Cannabidiol Attenuates Cardiac Dysfunction, Oxidative Stress, Fibrosis, and Inflammatory and Cell Death Signaling Pathways in Diabetic Cardiomyopathy

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Abstract

Objectives In this study, we have investigated the effects of cannabidiol (CBD) on myocardial dysfunction, inflammation, oxidative/nitrative stress, cell death, and interrelated signaling pathways, using a mouse model of type I diabetic cardiomyopathy and primary human cardiomyocytes exposed to high glucose.

Background Cannabidiol, the most abundant nonpsychoactive constituent of Cannabis sativa (marijuana) plant, exerts anti-inflammatory effects in various disease models and alleviates pain and spasticity associated with multiple sclerosis in humans.

Methods Left ventricular function was measured by the pressure-volume system. Oxidative stress, cell death, and fibrosis markers were evaluated by molecular biology/biochemical techniques, electron spin resonance spectroscopy, and flow cytometry.

Results Diabetic cardiomyopathy was characterized by declined diastolic and systolic myocardial performance associated with increased oxidative/nitrative stress, nuclear factor-κB and mitogen-activated protein kinase (c-Jun N-terminal kinase, p-38, p38α) activation, enhanced expression of adhesion molecules (intracellular adhesion molecule-1, vascular cell adhesion molecule-1), tumor necrosis factor-α, markers of fibrosis (transforming growth factor-β, connective tissue growth factor, fibronectin, collagen-1, matrix metalloproteinase-2 and -9), enhanced cell death (caspase 3/7 and poly(adenosine diphosphate-ribose) polymerase activity, chromatin fragmentation, and terminal deoxynucleotidyl transferase dUTP nick end labeling), and diminished Akt phosphorylation. Remarkably, CBD attenuated myocardial dysfunction, cardiac fibrosis, oxidative/nitrative stress, inflammation, cell death, and interrelated signaling pathways. Furthermore, CBD also attenuated the high glucose-induced increased reactive oxygen species generation, nuclear factor-κB activation, and cell death in primary human cardiomyocytes.

Conclusions Collectively, these results coupled with the excellent safety and tolerability profile of CBD in humans, strongly suggest that it may have great therapeutic potential in the treatment of diabetic complications, and perhaps other cardiovascular disorders, by attenuating oxidative/nitrative stress, inflammation, cell death and fibrosis.
Cardiovascular complications are the leading cause of morbidity and mortality in diabetic patients. Diabetic cardiomyopathy characterized by myocardial left ventricular dysfunction (both diastolic and later systolic), independent of atherosclerosis and coronary artery disease, has been well documented in both humans and animals (1-3). The mechanism of diabetic cardiac dysfunction is complex and involves increased oxidative/nitrative stress (4-7), activation of various downstream transcription factors, pro-inflammatory and cell death pathways such as nuclear factor-κB (NF-κB) (8-9), poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) (10), and mitogen-activated protein kinase (MAPK) (11-12), inactivation of pro-survival pathways such as Akt (13), eventually culminating in cell death (14) and changes in the composition of extracellular matrix with enhanced cardiac fibrosis and increased inflammation (15).

Various components of the Cannabis sativa (marijuana) plant, termed cannabinoids (e.g., the most characterized active ingredient, the delta 9-tetrahydrocannabinol [THC]), exert potent analgesic effects through the activation of classic CB1 receptors located in the central nervous system and anti-inflammatory properties through the activation of CB2 cannabinoid receptors on immune cells (16). However, the major limitation of the therapeutic utility of THC is the development of centrally mediated CB1-dependent psychoactive effects (16). Furthermore, the CB1 receptor activation in the cardiovascular system by endocannabinoids may also contribute to the pathophysiology of multiple cardiovascular

### Abbreviations And Acronyms

- **ADP**: adenosine diphosphate
- **CBD**: cannabidiol
- **HCM**: human cardiomyocytes
- **HG**: high glucose
- **HNE**: hydroxynonenal
- **ICAM**: intercellular adhesion molecule
- **IκB-α**: inhibitor of nuclear transcription factor nuclear factor-κB
- **iNOS**: inducible nitric oxide synthase
- **JNK**: c-Jun N-terminal kinase
- **MAPK**: mitogen-activated protein kinase
- **MMP**: matrix metalloproteinase
- **NADPH**: nicotinamide adenine dinucleotide phosphate
- **NF-κB**: nuclear factor kappa B
- **NT**: nitrotyrosine
- **PARP**: poly(ADP-ribose) polymerase
- **ROS**: reactive oxygen species
- **SOD**: superoxide dismutase
- **THC**: delta 9-tetrahydrocannabinol
- **TNF**: tumor necrosis factor
- **TUNEL**: terminal deoxynucleotidyl transferase dUTP nick end labeling
- **VCAM**: vascular cell adhesion molecule
diseases, including heart failure and atherosclerosis (17). In contrast to THC, cannabidiol (CBD), the most abundant cannabinoid of Cannabis sativa, which has been approved for the treatment of inflammation, pain, and spasticity associated with multiple sclerosis in humans since 2005 in Canada (18), does not bind to these receptors (19); therefore, it is devoid of psychoactive properties and has no potential to cause adverse cardiac toxicity (20). Importantly, CBD is well tolerated without side effects when chronically administered to humans (21-22).

A previous study has demonstrated cardiac protection by CBD in myocardial ischemic reperfusion injury (23); therefore, we have investigated the potential protective effects of CBD in diabetic hearts and in primary human cardiomyocytes exposed to high glucose. Our findings underscore the potential of CBD for the prevention/treatment of diabetic complications.

Methods

Animals and treatment

All the animal protocols conformed to the National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee of National Institute on Alcohol Abuse and Alcoholism (NIAAA)-NIH. Diabetes mellitus was induced in male C57/BL6J mice 8 to 12 weeks old, weighing 23 to 25 g (Jackson Laboratories, Bar Harbor, Maine) by intraperitoneal injection of streptozotocin (Sigma, St. Louis, Missouri) at the dose of 50 mg/kg dissolved in 100 mM citrate buffer pH 4.5 for 5 consecutive days. After 1 week, blood glucose levels were measured using Ascensia Coutour Glucometer (Bayer HealthCare, Tarrytown, New York) by mandibular vein puncture blood sampling. Mice that had blood sugar values >250 mg/dL were used for the study. In the first set of experiments 1-week diabetic mice were treated with CBD (1, 10, or 20 mg/kg intraperitoneally) or vehicle for 11 weeks (5). In another set of experiments, 8-week diabetic mice were treated with CBD or vehicle for 4 weeks (5). The CBD was isolated as described earlier (24). The corresponding control groups were treated with either vehicle or CBD alone for the same duration. All the animals were provided with food and water ad libitum.

Hemodynamic measurements in mice

Left ventricular performance was measured in mice anesthetized with 2% isoflurane as previously described (25-26).

Determination of superoxide dismutase activity, malondialdehyde, reduced glutathione, oxidized glutathione, 4-HNE, and protein carbonyl content

The superoxide dismutase (SOD) activities, and reduced glutathione and oxidized glutathione, malondialdehyde, 4-HNE, and protein carbonyl levels in the myocardial tissues were determined as described in the Online Appendix.

Determination of myocardial reactive oxygen species (ROS) by electron paramagnetic resonance spectrometer is described in the Online Appendix.

Reverse transcription and real-time polymerase chain reaction

Preparation of samples and reverse transcription and real-time polymerase chain reaction (PCR) experiments from heart tissues and the primers are described in the Online Appendix and 5.

Determination of PARP, caspase 3/7 activities, chromatin fragmentation, TUNEL, and 3-NT content

The PARP and caspase 3/7 activities, chromatin fragmentation, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and 3-nitrotyrosine (3-NT) content in the heart homogenates and/or human cardiomyocyte extracts are described in the Online Appendix.

Western immunoblot analysis

Sample preparations, Western immunoblot analysis, and sources of antibodies are described in the Online Appendix.

Immunohistochemistry

The immunohistochemistry/staining from frozen or formalin-fixed myocardial tissues (nitrotyrosine, TUNEL, Sirius red) is described in the Online Appendix.

Cell culture studies

Human cardiomyocytes (HCM) along with the culture medium were purchased from ScienCell Research.
Simultaneous determination of cytosolic and mitochondrial ROS generation and apoptosis by flow cytometry

Mitochondrial superoxide/ROS generation and cell death were determined as described (27) and are detailed in the Online Appendix.

Statistical analysis

Results are expressed as mean ± SEM. Statistical comparisons were made by 1-way analysis of variance followed by Newman-Keuls post-hoc analysis using GraphPad Prism 5 software (San Diego, California). When heterogeneity of variance was present, analysis of variance was performed after logarithmic transformation of the data. Probability values of p < 0.05 were considered significant.

Results

Blood glucose levels, pancreas insulin content, and body weights

Diabetic animals exhibited increased blood glucose levels (5) with the decrease in the body weight (5). Diabetic animals also had increased glycosylated hemoglobin (HbA1c) levels with concomitant decline in the pancreas insulin content (5). The CBD or vehicle treatment (1, 10, or 20 mg/kg intraperitoneally) for 11 or 4 weeks did not significantly alter the body weight, blood glucose level, or pancreas insulin content in either control or diabetic animals (5).

CBD treatment attenuates diabetes-induced hemodynamic alterations

Twelve weeks of established diabetes was associated with impaired diastolic and systolic left ventricular function, which was largely attenuated by the treatment with CBD for 11 weeks (starting 1 week after the establishment of diabetes) (Figure 1). The CBD treatment also improved the diabetes-induced myocardial dysfunction when it was given for 4 weeks in 8-week diabetic mice (5).

CBD treatment attenuates diabetes-induced myocardial oxidative stress

There was increased accumulation of lipid peroxides (Figure 2A and Figure 2B), protein carbonyls (Figure 2C), ROS generation (Figure 2D), expression of messenger ribonucleic acid of various ROS-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (p22phox, p67phox, gp91phox) (Figure 2E) with concordant decrease of reduced/oxidized glutathione ratio (Figure 2F) and attenuated activity of the...
superoxide-eliminating enzyme, the SOD (Figure 2G), in hearts of diabetic mice. These changes were attenuated when mice were treated with CBD for 11 weeks during the course of the diabetes (Figure 2A to Figure 2G).

**Figure 2**

CBD Attenuates Diabetes-Induced Myocardial Oxidative Stress

Oxidative stress in the myocardial tissues were determined by measuring (A) malondialdehyde (MDA), (B) 4-HNE, (C) protein carbonyl content, and (D) reactive oxygen species (ROS) levels by electron paramagnetic resonance spectrometer, as described in the Methods section, and the (E) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits messenger ribonucleic acid (mRNA) expression by real-time reverse transcriptase-polymerase chain reaction, (F) endogenous antioxidants (reduced glutathione [GSH] and oxidized glutathione [GSSG]) content, and (G) superoxide dismutase (SOD) activity. *p < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #p < 0.05 versus diabetes (D), n = 6 to 9 per group.

CBD treatment attenuates diabetes-induced myocardial nuclear factor-κB activation and inflammation

As shown in Figure 3A, there was a marked inhibitor of nuclear transcription factor NF-κB (IκB-α) degradation in the cytosol of diabetic hearts, with increased phosphorylation of IκB-α leading to release of active p65 NF-κB, which subsequently translocates to the nucleus to induce the inflammatory and apoptotic gene expressions (Figure 3B). Gel shift assay also confirmed the NF-κB activation in diabetic hearts (Figure 3C). The CBD treatment of diabetic mice inhibited the IκB-α and subsequent p65NF-κB nuclear translocation (Figure 3A to Figure 3C). The CBD treatment also inhibited the NF-κB–dependent mRNA and/or protein expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Figure 3D and Figure 3F) and pro-inflammatory cytokine tumor necrosis factor (TNF)-α (Figure 3E and Figure 3G), respectively, in the diabetic myocardial tissues.

**Figure 3**

CBD Attenuates Diabetes-Induced Myocardial NF-κB Activation

(A) Western blot analysis demonstrates inhibitor of nuclear transcription factor NF-κB (IκB-α) expression and its phosphorylation in the cytosolic fraction and (B) the nuclear translocation of p65 nuclear factor (NF)-κB in the nuclear fraction of the heart tissue homogenates. (C) The gel shift assay demonstrates NF-κB activation. (D) The messenger ribonucleic acid (mRNA) expression of ICAM (intercellular adhesion molecule)-1 and VCAM (vascular cell adhesion molecule)-1. (E) Tumor necrosis factor (TNF)-α in the respective groups, as indicated. (F) Western blot analysis for the protein expression of ICAM-1/VCAM-1, and (G) TNF-α protein in the myocardial tissues. *p < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #p < 0.05 versus diabetes (D), n = 6 to 9 per group.
CBD treatments attenuates diabetes-induced nitrative stress

There was significant increase in inducible nitric oxide synthase (iNOS) expression (Figure 4A) and 3-NT accumulation (Figure 4B to Figure 4E) in hearts of diabetic mice compared to vehicle or CBD alone treated mice. The CBD treatment attenuated the diabetes-induced iNOS expression and 3-NT accumulation (marker of nitrative stress) (Figure 4B to Figure 4E).

Figure 4

CBD Inhibits Diabetes-Induced Myocardial, iNOS Expression, and 3-NT Accumulation

(A) Expression of inducible nitric oxide synthase (iNOS) was determined by Western immunoblot in the heart tissues. (B) Levels of 3-nitrotyrosine (3-NT) in the heart samples were quantitatively determined by enzyme-linked immunosorbent assay with indicated cannabidiol (CBD) concentration (mg/kg body weight), respectively. (C) Representative gel indicates the nitrated proteins analyzed by immunoprecipitation (I.P) with 3-NT specific antibody. (D) Representative images for the histochemical staining for 3-NT accumulation in the formalin-fixed myocardial tissues (400× magnification). (E) Immunofluorescence staining for 3-NT from frozen sections as described in Methods (400× magnification). *p < 0.05 versus vehicle control (Co) and CBD alone; #p < 0.05 versus diabetes (D), n = 6 to 8 per group.

CBD treatment attenuates diabetes-induced MAPK activation and apoptosis

There was marked increase in the p38MAPK (Figure 5A) and c-Jun N-terminal kinase (JNK) (Figure 5B) activation in the myocardial tissues of diabetic mice. In addition, there was marked activation p38αMAPK (Figure 5C) and slightly diminished p38βMAPK (Figure 5C) in the diabetic myocardium. There was also activation of MAPKAPK-2 in the diabetic heart (Figure 5D). CBD treatment for 11 weeks significantly mitigated p38MAPK, JNK, p38αMAPK, MAPKAPK-2 activation, while it was not effective in restoring the p38βMAPK levels. In addition, Akt activation was also significantly hampered in the diabetic myocardium, which was attenuated with CBD treatment (Figure 5E). In diabetic myocardium, there was marked increase in caspase 3 cleavage, caspase 3/7 activity (Figure 6A and Figure 6B), chromatin fragmentation, and PARP activity (Figure 6C and Figure 6D), and enhanced apoptosis (Figure 6E and Figure 7); all these changes in diabetes were attenuated by CBD treatment.

Figure 5

CBD Mitigates Diabetes-Induced Myocardial Activation of MAPKs and Augments Akt Activation

Western blot analysis shows the (A) p38 mitogen-activated protein kinase (MAPK), (B) c-Jun N-terminal kinase (JNK), (C) p38α MAPK, (D) MAPKAPK-2, and (E) Akt activation in the myocardial tissues. *p < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #p < 0.05 versus diabetes (D), n = 6 per group.
CBD Mitigates Diabetes-Induced Myocardial Apoptosis and Cell Death

(A) Western blot analysis for the cleaved (Cvcl) caspase (Casp) 3 and (B) caspase 3/7 activity, (C) chromatin fragmentation, and (D) poly(ADP-ribose) polymerase (PARP) activation and (E) quantitative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay were performed, as described in Methods. *p < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #p < 0.05 versus diabetes (D), n = 6 to 9 per group.

CBD Mitigates Apoptosis in the Diabetic Myocardium

Shown are the representative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) images in the diabetic myocardium and mice that were treated with cannabidiol (CBD) for 11 weeks. For details, see Online Appendix Supplemental Methods.

CBD treatment attenuates diabetes-associated myocardial fibrosis

Real-time reverse transcriptase-polymerase chain reaction analysis revealed significant increases in the pro-fibrotic gene expressions (Figure 8A) and in collagen deposition (Figure 8B) in diabetic hearts, and these were attenuated by CBD (Figure 8).

CBD Attenuates Diabetes-Induced Cardiac Fibrosis

(A) Messenger ribonucleic acid (mRNA) expression of the profibrotic genes in the myocardial tissues. *p < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #p < 0.05 versus diabetes (D), n = 9 per group. (B) Sirius red staining indicating collagen deposition and implying the extent of cardiac fibrosis. Images shown are representative from 4 independent experiments. *p < 0.05 versus vehicle control (Co) and cannabidiol (CBD) alone; #p < 0.05 versus diabetes (D), n = 4 to 6 per group. CTGF = connective tissue growth factor; MMP = matrix metalloproteinase; TGF = transforming growth factor.
CBD post-treatment after the establishment of diabetic cardiomyopathy attenuates diabetes-induced myocardial oxidative/nitrative stress, cell death, and fibrosis

Remarkably, CBD 20 (mg/kg) treatment also attenuated the diabetes-induced increased myocardial nitrative stress, cell death (5) and fibrosis (5) when it was given for 4 weeks in 8-week diabetic mice.

CBD treatment attenuates high glucose-induced cytosolic and mitochondrial ROS generation and 3-NT formation in HCM

High glucose (HG) treatment of HCM for 48 h markedly increased cytosolic (5) and mitochondrial (5) ROS/superoxide generation compared with cells treated with either D-glucose 5 mM, L-glucose 30 mM, or CBD (4 μM) alone for the same duration. The CBD markedly attenuated the HG-induced increased ROS generation (5) and 3-NT accumulation in HCM (5).

CBD mitigates HG-induced NF-κB activation and apoptosis in HCM

The HG treatment induced NF-κB activation (5) and increased apoptosis and PARP-dependent cell death in cardiomyocytes (5); the antiapoptotic activity of CBD was mediated, at least in part, by its ability to modulate Akt activity (5).

Discussion

Accumulating evidence suggests that increased oxidative/nitrative stress coupled with activation of various downstream pro-inflammatory and cell death pathways play pivotal roles in the development of complex biochemical, mechanical, and structural alterations associated with diabetic cardiomyopathy (3-4, 6, 11-12, 14-15). However, in spite of the accumulating knowledge obtained during the past decades, the treatment of diabetic cardiomyopathy still remains poor and largely symptomatic (1-2).

Cannabidiol, a nonpsychoactive component of marijuana, has been shown to exert anti-inflammatory and antioxidant effects both in vitro and in various preclinical models of neurodegeneration and inflammatory disorders, independent from classical CB1 and CB2 receptors (20). Furthermore, CBD has recently been reported to lower the incidence of diabetes among nonobese diabetic mice (28) and to preserve the blood-retinal barrier in experimental diabetes (29).

In the present study, we have evaluated the effects of CBD treatment (for 11 weeks administered after the destruction of pancreatic beta cells and development of frank type 1 diabetes mellitus, as well as in 8-week diabetic animals for 4 weeks) on myocardial dysfunction, inflammation, oxidative/nitrative stress, cell death, and interrelated signaling pathways, using a mouse model of type 1 diabetic cardiomyopathy or primary human cardiomyocytes exposed to HG. Because significant cardiac dysfunction in this model starts to develop from 4 weeks of established diabetes (4, 10), with gradually increasing fibrosis thereafter (12, 15) (peaking around 8 weeks of established diabetes), in the first treatment protocol (5), we aimed to study if CBD treatment can prevent the development of characteristic alterations of type 1 diabetic cardiomyopathy; in the second treatment protocol (5), we sought to determine if it is able to reverse these changes once they have already developed.

Consistent with previous reports, diabetic cardiomyopathy was characterized by declined diastolic and systolic myocardial performance associated with enhanced myocardial expression of NADPH oxidase isoforms p22phox, p67phox, gp91phox, attenuated antioxidant defense (decreased glutathione content and SOD activity) coupled with increased myocardial ROS generation and lipid peroxidation (4, 6, 10, 12, 15). The HG-induced ROS generation in addition to inducing lipid peroxidation may also initiate activation of various

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Collectively, our results strongly suggest that CBD may have tremendous therapeutic potential in the treatment of diabetic cardiovascular and other complications by attenuating diabetes-induced oxidative/nitrative stress, inflammation, cell death, and fibrotic pathways.

Author Information

Appendix

For a detailed discussion of the Methods, supplemental references, table, and figures, please see the online version of this article.

Appendix
References


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