RESEARCH

Necrostatin-1 reduces cardiac and mitochondrial dysfunction in prediabetic rats

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Abstract

High-fat diet (HFD) consumption induces prediabetes and left ventricular dysfunction through many pathways including cell death pathway like necroptosis. Although the benefit of necroptosis inhibitor (necrostatin-1 or Nec-1) in the brain of prediabetic rats was shown, the effects of Nec-1 on cardiac autonomic function, blood pressure, cardiac function, along with its mechanistic insight have not been investigated. Male Wistar rats were fed with either a normal diet (n = 8) or HFD (n = 24) for 12 weeks to induce prediabetes. Prediabetic rats were randomly assigned into three interventional groups (n = 8/group): (1) vehicle, (2) Nec-1 (1.65 mg/kg, sc injection), and (3) metformin (300 mg/kg, oral gavage feeding). Treatments lasted for 8 weeks. Normal saline was given to normal diet-fed rats and vehicle group. Metabolic parameters, cardiac function and biochemical parameters were assessed. Prediabetic rats exhibited peripheral metabolic impairment as indicated by increased body weight, hyperinsulinemia with euglycemia, and dyslipidemia. Prediabetic rats also had cardiac autonomic imbalance, high blood pressure, and cardiac dysfunction, together with cardiac mitochondrial dysfunction, mitochondrial dynamic imbalance, and increased necroptosis and apoptosis. Treatment with Nec-1 did not affect peripheral metabolic parameters, however, it effectively reduced cardiac autonomic imbalance, blood pressure, and cardiac dysfunction via reducing cardiac inflammation, necroptosis, mitochondrial dysfunction, and increased mitochondrial fusion. Treatment with metformin reduced peripheral metabolic impairment and cardiac dysfunction via decreased cardiac mitochondrial dysfunction, mitochondrial dynamic imbalance, and apoptosis. In summary, Nec-1 directly suppressed necroptosis, cardiac mitochondrial dysfunction, and increased mitochondrial fusion independent of peripheral metabolic function, leading to an improved cardiac function in prediabetic rats.

Introduction

Long-term high-fat diet (HFD) consumption is known to cause metabolic syndrome, which is the cluster of obesity, insulin resistance, dyslipidemia, and hypertension (Lai et al. 2014). Metabolic syndrome increases the cardiovascular risk by approximately 50–60%, when compared with normal individuals (Moreira et al. 2014).
In a preclinical setting, our previous studies have shown that 12 weeks of HFD consumption led to obesity, hyperinsulinemia, insulin resistance, dyslipidemia, and hypertension in rats; however, their plasma glucose levels were in the euglycemic stage, indicating a prediabetic status (Apaijai et al. 2019, Jinawong et al. 2020). In addition to peripheral metabolic impairment, prediabetic rats demonstrated cardiac complications including cardiac autonomic imbalance, hypertension, and left ventricular (LV) dysfunction (Apaijai et al. 2012, 2019). It has been proposed that oxidative stress- and inflammation-mediated mitochondrial dysfunction, mitochondrial dynamic alterations, and apoptosis were responsible for cardiac dysfunction in animal models of metabolic syndrome (Pashkow 2011, Ilkun & Boudina 2013).

Long-term HFD consumption was shown to induce systemic inflammation after 2 weeks of HFD feeding, which is predisposed to systemic oxidative stress (Saiyasit et al. 2020), suggesting that inflammation may attribute to cardiac complication in prediabetic rats. Among several types of cell death, necroptosis is principally induced by inflammation (Dhuriya & Sharma 2018). Currently, the roles of necroptosis in the heart of prediabetes are still unknown. Necroptosis or the programmed necrosis is elicited by tumor necrosis factor receptor, interferon receptors, and pathogen recognition receptor upon inflammatory cytokine activation, followed by the induction of receptor-interacting protein kinase 1 and 3 (RIPK1, 3) and oligomerization of mixed-lineage kinase domain-like protein (MLKL) (Ying et al. 2021). A translocation of necroptosome promotes plasma membrane rupture to execute necroptosis. Furthermore, RIPK3 activation is involved in mitochondrial fission in the setting of hypoxia/reoxygenation, and it mediated mitochondrial permeability transition pore (mPTP) opening, leading to mitochondrial dysfunction and subsequent cell death (Zhou et al. 2018, Ying et al. 2021). As necroptosis plays a role in cardiac dysfunction in prediabetes, inhibition of necroptosis would provide cardiometabolic benefits in this condition. The cardioprotective effects of RIPK 1 inhibitor, necrostatin-1 (Nec-1), are demonstrated in various preclinical settings. Acute treatment with Nec-1 prior to cardiac ischemia or during reperfusion therapy successfully reduced the infarct size in aged mice and female rats subjected to cardiac ischemia-reperfusion injury (Garvin et al. 2018, Li et al. 2020). Furthermore, chronic treatment with Nec-1 could preserve LV function in paraquat-induced cardiac contractile dysfunction. However, the potential cardioprotective effects of Nec-1 in the heart of prediabetic rats are still unknown. In obesity, necroptosis was found in the adipose tissue and hippocampus (Jinawong et al. 2020, Karunakaran et al. 2020). We previously reported that chronic treatment with Nec-1 significantly reduced cognitive dysfunction without altering insulin-resistant status in prediabetic rats (Jinawong et al. 2020). Due to these reported benefits, targeting necroptosis using Nec-1 could be a potential novel therapeutic approach to reduce cardiac dysfunction in prediabetic rats. In this study, we hypothesized that Nec-1 attenuates cardiac dysfunction by reducing cardiac inflammation, cardiac mitochondrial dysfunction, and necroptosis in prediabetic rats.

**Materials and methods**

Experimental protocol in this study was approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Chiang Mai University (No. 28/2018), and the experiments were also performed in accordance with ARRIVE guideline. Thirty-two male Wistar rats purchased from Nomura Siam Company (Bangkok, Thailand) were used in this study. Rats were housed in a temperature- and humidity-controlled room. After a week of acclimatization, rats received either a normal diet (ND) ($n=8$, a standard chow diet containing 19.77% energy from fat, CP082, Bangkok, Thailand) or HFD ($n=24$, a diet containing 59.28% energy from fat, mainly by lard and cholesterol extract) throughout the experiment. At week 12, HFD-fed rats were divided into three intervention groups: (1) vehicle (normal saline solution, s.c. injection), (2) Nec-1 (1.65 mg/kg/day, s.c. injection), and (3) metformin (used as a positive control, 300 mg/kg/day, oral gavage feeding). ND-fed rats received normal saline solution. Dosage of Nec-1 and metformin were chosen from the previous report (Jinawong et al. 2020). Chronic treatment with Nec-1 at the dose of 1.65 mg/kg for 4–8 weeks effectively suppressed necroptosis signaling in rats’ hippocampus and in the bone of ovariectomized rats (Cui et al. 2016, Jinawong et al. 2020). Moreover, administration of Nec-1 through either i.p. injection or s.c. injection causes no harm to the rats (Cui et al. 2016, Jinawong et al. 2020). Therefore, Nec-1 at 1.65 mg/kg was chosen to be subcutaneously injected into our HFD fed rats for additional 8 weeks after 12 weeks of HFD consumption. The HFD was continued throughout the treatment period. At the end of the study protocol, all rats were sacrificed by decapitation under deep anesthesia (5% isoflurane, Forane, Abbott).

Rats received their assigned intervention for 8 weeks, then they were sacrificed. Peripheral metabolic parameters including body weight, fasting plasma glucose, insulin,
triglyceride, total cholesterol, high-density lipoprotein (HDL-C), and low-density lipoprotein (LDL-C) were determined at baseline, pre-, and post-treatment. Oral glucose tolerance test (OGTT) was performed only post-treatment. Visceral fat weight was recorded after sacrifice. For cardiac function parameters, cardiac autonomic balance, blood pressure, %LV fractional shortening and ejection fraction were determined at baseline, pre-, and post-treatment. After decapitation, the heart was rapidly removed for molecular investigations. Each heart was separated into two parts. The first part was the LV apex, which was snap-frozen and stored at −85℃ until analysis. This section was used for protein expression analysis of mitochondrial dynamics, inflammation, necroptosis, and apoptosis cell death (Apaijai et al. 2019, Jinawong et al. 2020). The second part was the remaining LV, which was used for cardiac mitochondria isolation, which was done immediately after decapitation to determine the cardiac mitochondrial function (Apaijai et al. 2019, Maneechole et al. 2019). Metformin is a standard treatment for type 2 diabetes and has been shown to improve insulin sensitivity (Giugliano et al. 1993, Giannarelli et al. 2003). Since prediabetes has an insulin-resistant condition, metformin has been widely used as a positive control in obese-insulin resistant/prediabetes models (Apaijai et al. 2012, Sivasinprasasn et al. 2016, Masola et al. 2018). Metformin showed favorable effects in reducing insulin resistance and improving LV function in prediabetic rats (Apaijai et al. 2012, Sivasinprasasn et al. 2016). Therefore, metformin was chosen as a positive control in this study. The experimental protocol is depicted in Fig. 1.

**Metabolic parameters determination**

To determine fasting plasma glucose, insulin, triglyceride, total cholesterol, HDL-C and LDL-C levels, rats were fasted for 5 h, then the blood was collected from their tail tip and was stored in either NaF-coated tubes (for plasma glucose measurement) or EDTA-coated tubes (for other blood parameters). Blood was centrifuged at 876 g for 10 min, and plasma was collected to measure metabolic profiles. Fasting plasma glucose, triglyceride, and total cholesterol levels were measured using commercial colorimetric assay kits (Erba Mannheim, Germany). Fasting plasma insulin levels were measured using a commercial ELISA kit (Merck). Fasting plasma HDL-C levels were directly measured by a commercial colorimetric assay kit (BioVision). Plasma LDL-C levels were indirectly calculated from the levels of fasting plasma HDL-C and triglyceride levels according to the Friedewald equation (Jinawong et al. 2020). OGTT was performed to determine peripheral insulin sensitivity. In brief, 2 mg/kg of glucose was orally administered to the rats after overnight fasting. The series of blood collection was done prior to glucose administration, at 15, 30, 60, 90, 120 min after glucose loading. The area under the curve of plasma glucose levels was analyzed (Jinawong et al. 2020).

**Cardiac autonomic balance, blood pressure, and cardiac function determination**

Cardiac autonomic balance was determined by heart rate variability (HRV). Lead II ECG was recorded in conscious rats for at least 20 min through the PowerLab 4/25T system (AD instruments, Australia), and the HRV was analyzed from the ECG signals using an analytical program. The ratio of the low-frequency band and high-frequency band (LF/HF) of HRV was used to indicate cardiac autonomic balance (Apaijai et al. 2019).

Non-invasive blood pressure monitoring was performed using a tail-cuff method with a CODA-2 system (Kent Scientific, USA). Systolic and diastolic blood pressure (SBP and DBP) were automatically analyzed by the software (Apaijai et al. 2019).

Non-invasive cardiac function was measured using echocardiography (Vivid-i, GE technology). Rats were placed in light anesthesia (1% isoflurane with oxygen) through a rodent anesthesia machine. The s12 probe was placed on the rat’s chest in the parasternal short axis, the M-mode echocardiogram was recorded to analyze %LV ejection fraction (%LV EF) and %LV fractional shortening (%LVFS) (Apaijai et al. 2019).

**Cardiac mitochondrial function determination**

Cardiac mitochondria were isolated from the fresh heart tissue through the differential centrifugation technique
In brief, the heart tissue was chopped and homogenized using a homogenizer at 600 rpm in a buffer containing 300 mM sucrose, 5 mM TES sodium salt, and 0.2 mM EGTA (Sigma) (Thummasorn et al. 2011). The homogenate was centrifuged at 800 g for 5 min, followed by 8800 g for 5 min to obtain the pellet of isolated cardiac mitochondria. The cardiac mitochondrial pellet was resuspended in a respiration buffer containing 100 mM KCl, 10 mM HEPES, and 5 mM KH₂PO₄. Mitochondrial protein concentration was measured using a bicinchoninic acid assay kit (Sigma), and the concentration at 0.4 mg/mL was applied to all mitochondrial function measurements including mitochondrial reactive oxygen species (ROS) production, mitochondrial membrane potential changes, and mitochondrial swelling. The protocol of all mitochondrial function measurements was described in our previous studies (Thummasorn et al. 2011, Apaijai et al. 2019, Maneechote et al. 2019, Benjanuwattra et al. 2020).

Briefly, cardiac mitochondrial ROS levels were measured using DCFH-DA dye by incubating cardiac mitochondria with the dye for 20 min at room temperature. The fluorescent intensity of DCF was read at λex 485/λem 530 nm. For cardiac mitochondrial membrane potential changes, isolated cardiac mitochondria were incubated with JC-1 dye for 30 min at 37°C. Fluorescent intensity of JC-1 was measured at 485/520 nm regarded as green fluorescence, and 485/590 nm regarded as red fluorescence. Red/green fluorescent intensity ratio was used as an indicator of mitochondrial membrane potential, a decrease in this ratio represents mitochondrial membrane depolarization. For cardiac mitochondrial swelling, the absorbance of mitochondria was measured at 540 nm. Both fluorescent intensity and absorbance of mitochondria were measured using a microplate reader (BioTek H4 synergy). To confirm the alterations of cardiac mitochondrial morphology, isolated cardiac mitochondria were fixed with 2.5% glutaraldehyde/osmium, then they were embedded in the resin, cut with a diamond knife, and visualized with a transmission electron microscope (Jeol, Japan) (Apaijai et al. 2019).

Protein expression analysis

Frozen cardiac tissue was used for protein extraction, the tissue was homogenized in an ice-cold buffer containing 20 mM Tris HCl, 1 mM Na₂VO₄, and 5 mM NaF; the homogenate was centrifuged at 14263 g for 10 min, and the supernatant was collected. The protein concentration was measured using a Bradford technique (Maneechote et al. 2019). The protein (2 mg/mL) was mixed with a loading buffer, then loaded onto 10% acrylamide gel. The protein was separated through an electrophoresis protocol and transferred to the nitrocellulose membrane (GE life sciences) in a wet/tank blotting system (Bio-rad). The membranes were incubated with the following primary antibodies for at least 16 h: (1) primary antibodies against mitochondrial dynamic proteins and OXPHOS: p-DRP1ser636, DRP1, MFN1, Mfn2, OPA1 (Cell signaling), OXPHOS (Abcam), (2) primary antibodies against necroptosis proteins: TNF-α (Abcam), p-RIPK1ser166 (Cell signaling), RIPK1 (Cell signaling), p-RIPK3ser227 (Abcam), RIPK3 (Abcam), p-MLKLser345 (Cell signaling), MLKL (Cell signaling), (3) primary antibodies against apoptosis proteins: BAX (Cell signaling), BCL-2 (Abcam), Cleaved and pro-caspase-3 (Cell signaling), and GAPDH (Abcam) was used as a loading control. Then, the membranes were incubated with the HRP-conjugated secondary antibody (Cell signaling) for 1 h at room temperature, and they were incubated with the ECL solution (Bio-rad). The Western blot was visualized with a chemidoc touching system (Bio-rad) and analyzed using image J program (NIH) (Apaijai et al. 2019).

Statistical analysis

Data were presented as mean ± S.E.M. The normality of distribution was checked by D’Agostino and Pearson normality. The homogeneity of variance was analyzed using the Brown-Forsythe test, and the statistical difference among groups was analyzed using one-way ANOVA followed by LSD post hoc test. P < 0.05 was considered statistically significant.

Results

Chronic HFD feeding-induced prediabetes and Nec-1 attenuated visceral fat deposition in prediabetic rats

At baseline, all metabolic parameters including body weight, fasting plasma glucose, insulin, triglyceride, total cholesterol, HDL-C, LDL-C levels, and HOMA-IR index were not different between ND and HFD-fed rats (Figs 2A, C, D, E, F and 3A, B, C, D). Twelve weeks of HFD feeding successfully induced prediabetic condition as indicated by increased body weight, fasting plasma insulin, total cholesterol, LDL-C levels, and HOMA-IR index (Figs 2A, D, E and 3B, D). There were no changes in fasting plasma glucose, triglyceride, and HDL-C levels, compared with ND-fed rats (Figs 2C and 3A, C).

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The area under the curve (AUC) of plasma glucose levels during OGTT was measured after treatments in all groups, and visceral fat was weighted after being sacrificed. HFD-fed rats treated with vehicle (HFDV) rats had higher AUC of plasma glucose during OGTT and visceral fat weight, compared with normal diet-fed rats treated with

**Figure 2**
Nec-1 reduced visceral fat weight, but it did not affect other peripheral metabolic parameters in prediabetic rats. (A) Body weight, (B) Visceral fat weight, (C) Fasting plasma glucose levels, (D) Fasting plasma insulin levels, (E) HOMA-IR index, (F) Plasma glucose AUC during OGTT. *P < 0.05 vs ND/NDV. †P < 0.05 vs HFDV. ND, normal diet; HFD, high-fat diet; NDV, normal diet-fed rats treated with vehicle; HFDV, high-fat diet-fed rats treated with vehicle; HFDN, high-fat diet-fed rats treated with necrostatin-1; HFDM, high-fat diet-fed rats treated with metformin; HOMA-IR, homeostatic model assessment of insulin resistance; AUC, area under the curve; OGTT, oral glucose tolerance test.

**Figure 3**
Nec-1 did not affect plasma lipid profiles in prediabetic rats. (A) Fasting plasma triglyceride, (B) Fasting plasma total cholesterol, (C) Fasting plasma HDL-C, (D) Fasting plasma LDL-C. *P < 0.05 vs ND/NDV. †P < 0.05 vs HFDV. ND, normal diet; HFD, high-fat diet; NDV, normal diet-fed rats treated with vehicle; HFDV, high-fat diet-fed rats treated with vehicle; HFDN, high-fat diet-fed rats treated with necrostatin-1; HFDM, high-fat diet-fed rats treated with metformin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
vehicle (NDV), indicating that HFDV rats exhibited truncal obesity with impairment of peripheral insulin sensitivity (Fig. 2B and F).

Treatment with Nec-1 attenuated visceral fat weight, but it did not alter other peripheral metabolic parameters (Figs 2A, B, C, D, E, F and 3A, B, C, D), whereas metformin reduced body weight, visceral fat weight, fasting plasma insulin, AUC of plasma glucose during OGTT, total cholesterol and LDL-C levels, and HOMA-IR index (Figs 2A, B, D, E, F and 3B, D). However, metformin did not alter fasting plasma glucose, triglyceride, and HDL-C levels in HFD-fed rats (Figs 2C and 3A, C).

Nec-1 restored cardiac autonomic balance, blood pressure, and cardiac function

At baseline, ND and HFD-fed rats showed similar HR, cardiac autonomic balance, SBP, DBP, %LVEF, and %LVFS (Fig. 4A, B, C, D, E and F). Twelve weeks of HFD consumption led to cardiac autonomic imbalance, indicated by an increased LF/HF ratio, SBP, DBP, and reduced %LVFS (83.6 ± 1.1% vs 75.2 ± 0.7%, P < 0.05) and LVFS (46.8 ± 0.8% vs 39.3 ± 0.6%, P < 0.05), when compared with ND-fed rats (Fig. 4B, C, D, E and F). In the vehicle-treated group, HFDV rats had a higher LF/HF ratio, SBP, DBP, with lower%LVEF and %LVFS, when compared with NDV rats (Fig. 4B, C, D, E and F).

Treatment with Nec-1 had reduced LF/HF ratio, SBP, DBP, and increased %LVEF (77.3 ± 2 in vehicle group vs 86 ± 3 in Nec-1 group) and %LVFS (41 ± 1.9% in vehicle group vs 51.1 ± 3.4% in Nec-1 group, P < 0.05) in HFD-fed rats, when compared with HFDV and NDV rats, and effectively brought all cardiac function parameters back to the normal levels (Fig. 4B, C, D, E and F). These benefits were similar to those found in the metformin-treated group. The individual analysis regarding the effects of Nec-1 on HR, LF/HF ratio, SBP, DBP, %LV ejection fraction (%LVEF) and %LV fractional shortening (%LVFS) was performed, and the results demonstrated that Nec-1 reduced LF/HF ratio, SBP, DBP, together with increased %LVEF and %LVFS in HFD-fed rats. These findings were also observed in metformin-treated rats (Supplementary Fig. 1, see section on supplementary materials given at the end of this article). Moreover, HR was not altered in all treatment groups (Fig. 4A).

Nec-1 reduced cardiac mitochondrial dysfunction in prediabetic rats

Cardiac mitochondrial function was investigated after treatment. The data demonstrated that HFDV rats had cardiac mitochondrial dysfunction as indicated by increased mitochondrial ROS levels, mitochondrial membrane...
depolarization, and mitochondrial swelling, compared with NDV rats (Fig. 5A, B and C). Treatment with Nec-1 reduced cardiac mitochondrial ROS levels, mitochondrial membrane depolarization, and mitochondrial swelling in HFD-fed rats, when compared with HFDV and NDV rats (Fig. 5A, B and C). Treatment with metformin also reduced cardiac mitochondrial dysfunction in HFD-fed rats to a similar extent to the Nec-1-treated group.

The representative TEM pictures of cardiac mitochondria are shown in Fig. 5D.

**Nec-1 increased cardiac mitochondrial fusion, but it did not affect mitochondrial fission and OXPHOS protein in prediabetic rats**

A mitochondrial dynamic imbalance was observed in the heart of HFDV rats, indicated by increased p-DRP1ser616/DRP1 and decreased MFN2 protein levels, while MFN1 and OPA1 were not changed in HFDV rats, when compared with NDV rats (Fig. 6A, B, C and D).

Treatment with Nec-1 did not alter p-DRP1ser616/DRP1, whereas metformin attenuated p-DRP1ser616/DRP1 protein levels in HFD-fed rats, when compared with HVF and NDV rats (Fig. 6A). Regarding mitochondrial fusion, treatment with Nec-1 effectively enhanced MFN2 protein levels, with no alterations in MFN1 and OPA1 protein levels in HFD-fed rats, when compared with HVF rats (Fig. 6B, C and D). These results were also observed in the metformin-treated group.

The OXPHOS protein levels were analyzed, and our data showed that HFDV rats had lower complex IV protein levels, however, other complexes were not changed, when compared with NDV rats (Fig. 6E). Treatment with Nec-1 did not alter OXPHOS protein levels in HFD-fed rats, while metformin significantly reduced complex I protein levels in HFD-fed rats, when compared with HFDV rats (Fig. 6E).

**Chronic HFD feeding induced cardiac necroptosis, and Nec-1 reduced cardiac necroptosis in prediabetic rats**

Necroptosis is initiated by inflammation, and the necroptosis signal is sent through the RIPK1/RIPK3/MLKL pathway (Ying et al. 2021). Our results showed that TNF-α, p-RIPK1/RIPK1, p-RIPK3/RIPK3 and p-MLKL/MLKL protein levels were higher in HFDV rats than the NDV rats (Fig. 7A, B, C and D).

Treatment with Nec-1 significantly reduced TNF-α, p-RIPK1/RIPK1, p-RIPK3/RIPK3, and p-MLKL/MLKL protein levels in HFD-fed rats, when compared with HFDV and NDV rats (Fig. 7A, B, C and D). Although metformin reduced TNF-α and p-RIPK3/RIPK3 protein levels (Fig. 7A and C), it did not decrease p-RIPK1/RIPK1 and p-MLKL/MLKL protein levels in HFD-fed rats, when compared with HFDV and NDV (Fig. 7B and D).

**Chronic HFD feeding induced cardiac apoptosis, and Nec-1 reduced BAX and increased BCL-2 protein levels in the heart of prediabetic rats**

HFDV rats exhibited cardiac apoptosis, as indicated by increased BAX and cleaved caspase-3/procaspase-3, along with decreased BCL-2 protein levels (Fig. 8A, B and C).

Although treatment with Nec-1 attenuated BAX and increased BCL-2 protein levels in HFD-fed rats (Fig. 8A and B), it did not affect the ratio of cleaved caspase-3/procaspase-3.
Cardiac necroptosis in prediabetic rats
N Apaijai et al.

Figure 6
Nec-1 enhanced cardiac mitochondrial fusion, but it did not affect mitochondrial fission in prediabetic rats. (A) p-DRP1/DRP1, (B) MFN1/GAPDH, (C) MFN2/GAPDH, (D) OPA1/GAPDH, (E) OXPHOS proteins. *P < 0.05 vs NDV. †P < 0.05 vs HFDV. NDV, normal diet-fed rats treated with vehicle; HFDV, high-fat diet-fed rats treated with vehicle; HFDN, high-fat diet-fed rats treated with necrostatin-1; HFDM, high-fat diet-fed rats treated with metformin; DRP1, dynamin-related protein 1; MFN, mitofusin; OPA, optic atrophy; OXPHOS, oxidative phosphorylation; I, complex I; II, complex II; III, complex III; IV, complex IV; V, complex V; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

Figure 7
Nec-1 suppressed cardiac necroptosis protein levels in prediabetic rats. (A) TNF-α/GAPDH, (B) p-RIPK1/RIPK1, (C) p-RIPK3/RIPK3, (D) p-MLKL/MLKL. *P < 0.05 vs NDV. †P < 0.05 vs HFDV. NDV, normal diet-fed rats treated with vehicle; HFDV, high-fat diet-fed rats treated with vehicle; HFDN, high-fat diet-fed rats treated with necrostatin-1; HFDM, high-fat diet-fed rats treated with metformin; TNF-α, tumor necrosis factor alpha; RIPK, receptor interacting protein kinases; MLKL, mixed lineage kinase domain-like protein; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.
caspase-3/procaspase-3, which is a protein responsible for the final process of apoptosis, when compared with HFDV and NDV rats (Fig. 8C). On the other hand, metformin effectively decreased cleaved caspase-3/procaspase-3 ratio in HFD-fed rats (Fig. 8C), with alleviated BAX and increased BCL-2 protein levels (Fig. 8A and B), when compared with HFDV and NDV rats. BAX/BCL-2 ratio was increased in HFD-fed rats treated with vehicle (HFDV), and the ratio was decreased in the Nec-1 and metformin groups (Fig. 8C).

Discussion

Data from our study demonstrated that (1) HFD consumption caused prediabetes and cardiac dysfunction in rats via inducing peripheral insulin resistance, dyslipidemia, cardiac mitochondrial dysfunction, and cardiac mitochondrial dynamic imbalance, (2) HFD consumption caused both cardiac necroptosis and apoptosis, (3) Treatment with Nec-1 directly reduced cardiac dysfunction by suppressing necroptosis, cardiac mitochondrial dysfunction, and increased mitochondrial fusion in prediabetic rats, independent of peripheral metabolic function.

Long-term HFD consumption is an accepted model of obesity and metabolic impairment, in which the result may be translated to human disease (Lai et al. 2014). Following 12 weeks of HFD consumption, our rats developed prediabetes, which is characterized by obesity, peripheral insulin resistance, and dyslipidemia, while fasting plasma glucose levels were not raised. Moreover, our prediabetic rats had cardiac autonomic imbalance, increased SBP and DBP, and cardiac dysfunction, and these data were consistent with our previous reports (Tunapong et al. 2018, Apaijai et al. 2019, Maneechote et al. 2019). LV weight is used as one of the cardiac hypertrophy indicators; however, LV weight or heart weight was not recorded, and that was considered as a limitation in this study. Nevertheless, our previous report demonstrated that the LV weight was not affected in a prediabetic model, compared to a control group (Tanajak et al. 2016).

Regarding cell death, cardiac apoptosis has been reported in HFD-fed rats (Hsieh et al. 2016, Tanajak et al. 2017, Chen et al. 2019), while the report on cardiac necroptosis following HFD consumption is limited (Giricz et al. 2017). Necroptosis is initiated by inflammation (Dhuriya & Sharma 2018), as we showed that cardiac TNF-α protein levels were upregulated in our prediabetic rats. The interaction between TNF-α and TNF receptor 1 mediates RIPK1 activity (Kearney et al. 2015). RIPK1 serves as an upstream of necroptosis. Its phosphorylation instigated RIPK3 phosphorylation, followed by MLKL phosphorylation, oligomerization, and transferred to the plasma membrane to induce necroptosis (Ying et al. 2021). There is only one study, conducted by Giricz and colleagues investigating the involvement of cardiac necroptosis in the heart of hypercholesterolemic rats. They reported that RIPK1, RIPK3, and MLKL proteins levels

![Figure 8](https://joe.bioscientifica.com)
were not upregulated in hypercholesterolemic rats (Giricz et al. 2017). Although the levels of necrototic proteins were not affected by a high-cholesterol diet consumption in that study, our study is the first study to report that the activity of necroptosis proteins was markedly increased in prediabetic rats.

Nec-1 is a specific inhibitor for RIPK1, which binds to the hydrophobic pocket of kinase domain near the ATP-binding active center and potently inhibits RIPK1 phosphorylation (Degterev et al. 2008, Takahashi et al. 2012). It directly mediated cardioprotection in prediabetic rats without the modulation of peripheral metabolic parameters. The effects of Nec-1 on the peripheral organ were reported in our previous study, in which Nec-1 did not reduce hepatic insulin resistance in prediabetic rats (Jinawong et al. 2020). In addition to necroptosis inhibition, Nec-1 reduced cardiac mitochondrial dysfunction in prediabetic rats, suggesting the roles of RIPK1 on the regulation of cardiac mitochondrial function. The interaction between RIPK1 and mitochondrial function has been reported in the model of compression-induced rat nucleus pulposus cell injury, where Nec-1 directly inhibited mPTP opening, thereby reducing mitochondrial membrane depolarization and ROS generation (Chen et al. 2018). Our data also demonstrated that Nec-1 reduced mitochondrial ROS levels and membrane depolarization, thus decreasing mitochondrial swelling. This result implied that Nec-1 reduced mitochondrial dysfunction through inhibition of mPTP. Moreover, Nec-1 promoted mitochondrial fusion that may enhance mitochondrial ATP production in prediabetic rats, leading to improved cardiac function. The effects of Nec-1 on hemodynamic parameters in prediabetic rats are still unknown, and this should be investigated in a future study.

Regarding the interaction between Nec-1 and apoptosis, although Nec-1 attenuated BAX and increased BCL-2 expression, it did not suppress cleaved caspase-3 protein expression. Since BAX is activated by various stimulators including oxidative stress and BCL-2 is known as a negative regulator of BAX (Westphal et al. 2011), Nec-1 might reduce BAX and increase BCL-2 through a reduction of mitochondrial oxidative stress. In the previous studies, Nec-1 (1.65 mg/kg/day for 4 weeks) was not able to reduce cleaved caspase-3 in the bone of ovariectomized rats (Cui et al. 2016), and it did not reduce cardiac apoptosis in cardiac I/R injury when it was acutely injected at 3.5 mg/kg during ischemia (Smith et al. 2007). Thus, we speculated that a higher or longer dose of Nec-1 is required to reduce the activity of cleaved caspase-3 and apoptosis. The summary of our findings is shown in Fig. 9.

Metformin is a standard treatment for patients with type 2 diabetes (Bosi 2009), and it has been used as a standard treatment in preclinical studies of metabolic syndrome (Gou et al. 2020, Jinawong et al. 2020). Metformin

Figure 9
The summary of findings from this study. Our data suggested that Nec-1 effectively inhibited necroptosis, improved cardiac mitochondrial function and mitochondrial fusion, resulting in improved cardiac function in prediabetic rats. Nec-1, necrostatin 1; RIPK, receptor interacting protein kinases; MLKL, mixed lineage kinase domain-like protein; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2; LV, left ventricle.
effectively reduced peripheral insulin resistance and dyslipidemia in our prediabetic rats, and cardiac function was improved by metformin treatment. The mechanism of metformin on the improvement of cardiometabolic function was thoroughly investigated in many studies (Hardie et al. 2006, Apaijai et al. 2012, Driver et al. 2018, Lu et al. 2019, Du et al. 2020). Metformin is an activator for AMP-activated protein kinase (AMPK), which is a cellular energy sensor, and it could inhibit mitochondrial complex I activity, resulting in increased ATP production (Agius et al. 2020). Mitochondrial complex I substantially contributes to mitochondrial ROS production during electron transport (Vinothkumar et al. 2014, Diniz Vilela et al. 2016), therefore, a blockage of complex I by metformin potentially inhibited mitochondrial ROS production in our prediabetic rats. Our results demonstrated that the protein expressions of the cardiac mitochondrial complexes (complexes I–V of the electron transport chain) were not altered in prediabetic rats. The interesting data from the previous study reported that the activity of mitochondrial complex I and complexes II+III was reduced in the mitochondria from parotid and submandibular glands, along with increased mitochondrial ROS levels in HFD-fed rats, suggesting that they are potential sites of ROS production during HFD feeding (Zalewska et al. 2019). Therefore, future investigation regarding the roles of Nec-1 on mitochondrial complex activity and energy production is needed to warrant its mitochondrial protection in prediabetic rats. In addition, metformin has been reported to enhance antioxidants, such as manganese superoxide dismutase and catalase in myocardial ischemia–reperfusion injury (Wang et al. 2017). Thus, an increased antioxidant might be another factor related to a decreased mitochondrial oxidative stress, resulting in decreased mitochondrial dysfunction, mitochondrial dynamic imbalance, and apoptosis in our prediabetic rats. Noteworthy, metformin did not involve in the necroptosis process since it had no effect on MLKL activity; however, it markedly reduced p-RIPK3 levels. RIPK3 can regulate mitochondrial-dependent apoptosis via activating PGAMS/CypD/mPTP opening pathway (Ying et al. 2021). Therefore, suppression of RIPK3 is another proposed mechanism of metformin in reducing cardiac apoptosis, which was observed in this study.

This study contains some limitations. Although our data showed that HFD consumption led to both cardiac necroptosis and apoptosis, the effects of apoptosis inhibitor on cardiac function and molecular mechanisms were not investigated. Recently, several cell death pathways have been discovered including ferroptosis, pyroptosis, NETosis, mPTP-mediated necrosis, and parthanatos, and they were proposed as mechanisms responsible for cardiac dysfunction (Del Re et al. 2019). A future study is suggested to clarify the roles of each cell death pathways in the model of metabolic syndrome, and this would help the scientific community to develop a new therapeutic strategy for preventing/reducing cardiovascular complications in metabolic syndrome patients. In our prediabetic rats, Nec-1 and metformin exerted cardioprotective effects, and their mechanisms are not fully overlapped. Metformin also improved metabolic profile; however, a long-term high dose metformin treatment possibly causes lactic acidosis (Misbin 2004). Therefore, a combination of low-dose metformin with Nec-1 might provide synergistic effects. Future studies are needed to investigate this issue. Several studies suggested that necroptosis was observed in other cardiac diseases such as myocardial ischemia and ischemia–reperfusion injury (Garvin et al. 2018, Piamsiri et al. 2021). In addition to acute ischemia–reperfusion injury in obese condition, it is known that heart failure is usually a serious consequence after acute myocardial infarction. Therefore, chronic treatment with Nec-1 might exert cardioprotective effects against heart failure caused by chronic myocardial infarction. Therefore, future investigations are needed to clarify this point.

Conclusion

Nec-1 directly suppressed necroptosis, cardiac mitochondrial dysfunction, and increased mitochondrial fusion independent to peripheral metabolic function, leading to an improved cardiac function in prediabetic rats.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-21-0134.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S C C and N C conceived and designed the experiment. N A, K J, K S, T J, and S K performed the experiments. N A, S C C, and N C analyzed the data. S C C and N C contributed reagents/materials/analysis tools. N A, S C C, and N C wrote the manuscripts. All authors reviewed the final version of this manuscript.

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