Original Article

Whole-body hyperthermia attenuates experimental autoimmune myocarditis in the rat

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Abstract

Background: Heat stress prior to induction of various forms of cardiac injury has been shown to result in preconditioning and attenuation of subsequent damage. We evaluated the effects of whole-body hyperthermia (WBH) on experimental autoimmune myocarditis (EAM) induced either by injection of myosin or by adoptive transfer of lymphocytes.

Methods and Results: Lewis rats were pretreated with WBH either 24 h prior to EAM induction (Group A1) or 14 days following EAM induction (Group A2). The control group included myocarditic rats that were not exposed to WBH (Group A3). Rats from Group A1 exhibited significant protection from myocarditis as compared to rats from Group A3, evidenced by reduced myocarditis scores (1.60±0.96 vs. 2.88±0.35, \(P=.016\)). Rats from Group A2 also exhibited protection from myocarditis although not significantly. In a second experiment, we used adoptive transfer of myosin-reactive lymphocytes to study the mechanism of WBH effect on myocarditis. There was evidence of microscopic myocarditis in four out of five rats that were injected with active lymphocytes (the Control Group B3). Myocarditis was not observed in rats adoptively transferred with preheated rat lymphocytes (Group B1) nor in preheated rats, which underwent administration of nonheated rat lymphocytes (Group B2).

Conclusions: Whole-body hyperthermia attenuates experimental myocarditis in the rat. The beneficial effect of whole-body hyperthermia may be related to immunomodulatory effect and direct cardiomyocyte protection. © 2008 Published by Elsevier Inc.

Keywords: Whole-body hyperthermia; Myocarditis; Myosin; Rat

1. Introduction

Acute myocarditis consists of an inflammatory destruction of myocytes due to myocardial infection and/or autoimmune process. It is a potentially lethal disease in humans, with a 5-year survival rate (for patients with biopsy-proven myocarditis) that approximates 50% [1,2]. Currently, there is no effective treatment for myocarditis, as immunosuppression has not been shown to be effective as a routine treatment for infectious or postinfectious myocarditis [3]. Unique models for autoimmune myocarditis have been established by immunizing mice [4] or rats [5] with cardiac myosin. Experimental autoimmune myocarditis (EAM) in the rat resembles the fulminant form of human myocarditis. Kodama et al. [6] showed that injection of spleen cells or lymph node cells from rats with severe myocarditis into naive rats elicited myocarditis, while injection of immunoglobulin fraction of pooled sera from rats with severe myocarditis did not. This study led to the hypothesis that EAM is a T-cell-dependent myocarditis. Whole-body hyperthermia (WBH) refers to a procedure of raising body core temperature to 41.0–42.0°C for several minutes. Applying WBH prior to induction of various forms of tissue injury in animal models has been shown to attenuate the severity of injury. This prior sublethal heat stress, also called hyperthermic preconditioning, attenuated myocardial damage and infarct size in ischemia–reperfusion and myocardial

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infarction models in rabbits and rats [7,8]. The protective effect of prior WBH has also been established in arterial denudation models mimicking arterial restenosis [9]. Several studies showed that WBH can protect cardiomyocytes from reactive oxygen species and also has immunomodulatory effects, either proinflammatory or anti-inflammatory [10,11]. Therefore, we conducted two consecutive experiments in an EAM model in rats aiming to study the effect of applying WBH prior to induction of EAM on the extent of myocardial inflammation and to try to elucidate whether this effect involves cardiomyocytes or the immune system.

2. Methods

2.1. Animals

Experiments were performed on male 6-week-old Lewis rats, which were purchased from Harlan and maintained in the local animal house under conventional conditions. The rats’ weights were measured immediately before beginning of experiments and ranged from 280 to 310 g. The experimental protocol was approved by the animal subjects committee of our institute.

2.2. Induction of EAM

Cardiac porcine myosin (Sigma) was dissolved in a solution of potassium chloride, 0.3 mol/l, and phosphate-buffered saline (PBS), 0.2 mol/l, at a concentration of 10.0 mg/ml. Rats were anesthetized with ketamine (20 mg/100 g) and xylazine (6 mg/100 g) intraperitoneal and subsequently were injected subcutaneously in the footpads with 0.3 mg of cardiac myosin in an equal volume of Freund’s complete adjuvant containing 10.0 mg/ml of heat-killed Mycobacterium tuberculosis and were sacrificed 21 days later.

2.3. Application of WBH

Rats were anesthetized with ketamine (20 mg/100 g) and xylazine (6 mg/100 g) intraperitoneal and were placed in a ventilated metal chamber heated by a heating plate from beneath and by an incandescent lamp from above. A digital thermometer was positioned rectally for serial measurements every minute. Animals were heated until colonic temperature reached 41°C and were maintained at 41.5°C for 15 min. Animals were then removed from the chamber and were allowed to cool.

2.4. Experimental design

In the first experiment, three groups of rats were tested (Fig. 1):

- Group A1 (n=10)—Lewis rats (properly sedated) were heated 24 h prior to induction of myocarditis by myosin.
- Group A2—Rats were heated 14 days after induction of myocarditis by myosin.
- Group A3 (control group, n=8)—rats were induced to develop myocarditis by injection of myosin. Group B1—Activated lymphocytes originated from pre heated rats were transferred into naive rats. Group B2—Activated lymphocytes were transferred to preheated naive rats. Group B3—Activated lymphocytes were transferred to nonheated naive rats.

![Fig. 1. Experimental protocol. H=WBH (41.5° for 15 min); L=intravenous injection of activated lymphocytes; M=intraperitoneal injection of myosin; S=sacrifice of rats. Group A1—Rats were heated 24 h before induction of myocarditis by myosin. Group A2—Rats were heated 14 days after induction of myocarditis by myosin. Group A3—rats were induced to develop myocarditis by injection of myosin. Group B1—Activated lymphocytes originated from pre heated rats were transferred into naive rats. Group B2—Activated lymphocytes were transferred to preheated naive rats. Group B3—Activated lymphocytes were transferred to nonheated naive rats.](image-url)
The lymph node cells were obtained from other six rats that were injected with porcine myosin but were not heated.

Group B3 (control group) Lymphocytes were injected intravenously into five naive rats. The lymph node cells were obtained from six other naive Lewis rats that were injected with porcine myosin but were not heated.

2.5. Adoptive transfer of activated lymphocytes

Eighteen rats were anesthetized and subsequently immunized with 0.3 mg of porcine myosin (in the same solution mentioned above) in each leg, and 10 days later their inguinal lymph nodes were removed and the rats were sacrificed. Single-cell suspension was prepared by passing through a mesh screen. The lymph node cells were suspended at a density of 4.5×10^6 cells/ml in RPMI-1640 supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% nonessential amino acids (Bioproducts, Inc., MD), and 5×10^-5 M 2-mercaptoethanol and cultured for 3 days in the presence of 1 μg/ml of concavalin A (Con A; Sigma Chemical, St. Louis, MO). Cultured lymph node cells at a dose of 3×10^7 were injected intravenously into Lewis rats. Recipient rats were killed on Day 14 for histological examination.

2.6. Serum IgG antimyosin antibody levels by ELISA

Porcine myosin (10 μg/ml) in PBS was coated onto flat bottom 96-well ELISA plates (Nunc) by overnight incubation at 4°C. After washings with 0.02% PBS Tween and blocking with 2% BSA in PBS, sera were added in a dilution at 4°C. After washings with 0.02% PBS Tween and blocking with 2% BSA in PBS, sera were added in a dilution similar to the frozen section staining method using a primary antibody [rabbit antimouse P65 (NFκB); Santa Cruz Biotechnology, CA, USA] and a secondary antibody (goat antirabbit, DAKO, Denmark). Positive stained cells were counted in myocadial inflammatory areas at ×200 magnification. Stained myocytes were not included. An average number from five different 0.5-mm^2 fields was calculated.

For evaluation of nuclear factor-kappa B (NFκB) lymphocyte nuclei staining, we used parafin-embedded sections cut by microtome. The slides were incubated at 60°C overnight, followed by deparaffinization and heated in a pressure cooker in a microwave 750 W for 5 min. Then, the slides were immersed in citrate buffer for 10 min and were rinsed for 5 min in PBS-TBS-Tween. Additional steps were similar to the frozen section staining method using a primary antibody [rabbit antimmune P65 (NFκB); Santa Cruz Biotechnology, CA, USA] and a secondary antibody (goat antirabbit, DAKO, Denmark).

2.8. Immunohistochemical studies

Immunohistochemical studies were applied to four to seven samples from each group. Frozen sections were briefly dried under airstream for 30 min, then rinsed twice for 5 min in BSA-TBS-Tween. Primary antibodies [goat antirat tumor necrosis factor-α (TNF-α); R&D systems, Minneapolis, MN, USA. Rabbit anti-inducible nitric oxide synthase (iNOS); Santa Cruz Biotechnology, Santa Cruz, CA, USA and Goat antitransforming growth factor-β1 (TGF-β1); Santa Cruz Biotechnology Inc., CA, USA] diluted in TBS were applied for 60 min at room temperature. The slides were again rinsed twice for 5 min in BSA-TBS-Tween and then incubated for 30 min at room temperature with the appropriate secondary antibody (rabbit antigoat; Chemicon International Inc. Temecula, CA, USA; rabbit antigoat, Chemicon International, Inc., USA and goat antirabbit; DAKO, Denmark, respectively). For quenching endogenous peroxidase activity, slides were incubated for 30 min with 3% H₂O₂. Antibody binding was visualized with 3-amino-9-ethylcarbazole (Sigma). Finally, the slides were counterstained with Mayer’s hematoxylin and mounted with Glycergel (DAKO, Carpinteria, CA, USA).

2.9. Statistical analysis

Data are presented as mean±S.D. Baseline rat body weights were evaluated by one-way analysis of variance test; histopathological scores were analyzed using the Mann-Whitney test. Other results were statistically evaluated by Student’s unpaired t tests. Univariate correlations were performed using Pearson’s correlation coefficient. All tests were two tailed. Differences of P<.05 were considered significant.

3. Results

3.1. Body weight and heart weight of rats

Baseline rat body weight was not significantly different between groups: mean±S.D.=295±17, 294±12, 290±20, P=.724 for groups A1, A2, and A3, respectively.
In the first experiment, we have found a significant reduction in heart weight of rats treated with WBH either 24 h before induction of myocarditis or 14 days after, as compared with rats that were induced to develop myocarditis and were not exposed to WBH (mean ± S.D. = 1.15 ± 0.29 g, \(P = .002\) and 1.37 ± 0.17 g, \(P = .036\), respectively, Fig. 2).

### 3.2. Prior WBH attenuated myocarditis score and humoral response

Prior WBH treatment reduced the extent of myocarditis by 45% compared to control group as was evident from reduction in myocarditis scores. Applying WBH 14 days after induction of myocarditis also led to a reduction of myocarditis score when compared with nontreated rats; however, it was less prominent and did not reach statistical significance (mean ± S.D. = 1.60 ± 0.96 vs. 2.88 ± 0.35, \(P = .016\) and 2.60 ± 0.52 vs. 2.88 ± 0.35, \(P = .360\), respectively, Figs. 3 and 4).

Levels of IgG antimyosin antibodies were significantly reduced in rats treated with WBH prior to induction of myocarditis compared with nonheated rats. Levels of IgG antimyosin antibodies were also lower (although not significantly) in rats treated with WBH 14 days after induction of myocarditis compared with nonheated rats (mean ± S.D. OD = 1.60 ± 0.20 vs. 1.97 ± 0.17, \(P = .0007\) and 1.88 ± 0.19 vs. 1.97 ± 0.17, \(P = .321\), respectively, Fig. 4).

Fig. 2. Rats treated by WBH had reduced heart weight. There was a significant reduction in heart weight of rats (solid lines denote the mean value; squares and triangles denote individual values) treated with WBH either 24 h before induction of myocarditis or 14 days after, as compared with rats that were induced to develop myocarditis and were not exposed to WBH (\(P = .002\) and \(P = .036\), respectively).

Fig. 3. Myocarditis severity evaluated by histopathology. (A, B) Histopathological examination of ventricular wall from a nonheated rat induced to develop myocarditis by injection of myosin (original magnification ×20 and ×50, respectively). (C, D) A rat heated 14 days after induction of myocarditis (original magnification ×20 and ×50, respectively). (E, F) A rat heated 24 h before induction of myocarditis (original magnification ×20 and ×50, respectively).
3.3. Immunohistochemical studies

Treatment with WBH prior to induction of myocarditis resulted in a significant decrease in the percentage of iNOS expressing mononuclear cells out of total mononuclear cells within the myocardium compared with nonexposed rats (mean±S.D.=12.0±9.9 vs. 33.4±11.8%,  \( P=.025 \)) (Fig. 5).

A similar decrease in the percentage of mononuclear cells expressing TGF-β and NFkB (only cell nuclei expressing NFkB were counted) compared with nonheated rats was also evident (for TGF-β: 13.7±9.2 vs. 54.1±12.9%,  \( P=.0007 \) and for NFkB: 22.1±9.1 vs. 36.4±11.4%,  \( P=.025 \)).

Treatment with WBH 14 days after induction of myocarditis resulted in a significant decrease in the percentage of TNF-α expressing mononuclear cells compared with nonheated rats (8.1±5.7 vs. 29.3±16.5%,  \( P=.043 \)).

There were no significant changes in the number of mononuclear cells expressing iNOS, TGF-β, and NFkB in the group heated 14 days after myocarditis induction compared with nonexposed rats (for iNOS: 30.7±14.1 vs. 33.4±11.8%,  \( P=.749 \), for TGF-β: 32.7±18.7 vs. 54.1±12.9%,  \( P=.072 \), and for NFkB: 45.4±18.0 vs. 36.4±11.4%,  \( P=.290 \)).

There was a strong correlation between heart weight, IgG antimyosin levels in serum, iNOS, TGF-β, and NFkB expression in mononuclear cells compared with myocarditis pathological score (for heart weight:  \( r=0.74, P<.0001; \) for IgG antimyosin levels in serum,  \( r=0.65, P=.0002; \) for iNOS,  \( r=0.52, P=.059; \) for TGF-β:  \( r=0.43, P=.106; \) and for NFkB:  \( r=0.54, P=.012 \)).

3.4. Prior WBH abolished myocarditis induced by adoptive transfer of activated lymphocytes

In the second experiment, we used adoptive transfer of myosin-reactive lymphocytes to study the mechanism of WBH effect on myocarditis (Fig. 1). We have found evidence of microscopic myocarditis in four out of five rats that were injected with lymphocytes (the Control Group
Myocarditis was not evident neither in rats (n=5) that were injected with lymphocytes obtained from rats that were exposed to WBH prior to immunization with myosin (Group B1) nor in rats (n=5) that were heated prior to injection with lymphocytes removed from rats immunized with myosin (Group B2).

4. Discussion

Herein we show for the first time that application of WBH prior to induction of experimental autoimmune myocarditis in the rat significantly attenuate myocarditis severity. This favorable effect was evident whether myocarditis was induced by myosin injection or by adoptive transfer of disease-triggering lymphocytes.

Brief periods of heat stress prior to induction of various forms of tissue injury have been shown to result in preconditioning and attenuation of subsequent damage. Either prior local hyperthermia or prior WBH attenuate myocardial injury induced by ischemia–reperfusion [12,13]. It was suggested that WBH provides biphasic protection against ischemia–reperfusion injury, soon after, and 24 to 72 h after hyperthermia [8]. Yamashita et al. demonstrated that an increase in manganese superoxide dismutase, an intrinsic radical scavenger, plays a central role in the protective effect of WBH. This increase in superoxide dismutase levels and activity was shown to be mediated through the production of TNF-α and IL-1 brought about by WBH [10].

There are conflicting results regarding the immunoregulatory effects of WBH. Some investigators reported that WBH reduces T-cell levels and activity [14–16].

Conversely, another experiment in humans [17] showed that hyperthermia stimulates the expression of adhesion molecules on endothelial cells and lymphocytes and enhances lymphocyte delivery to secondary lymphoid tissues. Yet another study showed that the effects of WBH on antigen-responsive immune response are time-dependent [18].

In humans, WBH has been applied as an adjunctive therapy to cancer treatment such as radiotherapy and chemotherapy. Experimental and clinical studies have shown that hyperthermia resulted in a higher response rate and better overall survival rates in patients with many types of cancer [19,20]. These favorable effects were attributed mainly to antitumor selective effects due to hypersensitivity of tumor cells to hyperthermia in regions with hypoxia and low PH. It was also suggested that hyperthermia induces T-cell activation and a proinflammatory state that promotes immune surveillance [21]. However, these immunomodulatory effects of WBH in humans were investigated as an adjunct to radiotherapy or chemotherapy and therefore cannot be applied to patients with myocarditis.

Kanda et al. [22] have shown in mice that WBH (42.5°C for 30 min) applied 4 h after encephalomyocarditis virus inoculation aggravated the viral induced myocardial necrosis compared with control mice with myocarditis. WBH applied 4 h prior to inoculation had no effect on myocarditis severity compared to controls. The timing, duration, and temperature of WBH were different in our study. We investigated the effects of prior WBH (41.5°C for 15 min) on myocarditis severity in two different time points: 24 h before and 14 days after myosin injection. These time points were chosen because WBH provides protection when applied 24 h before ischemia–reperfusion injury [8] and because it is established that myocarditis onset (as indicated by cellular infiltration) is 14 days after immunization [23]. In the current study, there was a reduction in heart weight of rats treated with WBH either 24 h before induction of myocarditis or 14 days after, as compared to nonheated rats with myocarditis. This heart weight reduction represented a reduction of inflammatory cellular infiltration and edema of the myocard in rats treated by WBH. However, WBH significantly ameliorated the extent of myocarditis as evidenced by histopathology only in rats exposed to WBH before induction of the disorder. In a separate experiment, rats were also induced to develop myocarditis by transfer of lymphocytes. We examined two groups of adoptively transferred myocarditis (Fig. 1) in order to elucidate whether WBH exerts its beneficial effects through direct cardioprotection or through immunoregulatory effects. However, in both groups, myocarditis was abolished compared with a control group. It is therefore conceivable that WBH mechanism includes both direct cardioprotection and immunomodulatory effects.

Hyperthermia may protect cardiomyocytes against an inflammatory injury in a similar way as it protects against an ischemia–reperfusion injury [7,8,24]. WBH may increase the expression of heat shock proteins that have an intracellular role as cardioprotectants by stabilizing cytoskeletal structures or inhibiting apoptosis [25]. WBH may activate intrinsic radical scavengers in the myocardium and attenuate the inflammatory injury [10].

Hyperthermia may also have potential influences on lymphocytes. Some investigators previously reported that WBH reduces T helper and T-suppressor cell levels in peripheral blood and suppresses T cells and natural killer cells activity [14–16]. As EAM is a T-cell-dependent myocarditis, reducing T-cell level or activity may abolish myocarditis.

We also investigated the mechanism of WBH beneficial effects by measuring several factors which play important roles in inflammatory processes. We evaluated iNOS, TGF-β, NFkB, and TNF-α expression in lymphocytes in cardiac tissue. Overexpression of iNOS in cardiac tissue increases myocardial injury due to high levels of nitric oxide [26,27]. TGF-β is a cytokine that induces fibroblast proliferation and fibrosis, and NFkB is an important proinflammatory transcription factor. Herein, we showed that prior WBH rather than WBH applied after myocarditis induction reduced iNOS, TGF-B, and NFkB expression in cardiac tissue. These results are supported by the strong correlation between myocarditis severity (assessed by histopathological score) and iNOS, TGF-β, and NFkB expression in cardiac tissue. It is possible that hyperthermia
exerts its beneficial effects by reducing the levels of iNOS, TGF-β, and/or NFκB. We also evaluated TNF-α expression in cardiac tissue and found a trend for increased levels in the preheated group. It is possible that increased TNF-α levels by mediating expression of superoxide dismutase, an intrinsic radical scavenger, also play a role in the cardioprotection attributed to WBH as demonstrated by Yamashita et al. [10].

In conclusion, this study demonstrates for the first time a beneficial effect of applying WBH prior to induction of autoimmune myocarditis induced by both myosin immunization and adoptive transfer of myosin reactive lymph node cells. These favorable effects may be attributed to both direct cardioprotection and immunomodulatory effects.

References


