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Insulin Over Expression induces Heart Abnormalities via Reactive Oxygen Species Regulation, might be step towards Cardiac Hypertrophy

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Abstract

Insulin is known to regulate blood-glucose level and promote its utilization as an energy source in cardiac tissues under normal physiological conditions as well as stimulates signaling pathways that involved cell growth and proliferation. Although recently insulin generated free radicals via NAD(P)H has been documented but the molecular mechanism is still under investigation. The aim of present study is to elucidate the reactive oxygen species (ROS) dependent possible role of insulin in cardiac abnormalities, including hypertrophy by regulation of antioxidants enzyme (SOD) activity. In the current study, 60 cardiac patients and 50 healthy individuals as well as the rat model with insulin administration were under investigation. Oxidant, anti-oxidant biochemical assays, hypertrophic marker expression via immunobloting and histopathology were performed. We observed statistically significant elevation of the reactive oxygen species level in the serum of patients as well as in the insulin administrated rat model, a mild expression of cardiac marker in experimental models along with abnormal histopathology of hearts. However, super oxide dismutase free radical scavenger activity was down regulated upon insulin treatment compared to control rats. Conclusively, the present study showed that over expression of insulin might stimulate cardiac hypertrophic signal via up regulation of free radicals and down regulation of antioxidants enzymes including SOD activity.

Key words: Insulin, Cardiac Hypertrophy, ROS, SOD, ANF.

Introduction

Insulin is a multifunctional polypeptide; it reduces the glucose level in the blood as well as stimulates synthesis of fatty acid and glycogen, improves microcirculation, induces cell proliferation and promotes mitochondrial function (1,2) by activating different signaling pathways. Thousands of insulin receptors have been reported that are present on the surface of each cell, including cardiomyocyte (3). When insulin binds to its receptor (IR), it activates a network of pathways, including mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (P13K) pathway which then further participates in the development of myocardial hypertrophy (4,5). Furthermore, insulin also increases the transcription factor expression such as the nuclear factor of activated T-cells (NFAT), responsible for the pro-hypertrophic gene expression (5).

In biological systems, reactive oxygen species (ROS) are known to play a dual role, they may either be harmful or beneficial to living organism. Unregulated excessive ROS is cytotoxic, causing oxidative damage to numerous cellular macromolecules and plays an important role in the etiology of a number of pathological conditions and diseases (6) including cardiac hypertrophy (7). ROS stimulates a variety of hypertrophy signaling kinases, transcription factors and causes muscle dysfunction, which may in turn be associated with insulin resistance (8). Free radicals are routinely removed by cellular antioxidants, including superoxide dismutase (SOD) and catalase (9). Over production of oxidative stress, such as superoxide anions in mitochondria during hyperglycemia will be quenched by SOD (4).

Different sources are considered to be involved in the generation of ROS, including enzymatic source as NADPH oxidase (10). Biswas *et al.,* 2013 (11) reported that insulin induced ROS generation in an NADPH oxidase dependent manner, additionally stimulates PI3K and PKC signaling. Moreover, insulin growth factor 1 (IGF-1) a peptide hormone is involved in a variety of cell physiological processes, enhance insulin sensitivity and also stimulates the free-radical generation (12). There are also accumulating evidences that insulin resistant hearts are more sensitive to ROS and mediate deleterious effects due to impaired antioxidant status in the state of insulin resistance (13).

Natriuretic peptides such as atrial natriuretic factor (ANF), B-type natriuretic peptide (BNP) modulates cardiac hypertrophy and are potential therapeutic options for patients with heart failure. It has been reported that insulin stimulates the release of atrial natriuretic factor (ANF) via PI3 kinase and tyrosine manner and increase of insulin stimulated ANF release in diabetic rat atria may be due to up regulation of insulin receptors (14). In the present study cardiac hypertrophic marker ANF is considered as standard for checking the hypertrophic conditions along with the cell- surface area measurement.

Overall, the purpose of this study is to highlight the effect of insulin administration on the hearts of rat model and to elucidate the co-relation of free radicals with insulin both in the rat model as well as in human cardiac patients. Based on these supporting evidence, it is postulated that insulin stimulated pathways and ROS

are important players of cardiac hypertrophy. The study also suggests a possible ROS dependent antioxidant enzyme such as SOD activity regulation but it need further investigation during cardiac hypertrophy.

Materials and methods

Materials

In this experimental study, the drugs used were Insulin, N-Acetyl cysteine (NAC) from Sigma Aldrich (St. Louis, MO, USA). Atrial natriuretic factor (ANF) antibody, Goat anti-rabbit antibody IgG-AP and nitrocellulose membrane of pore size 0.45 µm, from Santa Cruz Biotechnology (Dallas, Texas). Alkaline phosphatase, Bromo chloro indolyl phosphate (BCIP) and Nitroblue tetrazolium (NBT) were products of Tianjin (Beijing, China). Sodium acetate, Diethyl-para-phenylenediamine (DEPPD), Ferrous sulfate ($FeSO_4$), Sodium chloride (NaCl), Potassium dihydrogen phosphate (KH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4), Potassium chloride (KCl), L-methionine, Triton X-100, and Riboflavin were the products of Merck Chemicals (Germany).

Patients Selection and Ethical statement

Hypertrophic patients under treatment at the Cardiology Department of Pakistan Institute of Medical sciences (PIMS), Islamabad and Lady reading hospital (LRH), Peshawar were selected. The control subjects comprised of healthy volunteers with age and social conditions similar to those of the patients. Blood samples were collected under a consent from patients and their guardians. Human sampling was carried out strictly under international ethical recommendations and approved by the institutional ethics committee. All animal procedures complied with standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC; 1996). Studies on human samples complied with the ethical principles stated in the "*Declaration of Helsinki*".

Serum Collection

The blood samples were drawn in 5 ml sterile syringes (B.D) using an aseptic vein puncture technique and transferred to gel tube. All samples were centrifuged at 4000 rpm for 10 minutes and serum was separated on dry ice prior to storage at -80 °C.

Hypertrophic animal model establishment

All experiments, including animal model performed under strict regulation of the institutional animal care committee. Laboratory bred Sprague Dawley rats (n=24) of 6-8 weeks old, weighting 180-200 gm were bought from Central Animal House, National Institute of Health, Islamabad. The animals were housed in cages inside a well-ventilated room under standard laboratory conditions of temperature 24-28 °C, relative humidity 60-70% and 12 hour light/dark cycle and were fed a standard chow diet and water. Rats were divided into four groups, Insulin, insulin + NAC, NAC alone and control groups. They received daily subcutaneous injections of human insulin (125 U/kg) and N-acetyl, L-cysteine (NAC, 50 mg/kg). All of them were sacrificed; blood was collected and centrifuged at 4000 rpm for 10 minutes to separate serum. Hearts of rats were stored at -80 °C after treating with liquid nitrogen.

ROS analysis of serum samples

A DEPPD spectrometry assay was carried out for ROS detection, and standard curve formation as described previously (15). Briefly, DEPPD (R1), final concentration of 100 µg/ml and ferrous sulfate (R2), final concentration of 4.37 µM were dissolved in 0.1 M sodium acetate buffer (pH 4.8). R1 and R2 were mixed in a ratio of 1:25. This solution was then added as a starter in the cuvette (3 ml) followed by Sodium acetate buffer. Hydrogen peroxide (H_2O_2) was then added in it to use as a positive control and serum samples were used for ROS analysis at 505 nm in (OD) by Agilant 8453 UV-Visible Spectrophotometer (UK). A calibration curve was also generated using the multiple serial dilutions of hydrogen peroxide (H_2O_2) 35 %.

SOD assay of serum samples

SOD assay of samples was carried out with modifications as described by (16). The reaction mixture was prepared by taking PBS buffer (NaCl, KH_2PO_4, Na_2HPO_4 and KCl), L-methionine, NBT (Nitro Blue Tetrazolium) and Triton X-100, followed by the addition of sample. Illumination of the samples with fluorescent lamp was carried out and riboflavin was added to initiate the reaction. Sample mixture was delivered into cuvettes, and absorbance was measured at 560 nm. Control sample without serum was also assayed in parallel.

Immunoblotting for stress marker detection

Serum samples were used for ANF detection by western blotting, while using Transferrin as loading control. Samples were subjected to SDS-PAGE and transferred to the nitrocellulose membrane of pore size 0.45 µm (Santa Cruz Biotechnology) at 100 V for two hours in the transfer buffer (25 mM Tris, 192 mM glycine, and 20 % methyl alcohol, pH 8.3) according to the manufacturer's instructions. After being blocked with nonfat milk, the membrane was incubated with primary antibody with diluting ratio of 1:1000 (Santa Cruz Biotechnology) overnight at 4 °C and then treated with goat anti-rabbit antibody IgG-AP (Santa Cruz Biotechnology) for 1 hour at room temperature. The membrane was stained for 30 minutes in AP (Alkaline Phosphtase) color development solution containing one ml of 1X AP reaction buffer with each 50 µl of NBT and BCIP solutions (Tiangen, Beijing, China).

Histological analysis

Formalin fixed heart specimens were embedded in paraffin, cut into 3 µm sections and subjected to hematoxylin-eosin staining for morphological evaluation. Polarized light and bright field microscopes were used to visualize cross sections of hearts under different magnified powers (17).

Cell-surface area measurement

To determine the cell arrangement and size of heart cells, slides were developed and observed under 40X magnification. Cell-surface areas were measured by SPOT camw 4.0.

Table. Clinical and basic parameters of cardiac hypertrophic and normal patients

Parameters	Patients (n=60)	Controls (n= 55)	P values
ROS (Absorbance)	0.28815 ± 0.0579	0.11367 ± 0.0520	0.0051*
AGE (Years)	58.37 ± 16.1922	30.37 ± 7.8338	1.000*
SOD (Units/minutes)	81.7 ± 5.35	96.11 ± 2.13	<0.0001*
Smoker/Non-smoker	28/17	15/25	1.000**
Gender	20 ± 11.317	20 ± 14.142	1.000**
Hemoglobin (g/dL)	10.52±0.0844	12.31±0.096	0.4219**
Platelets	200655±11	27225±66	0.3366**
Lymphocytes (%)	17.93±1	28.57±1	0.9974**

* *p* values were calculated by Mann-Whitney test. ** *p* values were calculated by Chi-square test. Data presented as mean±SD

Statistical Analysis

Statistical analysis was performed with SPSS 21. All the basic characteristics and parameters are presented as mean ± SD from the mean. ROS and SOD data were compared by histograms. The p value was considered as significant when less than 0.05.

Results

Clinical parameters of the subjects

As shown in table baseline characteristics including age, genders, smoking, lymphocytes percentage, platelets concentration and hemoglobin levels of patients (n = 60) as well as healthy individuals (n = 60) were statistically compared.

Free radicals elevate oxidative stress in cardiac hypertrophy patients

Free radicals induced oxidative stress contributes a significant role in maladaptive cardiac hypertrophy. To assess and measure the reactive oxygen species in cardiac hypertrophy, their relation with different parameters such as age, gender, smoking and serum free radical levels were measured. A significant elevation of ROS levels in the serum of cardiac patients was observed when compared to control subjects (Table). When ROS compared among gender, less/greater than 40 years of age cardiac hypertrophic patient's significant differences were observed. However, non significant co-relation between free radicals with hemoglobin level and lymphocytes were observed (Figure 1A, C, D). In

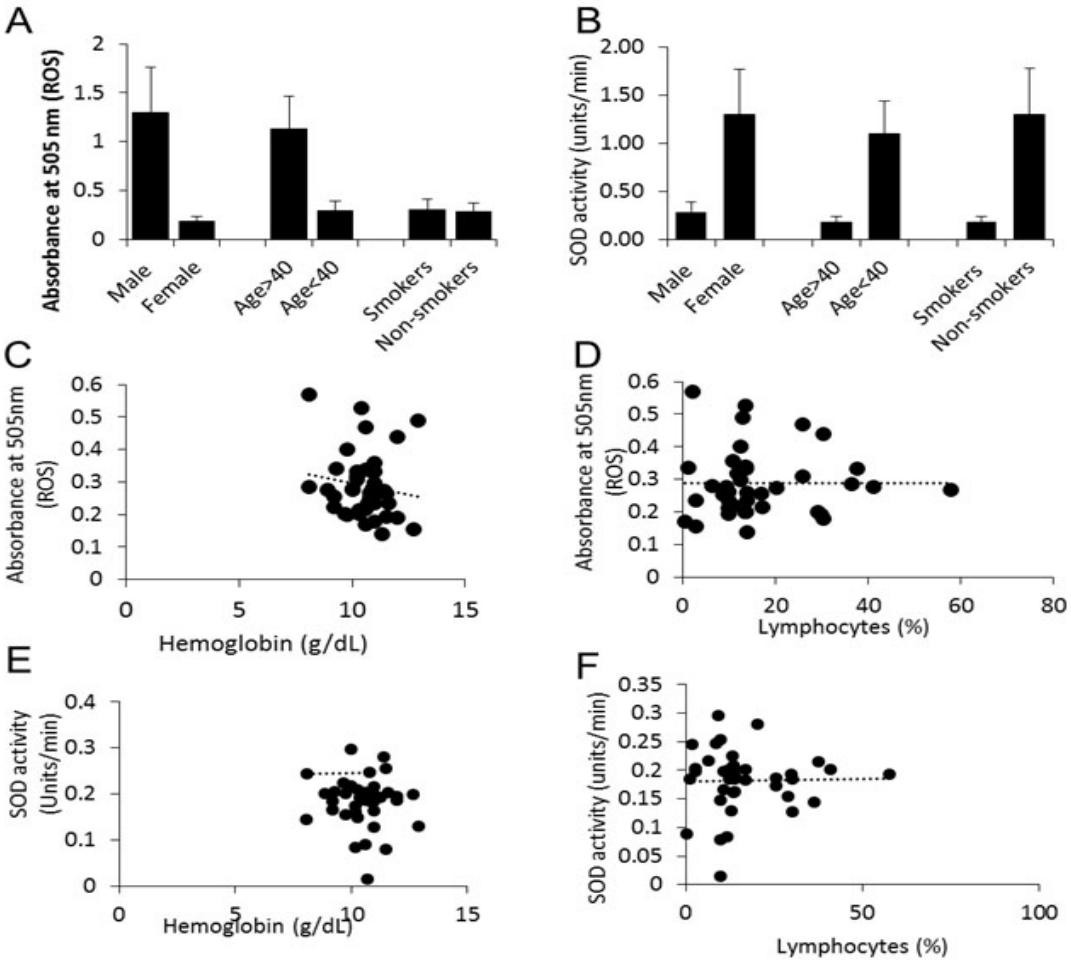

Figure 1. ROS and SOD levels and co-relation with different parameters in cardiac hypertrophy patients. A, B: ROS and SOD levels in the serum of cardiac hypertrophy patients with different parameters, respectively. **C, D:** Co-relation between ROS, hemoglobin and lymphocyte levels in patients. **E, F:** Co-relation between SOD activity, hemoglobin and lymphocyte levels in cardiac hypertrophy patients.

the light of the observed findings it is apparent that free radical induced oxidative stress plays an important role in cardiac hypertrophy.

Insulin induces ROS elevation in experimental models

Recently, insulin has been reported as free radical generative. To confirm the free-radical generation via insulin in our study, ROS levels in the insulin treated rat model were determined. We observed higher levels of free radical in the insulin treated model than that of the normal saline treated group (control subjects). ROS level elevation in case of the cardiac hypertrophic animal model, suggesting its importance in pathogenesis (Fig. 2, B).

Insulin alters the SOD activity in the experimental models

A significant decline of SOD levels was observed in the patients than that of control subjects (Table) when compared statistically. Similarl to free radicals, significant difference of SOD activity among genders, age (</>40 years) and smoking within the cardiac hypertrophic patients were observed. However, co-relation of SOD activity with hemoglobin and lymphocytes were non-significant (Figure 1 B, E and F). Further, to link and determine ROS dependent insulin down regulation of SOD activity, NAC was used as an antioxidant. Up regulation of SOD activity in NAC + Insulin as well as NAC treated rats were observed when compared with normal subjects (Fig. 2, C). The recovery of SOD activity after potent antioxidant, suggests free radical dependent regulation of SOD activity.

Insulin induces stress marker expression

ANF is considered a stress marker in hypertrophic condition. In the current study immunoblotting results showed elevated ANF expression in insulin treated animal model compared to control subjects (Fig. 2, E). Considering these results, it is apparent that elevated level of insulin may induce the expression of cardiac hypertrophic markers, including ANF, when compared with loading control (Transferrin).

Insulin induces histological changes in the heart of animal model

To address the ROS dependent role of insulin in heart pathology, experimental animal model heart histology was performed. Sections were studied under the light microscope (DIALUX 20 EB) at 40X magnifications. Insulin treated group leads to cellular enlargement and disorder of myocytes with increased interstitial fibrosis (Fig. 2, A), suggesting insulin's role in heart pathology. Cell-surface areas of cardiomyocytes and effects of insulin on the cardiomyocytes size, and cellular arrangements were measured by SPOT camw 4.0 (Fig. 2, D). About 100–200 cardiomyocytes were examined in 20–50 fields. Amplitudes of cross-sectional areas were also measured.

Discussion

The role of oxidative stress in the pathogenesis of different diseases, including cardiac disorders has been a part of debate for the past several decades. The previous clinical and experimental evidence revealed that oxidative stress, the imbalance of ROS production and efficient antioxidant system enhanced in cardiac disorders (18). Mitochondria and some enzymes, including NADPH oxidase are the major sources responsible for free radical generation (6, 10). NADPH oxidase through redox sensitive signaling is believed to be important in the pathogenesis of numerous cardiac remodeling (19). In different tissues, variety of signaling pathways such as RAS, c-Src, the MAPKs, the PI3 kinase (PI3K) / Akt pathway, NF-kB, AP-1, HIF-1 and some others are considered as the downstream targets of NADPH oxidase derived ROS. In addition, several studies had docu-

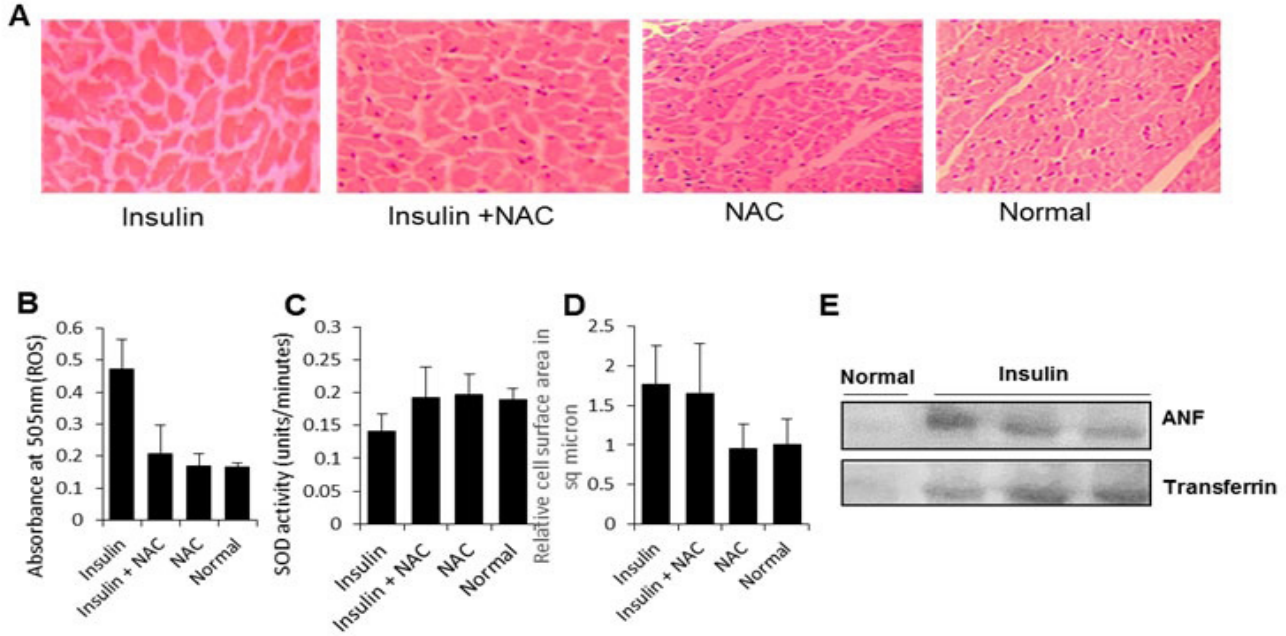

Figure 2. Insulin induced Free radicals (ROS) contributes in cardiac pathologies.

A: Cross sections of hearts analyzed by staining with hematoxylin-eosin at 40X magnification. **B:** Comparison of ROS levels in the serum of Insulin, NAC treated and normal rats. **C:** Comparison of SOD (U/min) activity in serum of Insulin, NAC treated and normal rats. **D:** Cell surface area measurement from cross sectioned histology by SPOT camw 4.0. **E:** Western blot for expression analysis of ANF in serum samples, Transferrin served as loading control. Data presented as mean ± SD. *P ≥0.05 considered as significant.

mented that NADPH oxidase involved in the angiotensin-II as well as endothelin-I induced isolated myocyte hypertrophy (20). In the current investigation, significant elevation of ROS levels in the cardiac patients suggesting its role and importance in cardiac hypertrophy. Similarly, in case of animal models, increase ROS level in insulin treated group compared to the insulin+NAC and NAC treated groups' postulates insulin and ROS co relation. As NADPH oxidase derived ROS is associated with the cardiac diseases, and insulin is one of the of ROS generative agent through NADPH oxidase (11). Based on this evidence, the present data suggest that insulin would be one of the cardiac hypertrophy inducing agents through NADPH oxidase derived free radicals, but it needs further investigation.

To counterbalance the free radical and its deleterious effects body defense system comprising different antioxidants enzymes including SOD and catalase, become activated upon stimulation (21). However, due to persistant oxidative stress their activities decelerated which further facilitates the ROS role in disease condition. In the present study decreased SOD activity in cardiac patients as well as in insulin treated rats postulates significant association of SOD activity with insulin and free radicals. The up regulation of SOD activity in insulin + NAC treated groups confirmed free radicals dependent insulin relation to SOD activity.

In the present study, heart histopathology was observed with increased cell-surface area and abnormal cell arrangement with insulin treatment. Previously it has been revealed that the myocardial mass may get increased in response to the insulin. In normal condition, the myocytes show normal arrangement, but in the hypertrophic state myocardium get distorted by the hypertrophic growth of masts, which leads to the enlarged shape of myocytes (22). Under the umbrella of present experimental observation, the abnormal histopathology, hypertrophied cell size with increased cell-surface area and enhanced expression of ANF in insulin administrated rat hearts than that of control, the study suggests a possible link of insulin with cardiac hypertrophy.

Conclusively, based on accumulative evidence, present study proposed interplay of insulin to cardiac hypertrophy via free radicals regulation; however, it needs further study to understand the physiological importance of insulin in cardiac hypertrophy. As the elevated level of insulin might be an inducer of cardiac disorders, the current experimental approach will present a better opportunity for designing future therapeutic interventions for cardiac hypertrophy.

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