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Phoenixin-14: detection and novel physiological implications in cardiac modulation and cardioprotection

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Phoenixin-14: detection and novel physiological implications in cardiac modulation and cardioprotection

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Abstract

Phoenixin-14 (PNX) is a newly identified peptide co-expressed in the hypothalamus with the anorexic and cardioactive Nesfatin-1. Like Nesfatin-1, PNX is able to cross the blood brain barrier and this suggests a role in peripheral modulation. Preliminary mass spectrography data indicate that, in addition to the hypothalamus, PNX is present in the mammalian heart. This study aimed to quantify PNX expression in the rat heart, and to evaluate whether the peptide influences the myocardial function under basal condition and in the presence of ischemia/reperfusion (I/R). By ELISA the presence of PNX was detected in both hypothalamus and heart. In plasma of normal, but not of obese rats, the peptide concentrations increased after meal. Exposure of the isolated and Langendorff perfused rat heart to exogenous PNX induces a reduction of contractility and relaxation, without effects on coronary pressure and heart rate. As revealed by immunoblotting, these effects were accompanied by an increase of Erk1/2, Akt and eNOS phosphorylation. PNX (EC_{50} dose), administered after ischemia, induced post-conditioning-like cardioprotection. This was revealed by a smaller infarct size and a better systolic recovery with respect to those detected on hearts exposed to I/R alone. The peptide also activates the cardioprotective RISK and SAFE cascades and inhibits apoptosis. These effects were also observed in the heart of obese rat. Our data provide a first evidence on the peripheral activity of PNX and on its direct cardiomodulatory and cadioprotective role under both normal conditions and in the presence of metabolic disorders.

Introduction

Phoenixin (PNX) is a predicted peptide identified in 2013 *via* a bioinformatic approach[1]. It derives from an uncharacterized precursor, C4orf52, whose cleavage generates a 14-residue peptide (DVQPPGLKVWSDPF-amide: PNX-14 amide) and an N-terminal extended form (AGIVQEDVQPPGLKVWSDPF-amide: PNX-20 amide). Both are highly conserved throughout vertebrate species (from fish to mammals and humans), and show a biological activity that has been mainly related to reproductive homeostasis[1].

A major site of PNX expression is the hypothalamus in which the peptide localizes in paraventricular and supraoptic nuclei and in cells of the median eminence[1]. This distribution partially overlaps that of Nesfatin-1 [2], another hypothalamic peptide with potent anorexic properties, with a role in cardiovascular modulation [3 and references therein]. PNX is also localized in extra-hypothalamic regions, including those linked to the autonomic control, in the spinal cord and in sensory neurons of the dorsal root, as well as in nodose and trigeminal ganglia [1,4]. Consistent with this wide distribution, PNX is regarded with interest as a putative multifaceted peptide with functions other than the modulation of the reproductive system. The limited literature so far available suggests that it is involved in sensory processing, anxiety depression [5a], and memory formation and preservation [5b]. In addition, PNX immunodetection in magnocellular neurons of the hypothalamus suggested that the peptide, released into the hypophyseal portal system, contributes to the hypothalamus-pituitary communication, acting as a general endocrine regulator [2].

So far, PNX targets, and the molecular pathways activated by the peptide are almost completely unrevealed. Only scant and fragmentary results have been provided, mainly in relation to its activity as an endocrine regulator. Preliminary data obtained by using a "deductive ligand receptor matching strategy" suggest that the peptide acts *via* an orphan G-protein coupled receptor (GPR173) to potentiate the effects of gonadotrophin-releasing hormone (GnRH) on reproductive hormones release [6].

Of relevance, PNX has been recently identified in the plasma of obese patients [7]. This suggests a peripheral activity, a possibility supported by mass spectrography data showing the presence of PNX in extracts from several organs [1]. In the heart, the 14 residues form of PNX was found to be expressed in an amount of approximately 485 pg/g tissue. These values make the cardiac tissue the second site of peptide production after the hypothalamus (2851 pg/g tissue) [1].

On the basis of these data, we designed the present study to investigate whether PNX influences the heart function. By using the Langendorff cardiac perfusion technique and biomolecular analyses, we analysed in the rat the presence of PNX in cardiac extracts and the effects of the peptide on the myocardial and coronary performance, under both basal conditions and in the presence of ischemia/reperfusion (I/R). We then explored the signal transduction mechanism activated by PNX in the rat heart. Cardioprotection was also

analysed in obese rats. Our data provide a first evidence concerning a cardiomodulatory and cardioprotective function of PNX in a mammalian model and contribute for the first time to clarify the peripheral role of PNX in cardiac homeostasis.

Materials and Methods

Animals. Male Wistar rats (250-300 g body weight) (Harlan Laboratories, Udine, Italy), identically housed under controlled lighting and temperature conditions, were fed for 12 weeks ad libitum with either a standard diet [(SD) (Diet 2018: 6.2% kcal fat, 18.6% kcal protein, and 44.2% kcal carbohydrate; Envigo, Udine, Italy)] or a high fat diet [(HFD) (Teklad Diet 06414: 60.3% kcal fat, 18.3% kcal protein, and 21.4% kcal carbohydrate; Envigo, Udine, Italy)], and had free access to water. All protocols were conducted in accordance with the Declaration of Helsinki, the Italian law (D.L. 26/2014), the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (2011) and the Directive 2010/63/EU of the European Parliament on the protection of animals used for science. The project was approved by the Italian Ministry of Health, Rome and by the ethics review board.

Drugs. Phoenixin-14 Amide (PNX) was provided from Phoenix Pharmaceuticals, Inc (Burlingame, CA, USA). Wortmannin (WT) [phosphatidylinositol 3-kinase (PI3K) inhibitor], N(5)-(1-immino-3-butenyl)-L-ornithine (L-NIO)[eNOS inhibitor], PD-98059 (PD) [specific inhibitor of ERK1/2], and 5-hydroxydecanoate (5HD) [mitoKATP channels blocker], were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All drug-containing solutions were freshly prepared just before use.

Isolated heart perfusion. Rats were heparinized (2.500 U i.m.) and anesthetised with ethyl carbamate (2 g/kg body weight, i.p.). Hearts were rapidly excised and transferred in ice-cold buffered Krebs-Henseleit solution (KHs) and weighed. Heart performance was evaluated according to the Langendorff technique. The aorta was immediately cannulated with a glass cannula and connected with the Langendorff apparatus to start the retrograde perfusion at a constant flow-rate of 12 ml/min with a modified KHs [gassed with 95% O_2 and 5% CO_2 (pH 7.4) and containing (in mmol/L): NaCl 113.0; KCl 4.7; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; KH_2PO_4 1.2; $CaCl_2$ 1.8; glucose 11; mannitol 1.1; and Na-pyruvate 5.0 (Sigma Aldrich, Saint Louis, Missouri, USA)] [8]. The perfusion pressure was set to 100 mmHg and kept constant throughout the experiments. Hearts were kept in a temperature-controlled chamber (37°C). To avoid fluid accumulation, the apex of the left ventricle (LV) was pierced. A water-filled latex balloon, connected to a BLPR gauge (WRI, Inc., Sarasota, FL, USA), was inserted through the mitral valve into the LV to allow isovolumic contractions and to continuously record mechanical parameters. The balloon was progressively filled with water up to 80µl to obtain an initial left ventricular end diastolic pressure (LVEDP) of 5–8mmHg. Haemodynamic parameters [heart rate (HR, beats

min^{-1}), LVP (mmHg), maximal values of the first derivative of LVP, [+LV(dP/dt)max, mmHg s^{-1}], maximal rate of LVP decline [–LV(dP/dt)max, mmHg s^{-1}], coronary pressure (CP, mmHg)] were assessed by PowerLab data acquisition system (AD Instruments, Oxford-UK). Hearts were equilibrated at basal level for 40 min before the specific protocol [3].

Experimental protocols

PNX stimulated preparations

Preliminary experiments (data not shown), obtained by repetitive exposure of each heart to one concentration of PNX (0.5 nmol/L), revealed the absence of desensitization. Thus, concentration–response curves were obtained by perfusing the cardiac preparations with KHs enriched with increasing concentrations of PNX (1 pmol/L–100 nmol/L), each concentration lasting 10 min.

Ischemia/reperfusion (I/R) protocols

After stabilization, the baseline parameters of each heart were recorded. Then, hearts were randomly assigned to one of the groups described below.

Cardiac function and infarct size studies

Animal treatment: SD and HFD groups

Animals fed with the SD were randomly assigned to the following protocols:

- Group 1 (SHAM-SD): hearts were stabilized and perfused with KH for 190 min.
- Group 2 (I/R-SD): hearts were stabilized and subjected to I/R alone.
- Group 3 (PNX Post-SD): at the beginning of 120 min reperfusion, hearts were perfused with PNX (0.5 nmol/L) for 20 min.
- Group 4 (PNX Post+inhibitors-SD): 5 min before ischemia and during the early 20 min of reperfusion, hearts were perfused with one of the following inhibitors: WT (100 nmol/L; PNX+WT), or L-NIO (10 µmol/L; PNX+ L-NIO), or PD (10 nmol/L; PNX+PD98059), or 5HD (10 µmol/L; PNX+5HD). At the beginning of 120 min reperfusion they were perfused with PNX (0.5 nmol/L) for 20 min.

Animals fed with the HFD were randomly assigned to the following protocols:

- Group 1 (SHAM-HFD): hearts were stabilized and perfused with KH for 190 min.
- Group 2 (I/R-HFD): hearts were stabilized and subjected to I/R alone.
- Group 3 (PNX Post group-HFD) at the beginning of 120 min reperfusion, hearts were perfused with PNX (1.0 nmol/L).

In SD groups, PNX concentration (0.5 nmol/L) used in cardioprotection protocols corresponded to the EC_{50} dose determined on the basis of dose-response (1 pmol/L–100 nmol/L) curves. In HFD groups, PNX concentration of 1.0 nmol/L was used since preliminary experiments (data not shown) showed that PNX EC_{50} dose unable to induce cardioprotection.

In all experiments, antagonist concentrations were selected on the basis of previous reports [9]. Preliminary data showed that in hearts perfused only with inhibitors, the dLVP recovery, the development of contracture (LVEDP) and the infarct size were similar to those obtained in the I/R group (data not shown). Cardiac performance before and after ischaemia was evaluated by analysing dLVP recovery as an index of contractile activity, and LVEDP for contracture [an increase in LVEDP of 4 mmHg above the baseline level [10]].

Infarct size (IS)

To measure infarct areas, hearts were rapidly removed from the perfusion apparatus at the end of reperfusion. Left ventricles were transversely dissected into 2-3 mm slices. After 20 min of incubation at 37°C in 0.1% nitro blue tetrazolium in phosphate buffer (59.8 mmol/L NaH_2PO_4, 484.9 mmol/L Na_2HPO_4, pH: 7.4), unstained necrotic tissues were carefully separated from stained viable tissues by an independent observer who was not aware of the nature of the intervention. Then, the weights of the necrotic and non-necrotic tissues were determined, and the necrotic mass was expressed as a percentage of total LV mass, including septum [8].

Immunoblotting analysis

Hearts subjected to SHAM, I/R, PNX-PostC protocols, and those perfused with increasing concentrations of PNX (1 pmol/L–100 nmol/L) for 10 min, were homogenized in ice-cold radio-immunoprecipitation assay buffer (Sigma-Aldrich, Saint Louis, Missouri, USA) containing a mixture of protease inhibitors (1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate). Then, homogenates were centrifuged at 15000 x g for 20 min at 4°C for debris removal. Protein concentration was determined using a Bradford reagent according to the manufacturer (Sigma-Aldrich, Saint Louis, Missouri, USA). Equal amounts of proteins were loaded on 8% SDS-PAGE gels (30 µg for p-eNOS, eNOS, p-nNOS and nNOS), or 10% SDS-PAGE gels (30 µg for p-Akt, Akt, p-Erk 1/2, Erk 1/2, p-GSK-3 α/β, GSK-3 α/β, p-STAT3, STAT3, p-p38MAPk and p38MAPk), or 12% SDS-PAGE gels (30 µg for Bcl-2, Bax, Cytochrome c (Cyt c) and active Caspase 3), subjected to electrophoresis and transferred to polyvinyl difluoride membranes. Membranes were blocked with non-fat dried milk and incubated overnight at 4°C with one of the following different antibodies: monoclonal rabbit antibody against β-actin, polyclonal rabbit antibodies against p-Akt, Akt, Erk 1/2, p-GSK3α/β, eNOS, nNOS, p-p38MAPK, p38MAPK and Active Caspase 3, monoclonal mouse

antibodies against p-Erk 1/2, GSK3α/β, p-STAT-3, STAT-3, Bax, Bcl-2, β-actin and Cyt c, monoclonal goat antibody against p-eNOS, polyclonal goat antibody against p-nNOS (Santa Cruz Biotechnology, Santa Cruz, California, USA, Sigma Aldrich Saint Louis, Missouri, USA, Cell Signaling Technology, Danvers, Massachusetts, USA, Thermofisher Scientific, Waltham, Massachusetts, USA). Antibodies were diluted 1:1000 in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk (TBSTM). Polyclonal rabbit antibodies against Akt, Erk1/2, eNOS and nNOS or monoclonal mouse antibody against GSK-3α/β, or monoclonal mouse against STAT-3 and β-actin were used as loading controls. Anti-rabbit, anti-goat and anti-mouse peroxidase-linked secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, California USA) were diluted 1:2000 in TBSTM. Immunodetection was performed by using the ECL PLUS enhanced chemiluminescence kit (Amersham, Little Chalfont, UK). Autoradiographs were obtained by membrane exposure to X-ray films (Hyperfilm ECL; Amersham, Little Chalfont, UK). Immunoblots were digitalized; densitometric analyses of the bands were performed evaluating the areas and the pixel intensity represented by 256 Gray values (0 = white; 256 = black) and the background was subtracted. Analyses were carried out using NIH IMAGE 1.6 (National Institutes of Health, Bethesda, Maryland).

Enzyme-Linked Immunosorbent Assay (ELISA)

PNX detection in hypothalamus, heart, testis and plasma was performed by ELISA using a commercial kit (Phoenix Phaceuticas, Inc. Burlingame, CA, USA). For the plasma collection in pre-prandial phase, the food has been withdrawn from the animals three hours before the blood sample removal, while for the plasma collection in post-prandial phase, making sure of the food intake, the blood was collected immediately following an access to the food for three hours. The PNX tissue expression was evaluated as follows: tissue samples were homogenized by using Ultra-Turrax® in Phosphate-Buffered Saline (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4; pH 7.4) plus a mixture of protease inhibitors (1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate) and centrifuged at 15000 x g for 20 min (4°C). Supernatants were then tested by ELISA kit.

Statistics

All data were expressed as mean ± SEM. One-way ANOVA, non-parametric Newman-Keuls Multiple Comparison Test (for post-ANOVA comparisons) was used for western blot and ELISA analysis. *p =<0.05, **p =<0.01, ***p =<0.001 were considered statistically significant. The statistical analysis was carried out using Graphpad Prism5.

Results

Tissue and plasma detection of PNX

PNX tissue detection by ELISA assay showed that, in control rats, the peptide was present in hypothalamus, but was undetected in the testis. Accordingly, the hypothalamus was considered as a positive control, while the testis was used a negative control for PNX detection.

PNX cardiac detection was examined under different experimental and metabolic conditions. We found that in the control groups (not perfused hearts and SHAM perfused animals), PNX concentration was ~500 pg/g and ~580 pg/g, respectively. A significant increased PNX cardiac production was detected in the I/R-SD group, but in heart extracts from I/R-HFD animals the expression of the peptide was significantly reduced **(Fig. 1A)**. ELISA assay, used to evaluate PNX plasma levels, showed in the SD group pre-prandial values of ~15 pg/ml and post-prandial values of ~50 pg/ml. To evaluate the influence of obesity, PNX plasma levels were analyzed in the HFD groups, revealing not significant differences between pre- and post-prandial phases (~8 pg/ml and ~15 pg/ml, respectively) **(Fig. 1B)**.

Basal cardiac parameters

After 40 min of stabilization, for all SD experimental groups, basal parameters of the isolated and Langendorff perfused hearts were: dLVP= 75±4 mmHg; LVEDP= 5–8 mmHg; CP= 66±1 mmHg; HR= 247±5 mmHg. Endurance and stability of the preparations were assessed by measuring each performance variable every 10 min. The stability was up to 190 min.

PNX effects on myocardial contractility and relaxation

Heart perfusion with increasing concentrations (1 pmol/L-100 nmol/L) of PNX (each concentration lasting 10 min) revealed that, in the animals fed with SD, the peptide depressed myocardial contractility and relaxation. Negative inotropism, evaluated as the decrease of LVP and +(LVdP/dt)max, and lusitropism, evaluated as -(LVdP/dt)max reduction, were significant from 100 pmol/L and occurred without changes in HR and CP **(Fig. 2A)**.

Mechanisms of action elicited by PNX

Western Blotting analysis was used to explore the mechanism of action activated by PNX on the rat heart. As illustrated by the immunoblots and densitometric analyses reported in **Fig. 2B**, hearts perfused with increasing concentrations of PNX showed Akt, eNOS and Erk 1/2 phosphorylation levels significantly higher than those detected in the SHAM group. Contrarily, nNOS phosphorylation was similar in both groups.

PNX effects on post-ischemic cardiac function and mechanism of action

The cardioprotective potential of PNX was investigated by comparing the effects induced by I/R maneuvers with those elicited by the peptide used as post-conditioning agent (PostC) (see protocols: Fig. 3). PNX effects on both systolic and diastolic function were analyzed at the EC50 dose of 0.5 nmol/L, obtained by dose-response curves previously described. Systolic function was described by the level of inotropic activity (i.e., dLVP recovery). Hearts from I/R group showed a limited dLVP recovery that, at the end of reperfusion, was of 34±3 mmHg. PNX, administered at the reperfusion, significantly improved dLVP recovery, being dLVP at the end of reperfusion of 74±16 mmHg **(Fig. 3A)**. The level of contracture was used as an expression of the diastolic function (i.e., LVEDP 4 mmHg or more above baseline level) [8]. I/R markedly increased LVEDP (from 7.6±0.8 mmHg in the baseline to 35.9±4.9 mmHg at the end of reperfusion), while in the presence of PNX administration, contracture was not observed (LVEDP at the end of reperfusion: 4.8±1.3 mmHg) **(Fig. 3A)**.

To evaluate the mechanism of action involved in PNX-dependent post-ischemic recovery, at the reperfusion, Langendorff perfused hearts were exposed to PNX plus the following specific inhibitors of intracellular pathways involved in the cardioprotection: wortmannin (WT), L-NIO, PD098059 (PD), and 5 hydroxy decanoate (5HD). All inhibitors abolished the post-ischemic systolic and diastolic recovery induced by PNX, as revealed by the dLVP and LVEDP values measured at the end of reperfusion **(Fig. 3B)**.

Total IS was expressed as a percentage of LV mass. LV mass was similar in all groups. IS was ~73% in I/R and ~34% in heart perfused with PNX, while IS was ~60% for PNX+WT, ~68% for PNX+L-NIO, ~65% for PNX+PD and ~62% for PNX+5HD **(Fig. 3B)**. For the hearts perfused with inhibitors alone, the recovery of systolic function, the development of contracture and the IS were similar to those in I/R group (data not shown).

PNX influence on cardioprotective pathways

Activation of intrinsic cardiac pro-survival RISK and SAFE cascades was evaluated by WB. In the presence of I/R, phosphorylation of Akt, Erk 1/2, GSK-3α/β and eNOS was lower than that detected in the SHAM-SD group. A significant increased phosphorylation of all above RISK components was observed in the hearts exposed to PNX Post-SD group **(Fig. 4).** A similar trend was observed also in the case of STAT3, a component of the SAFE pathway, that was reduced in I/R group, but increased in the PNX Post-SD group **(Fig. 4)**.

PNX influence on apoptotic signaling

The effect of PNX administration after ischemia on pro- and anti-apoptotic factors expression was evaluated by WB **(Fig. 5)**, showing that I/R was accompanied by an increase of Bax, active Caspase 3, Cyt C, and phosphorylated p38 MAPK expression. In contrast, PostC with PNX induced a significant reduction of all the above pro-apoptotic factors. An inverse trend was observed for the anti-apoptotic protein Bcl-2 **(Fig. 5)**.

Basal cardiac parameters in an obesity rat model

After 40 min of equilibration, basal cardiac parameters for all groups of animals fed with the HFD were: dLVP= 76±5 mmHg; LVEDP= 5–8 mmHg; CP= 90±5 mmHg; HR= 150±7 mmHg. Endurance and stability of the preparations were assessed by measuring the performance variables every 10 min. The stability was up to 190 min.

Cardioprotective effects of PNX in an obesity rat model

The influence of PNX in a rat model of cardio-metabolic disorder was analysed by evaluating the ability of the peptide to revert the HFD-dependent cardiac dysfunction. Since PNX was unable to elicit protection at the concentration of 0.5 nmol/L used on the isolated heart of SD-rats, experiments on HFD animals were performed at a PNX concentration of 1 nmol/L. Analysis of the post-ischemic performance showed that heart exposed to PNX at the reperfusion had a better response to ischemia. This was shown by dLVP values that, with respect to the I/R-HFD group (22±5.5 mmHg), were significantly higher in PNX Post-HFD group (53±6.6 mmHg) **(Fig.6A)**. In parallel, in PNX Post-HFD group, LVEDP, at the end of reperfusion, was 14±1.5 mmHg, while in I/R-HFD group it was 35±2.0 mmHg. The systolic and diastolic improvement was corroborated by a significant reduction of the IS in PNX Post-HFD group (42±1%), compared to that detected in the I/R-HFD group (73±4%) **(Fig.6A)**.

PNX influence on RISK and apoptotic pathways in an obesity rat model

WB analysis provided information on the mechanism of action activated by PNX to elicit protection in an obesity rat model. The densitometric evaluation of the blots showed that in the PNX Post-HFD group, p-Akt and p-Erk 1/2 were significantly higher with respect to the levels detected in the I/R-HFD group. A significantly reduced expression of active Caspase 3 and a significant enhancement of Bcl-2 expression was observed in the PNX Post-HFD group, in comparison with the I/R-HFD group **(Fig 6B)**.

Discussion

In the present study, we demonstrated for the first time that PNX directly affects the mammalian cardiac performance. We observed on the isolated and Langendorff perfused rat heart that it induces a reduction of contractility and relaxation in a dose-dependent manner by recruiting the AKT/p-eNOS and ERK1/2 pathways. We also found that PNX is produced by the heart and, in normo-weighted animals, its levels are influenced by food intake, since they significantly increase during the post-prandial phase. By exposing the isolated and perfused rat heart to ischemia, we observed that PNX protects against I/R injury. It acts as a Post conditioning agent through activation of the cardioprotective SAFE and RISK pathways, and the modulation of MitoK_{ATP}

channels. PNX cardioprotection is accompanied by inhibition of apoptosis and is maintained also in the presence of obesity.

PNX: tissue production and plasma levels

Few literature data so far available indicate that PNX is produced in the hypothalamus in which it is co-expressed with Nesfatin-1 [1,2]. Its presence is also reported in the heart [2]. In the present study we observed that, under normal conditions, PNX is produced in the rat heart and its production is increased after ischemic challenges. We also detected PNX in the plasma, showing that, in normo-weight, but not in obese rats, its levels increase during the post-prandial phase. This observation is consistent with the ability of PNX to cross the blood-brain barrier to elicit peripheral effects, similar to those reported for the co-expressed hypothalamic cardiovascular peptide Nesfatin-1 [3].

Recently, Hofmann and collaborators showed in obese patients that the levels of circulating PNX are around 0.7 ng/mL and are negatively associated with anxiety [7]. In the present study, we observed that in normo-weighted rats, PNX plasma levels are higher during the post-prandial phase with respect to those detected before a meal. These variations cannot be observed under obese conditions. This is interesting since suggests that the peptide, thanks to its anorexigenic properties, under normal conditions may contribute to turn off appetite and stop food intake. During obesity, this negative feedback on food intake and satiety is compromised, and this may contribute to the pathology.

Cardioinhibitory role of PNX

On the isolated and Langendorff perfused rat heart, we observed that exogenous PNX negatively affects contractility and relaxation in a dose-dependent manner. These effects do not involve HR and CP. The direct cardiac modulation is obtained starting from 100 pmol/L concentration of the peptide. This suggests that the heart is highly sensitive to PNX. In fact, the peptide concentration is lower than that detected in the plasma of human obese (0,7 ng/ml [7]), although being higher than that detected in the plasma of ad libitum-fed animals (52 pg/ml, see Fig.1). Since the rat heart expresses PNX [1], it is presumable that the locally generated peptide contributes to control the myocardial performance *via* an autocrine/paracrine circuit that may act in synergy and/or in alternative to the circulating and the nerve released peptide. In addition, PNX may interact with cardiac Nesfatin-1 [3] to modulate the heart function.

Our biomolecular analyses revealed that PNX-dependent cardiac effects involve the AKT/NOS-NO signal transduction pathway. In fact, PNX exposure is accompanied by an increased AKT and eNOS phosphorylation with final depressant effects on contractility and relaxation. The role of NO in the modulating cardiac performance is well acknowledged. In the mammalian heart, activation of the NO pathway decreases L-type

Ca^{2+} currents [11] and troponin I phosphorylation [12], thus contributing to the inhibition of myocardial contractile performance. Of note, PNX affected also cardiac relaxation (lusitropism), a crucial component of the cardiac cycle which allows the ventricle to be adequately filled with blood, with impact on the subsequent contraction [13]. We observed that PNX depresses the lusitropic performance of the rat heart. This is particularly important since an impaired relaxation contributes to cardiac dysfunction, such as in heart failure with normal ejection fraction [14].

PNX-dependent cardioprotection

PNX, given in the early reperfusion at 0.5 nM, limits the I/R-dependent myocardial damage. In particular, it reduces infarct size and markedly improves the post-ischemic contractile function without affecting the cardiac contracture. These effects, similar to those obtained by ischemic PostC maneuvers [15], are the goal of cardioprotective protocols [16]. Our results pave the way for analyzing the applicative potential of this peptide as pharmacological PostC agent. This is of relevance, due to the need of identifying novel cardioprotective molecules to be used in clinic and therapy. Considering the negative influence of alimentary disorders on normal cardiac performance, the characterization of the cardioprotective properties of cardio-depressant peptides also involved in alimentary homeostasis (as in the case of Glucagon-like peptide 2 [17] and Nesfatin-1 [3]), must be regarded with interest.

Like PostC maneuvers, many substances protect the heart by recruiting pro-survival intrinsic signaling cascades, such as RISK and SAFE pathways [16,18,19]. In our study, we observed that these cascades are activated in hearts exposed to PNX during the early reperfusion. By using selective inhibitors of specific targets of cardioprotection, such as IP3K, eNOS, ERK1/2, and mitoKATP channels, and by performing WB analysis of phosphorylated proteins of upstream (AKT, ERK1/2) and downstream (GSK-3β) kinases belonging to the RISK pathway, we revealed the involvement of this cascade in PNX-dependent cardioprotection. This agrees with previous data showing that these kinases are recruited by pharmacological PostC elicited by many protective substances [3,9,20]. During the reperfusion, both PI3K-Akt and ERK1/2 are activated and converge on GSK3β, inducing phosphorylation/inactivation with a final control on mitoKATP channels, one of the terminal elements of PostC protection [21,22].

Notably, we found that in post-ischemic heart treated with PNX, also STAT3 phosphorylation is increased. STAT is an important membrane-to-nucleus signaling in many stress responses such as I/R, oxidative stress and hypoxia conditions [23]. It is a crucial member of the SAFE pathway and, when activated, induces survival signals in the infarcted myocardium [24]. Our results suggest that, in addition to RISK, the efficacy of PNX PostC can be attributed to the parallel activation of the SAFE pathway.

Of note, we observed that PNX Post-conditioning administration is accompanied by a reduction of apoptosis. This was revealed by changes in the expression of Bcl-2, an apoptosis inhibitor [25], and of BAX, Caspase3,

cytochrome C and p38MAPK, all of them apoptosis promoters [26,27]. Compared to the SHAM and I/R group, the heart of animals exposed to PNX showed an enhanced expression of Bcl-2 and a reduction of BAX, caspase3, cytocrome C and p38MAPK [26,27].

PNX-dependent cardioprotection in obese conditions

Epidemiological evidence suggests a strong correlation between a diet rich in fat and a significant cardiovascular risk [28,29]. Under obese conditions, several anorexigenic peptides are unable to reduce food intake. This may be related either to decreased plasma levels in post-prandial phase, or to a reduced action of central and/or peripheral receptors [6,30].

Here, we observed in obese HFD rats that during the post-prandial phase PNX plasma levels do not increase with respect to the pre-prandial phase. This suggests are that, under obese conditions, the anorexigenic effect of the peptide is depressed. These date prompted us to evaluate the cardioprotective effects of PNX in the presence of obesity. We found that the heart of HFD rats perfused with PNX during PostC is protected by I/R injury. This was indicated by a significant reduction of IS and by a marked improvement of the post-ischemic contractile function expressed by an increased LVP and a decreased contracture development. Under these conditions, the cardiac protection required the activation of the pro-survival RISK pathway and the inhibition of apoptosis. These data suggest that in obesity the cardioprotection induced by exogenous PNX is preserved. However, we observed that in the heart of HFD rat, PNX elicits protection at a concentration that is higher than the concentration which is able to protect the heart of SD rats. It is possible that the lower sensitivity of the HFD heart to PNX is due to a lower efficacy of the endogenous peptide, whose plasma levels are altered in the presence of obesity. This may reduce the synergy between intracardiac autocrine/paracrine circuit and the circulating peptide. Our results suggest that, after an I/R damage, the compromised obese heart is unable to induce a compensatory PNX expression in response to the injury and that the loss of this important protective factor may exacerbate the obesity-dependent cardiac dysfunction.

Conclusions

This study provides the first evidence that PNX directly affects the heart by eliciting NO-dependent negative inotropism and lusitropism. The peptide is able to protect the myocardium against I/R injury by activating both RISK and SAFE pathways, and by switching off apoptosis. Our data are of physiological relevance since contribute to clarify the peripheral role of PNX in the modulation of the cardiac response. The preservation of the post-ischemic LV function elicited by the peptide under normal and obese conditions is of remarkable

applicative interest [31,32].In the search of novel endocrine modulators, able to attenuate reperfusion-induced cell death, PNX may represent a good strategy to limit myocardial infarction, particularly in the presence of a deteriorated cardiac performance, as occurring in obesity.

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FIGURE LEGENDS

Figure 1 A) ELISA PNX detection in hypothalamus (positive control), heart, and testis (negative control) and in perfused heart of SHAM, I/R-SD and I/R-HFD rat (n=3 for each group). **B)** ELISA PNX detection in plasma of SD and HFD rats in pre- and post-prandial conditions (n=3 for each group). Changes were evaluated as mean ± SE. Significant difference from control values (one-way ANOVA, Newman-Keuls test): *=p <0.05.

Figure 2 A) Concentration-response curves (1 pmol/L -100 nmol/L) of PNX on LVP, +LVdP/dT max, -LVdP/dT max, HR, CP and LVEDP (n=5). **B)** Western Blots and relative densitometric analysis of extracts of SHAM and PNX treated heart: ratio of p-Akt/Akt, p-Erk 1/2/Erk1/2, p-eNOS/eNOS and p-nNOS/nNOS (n=3 for each group). Changes were evaluated as mean ± SE. Significant difference from control values (one-way ANOVA, Newman-Keuls test): *=p <0.05.

Figure 3 Developed Systolic (dLVP) and Diastolic (LVEDP) function at the end of reperfusion **(A)**, and Infarct Size (IS: percentage of necrotic tissue with respect to total LV weight) **(B)** in I/R–SD (n=5), PNX Post-SD(n=5), and PNX Post-SD plus Inhibitors (n=3 for each group). Changes were evaluated as mean ± SE. Significant difference from control values (one-way ANOVA, Newman-Keuls test): *=p <0.05.**=p <0.01 ; ***=p<0.001

Figure 4 Western Blots and relative densitometric analysis on cardiac extracts of SHAM-SD, I/R-SD and PNX Post-SD: ratio of p-Akt/Akt, p-Erk 1/2/Erk 1/2, p-Gsk3 α-β/Gsk3 α-β, p-eNOS/eNOS, and p-STAT3/STAT3 (n=3 for each group). Changes were evaluated as mean ± SE. Significant difference from control values (one-way ANOVA, Newman-Keuls test): *=p <0.05; **=p < 0.01 ; ***=p < 0.001.

Figure 5 Western Blots and relative densitometric analysis on cardiac extracts of SHAM-SD, I/R-SD and PNX Post-SD: ratio of cyt c/β-actin, p-p38 MAPK/p38 MAPK, Bax/β-actin, Active Caspase 3/β-actin, and Bcl-2/β-actin (n=3 for each group). Changes were evaluated as mean ± SE. Significant difference from control values (one-way ANOVA, Newman-Keuls test): *=p <0.05; **=p < 0.01; ***=p < 0.001.

Figure 6 A) Developed Systolic (dLVP) and Diastolic (LVEDP) function at the end of reperfusion, and Infarct Size (IS: percentage of necrotic tissue with respect to total LV weight) in I/R–HFD, and PNX Post-HFD (n=4 for each group). **B)** Western Blots and relative densitometric analysis on cardiac extracts of SHAM-HFD, I/R-HFD and PNX Post-HFD: ratio of p-Akt/Akt, p-Erk 1/2/Erk 1/2, Active Caspase 3/β-actin, and Bcl-2/β-actin (n=3 for each group). Changes were evaluated as mean ± SE. Significant difference from control values (one-way ANOVA, Newman-Keuls test): *=p <0.05; **=p < 0.01; ***=p < 0.001.

Figure1

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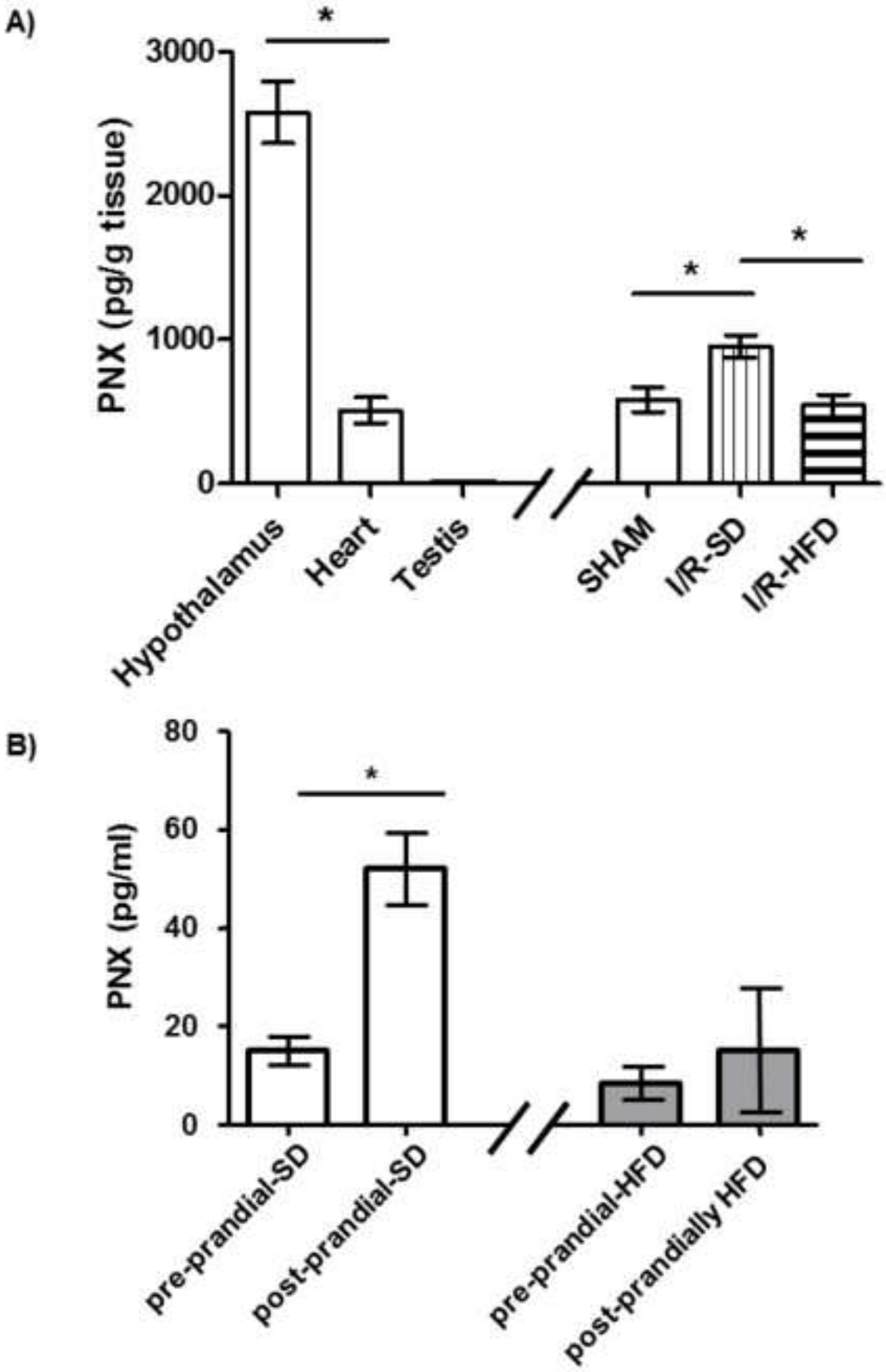

Figure 1

Figure2

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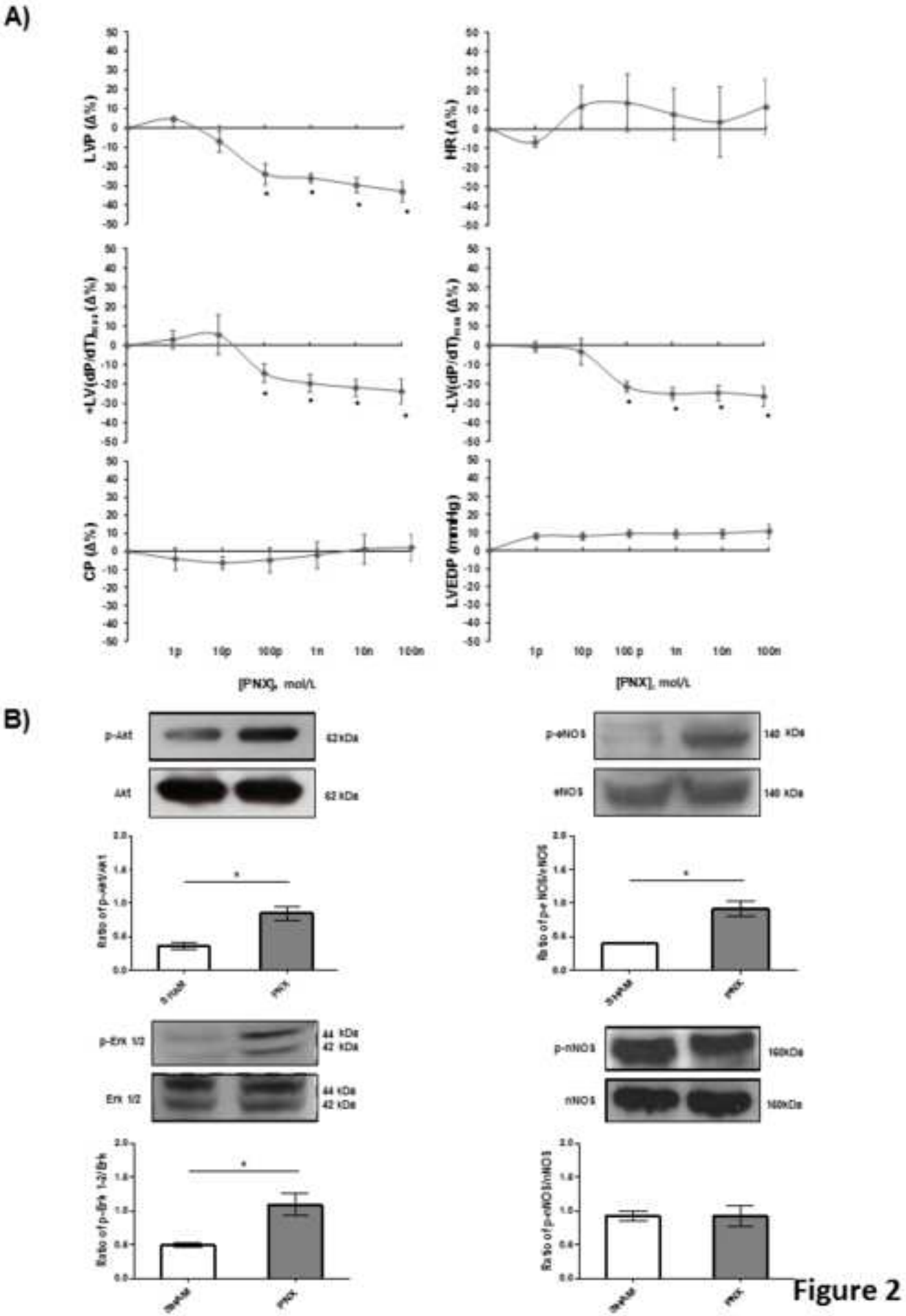

Figure 2

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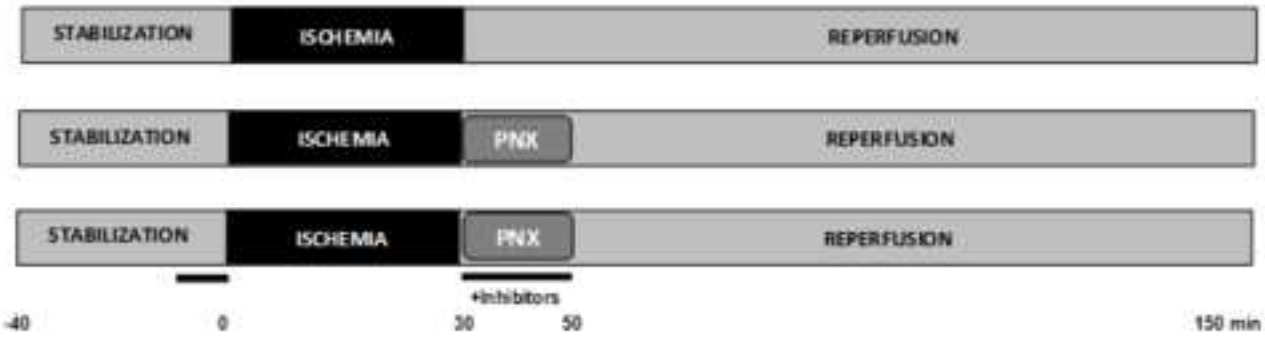

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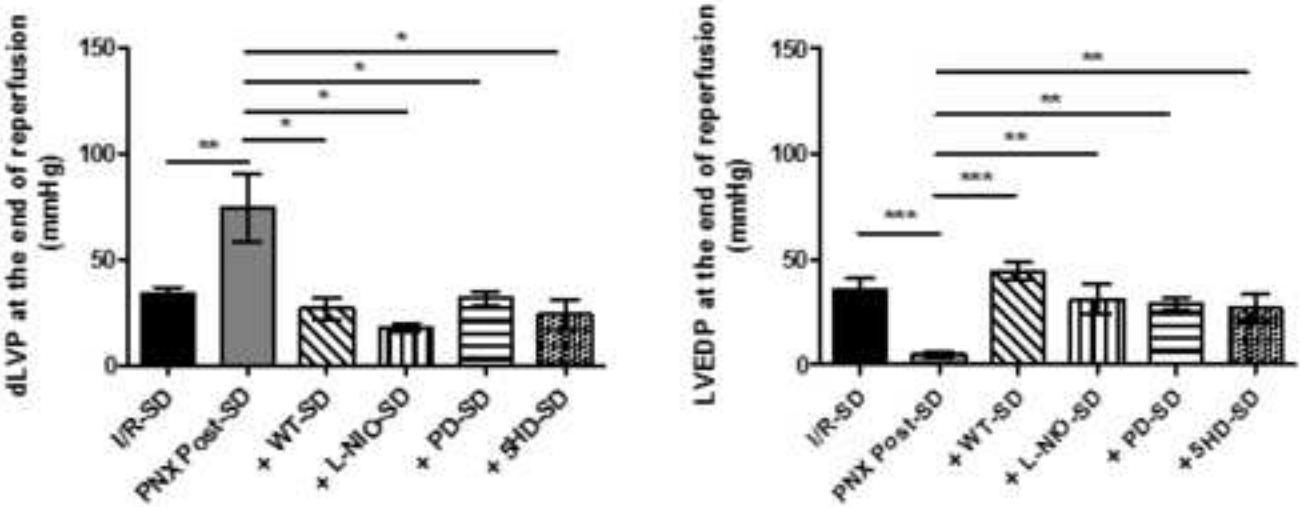

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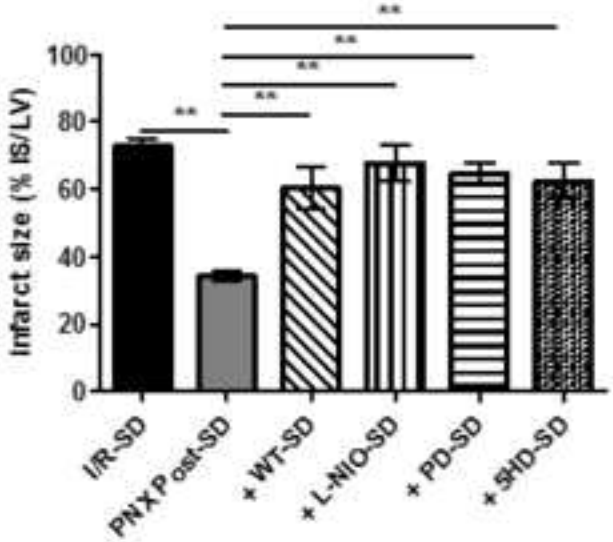

Figure 3

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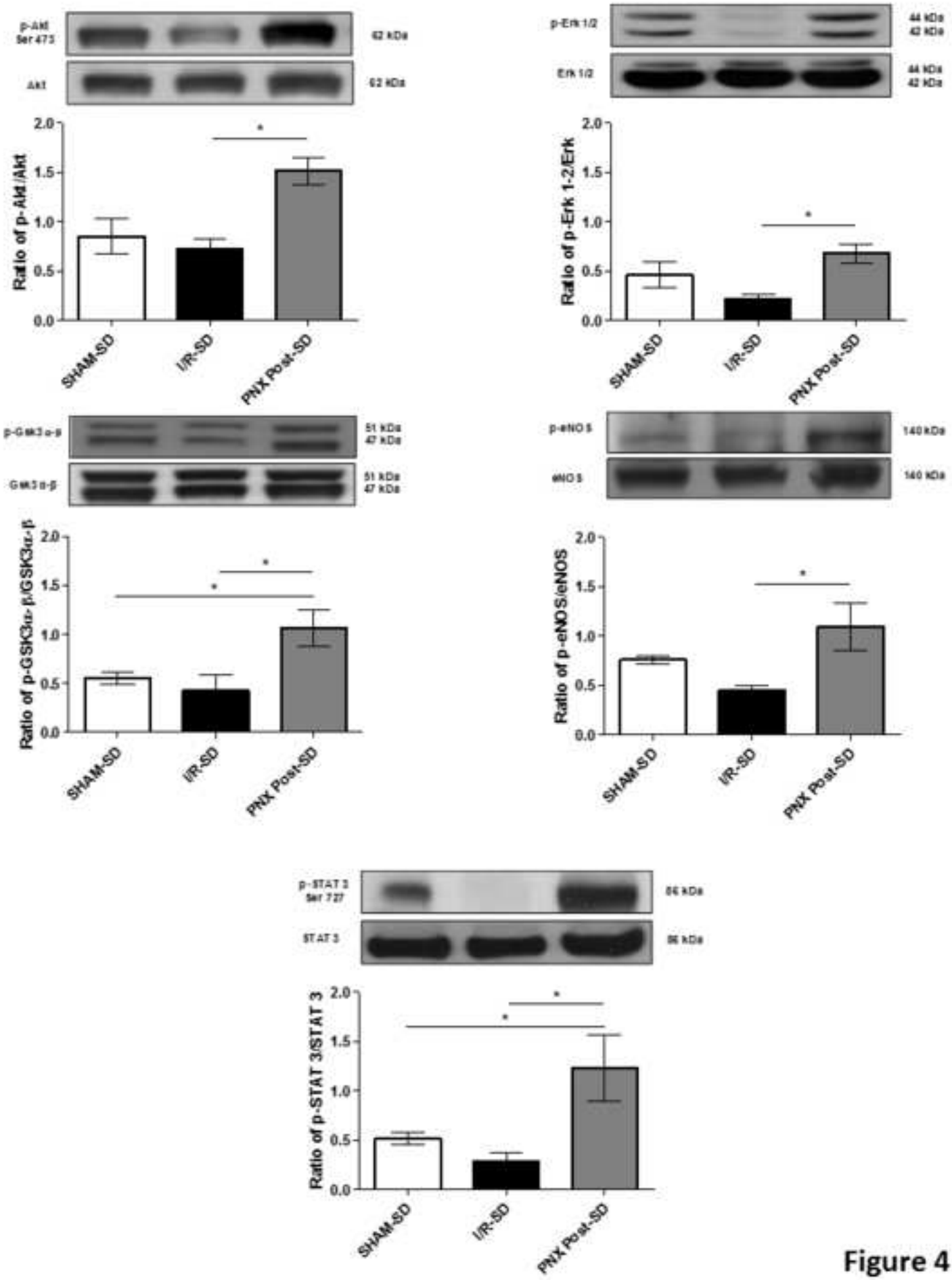

Figure 4

Figure5

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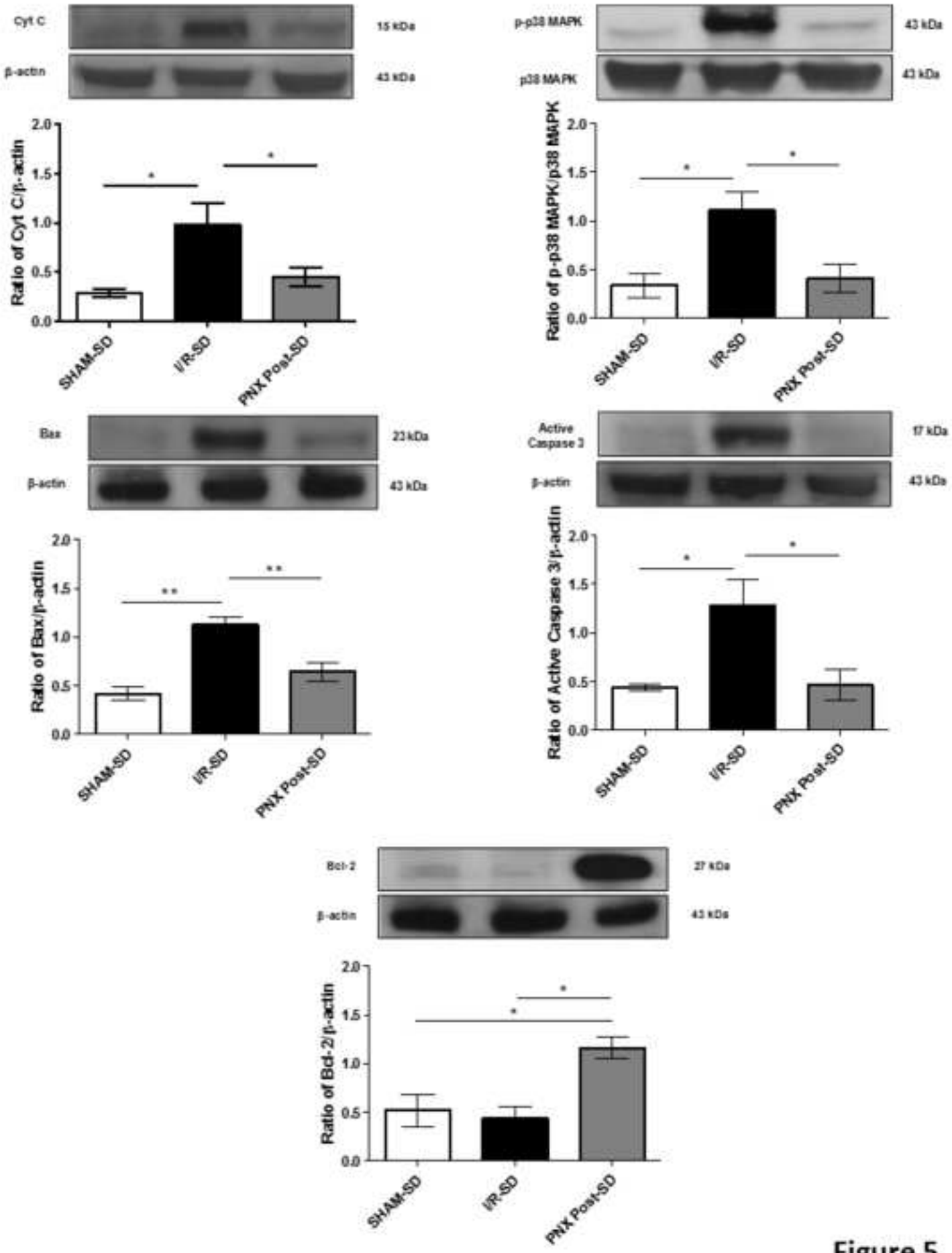

Figure 5

Figure6

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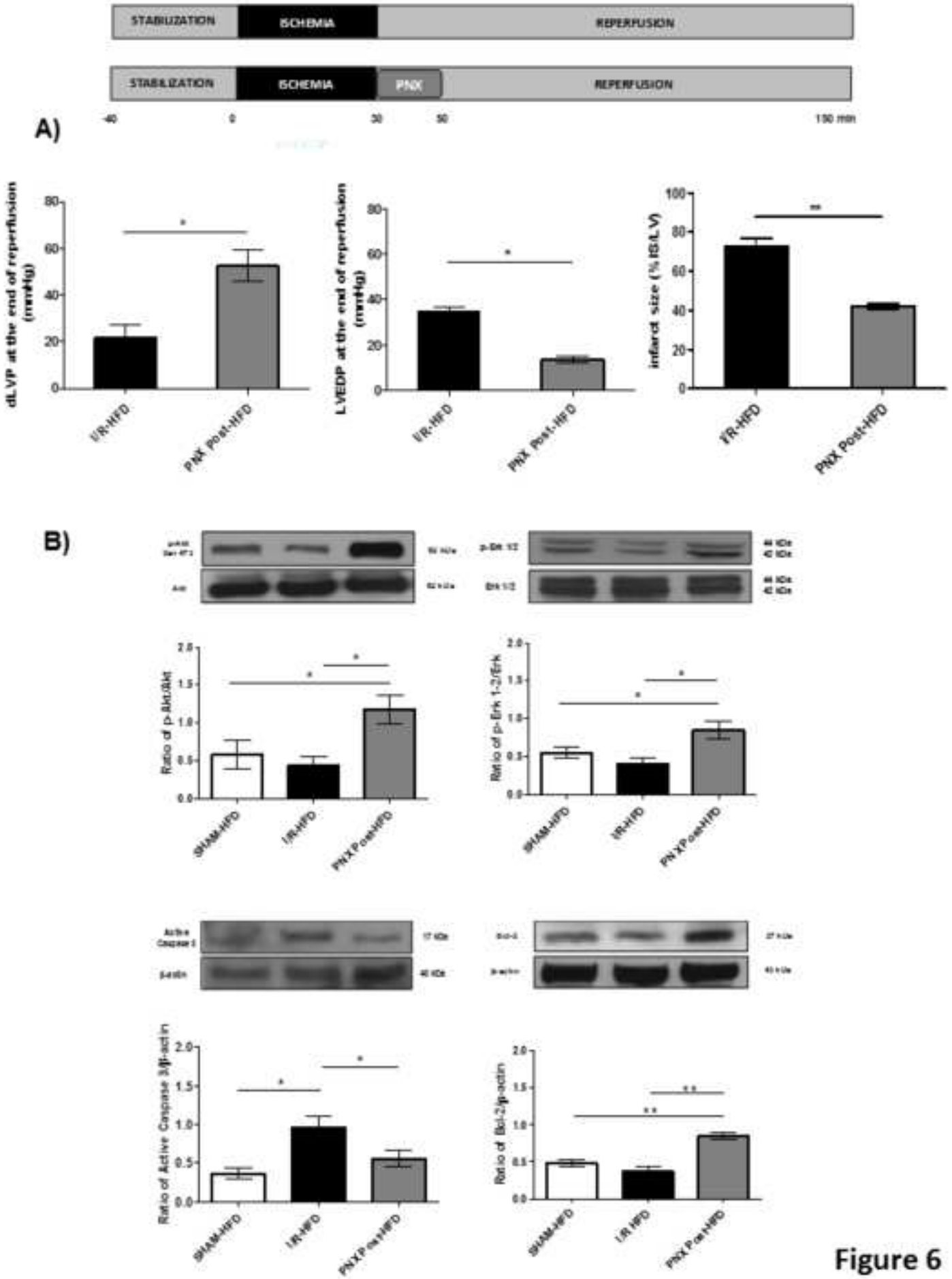

Figure 6