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CB_1 cannabinoid receptors promote oxidative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human cardiomyocytes

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Aims Here we investigated the mechanisms by which cardiovascular CB_1 cannabinoid receptors may modulate the cardiac dysfunction, oxidative stress, and interrelated cell death pathways associated with acute/chronic cardiomyopathy induced by the widely used anti-tumour compound doxorubicin (DOX).

Methods and results Both load-dependent and -independent indices of left-ventricular function were measured by the Millar pressure–volume conductance system. Mitogen-activated protein kinase (MAPK) activation, cell-death markers, and oxidative/nitrosative stress were measured by molecular biology/biochemical methods and flow cytometry. DOX induced left-ventricular dysfunction, oxidative/nitrosative stress coupled with impaired antioxidant defense, activation of MAPK (p38 and JNK), and cell death and/or fibrosis in hearts of wide-type mice ($CB_1^{+/+}$), and these effects were markedly attenuated in CB_1 knockouts ($CB_1^{-/-}$). In human primary cardiomyocytes expressing CB_1 receptors (demonstrated by RT–PCR, western immunoblot, and flow cytometry) DOX, likewise the CB_1 receptor agonist HU210 and the endocannabinoid anandamide (AEA), induced MAPK activation and cell death. The DOX-induced MAPK activation and cell death were significantly enhanced when DOX was co-administered with CB_1 agonists AEA or HU210. Remarkably, cell death and MAPK activation induced by AEA, HU210, and DOX $\pm$ AEA/HU210 were largely attenuated by either CB_1 antagonists (rimonabant and AM281) or by inhibitors of p38 and JNK MAPKs. Furthermore, AEA or HU210 in primary human cardiomyocytes triggered increased reactive oxygen species generation.

Conclusion CB_1 activation in cardiomyocytes may amplify the reactive oxygen/nitrogen species-MAPK activation-cell death pathway in pathological conditions when the endocannabinoid synthetic or metabolic pathways are dysregulated by excessive inflammation and/or oxidative/nitrosative stress, which may contribute to the pathophysiology of various cardiovascular diseases.

Keywords Heart failure • CB_1 receptor • Endocannabinoids • Rimonabant • SR141716 • AM281

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1. Introduction

Numerous preclinical and clinical studies have reported increased endocannabinoid levels and implicated cannabinoid-1 (CB_1) receptor activation by endocannabinoids in the pathogenesis of hypotension and/or cardiac dysfunction associated with various forms of shock and cardiomyopathies, as well as in the development of obesity, increased cardiometabolic risk, atherosclerosis, and impaired glucose tolerance.[1–5] Multiple studies found beneficial effects of CB_1 antagonists in these pathological conditions, both in animal models as well as in human trials. CB_1 activation may also lead to undesirable haemodynamic consequences in healthy volunteers depending on the duration of the use (bradycardia/tachycardia, hypotension, arrhythmias),[6] which can be attenuated by CB_1 blockade.[7,8] Furthermore several clinical cases of myocardial damage/cardiomyopathy associated with cannabis use have also been reported[9] (see also references within).

The quinone-containing anthracycline doxorubicin (DOX) is a potent anti-tumour compound commonly used to treat several malignancies. The major limitation of this class of drug is its dose-dependent cardiotoxicity, resulting in the death of cardiomyocytes which often leads to irreversible myocardial dysfunction.[10] The pathogenesis of DOX-induced cardiomyopathy/heart failure is complex and may involve multiple mechanisms including increased oxidative/nitrosative stress,[11,12] mitochondrial dysfunction/damage,[12] alterations in the excitation–contraction (EC) coupling,[13] and activation of mitogen-activated protein(MAP)kinases,[14] just to name a few.

We have recently demonstrated that DOX increased endocannabinoid levels in cardiomyocytes both *in vivo* and *in vitro*, and that the pharmacological inhibition of CB_1 attenuated the DOX-induced myocardial dysfunction in a clinically relevant mouse model of acute cardiomyopathy.[15] However, the role of the cardiovascular CB_1 cannabinoid receptors and its possible interplay with the oxidative/nitrosative stress and interrelated cell death signalling pathways, eventually culminating into cardiac dysfunction, have not been addressed; likewise the fundamentals of CB_1-mediated signalling in human cardiomyocytes, and its possible role in cell death and reactive oxygen species generation are also elusive. Therefore, using human primary cardiomyocytes and CB_1 knockout mice we aimed to investigate the CB_1-mediated signalling in cardiomyocytes and its possible role in oxidative stress, interrelated signalling and cell death pathways associated with clinically relevant acute and chronic cardiomyopathy models induced by DOX. In the chronic cardiomyopathy model, we also investigated the possible involvement of CB_1 receptors in myocardial fibrosis.

2. Methods

2.1 Animals

Protocols involving the use of animals were approved by the Institutional Animal Care and Use Committees and were performed in line with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996). Male $CB_1^{+/+}$ or $CB_1^{-/-}$ mice (25–30 g), maintained at NIAAA's breeding facility, were administered with single dose (20 mg/kg) of DOX-HCl (Sigma Chemicals, St Louis, MO) intraperitoneally (i.p.) or with multiple doses (5 mg/kg) at days 1, 7, 14, and 21. After 5 or 35 days, mice were subjected to haemodynamic measurements or hearts were excised and snap frozen in liquid nitrogen for biochemical measurements as described.[15,16]

2.2 Reagents and cell culture

DOX was purchased from Sigma chemicals (MO, USA); p38 myocardial mitogen-activated protein kinase (MAPK), JNK inhibitors (SB203580 and JNK inhibitor II, respectively) was obtained from Calbiochem, EMD (Gibbstown, NJ). Anandamine (AEA), (6aR,10a*R*)-9-(hydroxymethyl)-6,6dimethyl-3-(2-methyloctan-2-yl)-6a,7,10,10a-tetrahydrobenzo[c] chromen-1-ol (HU210), and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-moepholinyl-1*H*-pyrazole-3-carboxamide (AM 281) were purchased from Tocris Bioscience (Ellisville, MO). *N*-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR 141716A-Rimonabant;indicated as SR1) was obtained from NIDA Drug Supply Program (Research Triangle Park, NC) as described.[15] β-Actin antibody was purchased from Chemicon (Temecula, CA), cleaved caspase-3, phospho(Thr180/Tyr182)-p38MAPK/p38MAPK, phospho(Thr183/Tyr185)-JNK/JNK, and cytochrome c (Cyt-c), cytochrome c oxidase (COX-IV) rabbit polyclonal antibodies were from Cell Signaling Technology (Danvers, MA). CB_1 rabbit C-terminal polyclonal antibody for the Western was developed as described.[17] CB_2 rabbit polyclonal antibody for the Western was from Cayman Chemicals (Ann Arbor, MI). Flow cytometry reagents-Sytox Green and AnnexinV-APC were obtained from Molecular Probes (Invitrogen, Carlsbad, CA). Human cardiac myocytes (HCM) were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured in poly-lysine coated plates using complete cardiac myocyte medium according to the manufacturer's recommendation as described.[18]

2.3 Haemodynamic measurements in mice

Left-ventricular performance was analysed in mice anesthetized with 2% isoflurane using Millar pressure–volume conductance system (Millar Instruments, TX) as described.[15,16,19]

2.4 Western blot analysis

Protein extraction from myocardial tissue homogenates or cardiomyocytes, loading, incubations with antibodies, development, and analysis were as described.[12]

2.5 Real-time PCR and semi-quantitative RT–PCR

Total RNA was isolated from heart homogenate or cardiac myocytes using Trizol reagents (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Analysis of mRNA expressions was performed as previously described[15,20] and all primers are indicated in the supplements (see Supplementary material online).

2.6 Determination of myocardial superoxide dismutase, glutathione peroxidase, PARP and caspase 3/7 activities, nitrotyrosine and GSH content, and DNA fragmentation

Myocardial PARP, caspase 3/7, superoxide dismutase (SOD), glutathione peroxidase (GPx) activities, nitrotyrosine and GSH content, DNA fragmentation were determined as previously described.[12,15,21]

2.7 TUNEL staining

Paraffin sections were dewaxed and *in situ* detection of apoptosis in the myocardial tissues was performed by terminal

deoxynucleotodyltransferase mediated nick-end labelling (TUNEL) assay as per the instruction provided with the kit (Roche Diagnostics, Indianapolis). After TUNEL labelling, sections were stained with monoclonal α-actinin antibody (cardiomyocyte marker, 1:100 dilution, DBS, CA) for 1 h, followed by incubation with appropriate secondary antibody conjugated with Texas Red. Nucleus was labelled with Hoechst 33258 (Molecular probes, Invitrogen, CA) and the TUNEL positive labelled cardiomyocyte/endothelial cells were observed using confocal imaging microscope (Carl Zeiss, NY) using 40× objective at 2048 × 2048 resolution.

2.8 Myocardial 4-hydroxynonenal content

4-Hydroxynonenal (4-HNE) in the myocardial tissues was determined using the kit (Cell Biolabs, San Diego). In brief, BSA or myocardial tissue extracts (10 μg/mL) are adsorbed on to a 96-well plate for 12 h at 4°C. 4-HNE adducts present in the sample or standard are probed with anti-HNE antibody, followed by an HRP-conjugated secondary antibody. The HNE-protein adducts content in an unknown sample is determined by comparing with a standard curve.

2.9 Fatty acid amide hydrolase activity

Fatty acid amide hydrolase (FAAH) activities in the myocardial tissues were determined using the kit from Cayman (Ann Harbor, MI). FAAH hydrolyses AMC arachidonoyl amide resulting in the release of the fluorescent product, 7-amino-4-methylcoumarin (AMC). The flurophore was analysed using an excitation wavelength of 340–360 nm and an emission wavelength of 450–465 nm.

2.10 $CB_{1/2}$ receptor expression in HCM

$CB_{1/2}$ receptor expression in HCM was determined by flow cytometry using rabbit anti-human CB_1 N-terminal and CB_2 rabbit polyclonal antibody from Cayman Chemicals (Ann Arbor, MI). In addition, α-actinin (DBS, Pleasanton, CA) expression was also determined by intracellular staining followed by flow cytometry to confirm the phenotype of human cardiomyocytes.

2.11 Determination of apoptosis and ROS generation by flow cytometry

After the treatments, apoptosis/necrosis of HCM was determined using flow cytometry as described.[15,22,23]

2.12 Statistical analysis

Results are expressed as mean ± SEM. Statistical significance among groups was determined by one-way ANOVA followed by Newman–Keuls *post hoc* analysis using GraphPad Prism 5 software (San Diego, CA). Probability values of $P < 0.05$ were considered significant.

3. Results

3.1 Genetic deletion of CB_1 attenuates acute DOX-induced cardiac dysfunction

Treatment of $CB_1^{+/+}$ mice with acute DOX, 20 mg/kg i.p. (DOX; *Figure 1*) induced marked decrease in LVSP, +d*P*/d*t*, stroke work, ejection fraction, cardiac output, and load-independent indexes of contractility (PRSW, d*P*/d*t*-EDV, and Emax, respectively) 5 days following the drug administration, and an increase in LVEDP and prolongation of relaxation time constants (τ Weiss and Glantz). These changes were significantly attenuated in CB_1 knockouts (*Figure 1*).

3.2 Genetic deletion of CB_1 attenuates acute DOX-induced myocardial oxidative/nitrosative stress, impaired antioxidant defense, and inactivation of endocannabinoid metabolizing enzyme FAAH

As shown in *Figure 2*, acute DOX administration increased markers of oxidative/nitrosative stress [4-hydroxy-*trans*-2-nonenal (HNE) and nitrotyrosine; *Figure 2A*] by ∼5.3 and 7.8-fold in the myocardium of $CB_1^{+/+}$ mice, decreased myocardial total glutathione content, SOD, and GPx activities (markers of antioxidant defense) by ∼48, 30, and 36%, respectively (*Figure 2B*), and attenuated the activity of the endocannabinoid AEA metabolizing enzyme FAAH by ∼42% (*Figure 2C*). These changes were significantly less pronounced in $CB_1^{-/-}$ mice (*Figure 2C*).

3.3 The DOX-induced MAPKs activation, Cyt-c release, caspase 3/7, and PARP activity and cell death are attenuated in the myocardium of $CB_1^{-/-}$ mice

As shown in *Figure 3*, DOX-induced activation of p-38 and JNK MAPKs (*Figure 3A*), and Cyt-c release (*Figure 3B*) in the myocardium of $CB_1^{+/+}$ mice 5 days following administration. These changes were significantly attenuated in $CB_1^{-/-}$ mice (*Figure 3*). DOX also markedly increased myocardial apoptotic and PARP-dependent cell death (caspase 3 cleavage, caspase3/7, and PARP activity by ∼8.7, 4.2, and 6.4-fold, respectively) in $CB_1^{+/+}$ mice. These changes were significantly attenuated in $CB_1^{-/-}$ mice (*Figure 4A–C*). $CB_1^{-/-}$ mice treated with DOX had decreased number of TUNEL positive cardiomyocytes and endothelial cells (*Figure 4D*).

3.4 Genetic deletion of CB_1 attenuates chronic DOX-induced cardiac dysfunction, fibrosis, oxidative stress, and cell death

Treatment of $CB_1^{+/+}$ mice with multiple doses of DOX (5 mg/kg) at days 1, 7, 14, and 21 (*Figure 5D*), induced a significant decrease in LVSP, +d*P*/d*t*, stroke work, ejection fraction, cardiac output, and load-independent indexes of contractility (PRSW, d*P*/d*t*-EDV, and Emax, respectively) 35 days following the drug administration, and an increase in LVEDP and prolongation of relaxation time constants (τ Weiss and Glantz). These changes were significantly attenuated in CB_1 knockouts (*Figure 5A*). Chronic (but not acute) DOX administration (*Figure 5B*; see Supplementary material online, *Figure S1*) was associated with increased myocardial fibrosis in $CB_1^{+/+}$ mice, which was almost completely absent in knockouts.

Chronic DOX (cDOX) administration was also associated with marked increases in myocardial HNE and nitrotyrosine formation (∼8.4 and 5.5-fold; *Figure 6A*), caspase 3/7 activity, and DNA fragmentation (∼5.4 and 5.1-fold; *Figure 6B*), mRNA expression of NADPH oxidase isoforms gp91phox, NOX4, and p67phox (∼2.6, 2.5, 2.1-fold) and matrix metalloproteinases 2 and 9 (2.7

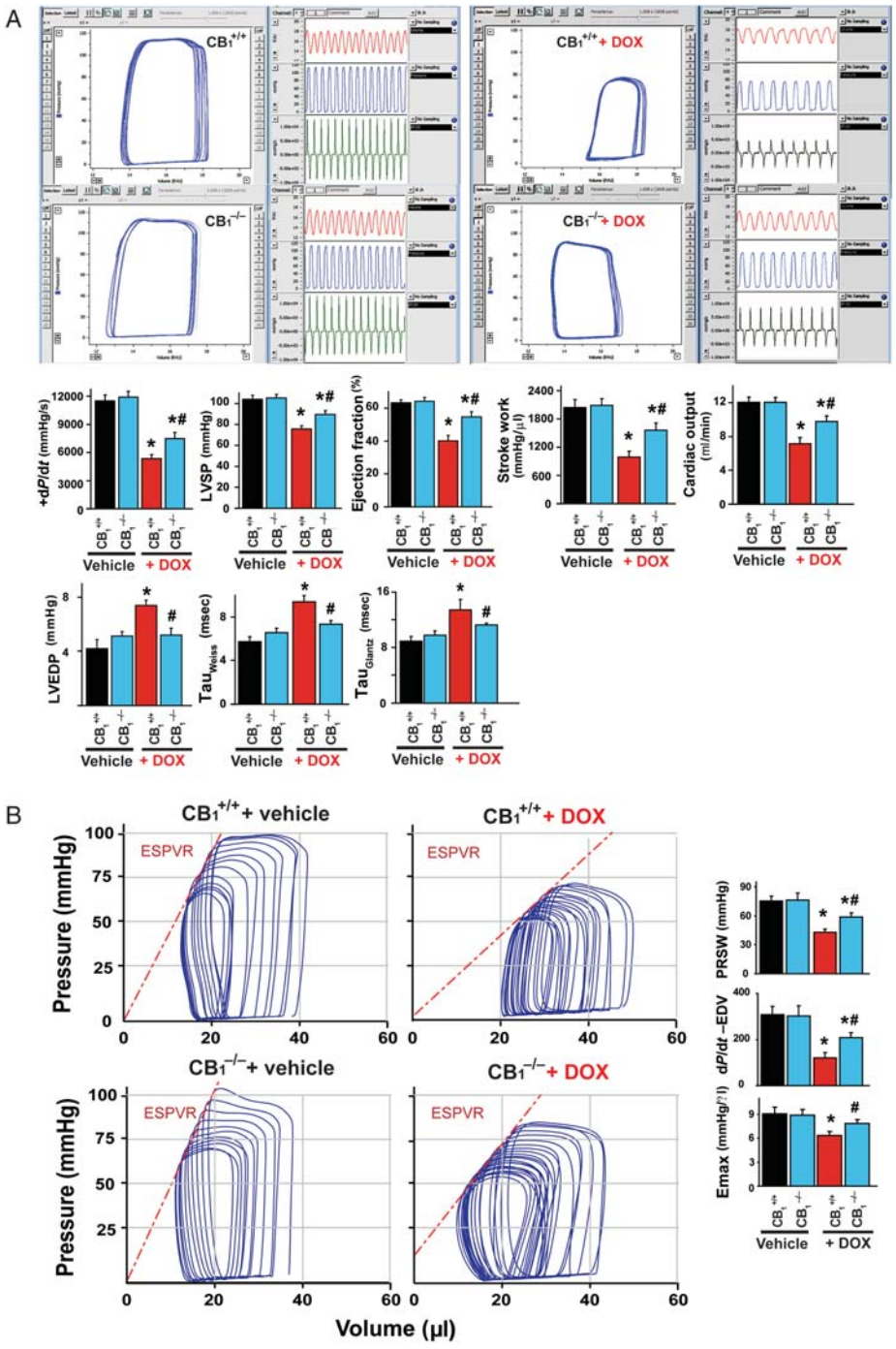

Figure 1 Improved DOX-induced acute cardiac dysfunction in CB_1 knockout mice. (*A*) Representative baseline pressure–volume (P–V) loops and derived parameters. Panels at right show volume (red), pressure (blue), and $\pm$ d*P*/d*t* (green) derived from the pressure signal. DOX-induced profound cardiac dysfunction in $CB_1^{+/+}$ mice evidenced by rightward shift of P–V relations, decrease in left-ventricular systolic pressure (LVSP), maximum first derivative of ventricular pressure with respect to time (+d*P*/d*t*), stroke work, ejection fraction, cardiac output, and an increase in left-ventricular end-diastolic pressure (LVEDP), and prolongation of relaxation time constants (τ Weiss and Glantz) 5 days following the administration of 20 mg/kg intra-peritoneally. These changes were largely attenuated in $CB_1^{-/-}$ mice. (*B*) Representative pressure–volume (P–V) loops at different preloads after vena cava occlusion, showing differences in the end-systolic P–V relation (ESPVR) in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice treated with vehicle or DOX. The less steep ESPVR in DOX-treated mice indicates decreased contractile function, which was less pronounced in $CB_1^{-/-}$ mice treated with DOX compared with $CB_1^{+/+}$ mice treated with the drug. DOX markedly decreased load-independent indexes of contractility [preload-recruitable stroke work (PRSW), d*P*/d*t*–end-diastolic volume relation (d*P*/d*t*–EDV), and end-systolic pressure–volume relation (Emax), respectively] in $CB_1^{+/+}$ mice, which was attenuated in knockouts. Results are mean $\pm$ SEM of 8–11 experiments/group. $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ $CB_1^{+/+}$ + DOX.

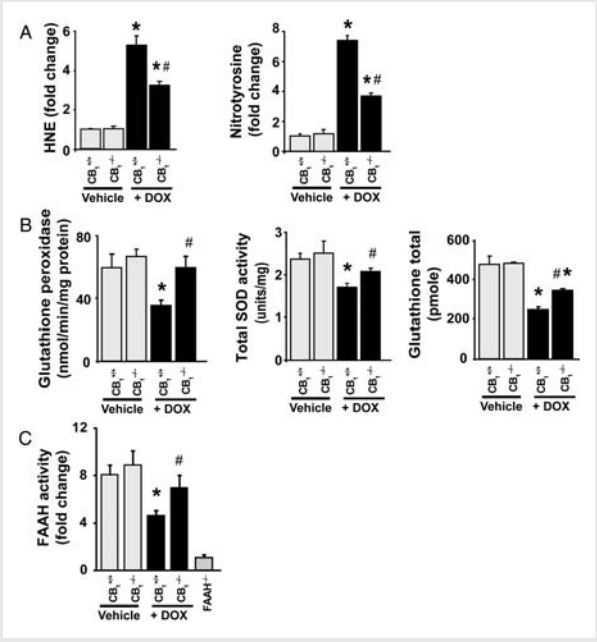

Figure 2 Acute DOX-induced myocardial oxidative/nitrosative stress, impaired antioxidant defense is attenuated in $CB_1^{-/-}$ mice. (*A*) Shows the lipid peroxides (4hydroxynonenal—4HNE) and nitrotyrosine (NT) accumulation in the groups as indicated. (*B*) Depicts myocardial glutathione peroxidase, superoxide dismutase (SOD) activities, and glutathione content. (*C*) Myocardial endocannabinoid metabolizing enzyme (FAAH) activity as indicated. $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ vs. $CB_1^{+/+}$ + DOX ($n = 6$–10/group).

and 2.1-fold, respectively) in $CB_1^{+/+}$ mice. These changes were significantly attenuated in knockouts (*Figure 6C* and *D*).

3.5 CB_1 and CB_2 receptors expressed in cultured human primary cardiomyocytes

CB_1 and to a less extent CB_2 receptors were expressed in human cardiomyocytes as determined by RT–PCR, real-time PCR, western blot, and flow cytometry (see Supplementary material online, *Figure S2*). $CB_{1/2}$ expressions were not affected by the treatment with 500 nM DOX (see Supplementary material online, *Figure S2*).

3.6 CB_1 agonist HU210 and the endocannabinoid AEA induce MAPK activation

Figure 7A and *B* show dose- and time-dependent activation of MAPKs by HU210 and AEA in HCM, respectively. Combination of DOX with CB_1 agonists markedly enhanced the activation of MAPKs (*Figure 7C*). These effects were attenuated by CB_1 antagonist (*Figure 7C*). Collectively, these observations suggest that CB_1 receptor activation is coupled with activation of MAPKs in HCM.

3.7 CB_1 receptor antagonists attenuate the DOX/HU210/AEA- or their combination-induced cell death in HCM

As shown in *Figure 8A* and *B*; see Supplementary material online, *Figures 3* and *4*, DOX, HU210, or AEA treatment(16 h) induced cell death in HCM, which were attenuated by the CB_1 antagonists

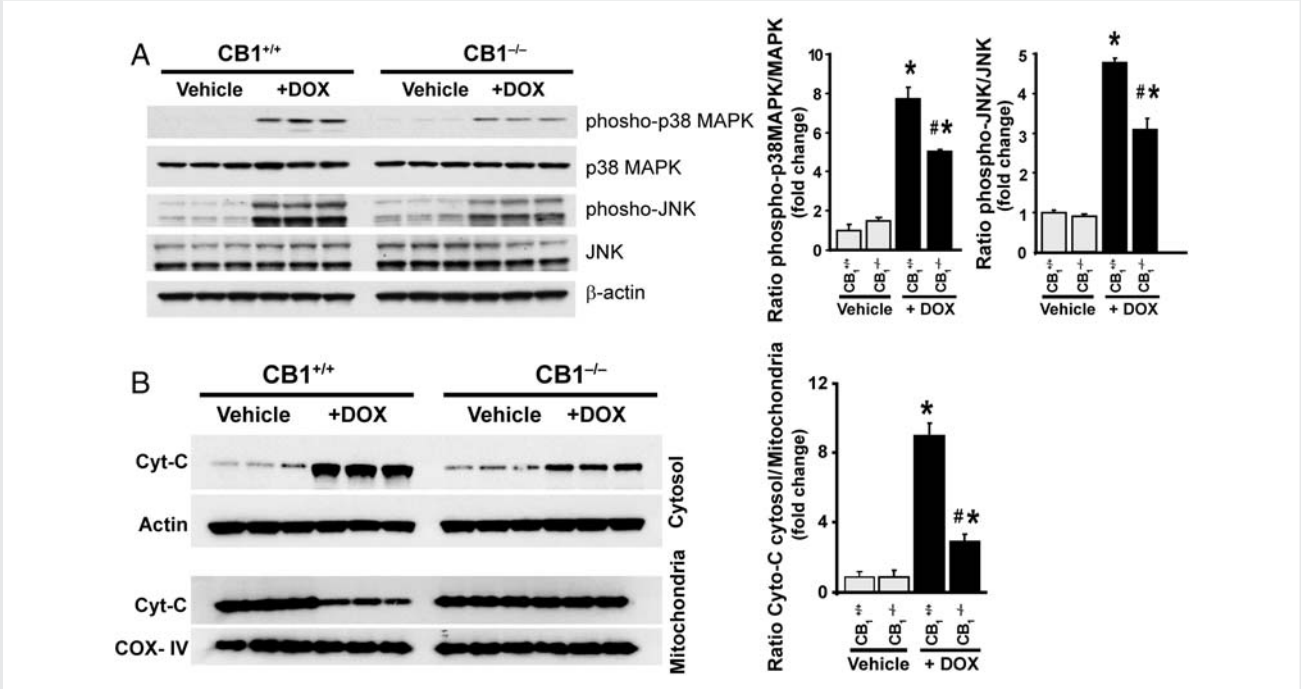

Figure 3 Acute DOX-induced myocardial MAPKs activation and cytochrome c release is attenuated in $CB_1^{-/-}$ mice. (*A*) Representative blots depicting the MAPK activation in $CB_1^{+/+}$ or $CB_1^{-/-}$ mice treated with DOX. (*B*) Representative western immunoblot depicting the cytochrome c release in the cytosol, 5 days after DOX administration. $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ vs. $CB_1^{+/+}$ + DOX ($n = 6$/group).

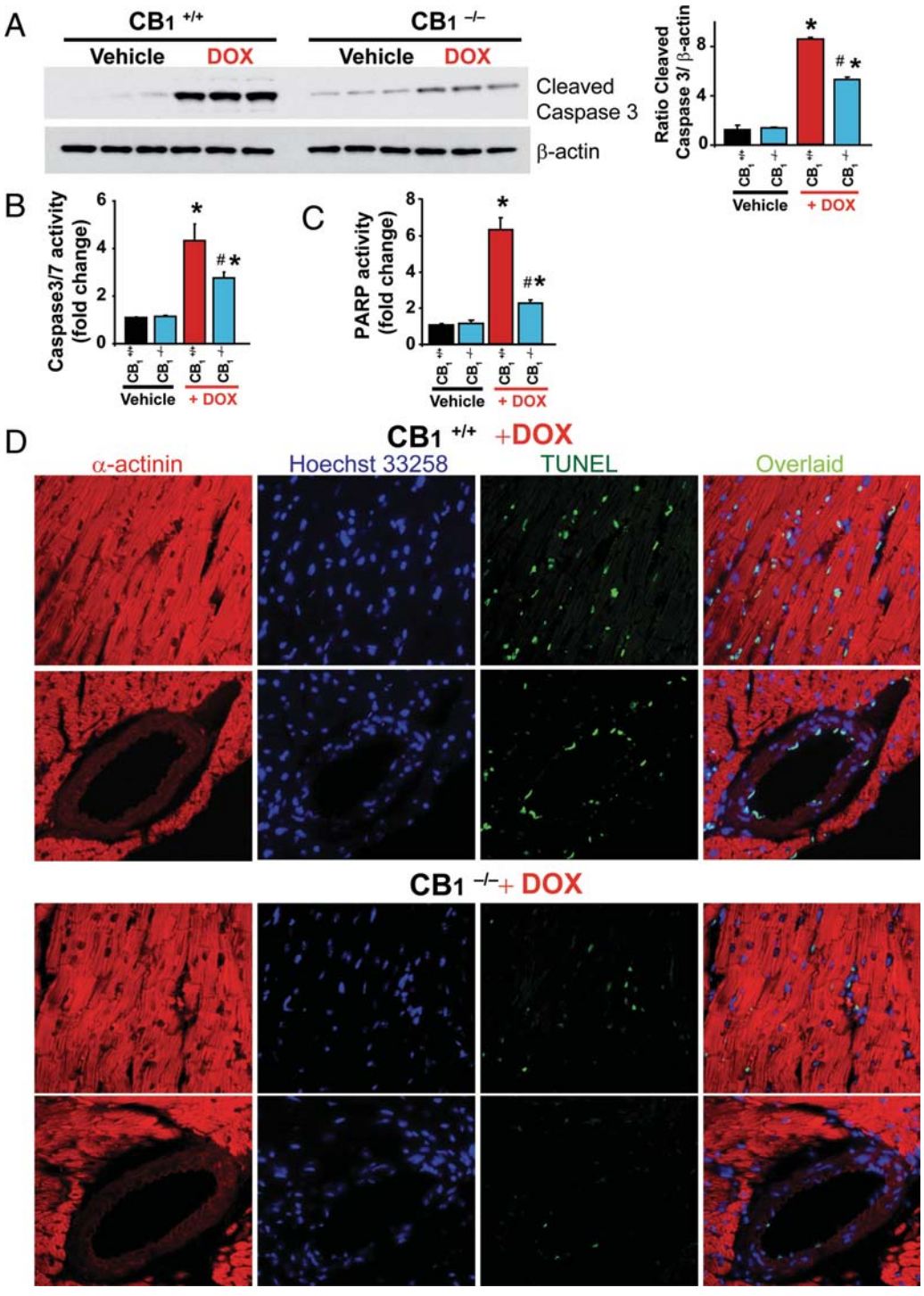

Figure 4 Acute DOX-induced myocardial apoptosis/necrosis is attenuated in $CB_1^{-/-}$ mice. (*A*) Cleaved caspase 3 activation by western blot analysis and (*B*) caspase 3/7 and (*C*) PARP activity in myocardial tissues from $CB_1^{+/+}$ or $CB_1^{-/-}$ mice treated with vehicle or DOX 5 days following the exposure. $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ vs. $CB_1^{+/+}$ + DOX ($n = 6$/group). (*D*) Representative TUNEL staining (green), showing that the DOX-induced apoptosis was present both in cardiomyocytes and endothelial cells, which was attenuated in $CB_1^{-/-}$ mice, irrespective of the cell type. Red colour represents cardiomyocyte specific α-actinin staining, blue nuclei of all cells, green or light green/white TUNEL positive cells (the latter in overlaid images). There were no TUNEL positive cells in vehicle treated mice (data not shown).

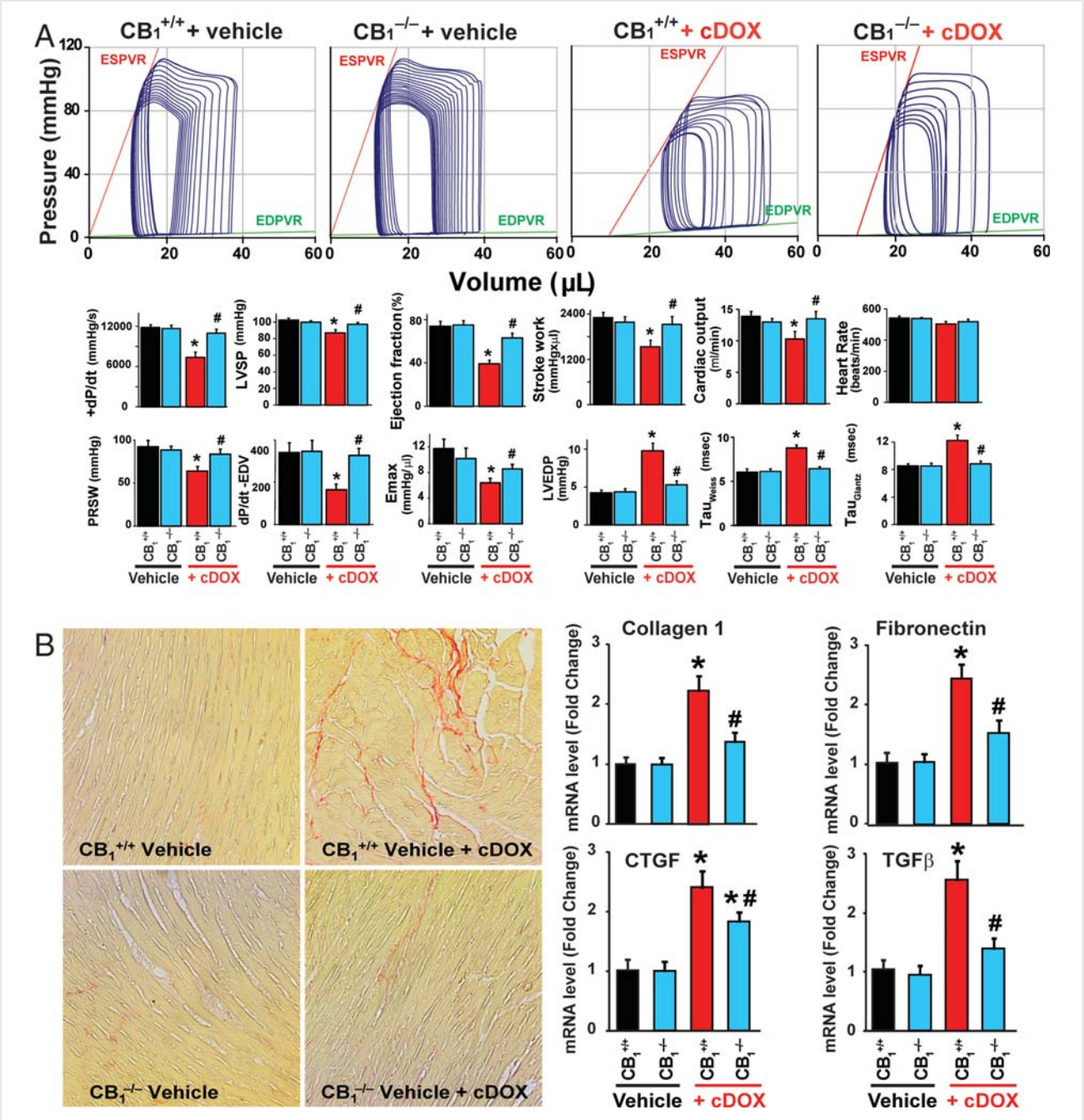

Figure 5 Improved DOX-induced chronic cardiac dysfunction and decreased myocardial fibrosis in CB_1 knockout mice. (*A*) Treatment of wide type $CB_1^{+/+}$ mice with (cDOX), 5 mg/kg intra-peritoneally at days 1, 7, 14, and 21 induced a significantly decreased left-ventricular systolic pressure, maximum first derivative of ventricular pressure with respect to time (+d*P*/d*t*), stroke work, ejection fraction, cardiac output, and load-independent indexes of contractility (PRSW, d*P*/d*t*−EDV, Emax), and an increase in left-ventricular end-diastolic pressure (LVEDP) and prolongation of relaxation time constants (τ Weiss and Glantz) 35 days following its administration, which was attenuated in $CB_1^{-/-}$ mice. (*B*) Genetic deletion of CB_1 attenuates chronic DOX(cDOX)-induced myocardial fibrosis Left: Sirius red staining of paraffin sections of heart tissues from respective groups and treatments as shown. Right: quantitative real-time PCR for the mRNA expression of fibrosis markers in the myocardium as indicated. Results are mean ± SEM of 10–14 experiments in each group for haemodynamics measurements, and 6/group for gene expressions. $^{*}P < 0.05$ vs. vehicle in $CB_1^{+/+}/CB_1^{-/-}$ mice; $^{\#}P < 0.05$ vs. $CB_1^{+/+}$ + DOX.

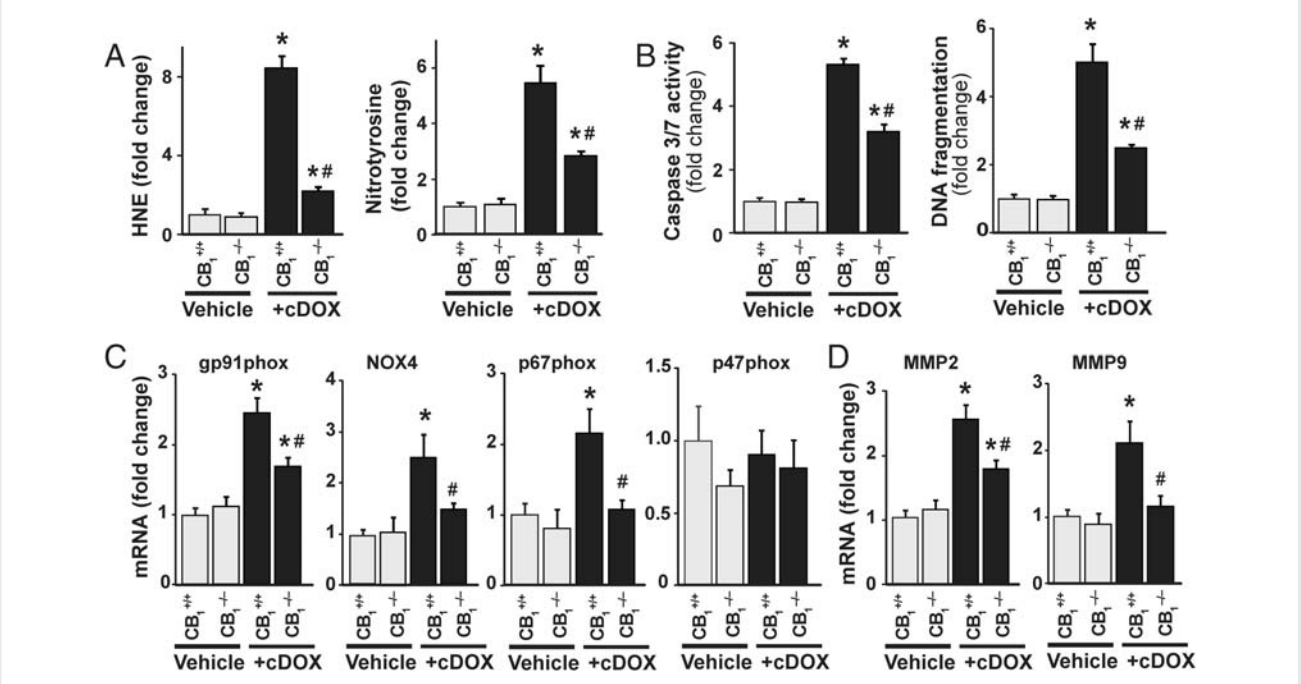

Figure 6 cDOX-induced myocardial oxidative/nitrosative stress and cell death is attenuated in $CB_1^{-/-}$ mice. Myocardial (*A*) HNE and nitrotyrosine accumulation, (*B*) apoptosis markers and mRNA expressions of (*C*) NADPH oxidase isoforms (gp91phox, NOX4, p67, and p47phox) and (*D*) metalloproteinases 2 and 9 (MMP2/9). $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ vs. $CB_1^{+/+}$ + DOX ($n = 6–10$/group).

SR1/AM281, respectively. Combination of DOX with CB_1 agonists markedly enhanced the cell death (*Figure 8B*; see Supplementary material online, *Figure S4*). Cell death induced by DOX/HU210/AEA- or their combination could also be attenuated by CB_1 inhibitors in HCM (*Figure 8B*; see Supplementary material online, *Figure S4*), likewise with inhibitors of p38 and JNK MAPKs (see Supplementary material online, *Figure S5*).

3.8 CB_1 activation increases ROS generation in HCM

AEA, HU210 induced ROS generation in HCM (measured 2 h after the treatment), which was attenuated by CB_1 antagonists SR1 and AM 281, respectively (*Figure 8C*).

4. Discussion

The primary cannabinoid receptors in the CNS are CB_1 receptors that predominantly couple to inhibitory G proteins (G_i and G_o) and under certain conditions also to G_s or $G_{q/11}$ leading to the inhibition of adenylyl cyclase and certain calcium channels together with the activation of inwardly rectifying potassium channels and several different MAP kinases.[24] Indeed, there is increasing recent recognition that CB_1 signalling involves activation of MAP kinases in a variety of cell types;[24] however, the existence and possible role of this pathway in cardiomyocytes is unknown. CB_1 receptors are also present, along with their natural ligands, the endocannabinoids (AEA and 2-AG), and metabolizing enzymes in rodent[15,25] and human myocardium[26] or cardyomyocytes (see Supplementary material online, *Figure S2*), and these receptors may mediate various cardiovascular depressive effects and contribute to the cardiovascular pathological alterations associated with multiple cardiovascular disorders via largely unexplored mechanisms.[5,6] Indeed, numerous experimental studies have demonstrated that activation of the endocannabinoid system contributes to hypotension and compromised cardiovascular function in a variety of pathophysiological states (e.g. endotoxic, haemorrhagic, and cardiogenic shock, advanced liver cirrhosis, and cirrhotic cardiomyopathy) through the activation of cardiovascular CB_1 receptors,[6] however the underlying signalling mechanism were not explored. Sustained activation of CB_1 receptors by endocannabinoids appears to increase cardiovascular risk factors in obesity/metabolic syndrome and diabetes.[3,5] These risk factors include plasma lipid alterations, abdominal obesity, hepatic steatosis, and insulin and leptin resistance.[1–3] Previously we have provided evidence that endocannabinoid AEA levels were elevated in the hearts from the well-established mouse model of acute DOX-induced cardiomyopathy and *in vitro* in rat-derived cardiomyocytes exposed to the drug, and that CB_1 antagonists improved compromised contractile function and protected against DOX-induced apoptosis, however the role of the underlying signalling mechanisms and the specificity of these effects were not addressed.

In this study, using CB_1 knockout mice and human primary cardiomyocytes, we provide evidence on the important role of CB_1-mediated signalling in cardiomyocytes in oxidative stress, interrelated signalling and cell death associated with a clinically relevant acute and chronic model of cardiomyopathy induced by DOX. We demonstrate that the activation of CB_1 receptors in human

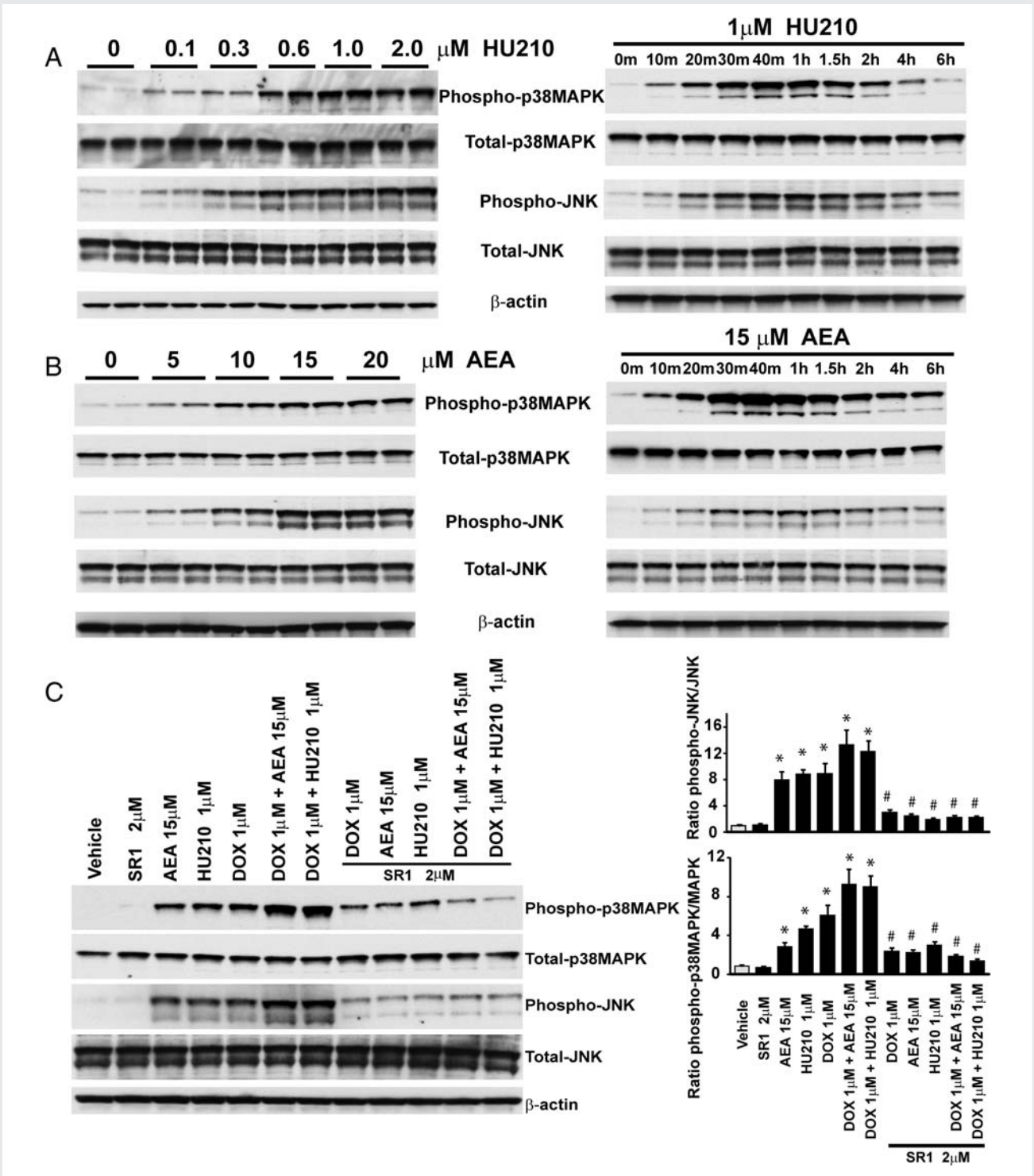

Figure 7 CB_1 receptor activation with synthetic/endogenous agonists or DOX induces CB_1-dependent MAPK activation in human cardiomyocytes (HCM). Concentration- and time-dependent effects of HU210 (*A*) and AEA (*B*) on activation of p38 and JNK MAPKs. (*C*) DOX-, AEA-, HU210, or DOX $\pm$ AEA/HU210-induced MAPKs activation is attenuated by CB_1 receptor antagonist SR141716(SR1). HCM were treated with HU210, AEA, DOX, or SR1 alone at indicated concentrations for 40 min or first treated with SR1 for 1 h, followed by continued incubation with DOX alone or in combination with AEA/HU210 for 40 min, and MAPK activation was determined by western blot. Shows MAPK activation upon respective treatments. Adjacent panels describe the quantification (represented as fold change). $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ vs. cells treated without SR1 with indicated treatments ($n = 4$).

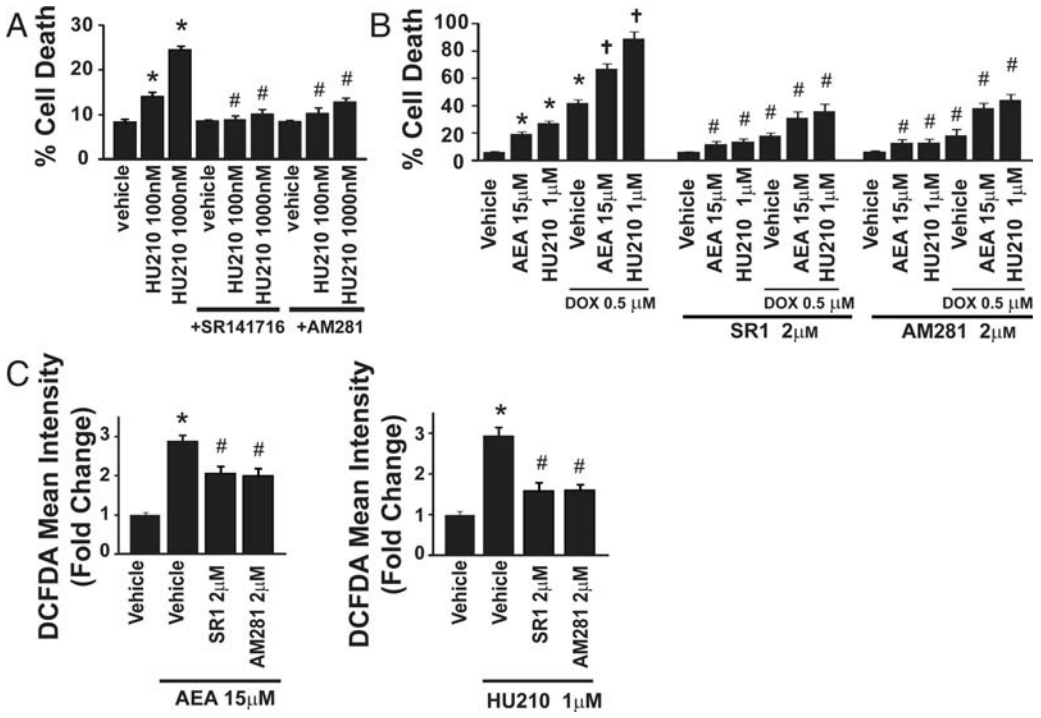

Figure 8 HU210/AEA/DOX or their combination-induced cell death is attenuated by CB_1 antagonists in human cardiomyocytes (HCM); CB_1 activation increases ROS generation in HCM. (*A* and *B*) Cells were treated as indicated and cell death was determined by flow cytometry as described in the methods (*A*) $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ vs. HU210 ($n = 4$) (*B*) $^{*}P < 0.05$ vs. vehicle; $^{\dagger}P < 0.05$ vs. AEA/HU210 alone; $^{\#}P < 0.05$ vs. AEA $\pm$ HU210 $\pm$ DOX treated cells ($n = 4$). (*C*) Shows the quantification of ROS generation by flow cytometry as per the treatments indicated. $^{*}P < 0.05$ vs. vehicle; #$P < 0.05$ vs. AEA/HU210 ($n = 4$). (see also Supplementary material online, *Figure S4*).

cardiomyocytes by the endocannabinoid AEA or the CB_1 agonist HU210 or DOX results in increased ROS generation, activation of various MAP kinases (p38 and JNK), and consequent cell death, which are attenuated by both selective CB_1 antagonists and MAPK inhibitors. Furthermore, DOX-induced MAPK activation and cell death in human cardiomyocytes were significantly enhanced when DOX was co-administered with AEA or HU210, an effect which could also be attenuated by both CB_1 antagonists and MAPK inhibitors. There is increasing recognition that MAPK activation contributes to DOX-induced cell death and cardiotoxicity,[14] which is consistent with our observation in human cardiomyocytes, as well as marked activation of p38 and JNK in the myocardium of wide type mice ($CB_1^{+/+}$) in our DOX-induced acute cardiomyopathy model. Marked MAPKs activation has also been reported in humans with advanced heart failure/dilated cardiomyopathy.[27] Notably, reactive oxygen and nitrogen species (e.g. superoxide, peroxynitrite, and hydrogen peroxide) importantly implicated in the development of DOX-induced cardiomyopathy, as well as in the development of numerous other cardiovascular pathologies by multiple mechanisms,[28–32] also dose-dependently activate MAPKs in cardiomyocytes leading to cell demise.[33] In addition to promoting cell death, these reactive oxidants may also affect Ca^{2+} transients and/or β-adrenergic response in cardiomyocytes leading to impaired contractility.[31,34,35] Indeed, the DOX-induced alterations in EC coupling in cardiomyocytes can be prevented by antioxidant *N*-acetylcystein, supporting this notion.[13] There is also increasing recognition that oxidative/nitrosative stress leads to increased expression and activation of matrix metalloproteinases (MMPs) and other signalling pathways promoting myocardial remodelling/fibrosis.[36,37]

In the acute DOX-induced cardiomyopathy model the severe cardiac dysfunction starts to develop 3 days after the DOX administration and peaks at day 5 parallel with the marked increases in myocardial nitrotyrosine generation and cell death followed by severe mortality in mice shortly thereafter.[15,16,38,39] In this model, time-dependent increases of myocardial MMP2/9 gene expression/activity were also reported[12,40,41] coinciding with the increased myocardial nitrotyrosine generation in mice,[12] underlying the importance of peroxynitrite/nitrosative stress in triggering the activation of MMPs.[36] At a time of the functional and biochemical measurements we could not detect myocardial fibrosis in the acute DOX-induced cardiomyopathy model (see Supplementary material online, *Figure S1*). Consistently with previous reports, acute administration of DOX increased myocardial oxidative/nitrosative stress and MAPK activation, impaired antioxidant defense, and lead to left-ventricular dysfunction and increased apoptotic and PARP-mediated cell death.[12,15,16,38,39] Remarkably, the DOX-induced myocardial MAPK activation, oxidative/nitrosative stress, mitochondrial dysfunction, cell death, and cardiac dysfunction were significantly attenuated in $CB_1^{-/-}$ mice, suggesting an

important role of these receptors in the development of the above-mentioned pathophysiological processes.

Because previous studies reported marked antifibrotic effects of CB_1 receptor deletion or pharmacological inhibition in various experimental models of liver fibrosis,[42] we hypothesized that CB_1 deletion may also influence the fibrosis in the myocardium. To investigate this we also utilized a chronic model of DOX-induced cardiomyopathy induced by multiple low doses of the drug.[16,39] In this model, genetic deletion of CB_1 was associated with almost complete absence of DOX-induced myocardial fibrosis (in addition to decreased oxidative nitrosative/stress, MMP2/9 gene expression, and cell death). Although we have recently identified mitochondria as the primary source of myocardial DOX-induced ROS generation both *in vitro* and *in vivo* in the acute cardiomyopathy model,[12] we did not exclude additional contribution of NADPH oxidases to this process *in vivo*, since mitochondrial ROS generation may also trigger secondary increased NADPH oxidase expression/activity and interrelated ROS production.[43] In fact, we implicated for the first time a possible role of NADPH oxidase-derived ROS in the cardiotoxicity of DOX *in vivo*,[16] even though the expression of various NADPH oxidase isoforms was not altered in the acute model of cardiomyopathy used,[12] which probably require more time to develop. Indeed, in the cDOX-induced cardiomyopathy model, we found increased mRNA expressions of various NADPH oxidase isoforms (gp91phox, NOX4, and p67phox; *Figure 6C*) suggesting a more pronounced role of NADPH oxidase-derived ROS in the development of cDOX-induced cardiotoxicity. Remarkably, these changes were also attenuated in CB_1 knockouts, consistently with decreased oxidative stress.

We propose that DOX initially rapidly increases mitochondrial reactive oxygen species generation in cardiomyocytes and/or endothelial cells by redox cycling triggering a cascade of events impairing mitochondrial function and resulting in more sustained oxidative/nitrosative stress, which persists even after the DOX is completely metabolized. In turn, the oxidative/nitrosative stress triggers the activation of MAPKs, and inactivates important enzymes/proteins involved in normal cardiac homeostasis and contractile function, eventually culminating into cell death of cardiomyocytes/endothelial cells and cardiac dysfunction. The oxidative/nitrosative stress may also trigger secondary expression of various NADPH oxidases further amplifying the vicious circle of ROS generation.

Oxidative/nitrosative stress also triggers increased endocannabinoid production[44,45] and/or inactivation of the endocannabinoid metabolizing enzyme FAAH (the main regulator of the tissue endocannabinoid levels because of the rapid metabolism) as observed in our recent study. Consequently, the endocannabinoids by activating CB_1 receptors in cardiomyocytes can trigger increased reactive oxygen species production, MAPK activation, and cell death. Consistent with our results in cardiomyocytes, a recent study has elegantly demonstrated a pivotal role of CB_1 receptors in reactive oxygen species generation by macrophages.[46]

Collectively, these data suggest that CB_1 activation in cardiomyocytes amplifies the reactive oxygen/nitrogen species-MAPK activation-cell death pathway in pathological conditions when the endocannabinoid synthetic or metabolic pathways are dysregulated by excessive inflammation and/or oxidative/nitrosative stress (e.g. in various forms of shock, cardiomyopathy/heart failure, and atherosclerosis), which contributes to the pathophysiology of these cardiovascular diseases (as also demonstrated presently in case of DOX-induced cardiomyopathy models). In these diseases, CB_1 receptor antagonists with limited CNS penetration may offer a cardioprotective strategy. Our results also unravel the fundamentals of the CB_1 signalling in human cardiomyocytes facilitating the better understanding of the beneficial effects of CB_1 antagonists observed in preclinical cardiovascular disease models, as well as in clinical trials. The resistance of CB_1 knockout mice against myocardial fibrosis in the chronic cardyomyopathy model may also have very important clinical implications and should be explored in the future studies. Furthermore these CB_1-mediated signalling mechanisms may also be involved in cardiotoxicity of cannabis occasionally reported in some users.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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P.P. wants to dedicate this study to his beloved mother Iren Bolfert who died from the cardiovascular complications of chemotherapy.

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