BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 688–700

0007-1188/10
www.brjpharmacol.org

THEMED ISSUE: CANNABINOIDS

RESEARCH PAPER

Cannabinoid-1 receptor activation induces reactive oxygen species-dependent and -independent mitogen-activated protein kinase activation and cell death in human coronary artery endothelial cells

Mohanraj Rajesh[1]*, Partha Mukhopadhyay[1]*, György Haskó[2], Lucas Liaudet[3], Ken Mackie[4] and Pál Pacher[1]

[1]*Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD, USA,* [2]*Department of Surgery, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, NJ, USA,* [3]*Department of Intensive Care Medicine, University Medical Center and Faculty of Biology and Medicine, Lausanne, Switzerland, and* [4]*Gill Center and the Department of Psychological & Brain Sciences, Indiana University, Bloomington, IN, USA*

Background and purpose: Impaired endothelial activity and/or cell death play a critical role in the development of vascular dysfunction associated with congestive heart failure, diabetic complications, hypertension, coronary artery disease and atherosclerosis. Increasing evidence suggests that cannabinoid 1 (CB_1) receptor inhibition is beneficial in atherosclerosis and cardiovascular inflammation both in experimental models, as well as in humans. Here, we investigated the effects of CB_1 receptor activation with the endocannabinoid anandamide (AEA) or synthetic agonist HU210 on cell death and interrelated signal transduction pathways in human primary coronary artery endothelial cells (HCAECs).
Experimental approach: Cell death, CB_1 receptor expression, reactive oxygen species (ROS) generation and activation of signal transduction pathways in HCAECs were determined by flow cytometry and molecular biology tools.
Key results: In HCAECs expressing CB_1 receptors (demonstrated by Western immunoblot and flow cytometry) AEA (5–15 μM) or HU210 (30–1000 nM) triggered concentration- and time-dependent activation of p38 and c-Jun NH_2-terminal protein kinase (JNK)–mitogen-activated protein kinases (MAPKs), cell death and ROS generation. The AEA- or HU210-induced cell death and MAPK activation were attenuated by CB_1 antagonists [SR141716 (rimonabant) and AM281], inhibitors of p38 and JNK–MAPKs or the antioxidant *N*-acetylcysteine. *N*-acetylcysteine alone prevented AEA- or HU210-induced ROS generation, but only partially attenuated MAPK activation and cell death. In contrast, in combination with CB_1 antagonists, *N*-acetylcysteine completely prevented these effects.
Conclusions and implications: CB_1 receptor activation in endothelial cells may amplify the ROS–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, thereby contributing to the development of endothelial dysfunction and pathophysiology of multiple cardiovascular diseases.
British Journal of Pharmacology (2010) **160,** 688–700; doi:10.1111/j.1476-5381.2010.00712.x
This article is part of a themed issue on Cannabinoids. To view the editorial for this themed issue visit http://dx.doi.org/10.1111/j.1476-5381.2010.00831.x

Keywords: endothelial cells; atherosclerosis; vascular dysfunction; endocannabinoids; cannabinoid receptors

Abbreviations: CB_1 receptor, cannabinoid-1 receptor; HCAECs, human coronary artery endothelial cells; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species

Correspondence: Pál Pacher, Section on Oxidative Stress Tissue Injury, Laboratory of Physiological Studies, National Institutes of Health/NIAAA, 5625 Fishers Lane, MSC-9413, Bethesda, MD 20892-9413, USA. E-mail: pacher@mail.nih.gov
*Authors contributed equally to this work.
Received 30 October 2009; revised 22 December 2009; accepted 21 January 2010

Introduction

In response to a variety of chemical and physical stimuli (e.g. change in pressure and pH, shear stress, cytokines, circulating hormones, drugs and substances released by sensory and autonomic nerves or platelets), endothelial cells produce both vasoconstricting (e.g. angiotensin II, endothelin-1, thromboxane A_2, isoprostanes) and vasodilating (e.g. nitric oxide, prostacyclin, adenosine, endothelium-derived hyperpolarizing factor, C-natriuretic peptide) substances that regulate vascular tone and permeability, hemostasis, angiogenesis and inflammation (Vanhoutte, 2009). The intact vascular endothelium sustains the balance between prevention and stimulation of platelet aggregation, thrombogenesis and fibrinolysis, promotion and inhibition of smooth muscle cell proliferation and migration and also the balance between vasoconstriction and vasodilation. Activated endothelial cells also play a very important role in the generation of various pro-inflammatory (cytokines, chemokines, adhesion molecules) mediators and reactive oxygen/nitrogen species (ROS/RNS), as well as in the orchestration of immune cell migration and activation to the site of the tissue injury (Osterud and Bjorklid, 2003; Pacher *et al*., 2007; Vanhoutte, 2009). Impaired endothelial homeostasis and/or cell death play a critical role in the development of vascular inflammatory responses and dysfunction associated with multiple diseases ranging from congestive heart failure, hypertension, diabetic complications to coronary artery disease, atherosclerosis and cardiovascular ageing (Csiszar *et al*., 2005; Armstrong *et al*., 2006a,b,c; Rao *et al*., 2007; Csiszar *et al*., 2008; Szabo, 2009).

In pre-clinical models of atherosclerosis, CB_1 inactivation or pharmacological inhibition limits the vascular inflammation and interrelated disease progression, and also decreases smooth muscle proliferation (Rajesh *et al*., 2008b; Dol-Gleizes *et al*., 2009; Sugamura *et al*., 2009). Certain beneficial effects of CB_1 antagonists on atherosclerosis have also been reported in humans with obesity and/or diabetes and the metabolic syndrome (Mach *et al*., 2008; Pacher, 2009). CB_1 receptors are expressed both in endothelial cells (Hillard, 2000; Liu *et al*., 2000; Rajesh *et al*., 2007), as well as in cardiomyocytes (Bonz *et al*., 2003; Mukhopadhyay *et al*., 2007a; Mach *et al*., 2008; Hiley, 2009). Activation of cardiovascular CB_1 receptors by excessive levels of endocannabinoids has importantly been implicated in the development of pathophysiological alterations associated with septic, haemorrhagic and cardiogenic shock; cirrhotic cardiovascular dysfunction; and heart failure (Pacher *et al*., 2006; Mendizabal and Adler-Graschinsky, 2007). There is also increasing recognition that in various pathological conditions, CB_1 receptor activation by endocannabinoids may promote activation of signalling pathways [e.g. p38 and c-Jun NH_2-terminal protein kinase (JNK)–mitogen-activated protein kinases (MAPKs)] promoting cell death (Howlett *et al*., 2002; Di Marzo, 2008; Dalton *et al*., 2009). Herein, we investigated the effects of CB_1 receptor activation on cell death, ROS generation and interrelated signal transduction pathways in human primary coronary artery endothelial cells (HCAECs).

Materials and methods

Reagents

p38 MAPK, JNK inhibitors (SB203580 and JNK inhibitor II, respectively) were obtained from Calbiochem, EMD (Gibbstown, NJ, USA). Anandamide (AEA) (6aR,10aR)-9-(hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-6a,7,10,10a-etrahydrobenzo[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, USA). (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR 141716A-rimonabant) (indicated as SR1 in this study) was obtained from NIDA Drug Supply Program (Research Triangle Park, NC, USA). β-Actin monoclonal antibody was purchased from Chemicon (Temecula, CA, USA), while phospho(Thr180/Tyr182)-p38MAPK/p38MAPK, phospho(Thr183/Tyr185)-JNK/JNK, phospho(Ser 473) rabbit polyclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). *N*-acetyl-L-cysteine (NAC) was obtained from Sigma Chemicals (Saint Louis, MO, USA). CB_1 rabbit C-terminal polyclonal antibody used for the Western blot was described previously (Rajesh *et al*., 2007; 2008a). Flow cytometry reagents Sytox Green and Annexin V-APC were obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA) (Mukhopadhyay *et al*., 2007a,b). The drug/molecular target nomenclature (e.g. receptors, ion channels and so on) conforms to BJP's *Guide to Receptors and Channels* (Alexander *et al*., 2008).

Cell culture

HCAECs and growth medium was purchased from Cell Applications, Inc. (San Diego, CA, USA). HCAECs were grown in HCAEC growth medium (Cell Applications) in cell culture dishes coated with 0.2 % gelatin (Sigma). HCAECs were used for the experiments between passages 4 and 7 as described (Rajesh *et al*., 2007).

Western blot analysis

Total protein was extracted from cells with radioimmunoprecipitation assay lysis buffer, containing protease inhibitor cocktail set III and phosphatase inhibitor cocktail set I (Calbiochem, EMD Biosciences, San Diego, CA, USA). Protein content was determined using Bio-Rad DC protein assay kit (Hercules, CA, USA). Then, 30 mg of protein was resolved on 4–12% gradient gels and transferred onto nitrocellulose membrane (GE-Biosciences, Niskayuna, NY, USA). After blocking, the blots were probed with the primary antibodies (1:1000) 4°C overnight. After washing, the blots were incubated with HRP conjugated secondary antibody for 1 h at room temperature. After final washing, blots were incubated with Super-Signal West Pico chemiluminescense substrate reagent (Pierce Biotechnology, Inc. Rockford, IL, USA) and developed using Kodak Biomax film (PerkinElmer, Wellesley, MA, USA). To verify equal loading, the blots were stripped and probed with β-actin antibody. Developed blots were scanned,

and the band intensities were determined using quantity one image analysis program (Bio-Rad).

CB_1 receptor expression in HCAEC
CB_1 receptor expression in HCAECs was determined by flow cytometry using CB_1 antibody (Cayman Chemical, Ann Arbor, MI, USA). CB_1 expression in HCAEC was also confirmed by Western immunoblot assay using CB_1 antibody developed by Dr Mackie's laboratory as described (Rajesh *et al*., 2007).

Determination of apoptosis by flow cytometry
After the treatments, apoptosis/necrosis of HCAEC was determined using flow cytometry as described (Mukhopadhyay *et al*., 2007a,b). For the simplicity annexin V-positive cells representing early apoptotic cell population (lower right squares in flow diagrams) were termed as 'apoptosis', while both annexin V and Sytox Green-positive cells (upper right squares in flow diagrams), representing both late apoptotic and necrotic cells, as 'necrosis' (see Figures 1D, 2B and 3B) as described (Mukhopadhyay *et al*., 2007a,b).

Caspase 3 activity
Caspase 3 activity in the cell lysates was performed using the Caspase 3 assay kit according to manufacturer's instructions (BioVision, Mountain View, CA, USA). In brief, caspase 3 in the samples in the assay buffer incubated at 37°C for 2 h cleaves the caspase 3 substrate pNA from DEVD. The pNA light emission is quantified using microplate spectrophotometer at 405 nm (Molecular Devices, Sunnyvale, CA, USA).

Detection of ROS generation by flow cytometry
Following the treatments, ROS generation in HCAEC was determined after 3 h of incubation with agonists/antagonists or with NAC. In brief, cells were loaded with 5 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Molecular Probes, Invitrogen) and incubated for 15 min. The carboxy-H_2DCFDA is an acetate ester of the fluorescent indicator 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein, is cell membrane permeable and remains non-fluorescent until hydrolysed. Once the cells uptake this redox dye, the acetate groups are cleaved by esterases, resulting in a charged species that sequesters the ROS generated and fluoresces upon excitation. ROS generation in endothelial cells was measured by the fluorescence intensity of carboxy-H_2DCFDA excited at 488 nm following the standard protocol using FACS Callibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

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. Statistical analysis of the data was performed using GraphPad Prism 5 software (San Diego, CA, USA). Probability values of $P < 0.05$ were considered significant.

Results

CB_1 receptors are expressed in HCAECs
Analysis by Western blot (Figure 1A) and by flow cytometry (Figure 1B) revealed expression of CB_1 receptors in HCAEC.

CB_1 receptor activation promotes MAPK-dependent cell death in HCAECs
Incubation of endothelial cells with either synthetic (HU210; Figure 1C,D) or endocannabinoid anandamide (AEA; Figure 2A,B), resulted in concentration-dependent cell death. The cell death was abrogated upon treatment with selective CB_1 receptor antagonists SR141716 (SR1) or AM281, respectively (Figures 1C,D and 2A,B). These observations suggest that CB_1 receptor activation can induce cell death in endothelial cells. To test whether MAPKs are involved in CB_1 receptor-mediated cell death, we pretreated endothelial cells with selective inhibitors of p38 (SB203580) or JNK (JNK II inhibitor) MAPKs for 1 h, followed by AEA or HU210 treatments, and analysed the cell death by flow cytometry. Inhibitors of p38 and JNK–MAPKs significantly attenuated the cell death induced by CB_1 agonists, suggesting that MAPKs are involved in CB_1 receptor-mediated cell death (Figures 1C,D and 2A,B).

CB_1 receptor stimulation triggers p38 and JNK–MAPKs, and caspase 3 activation in HCAEC
HU210 or AEA treatment of HCAECs elicits caspase 3 activation (Figure 4A), which is attenuated by CB_1 antagonists (SR1 or AM281), and concentration- (Figures 4B,C and 5A,B) and time-dependent (Figures 6A,B and 7A,B) increases in p38 and JNK–MAPKs activation. When cells were treated with CB_1 agonists in the presence of selective antagonists (SR1 or AM281), the activation of p38/JNK–MAPKs is attenuated (Figure 8A,B). Similarly, incubation of the cells with specific pharmacological inhibitors of either p38 (SB203580) (Figure 9A) or JNK–MAPKs (Figure 9B) (JNK II inhibitor) attenuates MAPK activation upon CB_1 receptor stimulation. These observations reveal that CB_1 receptor engagement leads to MAPK activation.

CB_1 receptor stimulation triggers ROS generation in HCAEC
As shown in Figure 3A, CB_1 receptor stimulation with AEA or HU210 in HCAECs increases ROS generation (shown 3 h following the treatments), which is attenuated by treatment with CB_1 receptor antagonists or antioxidant NAC. NAC or CB_1 antagonists alone only partially attenuate AEA- or HU210-induced cell death in HCAECs, while in combination with CB_1 antagonists NAC almost completely prevents these events (Figure 3B).

CB_1 receptor-triggered MAPK activation involves ROS-dependent and -independent mechanisms in HCAECs
As shown in Figure 10A,B, CB_1 receptor stimulation with AEA or HU210 increases ROS-dependent and -independent activation of p-38 and JNK–MAPKs in HCAECs, because NAC or CB_1 antagonists alone only partially attenuates AEA- or HU210-

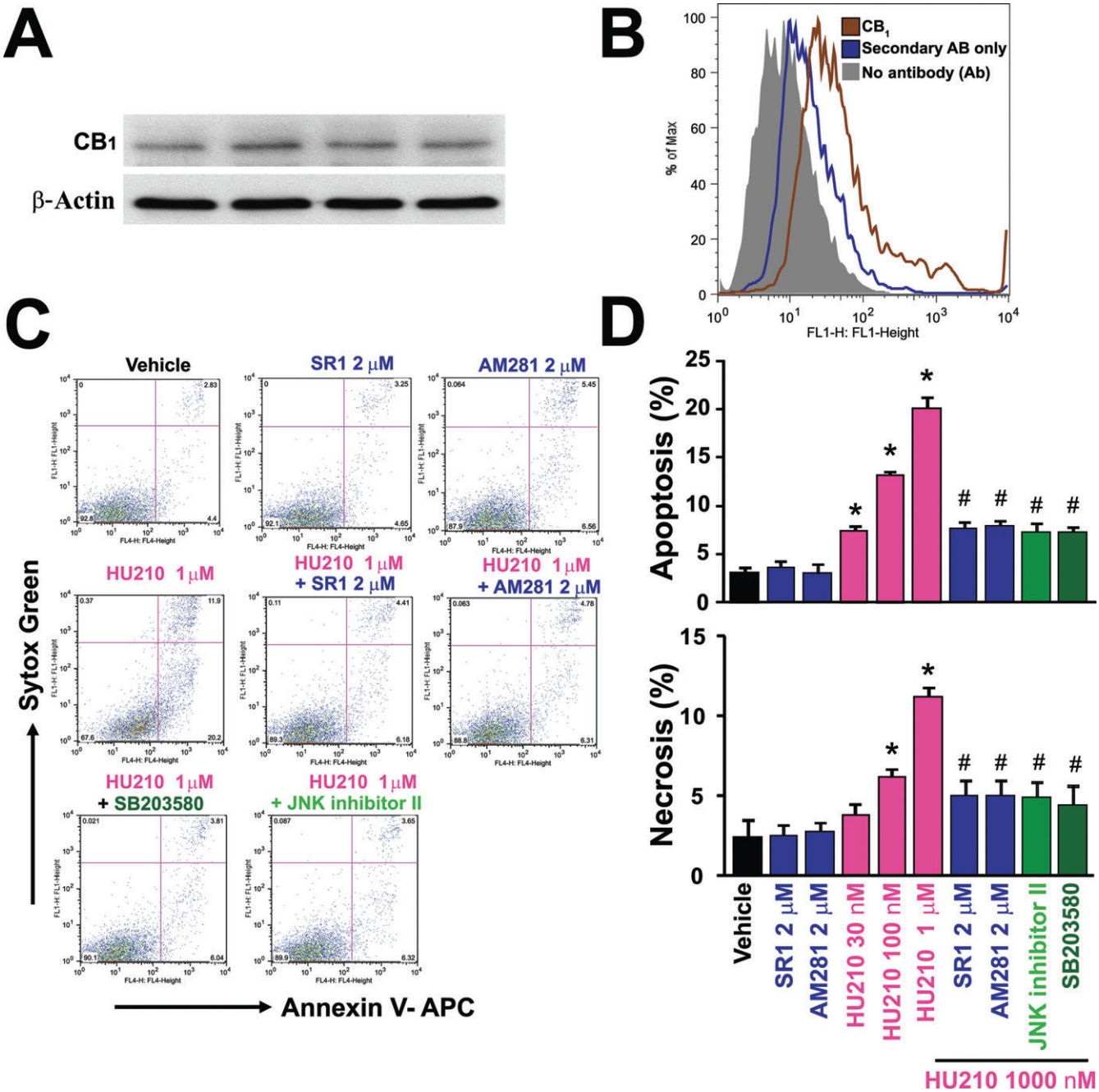

Figure 1 CB_1 receptor activation with HU210 triggers cell death in HCAECs. (A) Western blot depicts the expression of CB_1 receptors in HCAEC. (B) Flow cytometry analysis demonstrates the CB_1 receptors expression in HCAEC. (C) Flow cytometric analysis of cell death. Cells were treated as indicated with either agonist for 14–15 h or first treated with CB_1 antagonists/MAPK inhibitors for 1 h, followed by incubation with agonist for 14–15 h in the continuous presence of CB_1 antagonists/MAPK inhibitors, and cell death analyses were performed with flow cytometry. (D) CB_1 receptor-mediated cell death requires activation of both p38 and JNK kinases. Summary of results after cells were treated as described in (C). *$P < 0.05$ versus vehicle; #$P < 0.05$ versus HU210 alone ($n = 5$/treatment).

induced MAPK activation, while in combination with CB_1 antagonists NAC almost completely prevents these processes (Figures 8 and 10A,B).

Discussion

Pathological disruption of the highly regulated endothelial homeostasis leads to the development of endothelial dysfunction, which represents a predominant and unifying early feature of diabetes, hypertension, heart failure and atherosclerosis, and directly leads to cardiovascular complications and microthrombus formation. Although the hallmark of endothelial dysfunction is impairment of endothelium-dependent vasodilation, it is increasingly recognized that other alterations (e.g. inflammation, increased lipoprotein oxidation, vascular smooth muscle proliferation and migration from the media into the intima, extracellular matrix deposition or lysis,

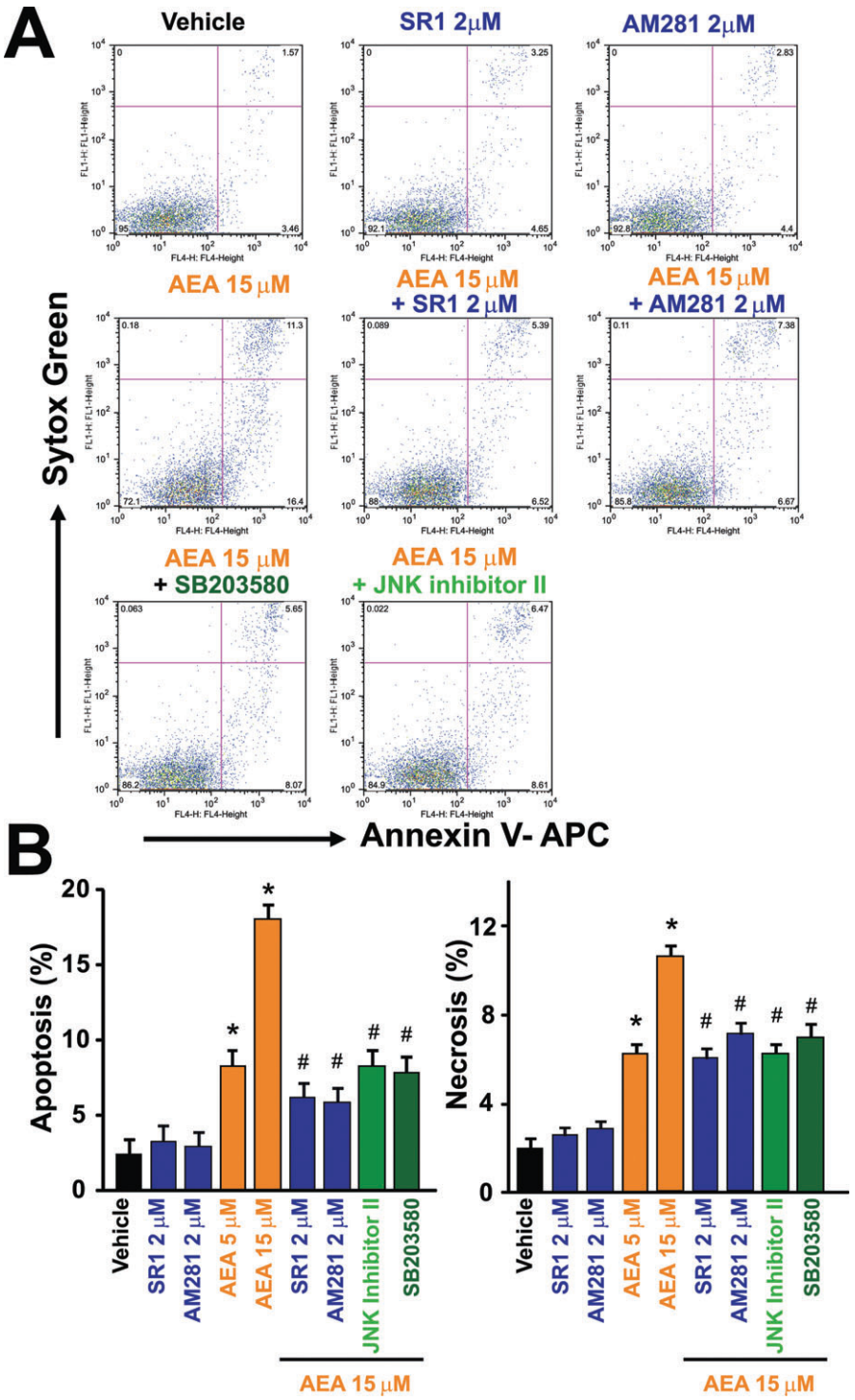

Figure 2 CB_1 receptor activation by AEA triggers cell death in HCAECs. (A) Cells were treated as indicated, and cell death analyses were performed 14–15 h following the administration of the drugs with flow cytometry as described in Figure 1. (B) AEA increases cell death via CB_1 receptors which requires both JNK and p38 MAP kinases. Cells were treated as above and results averaged. *$P < 0.05$ versus vehicle; # $P < 0.05$ versus AEA alone ($n = 5$/treatment).

platelet activation and thrombus formation) are also critically involved (Armstrong *et al*., 2006a,b,c; Rao *et al*., 2007). Accumulating evidence supports the view that the endothelial dysfunction associated with diabetes, hypertension, heart failure and atherosclerosis is related to the local formation of ROS and RNS (Pacher *et al*., 2007; Vanhoutte, 2009) in the vicinity of the vascular endothelium in concert with activation of various chronic inflammatory processes (Hansson and Libby, 2006; Tedgui and Mallat, 2006; Rao *et al*., 2007; Csiszar *et al*., 2008)

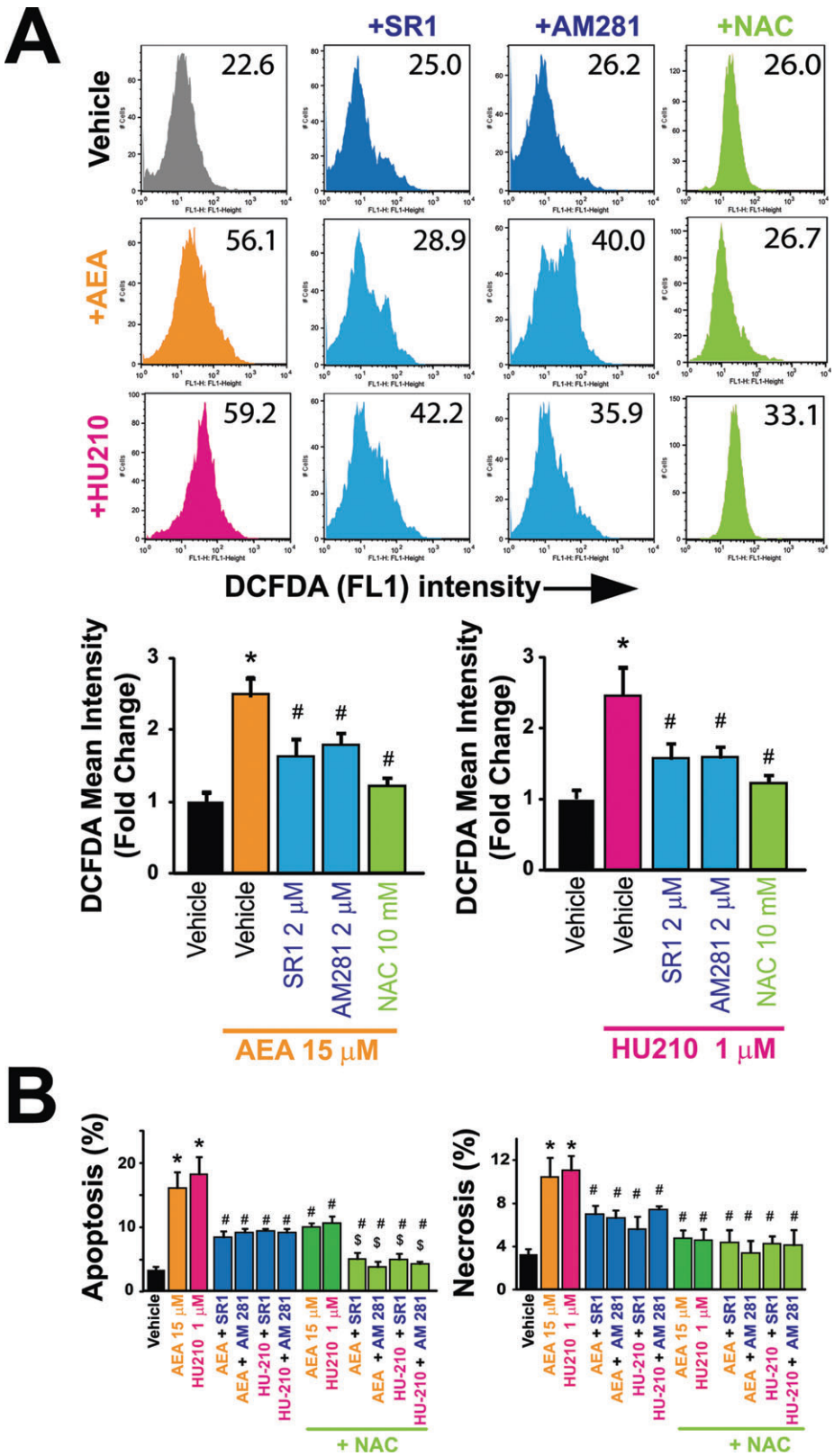

Figure 3 Cannabinoid receptor 1 agonists increase ROS generation in HCAEC cells; ROS generation and cell death is attenuated by NAC. Cells were treated as indicated for 3 h, and ROS generation was analysed using carboxy H2DCFDA with flow cytometry as described in the methods section. (A) Representative histograms (numbers in representative histograms indicate mean fluorescence intensity of DCFDA from the indicated representative treatment condition) and summary data (B) shown from four to six independent experiments. Cells were treated as indicated for 14–15 h, and cell death was determined by flow cytometer. The combination of CB_1 antagonists with NAC almost completely prevents AEA/HU210-induced cell death in HCAECs, while NAC or CB_1 antagonists alone are only partially protective. *$P < 0.05$ versus vehicle; #$P < 0.05$ versus AEA/HU210 alone; \$$P < 0.05$ versus AEA/HU210 ± CB_1 antagonists.

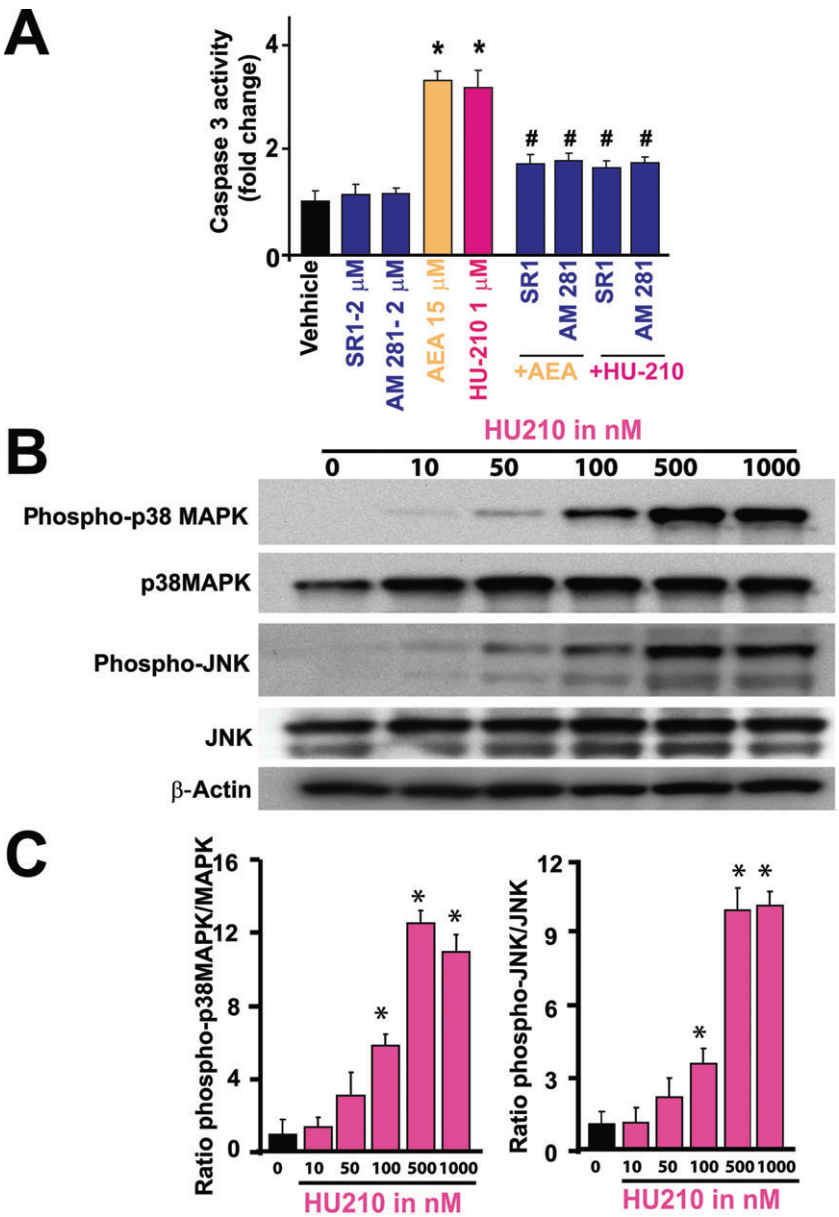

Figure 4 AEA or HU210 activates caspase 3, and HU210 triggers a concentration-dependent activation of p38 and JNK–MAPKs in HCAECs. (A) Caspase 3 activity assay demonstrates increased AEA- or HU210-induced caspase 3 activity 14–15 h following the indicated treatments, which is attenuated by CB_1 antagonists. $P < 0.05$ versus vehicle or SR1 or AM 218 alone; #$P < 0.05$ versus AEA or HU210 alone, n = 5–6/treatment. (B) Western blot analysis for p38 and JNK–MAPKs demonstrates their dose-dependent activation by HU210 after 40 min of treatment. A representative blot is presented from four identical experiments. (C) The quantification of MAPK activation. *$P < 0.05$ versus vehicle (n = 4/treatment).

In this study, we investigated the role of CB_1 receptors in ROS generation, cell death and interrelated signalling pathways in primary HCAECs. We show that the endocannabinoid AEA (5–15 μM) and synthetic cannabinoid receptor agonist HU210 (30–1000 nM) trigger concentration- and time-dependent activation of p38 and JNK–MAPKs and cell death (predominantly apoptotic). We also demonstrate that AEA or HU210 increases ROS generation in HCAECs. Importantly, the AEA- or HU210-induced cell death, MAPK activation and ROS generation were partially attenuated by CB_1 antagonists [SR141716 (rimonabant) and AM281], inhibitors of p38 and JNK–MAPKs or antioxidant *N*-acetylcysteine. Although in our study we did not identify the precise mechanisms and sources of AEA- or HU210-triggered increased ROS generation in HCAECs, our results suggest that this process, at least in part, involves CB_1 receptor-dependent signalling events. This is also supported by a recent study demonstrating that CB_1 receptor stimulation with AEA or the synthetic agonist ACEA induced a transient increase in human macrophage ROS generation, which could be inhibited by pretreatment with the CB_1 antagonist SR141716 (Han *et al.*, 2009). Furthermore, ACEA also induced increased ROS generation in murine CB_1 knock-out peritoneal macrophages transfected with human CB_1 recep-

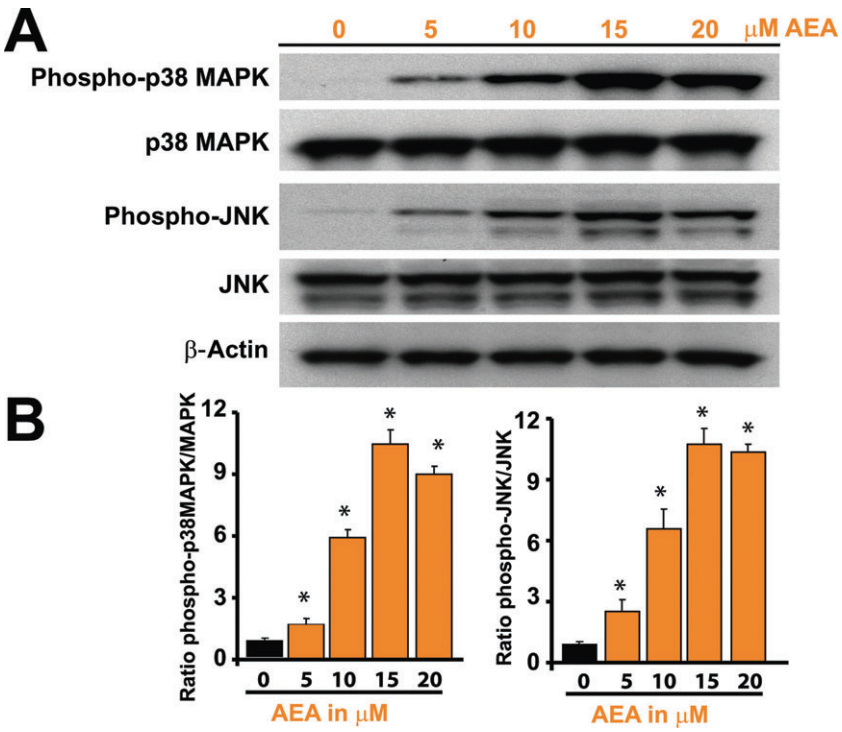

Figure 5 AEA triggers concentration-dependent activation of p38 and JNK–MAPKs in HCAECs. (A) Western blot analysis for p38 and JNK–MAPKs demonstrates their dose-dependent activation by AEA after 40 min of treatment. A representative blot is presented from four identical experiments. (B) The quantification of MAPK activation. *$P < 0.05$ versus vehicle ($n = 4$/treatment).

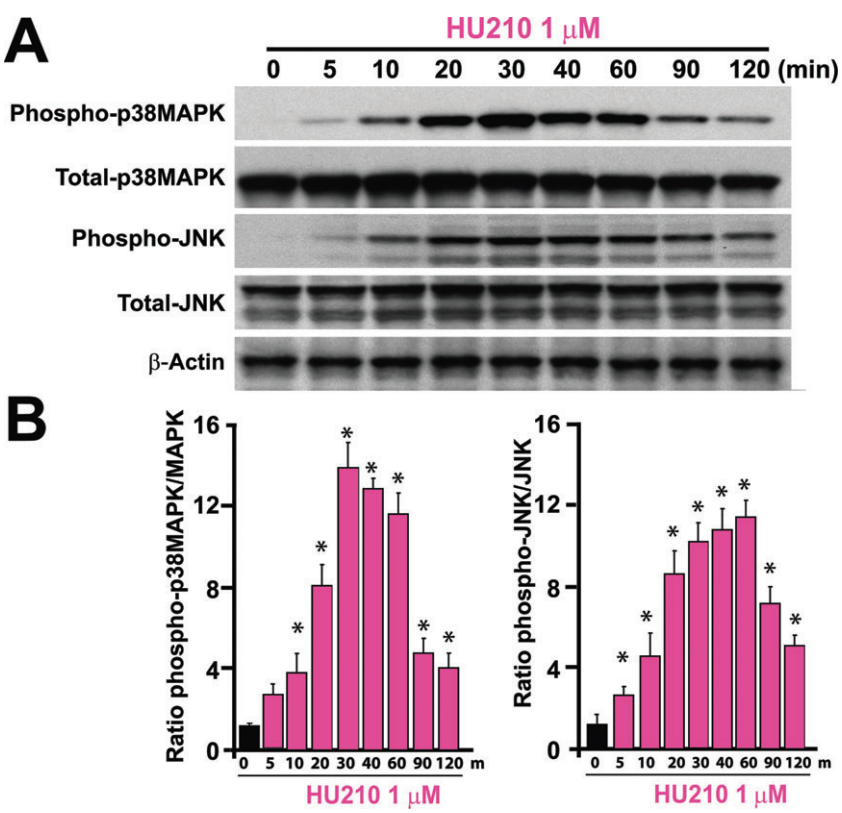

Figure 6 HU210 triggers time-dependent activation of p38 and JNK–MAPKs in HCAECs. (A) Western blot analysis for p38 and JNK–MAPKs demonstrates their time-dependent activation by HU210 treatment. Activation is maximal by 30 min and declines towards control levels by 90 min. A representative blot is presented from four identical experiments. (B) The quantification of MAPK activation. *$P < 0.05$ versus vehicle ($n = 4$/treatment).

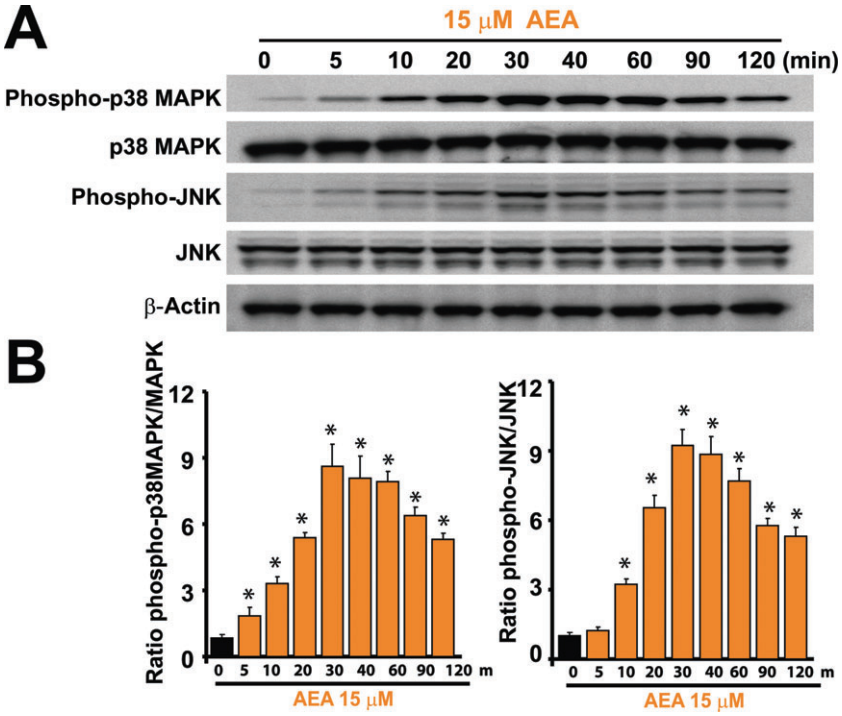

Figure 7 AEA triggers time-dependent activation of p38 and JNK–MAPKs in HCAECs. (A) Western blot analysis for p38 and JNK–MAPKs demonstrates their time-dependent activation by AEA treatment. Activation is maximal by 30 min and declines towards control levels by 90 min. A representative blot is presented from four identical experiments. (B) The quantification of MAPK activation. *$P < 0.05$ versus vehicle ($n = 4$/treatment).

tors. The CB_1-dependent ROS generation in human macrophages was dependent on the p38 MAPK pathway and could be attenuated by its inhibition with SB203580 (Han *et al.*, 2009). This study has also elegantly demonstrated that CB_1 activation in human macrophages directly modulated inflammatory activities [e.g. tumour necrosis factor-α (TNF-α) and MCP-1 secretion] through ROS generation (Han *et al.*, 2009). In contrast, CB_2 activation had opposing effects. They proposed that Rap1, a member of the Ras small G protein family, was responsible for the CB_2-dependent inhibition of CB_1-stimulated ROS production in macrophages (Han *et al.*, 2009). A similar mechanism may also be functional in endothelial cells because increased ROS generation [NADPH oxidases are pivotal sources of ROS generation both in macrophages and endothelial cells (Griendling *et al.*, 2000; Harrison *et al.*, 2003)] also leads to activation of endothelial cells and an inflammatory phenotype (secretion of pro-inflammatory cytokines/chemokines (e.g. TNF-α and MCP-1), expression of adhesion molecules, etc.), which is attenuated by CB_2 agonists (Rajesh *et al.*, 2007).

It is well known that ROS generation may also lead to activation of p38 and JNK–MAPKs promoting cell death, but as demonstrated in the above-mentioned study ROS generation may also depend on p38 MAPK activation. In our study, we found a rapid activation of p38 and JNK–MAPKs several minutes following AEA or HU210 treatment of HCAECs, which peaked around 30–60 min thereafter. The AEA- or HU210-induced ROS generation peaked between 1 and 3 h following the agonist exposure (data not shown) and gradually declined thereafter. Keeping in mind the multiple limitations of ROS detection using fluorescent probes in live cells (e.g. large experimental variability making the detection of small signals very difficult, rapid decline of fluorescent signal because of bleaching, etc.), it is not possible to exclude that CB_1 receptor activation-triggered ROS generation was directly involved in the activation of MAPKs in endothelial cells based only on these measurements, although no significant increase in ROS could be detected by our method a few minutes following AEA or HU210 treatment of HCAECs (MAPK activation was evident within 5 min). However, supporting some role of ROS in CB_1 activation, *N*-acetylcysteine pretreatment of endothelial cells (which prevented the AEA- or HU210-induced ROS generation) partially attenuated the CB_1-induced MAPK activation and cell death. In contrast, in combination with CB_1 antagonists, *N*-acetylcysteine almost completely prevented these events. These results imply that CB_1 receptor activation may promote ROS-dependent and -independent MAPK activation and cell death in HCAECs. This is further supported by observation that administration of p38 and JNK inhibitors together with *N*-acetylcysteine also completely abolished AEA- or HU210-induced cell death (data not shown). We also found that AEA or HU210 triggered CB_1-dependent ERK1/2 activation in HCAECs (data not shown), but ERK1/2 activation did not appear to be involved in endothelial cell death (data not shown).

The above-mentioned findings, coupled with a recent report demonstrating that CB_1 receptors promote oxidative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human cardiomyocytes (Mukhopadhyay *et al.*, 2010a), suggest that CB_1 receptor acti-

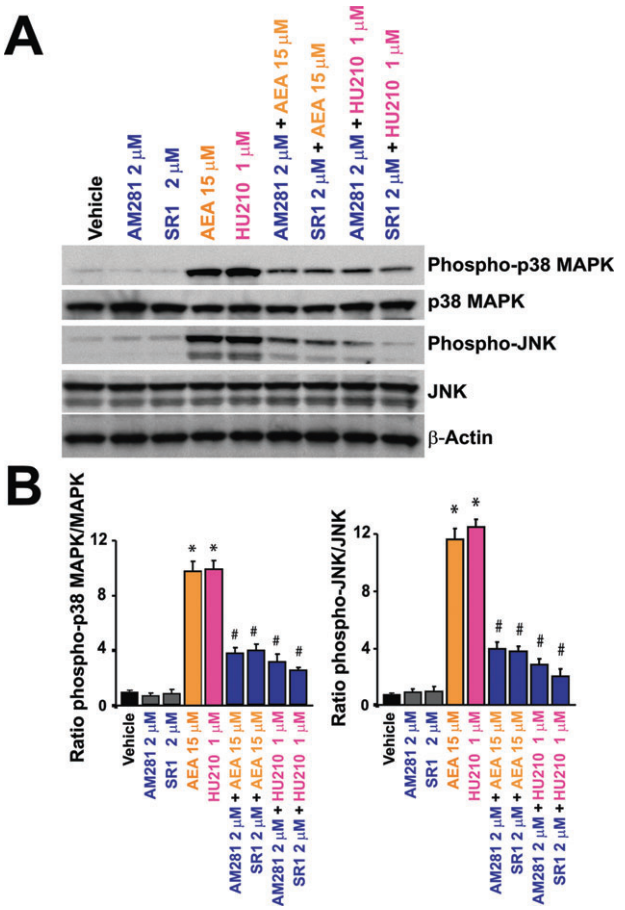

Figure 8 CB_1 antagonists attenuate AEA- or HU210-induced activation of MAPKs. (A) Cells were incubated with the indicated agonists alone for 40 min or first treatment with antagonist for an hour followed by co-incubation with agonist for 40 min, and Western blot analysis was performed to determine the MAPK activation. (B) The quantification of MAPK activation. *$P < 0.05$ versus vehicle; # $P < 0.05$ versus AEA/HU210 alone.

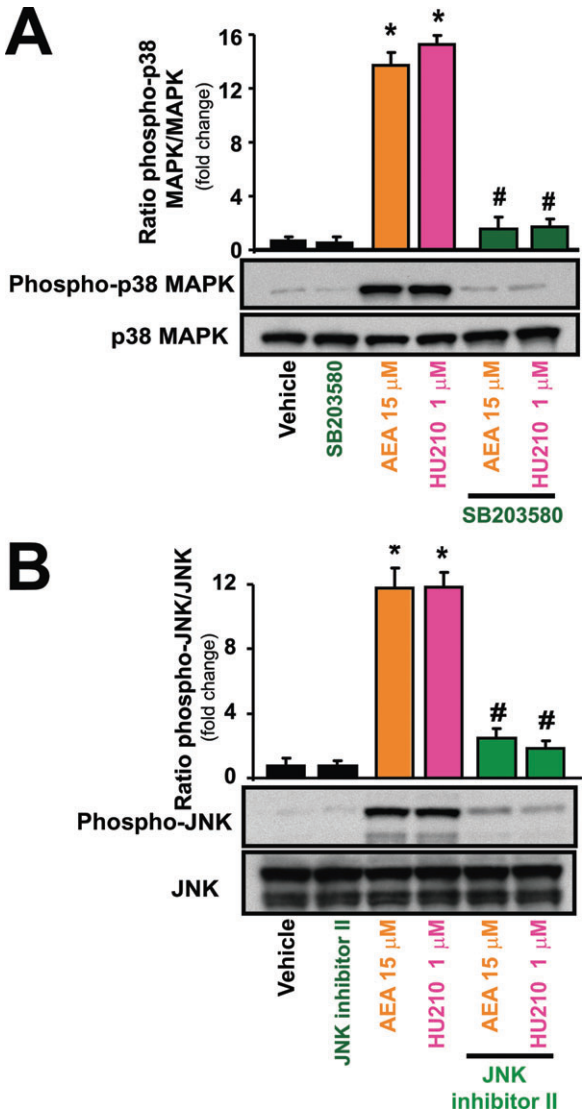

Figure 9 MAPK inhibitors attenuate AEA- or HU210-induced activation of MAPKs. Cells were treated as indicated with agonist for 40 min or first treated with MAPK inhibitors for 1 h, followed by incubation with agonist for 40 min, and MAPK activation was determined by Western blot analysis. (A) p38 MAPK activation. (B) JNK activation. *$P < 0.05$ versus vehicle; # $P < 0.05$ versus AEA/HU210 alone.

vation by endocannabinoids in cardiac and endothelial cells may facilitate cardiovascular dysfunction by amplifying oxidative stress, MAPK activation and cell death, and the subsequent inflammatory response. Consistent with this notion, pharmacological inhibition of CB_1 receptors was associated with numerous cytoprotective and anti-inflammatory effects in pre-clinical disease models of ischaemia–reperfusion injury (Berger *et al.*, 2004; Muthian *et al.*, 2004; Sommer *et al.*, 2006; Caraceni *et al.*, 2009; Lim *et al.*, 2009; Zhang *et al.*, 2009), septic shock (Kadoi *et al.*, 2005a,b; Kadoi and Goto, 2006; Villanueva *et al.*, 2009), cardiomyopathy (Mukhopadhyay *et al.*, 2007a; 2010a), kidney (Janiak *et al.*, 2007; Mukhopadhyay *et al.*, 2010b) and liver (Teixeira-Clerc *et al.*, 2006; Gary-Bobo *et al.*, 2007; Mallat and Lotersztajn, 2008) diseases, and atherosclerosis (Dol-Gleizes *et al.*, 2009; Sugamura *et al.*, 2009) just to mention a few. Chronic treatment of obese Zucker rats with the CB_1 antagonist/inverse agonist rimonabant (SR141716) (Pertwee, 2005) not only decreased body weight gain, insulin resistance and sympathetic activity, and increased adiponectin levels, but also reduced the increase in blood pressure, and beneficially altered the balance of COX-derived vasoactive products (Mingorance *et al.*, 2009). Remarkably, the CB_1 antagonists SR141716A or AM251 improved vascular hyporeactivity associated with haemorrhagic shock in rats and prolonged survival (Hou *et al.*, 2009), and chronic CB_1 receptor inhibition also decreased vascular angiotensin II type-1 receptor expression, decreased oxidative stress and improved endothelial function in a model of atherosclerosis (Tiyerili *et al.*, 2009). Importantly, rimonabant also attenuated inflammatory markers (e.g. TNF-α, C-reactive protein, etc.), plasma leptin and insulin levels, and increased plasma adiponectin in obese patients with metabolic syndrome and/or type 2 diabetes (reviewed in Di Marzo, 2008; Engeli, 2008; Mach *et al.*, 2008; Pertwee, 2009). Moreover, a recent study has also found visceral obesity to be associated with increased perirenal endocannabinoid tone, which positively correlated with increased kidney microvascular damage, suggesting a possible involvement of endocannab-

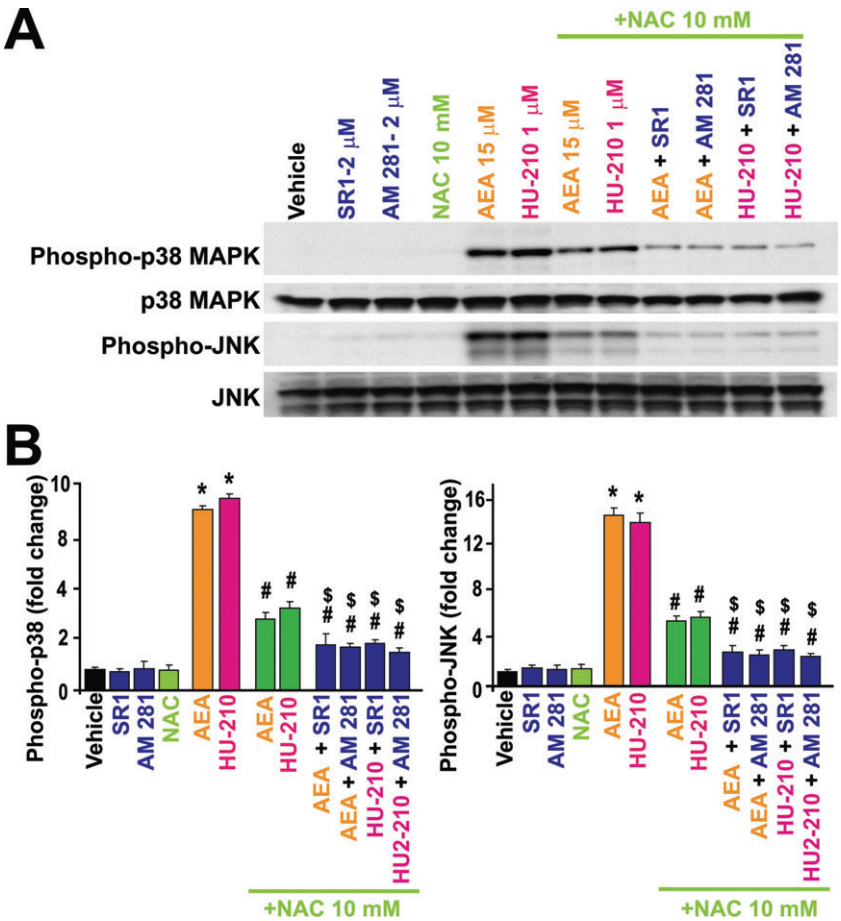

Figure 10 The AEA/HU210-induced activation of p38 and JNK–MAPKs involves ROS-dependent and -independent mechanisms in HCAECs. Cells were treated as indicated with agonist for 40 min or first treated with CB_1 inhibitors, NAC, or both for 1 h, followed by incubation with agonist for 40 min. MAPK activation was then determined by Western blot analysis. (A) p38 and JNK–MAPK activation as per treatments indicated. (B) Quantification of MAPK activation. The combination of CB_1 antagonists with NAC almost completely prevents p38 and JNK–MAPKs activation in HCAECs, while NAC alone is only partially effective in preventing MAPK activation. *$P < 0.05$ versus vehicle; #$P < 0.05$ versus AEA/HU210 alone; \$$P < 0.05$ versus AEA/HU210 ± CB_1 antagonists; $n = 4$/treatment.

inoid excess and CB_1 receptors in human visceral obesity and its renal microvascular complications (Bordicchia *et al.*, 2009).

In contrast to the above described effects of CB_1 agonists, activation of CB_2 receptors on endothelial and inflammatory cells appears to attenuate vascular inflammation (Rajesh *et al.*, 2007) and atherosclerosis progression (Steffens *et al.*, 2005), as well as the interplay of activated endothelium with inflammatory cells, interrelated oxidative/nitrosative stress and consequent cell death associated with ischaemia–reperfusion injury (Batkai *et al.*, 2007; Pacher and Hasko, 2008). Consistent with the opposing regulatory roles of CB_1 and CB_2 receptors, CB_1 activation triggers increased ROS generation in macrophages, which is attenuated by CB_2 activation as described earlier (Han *et al.*, 2009). Other examples of opposing regulation of pathological processes by cannabinoid receptors are the development of liver fibrosis (Julien *et al.*, 2005; Teixeira-Clerc *et al.*, 2006) and cisplatin-induced nephropathy (Mukhopadhyay *et al.*, 2010b,c).

Collectively, CB_1 receptor activation in endothelial cells may amplify the ROS–MAPK activation–cell death pathway in pathological conditions if the endocannabinoid synthetic or metabolic pathways are dysregulated by excessive inflammation and/or oxidative/nitrosative stress, thereby contributing to the development of endothelial dysfunction and pathophysiology of multiple cardiovascular diseases. The results reported in this study may further facilitate a better understanding of the multiple beneficial effects of CB_1 antagonism on cardiovascular dysfunction, observed both in pre-clinical models of various cardiovascular disorders, as well as in patients with obesity and cardiometabolic syndrome.

Acknowledgements

This study was supported by the Intramural Research Program of NIH/NIAAA (P.P.), and DA11322 and DA21696 (K.M.).

Conflict of interest

No conflicts to disclose.

References

Alexander SP, Mathie A, Peters JA (2008). *Guide to Receptors and Channels (GRAC)*, 3rd edition. *Br J Pharmacol* **153**: S1–S209.

Armstrong EJ, Morrow DA, Sabatine MS (2006a). Inflammatory biomarkers in acute coronary syndromes: part I: introduction and cytokines. *Circulation* **113**: e72–e75.

Armstrong EJ, Morrow DA, Sabatine MS (2006b). Inflammatory biomarkers in acute coronary syndromes: part II: acute-phase reactants and biomarkers of endothelial cell activation. *Circulation* **113**: e152–e155.

Armstrong EJ, Morrow DA, Sabatine MS (2006c). Inflammatory biomarkers in acute coronary syndromes: part III: biomarkers of oxidative stress and angiogenic growth factors. *Circulation* **113**: e289–e292.

Batkai S, Osei-Hyiaman D, Pan H, El-Assal O, Rajesh M, Mukhopadhyay P *et al*. (2007). Cannabinoid-2 receptor mediates protection against hepatic ischemia/reperfusion injury. *FASEB J* **21**: 1788–1800.

Berger C, Schmid PC, Schabitz WR, Wolf M, Schwab S, Schmid HH (2004). Massive accumulation of *N*-acylethanolamines after stroke. Cell signalling in acute cerebral ischemia? *J Neurochem* **88**: 1159–1167.

Bonz A, Laser M, Kullmer S, Kniesch S, Babin-Ebell J, Popp V *et al*. (2003). Cannabinoids acting on CB1 receptors decrease contractile performance in human atrial muscle. *J Cardiovasc Pharmacol* **41**: 657–664.

Bordicchia M, Battistoni I, Mancinelli L, Giannini E, Refi G, Minardi D *et al*. (2009). Cannabinoid CB1 receptor expression in relation to visceral adipose depots, endocannabinoid levels, microvascular damage, and the presence of the Cnr1 A3813G variant in humans. *Metabolism* doi:10.1016/j.metabol.2009.09.018.

Caraceni P, Pertosa AM, Giannone F, Domenicali M, Grattagliano I, Principe A *et al*. (2009). Antagonism of the cannabinoid CB-1 receptor protects rat liver against ischaemia–reperfusion injury complicated by endotoxaemia. *Gut* **58**: 1135–1143.

Csiszar A, Pacher P, Kaley G, Ungvari Z (2005). Role of oxidative and nitrosative stress, longevity genes and poly(ADP-ribose) polymerase in cardiovascular dysfunction associated with aging. *Curr Vasc Pharmacol* **3**: 285–291.

Csiszar A, Wang M, Lakatta EG, Ungvari Z (2008). Inflammation and endothelial dysfunction during aging: role of NF-kappaB. *J Appl Physiol* **105**: 1333–1341.

Dalton GD, Bass CE, Van Horn C, Howlett AC (2009). Signal transduction via cannabinoid receptors. *CNS Neurol Disord Drug Targets* **8**: 422–431.

Di Marzo V (2008). Targeting the endocannabinoid system: to enhance or reduce? *Nat Rev Drug Discov* **7**: 438–455.

Dol-Gleizes F, Paumelle R, Visentin V, Mares AM, Desitter P, Hennuyer N *et al*. (2009). Rimonabant, a selective cannabinoid CB1 receptor antagonist, inhibits atherosclerosis in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol* **29**: 12–18.

Engeli S (2008). Dysregulation of the endocannabinoid system in obesity. *J Neuroendocrinol* **20**: 110–115.

Gary-Bobo M, Elachouri G, Gallas JF, Janiak P, Marini P, Ravinet-Trillou C *et al*. (2007). Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker fa/fa rats. *Hepatology* **46**: 122–129.

Griendling KK, Sorescu D, Ushio-Fukai M (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* **86**: 494–501.

Han KH, Lim S, Ryu J, Lee CW, Kim Y, Kang JH *et al*. (2009). CB1 and CB2 cannabinoid receptors differentially regulate the production of reactive oxygen species by macrophages. *Cardiovasc Res* **84**: 378–386.

Hansson GK, Libby P (2006). The immune response in atherosclerosis: a double-edged sword. *Nat Rev* **6**: 508–519.

Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H (2003). Role of oxidative stress in atherosclerosis. *Am J Cardiol* **91**: 7A–11A.

Hiley CR (2009). Endocannabinoids and the heart. *J Cardiovasc Pharmacol* **53**: 267–276.

Hillard CJ (2000). Endocannabinoids and vascular function. *J Pharmacol Exp Ther* **294**: 27–32.

Hou LC, Li N, Zheng LN, Lu Y, Xie KL, Wang YM *et al*. (2009). CB1 cannabinoid receptor participates in the vascular hyporeactivity resulting from hemorrhagic shock in rats. *Chin Med J (Engl)* **122**: 950–954.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA *et al*. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54**: 161–202.

Janiak P, Poirier B, Bidouard JP, Cadrouvele C, Pierre F, Gouraud L *et al*. (2007). Blockade of cannabinoid CB1 receptors improves renal function, metabolic profile, and increased survival of obese Zucker rats. *Kidney Int* **72**: 1345–1357.

Julien B, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M *et al*. (2005). Antifibrogenic role of the cannabinoid receptor CB2 in the liver. *Gastroenterology* **128**: 742–755.

Kadoi Y, Goto F (2006). Effects of AM281, a cannabinoid antagonist, on circulatory deterioration and cytokine production in an endotoxin shock model: comparison with norepinephrine. *J Anesth* **20**: 284–289.

Kadoi Y, Hinohara H, Kunimoto F, Kuwano H, Saito S, Goto F (2005a). Effects of AM281, a cannabinoid antagonist, on systemic haemodynamics, internal carotid artery blood flow and mortality in septic shock in rats. *Br J Anaesth* **94**: 563–568.

Kadoi Y, Hinohara H, Kunimoto F, Saito S, Goto F (2005b). Cannabinoid antagonist AM 281 reduces mortality rate and neurologic dysfunction after cecal ligation and puncture in rats. *Crit Care Med* **33**: 2629–2636.

Lim SY, Davidson SM, Yellon DM, Smith CC (2009). The cannabinoid CB1 receptor antagonist, rimonabant, protects against acute myocardial infarction. *Basic Res Cardiol* **104**: 781–792.

Liu J, Gao B, Mirshahi F, Sanyal AJ, Khanolkar AD, Makriyannis A *et al*. (2000). Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem J* **346**: 835–840.

Mach F, Montecucco F, Steffens S (2008). Cannabinoid receptors in acute and chronic complications of atherosclerosis. *Br J Pharmacol* **153**: 290–298.

Mallat A, Lotersztajn S (2008). Cannabinoid receptors as therapeutic targets in the management of liver diseases. *Drug News Perspect* **21**: 363–368.

Mendizabal VE, Adler-Graschinsky E (2007). Cannabinoids as therapeutic agents in cardiovascular disease: a tale of passions and illusions. *Br J Pharmacol* **151**: 427–440.

Mingorance C, Alvarez de Sotomayor M, Jimenez-Palacios FJ, Callejon Mochon M, Casto C, Marhuenda E *et al*. (2009). Effects of chronic treatment with the CB1 antagonist, rimonabant on the blood pressure, and vascular reactivity of obese Zucker rats. *Obesity (Silver Spring)* **17**: 1340–1347.

Mukhopadhyay P, Batkai S, Rajesh M, Czifra N, Harvey-White J, Hasko G *et al*. (2007a). Pharmacological inhibition of CB1 cannabinoid receptor protects against doxorubicin-induced cardiotoxicity. *J Am Coll Cardiol* **50**: 528–536.

Mukhopadhyay P, Rajesh M, Hasko G, Hawkins BJ, Madesh M, Pacher P (2007b). Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. *Nat Protoc* **2**: 2295–2301.

Mukhopadhyay P, Rajesh M, Batkai S, Patel V, Kashiwaya Y, Liaudet L *et al*. (2010a). CB1 cannabinoid receptors promote oxidative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human cardiomyocytes. *Cardiovasc Res* **85**: 773–784.

Mukhopadhyay P, Pan H, Rajesh M, Batkai S, Patel V, Harvey-White J *et al*. (2010b). CB1 cannabinoid receptors promote oxidative/

nitrosative stress, inflammation and cell death in a murine nephropathy model. *Br J Pharmacol* in press.

Mukhopadhyay P, Rajesh M, Pan H, Patel V, Mukhopadhyay B, Batkai S *et al*. (2010c). Cannabinoid-2 receptor limits inflammation, oxidative/nitrosative stress and cell death in nephropathy. *Free Radic Biol Med* **48**: 457–467.

Muthian S, Rademacher DJ, Roelke CT, Gross GJ, Hillard CJ (2004). Anandamide content is increased and CB1 cannabinoid receptor blockade is protective during transient, focal cerebral ischemia. *Neuroscience* **129**: 743–750.

Osterud B, Bjorklid E (2003). Role of monocytes in atherogenesis. *Physiol Rev* **83**: 1069–1112.

Pacher P (2009). Cannabinoid CB1 receptor antagonists for atherosclerosis and cardiometabolic disorders: new hopes, old concerns? *Arterioscler Thromb Vasc Biol* **29**: 7–9.

Pacher P, Hasko G (2008). Endocannabinoids and cannabinoid receptors in ischaemia–reperfusion injury and preconditioning. *Br J Pharmacol* **153**: 252–262.

Pacher P, Batkai S, Kunos G (2006). The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev* **58**: 389–462.

Pacher P, Beckman JS, Liaudet L (2007). Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* **87**: 315–424.

Pertwee RG (2005). Pharmacological actions of cannabinoids. *Handb Exp Pharmacol* **168**: 1–51.

Pertwee RG (2009). Emerging strategies for exploiting cannabinoid receptor agonists as medicines. *Br J Pharmacol* **156**: 397–411.

Rajesh M, Mukhopadhyay P, Batkai S, Hasko G, Liaudet L, Huffman JW *et al*. (2007). CB2-receptor stimulation attenuates TNF-alpha-induced human endothelial cell activation, transendothelial migration of monocytes, and monocyte–endothelial adhesion. *Am J Physiol Heart Circ Physiol* **293**: H2210–H2218.

Rajesh M, Mukhopadhyay P, Hasko G, Huffman JW, Mackie K, Pacher P (2008a). CB2 cannabinoid receptor agonists attenuate TNF-alpha-induced human vascular smooth muscle cell proliferation and migration. *Br J Pharmacol* **153**: 347–357.

Rajesh M, Mukhopadhyay P, Hasko G, Pacher P (2008b). Cannabinoid CB1 receptor inhibition decreases vascular smooth muscle migration and proliferation. *Biochem Biophys Res Commun* **377**: 1248–1252.

Rao RM, Yang L, Garcia-Cardena G, Luscinskas FW (2007). Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. *Circ Res* **101**: 234–247.

Sommer C, Schomacher M, Berger C, Kuhnert K, Muller HD, Schwab S *et al*. (2006). Neuroprotective cannabinoid receptor antagonist SR141716A prevents downregulation of excitotoxic NMDA receptors in the ischemic penumbra. *Acta Neuropathol* **112**: 277–286.

Steffens S, Veillard NR, Arnaud C, Pelli G, Burger F, Staub C *et al*. (2005). Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice. *Nature* **434**: 782–786.

Sugamura K, Sugiyama S, Nozaki T, Matsuzawa Y, Izumiya Y, Miyata K *et al*. (2009). Activated endocannabinoid system in coronary artery disease and antiinflammatory effects of cannabinoid 1 receptor blockade on macrophages. *Circulation* **119**: 28–36.

Szabo C (2009). Role of nitrosative stress in the pathogenesis of diabetic vascular dysfunction. *Br J Pharmacol* **156**: 713–727.

Tedgui A, Mallat Z (2006). Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* **86**: 515–581.

Teixeira-Clerc F, Julien B, Grenard P, Tran Van Nhieu J, Deveaux V, Li L *et al*. (2006). CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat Med* **12**: 671–676.

Tiyerili V, Zimmer S, Jung S, Wassmann K, Nickening G, Wassmann S (2009). CB1 receptor inhibition leads to decreased vascular AT1 receptor expression, inhibition of oxidative stress and improved endothelial function. *Circulation* **120**: S1073.

Vanhoutte PM (2009). Endothelial dysfunction: the first step toward coronary arteriosclerosis. *Circ J* **73**: 595–601.

Villanueva A, Yilmaz MS, Millington WR, Cutrera RA, Stouffer DG, Parsons LH *et al*. (2009). Central cannabinoid-1 receptor antagonist administration prevents endotoxic hypotension affecting norepinephrine release in the preoptic anterior hypothalamic area. *Shock* **32**: 614–620.

Zhang M, Martin BR, Adler MW, Razdan RJ, Kong W, Ganea D *et al*. (2009). Modulation of cannabinoid receptor activation as a neuroprotective strategy for EAE and stroke. *J Neuroimmune Pharmacol* **4**: 249–259.