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Original Article

Protective effect of Momordica charantia fruit extract on TNFα-induced NF-κB activation and cardiomyocyte apoptosis

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Abstract: The aim of the present study was to investigate the effect of Momordica charantia fruit extract on tumor necrosis factor-α (TNF-α)-induced cardiac injury. Momordica charantia is reported to possess a number of pharmacological actions, including anti-inflammatory, anti-oxidative and anti-hyperglycemic effects. However, the role of Momordica charantia in TNFα-induced cardiac apoptosis and oxidative stress has remained elusive. In the present study, H9c2 rat cardiomyocytes were stimulated by TNFα and treated with Momordica charantia. The results showed that Momordica charantia regulated the expression of Bcl-2 and Bax, and rescued H9c2 cells from apoptosis induced by TNF-α. In addition, Momordica charantia inhibited the ROS production induced by TNFα and increased the mRNA expression levels of antioxidant including superoxide dismutase, catalase and glutathione peroxidase which were suppressed by TNFα. Furthermore, the activation and nuclear translocation of nuclear factor-κB (NF-κB) in TNFα-treated H9c2 cells were blocked by Momordica charantia treatment which was assessed by western blot and immunofluorescence staining. These results suggest that Momordica charantia protects cardiomyocytes from TNFα-induced apoptosis and oxidative stress, which may partly through the inhibition of NF-κB pathway.

Keywords: Momordica charantia, TNFα, apoptosis, antioxidant, NF-κB, H9c2 cells

Introduction

TNF-α, a pro-inflammatory cytokine, is elevated in many pathogenic conditions including cardiovascular injury and disease states [1, 2]. Evidence confirmed that prolonged overexpression of TNF-α provokes the induction of cardiomyocyte apoptosis [3, 4], which contributes to the pathophysiology of several heart diseases, including cardiac hypertrophy [5], myocardial infarction [6], and heart failure [7]. A series of studies have illustrated that many signaling pathways, including mitogen activated protein kinases (MAPKs) [8], phosphatidylinositol 3-kinase (PI3K)/AKT [3, 9] and especially, NF-κB [10, 11], play important roles in TNF-α induced cardiovascular injury. Thus, therapies focused on the mechanisms that regulate these pathways, may become a new strategy to blur the TNF-α induced cardiac injury.

Momordica charantia (MC) fruit, commonly known as bitter gourd or bitter melon, belongs to the family of Cucurbitaceae [12]. Various species of MC are used traditionally in many countries of the world for several human ailments including constipation, fever, hemorrhage, epilepsy, gout, boils, snakebites, diabetes, malaria, hypertension, difficult childbirth, bronchitis, protozoal, bacterial and fungal infections, and so forth [13]. Recently, studies reported that extract from MC exhibited remarkable anti-inflammatory and antioxidants activities [14, 15]. MC fruit extract also be reported to possesses anti-hyperglycemic, anti-oxidative, and cardio-protective properties which can protect rat heart from hyper-glycaemia-induced cardiac fibrosis [16]. This suggests that MC has a cardio-protective role. However, it has yet to be elucidated whether MC could protect TNFα-induced cardiomyocytes injury.

In the present study, TNFα was used to induce cardiac injury in H9c2 rat cardiomyocytes treated with MC, and the effects on cell viability, apoptosis and oxidative stress were investigated. In addition, the possible mechanisms underlying the effects of MC on these processes were examined.

Materials and methods

Materials

MC fruit extract was obtained from Ronghe Medical Technology Development Company (Shanghai, China). TNF-α was obtained from Biovision (San Francisco, USA). TRIzol® was obtained from Invitrogen Life Technologies. Rat cardiac H9c2 cells were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin and streptomycin were obtained from Gibco-BRL (Invitrogen Life Technologies, Carlsbad, CA, USA) Transcriptor First Strand cDNA Synthesis kit and Light Cycler® 480 SYBR Green I Master mix were obtained from Roche Diagnostics (Basel, Switzerland). The primary antibodies included antibodies specific for P-IκB, T-IκB, P-P65 and T-P65, Bax, Bcl-2, c-caspase3 and GAPDH were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The secondary antibodies were obtained from LI-COR Biosciences (Lincoln, NE, USA). RCL2 was obtained from ALPHELYS (Plaisir, France). Alexa FluorH 488 goat anti-mouse immunoglobulin (Ig)G and DAPI were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

H9c2 cardiomyocyte culture

Rat cardiac H9c2 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin/100 mg/ml streptomycin and 5% CO_2 at 37°C. Cells were seeded at a density of 1×10^6 per well in six-well culture plates for mRNA extraction, at a density of 10×10^7 per well in 10-cm-diameter culture plates for protein extraction, and at a density of 5.0×10^3 per well in 24-well plates for staining. Cells were cultured in serum-free medium for 6-8 h and treated with MC for 12 h in the presence of TNF-α (10 ng/ml) stimulation.

Cell viability

The cell viability was evaluated using a CCK-8, according to the manufacturer's instructions. Briefly, following MC treatment for 12 h, 10 μL CCK-8 solution was added to each well of 96-well plate and, after 4 h incubation the absorbance was measured at 450 nm using an ELISA reader (Synergy HT, Bio-tek, Vermont, USA). The effect of MC on cell viability was expressed as the percentage cell viability compared with the vehicle group, which was set at 100%.

ROS measurement

The level of intracellular ROS generation was accessed using a fluorescent dye 2',7'-dichlorofluorescin diacetate (DCFH-DA). After the indicated treatments, cells were washed twice with PBS and then incubated with serum-free DMEM and 1×10^{-5} mol/L DCFH-DA in a 37°C incubator for 30 minutes. After that, cells were washed with PBS for three times in order to eliminate the residues DCFH-DA. Cells from each group were analyzed by excitation and emission spectrum at 488 and 525 nm with Synergy HT, respectively. Data were collected and analyzed, the means of fluorescence intensity (FI) of the five wells were used to measure the percentage of ROS level according to the formula: ROS level (%) = $(FI_{treatment}\text{-}FI_{control\ group}) \times 100\%$. A florescence microscope (BX51; Olympus Corp.) was also used to evaluate the DCF florescence of cells on coverslips.

Quantitative real-time PCR

Total RNA was extracted from H9C2 cells with TRIzol reagent according to the manufacturer's instructions (Invitrogen). Their yields and purities were spectrophotometrically estimated using the A260/A280 andA230/260 ratios via a SmartSpec Plus Spectrophotometer (Bio-Rad). The RNA (2 μg of each sample) was reverse-transcribed into cDNA using oligo (dT) primers and the Transcriptor First Strand cDNA Synthesis Kit with a 96-well thermal cycler. (Applied Biosystems). We then used SYBR Green PCR Master Mix to quantify PCR amplifications with the Light Cycler 480 instrument (Software version 1.5, Roche) and examined the relative mRNA expression of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx), which were normalized to the GAPDH gene. The PCR cycling conditions were as follows: Initial activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The primer sequences used were as follows: SOD, F 5'-CAAAGGAGA-

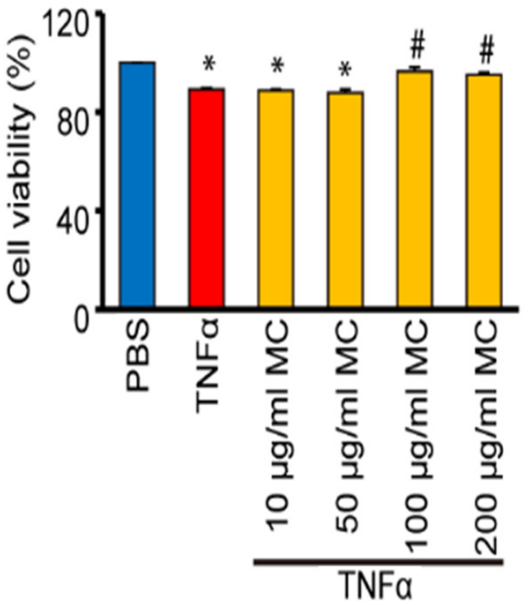

Figure 1. Effect of MC fruit on TNFα-induced cardiomyocytes cytotoxicity. The cell viability in cells treated with TNFα and indicated concentration of MC (10, 50, 100, 200 μg/ml) for 12 h. *P<0.05, compared with PBS group; #P<0.05, compared with TNFα group.

GTTGCTGGAGG-3'; R 5'-AGCAGTGGAATAAGGCCTGT-3'; CAT, F 5'-ATGAGGAGGAGAGGAAACGC-3'; R 5'-TTCCCTTGGCAGCTATGTGA-3'; Gpx, F 5'-GGTTTCCCGTGCAATCAGTT-3'; R 5'-GTACTTGGGGTCGGTCATGA-3'; GAPDH, F 5'-GACATGCCGCCTGGAGAAAC-3'; R 5'-AGCCCAGGATGCCCTTTAGT-3'.

Western blot analysis

Cultured cardiac H9c2 cells were lysed in radioimmuno precipitation (RIPA) lysis buffer (720 μL RIPA, 20 μL PMSF (1 mM), 100 μL complete, 100 μL cOmplete (Roche, Indianapolis, IN, USA, 04693124001), 100 μL phostop (Roche, 04906837001), 50 μL NaF (1 mM), 10 μL Na3V04, per ml) and 30 μg cell lysate was used for protein separation by 10% SDS-PAGE. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) and incubated with specific primary antibodies overnight at 4°C prior to incubation with IRDye 800CW-conjugated secondary antibodies. The blots were scanned using a two-color infrared imaging system (Odyssey, LI-COR Biosciences).

Immunoflorescence

Cells were analyzed for cardiac c-caspase3 expression by immunofluorescence. The cells were washed with phosphate-buffered saline (PBS), fixed with RCL2 and permeabilized in 0.1% Triton™ X-100 in PBS. The cells were then incubated with anti-c-caspase3 at a dilution of 1:100 in 1% goat serum. The cells were then incubated with Alexa FluorH 488 goat anti-mouseIgG secondary antibody. The cells on cover slips were mounted onto glass slides with Slow Fade Gold antifadereagent with DAPI. A florescence microscope (BX51; Olympus Corp.) was also used to evaluate the florescence of cells on coverslips.

Statistical analysis

Values are presented as the mean ± standard error of the mean. Differences among the groups were determined by two-way analysis of variance followed by a post hoc Turkey test. Comparisons between two groups were performed using the unpaired Student's t test. $P<0.05$ was considered to indicate a statistically significantly difference.

Results

Effect of MC fruit on TNFα-induced cardiomyocytes cytotoxicity

The effects of MC on TNFα-induced cytotoxicity on H9c2 cells were assessed by CCK-8 assay. TNFα caused a remarkable decrease in cell viability. Treatment with 10 and 50 μg/ml MC could not rescue the cytotoxicity on H9c2 cells induced by TNFα, while 100 and 200 μg/ml MC significantly inhibited the TNFα-induced cytotoxicity on H9c2 cells (**Figure 1**). Thus, two dosages of 100 and 200 μg/mL MC were used in the following study.

MC fruit attenuates TNFα-induced apoptosis in cardiomyocytes

To investigate the possible protective role of MC in moderating TNFα-induced apoptosis of H9c2 cardiomyocytes, c-caspase3, the apoptosis actuator, staining was used to identify the apoptotic cells. As expected, TNFα-induced a significant increase in the number of apoptotic cells, and 100 and 200 μg/mL MC treatment markedly reduced TNFα-induced cell apoptosis (**Figure 2A**). In addition, the apoptosis associated proteins were determined by western blot. As a result, a noticeable increase in pro-apoptosis protein Bax expression and decrease in anti-apoptosis protein Bcl-2 expression were observed in cells incubated with TNFα. Both

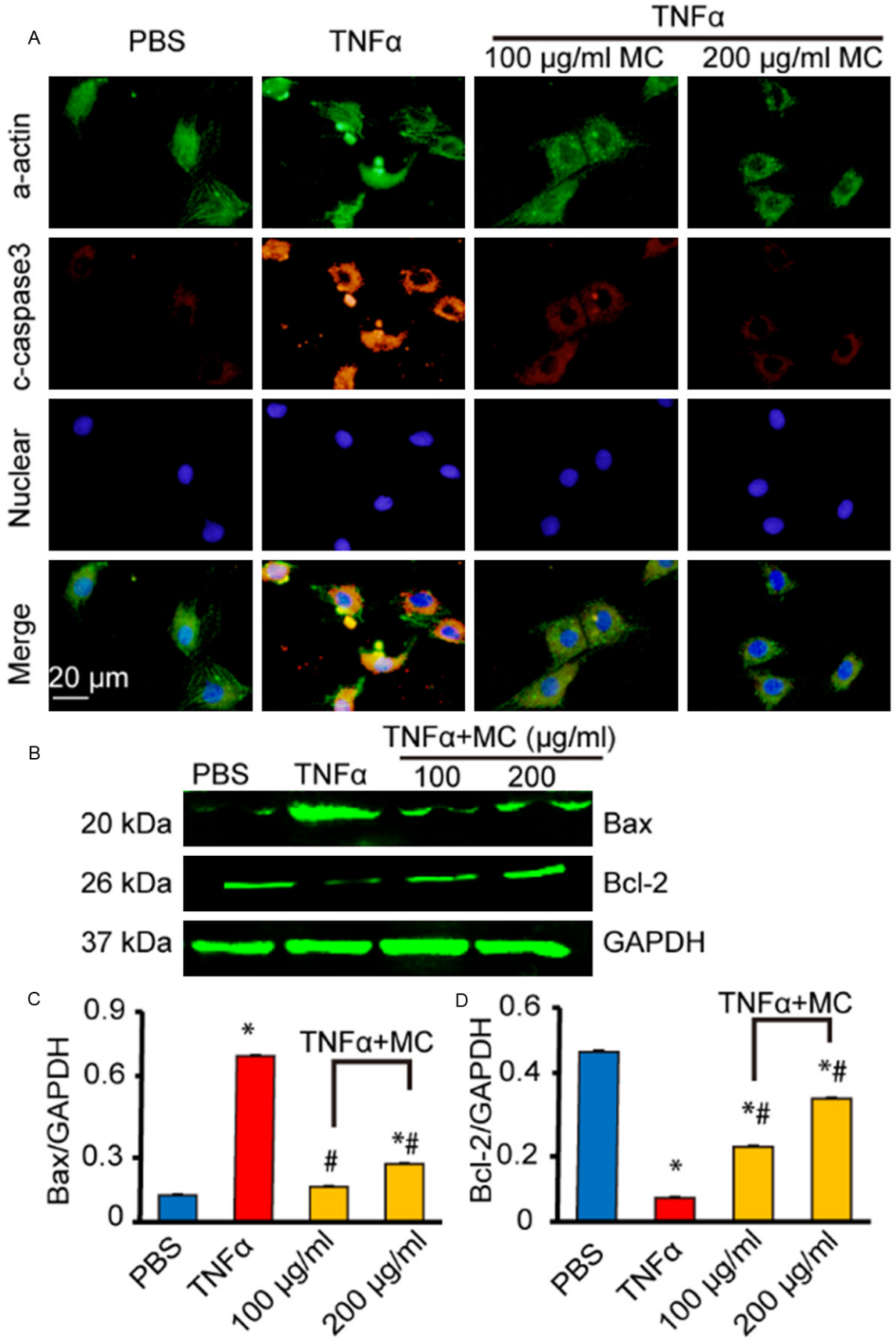
A
PBS
TNFα
TNFα
100 μg/ml MC
200 μg/ml MC
a-actin
c-caspase3
Nuclear
Merge
20 μm
B
TNFα+MC (μg/ml)
PBS
TNFα
100
200
20 kDa
Bax
26 kDa
Bcl-2
37 kDa
GAPDH
C
Bax/GAPDH
0.9
0.6
0.3
0
*
TNFα+MC
#
*#
PBS
TNFα
100 μg/ml
200 μg/ml
D
Bcl-2/GAPDH
0.6
0.4
0.2
0
TNFα+MC
*
*#
*#
PBS
TNFα
100 μg/ml
200 μg/ml

Figure 2. MC fruit attenuates TNFα-induced apoptosis in cardiomyocytes. A. Effect of MC (100, 200 μg/ml) on the TNFα-induced c-caspase3 activation among cardiomyocytes detected by immunofluorescence. B-D. The effect of MC (100, 200 μg/ml) on the activation of apoptotic signaling pathways including Bax and Bcl-2 determined by Western blot analysis. B. Representative blots. C and D. Quantitative results. **P*<0.05, *vs*. PBS group; #*P*<0.05, *vs*. TNFα group.

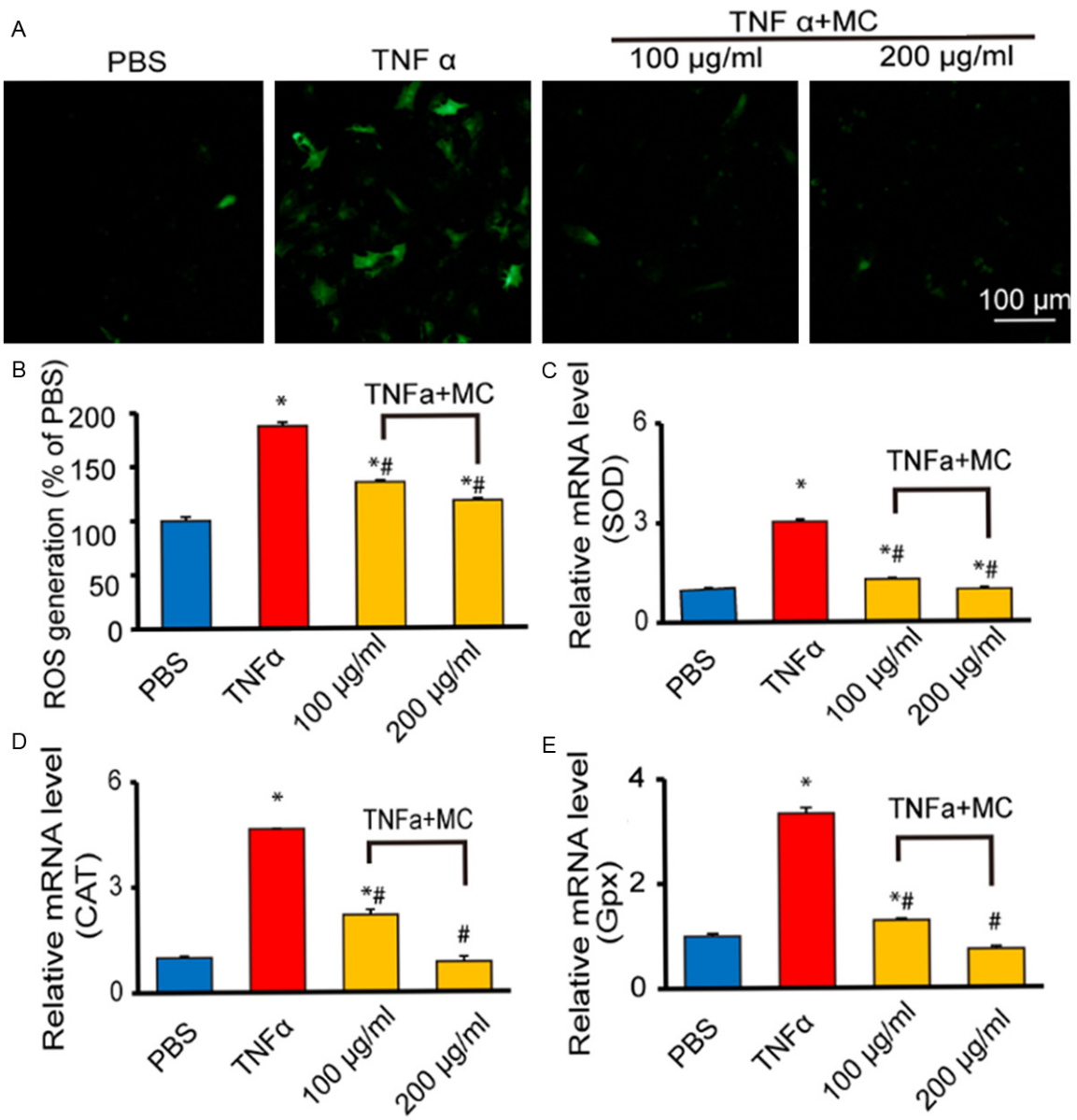

Figure 3. MC fruit inhibits the oxidative stress induced by TNFα in cardiomyocytes. A and B. MC (100, 200 μg/ml) inhibits the ROS production induced by TNFα. A. Detected using a florescence microscope; B. Detected by a florescence reader. C-E. Real-Time PCR analysis of the mRNA levels of SOD, CAT and Gpx induced by MC (100, 200 μg/ml) with TNFα for 12 h. **P*<0.05 *vs*. PBS group; #*P*<0.05 vs. TNFα group.

100 and 200 μg/mL MC decreased the levels of expression of Bax protein, while increase the Bcl-2 protein expression levels in cells following TNFα stimulation (**Figure 2B-D**). This mechanism may in part mediate the anti-apoptotic effect of MC.

MC fruit inhibits the oxidative stress induced by TNFα in cardiomyocytes

Cells incubated with DCFH-DA were used to determine the ROS production. A remarkable increased ROS was observed in H9c2 cells

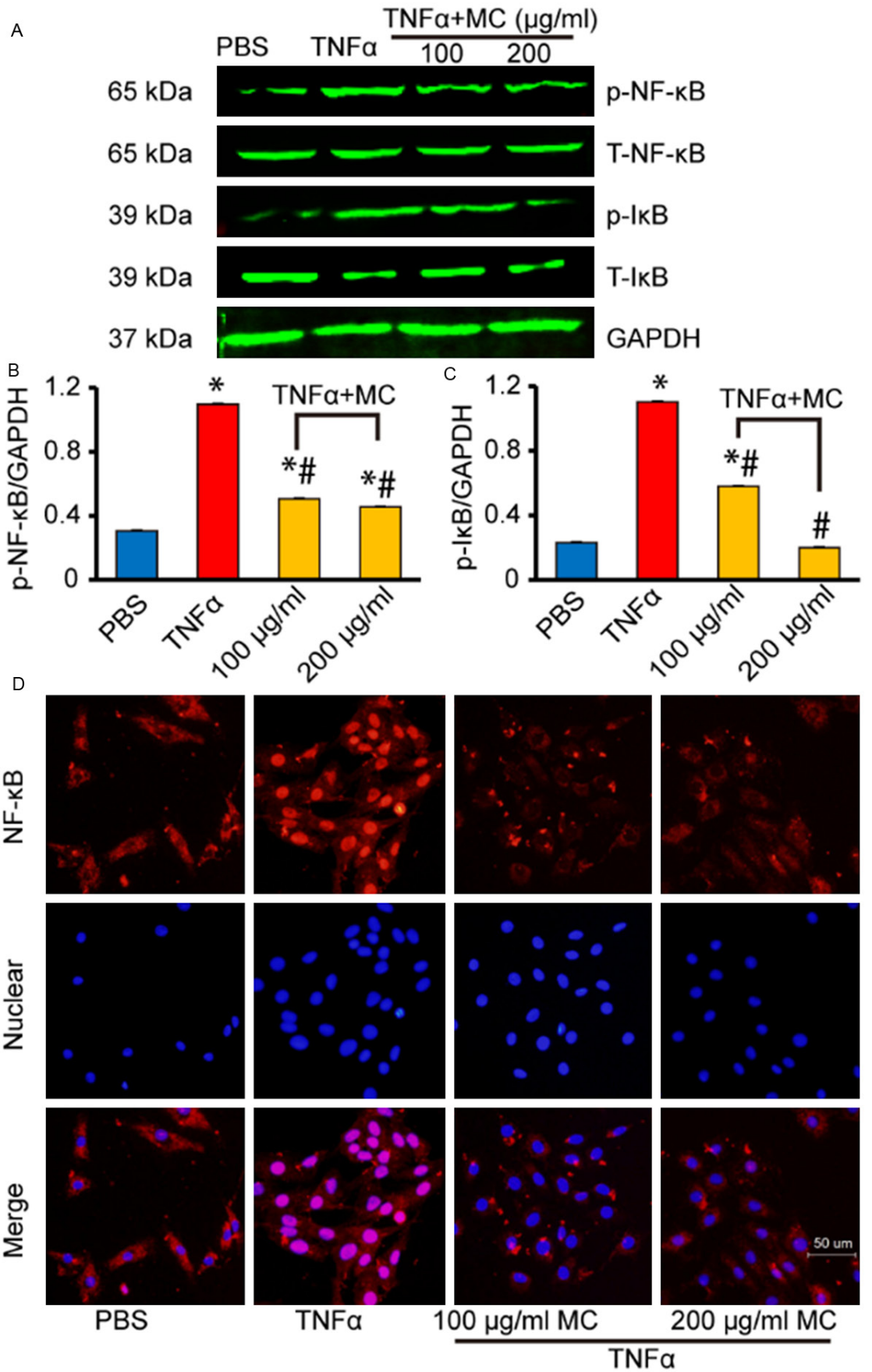
A
TNFα+MC (μg/ml)
PBS
TNFα
100
200
65 kDa
p-NF-κB
65 kDa
T-NF-κB
39 kDa
p-IκB
39 kDa
T-IκB
37 kDa
GAPDH
B
p-NF-κB/GAPDH
1.2
0.8
0.4
0
*
TNFα+MC
*#
*#
PBS
TNFα
100 μg/ml
200 μg/ml
C
p-IκB/GAPDH
1.2
0.8
0.4
0
*
TNFα+MC
*#
#
PBS
TNFα
100 μg/ml
200 μg/ml
D
NF-κB
Nuclear
Merge
50 um
PBS
TNFα
100 μg/ml MC
200 μg/ml MC
TNFα

Figure 4. MC fruit inhibits the activation of NF-κB in response to TNFα in cardiomyocytes. A-C. Western blot analysis the effect of MC (100, 200 μg/ml) on the activation of NF-κB pathways including phosphorylated (p-) NF-κB, IκB and total (T-) NF-κB, IκB. A. Representative blots. B and C. Quantitative results. **P*<0.05 vs. PBS group; #*P*<0.05 vs. TNFα group. D. Effect of MC (100, 200 μg/ml) on the TNFα induced nuclear translocation of NF κB detected by immunofluorescence.

treated with TNFα, while different concentrations of MC fruit (100 and 200 μg/ml) dramatically suppressed the increased ROS generation induced by TNFα (**Figure 3A** and **3B**). The effect of MC on the induction of antioxidants genes expression was also measured by RT-PCR. Stimulation with TNFα for 12 h significantly decreased the mRNA levels of SOD, CAT and Gpx in H9c2 cells. And both the 100 and 200 μg/mL MC significantly blurred this decrease, which exhibited no difference between these two concentrations (**Figure 3C-E**).

MC fruit inhibits the activation of NF-κB in response to TNFα in cardiomyocytes

It is well recognized that NF-κB plays important roles in TNF-α-induced cardiovascular injury. To further investigate the mechanisms underlying the effects of MC on TNFα induced cardiomyocytes injury, western blot analysis and immunofluorescence were used to detect the NF-κB pathway. The results showed that the phosphorylation and degradation of IκB in H9c2 cells were significantly elevated by TNFα stimulation. MC treatment (100 and 200 μg/ml) significantly inhibited this TNFα-induced phosphorylation and degradation of IκB and subsequently decreased the phosphorylation and nuclear translocation of NF-κB (**Figure 4A-D**).

Discussion

MC fruit has been reported in previous studies to possess multiple biological activities including anti-hyperglycemic, anti-oxidative, and preventing cardiac fibrosis. The present study examined the anti-apoptosis, anti-oxidative and cardiomyocytes protective effects of MC fruit. Our data showed that TNFα induced apoptosis and suppression of antioxidants in cardiomyocytes by an activation of NF-kB pathway. MC fruit prevented the TNFα-induced apoptosis and suppression of antioxidants by down-regulating the IKK phosphorylation and in turns NF-kB activation.

Evidence proofs that apoptosis plays a key role in the pathogenesis of cardiovascular disease due to loss of terminally differentiated cardiomyocytes [5]. TNFα is the canonical member of the TNF superfamily, which mediates both inflammation and apoptosis [17]. TNF-α, binding to its cell surface receptors, triggers apoptotic signaling through caspase-3 activation and causes apoptosis, which named extrinsic apoptosis pathway. In addition, TNF-α can also increases the production of reactive oxygen species and mitochondrial injury, which named intrinsic apoptosis pathway [3]. Both these pathway disturbs the balance between anti- and pro-apoptotic proteins of Bcl-2 family, which determines whether cells undergo apoptosis or survive [18]. MC fruit down-regulated the expression of pro-apoptosis protein Bax but up-regulated the anti-apoptosis protein Bcl-2 in TNF-α-stimulated cardiomyocytes. These changes may contribute to the anti-apoptotic effect of MC fruit.

The intimate link between inflammatory cytokines and oxidative stress has long been established [19]. TNFα interacted with other hypertrophic factors such as Ang II to induce oxidative stress, contributing to cardiac pathophysiology [20]. Previous studies have shown that MC fruit protects diabetic mice against global cerebral ischemia-reperfusion- induced cerebral oxidative stress and damage [21], and inhibits oxidative stress in chronic streptozotocin-induced diabetic rats. These findings indicated that MC fruit has anti-oxidative effects. The present study showed that MC fruit decreased ROS production and increased antioxidants genes expression, which may partly explain the anti-apoptosis effect of MC fruit.

The NF-κB, a family member of transcription factors, is a key molecule that participate in the regulation of the inflammatory response, as well as cell growth, proliferation and apoptosis [22]. NF-κB dimmers are inactivated by binding to IκBs in the cytoplasm, which inhibits the translocation of NF-κB to the nucleus. The phosphorylation of IκBs induced by various stimulations, causes themselves ubiquitination and degradation, and subsequently leads to the release and activation of NF-κB [23]. It has

been reported that NF-κB plays an important role in TNF-α-induced cardiovascular injury [10, 11]. The present study indicated that MC fruit inhibited the phosphorylation and degradation of IκBs and suppressed subsequent the activation and nuclear translocation of NF-κB induced by TNF-α, suggesting that MC fruit attenuated TNF-α induced apoptosis and oxidative stress by inhibiting the NF-κB signaling pathways.

In conclusion, our study has clearly demonstrated a previously unknown effect of MC fruit on TNF-α-induced cardiomyocyte apoptosis and oxidative stress. Indeed, MC fruit is able to trigger a down-regulation of the NF-κB pathways. Moreover, our results provide experimental evidence for the use of MC fruit in the treatment of inflammatory disorders in heart.

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Disclosure of conflict of interest

None.

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