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 the 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 nuclear factor-κB in both H9c2 cells and neonatal cardiomyocytes. Furthermore, we showed that the inhibition of Jun NH2-terminal kinase (JNK) phosphorylation contributed to the protection of C66 from inflammation and cell apoptosis, which was validated by the use of SP600125 and dominant-negative JNK. The molecular docking and kinase activity assay confirmed direct binding of C66 to and inhibition of JNK. In mice with type 1 diabetes, the administration of C66 or SP600125 at 5 mg/kg significantly decreased the levels of plasma and cardiac tumor necrosis factor-α, accompanied by decreasing cardiac apoptosis, and, finally, improved histological abnormalities, fibrosis, and cardiac dysfunction without affecting 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.

Diabetic cardiomyopathy is a leading cause of the increased morbidity and mortality in diabetic patients. The pathophysiology of diabetic cardiomyopathy includes microangiopathy, cardiac fibrosis, and disruption of the intracellular transport of Ca2+ (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 the increased expression of cytokines in local cardiac myocytes or immunocompetent cells recruited into the heart (4). Overproduced proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, 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 the 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 proapoptotic proteins from the mitochondria (11–13). Sustained inflammation may lead to the activation of multiple pathways that lead to 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 in the pressure overload state, respectively (16,17).

Intervention against TNF-α 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 (2E,6E)-2,6-bis[(trifluoromethyl)benzylidene]cyclohexanone (C66) (Fig. 1A) is a synthetic derivative of natural active curcumin (19,20). Our previous studies (19,20) demonstrated that C66 could inhibit the expression of inflammatory cytokines in mouse macrophages stimulated by both high glucose (HG) and lipopolysaccharide. The current 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 antiapoptosis, by C66 was found to be mediated most likely by its direct inhibition of Jun NH2-terminal kinase (JNK).

RESEARCH DESIGN AND METHODS

Reagents, Cell Culture, and Treatment
Glucose, mannitol, JNK inhibitor SP600125 (SP), nuclear factor-κB (NF-κB) inhibitor Bay 11–7085 (Bay), and fenofibrate (FE) were purchased from Sigma (St. Louis, MO). Compound C66 was synthesized and purified (>98.4%) as described in our previous publication (20). C66 was dissolved in DMSO for in vitro experiments and in CMCNa (1%) for in vivo experiments. Antibodies for phospho-JNK (p-JNK)/JNK, NF-κB p65, inhibitor of κB (IkB), MCP-1, CD68, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for caspase-3/9 were bought from Abcam (Cambridge, MA). A 3.3-kb cDNA fragment (dominant-negative type) encoding hemagglutinin-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 a gift from Professor Aimin Xu (School of Medicine, 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, People’s Republic of China) and cultured in DMEM medium (Gibco, Eggenstein, Germany) containing 5.5 mmol/L d-glucose (low glucose [LG]) supplemented with 10% FBS, 100 unit/mL penicillin, and 100 mg/mL streptomycin. In the HG-treated group, cells were incubated with DMEM containing 22 mmol/L 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 DMEM that contained 5.5 mmol/L D-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/AFWC/0031). Male C57BL/6 mice, weighing 18–22 g at 8 weeks of age, were obtained from the Animal Center of Wenzhou Medical College (Wenzhou, People’s Republic of China). Animals were housed at 22°C with a 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 with a glucometer on days 3 and 7 after the STZ injection. Seven days after STZ injection, mice with a fasting blood glucose concentration >12 mmol/L were considered to be diabetic. All mice had free access to food and water at all times. In the first set of experiments, 1-week-old diabetic mice (DM) were randomly divided into the following two groups (n = 8): DM and C66-treated DM (DM+C66). In the DM+C66 group, mice received 5 mg/kg C66 orally once every 2 days for 12 weeks. The DM group and age-matched control group (n = 8) received 1% CMCNa solution alone in the same schedule as the DM+C66 group. Body weight and blood glucose level were recorded on days 7, 17, 27, 47, 57, and 67 after STZ induction. On the 67th day after STZ induction, mice were killed under anesthesia. In the second set of experiments, DM were randomly divided into the following three groups (n = 8): DM, DM+C66, and SP-treated
DM. Besides, another two groups were set as C66- and SP-treated control mice. Then C66 or SP at 5 mg/kg was administered orally once every 2 days for 12 weeks. The DM group and the age-matched control group were administered a 1% CMCNa solution alone on the same schedule as the treated DM groups. Animals were killed under sodium pentobarbital anesthesia. After the mice were 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 FE on type 1 diabetic cardiomyopathy. After type 1 diabetes mouse models were established using the same method described above, DM were randomly divided into the following two groups (n = 6): DM and FE-treated DM (DM+FE). In the DM+FE group, mice were administered FE orally at 100 mg/kg every day for 12 weeks. The DM group and the age-matched control group (n = 6) were given a 1% CMCNa solution alone on the same schedule as the DM+FE group. At the 12-week end point, cardiac function was detected via echocardiography. Then mice were killed, and heart tissue and blood were collected for related analysis.

Cardiac Function Measurements by Echocardiography
To assess cardiac function, transthoracic echocardiography was performed in mice using a Vevo 770 High-Resolution Imaging System (Visual Sonics, Toronto, ON, Canada) equipped with a high-frame rate RMV 707B Scanhead (focal length 12.7 mm, frequency 30.0 MHz), as described previously (22). The indices directly measured...
included left ventricle (LV) internal dimension (LVID) in diastole (LVID,d) and systole (LVID,s), LV posterior wall thickness in diastole and systole, and interventricular septum thickness in diastole (IVS,d) and systole (IVS,s) (Supplementary Table 1). LV fractional shortening (FS) percentage = [(LVID,d − LVID,s)/LVID,d] × 100; LV ejection fraction (EF) percentage = [(LV end-diastolic volume − LV end-systolic volume)/LV 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, 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). Real-time quantitative PCR was performed using an M-MLV Platinum real-time quantitative PCR Kit (Invitrogen). Real-time quantitative PCR was carried out using the RealPlex4 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 (Shanghai, People’s Republic of China). The primer sequences used are shown in Supplementary Table 1. 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% SDS-PAGE, and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). After being blocked in blocking buffer (5% milk in Tris-buffered saline solution 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 Tris-buffered saline with Tween and reacted with secondary horseradish peroxidase–conjugated antibody (1:5,000; Santa Cruz Biotechnology) for 1–2 h at room temperature. Antigen-antibody complexes were then visualized using enhanced chemiluminescence reagents (Bio-Rad). The density of the immunoreactive bands was analyzed using ImageJ software (National Institutes of Health, 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 Biotechnology) and then with fluorescent isothiocyanate-labeled secondary antibody (1:500) (Santa Cruz Biotechnology) 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 (original magnification ×400; Nikon, Tokyo, Japan).

Transient Transfection
H9c2 cells were incubated for 6 h in 1 mL serum-free medium containing 10 μL Lipofectamine 2000 reagent (Invitrogen) and 2.5 μg dominant-negative JNK (dn-JNK) or vector. After 24 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, People’s Republic of 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 milligram of protein.

TUNEL Assay
Tissue sections of 5 μm were used for the TUNEL apoptosis detection kit (R&D Systems, Minneapolis, MN). Cells cultured on six-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 magnification; Nikon).

Histopathology
Hearts were fixed in a 4% paraformaldehyde solution, embedded in paraffin, and sectioned at 5 μm. After dehydration, sections were stained with hematoxylin-eosin (H-E). To evaluate the histopathological damage, each image of sections was obtained using a light microscope (original magnification ×400; Nikon).

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 were then viewed using a Nikon fluorescence microscope (original magnification ×400).

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. Values were normalized to protein in homogenate before extraction determined by the Bradford assay (Bio-Rad).

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 (JNK1:1UKJ; JNK2:3NPC). The ligand-binding groove on JNK1/2 was cited from the Protein Data Bank (JNK1:1UKI; JNK2:3NPC) and its trypsin-like catalytic site was examined as described in our previous publication.
kept rigid, whereas all torsible bonds of the ligands were freed to perform flexible docking to produce >100 structures. The final, docked conformations were clustered within the tolerance of a 1 Å root mean square deviation.

**Kinase Activity Inhibition Assays**

The JNK1/2 kinase activities were detected by Caliper Mobility Shift Assay on EZ Reader. The recombinant kinases JNK1/2 were purchased from Carma Biosciences. The ATP concentration was set at the \( K_m \) value of JNK2 (16 \( \mu \)mol/L). For the determination of the half-maximal inhibitory concentration (IC\(_{50}\)), compounds were tested in duplicate at 10 concentrations from 5 \( \mu \)mol/L to 100 \( \mu \)mol/L. Specific details of each assay are available in the supporting information. In electrophoretic mobility shift assays, product accumulation was expressed as the percentage conversion, product peak height/(product peak height + substrate peak height).

**Statistical Analysis**

Data were presented as the mean ± SD. The statistical significance of differences between groups was obtained by the Student t test or multiple-comparison ANOVA in GraphPad (San Diego, CA) Pro. 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 \( \mu \)mol/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 Fig. 1B and C, HG treatment significantly increased TNF-α expression in both protein and mRNA levels, which were remarkably inhibited by pretreatment with C66. The production of TNF-α was not affected by treatment with mannitol, indicating that the osmotic effect did not induce inflammation (Fig. 1B). C66 also decreased HG-induced IL-12 (Fig. 1D), IL-6 (Fig. 1E), and IL-1β (Fig. 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 (Fig. 1G and H).

**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 IkBα has a key role in mediating the activation and nuclear translocation of NF-κB, we first examined the effect of C66 on IkBα degradation in HG-exposed H9c2 cells and neonatal cardiomyocytes. HG treatment for 2 h increased IkBα degradation, and treatment of HG-exposed cardiac cells with C66 markedly reversed HG-induced IkBα degradation in a dose-dependent manner (Fig. 2A). Interestingly, similar results also were observed in the cells exposed to HG for 24 h (Fig. 2B), indicating the persistence of HG proinflammatory and C66 anti-inflammatory effects. These changes (Fig. 2A and B) were also observed in primary cardiomyocytes (Supplementary Fig. 1A and B). Degradation of IkBα 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 (Fig. 2C). These data suggest that C66 significantly suppressed the HG-induced activation of NF-κB. The fact that the NF-κB-specific inhibitor Bay (at 10 \( \mu \)mol/L) significantly reduced HG-increased TNF-α expression (Fig. 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. Figure 3A and B shows 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 (Fig. 3C). Similar results also were observed in the primary cardiac cells exposed to HG (Supplementary Fig. 1C and D). Furthermore, HG treatment for 72 h induced a significant increase in cardiac cell apoptosis, shown by an increased TUNEL-positive cells (Fig. 3D). Subsequently, HG treatment with C66 significantly reduced HG-increased apoptosis, shown by an increased TUNEL-positive cells (Fig. 3E). According to the hypotrophy, ANP, a peptide released by cardiac myocytes to regulate blood pressure and natriuresis, is generally considered to be a hallmark of cardiomyocyte hypertrophy (24). We showed that HG increased the ANP mRNA transcription, which was significantly reduced by C66 pre-treatment (Supplementary Fig. 2A). Subsequently, fluorescence microscopic study showed that C66 markedly reduced the HG-induced hypertrophy in primary neonatal cardiomyocytes (Supplementary Fig. 2B).

**JNK Inactivation Has a Critical Role in Prevention of HG-Induced Cardiac Inflammation and Apoptosis by C66**

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 the inhibition of C66 in HG-induced NF-κB activation and inflammation in cardiac cells. Figure 1B–H showed that JNK-specific inhibitor SP (10 \( \mu \)mol/L) could significantly decrease HG-induced inflammatory cytokine expression in either the H9c2 cell line (Fig. 1B–F) or primary cardiomyocytes (Fig. 1G and H). Treatment with
SP also inactivated NF-κB in terms of both IκB degradation (Fig. 2A and B) \( (P < 0.05) \) and p65 translocation (Fig. 2C) \( (P < 0.05) \). Similar results were observed in primary cardiomyocytes (Supplementary Fig. 1A and 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 SP significantly inhibited HG-induced caspase-3 and caspase-9 activation in either H9c2 cells (Fig. 3A and B) or primary cardiomyocytes (Supplementary Fig. 1C and D), which further resulted in a prevention of apoptotic cell death based on examination by TUNEL and DAPI-stained nuclear fragmentation (Fig. 3D–F).

To exclude possible nonspecific 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 (Fig. 4A) and the degradation of IκB (Fig. 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 (Fig. 4C). These data further suggest that JNK, as an upstream regulator of NF-κB and caspase-3, can upregulate and C66 may inhibit the activation of JNK to prevent HG-induced inflammation and apoptosis.

C66 May Target JNK 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 the JNK inhibitor SP, implying the possibility that the potent anti-inflammatory effect of C66 is mediated by JNK inactivation (Fig. 4D). A similar result was observed in mouse primary cardiomyocytes (Supplementary Fig. 3A).

Based on the above evidence, we proposed a docking model of C66 to the crystal structure of JNK (Supplementary Fig. 3B). Comparison of C66 with SP showed that the C66 docking sites in JNK overlapped with the SP binding pockets. C66 is located in the hydrophobic region of the JNK1 receptor-binding pocket and interacts with the hydrophobic residues leu-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.
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 by Caliper mobility shift assay. As shown in Supplementary Fig. 3C, C66 dose-dependently inhibited the kinase activity of both JNK1

Figure 3—C66 treatment attenuated apoptosis in H9c2 cells exposed to HG. A and B: Cells were pretreated with C66 (2.5, 5, or 10 μmol/L) or SP (10 μmol/L) for 2 h, followed by incubation with HG (22 mmol/L) for 48 h. The levels of cleaved caspase-3 (A) and caspase-9 (B) in total lysates were determined by Western blot analysis. The columns show the normalized optical density for data from more than three independent experiments. C–E: Cells were pretreated with C66 (2.5, 5, or 10 μmol/L) for 2 h, followed by incubation with HG (22 mmol/L) for 72 h. C: Cell lysates were collected and subjected to caspase-3 enzymatic assay. Ac-DEVD-pNA is the substance of caspase-3 in the assay kit. The apoptotic cells were detected using staining with TUNEL (D) and DAPI (E), as described in RESEARCH DESIGN AND METHODS. Similar results were observed in three independent experiments. F: The column figure shows the semiquantitative analysis for TUNEL staining. *P < 0.05, **P < 0.01 vs. HG group.
and JNK2, with IC₅₀ values of 81.9 and 2.72 μmol/L, respectively, on JNK1 and JNK2. We also tested the inhibitory effects of C66 on ERK and p38 kinase, while the IC₅₀ values of both kinases are around 100 μmol/L, 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.

**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 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 (Fig. 5A), confirming that JNK activation is directly involved in mediating TNF-α–induced cardiac cell apoptosis. Because of 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 (Fig. 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 (Fig. 5C). Western blotting of the cleavage of caspase-3 confirmed the proapoptotic effect of TNF-α, which was prevented by treatment with either C66 or SP (Fig. 5D). It also was observed that C66 inhibited JNK phosphorylation at 24 h after HG treatment in H9c2 cells (Fig. 5E) and primary cardiomyocytes (Supplementary Fig. 3D) when a majority of the glucose had been consumed and the TNF-α protein had been increased in the culture medium (Fig. 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 DM, 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 DM (Supplementary Fig. 4A). Diabetes reduced the body weight and heart weight in the untreated and C66-treated diabetic groups (Supplementary Fig. 4B and C). However, C66 treatment significantly reduced the increased ratio of heart weight to body 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 DM by C66 Treatment**

As shown in Fig. 6A–C, the administration of C66 at 5 mg/kg for 2 months significantly reduced diabetic increases in 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 DM. We also detected MCP-1 and CD68, two markers for macrophage infiltration (29). Supplementary Fig. 5 shows that diabetic hearts have marked increases in MCP-1 and CD68 expression, while the administration of C66 or SP resulted in significant reductions. The data in Fig. 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 hearts of DM, while C66 administration markedly reduced this translocation (Fig. 6D–E). C66 treatment also significantly reversed diabetes-induced degradation of cardiac IκBα (Fig. 6F and G).

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

**C66 Treatment Prevented Diabetes-Induced Cardiac Cell Death and Remodeling**

In the next study, we examined the antiapoptotic effect of C66 in diabetic hearts. In the diabetic hearts, there was a marked increase in caspase-3 cleavage (Fig. 7A), which was further confirmed by the TUNEL-positive cells (Fig. 7B and C and Supplementary Fig. 7A and B). However, the
treatment of DM with C66 or SP 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 DM, while C66 treatment significantly reduced the degree of collagen deposition ($P < 0.05$) (Fig. 7D and E). Real-time quantitative PCR analysis revealed significant increases in the profibrotic gene TGF-$\beta$ expression (Fig. 7F) and hypertrophic marker ANP expression (Fig. 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

![Figure 7](https://diabetes.journals.org/pan-and-associates/3507)

**Figure 7**—C66 administration improved cell apoptosis, histological abnormalities, and fibrosis in diabetic hearts. 

*Panel A*: Total proteins extracted from heart tissues were subjected to Western blot analysis for the determination of cleaved caspase-3. *Panel B*: Heart tissues were sectioned at 5 $\mu$m, and the slides were processed for TUNEL assay to detect apoptotic cells ($n = 3$ in each group). *Panel C*: The column figure shows the relative TUNEL-positive cell number ($n = 3$; $^{*}P < 0.05$ vs. DM group). *Panel D*: Heart tissues were sectioned at 5 $\mu$m, and the slides were processed for Sirius Red staining to detect type IV collagen. *Panel E*: A quantitative analysis of the relative amount of collagen IV in D, F and G: Heart tissues from mice were individually processed for RNA extraction and real-time quantitative PCR. The mRNA levels of TGF-$\beta$ (F) and ANP (G) were determined ($n = 5$ per group; $^{**}P < 0.01$ vs. DM group). *Panel H*: A representative H-E staining for heart tissue from the six mice studied in each group is shown. All images were obtained by microscope with original magnification $\times 400$. 

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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 DM that had been treated with C66 or SP (Fig. 7H and Supplementary Fig. 7C).

**C66 Treatment Attenuated Diabetes-Induced Alterations of Cardiac Function**

The preventive effect of C66 on cardiac pathological changes in DM may result in an improvement of diabetes-induced cardiac dysfunction; therefore, the protection from diabetes-induced heart dysfunctions in DM due to the administration of C66 was examined at 13 weeks after the onset of diabetes. Diabetes significantly impaired the functioning of the diabetic and systolic LV, which was largely attenuated by the treatment with C66 for 12 weeks (starting 1 week after the onset of diabetes) (Table 1). Treatment with SP also showed a similar improvement of cardiac function in DM (Supplementary Table 2).

**DISCUSSION**

It is intriguing to speculate that a reduction of cardiac cytokine levels 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, despite 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 have 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 current 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, the 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 current study observed that C66 treatment effectively suppressed the expression of inflammatory cytokines in both HG-incubated cardiac cells (Fig. 1B–H and Supplementary Fig. 1A and B) and diabetic hearts (Fig. 6A–C and Supplementary Fig. 5), which was accompanied with the subsequent decrease in apoptotic cell death (Figs. 3A–E and 7A–C, and Supplementary Figs. 1C and D and 7A and B). Hypertrophy (Fig. 7G), fibrosis (Fig. 7D–F), pathological changes (Fig. 7H and Supplementary Fig. 7C), and cardiac dysfunction (Table 1 and Supplementary Table 2). Similar to previous reports (9), the current study observed serum and cardiac lipid (triglyceride) accumulation in the DM, while

![Table 1 - Echocardiographic parameters of DM with type 1 diabetes for 12 weeks](chart.png)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>C66</th>
<th>DM</th>
<th>DM+C66</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic</td>
<td>72 ± 1.58114</td>
<td>72.6 ± 4.77493</td>
<td>77.85714 ± 3.13202</td>
<td>76.83333 ± 4.02078</td>
</tr>
<tr>
<td>Systolic</td>
<td>110.6 ± 4.219</td>
<td>107 ± 3.60555</td>
<td>110.57143 ± 3.35942</td>
<td>107.66667 ± 3.93277</td>
</tr>
<tr>
<td>Mean</td>
<td>83.4 ± 3.36155</td>
<td>83.8 ± 2.28035</td>
<td>87.85714 ± 2.1157</td>
<td>87.16667 ± 3.54495</td>
</tr>
<tr>
<td>HR</td>
<td>576.4 ± 17.00882</td>
<td>586.8 ± 12.33694</td>
<td>595.7 ± 23.9145</td>
<td>589.7 ± 19.01229</td>
</tr>
<tr>
<td>IVS,d</td>
<td>0.642 ± 0.00837*</td>
<td>0.644 ± 0.00548*</td>
<td>0.59714 ± 0.02928</td>
<td>0.635 ± 0.01049*</td>
</tr>
<tr>
<td>LVID,d</td>
<td>3.734 ± 0.08678</td>
<td>3.724 ± 0.02881</td>
<td>3.766 ± 0.13088</td>
<td>3.69 ± 0.03098</td>
</tr>
<tr>
<td>LVWP,d</td>
<td>0.764 ± 0.02702*</td>
<td>0.75 ± 0.01732*</td>
<td>0.644 ± 0.03409</td>
<td>0.732 ± 0.01941*</td>
</tr>
<tr>
<td>IVS,s</td>
<td>1.096 ± 0.0114*</td>
<td>1.102 ± 0.00447*</td>
<td>0.97429 ± 0.05473</td>
<td>1.09333 ± 0.00816*</td>
</tr>
<tr>
<td>LVID,s</td>
<td>1.872 ± 0.6573*</td>
<td>1.856 ± 0.03467*</td>
<td>2.30714 ± 0.25934</td>
<td>2.00333 ± 0.00816*</td>
</tr>
<tr>
<td>LVWP,s</td>
<td>1.388 ± 0.02387*</td>
<td>1.384 ± 0.00894*</td>
<td>1.09 ± 0.011776</td>
<td>1.32167 ± 0.02229*</td>
</tr>
<tr>
<td>LV vol,d</td>
<td>59.0044 ± 1.02313</td>
<td>59.0044 ± 1.02313</td>
<td>60.78475 ± 5.14001</td>
<td>57.843 ± 1.10401</td>
</tr>
<tr>
<td>LV vol,s</td>
<td>10.5604 ± 0.55192</td>
<td>10.5604 ± 0.55192</td>
<td>18.62571 ± 5.12945</td>
<td>12.77144 ± 0.13072*</td>
</tr>
<tr>
<td>EF%</td>
<td>81.9392 ± 0.72979*</td>
<td>82.0992 ± 0.90125*</td>
<td>69.618 ± 6.53716</td>
<td>77.90967 ± 0.38012*</td>
</tr>
<tr>
<td>FS%</td>
<td>49.9252 ± 0.74005*</td>
<td>50.086 ± 0.98208*</td>
<td>38.84229 ± 5.43694</td>
<td>45.76289 ± 0.38832*</td>
</tr>
<tr>
<td>LV mass</td>
<td>88.136 ± 5.37411*</td>
<td>86.2624 ± 1.29667*</td>
<td>76.08829 ± 4.310851</td>
<td>83.40567 ± 3.1431*</td>
</tr>
<tr>
<td>LV mass,Co</td>
<td>70.5092 ± 4.29885*</td>
<td>69.4612 ± 1.03794*</td>
<td>60.87029 ± 3.44823</td>
<td>66.72489 ± 2.51503*</td>
</tr>
</tbody>
</table>

n = 8 per group. Co, corrected; d, diastole; HR, heart rate; LVWP, left ventricular posterior wall; s, systole; vol, volume. *P < 0.05 vs. DM group.
treatment with C66 and SP for 12 weeks effectively decreased the triglyceride deposit in the serum and cardiac tissues of the DM (Supplementary Fig. 6A and B). We also observed that C66 did not affect the blood glucose profile in DM (Supplementary Fig. 4A). 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 antiapoptotic actions.

Although several anti-inflammatory medications have been reported (5,30–32) 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 activation of several transcription factors, such as NF-κB, activator protein 1, and STAT protein (19,34,35). The body of evidence suggests that the activation of NF-κB is an important step for cardiac injuries (36). Compounds that inactivate NF-κB may become an effective and powerful approach for the prevention of cardiac apoptosis in diabetic patients (37). Curcumin has been identified as an NF-κB inhibitor. Recently, we have proven that C66 inhibited NF-κB activation in HG-treated macrophages (19). The specific NF-κB inhibitor Bay significantly suppressed HG-induced TNF-α expression (Fig. 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 DM via inactivation of NF-κB. Both in vitro (Fig. 2 and Supplementary Fig. 1) and in vivo (Fig. 6D–E) data further indicated that the anti-inflammatory effect of C66 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 β-subunit of IκB kinase complex (IKKβ) showed that C66 did not bind to the IKKβ directly (data not shown). Thus, we investigated the possible NF-κB upstream target of C66. JNK belongs to the family of mitogen-activated protein kinases involved in the regulation of cell proliferation, stress, and apoptosis (38). Recently, it also has been reported (38) that JNK is activated in response to inflammatory and stressful stimuli, including HG or a diabetic situation. Reports (25,39) have shown that JNK activation may upregulate NF-κB by the phosphorylation of IKKβ in lipopolysaccharide-stimulated macrophages. In the current study, both JNK inhibition by specific inhibitors (i.e., SP and dn-JNK) significantly attenuated the HG-induced expression of inflammatory cytokines (Fig. 1B–H) and NF-κB activation (Figs. 2 and 4A and Supplementary Fig. 1), 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 SP against HG-induced JNK phosphorylation (Fig. 4D and Supplementary Fig. 2A and D), 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 toward 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 (Supplementary Fig. 3B and 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 SP could improve cardiac structural disorders and dysfunction (Supplementary Fig. 7 and Supplementary Table 2).

In addition to inflammatory response, JNK has an important role in the apoptotic pathway initiated by death receptors, such as TNF-α, TNF-related apoptosis-inducing ligand, and Fas ligand (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 SP (Fig. 3) and dn-JNK (Fig. 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 complex II, a classic death receptor (18). The binding between TNF-α and TNF-α type 1 receptor 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 current study, treatment with either dn-JNK (Fig. 5A) or SP (Fig. 5D and 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. Figure 5B–E shows 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 (Fig. 5E and Supplementary Fig. 3D) 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 hypertriglyceridermia, C66 treatment could decrease diabetes-induced hypertriglyceridermia in mouse serum and hearts (Supplementary Fig. 6A and B). It is unclear whether this compound is protecting the heart against hyperglycemia-induced damage, hypertriglyceridermia-induced damage, or both. We used FE to investigate the role of lipotoxicity in type 1 diabetic cardiomyopathy. As shown in Supplementary Fig.
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, using C66 and pharmacologically specific inhibitors, we demonstrated that the exposure of cardiac cells to HG activated the JNK-mediated NF-κB pathway and upregulated the expression of inflammatory cytokines, including TNF-α, which then triggers the JNK-dependent caspase activation and cell apoptosis (Supplementary Fig. 8B). Thus, as an important therapeutic target, JNK regulates the inflammatory transcription and mediates the apoptotic effect of the increased TNF-α levels in cardiac cells exposed to HG (Supplementary Fig. 8B). 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.

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Duality of Interest. No potential conflicts of interest relevant to this article were reported.

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