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Pharmacological Inhibition of CB_1 Cannabinoid Receptor Protects Against Doxorubicin-Induced Cardiotoxicity

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Abstract

Objectives—We aimed to explore the effects of pharmacologic inhibition of cannabinoid-1 (CB_1) receptor in in vivo and in vitro models of doxorubicin (DOX)-induced cardiotoxicity.

Background—Doxorubicin is one of the most potent antitumor agents available; however, its clinical use is limited because of the risk of severe cardiotoxicity. Endocannabinoids mediate cardiodepressive effects through CB_1 receptors in various pathophysiological conditions, and these effects can be reversed by CB_1 antagonists.

Methods—Left ventricular function was measured by Millar pressure-volume system. Apoptosis markers, CB_1/CB_2 receptor expression, and endocannabinoid levels were determined by immunohistochemistry, Western blot, reverse transcription-polymerase chain reaction, real-time polymerase chain reaction, flow cytometry, fluorescent microscopy, and liquid chromatography/in-line mass spectrometry techniques.

Results—Five days after the administration of a single dose of DOX (20 mg/kg intraperitoneally) to mice, left ventricular systolic pressure, maximum first derivative of ventricular pressure with respect to time (+dP/dt), stroke work, ejection fraction, cardiac output, and load-independent indexes of contractility (end-systolic pressure–volume relation, preload-recruitable stroke work, dP/dt–end-diastolic volume relation) were significantly depressed, and the myocardial level of the endocannabinoid anandamide (but not CB_1/CB_2 receptor expression) was elevated compared with vehicle-treated control mice. Treatment with the CB_1 antagonists rimonabant or AM281 markedly improved cardiac dysfunction and reduced DOX-induced apoptosis in the myocardium. Doxorubicin also decreased cell viability and induced apoptosis in the H9c2 myocardial cell line measured by flow cytometry and fluorescent microscopy, which were prevented by the preincubation of the cells with either CB_1 antagonist, but not with CB_1 and CB_2 agonists and CB_2 antagonists.

Conclusions—These data suggest that CB_1 antagonists may represent a new cardioprotective strategy against DOX-induced cardiotoxicity.

Doxorubicin (DOX) (adriamycin) is one of the most potent broad-spectrum antitumor anthracycline antibiotics commonly used to treat a variety of cancers, including severe

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leukemias, lymphomas, and solid tumors (1–3). However, the clinical use of DOX is limited because of its serious cardiotoxicity, which often leads to irreversible degenerative cardiomyopathy and heart failure (3).

The mechanism of DOX-induced cardiotoxicity involves increased oxidative/nitrosative stress (4–7), matrix metalloproteinase activation (8,9), and alteration of cardiac energetics (10), which eventually lead to cell death by apoptosis or cell necrosis (11–15). However, the exact mechanisms have not been fully established, and optimal therapeutic approaches for cardioprotection remain undefined (3).

Endocannabinoids and their synthetic analogs exert powerful cardiodepressive effects mediated through cannabinoid-1 (CB_1) receptors (16,17), which have recently been implicated in the mechanism of hypotension associated with hemorrhagic, endotoxic, and cardiogenic shock, and advanced liver cirrhosis, and these effects can be prevented or reversed by treatment with CB_1 antagonists (16,17). Furthermore, the CB_1 antagonist rimonabant is emerging as a novel therapeutic agent for obesity and related cardiometabolic risk factors in humans (17–20). Here, we explore the effects of 2 CB_1 antagonists, rimonabant and AM281, on DOX-induced cardiac dysfunction and cardiotoxicity both in vivo and in vitro.

Materials and Methods

Animals

All protocols were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Male C57BL/6J mice weighing 25 to 35 g were administered a single dose of DOX HCl (Sigma/Aldrich, St. Louis, Missouri) at 20 mg/kg intraperitoneally, and used for functional measurements 5 days later when severe cardiac dysfunction is well established (6,14,15,21,22). Treatment with the CB_1 antagonists AM281 or rimonabant (SR141716; 10 mg/kg intraperitoneally, respectively) started 1.5 h before the DOX injection and continued (10 mg/kg intraperitoneally/day) until the hemodynamic measurements were made.

Hemodynamic measurements in mice

Left ventricular performance was analyzed in mice anesthetized with 2% isoflurane by using 1-F microtip pressure-volume catheter (PVR 1045) and ARIA pressure–volume conductance system (Millar Instruments, Houston, Texas) coupled to a Powerlab/4SP A/D converter (AD Instruments, Mountain View, California) as described (6) (also see the Appendix).

Reagents, antibodies, and cell culture

Doxorubicin was purchased from Sigma Chemical. AM281, AM630, JWH133, and HU210 were purchased from Tocris (Baldwin, Missouri). SR144528 and rimonabant (SR141716) are from the National Institute on Drug Abuse Drug Supply Program (Research Triangle Park, North Carolina). Antibodies used are anti-actin mAb (Chemicon, Temecula, California), anti-caspase 3 mAb (Cell Signaling, Danvers, Massachusetts), and anti-active caspase-3 (Cell Signaling).

Rat embryonic ventricular myocardial H9c2 cells were obtained from American Type Culture Collection (Manassas, Virginia). Cells were cultured in DMEM (GIBCO, Invitrogen, Carlsbad, California) containing 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 as described (23). Cells were always used at <80% of confluence.

Real-time polymerase chain reaction and reverse transcription-polymerase chain reaction analyses were performed from hearts and H9c2 cells for caspase-3, caspase-9, and $CB_{1/2}$ receptor gene expression as detailed in the Appendix.

Western immunoblot analyses

Protein was extracted from tissue homogenates using radioimmunoprecipitation assay lysis buffer, containing protease inhibitor cocktail set III and phosphatase inhibitor cocktail set I (Calbiochem, EMD Biosciences, San Diego, California). Protein was measured by Dc protein assay kit (Bio-Rad, Hercules, California), and equal amounts (40 μg per lane) were fractionated on NuPAGE 4% to 12% Bis-Tris gel (Invitrogen) and transferred onto nitrocellulose membrane (Invitrogen) using a semidry transfer apparatus (Bio-Rad). The blots were detected with Supersignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, Illinois) and developed using Kodak Biomax film (PerkinElmer, Wellesley, Massachusetts). Immunoblots were scanned with Epson V750 Pro scanner and quantification after background correction was carried out by ImageQuant5.1 software (GE Healthcare, Piscataway, New Jersey); all values were normalized to beta-actin.

Cell viability assay

Cells were seeded at a density of 1×10^5 cells in 96 well plates, and cell viability was assessed via conventional XTT assays (Roche, Indianapolis, Indiana). After incubation, the cells were treated for 6 h with XTT solution at 37°C. The absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay reader (SpectramaxPro, Molecular Devices, Union City, California).

Flow cytometry

Early apoptosis and cytotoxity is determined by flow cytometry using propidium iodide and Annexin V staining (Molecular Probes, Invitrogen, Carlsbad, California) according to manufacturer's recommendation. The cells were trypsinized for a very short time and collected via centrifugation at 1,000 μg for 5 min. The harvested cells were then washed with phosphate-buffered saline. The cells were resuspended at a density of 1×10^6 cells/ml in Hank's balanced salt solution buffer containing calcium and magnesium. Flow-cytometry analyses included 10,000 events using a FACSCalibur (Becton Dickinson, San Jose, California). The data were acquired and analyzed using Cell Quest program (Becton Dickinson).

Fluorescence microscopy, DNA fragmentation assay, myocardial terminal deoxynucleotidyltransferase-mediated nick-end labeling (TUNEL) staining, and assay and caspase 3 activity were used according to manufacturer's instructions as described in the Appendix.

Measurement of endocannabinoid levels

The levels of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) were quantified by liquid chromatography/in-line mass spectrometry as detailed in the Appendix.

Statistical analysis

Results are reported as mean ± standard error of the mean. Probability values of $p < 0.05$ were considered significant. For detailed statistical analysis see the Appendix.

Results

CB_1 antagonists improve DOX-induced cardiac dys-Function

Treatment of mice with DOX, 20 mg/kg intra-peritoneally, induced a significant decrease in heart rate, left ventricular systolic pressure, maximum first derivative of ventricular pressure with respect to time (+dP/dt), −dP/dt, stroke work, ejection fraction, cardiac output, and load-independent indexes of contractility (preload-recruitable stroke work, dP/dt–end-diastolic volume relation, and end-systolic pressure–volume relation, respectively), and an increase in left ventricular end-diastolic pressure and prolongation of relaxation time constants (τ Weiss and Glantz) (Figs. 1 and 2). Treatment with rimonabant or AM281 (10 mg/kg^{-1} intraperitoneally) immediately before and daily for 5 days after DOX treatment significantly attenuated the DOX-induced changes in ventricular function (Figs. 1 and 2). In vehicle-treated control animals, rimonabant or AM281 exerted no significant effects on the hemodynamic parameters studied (Fig. 1).

CB_1 antagonists protect against DOX-induced cell death in rat embryonic ventricular myocardial-derived H9c2 cells in vitro

Incubation of cells with 1 or 5 μmol/l DOX for 18 h resulted in significant decreases in cell viability, measured by a colorimetric XTT-based cell viability assay to 78.6 ± 1.6% or 74.4 ± 1.2%, respectively. Doxorubicin-induced cell death at 1 and 5 μmol/l was completely prevented by 2 h of preincubation (followed by continuous treatment during the 18 h DOX exposure) with 1 μmol/l AM281 (98.0 ± 4.5% and 95.3 ± 5.1%) or rimonabant (96.5 ±3.6% and 102.0 ± 4.3%, respectively) (Fig. 3A). Cannabinoid-1 receptor antagonists alone did not have any effect on cell viability (data not shown).

CB_1 antagonists, but not CB_1 and CB_2 agonists and CB_2 antagonists, prevent DOX-induced apoptosis in vitro

The H9c2 cell line was subjected to flow cytometric analysis for apoptotic and total dead cells by Annexin V and propidium iodide staining. Early apoptotic marker Annexin V was significantly increased in cells exposed to 1 μmol/l DOX for 18 h but remained at control levels in cells pretreated with either 1 μmol/l AM281 or rimonabant starting from 2 h before DOX administration (Fig. 3B). The DOX-induced total cell death as indicated by positive propidium iodide staining (Fig. 3B) confirmed recent cell viability data, including the higher level of propidium iodide staining in apoptotic compared with normal cells (14). Activation of caspase is a key downstream event of apoptosis. We measured the active form of caspase by fluorescence microscopy. As an indication of caspase activity, green color is generated by cleavage of a generic caspase substrate (rhodamine 110, bis-[L-aspartic acid amide], trifluoroacetic acid salt) introduced into live cells. The green color was conspicuously present in all DOX-treated H9c2 cells (Fig. 3C). In contrast, the green fluorescence was completely absent in cells treated with 1 μmol/l AM281 or rimonabant. We simultaneously measured the late apoptotic marker represented as fragmented nuclei using Hoechst 33342 staining (Molecular Probes, Invitrogen). Few fragmented nuclei were observed in DOX-treated cells whereas no such nuclei were present in normal, AM281, or rimonabant pretreated samples. Thus, pretreatment with either AM281 or rimonabant protects H9c2 cells from DOX-induced apoptosis as shown by early-to-late apoptotic markers.

Pretreatment for 2 h (followed by continuous treatment during the 18 h DOX exposure) with CB_2 agonist JWH133, CB_1 agonist HU210, CB_2 antagonists (AM630 and SR144528) (1 μmol/l each) did not have any protective effect against DOX-induced cell death and apoptosis (Fig. 4). Interestingly, CB_1 agonist HU210 by itself significantly enhanced apoptosis (Fig. 4).

CB_1 antagonists prevent DOX-induced apoptosis of cardiomyocytes in vivo

Myocardial caspase-3-dependent apoptosis was previously described in the mouse model of DOX-induced heart failure (14,24). We have observed significant level of cleaved caspase-3 in samples of heart tissue from DOX-treated mice. Two representative samples from each group are shown in Figure 5A. The total absence of cleaved caspase-3 in AM281 or rimonabant pretreated mice indicates the protective effect of CB_1 antagonists against apoptosis.

As shown in Figure 5B, DOX treatment induced marked elevation in the caspase-3 activity in the myocardial tissues (~2.8-fold) compared with control mice. Rimonabant/AM281 pretreatment of mice prevented the DOX-induced increase in caspase-3 activity.

We also analyzed gene expression of 2 apoptotic markers, caspase-3 and caspase-9, from the same group of heart samples. In agreement with the increased protein expression, caspase-3 mRNA was also increased significantly by DOX treatment to 4.5 ± 0.7-fold over control group, and this increase was markedly attenuated by AM281 or rimonabant pretreatment (1.6 ± 0.2 and 0.9 ± 0.2, respectively) (Fig. 5C). Doxorubicin also induced a 10.4 ± 1.6-fold increase in caspase-9 gene expression compared with normal hearts, which was reduced by pretreatment with AM281 or rimonabant to a 1.8 ± 0.6 and 2.1 ± 0.6-fold change, respectively. In vehicle-treated control mice, AM281 or rimonabant treatment did not affect caspase-3 and caspase-9 gene expression.

CB_1 receptor antagonists attenuate DOX-induced DNA fragmentation

Doxorubicin also increased myocardial TUNEL staining in the myocardium, which was largely attenuated by either rimonabant or AM281 pretreatment (Fig. 6A).

To strengthen the preceding conclusion, we used 2 additional quantitative methods. Doxorubicin-induced DNA fragmentations in the myocardial tissue was increased 2.9- and 4.5-fold compared with sham control measured by quantitative TUNEL and DNA fragmentation assays, and was largely attenuated by pretreatment with CB_1 antagonists (Figs. 6B and 6C).

Effects of DOX on myocardial CB_1 and CB_2 receptors

Myocardial CB_1 receptor protein levels were unchanged in DOX-treated mice as documented by using Western blots (Fig. 7A). The specificity of the antibodies used was confirmed by the absence of a specific band using myocardial tissue from a CB_1 knockout mouse. Myocardial CB_1 or CB_2 receptor gene expression was not affected by DOX treatment in mice as verified using reverse transcription-polymerase chain reaction (Fig. 7B) as well as quantitative real-time polymerase chain reaction where the CB_1 or CB_2 receptor gene expressions were normalized to beta-actin gene expression (Fig. 7C). Similar results were obtained in cardiomyocytes (Fig. 7D, lower part).

DOX increased endocannabinoid AEA content both in vivo and in vitro

Doxorubicin treatment resulted in increased AEA but not 2-AG content in the myocardium (Fig. 7E). Similarly, elevated AEA levels were detected in DOX-treated H9c2 cells as compared with the vehicle-treated control cells (Fig. 7F).

Discussion

The natural ligands of CB_1 receptors are the endocannabinoids AEA and 2-AG, both of which are present in the myocardium, along with CB_1 receptors (16,17). Numerous previous experimental studies have demonstrated that activation of the endocannabinoid system may contribute to the hypotension and compromised cardiovascular function in a variety of pathophysiological states (e.g., hemorrhagic, endotoxic, and cardiogenic shock, advanced liver cirrhosis, and cirrhotic cardiomyopathy) through the activation of myocardial and vascular CB_1 receptors. Importantly, in all these conditions, the cardiovascular depressive effects could be prevented or reversed by various CB_1 antagonists (16,17).

Our present findings provide evidence that endocannabinoid AEA is overproduced in a well-established model of DOX-induced acute heart failure, and CB_1 antagonists improve compromised contractile function. Furthermore, we show that CB_1 antagonists exert powerful cytoprotective effect in cardiomyocytes against DOX-induced cardiotoxicity both in vivo and in vitro by reducing apoptosis, offering a novel approach for the prevention of this devastating complication of chemotherapy.

There is limited and conflicting information about the role of cannabinoid receptor activation in cell-protective mechanisms against ischemia/reperfusion (I/R) damage in the heart (16,17, 25). A major limitation of these studies is the use of buffer-perfused isolated heart preparations, in which the effects of endocannabinoids and synthetic agonists on immune cells, which are pivotal in reperfusion damage, cannot be studied, as well as the use of nonselective cannabinoid ligands. In a more relevant in vivo model of myocardial I/R injury induced by coronary occlusion/reocclusion in anesthetized mice, the published evidence points to the protective role of CB_2 but not CB_1 receptor activation (CB_2 activation on immune cells was responsible for the reduced leukocyte-dependent myocardial damage associated with reperfusion) (26). The presence of CB_2 receptors in the myocardium and their function has never been convincingly demonstrated so far (selective CB_2 receptor agonists do not decrease blood pressure and myocardial contractility in vivo, in contrast to the well-known hypotensive and cardiodepressive effects of CB_1 agonists). Two studies using rat models of acute and chronic myocardial infarction have demonstrated that endocannabinoids contribute to the hypotension and cardiodepression associated with acute cardiogenic shock, which could be attenuated by CB_1 antagonists (27,28). Cannabinoid-1 receptor antagonist AM251 was suggested to promote remodeling in the later chronic heart failure study, but it tended to improve survival. In contrast, the treatment with CB_1 agonist HU210 was shown to prevent endothelial dysfunction, but it increased left ventricular end-diastolic pressure (28). However, the major limitation of the previous study (28) is the use of high dose of HU210, which was previously reported to cause severe psychotropic side effects and hypothermia, and the use of suboptimal doses of CB_1 antagonist AM251 (17). Furthermore, HU210 was previously demonstrated to exert antiarrhythmic, anti-inflammatory properties, and to decrease myocardial damage in perfused hearts by mechanisms not related to CB_1 activation, complicating the interpretation of these results (17).

In our present study, we used a well-established mouse model of DOX-induced acute heart failure in which the cardiac dysfunction has been characterized by using either echocardiography (14,21), tissue Doppler imaging (22), or pressure–volume systems (6,15). Importantly, in this model, a direct correlation between the degree of myocardial apoptosis and the severity of DOX-induced heart failure has been established (22). Apoptotic cell death is a key component in DOX-induced cardiotoxicity as established by numerous studies (11,13, 14,24,29), and also indicated by the present findings.

Here we demonstrate that pharmacologic inhibition of CB_1 receptors with rimonabant and AM281 conferred marked protection against DOX-induced cardiac dysfunction and cell death both in vivo and in vitro. The protective effect against DOX-induced cell death was not observed with CB_1 and CB_2 agonists and CB_2 antagonists in vitro. Thus, CB_1 antagonists may protect against DOX-induced cardiotoxicity by exerting potent cytoprotective effects on the one hand, and by antagonizing the cardiodepression elicited by endocannabinoid AEA on the other.

The observed protective effect of CB_1 antagonists and the DOX-induced increase in myocardial AEA content may, therefore, suggest that DOX-induced cardiotoxicity is associated with and mediated, at least in part, by activation of the endocannabinoid system. Collectively, the present findings suggest that CB_1 antagonists may represent a new cardioprotective strategy against DOX-induced cardiotoxicity and perhaps other cardiovascular pathologies associated with increased cell death. Further studies are warranted to investigate the underlying signaling mechanisms of these beneficial effects.

Supplemental Materials

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations and Acronyms

AEA
: anandamide

AG
: arachidonoylglycerol

CB_1/CB_2
: cannabinoid-1/-2

DOX
: doxorubicin/adriamycin

I/R
: ischemia/reperfusion

TUNEL
: terminal deoxynucleotidyltransferase-mediated nick-end labeling

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APPENDIX

For expanded Materials and Methods, please see the online version of this article.

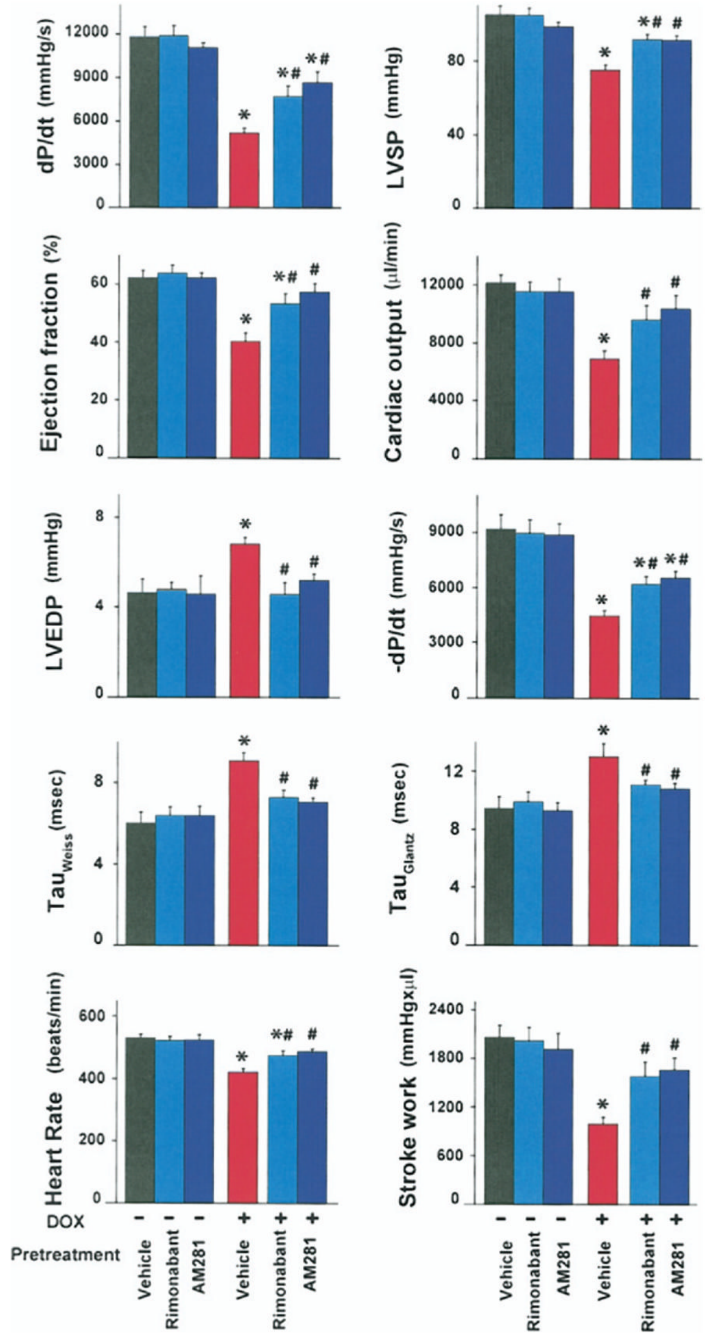

Figure 1. Effects of CB_1 Antagonists on DOX-Induced Cardiac Dysfunction

Effect of doxorubicin (DOX) on left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), LV maximum first derivative of ventricular pressure with respect to time (+dP/dt), LV −dP/dt, heart rate, stroke work, ejection fraction, and cardiac output and tau (Weiss and Glantz) in mice. Mice were pretreated either with vehicle, rimonabant, or AM281, and treated with either vehicle or DOX, as indicated in the Methods section. Hemodynamic parameters were measured 5 days after DOX administration. Results are mean ± standard error of the mean of 7 to 13 experiments in each group. *$p < 0.05$ versus vehicle; #$p < 0.05$ versus DOX. CB_1 = cannabinoid-1.

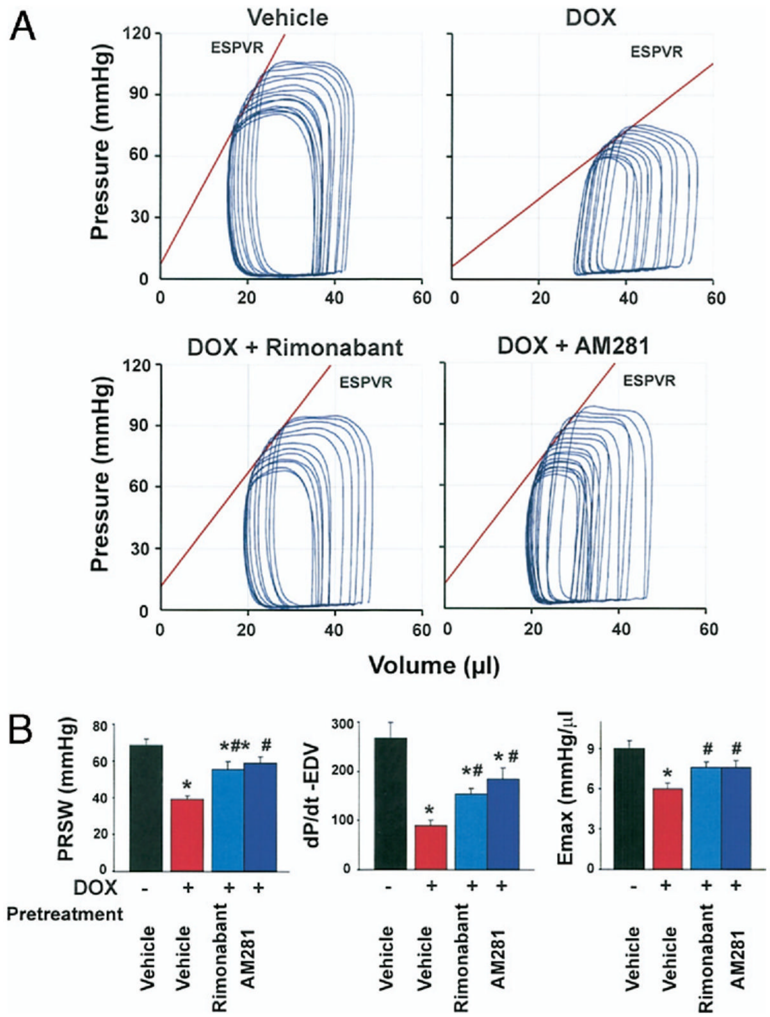

Figure 2. Effect of CB_1 Antagonists on DOX-Induced Depression of Load-Independent Indexes of Cardiac Contractility

(A) Representative pressure–volume (P–V) loops obtained with a P–V conductance catheter system at different preloads after vena cava occlusion, showing differences in the end-systolic P–V relation (ESPVR) and between mice pretreated with vehicle, rimonabant, or AM281 and treated with vehicle or DOX. The less steep ESPVR in DOX-treated mice indicates decreased contractile function, which was improved by CB_1 antagonists. **(B)** Effects of CB_1 antagonists on DOX-induced depression of load-independent indexes of cardiac contractility. Results are mean ± standard error of the mean of 9 to 18 experiments in each group. *$p < 0.05$ versus vehicle; #$p < 0.05$ versus DOX. EDV = end-diastolic volume relation; ESPVR or Emax = end-systolic pressure–volume relation; PRSW = preload-recruitable stroke work; other abbreviations as in Figure 1.

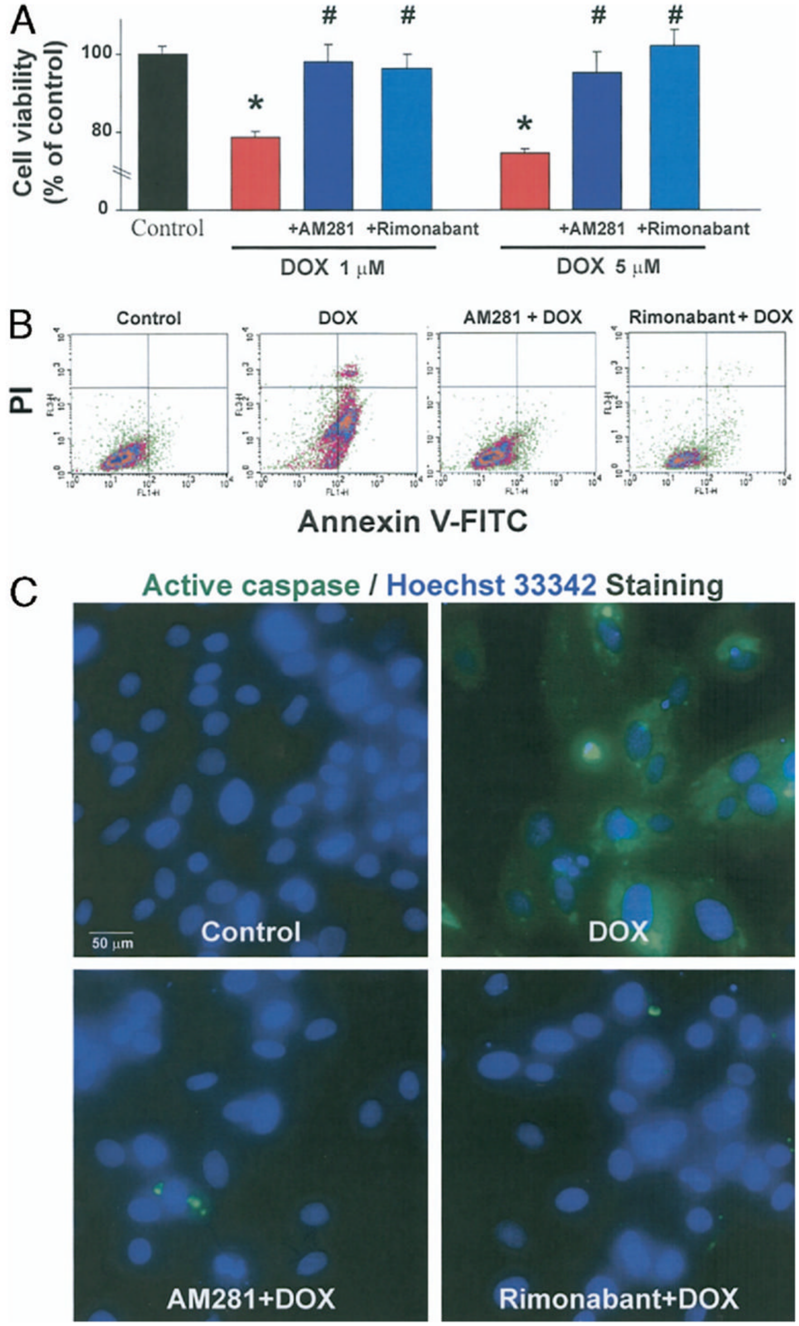

Figure 3. Effects of CB_1 Antagonists on DOX-Induced Cell Death and Apoptosis In Vitro

(A) Effects of CB_1 antagonists on cell viability measured by XTT assays. AM281 and rimonabant (1 μmol/l) prevent cell death induced by 1 or 5 μmol/l of DOX. *$p < 0.05$ versus control group; #$p < 0.05$ versus DOX (n = 4). **(B)** Effects of CB_1 antagonists on the early apoptosis marker fluorescent annexin V conjugate (Annexin V-FITC) and cell death detection dye propidium iodide (PI) measured by flow cytometric analysis of H9c2. Representative data from 3 experiments analyzed. **(C)** Effects of CB_1 antagonists on active caspase expression **(green)** and nuclear staining pattern by Hoechst 33342 dye **(blue)**. Please also note that some of the nuclei are fragmented in DOX-treated cells (indicative of late apoptosis), but not in

CB_1 antagonist-treated or control cells. Representative data from at least 15 experiments analyzed. Abbreviations as in Figure 1.

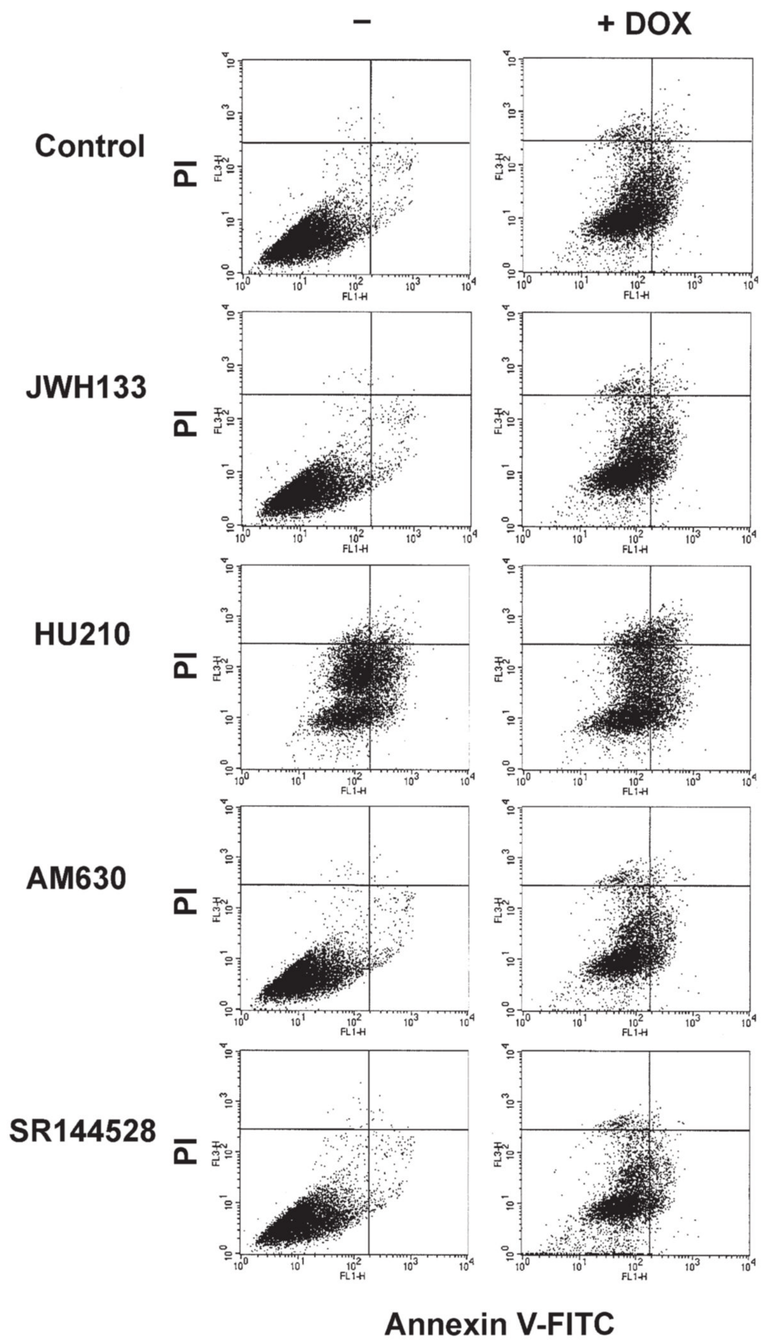

Figure 4. Effects of CB_1 Agonist, CB_2 Antagonists, and Agonists on DOX-Induced Apoptosis in H9c2 In Vitro

Effects of CB_1 agonist, CB_2 antagonists, and agonists on the early apoptosis marker Annexin V-FITC and cell death detection dye propidium iodide (PI) measured by flow cytometric analysis of H9c2. Representative data from 3 experiments analyzed. Abbreviations as in Figures 1 and 3.

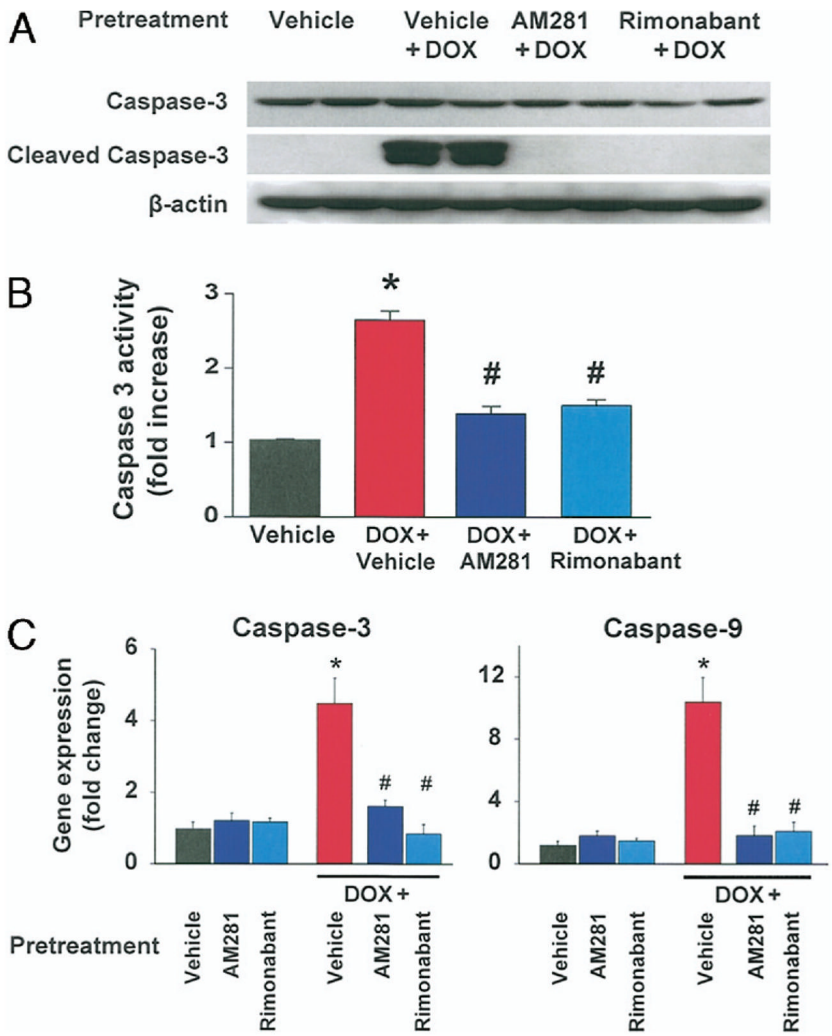

Figure 5. Effects of CB_1 Antagonists on DOX-Induced Apoptosis In Vivo

(A) Effects of CB_1 antagonists on DOX-induced caspase-3 activation analyzed by Western blot from heart tissue homogenates. Treatment with rimonabant or AM281 reduced myocardial caspase-3 activation in DOX-treated mice. The blot was also probed for beta-actin level for loading control. Representative blot from at least 5 experiments. **(B)** Effects of CB_1 antagonists on DOX-induced caspase-3 activity analyzed by colorimetric method from heart tissue homogenates. Treatment with rimonabant or AM281 reduced caspase-3 activity in DOX-treated mice. *$p < 0.05$ versus vehicle; #$p < 0.05$ versus DOX (n = 4 per group). **(C)** Effects of CB_1 antagonists on DOX-induced caspase-3 and caspase-9 gene expression. Data were analyzed with 2 housekeeping genes, and data presented here were normalized with beta-actin. Treatment with rimonabant or AM281 reduced myocardial caspase-3 and caspase-9 gene expression in DOX-treated mice. *$p < 0.05$ versus vehicle; #$p < 0.05$ versus DOX (n = 6 to 15 per group). Abbreviations as in Figure 1.

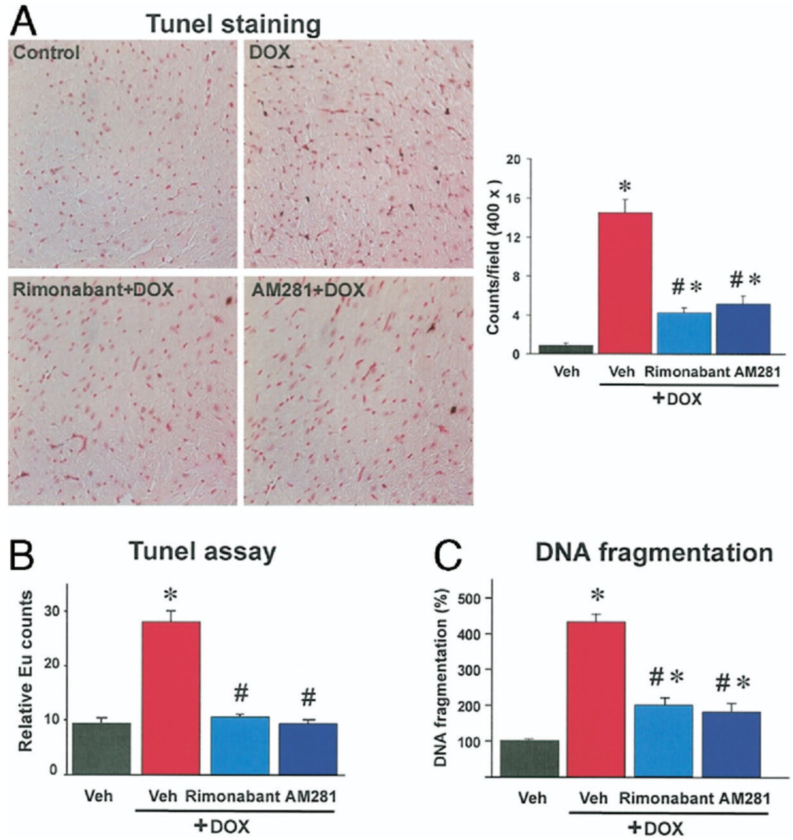

Figure 6. Effects of Rimonabant and AM281 on DOX-Induced Myocardial Apoptosis In Vivo Determined by TUNEL and DNA Fragmentation Assay

(A) Note the increased myocardial terminal deoxynucleotidyltransferase-mediated nick-end labeling (TUNEL) staining from doxorubicin (DOX)-treated mice **(brown)**. Treatment with cannabinoid-1 antagonist rimonabant and AM281 reduced myocardial TUNEL staining in DOX-treated mice. Similar immunohistochemical profiles were seen in n = 3 hearts per group. Quantitative measurements were carried out in 20 fields per group. *$p < 0.05$ versus vehicle (Veh); #$p < 0.05$ versus DOX. **(B)** Effects of rimonabant and AM281 on DOX-induced myocardial apoptosis in vivo by TUNEL assay. *$p < 0.05$ versus vehicle; #$p < 0.05$ versus DOX (n = 4 per group). **(C)** Effects of rimonabant and AM281 on DOX-induced deoxyribonucleic acid (DNA) fragmentation in vivo. Changes are expressed in % of myocardial DNA fragmentation in vehicle-treated mice (100%). *$p < 0.05$ versus vehicle; #$p < 0.05$ versus DOX (n = 4 per group). Eu = Europium.

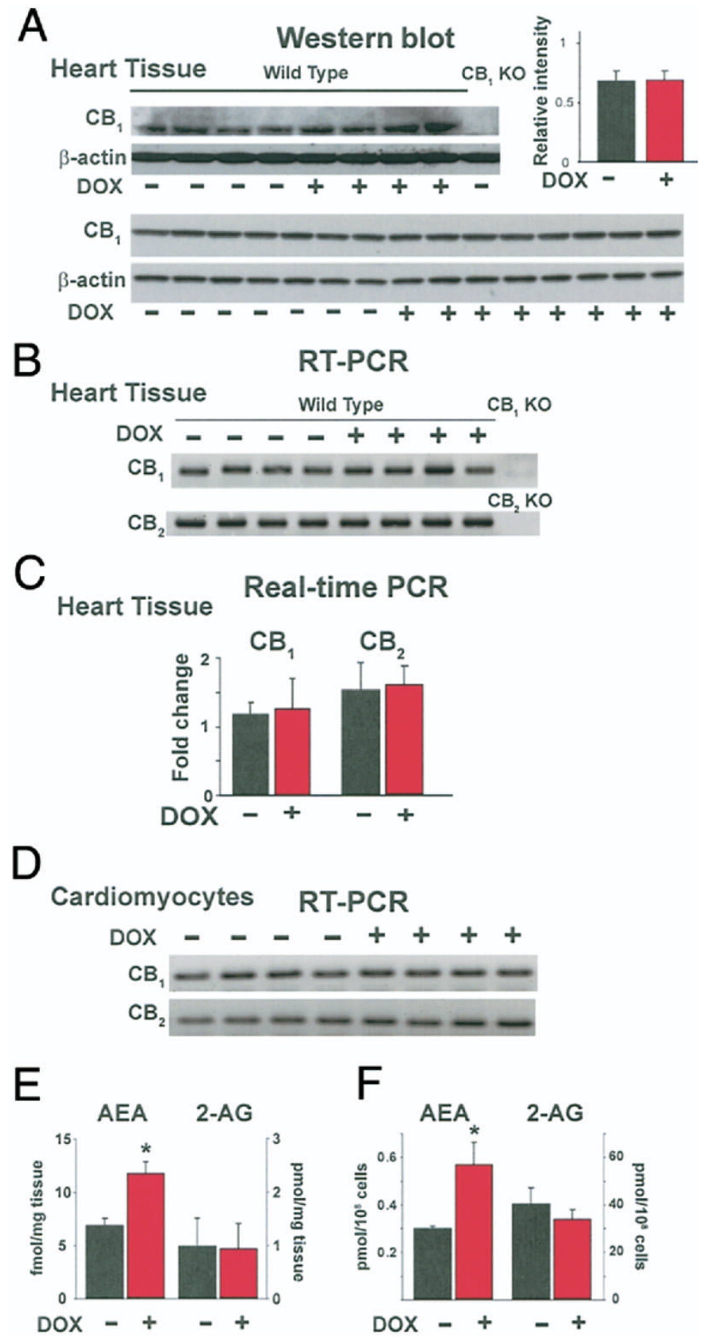

Figure 7. Effects of DOX on Myocardial Endocannabinoid Content and CB $_{1/2}$ Receptor Expression

(A) Evidence of CB_1 receptor protein expression in total lysate of DOX-treated mouse heart tissue homogenate by Western blot analysis. **(B and D)** Evidence of CB_1 and CB_2 receptor gene expression by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) from complementary deoxyribonucleic acid of both untreated and DOX-treated mouse heart samples **(B)** and cardiomyocytes **(D). (C)** Evidence of CB_1 receptor and CB_2 receptor gene expression in heart tissue by quantitative real-time PCR after normalization to beta-actin (n = 6 to 15). **(E)** Effect of DOX on anandamide (AEA) and 2-arachidonylglycerol (2-AG) production in vivo by liquid chromatography mass spectrometry (LCMS) analysis of heart tissue samples. *$p < 0.05$ versus vehicle (n = 6). **(F)** Effect of DOX (1 μM for 2 h) on AEA and 2-AG production in vitro by LCMS analysis of H9c2 cells. Increased AEA levels were observed in doxorubicin treated samples. *$p < 0.05$ versus vehicle ($n = 3$). KO = knockout; other abbreviations as in Figure 1.