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The effect of IGF-1 on symptoms of sleep deprivation in a rat model of inflammatory heart disease and metabolic syndrome

Haiyan Chen [a,1], Zhengqing Zhao [b,1], Jiaoyang Zheng [a], Xiangfang Chen [a], Junjie Zou [a], Yongquan Shi [a,*], Zhimin Liu [a,*]

[a] Department of Endocrinology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China
[b] Department of Neurology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China

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ABSTRACT

Sleep deprivation (SD) has become a worldwide public health concern due to the many negative health consequences associated with suboptimal sleep. SD has been linked to a catabolic hormone signature, heart disease, hypertension, diabetes, and an increase in morbidity and mortality. Herein, we investigated the effects and mechanism of SD on cardiac and metabolic health and evaluated the impact of exogenously supplied IGF-1 on these symptoms. In the present study, we show that 5 days of acute SD negatively impacted all of the various indicators of cardiac and metabolic health. All symptoms of SD were ameliorated by daily administration of IGF-1, however. IGF-1 administration also reduced the phosphorylation of Akt and expression of Bax, a promoter of apoptosis. Conversely, the expression of Bcl-2, an inhibitor of apoptosis, was elevated by IGF-1, and all IGF-1 effects were suppressed by the PI3K/Akt inhibitor LY294002, reaffirming the importance of the PI3K/Akt pathway in the maintenance of cardiac and metabolic health.

1. Introduction

Negative health consequences resulting from chronic sleep deprivation (SD) have become a growing health concern worldwide in recognition of the significant and growing proportion of the population reporting suboptimal sleep (<7 in 24 h) [1,2]. Indeed, many different studies have drawn links between both acute and long-term SD and heart disease, inflammation, hypertension, diabetes, obesity and a general increase in morbidity and mortality [3,4]. Long-term, low-level inflammation is a known risk factor for heart disease, and reduced sleep duration has been linked to coronary calcification, a precursor of coronary heart disease [5]. A number of studies have identified a connection between sleep deprivation and an increase in IL-6 and IL-1β levels among other markers of inflammatory activation, such as CRP, IL-7 and TNF-α [6]. For example, van Leeuwen et al. [7] demonstrated that 5 days of sleep restricted to 4 h in 24 increased lymphocyte activation, heart rate and IL-6, IL-17 and C-reactive protein levels, suggesting that prolonged SD may increase the risk of cardiovascular disease.

In addition to inflammation and heart disease, diabetes and obesity have also been linked to SD [8]. This connection was first probed by Spiegel et al. who observed an increase in the ghrelin-to-leptin ration concomitant with increased hunger in subjects following sleep restriction to 4 h/night.

Numerous lines of evidence point to the central role played by the PI3K/AKT pathway in the maintenance of cardiac and metabolic health [9,10]. This pathway is regulated by a wide variety of external stimuli, including nutrients, cellular stressors, insulin and insulin-like growth factor-1 (IGF-1) [11,12]. IGF-1, a hormone similar in structure to insulin, is the primary mediator of the effects of growth hormone. This hormone can promote the growth and repair of heart tissues [13].

The purpose of our study was to evaluate the severity of myocardial damage resulting from SD as indicated by cardiac function, apoptosis levels and general tissue integrity. We also explored the mechanism of this damage, and determined how SD affects blood glucose, glucagon and insulin in our rat model of inflammatory heart disease. We then investigated the effects of IGF-1 on these indicators of heart disease and metabolic disorder. This study should help elucidate the molecular mechanisms of SD pathology and, more specifically, clarify the role played in SD by the PI3K/AKT pathway.

* Corresponding authors. Fax: +86 021 63586228 (Y. Shi).
E-mail addresses: young.stone@163.com (Y. Shi), zmliu_cz@hotmail.com (Z. Liu).
[1] The first two authors contributed equally to the work.

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2. Materials and methods

2.1. Animals and housing

All experiments were performed on adult male Wistar rats (Charles River Canada, St. Constant, QC, Canada) weighing between 220 and 300 g. The rats were housed individually in stainless steel cages in a climate-controlled room (22 °C ± 1) under a 12:12 h light:dark cycle (lights on at 7:00 am) with rat chow and water available *ad libitum*. Rats were allowed to acclimate to their new environment for at least 2 weeks prior to experimentation.

All animal experiments were approved by the medical ethics committee of Shanghai Changzheng Hospital and were carried out in accordance with the International Guiding Principles for Animal Research.

2.2. Sleep deprivation

Rats were deprived of sleep by placing them in rolling drums of 33 cm in diameter that rotated at one revolution every 3 min. Each wheel was divided into four compartments, allowing the simultaneous treatment of 4 rats. The slow rotation forced the rats to move continuously, albeit with minimal effort, and permitted consumption of food and water *ad libitum* from a central tube.

In experiment 1, rats were divided randomly in 5 SD groups (SD-1d–SD-5d; $n = 4$ for each group) and subjected to sleep deprivation for 1–5 d. Control rats (NC; $n = 4$) were allowed to sleep normally in plastic cages placed beside the SD apparatus. Sleep deprivation was started at 7:00 am and ended in one-day intervals for each of the five groups out to day 5.

In experiment 2, rats were randomly divided into 4 groups ($n = 4$ for each group) and treated in the following ways: (1) rats were allowed to sleep normally for 5 d (NC group); (2) rats were sleep deprived for 5 d, and normal saline was injected into their jugular veins between 7:00 and 8:00 am every day (SD-5d [NS] group); (3) rats were sleep deprived for 5 d, and IGF-1 (Pharmacia and Upjohn, Inc., Stockholm, Sweden) was injected intravenously (i.v.) at a dose of 1 μg/kg/day between 7:00 and 8:00 am (SD-5d [IGF-1] group); or (4) rats were sleep deprived for 5 d, and 0.3 mg/kg/day of the PI3 K/Akt inhibitor LY294002 (Sigma, USA) was i.v. injected 15 min before IGF-1 injection between 7:00 and 8:00 am (SD-5d [IGF-1 + LY] group).

At the end of SD treatment, the heart rate (HR) and mean arterial pressure (MAP) of each rat was measured, and 5 mL of blood was collected with an arterial catheter. Blood glucose levels were measured with an Ascensia Counter Glucometer (Bayer health care, NY), and serum was separated and stored at −80 °C for the measurement of glucagon, insulin and IGF-1. Animals from each group were killed by decapitation for cardiac-tissue sample collection. The cardiac-tissue samples were cut into sections and either fixed in 4% paraformaldehyde (pH 7.4) and stored at 4 °C or flash-frozen in liquid nitrogen and stored at −80 °C for future analysis.

2.3. Hemodynamic measurements

All rats were anesthetized via i.v. injection of chloral hydrate (0.4 mL/kg) and placed on a heating pad to maintain a normal body temperature of 37 °C. A polyethylene catheter inserted into the right femoral artery was connected to a Gould model P23 ID pressure transducer (Glen Burnie, MD, USA), and the pressure output signals were sent to a MP-36 data acquisition system (Biopac Systems, Santa Barbara, CA). All signals were analyzed with Biopac Student Lab Pro version 3.7.3 (Biopac Systems) software to obtain the MAP and HR.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Following hemodynamic measurements, 5 mL of blood was collected from the abdominal artery. Serum was then separated for the measurement of glucagon, insulin and IGF-1 using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA).

Interleukin (IL)-6 and IL-1β levels in cardiac tissue were also determined using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. IL-6 and IL-1β levels in cardiac tissue were expressed as pg/mg of protein.

2.5. Histological analysis

Serial sections of the left ventricle were taken from tissues fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin blocks were cut into 5 μm slices and stained with hematoxylin–eosin (H&E). A pathologist blinded to the treatments carried out the histopathological examination using an optical microscope (Olympus, Tokyo, Japan).

2.6. Immunohistochemistry (IHC)

Slides were blocked with 5% normal serum diluted 1:200 and incubated with mouse anti-rat IGF-1 monoclonal antibody (1:200 dilution; Abcam, Cambridge, UK) overnight at 4 °C. Slides were washed three times in PBS for 5 min each and then incubated for 20 min at 37 °C with a biotin-labeled, anti-mouse detection antibody (Santa Cruz, California, USA). Slides were then developed with diaminobenzidine (DAB) reagent (Sigma), counterstained with hematoxylin (Sigma) for 30 s, dehydrated with graded ethanol washes and mounted on Canadian balsam. Captured fields were analyzed using an HPIAS-2000 image analysis system (Huahai Medical Info-Tech Co., Ltd., China). The integrated optical density (IOD/area) was measured to characterize the nucleus.

2.7. TUNEL assay

We applied the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) technique to paraformaldehyde-fixed cardiac samples in paraffin blocks using a Colorimetric TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's directions. The percentage of apoptotic cardiomyocytes, also know as the apoptotic index, was calculated as the number of TUNEL-positive cells over the total number of cells in 5 representative microscope views. As a positive control, we used tissue sections from a normal rat heart treated with 10 U/mL of DNase I for 10 min at room temperature.

2.8. Western blot analysis

Frozen heart tissue was homogenized in the following lysis buffer on ice: 20 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM b-glycerolphosphate; 1 mM Na3VO4; 1 mg/mL each of aprotinin, leupeptin and pepstatin; and 1 mM phenylmethylsulfonyl fluoride (PMSF). Equalized amounts of protein were separated with 8–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were then transferred to nitrocellulose membranes and probed with the following rabbit anti-rat antibodies: Bcl-2 (1:500), Bax (1:1000), phospho-Akt (Ser473; 1:5000), Akt (1:500) and b-actin (1:1000) (Abcam, Cambridge, England). The transferred membranes were then washed three times with 50 mM PBS and 0.05% Tween, pH 7.6 and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at 37 °C. Protein blots were then developed with Pierce ECL western

blotting substrate (Waltham, MA, USA). The expression of β-actin was determined as a normalization control.

2.9. Statistical analysis

Data were expressed as the mean ± SD. All statistical analyses were performed with SPSS statistical software (SPSS for Windows, version 19.0). The two-tailed student's *t*-test was used to determine the statistical significance of differences between the experiment conditions. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Sleep deprivation and markers of metabolic and cardiovascular activity

We evaluated the effects of sleep deprivation on sugar metabolism using ELISAs to quantify serum glucagon and insulin. Both blood glucose and glucagon levels rose with increasing duration of sleep deprivation. The blood glucose concentration rose 41.7% by day 5 relative to the NC (9.36 ± 0.8 mmol/L versus 6.45 ± 0.2 mmol/L, respectively [$p < 0.05$]) (Fig. 1A), and a similar trend was observed for the glucagon concentration, which rose 72% (129.94 ± 11.2 ng/mL versus 75.48 ± 4.01 ng/mL, respectively [$p < 0.01$]) (Fig. 1B). Insulin increased for the 1–3 day SD, then followed the inverse trend that fell with increasing SD and reached a recorded minimum at day 5 (Fig. 1C). These data demonstrate that prolonged SD leads depressed insulin production and elevated blood sugar.

Similar trends were observed in both the HR and MAP data. Both hemodynamic measurements rose with increasing duration of sleep deprivation and reached peak levels at days 4 or 5 (Fig. 1E and F). The HR levels rose to a maximum of 461 ± 8.3 bmp at day 4, a number 17.3% higher than the NC (393 ± 3.2 bmp).

We also measured the concentration of IGF-1 during the course of prolonged SD to determine the baseline level of this circulating growth hormone. IGF-1 levels declined from 381.5 ± 10.1 ng/mL in the NC case to 194.4 ± 10.2 ng/mL at day 5 of SD, a 49.0% drop ($p < 0.01$) (Fig. 1D). These data reinforced the connection between IGF-1, sugar metabolism and cardiovascular health in the context of SD. Notably, all six indicators reached their respective extremes at or near day 5. Therefore, further investigations focused on rats deprived of sleep for 5 days.

3.2. The effects of IGF-1 on metabolic and cardiovascular activity during sleep deprivation

Measures of cardiovascular activity (HR and MAP) and sugar metabolism (blood sugar, glucagon and insulin levels) in rats subjected to sleep deprivation for 5 days and i.v. injection of IGF-1, either alone or in combination with the PI3K/Akt inhibitor LY294002, are shown in Fig. 2.

The SD-5d levels of glucagon and blood sugar declined to close to NS levels when the rats were injected daily with IGF-1 (glucagon: 126.52 ± 15.13 versus 93.24 ± 11.3 ng/mL for SD-5d [NS] and SD-5d [IGF-1] [$p < 0.05$], respectively and blood glucose: 9.83 ± 0.65 versus 7.11 ± 0.57 mmol/L for SD-5d [NS] and SD-5d [IGF-1] [$p < 0.05$]). This effect was partially reversed in the presence of the PI3K/Akt inhibitor LY294002 (Fig. 2A and C).

Insulin levels declined significantly in response to sleep deprivation, while IGF-1 partially rescued insulin levels (0.79 ± 0.13 versus 1.41 ± 0.2 ng/mL for SD-5d [NS] and SD-5d [IGF-1], respectively [$p < 0.05$]). The effect of IGF-1 was again reversed by the presence of LY294002 (Fig. 2B).

HR and MAP followed very similar patterns. Both were elevated as before by 5 days of SD plus the injection of saline. The effects of SD were suppressed by daily injection of IGF-1 (HR: 483 ± 15 versus 419 ± 12 bmp for SD-5d [NS] and SD-5d [IGF-1] [$p < 0.05$], respectively and MAP: 179 ± 12 versus 147 ± 10 mmHg for SD-5d [NS] and SD-5d [IGF-1], respectively [$p < 0.05$]), and again the IGF-1 effect was suppressed by the presence of LY294002 (Fig. 2F and G). Overall, these data demonstrate that exogenous IGF-1 can partially suppress the deleterious effects of SD on glucose metabolism and cardiac performance. Also, the suppression of the protective effects of IGF-1 by the PI3K/Akt inhibitor LY294002 suggests that IGF-1 operates through the PI3K/Akt pathway in this system.

3.3. IHC analysis of IGF-1

We performed anti-IGF-1 IHC to determine to what extent IGF-1 was localized to ventricular tissue under different experimental conditions. Notably, IGF-1 staining of cardiomyocytes was sporadic, with a minority of cells staining more brightly (Fig. 2D). IGF-1 staining was elevated in response to SD plus saline, in agreement with the ELISA results presented in Fig. 1. Injecting rats with IGF-1 elevated IGF-1 levels in ventricular tissue further (0.066 ± 0.005 versus 0.089 ± 0.006 IOD/area for SD-5d [NS] and SD-5d [IGF-1], respectively [$p < 0.05$]), while LY294002 suppressed IGF-1 staining to close to SD-5d (NS) levels (Fig. 2E). These results directly demonstrated that the pool of IGF-1 available in cardiac tissue was enhanced by daily IGF-1 injection and the inhibitor LY294002 significantly suppressed IGF-1 levels in tissue.

3.4. Markers of inflammation: histopathological analysis of rat ventricular tissue and expression of IL-6 and IL-1β

In Fig. 3A, H&E stained rat ventricular tissues from the NC, SD-5d (NS), SD-5d (IGF-1) and SD-5d (IGF-1 + LY) treatment groups are presented. In these representative views of cardiac tissue, sleep deprivation has visibly reduced tissue integrity, with an increase in cross-grain lesions present in the myocardium. This phenomenon was partially suppressed by daily IGF1 injection. Again, LY294002 visibly suppressed the protective effects of IGF-1 on tissue integrity.

Five days of SD led to robust increases in the concentration of both IL-6 (Fig. 3B) and IL-1β(Fig. 3C), with IL-6 levels rising from 25.12 ± 2.83 to 92.05 ± 8.01 pg/mg total protein ($p < 0.01$), an increase of 266%. IL-1β levels rose 312%, from 8.05 ± 1.54 to 33.18 ± 2.87 pg/mg total protein ($p < 0.01$). Daily IGF-1 injection partially suppressed the expression of these two pro-inflammatory cytokines to 64.88 ± 6.16 and 16.19 ± 2.31 pg/mg total protein for IL-6 and IL-1β, respectively, but levels for both cytokines were still significantly higher than in the NC. When the PI3K/Akt inhibitor LY294002 was included in the daily IGF-1 injection, cytokine levels again rose to nearly the same levels as observed in the absence of IGF-1. Thus, SD provoked significant expression of pro-inflammatory cytokines in the heart and also caused a general disruption in cardiac tissue integrity. Exposure to experimental levels of IGF-1 was not sufficient to completely suppress this effect.

3.5. TUNEL staining and the apoptotic index in rat heart tissue

We used the TUNEL assay to quantify apoptosis of rat cardiomyocytes in response to SD. In Fig. 4A, arrows indicate the dark brown staining of apoptotic cardiomyocyte. Little staining was visible in NC cells, but the number of visibly stained nuclei rose significantly after 5 days of SD (Fig. 4A). This change was reflected in the apoptotic index, which rose from 3.5 ± 0.28% for the NC to 11.8 ± 0.62% in SD-5d (NS) tissue ($p < 0.01$). Daily IGF-1 injection

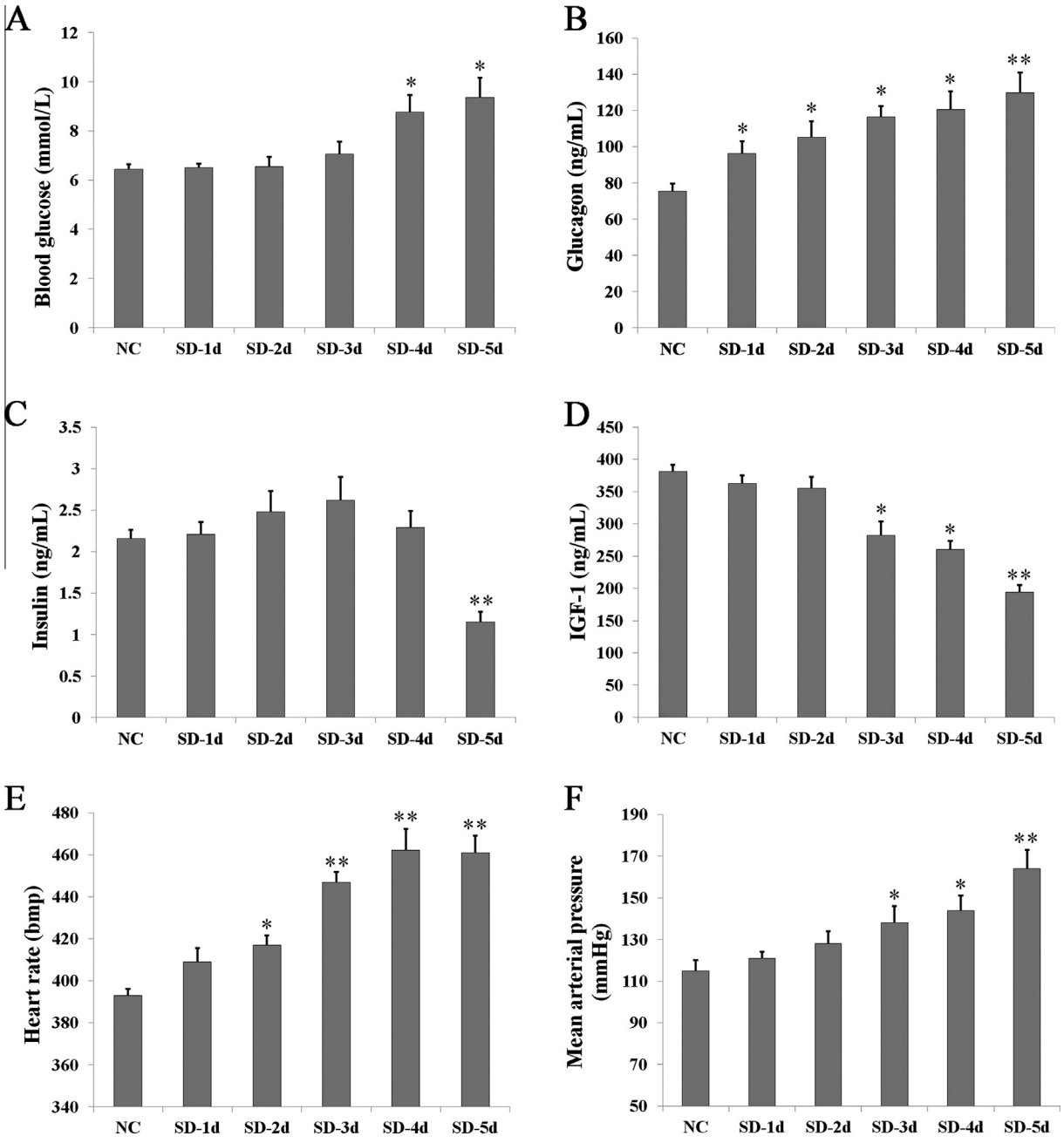

Fig. 1. Markers of metabolic and cardiovascular activity of sleep deprivation rats. Rats were subjected to sleep deprivation for 1–5 days, histograms depict blood glucose (A), glucagon (B), insulin (C) and IGF-1 (D) levels in serum, heart rate (HR) (E) and mean arterial pressure (MAP) (F) of normal control (NC) and sleep-deprived (SD) rats. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with normal control (NC).

reduced the apoptotic index to 6.5 ± 0.58%, an effect that was partially suppressed by the inhibitor LY294002 (Fig. 4B).

3.6. Western blot analysis of Bax, Bcl-2, Akt and p-Akt in rat heart tissue

To further explore the role of IGF-1 in the suppression of apoptosis during SD, we examined the expression of Bax, a key driver of apoptosis, using western blotting. Five days of SD caused 3.41 ± 0.1-fold overexpression ($p < 0.01$) of Bax relative to the NC (Fig. 4C and D). This expression was partially suppressed by IGF-1 injection, in agreement with other measures of apoptosis.

We also used Western blotting to examine the phosphorylation, and thus activation, level of Akt in heart tissue of sleep-deprived rats. Akt expression remained more or less constant across all of the treatments tested, and 5 days of SD had no significant effect on phosphorylation at Serine 473. However, phosphorylation rose 2.5 ± 0.2-fold ($p < 0.01$) (Fig. 4C and D) relative to the NC with daily injection of IGF-1, demonstrating that IGF-1 contributed to Akt activation via phosphorylation. This effect was again partially suppressed by the inhibitor LY294002. Bcl-2, a potent suppressor of apoptosis, exhibited a nearly identical pattern to phosphorylation of Akt, suggesting that the two proteins function in concert to protect heart tissue from damage resulting from SD.

4. Discussion

The purpose of this study was to evaluate the severity of myocardial damage resulting from SD and to explore the mechanisms causing such damage. We also wanted to evaluate the effects of

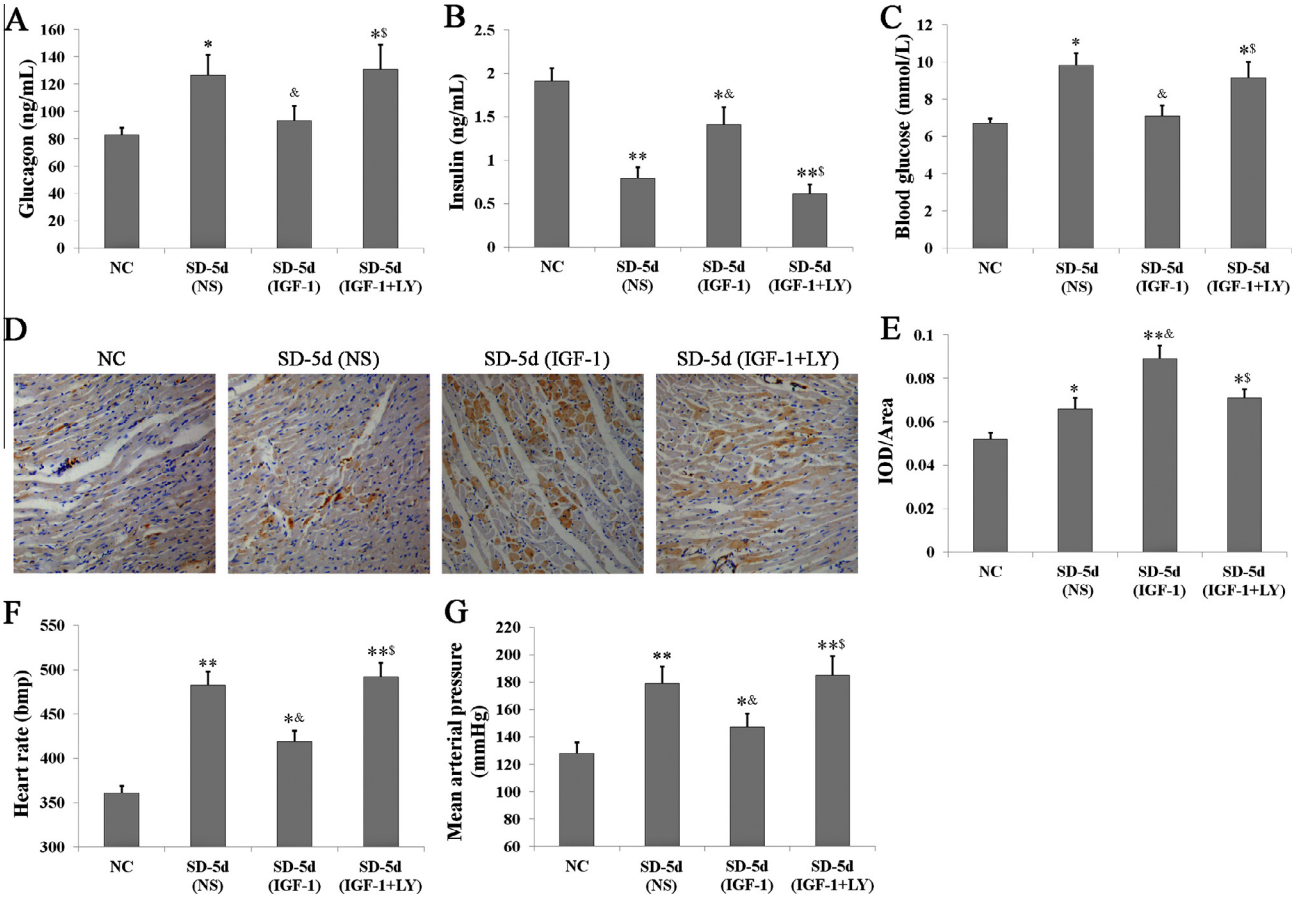

Fig. 2. The effects of IGF-1 on metabolic and cardiovascular activity during sleep deprivation. Rats received different treatment during the course of 5 days of sleep deprivation. Glucagon (A) and insulin (B) levels in serum, blood glucose (C), Immunohistochemistry of IGF-1 (D and E) in left ventricular tissue, heart rate (HR) (F) and mean arterial pressure (MAP) (G). $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with NC; $^{\&}p < 0.05$ as compared with SD-5d (NS); $^{\$}p < 0.05$ as compared with SD-5d (IGF-1).

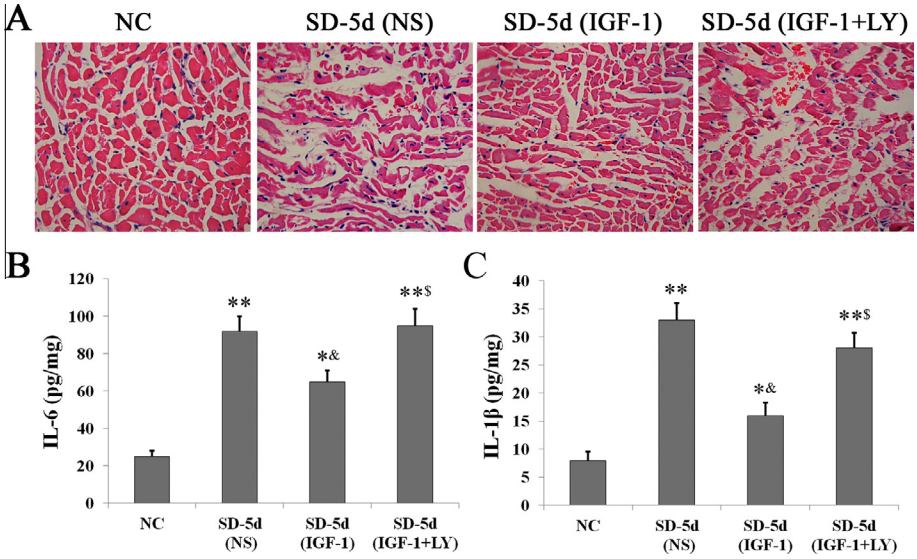

Fig. 3. Histopathological analysis of rat ventricular tissue and expression of IL-6 and IL-1β. (A) Histopathological changes in rat cardiac tissue (HE, ×200); interleukin (IL)-6 (B) and IL-1β (C) levels in cardiac tissue. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with NC; $^{\&}p < 0.05$ as compared with SD-5d (NS); $^{\$}p < 0.05$ as compared with SD-5d (IGF-1).

exogenously administered IGF-1 on the cardiovascular and metabolic symptoms of acute sleep deprivation.

Sleep deprivation has a significant effect on hormone secretion in both humans and rats, inducing a catabolic profile through the secretion of corticosterone in rats and the suppression of anabolic hormones such as IGF-1 [14] and testosterone [15]. Therefore, we sought to determine the effect of the catabolic state induced by acute SD on the tissue integrity and function of the heart. In this

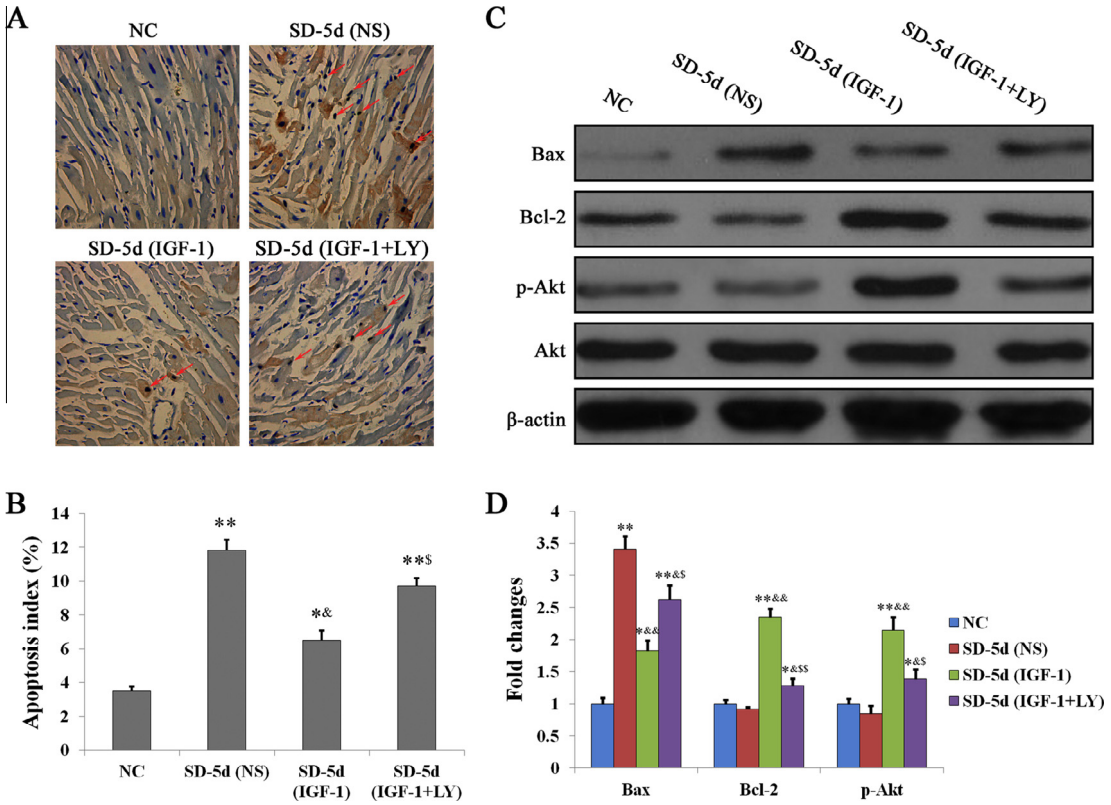

Fig. 4. TUNEL staining and the apoptotic index in rat heart tissue. (A) TUNEL staining in rat hearts (×200); (B) apoptosis index; (C and D) Western blot analysis of Bax, Bcl-2 and p-Akt in rat hearts. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with NC; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ as compared with SD-5d (NS); $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ as compared with SD-5d (IGF-1).

study, we verified that secretion of IGF-1 was suppressed, and our IHC results clearly demonstrated that the gross tissue integrity of the heart was compromised as a result of the catabolic hormonal conditions induced by 5 days of acute SD. Gross tissue damage was also accompanied by increased levels of cardiomyocyte apoptosis as indicated by the TUNEL assay.

As a circulating hormone, exogenously supplied IGF-1 has been shown to protect against neuronal damage in a rat model of ischemic stroke [16]. Exogenously supplied IGF-1 has also been shown to improve left ventricular function and reduce cardiomyocyte apoptosis after experimental coronary-artery ligation in Sprague–Dawley rats [17]. We adopted a similar strategy to investigate the effects of exogenously supplied IGF-1 in heart tissues damaged by SD. IGF-1 had significant effects on all of markers of heart function and integrity tested, including blood pressure and heart rate, and also reduced the level of cardiomyocyte apoptosis, in agreement with Rong et al.

The hormonal state of both rats and humans impacts muscle condition primarily through the PI3K/Akt pathway [15,18]. Enhanced phosphorylation of Akt has been correlated with muscle hypertrophy in a rat model of compensatory muscle hypertrophy in rats [19]. Activation of this pathway enhances protein synthesis through various molecular interactions, including the inhibition of glycogen synthase kinase 3β (GSK-3β) [20] and activation of $p70^{S6K}$ by Akt [21]. To verify that this pathway is active in SD, we antagonized the action of IGF-1 with the PI3K/Akt inhibitor LY294002, and this inhibitor did in fact suppress the protective effects of IGF-1.

Strikingly, all of the deleterious effects associated with SD in this study were ameliorated by IGF-1, and the effects of IGF-1 were inhibited by LY294002. Also, the magnitude of the effects, both cardiac and metabolic, was remarkably similar. Therefore, IGF-1 and the PI3K/Akt pathway clearly occupy a central pathway in the maintenance of cardiac and metabolic health, and increased IGF-1 concentrations following both surgical and environmental insults are restorative for cardiac and metabolic function.

Although LY294002 fully inhibited the protective cardiac and metabolic effects of IGF-1, it did not fully inhibit the enhanced Akt phosphorylation caused by exogenous IGF-1 as is seen in Fig. 4D. This may be because there is a threshold level of Akt phosphorylation that provides a fully protective effect [22]. It is also possible that the Akt phosphorylation response to the IGF-1 level lags behind the hormone response [23].

In summary, our results demonstrate that acute sleep deprivation causes significant degradation of the cardiac tissue accompanied by elevated levels of cardiomyocyte apoptosis and markers associated with inflammation. These effects were accompanied by elevated blood pressure and heart rate. Changes in glucagon, insulin and blood sugar levels also indicated that acute SD may increase the risk of metabolic dysfunction. All of these deleterious symptoms were suppressed by exogenously administered IGF-1 in a LY294002-sensitive manner, implicating the PI3K/Akt pathway in this global protective system.

Acknowledgments

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