Original Paper

Protective Effect of 2-Dodecyl-6-Methoxycyclohexa-2, 5-Diene-1, 4-Dione, Isolated from *Averrhoa Carambola L.*, Against Palmitic Acid-Induced Inflammation and Apoptosis in Min6 Cells by Inhibiting the TLR4–MyD88–NF- κ B Signaling Pathway

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Key Words

DMDD • Min6 cell • Inflammation • Apoptosis • TLR4 • MyD88 • NF- κ B

Abstract

Background/Aims: Studies have demonstrated that 2-dodecyl-6-methoxycyclohexa-2, 5-diene-1, 4-dione (DMDD), isolated from the roots of *Averrhoa carambola L.*, has significant therapeutic potential for the treatment of diabetes. However, the protective effect of DMDD against pancreatic beta cell dysfunction has never been reported. We investigated whether DMDD protected against palmitic acid-induced dysfunction in pancreatic β -cell line Min6 cells by attenuating the inflammatory response and apoptosis and to shed light on its possible mechanism. **Methods:** Cell viability was assessed by CCK-8. Glucose-stimulated insulin secretion levels and inflammatory cytokines levels were examined by ELISA. Apoptosis was assessed by Annexin V-FITC/PI Flow cytometry assay, Hoechst 33342/PI double-staining assay, and Transmission electron microscopy assay. Relative quantitative real-time PCR and western blot were used to determine the expressions of genes and proteins. **Results:** Cell viability and glucose-stimulated insulin secretion levels were increased in DMDD-pretreated Min6 cells. DMDD inhibited inflammatory cytokines IL-6, TNF- α and MCP-1 generations in palmitic acid (PA)-induced Min6 cells. Moreover, DMDD protected against PA-induced Min6 cells apoptosis and the expression of Cleaved-Caspase-3, -8 and -9 were down-regulated and the Bcl-2/Bax ratio was increased in DMDD-pretreated Min6 cells. In addition, the expression of TLR4, MyD88 and NF- κ B were down-regulated in DMDD-pretreated Min6 cells and TAK-242-pretreated group cells. **Conclusions:** DMDD protected Min6 cells against PA-induced dysfunction by attenuating the inflammatory response and apoptosis, and its mechanism of this protection was associated with inhibiting the TLR4–MyD88–NF- κ B signaling pathway.

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Introduction

Many complications result from the metabolic disorders of pancreatic β -cell dysfunction [1]. Inflammation and apoptosis play crucial roles in the progression of pancreatic β -cell dysfunction [2].

Many studies have demonstrated that decreases in pro-inflammatory cytokines that contribute to pancreatic β -cell dysfunction, such as IL-6, TNF- α and MCP-1 have a beneficial effect on pancreatic β -cell dysfunction [3]. TLR4 is an important molecular receptor in innate immunity and inflammatory responses. TLR4 is directly activated by palmitic acid (PA), one of free fatty acids, resulting in the activation of the distinct adaptor protein MyD88. MyD88-dependent signaling pathway leads to the release of NF- κ B from I κ B α , resulting in the increased levels of downstream inflammatory cytokines [4]. Apoptosis also plays a crucial role in the progression of pancreatic β -cell dysfunction. Apoptosis involves two major pathways, the extrinsic and intrinsic [5]. The extrinsic pathway, also known as the death receptor pathway, initiates the Cleaved-Caspase-8 dependent signal cascade [6]. The intrinsic pathway, also known as the mitochondrial pathway, involves the release of apoptotic proteins, such as cytochrome c, Cleaved-Caspase-3, and Cleaved-Caspase-9 [7]. Bcl-2 family proteins play an important role in intrinsic apoptosis pathway [8]. These proteins are either anti-apoptotic proteins such as Bcl-2 or pro-apoptotic proteins such as Bax, and the interactions among them may influence cell fate [9]. The Bcl-2 to Bax ratio is critical for determining whether apoptosis occurs [10]. A decrease in this ratio promotes the release of cytochrome c from the mitochondria into the cytosol, leading to the subsequent activation of Caspase-3 and Caspase-9 [11]. Apoptosis has a beneficial effect on pancreatic β -cell dysfunction.

A new drug or method to obstruct the process of Min6 cell dysfunction is significant. *Averrhoa carambola* L. is a perennial herb widely distributed in Southeast Asia. Its roots have been employed in Traditional Chinese Medicine (TCM) for thousands of years. *Averrhoa carambola* L. is commonly used to treat headaches, vomiting, coughing and hangovers [12]. Furthermore, it is used as an appetite stimulant, a diuretic, and as an antidiarrheal and febrifugal agent [13]. In our previous work, we found that the extract obtained by decocting the roots of *Averrhoa carambola* L. had been used in the treatment of streptozotocin-induced diabetic mice [14], and reduced the expression levels of TLR4 and NF- κ B [15]. We also found that 2-dodecyl-6-methoxycyclohexa-2, 5-diene-1, 4-dione (DMDD) (Fig. 1), isolated from the roots of *Averrhoa carambola* L., had a beneficial effect on advanced glycation end-product-mediated renal injury in type 2 diabetic KKAY mice [16]. However, the protective effect of DMDD against pancreatic β -cell line Min6 has never been reported. We investigated whether DMDD protected against PA-induced inflammation and apoptosis, and whether its mechanism of this protection was associated with inhibiting the TLR4–MyD88–NF- κ B signaling pathway.

Materials and Methods

Plant materials and extraction and isolation of DMDD

The *Averrhoa carambola* L. plant material was obtained from Lingshan County, Guangxi Autonomous Region, China, in October 2012 and was identified by Lai Mao-xiang at Traditional Chinese Medicine Research Institute of Guangxi. The voucher specimen (No. 20121016) was deposited in the Guangxi Institute of Chinese Medicine & Pharmaceutical Science herbarium (Guangxi, China). The isolation of DMDD was prepared using a method as described previously [17]. DMDD was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mmol/l. In all tests, the DMDD stock solution was further diluted in cell culture medium by 100 fold or more, and 0.5% DMSO was used as a vehicle control in the untreated cells.

Cell culture

Min6 cells, purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (CTCCAS, Shanghai, China), were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, New

York, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, New York, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA) in a humidified incubator at 37 °C with 5% CO₂.

Cell viability analysis

The cytotoxicity of DMDD was first measured by using a Cell Counting Kit-8 (CCK8) (Dojindo Laboratory, Kumamoto, Japan). Briefly, Min6 cells were seeded into a 96-well plate overnight and treated with concentrations (0 μmol/l, 10 μmol/l, 20 μmol/l, 30 μmol/l, 40 μmol/l, and 50 μmol/l) of DMDD. After incubation for 24 h, cell survival was measured by using a CCK8 as described previously [18].

Next, Min6 cells were seeded into a 96-well plate overnight. Cells were pretreated with concentrations (5 μmol/l, 10 μmol/l, 15 μmol/l, 20 μmol/l, 25 μmol/l and 30 μmol/l) of DMDD for 24 h and then exposed to 0.4 mmol/l PA (dissolved in dimethyl sulfoxide (DMSO) at a concentration of 40 mmol/l and further diluted in cell culture medium by 100 fold) for 24 h. 0.5% DMSO was used as a vehicle control in the untreated cells. After treatment, cell viability was measured by using a CCK8 (Dojindo Laboratory, Japan) as described previously [18].

Glucose-stimulated insulin secretion levels analysis

Glucose-stimulated insulin secretion levels were measured by using an Insulin ELISA Kit. Briefly, Min6 cells were seeded into a 24-well plate overnight. Cells were pretreated with concentrations (10 μmol/l, 15 μmol/l, and 20 μmol/l) of DMDD for 24 h and then exposed to 0.4 mmol/l PA for 24 h. After treatment, cells were incubated for 1 h in glucose-free Krebs-Ringer Bicarbonate (KRB) buffer (115 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, 20 mmol/l NaHCO₃, 16 mmol/l HEPES, 2.56 mmol/l CaCl₂, 0.2% BSA) (all from Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). Afterward, cells were incubated for 1 h in KRB buffer with basal (2.8 mmol/l) and high glucose levels (16.7 mmol/l). Insulin levels were measured by using an Insulin ELISA Kit (Sigma-Aldrich, St. Louis, Missouri, USA) as described previously [19].

Inflammatory cytokines IL-6, TNF-α, MCP-1 levels analysis

Inflammatory cytokines IL-6, TNF-α, and MCP-1 levels in DMDD-pretreated Min6 cells were measured by using IL-6, TNF-α, and MCP-1 ELISA Kits (all from Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) as previously described [20].

Annexin V-FITC/PI Flow cytometric analysis

The rate of apoptosis in PA-induced Min6 cells was measured by using the Annexin V-FITC/ PI Kit (Life Technologies, Carlsbad, CA, USA), and analyzed on a FACStar-Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) as previously described [21].

Hoechst 33342/ PI double-staining analysis

The numbers of apoptotic cells in PA-induced Min6 cells were also investigated by using the Hoechst 33342/PI double-staining Kit (Life Technologies, Carlsbad, CA, USA) as previously described [22], and cell images were captured using a fluorescence microscopy (Olympus, Tokyo, Japan). Apoptotic cells were counted using a cell counter under a fluorescence microscope at 200 × magnifications, and 4 randomly selected fields in each group were used to estimate the average numbers of apoptotic cells.

Transmission electron microscopy analysis

Ultrastructure changes of apoptosis were observed using a transmission electron microscope (Hitachi, Tokyo, Japan). Transmission electron microscopy analysis was conducted as previously described [23].

Relative quantitative real-time PCR analysis

Total RNA was extracted by using RNAiso Plus kits (Takara Biotechnology Co. Ltd, Dalian, China) and cDNA was synthesized from RNA with a PrimeScript™ RT reagent Kit (Takara Biotechnology Co. Ltd, Dalian, China) as described previously [24]. RT-PCR was performed on an ABI Prism 7300 real-time thermocycler (Applied Biosystems, Foster City, CA, USA) using the TransScript™ SYBR® Green Master Mix kit (Takara

Fig. 1 Chemical structure of DMDD.

Table 1. Primers information for RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
TLR4	CATGGATCAGAACTCAGCAAAGTC	CATGCCATGCCTTGTCTTCA	179bp
MyD88	TACAGGTGGCCAGAGTGGA	GCAGTAGCAGATAAAGGCATCGAA	119bp
NF- κ B	GAACGATAACCTTTGCAGGC	TTTCGATTCCGCTATGTGTG	130bp
IL-6	GACGTTTCAGAGGTTCTCAGAG	TAGTCCTTCTACCCCAATTTCC	180bp
TNF- α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	176bp
MCP-1	CCACAGCATGGACGAATTCA	AGCTTGCTTTGTGGCCTTCA	154bp
Bax	CAGGATGCGTCCACCAAGAA	CGTGTCCACGTCAGCAATCA	102bp
Bcl-2	AGCGTCAACAGGGAGATGTC	TTCCACAAAGGCATCCAGC	389bp
β -actin	AACCTTAAGGCCAACCGTGAAAAG	TCATGAGGTAGTCTGTCAGGT	186bp

Biotechnology Co. Ltd, Dalian, China) as described previously [25]. β -actin was used as an endogenous control. The amount of genes expression was normalized by the $2^{-\Delta\Delta CT}$ relative quantitative method. The primers information for PCR is given in Table. 1.

Western blotting analysis

Protein isolation and western blot was conducted as previously described [26]. Proteins were detected with primary antibodies to TLR4, MyD88, NF- κ B, I κ B α , Cleaved-Caspase-3, -8, -9, Bax, and Bcl-2 (all from Cell Signaling Technology, Boston, MA, USA).

Statistical analysis

Statistic analysis was performed using the SPSS 17 statistical program (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare the means among different groups. Multiple comparisons between the groups were performed using S-N-K method. The data are presented as the mean $\pm$ SD. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Results

DMDD increases cell viability in PA-induced Min6 cells

As shown in Fig. 2(a), DMDD showed a non-toxic range at concentrations of 0–30 μ mol/l for 24 h. As shown in Figure. 2(b), cell viability was significantly lower in cells only treated with PA, and this decrease was prevented by pretreating cells with DMDD. No significant difference was found between the 5 μ mol/l DMDD-pretreated group and the PA group. There was a similar viability among the 20 μ mol/l, 25 μ mol/l and 30 μ mol/l DMDD-pretreated groups. Therefore, we used the low-, medium-, and high-concentrations of DMDD (10 μ mol/l, 15 μ mol/l, and 20 μ mol/l) for the following studies with cultured Min6 cells.

DMDD increases glucose-stimulated insulin secretion levels in PA-induced Min6 cells

As shown in Fig. 3, glucose-stimulated insulin secretion levels were significantly lower in cells only exposed to PA, and this decrease was prevented by pretreating cells with DMDD at high glucose levels (16.7 mmol/l). No significant difference was found between basal (2.8 mmol/l) glucose-stimulated groups.

DMDD reduces inflammatory cytokines levels in PA-induced Min6 cells

As shown in Fig. 4(a), the expressions of IL-6, TNF- α , and MCP-1 mRNA were up-regulated in PA group cells. However, the expressions of these genes were down-regulated in DMDD-pretreated group cells. As shown in Fig. 4(b), these inflammatory cytokines levels were significantly higher in cells only exposed to PA, and this increase was prevented by pretreating cells with DMDD.

DMDD protects Min6 cell against PA-induced apoptosis

The representative images for AnnexinV-FITC/PI flow cytometry analysis were presented in Fig. 5(a). As shown in Fig. 5(d), the rate of apoptosis was significantly higher

Fig. 2. DMDD increases cell viability in PA-induced Min6 cells. (a) The cytotoxicity of DMDD on Min6 cells. (b) DMDD increases cell viability in PA-induced Min6 cells. All data are presented as the mean $\pm$ SD. ($n=3$). # $P \leq 0.05$, compared with untreated group. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$, compared with PA group.

Fig. 3. DMDD increases glucose-stimulated insulin secretion levels in PA-induced Min6 cells. All data are presented as the mean $\pm$ SD. ($n=3$). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Fig. 4. DMDD reduces inflammatory cytokines levels in PA-induced Min6 cells. (a) DMDD inhibits the expressions of IL-6, TNF- α and MCP-1 mRNA in PA-induced Min6 cells. (b) DMDD inhibits the inflammatory cytokines IL-6, TNF- α and MCP-1 generations in PA-induced Min6 cells. All Data are presented as the mean $\pm$ SD. ($n=3$). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

in cells only exposed to PA, and this increase was prevented by pretreating cells with DMDD.

The representative images for Hoechst 33342/PI double-staining analysis were presented in Fig. 5(b). High blue fluorescent indicated early apoptotic cells (bright blue arrow), and low blue indicated live cells (azury arrow). While high red represented necrotic cells (pink arrow), and low pink represented late apoptotic cells (bright pink arrow). As shown in Fig. 5(e), the numbers of

Fig. 5. DMDD protects Min6 cell against PA-induced apoptosis. (a) The representative images for AnnexinV-FITC/PI flow cytometry analysis. (b) The representative images (magnifications: $\times 200$) for Hoechst 33342/PI double-staining analysis. (c) The representative images (magnifications: $\times 15000$ and magnifications: $\times 30000$) for transmission electron microscopy analysis. Nu: nuclear; Mi: mitochondria; En: endoplasmic reticulum; Va: cytoplasm vacuolization. (d) DMDD decreases the rate of apoptosis in PA-induced Min6 cells using AnnexinV-FITC/PI flow cytometry analysis. (e) DMDD decreases the numbers of apoptotic cells using Hoechst 33342/PI double-staining analysis. All data are presented as the mean $\pm$ SD. ($n=3$). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

apoptotic cells, including early and late apoptotic cells, were significantly higher in cells only exposed to PA, and this increase was prevented by pretreating cells with DMDD.

As shown in Fig. 5(c), PA group cells displayed cell shrinkage, irregular nuclear outline, condensed chromosomes in apoptotic cells, a loose endoplasmic reticulum, morphologically abnormal mitochondrial structures, and cytoplasm vacuolization. The ultrastructural changes of apoptosis in the DMDD-pretreated group cells appeared to be improved compared with PA group cells.

Fig. 6. DMDD inhibits the expressions of Cleaved-Caspase-3, -8, -9, and increases the Bcl-2/Bax ratio in PA-induced Min6 cells. (a, b) DMDD inhibits the expression of Bax mRNA and increases the expression of Bcl-2 mRNA. (c, d, e, f, g, h, i, j) DMDD inhibits the expressions of Cleaved-Caspase-3, -8, -9 proteins, and increases the Bcl-2/Bax rate. All data are presented as the mean $\pm$ SD. (n=3). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

DMDD inhibits the expressions of Cleaved-Caspase-3, -8, -9, and increases the Bcl-2/Bax ratio in PA-induced Min6 cells

As shown in Fig. 6(c, e, f, g), the expressions of Cleaved-Caspase-3, -8, and -9 proteins were up-regulated in cells only exposed to PA. However, the expressions of these proteins were down-regulated in cells pretreated with DMDD. As shown in Fig. 6(a, b, d, h, i, j), the expressions of Bax mRNA and protein were up-regulated whereas the expressions of Bcl-2 mRNA and protein were down-regulated in PA group cells. However, the expressions of Bax mRNA and protein were down-regulated whereas the expressions of Bcl-2 mRNA and protein were up-regulated in cells pretreated with DMDD. The Bcl-2/Bax ratio was significantly lower in cells only exposed to PA, and this decrease was prevented by pretreating cells with DMDD.

DMDD inhibits the TLR4-MyD88-NF- κ B signaling pathway in PA-induced Min6 cells

TAK-242, a TLR4 pathway inhibitor, was a control drug to determine whether the protective effects of DMDD against PA-induced inflammation and apoptosis in Min6 cells and to further explore the impact of DMDD on the downstream targets of the TLR4-MyD88-

Fig. 7. DMDD inhibits the expressions of TLR4, MyD88 and NF-κB in PA-induced Min6 cells. PA group: PA (0.4 mmol/l). DMDD-pretreated group: DMDD (20 μmol/l) + PA (0.4 mmol/l). TAK-242-pretreated group: TAK-242 (1 ng/l) + PA (0.4 mmol/l). DMDD and TAK-242-pretreated group: DMDD (20 μmol/l) + TAK-242 (1 ng/l) + PA (0.4 mmol/l). (a, b, c) DMDD inhibits the expressions of TLR4, MyD88 and NF-κB mRNA in PA-induced Min6 cells. (d, e, f, g, h) DMDD inhibits the expressions of TLR4, MyD88, IκBα and NF-κB proteins in PA-induced Min6 cells. All data are presented as the mean ± SD. ($n=3$). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

NFκB signaling pathway. As shown in Fig. 7, the expressions of TLR4, MyD88, and NF-κB mRNA and proteins were up-regulated in PA group cells. However, the expressions of these genes and proteins were down-regulated in DMDD-pretreated group cells and TAK-242-pretreated group cells. DMDD and TAK-242 also suppressed the phosphorylation of IκBα, which leads to IκBα degradation and release of NF-κB.

Discussion

Natural products derived from plants have long been used in folk medicine, making the compounds derived from these plants good candidates for new therapeutic strategies. In our previous work, we provided evidence that the extract obtained by decocting the roots of

Averrhoa carambola L. had a beneficial effect on streptozotocin-induced diabetic mice [14], and reduced the expression levels of TLR4 and NF- κ B [15]. We also isolated 2-dodecyl-6-methoxycyclohexa-2, 5-diene-1, 4-dione (DMDD) from the roots of *Averrhoa carambola* L., and found it beneficial on advanced glycation end-product-mediated renal injury in type 2 diabetic KKAY mice [16]. However, the protective effect of DMDD against pancreatic β -cell line Min6 has never been reported. In this work, we provide evidence for and mechanistic insight into how DMDD exerts a beneficial effect on pancreatic β -cell line Min6 dysfunction.

Inflammation is believed to be a key feature in the pathogenesis of diabetes and cytokines are known to contribute to the failure and death of beta cells [27]. The immune system is activated by TNF- α , and leukotrienes and platelet-activating factor are then synthesized and mobilized, which results in an inflammatory response [28]. An extended duration of hyperglycemia produces glycation products that cause excess IL-6 synthesis and initiate β -cell dysfunction. Our work indicated that DMDD reduced PA-induced inflammation damage in Min6 cells by reducing the levels of IL-6, TNF- α , and MCP-1.

Apoptosis is widely recognized to play an important role in the pathogenesis of Min6 cell dysfunction. Apoptosis can be induced by either the intrinsic or the extrinsic pathway. Caspase-8 was the initiator caspase of the extrinsic pathway. Caspase-3 and Caspase-9 were the initiator caspase of the intrinsic pathway. Bcl-2 family members play an important role in intrinsic apoptosis pathway. The ratio of Bcl-2 to Bax is critical for determining whether apoptosis occurs. A decrease in this ratio promotes the release of cytochrome c from the mitochondria into the cytosol, leading to the subsequent activation of Caspase-9 and Caspase-3. Our work indicated that DMDD affected the extrinsic apoptotic pathway by suppressing Caspase-8, and affected the intrinsic apoptotic pathway by suppressing Cleaved-Caspase-3 and Cleaved-Caspase-9, and increasing the Bcl-2/Bax ratio. DMDD protected Min6 cell against PA-induced apoptosis involving both the extrinsic and intrinsic signaling pathways.

Numerous studies have shown that PA induces Min6 cell dysfunction by the TLR4 pathway [29]. With the stimulation of PA, TLR4 and MyD88 are activated, which leads to the degradation of I κ B α proteins and the subsequent translocation of the transcription nuclear factor κ B (NF- κ B) [30]. In our work, we used TAK-242, a TLR4 pathway inhibitor, as a control drug to determine whether the protective effects of DMDD against PA-induced inflammation and apoptosis in Min6 cells and to further explore the impact of DMDD on the downstream targets of the TLR4–MyD88–NF- κ B signaling pathway. The expression of TLR4, MyD88, and NF- κ B were down-regulated in DMDD-pretreated Min6 cells and TAK-242-pretreated group cells. Our work indicated that DMDD protected Min6 cells against PA-induced inflammation and apoptosis in Min6 cells by inhibiting the TLR4–MyD88–NF- κ B signaling pathway.

Collectively, we provide evidence that DMDD protects Min6 cells against PA-induced inflammation and apoptosis, and its mechanism of this protection is associated with inhibiting the TLR4–MyD88–NF- κ B signaling pathway. DMDD may be a promising candidate for the treatment of pancreatic β -cell Min6 dysfunction.

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Disclosure Statement

All authors declare that there is no duality of interest associated with this manuscript.

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