Inhibition of JNK phosphorylation by a novel curcumin analog prevents high glucose-induced inflammation and apoptosis in cardiomyocytes and the development of diabetic cardiomyopathy

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Abstract

Hyperglycemia-induced inflammation and apoptosis have important roles in the pathogenesis of diabetic cardiomyopathy. We recently found that a novel curcumin derivative, C66, is able to reduce high glucose (HG)-induced inflammatory response. This study was designed to investigate the protective effects on diabetic cardiomyopathy and its underlying mechanisms. Pretreatment with C66 significantly reduced HG-induced overexpression of inflammatory cytokines via inactivation of NF-κB in both H9c2 cells and neonatal cardiomyocytes. Furthermore, we showed that the inhibition of JNK phosphorylation contributed to the protection of C66 from inflammation and cell apoptosis, which was validated by the utilization of SP600125 and dn-JNK. The molecular docking and kinase activity assay confirmed C66’s direct binding to and inhibition of JNK. In mice with type 1 diabetes, administration of C66 or SP600125 at 5 mg/kg significantly decreased the plasma and cardiac TNF-α level, accompanied by decreasing cardiac apoptosis, and, finally, improved histological abnormalities, fibrosis, and cardiac dysfunction without affecting the hyperglycemia. Thus, this work demonstrated the therapeutic potential of the JNK-targeting compound C66 for the treatment of diabetic cardiomyopathy. Importantly, we indicated a critical role of JNK in diabetic heart injury, and suggested that JNK inhibition may be a feasible strategy for treating diabetic cardiomyopathy.

Keywords: inflammation; JNK; diabetic cardiomyopathy; apoptosis; NF-κB

Non-standard Abbreviations and Acronyms:
C66: (2E,6E)-2,6-bis(2-(trifluoromethyl)benzylidene)cyclohexanone;
HG: high glucose;
LG: low glucose;
SP: SP600125, a specific JNK inhibitor;
BAY: BAY-11-7085, a specific NF-κB inhibitor;
STZ: streptozotocin;
DM: diabetic mice;
dn-JNK: dominant negative JNK
Introduction

Diabetic cardiomyopathy is a leading cause of the increased morbidity and mortality in diabetic patients. Pathophysiology of diabetic cardiomyopathy includes microangiopathy, cardiac fibrosis, and disruption of the intracellular transport of Ca\(^{2+}\).(1-3) In addition, inflammation with increased cytokine levels in the heart was also found to have an important role in the pathogenic development of diabetic cardiomyopathy.(4; 5) Diabetic hyperglycemia is accompanied by increased expression of cytokines in local cardiac myocytes or immuno-competent cells recruited into the heart.(4) Overproduced pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-\(\alpha\) and interleukin (IL)-1\(\beta\), stimulate the expression of inflammatory mediators as a positive feedback mechanism and also stimulate cardiomyocyte apoptosis, which eventually leads to cardiac dysfunction.(5; 6)

Apoptosis of cardiomyocytes is one of important outcomes of hyperglycemia-induced inflammation and oxidative stress in the heart.(7) Increased cardiomyocyte apoptosis has been reported in diabetic animal models and patients as a predominant cause for the loss of contractile tissues, remodeling, and eventually dysfunction.(8-10) However, the mechanisms by which hyperglycemia induces apoptosis in cardiomyocytes are not fully understood. Generally, a few independent pathways may lead to cardiomyocyte apoptosis, including the extrinsic pathway initiated by ligands that bind to death receptors and the intrinsic pathway governed by the release of various pro-apoptotic proteins from the mitochondria.(11-13) Sustained inflammation may lead to the activation of multiple pathways that lead to the cell death (14; 15) Two groups have shown that TNF provokes cardiomyocyte apoptosis and cardiac remodeling through the activation of multiple cell-death pathways under diabetic conditions and pressure overload state, respectively.(16; 17) Intervention against TNF-\(\alpha\) using a specific antagonist was reported to protect against cardiac inflammation, apoptosis, and fibrosis in experimental diabetic cardiomyopathy.(18) This suggests that inhibition of inflammatory cytokines may be an effective strategy for the prevention of diabetes-induced pathogenic changes in the heart.

Compound (2\(E\),6\(E\))-2,6-bis(2-(trifluoromethyl)benzylidene) cyclohexanone (C66, Figure 1A) is a synthetic derivative of natural active curcumin.(19; 20) Our previous studies demonstrated that C66 could inhibit the expression of inflammatory cytokines in mouse macrophages stimulated by both high glucose (HG) and lipopolysaccharide.(19; 20) The present study was designed to determine whether C66 could prevent diabetes-induced cardiac damage that eventually induces cardiomyopathy.
Our data demonstrated that C66 treatment significantly ameliorated cardiac inflammation, apoptosis, fibrosis, and dysfunction associated with diabetic cardiomyopathy. The cardiac protection from diabetes, including anti-inflammation and anti-apoptosis, by C66 was found to be mediated most likely by its direct inhibition of c-Jun N-terminal kinase (JNK).

Methods

Reagents, cell culture and treatment

Glucose, mannitol, JNK inhibitor SP600125, NF-κB inhibitor BAY-11-7085, and fenofibrate were purchased from Sigma (St. Louis, MO, USA). Compound C66 was synthesized and purified (>98.4%) as described in our previous publication. C66 was dissolved in DMSO for in vitro experiments and in CMCNa (1%) for in vivo experiments. Antibodies for p-JNK/JNK, NF-κB p65, IκB, MCP-1, CD-68 and β-actin were purchased from Santa Cruz Technology (Santa Cruz, CA). Antibodies for caspase-3/9 were bought from Abcam (Cambridge, MA). A 3.3-kb cDNA fragment (dominant-negative type) encoding HA-tagged JNKK2 (KM)-JNK1 fusion protein, in which lysine 149 in the ATP domain of the JNKK2 moiety was replaced by methionine, and vector cDNA (control) were gifted from Prof. Aimin Xu (School of Medicine at the University of Hong Kong, Hong Kong). H9c2 embryonic rat heart-derived cell line was obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in DMEM medium (Gibco, Eggenstein, Germay) containing 5.5 mmol/L of D-glucose (low glucose, LG) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. In the high glucose-treated group (HG), cells were incubated with a DMEM medium containing 22 mmol/L of glucose.

Isolation and culture of neonatal cardiac cells

Primary cultures of neonatal rat cardiomyocytes were performed using a method published previously.(21) Cells were incubated with a DMEM medium that contained 5.5 mmol/L of D-glucose (low glucose, LG).

Animal studies

Protocols used for all animal studies were approved by the Wenzhou Medical College Animal Policy and Welfare Committee (Approved documents: 2009/APWC/0031). Male C57BL/6 mice,
weighing 18-22 g with 8 weeks of age, were obtained from Animal Center of Wenzhou Medical College (Wenzhou, China). Animals were housed in 22 °C with a 12:12 h light/dark cycle; water and mouse standard diet were consumed. To induce diabetes, mice were treated with a single intraperitoneal injection of streptozotocin (STZ; 150 mg/kg in citrate buffer, pH=4.5), while the control animals received the same volume of citrate buffer. The blood glucose level was monitored on day 3 and 7 from the STZ injection with a glucometer. Seven days after STZ injection, mice with fasting-blood glucose >12 mmol/L were considered diabetic. All mice had free access to food and water at all times. In the first set of experiment, 1-week diabetic mice were randomly divided into two groups (n=8): diabetic mice (DM) and C66-treated diabetic mice (DM+C66). In C66-treated DM group, mice were orally administrated with C66 at 5 mg/kg once every 2 days for 12-week. The DM group and age-matched control group (n=8) were received 1% CMCNa solution alone in the same schedule as the DM+C66 group. Body weight and blood glucose were recorded on day 7, 17, 27, 47, 57, and 67 after STZ induction. At 67th day after STZ induction, mice were killed under anaesthesia. In the second set of experiment, diabetic mice randomly divided into three groups (n=8): diabetic mice (DM), C66-treated diabetic mice (DM+C66) and SP600125-treated diabetic mice (DM+SP). Besides, another two groups were set as C66- and SP-treated control mice (Con+C66 and Con+SP). Then C66 or SP at 5 mg/kg were orally given once every 2 days for 12-week. The DM group and age-matched control group were received 1% CMCNa solution alone in the same schedule as the treated DM groups. Animals were sacrificed under sodium pentobarbital anaesthesia. After mice killed, heart tissues were embedded in 4% paraformaldehyde for pathological analysis and/or snap-frozen in liquid nitrogen for gene and protein expression analysis. In addition, the blood was collected from the right ventricle using a heparin-containing syringe with a needle at the time of death.

Another animal experiment was carried out to investigate the effect of fenofibrate on type 1 diabetic cardiomyopathy. After type 1 diabetic mouse models were established using the same method described above, diabetic mice were randomly divided into two groups (n=6): diabetic mice (DM) and fenofibrate-treated diabetic mice (DM+FE). In FE-treated DM group, mice were orally administrated with FE at 100 mg/kg every day for 12-week. The DM group and age-matched control group (n=6) were received 1% CMCNa solution alone in the same schedule as the DM+FE group. On 12-week endpoint, cardiac function was detected via echocardiography. Then mice were sacrificed, and heart tissues and blood were collected for related analysis.
Cardiac function measurements by echocardiography

To assess cardiac function, transthoracic echocardiograph (echo) was performed on mice using a Visual Sonics Vevo 770 high-resolution imaging system (Visual Sonics, Toronto, ON, Canada) and equipped with a RMV 707B High-Frame-Rate Scanhead (focal length 12.7 mm, frequency 30.0 MHz), as described before (22). The indices directly measured included left ventricle (LV) cavitory dimensions in diastole (LVID,d) and systole (LVID,s), LV posterior wall thickness in diastole and systole, and interventricle septum thickness in diastole and systole (Supplemental Table 1). LV fractional shortening (FS) %= \[(LVIDd − LVIDs)/LVIDd\] × 100; LV ejection fraction (EF) %= \[(LV \text{ end-diastolic volume} − LV \text{ end-systolic volume})/ LV \text{ end-diastolic volume}\] × 100.

Determination of TNF-α and IL-6

Levels of TNF-α and IL-6 in the cultured cell medium and mouse plasma were measured with specific ELISA kits (eBioscience Company, San Diego, CA) according to the manufacturer's instructions, as described in our previous publication.

Real-time quantitative PCR

Total RNA was isolated from cells and tissues (50-100 mg) using TRIZOL (Invitrogen, Carlsbad, CA). Reverse transcription and quantitative PCR (RT-qPCR) were performed using M-MLV Platinum RT-qPCR Kit (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was carried out using the Eppendorf Realplex instrument (Eppendorf, Hamburg, Germany). Primers of genes including TNF-α, IL-6, IL-1β, IL-12, TGF-β, atrial natriuretic peptide (ANP), and β-actin were synthesized from Invitrogen (Invitrogen, Shanghai, China). The primer sequences used were shown in Table S1. The relative amount of each gene was normalized to the amount of β-actin.

Western blotting

Cells and tissues lysate homogenates were prepared. Protein samples (30 – 80 µg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride membrane (Bio-Rad Laboratory, Hercules, CA). After blocked in blocking buffer (5% milk in tris-buffered saline containing 0.05% Tween 20) for 1.5 h at room temperature, membranes were incubated with different primary antibodies overnight at 4 °C. Then membranes were washed in TBS-T and reacted with secondary horseradish peroxidase-conjugated antibody (Santa Cruz, CA; 1:5000) for 1-2 h at room temperature. Antigen-antibody complexes were then visualized using enhanced chemiluminescence reagents (Bio-Rad, Hercules, CA). The density of the
immunoreactive bands was analyzed using Image J software (NIH, Bethesda, MD).

**Fluorescence immunocytochemistry for NF-κB p-65 translocation**

After deparaffinization and rehydration, 5-µm heart sections were treated with 3% H2O2 for 10 min and with 1% BSA in PBS for 30 min. The samples were incubated overnight at 4 °C with NF-κB p65 antibody (1:50) (Santa Cruz, CA) and then with fluorescent isothiocyanate-labeled secondary antibody (1:500) (Santa Cruz, CA) for 1 h at room temperature. The nuclei were stained with DAPI for 5 min. The NF-κB p65 protein and the nuclei were stained red and blue, respectively, and they were viewed with a fluorescence microscope (400×, Nikon, Tokyo, Japan).

**Transient transfection**

H9c2 cells were incubated for 6 h in 1 mL of serum-free medium containing 10 µL of Lipofectamine 2000 reagent (Invitrogen, City, CA) and 2.5 µg of dominant negative JNK (DN-JNK) or vector. After 24 h or 48 h of incubation in complete medium, the cells were treated with HG or TNF-α.

**Caspase-3/9 activity assay**

Caspase-3/9 activity was determined using a Caspase-3 activity kit (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer's protocol. The caspase-3 activity was normalized by the protein concentration of the corresponding cell lysate and was expressed in enzymatic units per mg of protein.

**TUNEL assay**

Tissue sections of 5 µm were used for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis detection kit (R&D Systems, Minneapolis, MN). Cells cultured on 6-well chamber slides were also performed for TUNEL assay using the same kit according to the manufacturer's instructions. TUNEL positive cells were imaged under a light microscope (400× amplification; Nikon Tokyo, Japan).

**Histopathology**

Hearts were fixed in 4% paraformaldehyde solution, embedded in paraffin, and sectioned at 5 µm. After dehydration, sections were stained with Hematoxylin and Eosin (H&E). To evaluate the histopathological damage, each image of sections was obtained using a light microscope (400× amplification; Nikon Tokyo, Japan).
**Sirius red staining**

Paraffin sections (5 µm) were stained with 0.1% Sirius Red F3B and 1.3% saturated aqueous solution of picric acid to evaluate the type IV collagen collection. The stained sections then were viewed by a Nikon fluorescence microscope (400× amplification; **Nikon Tokyo, Japan**).

**Measurement of triglyceride level**

Whole blood was collected from the anesthetized animals, and serum was prepared with the use of a serum separator apparatus (Becton Dickinson). Mouse hearts were homogenized in PBS. Tissue lipids were extracted with methanol: chloroform (1:2), dried in an evaporating centrifuge and resuspended in 5% fat-free BSA. The triglyceride levels in serum and heart tissue homogenates were measured following the instructions provided in the corresponding Sigma Diagnostics Triglyceride Kits (Sigma Chemical Co, St. Louis, MO). Values were normalized to protein in homogenate before extraction determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). **Molecular modeling**

Docking simulation was conducted using Sybyl-x.v1.1.083 software (**Tripos, St. Louis, MO**). The crystal structure of JNK1/2 was cited from the Protein Data Bank (JNK1:1UKI; JNK2:3NPC). The ligand-binding groove on JNK1/2 was kept rigid, whereas all torsible bonds of the ligands were freed to perform flexible docking to produce more than 100 structures. The final, docked conformations were clustered within the tolerance of 1Å root-mean-square deviation.

**Kinase activity inhibition assays**

The JNK1/2 kinase activities were detected by Caliper Mobility Shift Assay on EZ Reader (**Caliper Life Sciences, MA**) according to the instruction. The recombinant kinases JNK1/2 were purchased from Carna. The ATP concentration was set at the Km value of JNK2: 16 µM. For the IC50 determination, compounds were tested in duplicate at 10 concentrations from 5 nM to 100 µM. Specific details of each assay are available in the supporting information. In electrophoretic mobility shift assays, product accumulation was expressed as percentage conversion, product peak height / (product peak height + substrate peak height).

**Statistical analysis**

Data were presented as means ± SDs. The statistical significance of differences between groups was obtained by the student’s t test or ANOVA multiple comparisons in GraphPad Pro (**GraphPad, San Diego, CA**). Differences were considered to be significant at P < 0.05.

**Results**
Treatment with C66 inhibited HG-increased expression of cytokines in H9c2 and neonatal cardiomyocytes

To investigate the role of C66 in preventing HG-induced inflammation in cardiac cells, H9c2 cells were treated with C66 (2.5, 5, or 10 µmol/L) or vehicle (DMSO, 0.1% v/v) for 2 h and then incubated with either LG (5.5 mmol/L) or HG (22 mmol/L) for 22 h. As shown in Figures 1B and C, HG treatment significantly increased TNF-α expression in both protein and mRNA levels, which were remarkably inhibited by pre-treatment with C66. The production of TNF-α was not affected by treatment with mannitol, indicating that the osmotic effect did not induce inflammation (Figure 1B). C66 also decreased HG-induced IL-12 (Figure 1D), IL-1β (Figure 1E), and IL-6 (Figure 1F) mRNA expression in a dose-dependent manner. Similar results, indicating that pretreatment with C66 significantly decreased HG-induced protein expression of TNF-α and IL-6 in H9c2 cells, also were observed in neonatal cardiomyocytes (Figures 1G and 1H).

C66 treatment suppressed HG-induced NF-κB activation, apoptosis, and hypertrophy in vitro

The transcriptional factor NF-κB has been well appreciated as a main controller of transcription and expression of several inflammatory cytokines.(23) Thus, we examined the effect of C66 on the NF-κB signaling pathway. Since IκBα has a key role in mediating the activation and nuclear translocation of NF-κB, first, we examined the effect of C66 on IκBα degradation in HG-exposed H9c2 cells and neonatal cardiomyocytes. HG treatment for 2 h increased IκBα degradation, and treatment of HG-exposed cardiac cells with C66 markedly reversed HG-induced IκBα degradation in a dose-dependent manner (Figure 2A). Interestingly, similar results also were observed in the cells exposed to HG for 24 h (Figure 2B), indicating the persistence of HG pro-inflammatory and C66 anti-inflammatory effects. These changes (Figures 2A, B) were also observed in primary cardiomyocytes (Figures S1A, B). Degradation of IκBα leads to NF-κB p65 translocation from cytosol to nuclei for triggering its target gene transcription.(23) Therefore, we analyzed the amount of p65 protein in both nucleus and cytoplasm by western blot analysis. HG significantly accelerated the nuclear translocation of NF-κB p65, which was abolished by C66, depending on the dosage (Figure 2C). These data suggest that C66 significantly suppressed the HG-induced activation of NF-κB. The fact that NF-κB specific inhibitor BAY-11-7085 (Bay at 10 µM) significantly reduced HG-increased TNF-α expression (Figure 1B) further suggested that the inhibitory effect of C66 on
HG-stimulated TNF-α production was most likely via the inactivation of NF-κB.

We further examined the protective effect of C66 on HG-induced cardiac apoptosis. Figures 3A and 3B show that C66 treatment, depending on the dosage, could reduce HG-increased cleavage of caspase-3 and caspase-9. Treatment with C66 also reduced HG-increased caspase-3 activity, measured by a caspase-3 activity assay kit (Figure 3C). Similar results also were observed in the primary cardiac cells exposed to HG (Figures S1C and S1D). Furthermore, HG treatment for 72 h induced a significant increase in cardiac cell apoptosis, shown by increased TUNEL-positive cells (indicated by the white arrow) (Figure 3D) and DAPI-positive nuclei (Figure 3E), which was attenuated significantly by treatment with C66 (Figure 3F). Regarding the hypertrophy, ANP, a peptide released by cardiac myocytes to regulate blood pressure and natriuresis, is generally considered as a hallmark of cardiomyocyte hypertrophy.(24) We showed that HG increased the ANP mRNA transcription, which was significantly reduced by C66 pretreatment (Figure S2A). Subsequently, fluorescence microscopic study showed that C66 markedly reduced the HG-induced hypertrophy in primary neonatal cardiomyocytes (Figure S2B).

**JNK inactivation has a critical role in C66’s prevention of HG-induced cardiac inflammation and apoptosis**

JNK reportedly is a key transcriptional regulator of inflammatory cytokines, and it also has an important role in regulating the activity of NF-κB in various types of cell lines.(25) First, we examined the possible involvement of JNK in C66’s inhibition of HG-induced NF-κB activation and inflammation in cardiac cells. Figures 1B-H showed that JNK specific inhibitor SP600125 (SP at 10 µM) could significantly decrease HG-induced inflammatory cytokine expression in either H9c2 cell line (Figure 1B-1F) or primary cardiomyocytes (Figure 1G, 1H). Treatment with SP600125 also inactivated NF-κB in terms of both IκBα degradation (Figures 2A, 2B, p < 0.05) and p65 translocation (Figure 2C, p < 0.05). Similar results were observed in primary cardiomyocytes (Figures S1A, B).

In addition to inflammatory stress, JNK signaling also is involved in the extrinsic apoptotic pathway initiated by death receptors, including the TNF receptor.(26) Here, we also showed that the inhibition of JNK with its inhibitor SP600125 significantly inhibited HG-induced caspase-3 and caspase-9 activation in either H9c2 cells (Figures 3A, 3B) or primary cardiomyocytes (Figures S1C,
D), which further resulted in a prevention of apoptotic cell death based on examination by TUNEL and DAPI-stained nuclear fragmentation (Figures 3D-F).

To exclude possible non-specific inhibition by the pharmacological inhibitor, H9c2 cells also were transfected with a dn-JNK-containing plasmid and then exposed to HG for different times. We demonstrated that dn-JNK could significantly block the HG-induced phosphorylation of JNK (Figure 4A) and the degradation of IκB (Figure 4B), further confirming the function of JNK in regulating the activation of NF-κB. The mediating role of JNK in HG-induced apoptosis was further confirmed by no activation of caspase-3 after blocking JNK phosphorylation by dn-JNK transfection in HG-stimulated H9c2 cells (Figure 4C). These data further suggest that JNK, as an upstream regulator of NF-κB and caspase-3, can up-regulate, and C66 may inhibit the activation of JNK to prevent HG-induced inflammation and apoptosis.

**C66 may target JNK kinase and directly inhibit its phosphorylation**

To define the direct effect of C66 on HG-induced JNK activation, H9c2 cells were incubated with C66 or vehicle (DMSO, 3 µL) for 2 h and then treated with HG for 10 min. Western blot analysis revealed that JNK phosphorylation was inhibited significantly by C66 treatment, an effect comparable to that of JNK inhibitor SP600125, implying the possibility that the potent, anti-inflammatory effect of C66 is mediated by JNK inactivation (Figure 4D). A similar result was observed in mouse primary cardiomyocytes (Figure S3A).

Based on the above evidence, we proposed a docking model of C66 to the crystal structure of JNK (Figure S3B). Comparison of C66 with SP600125 showed that the C66-docking sites in JNK overlapped with the SP600125-binding pockets. C66 is located in the hydrophobic region of the JNK1 receptor-binding pocket and interacts with the hydrophobic residues Ieu-168, Ile-52, Val-40, and Ile-32. In another model, C66 was buried inside the JNK2 pocket and achieved three hydrogen bonds with Arg72 at the opening rims of the pocket. In addition, C66 also interacts with the hydrophobic residues in this JNK2 pocket in the most energetically-favorable simulation. These data suggested a possible kinase target (JNK1/2) for C66 biological effects.

Furthermore, we tested the kinase-inhibitory effect of C66 using recombined JNK1/2 kinase by caliper mobility shift assay. As shown in Figure S3C, C66 dose-dependently inhibited the kinase activity of both JNK1 and JNK2, with the IC$_{50}$ of 81.9 and 2.72 µM on JNK1 and JNK2, respectively.
We also tested the inhibitory effects of C66 on ERK and p38 kinase, while the IC₅₀s on both kinase are over 100 µM, indicating a high selectivity of C66 on kinase inhibition. The data from the cell-free kinase activity assay extraordinarily matched the above *in silico* docking results. C66 has a much higher binding affinity on JNK2 than JNK1. These data confirmed the specific inhibition of C66 on JNK and indicated that C66 exerts anti-inflammatory and anti-apoptosis effects, possibly through its direct inhibition on JNK and especially on JNK2.

**C66 also inhibited TNF-α-induced cell apoptosis by JNK inactivation**

The available evidence indicates that TNF-α can activate JNK/stress-activated protein kinases (SAPK) to promote apoptosis in cardiomyocytes and endothelial cells.(27; 28) Incubation of TNF-α for 24 h increased the cleavage of caspase-3, which also was markedly prevented by JNK inactivation in dn-JNK-transfected cells (Figure 5A), confirming that JNK activation is directly involved in mediating TNF-α-induced cardiac cell apoptosis. Due to the direct binding of C66 with JNK, we hypothesized that C66 also prevents TNF-α-induced JNK phosphorylation and the subsequent activation of caspase-3 in H9c2 cells. Western blot analysis indicated that C66 treatment dose-dependently inhibited TNF-α-induced JNK phosphorylation at 10 min after treatment (Figure 5B). Furthermore, treatment with TNF-α for 24 h dose-dependently induced caspase-3 activation, which was significantly reduced by treatment with C66 at a concentration of 10 µmol/L (Figure 5C). Western blotting of the cleavage of caspase-3 confirmed the pro-apoptotic effect of TNF-α, which was prevented by treatment with either C66 or SP600125 (Figure 5D). It also was observed that C66 inhibited JNK phosphorylation at 24 h after HG treatment in H9C2 cells (Figures 5E) and primary cardiomyocytes (Figure S3D) when a majority of the glucose had been consumed and the TNF-α protein had been increased in the culture medium (Figure 1B). These data suggested that inhibition of JNK by C66 also prevented TNF-α-mediated cardiac cell apoptosis.

**C66 treatment did not affect the profile of blood glucose in diabetic mice, but significantly prevented diabetes-induced pathogenic changes and dysfunction in the heart**

To validate the beneficial effect of C66 *in vivo*, an experimental type 1 diabetic model was established and used for C66 anti-inflammatory and cardioprotective studies. As expected, dynamic measurements of blood glucose showed that C66 treatment for 2 months did not affect the
hyperglycemic profile in diabetic mice (Figure S4A). Diabetes reduced the body weight and heart weight in untreated and C66-treated diabetic group (Figure S4B and C). However, C66 treatment significantly reduced the increased ratio of the heart’s weight to the body’s weight that is caused by diabetes, and this ratio is an important index of cardiac hypertrophy and remodeling.

**Attenuation of the diabetes-elicited inflammatory response and hyperlipidemia in diabetic mice by C66 treatment:** As shown in Figures 6A-C, administration of C66 at 5 mg/kg for 2 months significantly reduced diabetic increases of serum TNF-α level and also cardiac TNF-α and IL-6 mRNA expressions, indicating that C66 has an inhibitory effect on the inflammation in diabetic mice. We also detected MCP-1 and CD68, two markers for macrophage infiltration. (29) Figure S5 shows that diabetic hearts have marked increases in MCP-1 and CD68 expression, while the administration of C66 or SP600125 resulted in significant reductions. The data in Figures 6D-F validated the inactivation of NF-κB by C66 treatment *in vivo*. For the hyperglycemic condition, NF-κB p65 (green points) was shifted to the nuclei in the diabetic mice’s hearts, while C66 administration markedly reduced this translocation (Figures 6D-E). C66 treatment also significantly reversed diabetes-induced degradation of cardiac IκBα (Figure 6F-G).

Besides, it was observed that C66 treatment decreased diabetes-induced hyperlipidemia in plasma and myocardial tissues (Figure S6A, B). To demonstrate the possible role of hypertriglyceridemia in diabetic heart injury and C66’s protection, we investigated the heart-protective effects of an anti-hyperglycemia drug, fenofibrate (FE), the same diabetic models. Seven days after STZ injection, diabetic mice were randomly divided into two groups (n=6): diabetic mice (DM) and FE-treated diabetic mice (DM+FE). Then FE at 100 mg/kg/day was orally given every day for 12 weeks. As shown in Figure S6C-F, FE treatment effectively reduced diabetes-induced increase in the levels of plasma triglyceride and cholesterol and myocardial triglyceride but did not affect the blood glucose level (Figure S6C). FE treatment also inhibited diabetes-induced JNK phosphorylation (Figure S6G) and cardiac TNF-α and IL-6 expression (Figure S6H-I), indicating that hyperlipidemia may contribute to JNK signaling activation and cardiac inflammation. Cardiac ANP overexpression in diabetic mice was also significantly attenuated by FE treatment (Figure S6J). Furthermore, we detected both the diastolic function (LVID and LV volume) and the systolic function (EF%, and FS%) of the heart by transthoracic echocardiograph (Figure S6K-N). These results indicated that the decrease in hyperlipidemia could improve the cardiac
function in type 1 diabetic mouse model, accompanied with inhibiting JNK signaling and cardiac inflammation.

**C66 treatment prevented diabetes-induced cardiac cell death and remodeling:** In the next study, we examined the anti-apoptotic effect of C66 in the diabetic hearts. In the diabetic hearts, there was a marked increase in caspase-3 cleavage (Figure 7A), which was further confirmed by the TUNEL-positive cells (Figures 7B, 7C, S7A, and S7B). However, treatment of diabetic mice with C66 or SP600125 significantly prevented the diabetic activation of caspase-3 and induction of the TUNEL positive cells. Further, we examined the fibrosis and histopathology in diabetic hearts. Sirius red staining revealed a marked collagen accumulation in the heart of diabetic mice, while C66 treatment significantly reduced the degree of collagen deposition (p < 0.05, Figures 7D and 7E). Real-time q-PCR analysis revealed significant increases in the pro-fibrotic gene TGF-β expression (Figure 7F) and hypertrophic marker ANP expression (Figure 7G) in the diabetic hearts, but these changes were significantly blocked by the administration of C66. To further support the preventive effect of C66 on cardiac remodeling, H&E staining also showed that diabetic hearts displayed structural abnormalities, including broken fibers, deranged cellular structures, the existence of foci with necrotic myocytes, and the infiltration of inflammatory cells. There was no significant evidence of these abnormalities in the hearts of diabetic mice that had been treated with C66 or SP600125 (Figures 7H and S7C).

**C66 treatment attenuated diabetes-induced alterations of cardiac function:** The preventive effect of C66 on cardiac pathological changes in diabetic mice may result in an improvement of diabetes-induced cardiac dysfunction; therefore, the protection from diabetes-induced heart dysfunctions in diabetic mice due to the administration of C66 was examined at 13 weeks after the onset of diabetes. Diabetes significantly impaired the functioning of the diastolic and systolic left ventricle, which was largely attenuated by the treatment with C66 for 12 weeks (starting 1 week after the onset of diabetes, Table 1). Treatment with SP600125 also showed a similar improvement of cardiac function in diabetic mice (Table S2).

**Discussion**

It is intriguing to speculate that a reduction of cardiac cytokines by anti-inflammatory therapy might lead to preventing and/or treating heart injuries caused by diabetes. Several anti-inflammatory
compounds, such as tanshinone IIA (30), cannabidiol (31), caffeic acid (5), and atorvastatin (32), were demonstrated to be beneficial for the treatment of diabetic cardiomyopathy. However, in spite of the knowledge that has been acquired during past decades, the treatment of diabetic cardiomyopathy still remains ineffective, and many efforts have been directed at the development of new anti-inflammatory drugs.

Among these efforts, identifying novel agents from natural products has attracted significant attention recently. Several of our previous studies have focused on the development of new anti-inflammatory derivatives or analogs of curcumin, the main active component of *Curcuma* and *Ginger*, which has shown multiple pharmacological activities. (19; 33) C66, a synthetic curcumin analog developed in our laboratory, has been shown to exert anti-inflammatory effects both *in vitro* and *in vivo*. (20) Furthermore, C66 has recently been reported to reduce HG-induced inflammatory cytokines in macrophages and to attenuate diabetic renal injury in rats. (19) In the present study, we identified the preventive effect and mechanism of C66 on cardiac inflammation, apoptosis, and fibrosis *in vitro* and *in vivo* by using HG-exposed cardiac cells and a type 1 diabetic mouse model.

Consistent with previous reports, exposure of cells to HG led to an increase in the expression of inflammatory cytokines, which is subsequently accompanied with cardiac apoptosis followed by cardiac hypertrophic and fibrotic responses. (8; 9) Pharmacologically, the present study observed that C66 treatment effectively suppressed the expression of inflammatory cytokines in both HG-incubated cardiac cells (Figures 1B-1H and S1A, B) and diabetic hearts (Figures 6A-6C and S5), which was accompanied with the subsequent decrease in apoptotic cell death (Figures 3A-3E; 7A-7C; S1C, D; and S7A, B), hypertrophy (Figures 7G), fibrosis (Figures 7D-7F), pathological changes (Figures 7H and S7C), and cardiac dysfunction (Tables 1 and S2). Similar to previous reports (9), the present study observed serum and cardiac lipid (triglyceride) accumulation in the diabetic mice, while treatment with C66 and SP600125 for 12 weeks effectively decreased the triglyceride deposit in the serum and cardiac tissues of the diabetic mice (Figure S6A, B). We also observed that C66 did not affect the blood glucose profile in diabetic mice (Figure S4A). Collectively, our results strongly support the conclusion that C66 may have great potential for use in treating cardiac injuries caused by diabetes via anti-inflammatory and anti-apoptotic actions.

Although several anti-inflammatory medications have been reported to have important roles in the prevention of diabetic cardiomyopathy, the molecular mechanism and targets are still
controversial. In this study, we focused on the mechanism responsible for the beneficial effects of C66. It has been demonstrated that HG induced inflammatory gene expression via activating several transcription factors, such as NF-κB, AP-1 and STAT. The body of evidence suggests that the activation of NF-κB is an important step for cardiac injuries. Compounds that inactivate NF-κB may become an effective and powerful approach for the prevention of cardiac apoptosis in diabetic patients. Curcumin has been identified as an NF-κB inhibitor. Recently we have proven that C66 inhibited NF-κB activation in HG-treated macrophages. The specific NF-κB inhibitor, BAY, significantly suppressed HG-induced TNF-α expression (Figure 1B). Therefore, we hypothesized that C66 might also inhibit HG/hyperglycemia-induced inflammation in the cultured cardiac cells in vitro and in the hearts of diabetic mice via inactivation of NF-κB. Both in vitro (Figures 2 and S1) and in vivo (Figures 6D-F) data further indicated that C66’s anti-inflammatory effect is associated with its inhibitory effect on HG-induced and diabetes-induced NF-κB activation by blocking IκB degradation and p65 nuclear translocation.

To further explore mechanistically the effect of C66 on NF-κB activation, a molecular docking between C66 and the IκB-regulating kinase IKKβ showed that C66 did not bind to IKKβ directly (data not shown). Thus, we investigated the possible NF-κB-upstream target of C66. JNK belongs to the family of MAP-kinases involved in the regulation of cell proliferation, stress, and apoptosis. Recently, it also has been reported that JNK is activated in response to inflammatory and stressful stimuli, including HG or a diabetic situation. Reports have shown that JNK activation may up-regulate NF-κB by phosphorylation of IKK-β in lipopolysaccharide-stimulated macrophages. In the present study, both JNK inhibition by specific inhibitors, i.e., SP600125 and dn-JNK, significantly attenuated the HG-induced expression of inflammatory cytokines (Figures 1B-1H) and NF-κB activation (Figures 2, 4A, and S1), indicating that JNK is a critical upstream molecule of NF-κB and has an important role in HG-induced inflammation. As far as we know, this is the first report to find that JNK independently regulates NF-κB and inflammation in cardiac cells exposed to HG. C66 also showed a comparable (even better) inhibition with the specific inhibitor SP600125 against HG-induced JNK phosphorylation (Figures 4D, S2A, and S2D), indicating that JNK may be an intracellular target of C66. This assumption was validated further by computer-assisted molecular docking analysis and cell-free kinase activity assay. The actual binding and direct inhibition of C66
towards JNK1/2 was confirmed by using cell-free enzymological experiments, which indicated a direct molecular target (JNK, especially JNK2) for the biological effects of C66 (Figure S3B, C) and supported the critical role of JNK in regulating HG-induced inflammation. We also showed that the oral administration of the specific JNK inhibitor, SP600125, could improve cardiac structural disorders and dysfunction (Figure S7 and Table S2).

In addition to inflammatory response, JNK has an important role in the apoptotic pathway initiated by death receptors, such as TNF-α, TRAIL, and FAS-L.(27; 28) Increasing evidence has shown that JNK activation is required for cardiac cell apoptosis and diabetic cardiomyopathy.(27; 30) Consistent with previous reports, our results using the pharmacological inhibitor SP600125 (Figure 3) and dn-JNK (Figure 5A) supported the conclusion that JNK mediated HG-induced caspase-3 activation and intrinsic apoptotic pathways. TNF-α, which is increased by HG induction, also induces cardiac apoptosis through its type 1 receptor (TNFR1) complex II, a classic death receptor.(18) The binding between TNF-α and TNFR1 complex II results in the activation of the JNK-dependent caspase cascade and then commits cells to apoptosis.(40) Reportedly, under HG exposure, TNF-α-mediated JNK activation led to triggering caspase-3 cleavage and, consequently, apoptosis in endothelial cells.(28) In the present study, treatment with either dn-JNK (Figure 5A) or SP600125 (Figures 5D, E) inhibited TNF-α-induced caspase-3 activation. Further, we assessed whether C66, as a potential JNK inhibitor, could decrease TNF-α-induced JNK phosphorylation and caspase activation. Figures 5B-E show that C66 also blocked the apoptotic pathway directly induced by TNF-α via targeting JNK. C66 also inhibited JNK phosphorylation at 24 h after HG treatment (Figures 5E and S3D) when JNK phosphorylation was induced mainly by a series of inflammatory cytokines secreted in the culture medium. Taken together, these data strongly support the concept that the inhibition of JNK may be a critical and effective step in the mediation of cardiac inflammation and subsequent apoptosis induced by HG.

Regarding to the effect of C66 on hypertriglyceridemia, C66 treatment could decrease diabetes-induced hypertriglyceridemia in mouse serum and hearts (Figure S6A-B). It is unclear whether this compound is protecting the heart against hyperglycemia-induced damage or hypertriglyceridemia-induced damage or both. We utilized fenofibrate to investigate the role of lipotoxicity in type 1 diabetic cardiomyopathy. As shown in Figure S6C-N, the decrease in hyperlipidemia could improve the cardiac function accompanied with inhibiting JNK signaling and cardiac inflammation in type 1 diabetic mouse model. Thus, we may conclude that C66 protects the
diabetic hearts against both hyperglycemia-induced damages via targeting JNK and hyperlipidemia-induced damages via directly reducing lipid concentration. A schematic signaling pathway that was possibly involved in hyperglycemia- and hyperlipidemia-induced cardiomyopathy and C66’s biological effects is illustrated in Figure S8A. Further study should be performed to demonstrate the new questions “how dose C66 attenuate the diabetes-induced hyperlipidemia and whether C66 can prevent hyperlipidemia-induced cardiac damages via JNK inhibition”.

The novel curcumin derivative, C66, was able to reduce the HG-induced inflammatory response and cell apoptosis via direct inhibition of JNK kinase activity. The attenuation of the inflammatory process by C66 also contributed to the protection of the heart from diabetes in a mouse model. This work also suggests that there is significant potential for using the novel compound, C66, for the treatment of diabetic cardiomyopathy via its anti-inflammatory mechanism. More importantly, utilizing C66 and pharmacologically-specific inhibitors, we demonstrated that the exposure of cardiac cells to HG activated JNK-mediated NF-κB pathway and up-regulated the expression of inflammatory cytokines, including TNF-α which then triggers the JNK-dependent caspase activation and cell apoptosis (Figure S8B). Thus, as an important therapeutic target, JNK regulates the inflammatory transcription and mediates the apoptotic effect of the increased TNF-α in the cardiac cells exposed to HG (Figure S8B). In summary, these results provide a deeper understanding of the regulatory role of JNK in diabetic heart inflammation and apoptosis, indicating that JNK inhibition may be a feasible strategy for treating diabetic heart injury and cardiomyopathy.

Author Contributions


Acknowledgements

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Medical University (to X. L. and L.C.). We thank Prof. Aimin Xu in Departments of Medicine at the University of Hong Kong for providing us the cDNA fragment encoding HA-tagged JNKK2 (KM)-JNK1 fusion protein. Professor Guang Liang is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Conflict of interest statement**

All the authors declare no competing financial interest.

**References**

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**Figure legends**

**Table 1.** Echocardiographic parameters of type 1 diabetic mice for 12 weeks.

Notes: n = 8 per group; * p < 0.05 vs. DM group.
Figure 1. C66 reduced inflammatory cytokines production in HG-induced H9c2 and neonatal cardiac cells. A. Chemical structure of C66. B. H9c2 cells (1×10^6/plate) were pre-treated with C66 at indicated concentrations (2.5, 5, or 10 µmol/L), SP600125 (SP, 10 µmol/L), BAY-11-7085 (Bay, 10 µmol/L), or vehicle (DMSO) for 2 h and then incubated with high glucose (HG, 22 mmol/L) or mannitol (16.5 mmol/L) for 22 h. The levels of TNF-α in culture medium were detected as described in Methods and Materials. C-F. H9c2 cells were pre-treated with C66 (2.5, 5, or 10 µmol/L), SP (10 µmol/L) or DMSO for 2 h, followed by the incubation with 22 mmol/L HG for 22 h. The mRNA levels of TNF-α (C), IL-12 (D), IL-1β (E) and IL-6 (F) were examined by RT-qPCR and normalized by β-actin. G and H. Primary cardiac cells were isolated and pre-treated with C66 (10 µmol/L), SP (10 µmol/L) or DMSO for 2 h, followed by the incubation with 22 mmol/L HG for 22 h. The levels of TNF-α and IL-6 in culture medium were detected by ELISA method. Bars represent the mean ± SD of four independent experiments run in triplicate (* p<0.05, **p<0.01, v.s. HG group).

Figure 2. C66 inhibited NF-κB activation in HG-induced H9c2 cells. Cells were pre-treated with C66 (2.5, 5 or 10 µmol/L), SP (10 µmol/L), or BAY (10 µmol/L) for 2 h and then incubated with HG (22 mmol/L) for indicated times. A and B. The cell lysates were collected and IκBα protein was detected by western blot analysis using Actin as a loading control. The column figures show the normalized optical density from the data more than 3 independent experiments. C. The nuclear and cytosol proteins were isolated and p65 protein was detected by western blot analysis using Lamin B and actin as loading controls, respectively. A column figure for nuclear p65/ cytosol p65 shows the normalized optical density from the data more than 4 independent experiments as percentage of HG group. (* p<0.05, **p<0.01, v.s. HG group).

Figure 3. C66 treatment attenuated apoptosis in H9c2 cells exposed to HG. A and B. Cells were pre-treated with C66 (2.5, 5 or 10 µmol/L) or SP (10 µmol/L) for 2h, followed by incubation with HG (22 mmol/L) for 48 h. The levels of cleavaged caspase-3 (A) and caspase-9 (B) in total lysates were determined by western blot analysis. The column figures show the normalized optical density from the data more than 3 independent experiments. C-E. Cells were pre-treated with C66 (2.5, 5 or 10 µmol/L) for 2h, followed by incubation with HG (22 mmol/L) for 72 h. Cell lysates were
collected and subjected to caspase-3 enzymatic assay (C). The apoptotic cells were detected using TUNEL staining (D) and DAPI staining (E) as described in Materials and methods. Similar results were observed in 3 independent experiments. F. A column figure shows the semi-quantitative analysis for TUNEL staining. (*p<0.05, **p<0.01, v.s. HG group).

Figure 4. JNK may be an important target of C66. A. The dn-JNK blocked HG-induced IκB degradation and caspase 3 activation. H9c2 cells were transferred with dn-JNK plasmid or vector for 48 h before the incubation with HG for different time (10 min for p-JNK, 2 h for IκB, and 24 h for caspase-3). Total protein was extracted and then subjected to western blot analysis. B. Cells were pre-treated with C66 (2.5, 5 or 10 µmol/L) or SP (10 µmol/L) for 2 h and then incubated with HG (22 mmol/L) for 10 min. Total protein was extracted and then subjected to western blot analysis. (*p<0.05, **p<0.01, v.s. HG group).

Figure 5. C66 inhibited TNF-α-induced JNK phosphorylation and apoptosis in H9c2 cells. A. dn-JNK blocked caspase-3 activation in H9c2 cells exposed to HG. H9c2 cells were transferred with dn-JNK plasmid or vector for 24 h before the incubation with TNF-α (1 µg/mL) for 48 h. Total protein was extracted and then subjected to western blot analysis. B. Cells were pre-treated with C66 (2.5, 5 or 10 µmol/L) for 2 h and then incubated with TNF-α (1 µg/mL) for 24 h. Total protein was extracted and then subjected to western blot analysis. C. Cells were pre-treated with C66 (10 µmol/L) for 2 h, followed by incubation with TNF-α (0.25, 0.5 or 1 µg/mL) for 72 h. Cell lysates were collected and subjected to caspase-3 enzymatic assay. D. Cells were pre-treated with C66 (10 µmol/L) or SP (10 µmol/L) for 2 h, followed by incubation with TNF-α (1 µg/mL) for 48 h. Cleaved caspase-3 was detected by western blot. The column figures show the normalized optical density from the data more than 3 independent experiments. E. Cells were pre-treated with C66 (2.5, 5 or 10 µmol/L) or SP (10 µmol/L) for 2 h and then incubated with HG (22 mmol/L) for 24 h. P-JNK/JNK were detected by western blot. The column figures show the normalized optical density from the data more than 3 independent experiments. (*p<0.05, **p<0.01, ***p<0.001, v.s. HG group).
**Figure 6.** C66 suppressed TNF-α expression and NF-κB activation in diabetic mice. A. Effect of C66 on serum TNF-α expression at 2-month after C66 administration (n=8). B-C. C66 administration reduced TNF-α and IL-6 gene expression in diabetic hearts. Heart tissues from each group were individually processed for RNA extraction and RT-qPCR. The mRNA levels of TNF-α (B) and IL-6 (C) were normalized by β-actin (n=5-8). D. Heart tissues (5 µm section) from each group (n=4) were processed for p65 immunocytochemistry as described in Materials and methods. E. A quantitative analysis of relative amount of nuclei p65 in heart tissues (compared with control group). F and G. Cardiac proteins were extracted from heart tissues of each group and were subjected to western blot analysis for IkBα determination (n=3). (*p<0.05, **p<0.01, ***p<0.001, v.s. DM group).

**Figure 7.** C66 administration improved cell apoptosis, histological abnormalities and fibrosis in diabetic heart. A. Total proteins extracted from heart tissues were subjected to western blot analysis for the determination of cleaved caspase-3. B. Heart tissues were sectioned at 5 µm and the slides were processed for TUNEL assay to detect apoptotic cells (n=3 in each group). C. The column figures show the relative TUNEL positive cell number (n=3, *p<0.05, v.s. DM group). D. Heart tissues were sectioned at 5 µm, and the slides were processed for Sirius red staining to detect type IV collagen. E. A quantitative analysis of relative amount of collagen IV in D. F-G. Heart tissues from mice were individually processed for RNA extraction and RT-qPCR. The mRNA levels of TGF-β (F) and ANP (G) were determined (n=5 per group; **p<0.01, v.s. DM group). H. A representative H&E staining for heart tissues out of six mice studied in each group is shown. All images were obtained by microscope with 400× amplification.
Table 1. Echocardiographic parameters of type 1 diabetic mice for 12 weeks.

Notes: n = 8 per group; * p < 0.05 vs. DM group.

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<th>Parameters</th>
<th>Con</th>
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<th>DM</th>
<th>DM+C66</th>
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Figure 1

A

C66: (2E,6E)-2,6-bis(2-(trifluoromethyl)benzylidene)cyclohexanone

B

TNF-α protein

C

TNF-α mRNA

D

IL-12 mRNA

E

IL-6 mRNA

F

IL-1β mRNA

G

TNF-α in primary cardiomyocytes

H

IL-6 in primary cardiomyocytes
Figure 2

A

IkBα

Actin

2 h

HG incubating for 2 h

B

IkBα

Actin

24 h

HG incubating for 24 h

C

Nuclear p65

Lamin B

Cytosol p65

GAPDH

LG  DMSO  SP  2.5  5.0  10

C66 (uM)

HG incubating for 4 h

nuclear p65 / cytosol p65

HG incubating for 4 h
Figure 3

A. Cleaved caspase-3/Actin

B. Cleaved caspase-9/Actin

C. Caspase-3 activity

D. Tunel staining

E. DAPI staining
Figure 4

A. HG - + +
   DN-JNK - - +
   p-JNK
   JNK
   p-JNK/JNK

B. HG - + +
   DN-JNK - - +
   IkB
   GAPDH
   IkB/GAPDH

C. HG - + +
   DN-JNK - - +
   Cleaved caspase-3
   Actin
   Cleaved caspase-3 / Actin

D. HG incubating for 10 min
   p-JNK
   JNK
   p-JNK/JNK

C66 (uM)

* p-JNK/JNK
** p-JNK/JNK
Figure 5

A

TNF-α - + +
DN-JNK - - +
cleaved caspase-3
Actin

C

Caspase-3 activity

Ac-DEVD-pNA units/mg

0 5 10 15

MF
MN
FM

TNF-α (μg/ml) - 0.25 0.5 1.0 1.0
C66 (μM) - - - - 10

D

C66 10μM - - - +
SP 10μM - - + -
TNF-α 1μg/ml - + + +
Cleaved-caspase 3
Actin

Cleaved-caspase-3

0.0 0.5 1.0 1.5 2.0 2.5

* * **

** ** **

E

p-JNK/JNK

0.0 0.2 0.4 0.6 0.8

* ** ***

** ** ** ** **

TNF-α incubating for 10 min

TNF-α (μg/ml) - 0.25 0.5 1.0 1.0
C66 (μM) - - - - 10

DMSO

C66 (μM) 2.5 5.0 10

TNF-α incubating for 24 h

DMSO
Figure 6

(A) Serum TNF-α

(B) Cardiac TNF-α mRNA

(C) Cardiac IL-6 mRNA

(D) p65 translocation

(E) Relative amount of nuclei p65 (compared to Control%)

(F) Western blot for IκBα and Actin

(G) IκBα/Actin ratio
Figure 7

A. Caspase 3 and Actin Western Blot Analysis

B. Tunel Staining

C. TUNEL positive cell (%)

D. Sirius Red Staining

E. Relative amount of Sirius red stained collagen (compared to Control%)

F. TGF-β Relative amount of mRNA

G. ANP Relative amount of mRNA

H. H&E Staining

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