HAT) and characterized by hypotension, delirium, coma and convulsion. Despite adequate lowering of body temperature and aggressive treatment, heatstroke is always associated with high mortality rates. Understanding of the pathophysiological mechanisms of the hypotension associated with heatstroke is critical for establishing the methods to prevent or treat heatstroke. There are still a lot of arguments about the cause of heatstroke and consequent death. It was once believed that the main cause of death in heatstroke is related to the damage of central nervous system (CNS) after prolonged exposure to HAT. However, the accumulating evidence indicates that the cause of death is probably not CNS damage, but systemic hemodynamic dysfunction caused by HAT and consequent increased concentration of endotoxin is the fatal factor of decreased vasoconstrictor tone and profound hypotension, and interleukin-1β (IL-1β) is involved in this complex process. A recent study from Kharbanda et al. suggested that pretreatment with aspirin can provide the preventive effects against inflammation-induced endothelial dysfunction which may be through modulation of cytokine (such as IL-1β) cascade. IL-1β associated with inflammation or stress can induce inappropriate expression of inducible nitric oxide synthase (iNOS) in local vessel endothelium which may be responsible for the decreased tone of peripheral blood vessel, and administration of nonsteroidal anti-inflammatory drugs (NSAIDs) dose-dependently inhibited IL-1β-stimulated iNOS and nitric oxide (NO) production in rats with heatstroke, consequently may provide protective effects on rats with heatstroke. In the present study, we try to determine whether pretreatment with aspirin can reduce interleukin-1β concentration and result in preventive and anti-fatigue effects in heatstroke rats, and explore whether iNOS is involved in this process using the selective iNOS prohibitor aminoguanidine (AG).

1 MATERIALS AND METHODS

1.1 Experimental animals
All experiments and animal care were approved by the Institutional Animal Care and Use Committee of the Southern Medical University, China. Adult male Sprague-Dawley (SD) rats (weighing 250–300 g) were obtained from the animal resource center of the Southern Medical University of China (Guangzhou). The animals were housed individually in hanged wire cages at room temperature (24°C) on a normal light-dark schedule. The animals had free access to food and water and were allowed to acclimatize to the light-dark cycle and the room temperature for at least 2 weeks before the experiment began.

1.2 Effects of aspirin against heatstroke in rat

1.2.1 Surgery and measurement of cardiovascular parameters in rat heatstroke
Twenty Sprague-Dawley rats were randomly assigned of 1 of the following 2 groups (n=10 each): control group and aspirin group were treated with 0.5% CMC (10 ml/kg, gastric gavage) and 0.5% CMC+0.25% aspirin (10 ml/kg, gastric gavage; Sigma) respectively 12 h before the experiments and were kept away from food and water. Rats of both groups received the same following procedures. The right femoral arteries of rats under urethane (1.4 g/kg, i.p.) anesthesia were cannulated with polyethylene tubing (PE-50), which is connected to a pressure transducer (PowerLab, ADInstruments) for monitoring
arterial blood pressure or taking blood sample (for IL-1β assay). The rats were measured using a thermocouple inserted 6~7 cm into the colon and kept colonic temperature (Tco) at (34±0.5)°C before HE. The animals were then put in a supine position. Three stainless steel needles were used as the recording electrodes for electrocardiograph (ECG) and connected to the ECG preamplifier (PowerLab, ADInstruments) to record lead II ECG. Blood pressure (BP) and ECG data were analog-to-digital converted and analyzed by a physiological monitoring system with real-time graphics and data analysis capabilities (Chart v4.1, PowerLab, ADInstruments). Two groups of rats were put in a simulated HAT chamber (designed by Southern Medical University) in which ambient temperature (Ta) was kept at 41°C with a relative humidity of 65% to induce heatstroke; the moment at which mean arterial blood pressure (MAP) began to decrease from its peak value was taken as the onset of heatstroke, then we disconnected HE and recovered Tco to 24°C and continued monitoring the experimental parameters until death ensured by ECG. The interval between the start of HE and the death was defined as survival time (ST).

1.2.2 IL-1β assay during rat heatstroke

Additional control group and aspirin group of rats (n=7 each) were used for the experiment. All the rats were anesthetized and instrumented as described above. Blood samples were taken 0 and 60 min after HE, anticoagulated with EDTA and conserved at –20°C for determination of IL-1β levels. Rat-IL-1β ELISA kit (Biodinge, Beijing) was used for determining the levels of active IL-1β presented in rat plasma. This assay was carried out by quantitative sandwich enzyme immunoassay technique, making two monoclonal antibodies directly act on two different epitopes of IL-1β. During the first incubation step, IL-1β in the samples is simultaneously bound to the biotinylated antibody and the peroxidase. Conjugated detection antibody forms a complex that binds via the biotinylated antibody to the streptavidin-coated surface of the microtiter plate (one-step immunoreaction). The peroxides in the complex was visualized by tetramethylbenzidine (TMB) as a substrate in the samples was simultaneously bound to the biotinylated antibody and the peroxidase. Conjugated detection antibody forms a complex that binds via the biotinylated antibody to the streptavidin-coated surface of the microtiter plate (one-step immunoreaction). The peroxides in the complex was visualized by tetramethylbenzidine (TMB) as a substrate and determined photometrically. The developed color was proportional to the concentration of IL-1β. OD values were read at 450 nm from enzyme immunoassay analyzer (Bio-Rad 550).

1.3 Effects of specifically blocking iNOS against rat heatstroke by selective iNOS inhibitor aminoguanidine

1.3.1 Surgery and measurement of cardiovascular parameters in rat heatstroke

Twenty Sprague-Dawley rats were randomly assigned to control group or AG group (n=10 each) for this experiment. All rats were anesthetized and instrumented as described above. Rats of control group and AG group were treated with normal saline and 2.5% AG (3 ml/kg, i.p.) respectively and were induced heatstroke as described above. ECG, MAP, ST were recorded as described above.

1.3.2 Nitric oxide assay during rat heatstroke

Additional 20 Sprague-Dawley rats were randomly assigned to control group or AG group (n=10 each) for the experiment. The right femoral artery of rats under urethane anesthesia (1.4 g/kg, i.p.) were cannulated with polyethylene tubing (PE-50) which was for blood sampling (for nitric oxide assay), and Tco was kept at (34±0.5)°C before HE. Blood samples were taken 0 and 60 min after HE, anticoagulated with EDTA and conserved at –20°C for determination of NO levels. Nitric oxide detection kit (Jiancheng, Nanjing) was used for determining the levels of NO presented in rat plasma. The blood samples melted at room temperature, and then NO levels in the samples were detected by nitrate reductase method. NO is active chemically, and will be converted into NO3− and NO2− quickly in vivo. NO2− will be converted into NO3− further. This method uses nitrate reductase to reduce NO3− to NO2− specifically, and then determine the concentration by colorimetry.

1.4 Determination of anti-fatigue effects of aspirin at high ambient temperature

Tco of rats in aspirin and control groups (n=12 each) was kept at (34±0.5)°C before experiment. The rats were put in a barrel (high 0.75 m, diameter 0.5 m) with 41°C water (depth 0.5 m) and kept swimming until drowning over 10 s. The interval between the start of experiment and drowning was defined as survival time. After the experiments, the rats were killed with an overdose of urethane.

1.5 Statistical analysis

Data from experiments were analyzed by SPSS 10.0 and expressed as mean ± SD.

2 RESULTS

2.1 Effects of pretreatment with aspirin against rat heatstroke

2.1.1 The time of the onset of heatstroke (TOHS) and ST

As shown in Table 1, TOHS of the two groups were not significantly different (P>0.05), while ST of aspirin group was significantly longer than that of control group (P<0.05).
2.1.2 MAP and $T_{co}$
From 0 to 50 min after HE, MAPs of two groups were not significantly different ($P>0.05$), but after TOHS (55–60 min after HE), MAPs of control group were decreased significantly as compared with that of aspirin group ($P<0.01$). $T_{co}$ of both groups was increased with increase in HE time until $T_{co}$ increased to 42ºC (the onset of heatstroke), without significant difference between two groups ($P>0.05$) (Fig.1).

2.2 Effects of specifically reducing iNOS against rat heatstroke by aminoguanidine
2.2.1 MAP and $T_{co}$
From 0 to 50 min after HE, MAPs of two groups were not significantly different, but after TOHS (55–60 min after HE), MAPs of control group were decreased significantly as compared with that of AG group ($P<0.01$). $T_{co}$ of both groups was increased with increase in HE time until $T_{co}$ increased to 42ºC (the onset of heatstroke), but there was no significant difference between two groups (Fig.2).

2.2.2 TOHS and ST
TOHS and ST of AG group were significantly longer than that of control group ($P<0.05$) (Table 3).

2.2.3 Effects of specifically reducing iNOS on plasma NO in rat heatstroke
The plasma NO concentrations of the two groups were significantly higher 60 min after HE than 0 min after HE, and the plasma NO concentrations of control group were significantly higher than that of AG group 60 min after HE (Table 4).

2.3 Effects of pretreatment with aspirin on fatigue

### Table 1. Effects of pretreatment with aspirin on the time of the onset of heatstroke and survival time in rat heatstroke (min)

<table>
<thead>
<tr>
<th>Group (n=10)</th>
<th>TOHS</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>57.5±3.92</td>
<td>66.7±4.27</td>
</tr>
<tr>
<td>Aspirin group</td>
<td>59.7±5.56</td>
<td>88.7±17.33</td>
</tr>
</tbody>
</table>

mean ± SD. *$P<0.05$ vs control group.

### Table 2. Effects of pretreatment with anti-inflammatory dose of aspirin on plasma IL-1β in heatstroke rats (pg/ml)

<table>
<thead>
<tr>
<th>Group (n=7)</th>
<th>0 min after HE</th>
<th>60 min after HE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>43.3±10.11</td>
<td>102.4±44.44</td>
</tr>
<tr>
<td>Aspirin group</td>
<td>28.8±8.18</td>
<td>42.2±13.00</td>
</tr>
</tbody>
</table>

mean ± SD. *$P<0.01$ vs 0 min after HE, †$P<0.01$ vs control group. Interaction exists between administration of aspirin and HE, $P<0.05$. 

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Fig.1. Effects of pretreatment with aspirin on MAP and $T_{co}$ in rat heatstroke. mean ± SD. *$P<0.01$ vs control group.

Fig.2. Effects of reducing iNOS by AG on MAP and $T_{co}$ in rat heatstroke. mean ± SD. *$P<0.05$ vs control group.
associated with high ambient temperature
Survival time of aspirin group was significantly longer than that of control group [(39.25±6.98) min vs (49.58±21.21) min, P=0.007], showing that aspirin may have some protective effects against fatigue associated with HAT (Fig.3).

![Fig.3. Effects of pretreatment with aspirin on fatigue under HAT.](image)

\* P<0.01 vs control group.

3 DISCUSSION
The physiological mechanisms of producing heatstroke and consequent death are still unresolved, which were mostly thought as CNS damage induced by HAT, but Eshel reported that the acute cerebral derangements during and after lethal hyperthermia are reversible and the cause of death is probably not CNS damage, but systemic hemodynamic deterioration\cite{11}. Owing to the fact that mechanism of producing heatstroke is still unknown, methods for clinical prevention and treatment to heatstroke are limited.

Since 1990’s, it has been found that a series of cytokines (IL-1β, tumor necrosis factor α [TNF-α], interleukin-6 [IL-6], interferon γ [INFγ]) are involved in modulation of heatstroke, an imbalance between inflammatory cytokines (IL-1β, TNF-α, INFγ) and anti-inflammatory cytokines (interleukin-1ra, interleukin-2, interleukin-10, soluble TNF receptors p55 and p75) may contribute to circulatory dysfunction and CNS damage in heatstroke\cite{7-15}. In 1997, Lin et al. found that increased plasma levels of IL-1 followed heatstroke; animals injected with IL-1ra at the time of heatstroke induction were protected from some of the cardiovascular effects of heatstroke such as depressed ventricular depolarization, decreased stroke volume, and arterial hypotension; and the hemodynamic changes associated with heatstroke could be mimicked by IL-1 injection\cite{2}. The data suggest that heatstroke stimulates synthesis and release of IL-1 in the plasma, depresses ventricular depolarization and stroke volume, and results in arterial hypotension. It was believed that IL-1β, TNF-α, IL-6 exert important effects on production and development of heatstroke, and elevation of concentration of IL-1β, TNF-α, IL-6 in heatstroke is induced by endotoxin under the condition of high temperature, but not by heat stress alone\cite{16}.

The present experiment estimated the preventive effects of pretreatment with anti-inflammatory dose of aspirin against rat heatstroke induced by HAT. The results of present study showed that pretreatment with aspirin did not exert significant effects on the time of onset of heatstroke or influences on MAP before the onset of heatstroke. However the results showed that pretreatment with anti-inflammatory dose of aspirin attenuated the decrease in MAP after onset of heatstroke, and that survival time of aspirin group was significantly longer than that of control group. These results suggest that pretreatment with anti-inflammatory dose of aspirin may provide preventive effects against rat heatstroke. In the present study, IL-1β assay showed that pretreatment with aspirin significantly attenuated the increase of IL-1β in the plasma under the condition of prolonged HE. However, it is still unknown why the plasma IL-1β levels of the two groups were already significantly different 0 min after HE. We speculate that pretreatment with aspirin can contribute both to the decrease in the plasma levels of IL-1β in normal rats and to the prohibition of elevation of the plasma levels of IL-1β in heatstroke rats. Nevertheless, we still consider that aspirin can reduce the level of IL-1β in heatstroke rat through...
statistical analysis. Although it was reported that levels of IL-1β in heatstroke rat was elevated and aspirin reduced the elevation of IL-1β in inflammation, there are no data demonstrating that IL-1β can exert direct effects on blood pressure. We consider that there are more factors that contribute to the preventive effects against heatstroke provided by pretreatment with aspirin. In the present study, we also determined the effects of specifically reducing iNOS against rat heatstroke by aminoguanidine. We found that administration of aminoguanidine achieved the similar effects against rat heatstroke. Although we have not the direct evidence to show the relationship between iNOS and IL-1β in heatstroke, the results of the study show a clue that the similar effects against heatstroke achieved by reducing iNOS and inhibiting IL-1β are not coincidental. The promoter region of the rat iNOS gene contains several potential cis-elements for the binding of different transcription factors, including two putative binding sites for NF-κB[24]. IL-1β can activate nuclear factor-κB (NF-κB) and stimulate the transcription of iNOS mRNA via NF-κB, elevate the levels of iNOS and NO in a series of cells, such as macrophages, hepatocytes, vascular endothelial cells, smooth muscle cells, fibroblasts and myocardial cells. Activation of NF-κB by lipopolysaccharide (LPS) or cytokines requires either the degradation of IκB-α[19] or proteolytic cleavage of p105 through the ubiquitin-proteasome pathway after phosphorylation[20].

Aspirin may exert its effects on inhibition of iNOS through multiple mechanisms. One is that aspirin at low dose inhibits iNOS expression at the translational or post-translational level without prevention of NF-κB activation[21,22]; the other is that aspirin at anti-inflammatory dose inhibits iNOS protein expression and reduces the translocation of NF-κB[23]. Through which way aspirin is implicated in the present system is unknown. However, pretreatment with aspirin did not significantly affect the change of Tcorr, suggesting that aspirin does not exert its protective effects against heatstroke through antipyretic effect on body temperature. It was demonstrated that these effects appear to be independent of cyclooxygenase inhibition and may be dependent on the level of oxidative stress or activation of NF-κB[22].

In the experiment that estimate effects of pretreatment with aspirin on fatigue under the condition of HAT, we, for the first time, unexpectedly observed that pretreatment with anti-inflammatory doses of aspirin significantly improved fatigue tolerance of conscious rats under HAT. The result is fascinating, which indicates that aspirin exerts anti-heatstroke effects through a complex process, in which it, on one hand, blocks inflammatory cytokines, on the other hand, improves the exercise endurance under HAT. Our experiments suggest that pretreatment with anti-inflammatory dose of aspirin may provide protective effects on the population working in strong intensity under HAT. However, heatstroke is a complex process involved in multiple systems and multifactors, and aspirin is also an agent with broad utility, which behaves different pharmacological characters at different doses. It needs to be determined that whether other NAIDS drugs have similar utility in anti-heatstroke and at what dose and when it is administered aspirin can exert its protective effects against heatstroke. Indeed, we have found that administration of aspirin after HE or even just at the start of HE does nothing or even harms to heatstroke animals in our previous experiments (data not shown) which shows that the antipyretic and analgesic effects of aspirin are not useful for the heatstroke, and that is the reason why we chose the time of administration of aspirin 12 h before the start of HE in the present experiment.

In summary, our present results and previous study of others show that inflammation induced by elevation of endotoxin and cytokines in heatstroke can induce endothelial dysfunction and attenuate the peripheral vascular resistance and pretreatment with anti-inflammatory dose of aspirin can prevent these effects associated with the decrease in elevation of the plasma IL-1β levels. A preliminary study showed a novel usage of aspirin for anti-fatigue effect of rat heatstroke. Aspirin as an inexpensive and safe drug may produce a novel action on human heatstroke and may exert important protective effects on the population in industrial production, military training, etc. in summer or under HAT.

REFERENCES


