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Pharmacological evaluation of novel PKR inhibitor indirubin-3-hydrazone in-vitro in cardiac myocytes and in-vivo in wistar rats

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ABSTRACT

Aims: Double stranded protein kinase R cellular response is associated with various stress signals such as nutrients, endoplasmic stress, cytokines and mechanical stress. Increased PKR activity has been observed under diabetic and cardiovascular disease conditions. Most of the currently available PKR inhibitors are non-specific and have other effects as well. Thus, the aim of the present study was to examine the effect of novel PKR inhibitor indirubin-3-hydrazone (IHZ) in cultured rat H9C2 cardiomyocytes and wistar rats.

Materials and methods: PKR expression was determined by Q-PCR, immunofluorescence and immunoblotting. The expression of different gene markers for apoptosis was measured by RT-PCR. Apoptosis and oxidative stress were determined by flow cytometry. KEY FINDINGS: High glucose (HG) treated H9C2 cardiomyocytes and high fructose (HF) treated wistar rats developed a significant increase in PKR expression. A significant increase in apoptosis and generation of reactive oxygen species was also observed in HG treated H9C2 cells and HF treated rats. Reduced vacuole formation and prominent nuclei were also observed in high glucose treated cells. Cardiac hypertrophy and increased fibrosis were observed in HF treated rats. All these effects of HG and HF were attenuated by novel PKR inhibitor, indirubin-3-hydrazone.

Significance: Our results indicate IHZ as an effective inhibitor of PKR in vitro and in-vivo, thus it may prove very useful in blocking the multiple harmful effects of PKR.

1. Introduction

Diabetes and cardiovascular disorders constitute the most significant health burden to worldwide population [1]. Type I and type II diabetic patients are at increased risk of developing cardiovascular diseases [2]. Under obese and metabolic stress conditions several inflammatory pathways get activated which in turn lead to activation of downstream signaling molecules such as Jun NH2-terminal kinase (JNK) and NFKB. These pathways play a significant role in development of insulin resistance and cardiovascular diseases by controlling the inflammatory responses in metabolic tissues, inhibition of insulin receptor signaling and disruption of systemic glucose and lipid homeostasis [3–8]. Double stranded protein kinase R (PKR) is one such molecule that is integrated with both nutrient and pathogen response system, is activated by DsRNA, several stress signals, nutrients and pathogens [9]. PKR plays an important role in signal transduction and transcriptional control of several inflammatory pathways such as IkB/NFKB and JNK [10–12]. Previous studies have reported that PKR gene silencing (PKR−/−) in mice relates with improvement in insulin resistance [12,13]. Increased translocation and myocardial expression of PKR has been demonstrated in human subjects suffering from congestive heart failure (CHF) [14]. Congestive heart failure (CHF) is associated with cardiomyocyte hypertrophy, apoptosis and inflammation. PKR plays a significant role in development of CHF by intensifying apoptosis and inflammation of cardiomyocytes [15]. Based on this research pharmacologically targeting PKR may be an effective therapeutic strategy for treatment of diabetes and vascular complications.

Indirubin is one of the main active ingredients of Chinese herb drug and has been known for its anti-proliferative effect in inhibition of cyclic cyclin dependent kinase (CDK) and glycogen synthase kinase-3ß

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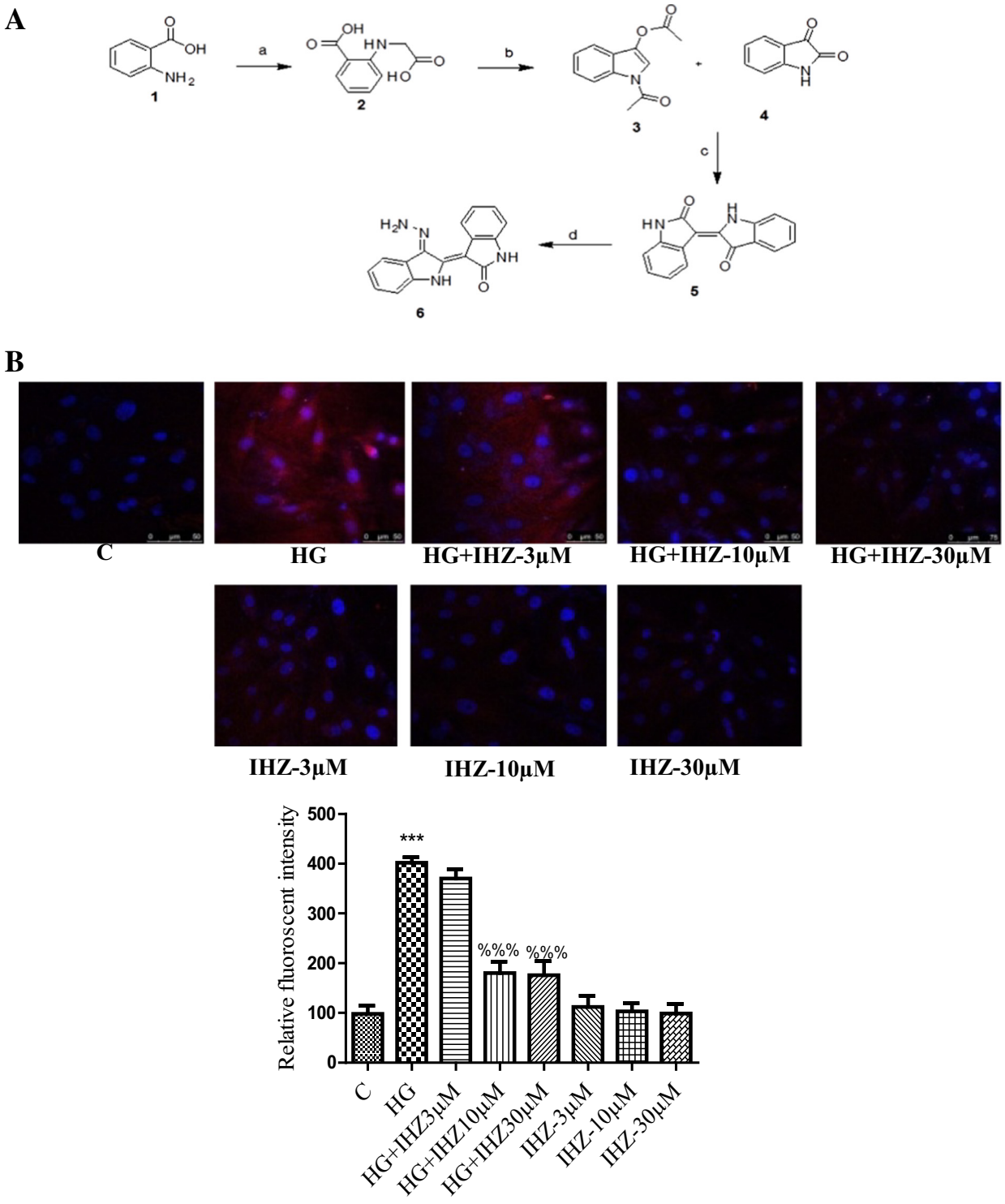
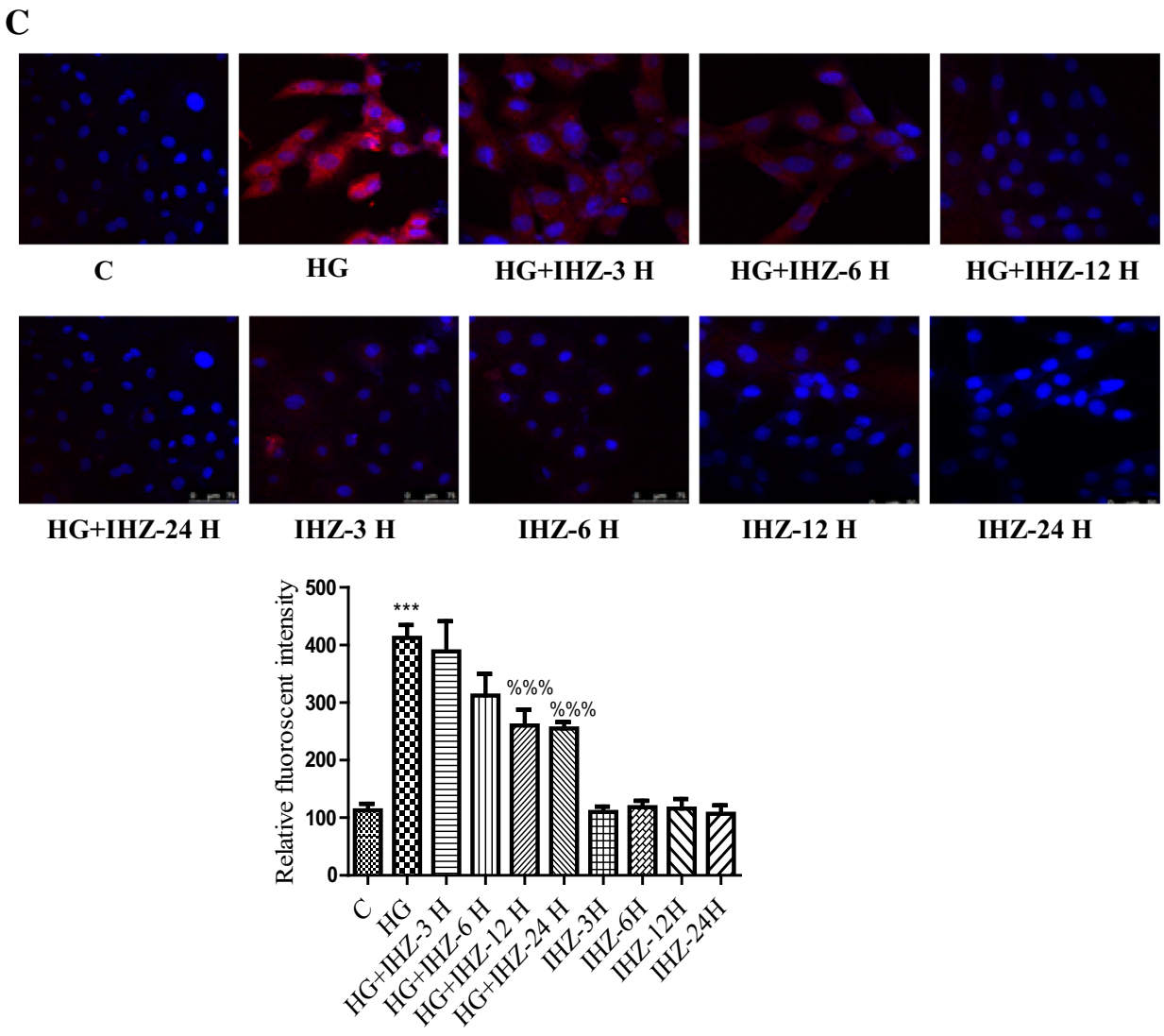


Fig. 1. Schematic representation for synthesis of novel PKR analogue (indirubin 3 hydrazone (IHZ)) and concentration and time dependent effect of IHZ on PKR levels. (A) Briefly describes the scheme developed for synthesizing indirubin 3 hydrazone (IHZ). The reagents and conditions for synthesis of IHZ were (a) 2-Bromoacetic acid, K_2CO_3, DMF, 4 h,110 °C; (b) Acetic anhydride, 135–140 °C, 10 h; (c) K_2CO_3, CH_3OH (d) $NH_2NH_2.H_2O$, C_2H_5OH, 80 °C, reflux. (B) Immunohistochemistry was done to determine the concentration dependent effect of IHZ on PKR levels using PKR specific antibody. Briefly, cultured cells were incubated with different treatment groups Control (C), high glucose (HG) (25 mM), and IHZ (3, 10, 30 μM) alone or co-incubated with HG. (C) Time dependent studies for determining the PKR expression. The cultured cells were incubated with different treatment groups Control (C), high glucose (HG) (25 mM), and the most effective dose of IHZ (10 μM) alone or co-incubated with HG. for 3, 6, 12 and 24 h. (D) Immunohistochemistry, western blotting and gene expression was studies were carried out for comparing the efficacy of IHZ (10 μM) with standard PKR inhibitor C16 (5 μm) on PKR activity. (E) PKR expression was significantly increased in HF treated rats, which was significantly attenuated by administration of IHZ. n = 6 for each group was taken for each study $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.5$ vs. control. $^{\%\%\%}p < 0.001$, $^{\%}p < 0.5$ vs. HG, $^{\%}p < 0.5$ vs. HF, $^{\#}p < 0.5$ vs. HG + C16.



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(GSK-3 ß) [16]. We have also reported recently that indirubin-3-oxime (I3O) has beneficial effects against high glucose induced oxidative stress and apoptosis in cultured rat cardiomyocytes. Further, we found that I3O is also capable of attenuating high glucose induced increased PKR expression [17]. Indirubin- 3-hydrazone (IHZ), a novel derivative of indirubin is a reported cyclin dependent kinase (CDK) inhibitor [16,18]. However, the effect of IHZ on PKR signaling is not yet reported, so the aim of the present study was to investigate the effect of IHZ on PKR signaling pathway and underlying molecular mechanism.

2. Materials and methods

2.1. Novel PKR inhibitor

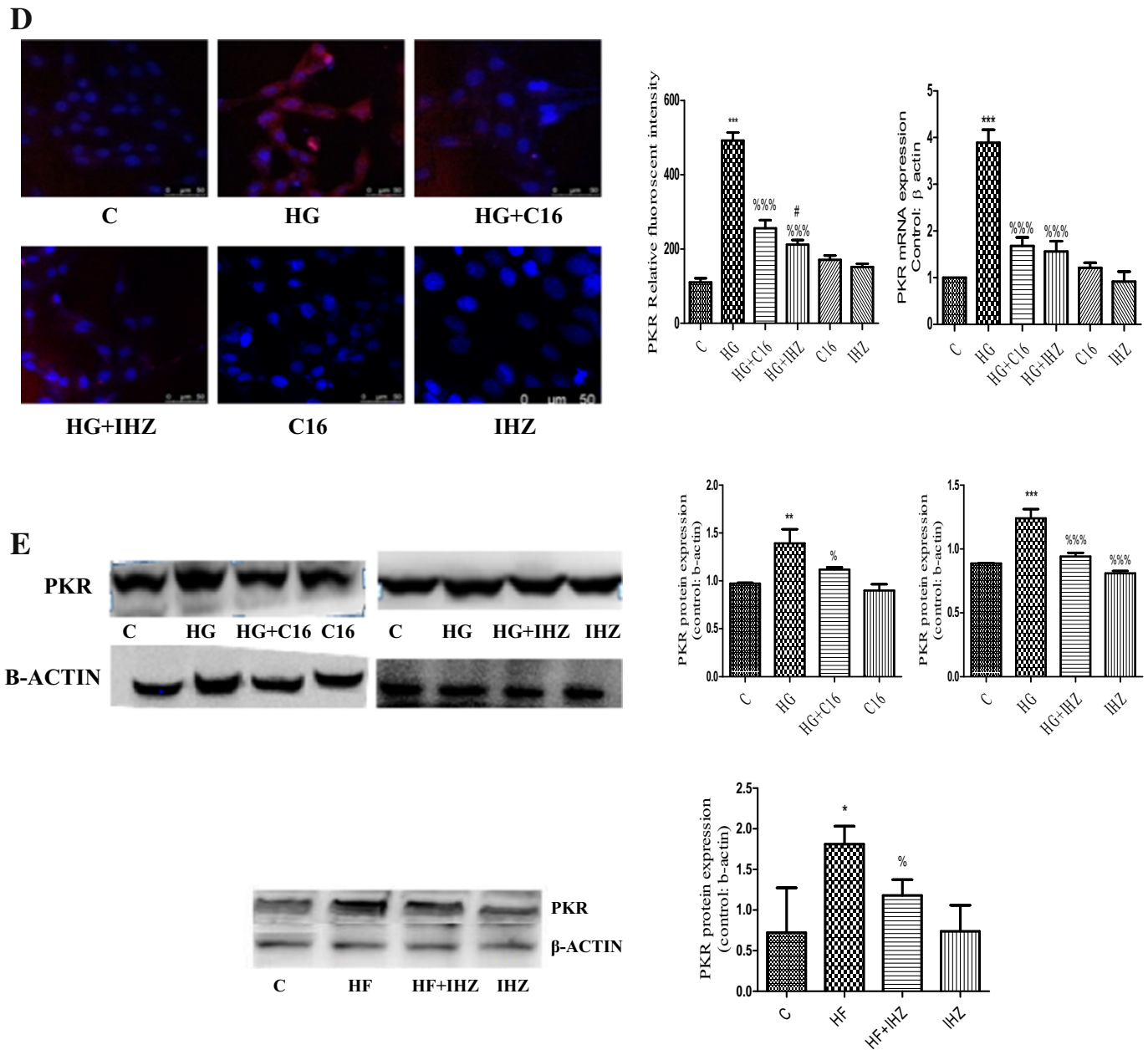
The synthesis of novel PKR inhibitor was given in Fig. 1. In the first step of reaction we used anthranilic acid (1) with bromoacetic acid and potassium carbonate in presence of ethyl acetate to yield corresponding (carboxymethylamino) benzoic acid (2) which was then filtered and purified through column chromatography to obtain 1-acetyl-1*H*-indol-3-yl acetate (3). Later to this reaction indoline-2,3-dione (commercially purchased) (4) was added and dissolved in methanol in presence of potassium carbonate to yield indirubin (5), resultant indirubin was dissolved in hydrazine and ethanol. Finally, resulting compound was washed and filtered by column chromatography to obtain indirubin 3 hydrazone (IHZ) (6).

2.2. Cell culture

H9C2 cells (a myoblast cell line purchased NCCS, Pune, India) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells at 3–4 passages were exposed to high glucose (25 mM) alone or in combination with PKR inhibitor, C16 (Sigma Aldrich, MO, USA).

2.3. Animals

5–6-week-old male wistar rats with a mean body weight (b.w.) of 180–220 g were randomly divided into 4 groups of 6 animals in each group as follows: Control (C), Diabetes Control with 20 g of fructose in drinking water (HF), high fructose and IHZ (HF + IHZ), IHZ alone. Animals were housed as 3 rats per polycarbonated cage in a temperature and humidity controlled room (22 ± 1 °C, 45–60% humidity) with a set of 12 h light-dark cycle. The rats were fed a commercially available rat pellet diet ad libitum throughout the 6 weeks experimental



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period. Control groups were supplied with normal drinking water ad libitum. Diabetes control group were fed with FR20 were supplied with 20% fructose solution. The animals were maintained according to the rules and regulations of the Institutional ethical committee (Ethical approval number: HYD/IAEC/2016/06).

2.4. Induction of hyperglycemia

Low dose (35 mg/kg b.w.) of streptozotocin (STZ), (Sigma Aldrich, MO, USA) was given to diabetic control and high fructose and IHZ group while the animals in normal control group were injected with vehicle buffer only and all biochemical parameters were measured in plasma.

2.5. Immunohistochemistry

Cells were plated on a 6 well plate (n = 6 for each group). After treatment for 24 h cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min, permeabilised in 0.1% triton-x, blocked with 3% BSA for 1 h at room temperature, incubated with primary antibodies for over-night at 4 °C, then incubated with secondary antibodies for 2 h and counterstained with DAPI.

2.6. Real time quantitative PCR (RT-PCR)

Total RNA from cultured cells was isolated using RNA isolation kit (HI Pura, Germantown, Md., USA). The real-time PCR (RT-PCR) was performed in CFX connect apparatus associated with the Bio-rad CFX manager software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad Laboratories Ltd., India).

2.7. Western blotting

Briefly, aliquots of cell lysates (50 μg each) (n = 6 for each group) were separated on 6–10% SDS-PAGE, electrotransferred to a poly-vinylidene difluoride (PVDF) membrane (Bio-Rad) and blocked with

5% nonfat milk in TBS-Tween buffer (20 mM Tris–HCl, pH 7.4, 135 mM NaCl, 0.1% Tween) for 1.5 h at room temperature. Next the membrane were incubated with the appropriate primary antibodies (Santa Cruze Biotechnology, CA, USA) respectively, followed by incubation with horseradish peroxidase conjugated secondary antibody for 1 h. After extensive washing, immunoreactive protein was detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp.) Each immunoblot experiment was repeated 3 times.

2.8. Estimation of lactate dehydrogenase activity and nitrite levels

Cells were incubated with high glucose, C16 and novel PKR inhibitor or high glucose alone for 24 h, later the supernatant was collected and incubated with working reagent for 5 min according to manufacturer's instructions and absorbance was taken using plate reader at 340 nm–356 nm.

2.9. Measurement of reactive oxygen species and apoptosis

The formation of peroxynitrite was determined by a DCFH assay. Cells were collected after incubation for 24 h with different treatment groups. For ROS cells were loaded with a membrane-permeable, non-fluorescent probe 2,7′-dichlorofluorescin diacetate (CM-H2DCFDA, 5 μmol/l) for 2 h at 37 °C in FBS-free DMEM in the dark. For apoptosis cells were incubated with annexin and propidium iodide for 15 min. After washing with PBS 3 times, apoptosis and ROS were measured using flow cytometry.

2.10. Labelling of H9c2 cells with rhodamine for autophagy and hand E staining

Cells were grown on coverslips and incubated with Tetra methyl rhodamine methyl ester (TMRM) (100 nm) and Hematoxylin (H) in separate wells for 30 min in dark at 37 °C. H coverslips were counter-stained with Eosin and observed under confocal microscope for autophagy and cell integrity.

2.11. Measurement of fibrosis and cardiac hypertrophy

Hearts were isolated and sectioned of 5 μm, weighed (cardiac hypertrophy) and stained with Sirius red for 1 h and washed with 2 changes of alcohol, later observed for collagen deposition or fibrosis.

2.12. Statistical analysis

Data obtained from separate experiments are expressed as mean ± SEM. Statistical analysis was performed using ANOVA with post hoc Bonferroni's test. A p value of < 0.05 was considered to be statistically significant (n = 6 for each group).

3. Results

3.1. Effect of novel PKR inhibitor (IHZ) on HG induced PKR expression

Novel PKR inhibitor at different concentrations (3, 10, 30 μm) was incubated with HG in-vitro in H9C2 cells (Fig. 1B). C16 was used as a standard for comparison of PKR inhibition levels. Incubation of HG with novel inhibitor at 3 μm for 24 h did not showed any significant reduction, whereas incubation of HG with 10 and 30 μm showed almost 75% of the PKR inhibition (Fig. 1B). So based on this observation we have selected 10 μm concentration and incubated for different time points 3,6,12 and 24 h however significant reduction in PKR expression was observed at 12 and 24 h time point (Fig. 1C).

3.2. IHZ inhibits HG induced PKR activity in vitro

We previously reported that HG induced PKR expression was attenuated by Indirubin 3-oxime, recently from docking analysis we found novel analogue of indirubin, indirubin hydrazone (IHZ) has inhibitory activity on PKR. In this study we investigated the in-vitro potential of IHZ in H9C2 cells on PKR by RT-PCR, immunohistochemistry and western blotting. The mRNA and protein expression of PKR, which is a key modulator involved in glucose homeostasis was significantly increased in HG incubated cells and was significantly attenuated by IHZ (10 μM) co-incubated with HG (Fig. 1D). In heart tissue also we found increased PKR expression (Fig. 1E). This was significantly decreased by IHZ co-incubated with HF.

3.3. Effect of IHZ on gene markers of apoptosis

We examined the effect of IHZ on gene markers associated with apoptosis. H9C2 cells were incubated with HG alone and in combination with C16 and IHZ for 24 h and analyzed for gene expression. The mRNA expression of NFKB (Fig. 2C), an oxidative stress marker, JNK (Fig. 2C) and caspase-3 (Fig. 2B) an apoptotic and inflammatory transcription factor was significantly increased in HG incubated H9C2 cells which was significantly decreased by IHZ (10 μM) co-incubated with HG (Fig. 2A). Protein expression studies using western blot was performed for JNK and phospho-JNK. There was no change in protein expression of JNK but increased expression of phospho-JNK was observed when incubated with HG for 24 h compared to untreated cells this was significantly attenuated by IHZ with HG (Fig. 2A). Immunofluorescence studies for JNK and caspase-3 (Fig. 2B) were performed and HG significantly increased the JNK expression which was attenuated by IHZ co-incubated with HG (Fig. 2A).

3.4. Effect of IHZ on HG induced apoptosis and ROS generation

Previously, we have reported that HG induces apoptosis and ROS, so here we have examined whether IHZ can prevent HG induced apoptosis and ROS in H9C2 cells and wistar rats or not. HG treated cells have shown more early apoptotic cells and ROS production which was significantly attenuated by C16 and IHZ co incubated with HG. IHZ along with HG show significant reduction in early apoptotic percentage of cells and ROS production compared to HG + C16 (3A). IHZ along with HF attenuated ROS produced by HF (Fig. 3A and B).

3.5. Effect of IHZ on iNOS production and LDH

Inducible nitric oxide (iNOS) production is a key mediator to promote apoptosis, LDH an important marker in cardiomyopathy so we examined whether HG can induce iNOS and LDH. so in H9C2 cells incubated with HG (25 mM) there was significant increase in iNOS and LDH production when compared with untreated cells which was significantly decreased by IHZ (10 μM) co-incubated with HG (Fig. 4A). However, it was not significant when compared to HG + C16.

3.6. Effect of IHZ on HG elicited autophagy in cardiomyocytes and cellular integrity

Autophagy is followed by oxidative stress, in the present study we have examined the reduced vacuole formation in HG treated cultured cells which may be due to autophagy using rhodamine staining. Prominent nuclei and irregular shaped cell structure was found in cells treated with HG, Moreover when cells were exposed to HG along with IHZ vacuole formation and cell structure was recovered when compared to HG alone treated cells (Fig. 4B).

3.7. Effect of IHZ on HF induced changes in biochemical markers, fibrosis and cardiac hypertrophy in heart

All the biochemical estimations were done in plasma samples (Fig. 5A). For fibrosis and cardiac hypertrophy, the heart sections were then deparaffinized with Xylene, rehydrated with alcohol and water. The rehydrated sections were stained using Sirius red, collagen deposition and heart size was significantly increased in HF treated rats this was significantly attenuated by IHZ (Fig. 5B and C).

4. Discussion

Metabolic disorders including obesity, insulin resistance, impaired glucose tolerance has limited treatment options and has become a growing health concern across world [19,20]. PKR a serine/threonine protein kinase is activated by cytokines, stress signals, interferon's and viral infection and plays an important role in the nutrient/pathogen sensing interface and acts as a key modulator of chronic metabolic inflammation, insulin sensitivity and glucose homeostasis [17]. PKR has been implicated in the pathogenesis of insulin resistance as well as congestive heart failure [14]. Thus selective and specific inhibitors of PKR can be very effective agents against the harmful effects of high glucose. Although, Lancaster et al. [21] have reported that PKR deletion does not interfere in the progression and development of high fat diet (HFD) induced obesity. Interestingly, they found that increased PKR levels or its activation causes inflammation of adipose tissue resulting in impairment of glucose metabolism. However, they also suggested that several independent studies need to be carried out to contradict the results obtained from Nakamura et al. Thus, further detailed studies are required to explore role of PKR in cardiovascular and metabolic complications. Moreover, currently there is a lack of selective PKR inhibitors and most of the compounds available, such as imoxin, 2-aminopurine used as PKR inhibitors are costly.

Thus in the present study we designed and synthesized novel PKR inhibitor, IHZ analogue using indirubin as a backbone (Fig. 1A). We report for the first time that IHZ attenuated high glucose and HF induced increased PKR expression in cultured rat cardiomyocytes. The attenuation was much more significant compared to standard PKR inhibitor C16 (Fig. 1B, C). Recent studies have reported that PKR integrates nutrient overload, ER stress and inflammation. Inhibition of PKR activation reduces JNK phosphorylation which is responsible for insulin resistance. Activated PKR also induces overexpression of NFKB

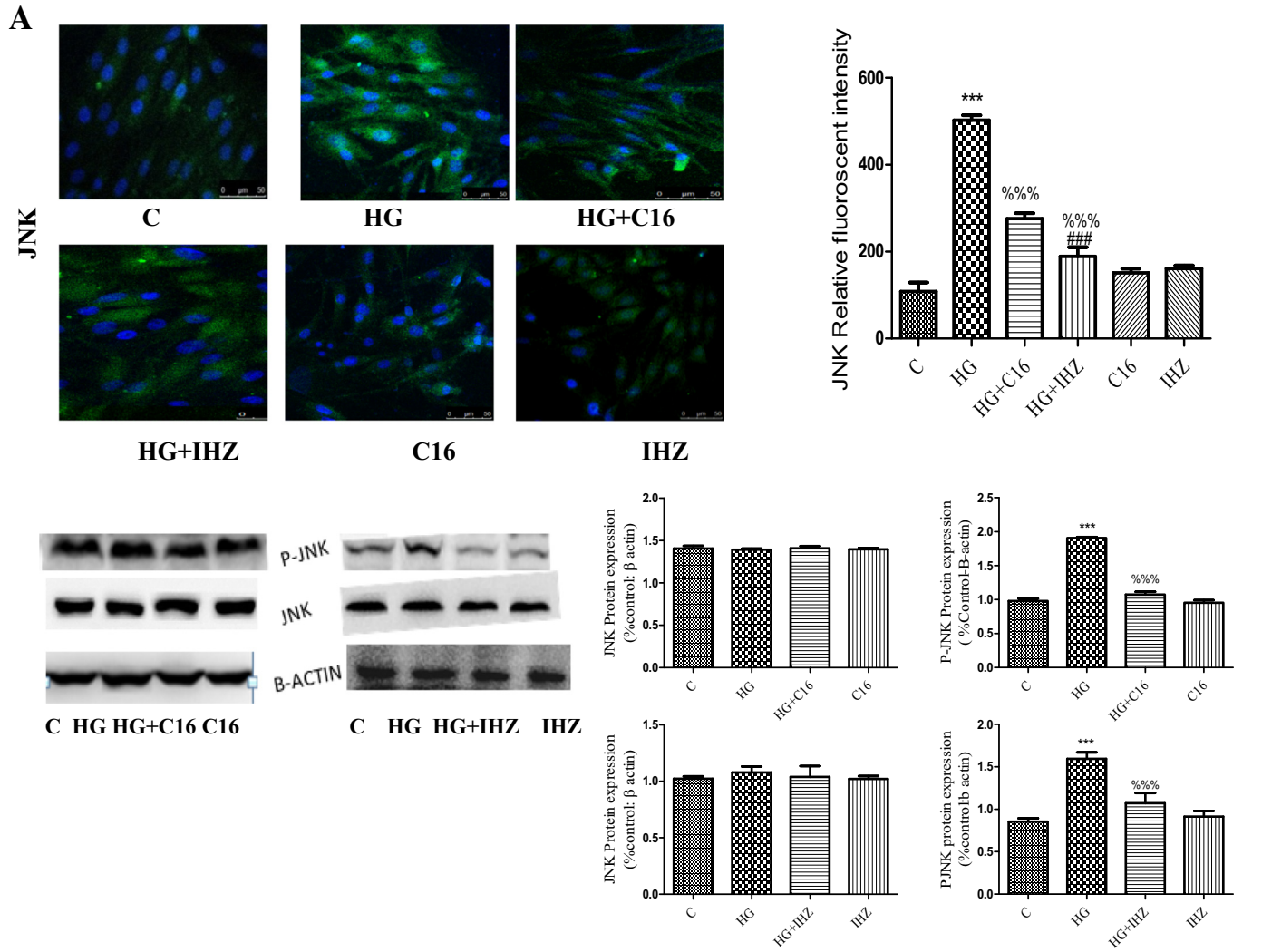
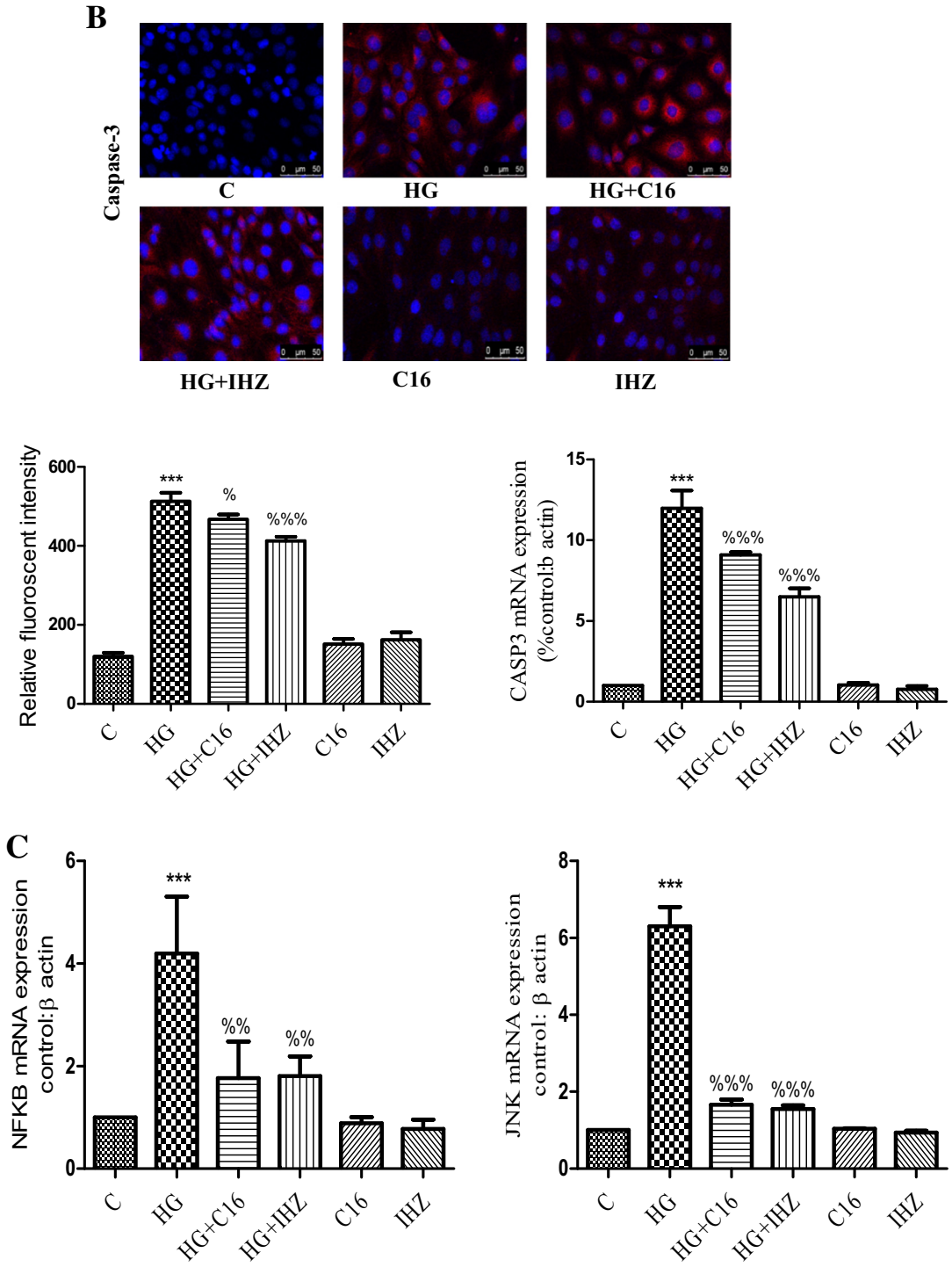


Fig. 2. IHZ attenuates HG induced expression of JNK, caspase-3 and NFKB.
Incubation of cultured cells with HG (25 mM) significantly increased expression of JNK, and caspase-3 which was significantly attenuated by IHZ (10 μM) on co-incubation with HG (A). Effect of IHZ on JNK expression was determined by performing immunohistochemistry, and western blotting using antibodies specific for JNK and phospho-JNK, (B) Representative results obtained from immunohistochemistry and mRNA expression studies for determining the expression of caspase-3 with specific caspase-3 antibody, (C) Demonstrates gene expression studies for NFKB and JNK. n = 6 for each group $^{***}p < 0.001$, $^{**}p < 0.01$ vs. control. $^{\%\%\%}p < 0.001$, $^{\%\%}p < 0.01$, $^{\%}p < 0.5$ vs. HG treated group. $^{\#}p < 0.5$ vs. HG + C16.



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causing transcription of many apoptotic markers such as caspase-3 [17,18]. So we investigated whether the novel molecule IHZ can attenuate the harmful effects of HG on JNK and NFKB expression (Fig. 2C). Here we report that IHZ attenuates HG induced JNK expression in cultured H9C2 cardiac myocytes (Fig. 2A). Moreover the effect was much more significant than the standard PKR inhibitor C16.

Oxidative stress and ROS production has negative influence on glucose tolerance, insulin resistance and hyperglycaemia [22]. HG is a known inducer of oxidative stress [17]. IHZ attenuates the HG and HF induced oxidative stress in cultured cardiomyocytes (Fig. 3A). The reduction in ROS production was ever more significant than standard PKR inhibitor C16 (Fig. 3A). Apoptosis plays a crucial role in various metabolic diseases such as ischemia, hypertension and diabetes [22]. Tissue injury, disease condition and stress signals ultimately lead to caspase-3 activation which in turn will induce cell death via activation of nucleases and proteases [23,24]. PKR mediates apoptosis through multiple mechanisms, via interacting with death ligands such as TNF-α and FasL [23]. IHZ attenuates HG induced early apoptosis in cultured cardiomyocytes (Fig. 3B). Moreover, as shown the FACS analysis, the effect was more significant compared to C16. HG induced increased caspase-3 immunofluorescence as well as mRNA expression was also attenuated by IHZ in cultured cardiomyocytes for 24 h (Fig. 2B). Lactate

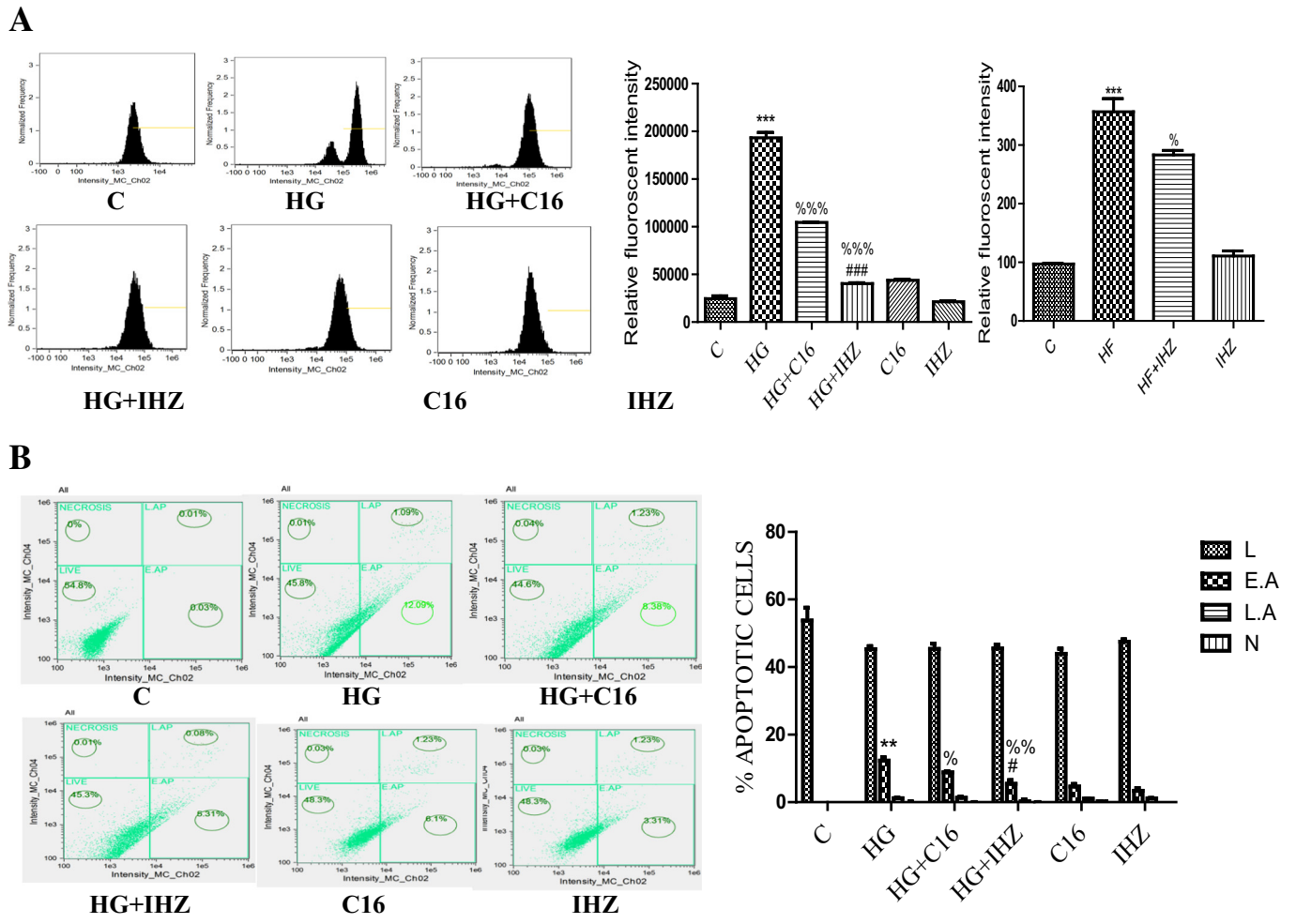

Fig. 3. IHZ attenuates HG induced increase reactive oxygen species production and apoptosis: incubation of cultured cardiomyocytes with HG and HF treated rats for 24 h induced increase in ROS generation which was significantly attenuated by IHZ when co-incubated with HG and HF which was analyzed by FACS and plate reader (A). Annexin V/alexa fluor 488 assay was performed for apoptosis. Cells were incubated with HG for 24 h and analysis was performed using annexin V alexa fluor 488, the results explain percentage of live, early, late apoptotic and necrosis cells falling in that particular gating (B). n = 5 for each group $^{***}p < 0.001$, $^{**}p < 0.01$ vs. control, $^{\%}p < 0.5$, $^{\%\%}p < 0.01$, $^{\%\%\%}p < 0.001$ vs. HF and HG. $^{\#\#\#}p < 0.001$ vs. HGC16. $^{\#}p < 0.5$ vs. HGC16 early apoptotic cells.

dehydrogenase (LDH), a soluble cytoplasmic enzyme is an important marker to detect necrosis. LDH is present in almost all the cells and is released into extracellular space when plasma membrane is gets damaged [25,26]. In the present study we found that in incubation of H9C2 cardiac myocytes with HG lead to increased LDH levels, whereas co-incubation of IHZ with HG significantly attenuated the increased LDH levels in cultured rat cardiomyocytes (Fig. 4A). NFκB is one of the key transcription factors involved in triggering the events that are associated with the inflammatory pathways. Further, it has also been reported that inducible nitric oxide synthase activation is coupled/linked with the activation of NFκB [27,28]. Therefore, we investigated the effect of IHZ on iNOS levels on HG treated cardiomyocytes. HG treated cells co-incubation with IHZ has significantly reduced iNOS levels compared to HG treated cultured cardiomyocytes (Fig. 4A).

Autophagy has been depicted as cellular degradation pathway playing an important role in cellular homeostasis. At basal levels autophagy is used to maintain biological function [29]. Upregulated autophagy is a prominent marker observed under cardiomyopathy [30]. In the present study interest has been generated in addressing the autophagic activity of cardiomyocytes in response to HG. Severe autophagy was observed in HG treated cells which was determined with the help of a specific tracer Tetra methyl rhodamine methyl ester (TMRM) for autophagic vacuoles [31]. Our novel molecules attenuated this vacuole formation when incubated with HG (Fig. 4B). Additionally, through H and E staining we have detected numerous other morphological changes (Fig. 4B) in cultured cardiomyocytes that could have occurred as a consequence of apoptosis [32]. We found that in HG treated cardiomyocytes, dark eosinophilic cytoplasm with fragmented nucleus was present, a well-known marker for apoptosis. Moreover, improvement in morphological changes was observed on co-incubating the HG treated cells with IHZ. Results obtained from biochemical studies done in rat plasma also confers it role in improving overall lipid profile and glucose levels and (Fig. 5A). We also measured collagen deposition in all rats using Sirius red staining, there was a significant

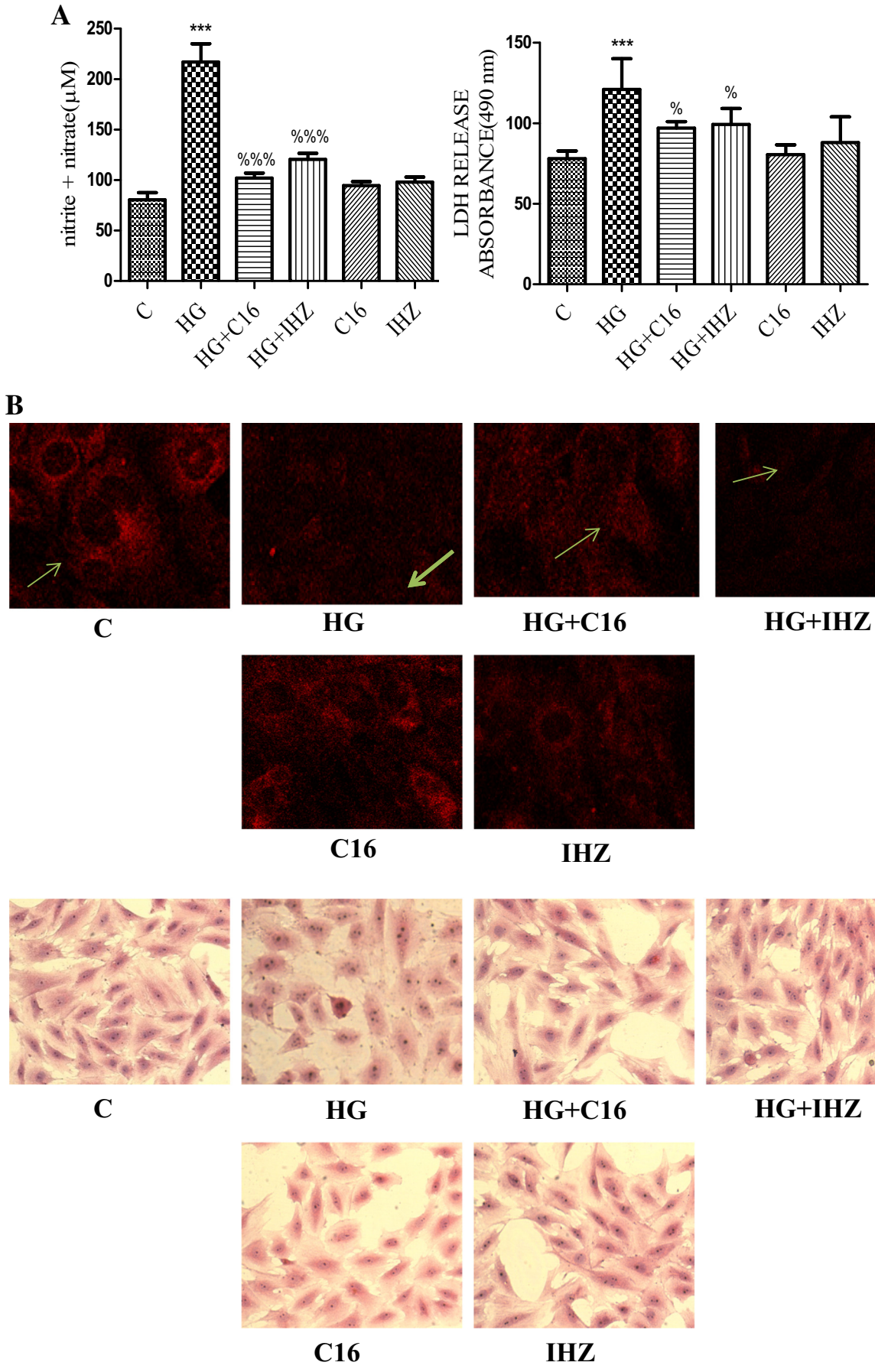

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Fig. 4. IHZ attenuates HG induced iNOS production, lactate dehydrogenase (LDH), morphological changes and autophagy.
Incubation of cultured cells with HG (25 mM) caused significant increase in nitrite levels and induced morphological changes along with the increased activity of LDH and autopahgy, which was significantly attenuated by IHZ (10 μM) incubated with HG, n = 8 for each group. Cells after incubating with HG has shown no vacuole formation which was attenuated by IHZ (10 μM), n = 6 for each group. ***$p < 0.001$ vs. control. $^{\%}p < 0.5$, $^{\%\%\%}p < 0.001$ vs. HG. (A) Nitrite levels were measured with the help of Griess reagent. LDH activity was measured by commercially available kit and autophagy was determined with the help of rhodamine staining. (B) Cellular integrity was assessed by staining the sections with H & E and autophagy was determined with the help of rhodamine staining.

increase in fibrosis in HF treated rats, this was significantly attenuated by IHZ on co-treatment with HF (Fig. 5B). As cardiac hypertrophy is a marker for cardiomyopathy [15] we next measured weight of hearts and we observed if there is any change in heart size. HF treated rat hearts showed increase in weight and size this was attenuated by IHZ along with HF (Fig. 5C). Therefore, the present study indicates that PKR is involved in the development of diabetes related cardiac complications and contribute an important role in progression of cardio-metabolic diseases. Hence, targeting PKR may present a novel approach in the discovery and development of therapeutic strategies which are aimed at correcting cardio-metabolic disorders.

A

Parameters	C	HF	HF+IHZ	IHZ
Total glucose (mg/dL)	106±9	322±71	209±27	90±14.68
Total cholesterol (mg/ldL)	89±0.894	613±10	421±3.01	63±5.1
High density lipoprotein (mg/dL)	24±0.81	6±0.27	11±0.71	23±0.91
Low density lipoprotein (mg/dL)	62.1±1	386±8.1	291±1	56±2.1
Triglycerides (mg/dL)	13.55±0.891	105±0.551	90±0.201	18.3±0.41

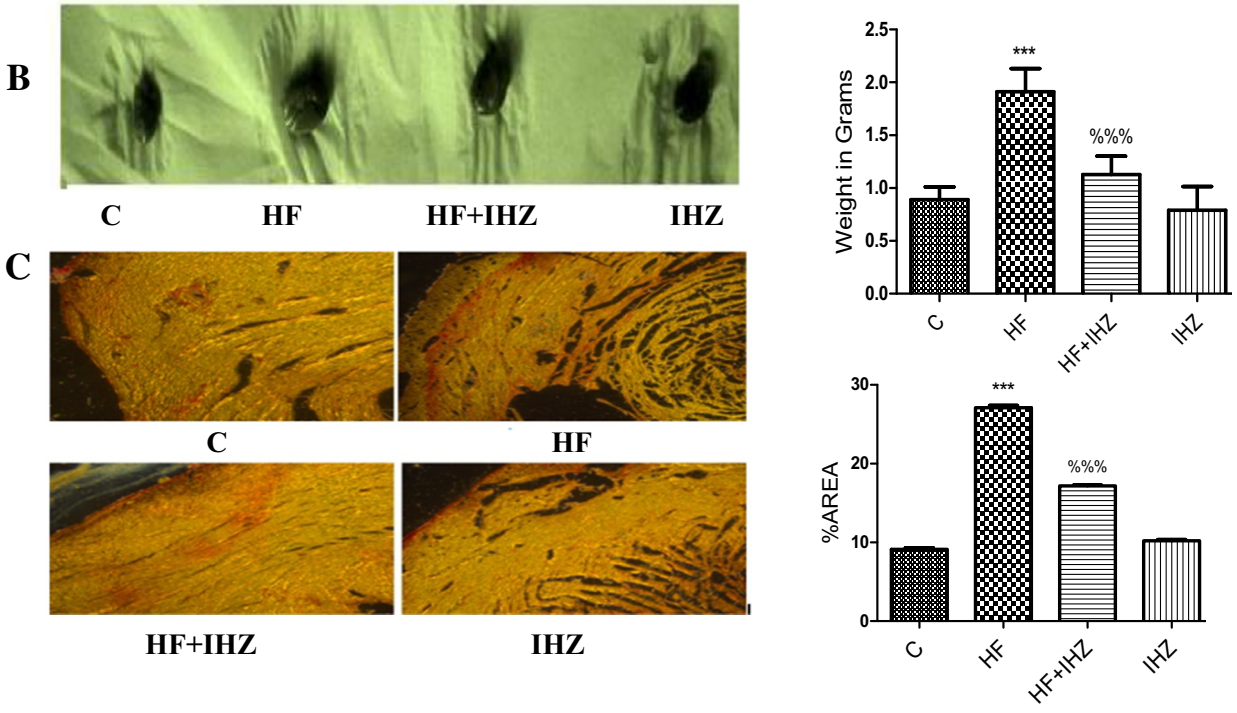
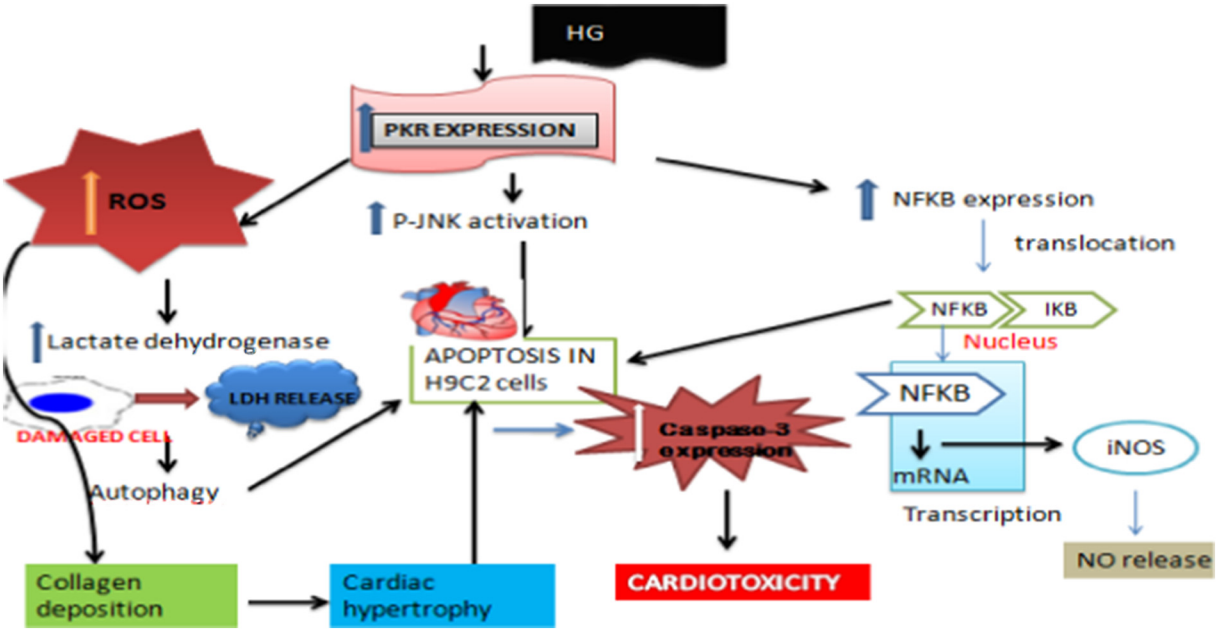


Fig. 5. IHZ attenuates HF induced biochemical changes, cardiac hypertrophy and collagen deposition in-vivo in Wistar rats.
After the experimental protocol all animals were sacrificed on same day and serum was collected and hearts were harvested. (A) Biochemical changes in lipid profile were assessed using commercially available kits. (B) Hearts were dehydrated and weighed and changes in heart were measured. (C) Paraffin embedded tissues were sectioned of 4–5 μm and sirius red staining was done to observe the collagen deposition. Positive staining was seen in red colour, n = 6 for each group. ***$p < 0.001$ vs. C, $^{\%\%}p < 0.01$, $^{\%\%\%}p < 0.001$ vs. HF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Conclusion



5.1. Potential mechanism of PKR activation and its link to cardiotoxicity

High glucose increases the protein level of PKR which stimulates the expression of p-JNK and NFKB, thus resulting in increased expression of iNOS and caspase-3 leading to apoptosis which in turn leads to cardio toxicity. On other hand increased production of reactive oxygen species causes autophagy and also collagen deposition in heart, this finally leads to cardiac hypertrophy and apoptosis.

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Conflict(s) of interest/disclosure(s) statement

None.

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