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RESEARCH PAPER

CB_2 cannabinoid receptor agonists attenuate TNF-α-induced human vascular smooth muscle cell proliferation and migration

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Background and purpose: Vascular smooth muscle proliferation and migration triggered by inflammatory stimuli are involved in the development and progression of atherosclerosis and restenosis. Cannabinoids may modulate cell proliferation in various cell types through cannabinoid 2 (CB_2) receptors. Here, we investigated the effects of CB_2 receptor agonists on TNF-α-induced proliferation, migration and signal transduction in human coronary artery smooth muscle cells (HCASMCs).
Experimental approach: HCASMCs were stimulated with TNF-α. Smooth muscle proliferation was determined by the extent of BrdU incorporation and the migration was assayed by modified Boyden chamber. CB_2 and/or CB_1 receptor expressions were determined by immunofluorescence staining, western blotting, RT-PCR, real-time PCR and flow cytometry.
Key results: Low levels of CB_2 and CB_1 receptors were detectable in HCASMCs compared to the high levels of CB_2 receptors expressed in THP-1 monocytes. TNF-α triggered up to ~80% increase (depending on the method used) in CB_2 receptor mRNA and/or protein expression in HCASMCs, and induced Ras, p38 MAPK, ERK 1/2, SAPK/JNK and Akt activation, while increasing proliferation and migration. The CB_2 agonists, JWH-133 and HU-308, dose-dependently attenuated these effects of TNF-α.
Conclusions and implications: Since the above-mentioned TNF-α-induced phenotypic changes are critical in the initiation and progression of atherosclerosis and restenosis, our findings suggest that CB_2 agonists may offer a novel approach in the treatment of these pathologies by decreasing vascular smooth muscle proliferation and migration.
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Keywords: cannabinoids; cannabinoid 2 receptor; smooth muscle; proliferation; migration; antibodies

Abbreviations: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; AM-630, (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone; CB_1 and CB_2 receptors, cannabinoid 1 and 2 receptors; HCASMCs, human coronary artery smooth muscle cells; HU-308, (+)-(1a*H*,3 *H*,5a*H*)-4-[2,6-dimethoxy-4-(1,1-dimethylheptyl) phenyl]-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-carbinol; JWH-133, 3-(1,1-dimethylbutyl)-1-deoxy-Δ8-tetrahydrocannabinol; MAPK, mitogen-activated protein kinase, SR2 or SR144528; THC, delta9-tetrahydrocannabinol; THP-1, human monocyte cell line; TNF-α, tumour necrosis factor alpha

Introduction

Vascular smooth muscle proliferation and migration are pivotal events in the pathogenesis of atherosclerosis and are directly implicated in the failure of clinical interventions used to treat patients with coronary heart disease. For example, percutaneous transluminal angioplasty (an invasive procedure aimed to repair a stenotic blood vessel by inflating a balloon-tipped catheter at the site of the vascular narrowing followed by an insertion of an expandable wire mesh or hollow perforated tube (stent) into the reconstructed/dilated blood vessel to protect against re-narrowing), often fails with the time due to the development of restenosis (Tanaka *et al.*, 1996; Zimmerman *et al.*, 2003; Cai, 2006; Tedgui and Mallat, 2006). Vascular smooth muscle cells are the principal cell type in both restenotic and atherosclerotic lesions (Tanaka *et al.*, 1996; Zimmerman *et al.*, 2003; Tedgui and Mallat, 2006), which are formed as a result of numerous pathological processes involving generation of reactive oxygen and

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nitrogen species (Pacher *et al*., 2006b, 2007), and the accumulation of inflammatory cells and the release of cytokines such as tumour necrosis factor alpha (TNF-α) (Patel *et al*., 2000; Harrison *et al*., 2003; Hansson and Libby, 2006; Pacher *et al*., 2007). The proinflammatory cytokine TNF-α is considered to play a key role in increasing the expression of endothelial-cell adhesion molecules and promoting the release of various chemokines recruiting monocytes to the site of injury and enhancing their adhesiveness to the endothelium. TNF-α also stimulates the release of variety of factors that induce smooth muscle migration and proliferation (from the media into the intima), which then synthesize and deposit extracellular matrix (Ip *et al*., 1990; Osterud and Bjorklid, 2003; Hansson, 2005; Tedgui and Mallat, 2006; Gerthoffer, 2007).

Endocannabinoids, which include arachidonoyl ethanolamide or anandamide (AEA) and 2-arachidonoylglycerol (2-AG), phytocannabinoids and their synthetic analogues exert various cardiovascular, metabolic, central nervous system and anti-inflammatory effects, predominantly, through CB_1 and CB_2 receptors (Mechoulam *et al*., 1998; Klein, 2005; Pacher *et al*., 2006a). The CB_1 receptor is highly expressed in the brain, but lower levels are also detectable in peripheral tissues including heart, vascular endothelium and liver (reviewed in Mackie, 2006; Pacher *et al*., 2006a). Initially, the CB_2 receptor was considered to be expressed primarily by immune and haematopoietic cells, however, recent studies have also intriguingly demonstrated these receptors in brain (Van Sickle *et al*., 2005), myocardium (Mukhopadhyay *et al*., 2007a), cardiomyoblasts (Shmist *et al*., 2006; Mukhopadhyay *et al*., 2007a) and endothelial cells of various origins (Blazquez *et al*., 2003; Zoratti *et al*., 2003; Golech *et al*., 2004; Mestre *et al*., 2006) (reviewed in Klein, 2005; Mackie, 2006; Pacher *et al*., 2006b).

Delta9-tetrahydrocannabinol (THC), a cannabis constituent, has recently been demonstrated to inhibit atherosclerosis progression in a mouse model of the disease, presumably by CB_2 receptor stimulation in inflammatory cells (Steffens *et al*., 2005). However, the role of CB_2 receptor in vascular smooth muscle cell activation in this phenomenon remains unexplored. There is considerable evidence that endocannabinoids and their synthetic analogues may differentially modulate cell proliferation in various cell types through CB_2 receptor-dependent and -independent mechanisms (Lopez-Rodriguez *et al*., 2005; McAllister *et al*., 2005; Blazquez *et al*., 2006; Ofek *et al*., 2006; Lombard *et al*., 2007; Maresz *et al*., 2007; Wilkinson and Williamson, 2007). Thus, we aimed to evaluate the effects of selective CB_2 receptor agonists on TNF-α-induced proliferation, migration and signal transduction of human coronary artery smooth muscle cells (HCASMCs). Given that the vascular smooth muscle proliferation and migration triggered by inflammatory stimuli are pivotal events in the pathogenesis and progression of atherosclerosis and restenosis, our findings may have important clinical implications. Since these are critical tools for assessing the role of CB_2 in any process, we will discuss some limitations of the presently available CB_2 antibodies and emphasize the importance of the use of proper controls and multiple methods to confirm the presence of CB_2 receptors.

Materials and methods

Materials

The CB_2 receptor agonist JWH-133 was either purchased from Tocris Bioscience (Ellisville, MO, USA) or synthesized as described earlier (Huffman *et al*., 1999). The selective CB_2 receptor agonist HU-308 (Hanus *et al*., 1999) was from Cayman Europe (Tallinn, Estonia). CB_2 antagonist AM-630 was purchased from Tocris Bioscience (Ellisville, MO, USA). SR141716A (SR1) and SR144528 (SR2) were purchased from NIDA Drug Supply (NC, USA). Human recombinant TNF-α was obtained from R&D Systems, (MN, USA). Sources of all the other reagents are mentioned in the text where appropriate.

Cell culture

Human coronary artery smooth muscle cells were obtained from Cambrex (MD, USA) and were cultured in smooth muscle growth medium (SmGM-2) (Cambrex). HCASMCs in passages 3–6 were used for the experiments. Human coronary artery endothelial cells and its growth medium were obtained from Cell Applications (CA, USA). Human monocytic cell line (THP-1) was obtained from American Type Culture Collection (ATCC, VA, USA) and was grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 10% fetal bovine serum, 100 U of penicillin and 100 μg of streptomycin per ml, respectively (Invitrogen, CA, USA).

Immunofluorescence staining

Human coronary artery smooth muscle cells were grown to confluence in chamber slides (Lab-Tek, Nalgene-Nunc Inc., New York, NY, USA). The growth medium was aspirated and cells were washed twice with phosphate buffered saline (PBS) and then were fixed with 4% paraformaldehyde for 20 min at 4 °C. After washing with PBS, cells were permeabilized in 0.2% Triton X-100 in PBS for 15 min at room temperature (RT). Following washing, the CB_1 and CB_2 expressions in the human vascular smooth muscle cells were determined by immunofluorescence staining technique using anti-CB_1 (rabbit polyclonal, Cayman Chemical, MI, USA) or CB_2 (rabbit polyclonal, Cayman Chemicals) respectively, used at 1:100 dilution for 5 h at 4 °C. Next, the cells were probed with goat-anti-rabbit FITC (1:250, Pierce, IL, USA) for 1 h at RT. After rinsing cells with PBS, the cells were probed with goat-anti-rabbit FITC (1:250, Pierce) for 1 h at RT. The nucleus was counterstained with DAPI (Molecular Probes, CA, USA). Images were obtained using fluorescent microscope (Olympus IX 81) at $\times 20$ objective with $\times 1.5$ optical zoom. To rule out the non-specific staining, the primary antibodies were preabsorbed with the corresponding blocking peptides for CB_1 (Cayman, Cat #10006591) or CB_2 (Cayman, Cat #301550), according to the protocol provided with the peptides. In brief, the blocking peptides were mixed with corresponding antibodies in 1:1 ratio and were incubated for 1 h at RT. Later, this preabsorbed antibody was used for the staining. This procedure essentially blocks the antibody–antigen (protein) formation during the

immunofluorescence staining and thus aids in confirming/evaluating specificity of the CB_1/CB_2 receptor staining.

Conventional RT-PCR and real-time PCR

Total RNA was isolated from the cells using Trizol LS reagent (Invitrogen) according to the manufacturer's instruction. The RNA was treated with RNase-free DNase (Ambion, TX, USA) to remove traces of genomic DNA contamination. Total RNA was then reverse-transcribed to cDNA using the Super-Script II (Invitrogen) and the target genes were amplified using the standard PCR kit (BioRad, CA, USA). The PCR conditions were as follows: after initial denaturation at 95 °C for 2 min, 35 cycles were performed at 95 °C for 30 s and at 60 °C for 30 s. Primers used were as follows:

CB_1. 5′-TTCCCTCTTGTGAAGGCACTG-3′ (forward)
5′-TCTTGACCGTGCTCTTGATGC-3′ (reverse)

CB_2. 5′-TTTGCTTTCTGCTCCATGCTG-3′ (forward)
5′-TTCTTTTGCCTCTGACCCAAG-3′ (reverse)

β-*Actin*. 5′-ATTGCCGACAGGATGCAGAAG-3′ (forward)
5′- TAGAAGCATTTGCGGTGGACG-3′ (reverse).

The amplified products were separated on 1.5% agarose gels stained with ethidium bromide and documented using phosphor-imaging system (GE Healthcare, NJ, USA).

In a separate set of experiments, HCASMCs were treated with TNF-α (50 ng ml^{-1}) for 0−6 h and real-time PCR was performed with identical conditions except the amplification and quantification of the target gene expression was carried out using iTaq Syber green mix (BioRad) using BioRad chromo4/opticon system. Relative quantification was carried out using the relative (comparative) C_T method.

Western immunoblot assay

Human coronary artery smooth muscle cells were grown to confluence in 100 mm culture dishes coated with 0.2% gelatin and the cell lysates were prepared using lysis buffer (Pierce Biotechnology, IL, USA) supplemented with protease inhibitors (Roche, GmbH, IN, USA). The lysates were prepared by sonication (15k for 20 s) on ice. Later, the lysates were clarified to remove the cellular debris by centrifuging at 10 000 r.p.m. for 15 min at 4 °C. Protein content in the lysates was determined using the Lowry assay (BioRad). A measure of 25 μg protein was resolved in 12% SDS-PAGE and was transferred onto nitrocellulose membranes (GE Healthcare). Blocking was performed for 2 h at RT with 5% non-fat skimmed milk powder prepared in PBS containing 0.1% Tween 20 (Sigma, CA, USA). After washing with phosphate-buffered saline 0.1% Tween 20 (PBST), the membranes were probed with either rabbit polyclonal CB_1 (Cayman Chemicals; 1:1000 dilution) or with an antibody raised against the last 15 residues of rat CB_1 or CB_2 antibody (Cayman Chemicals; 1:1000 dilution) overnight at 4 °C. After subsequent washing with PBST, the secondary antibody—goat-anti-rabbit was added to HRP (Pierce Biotechnology) was incubated at RT for 1 h. Similar experiments were also performed with CB_1 antibody from Ken Mackie's laboratory (Figure 1d, lower blot). Later, the membranes were developed using chemiluminescence detection kit (Super Signal -West Pico Substrate, Pierce). To confirm uniform loading, the membranes were stripped and re-probed with β-actin (Chemicon, CA, USA). In a separate set of experiments, the cells were treated with TNF-α (50 ng ml^{-1}) for different time periods (0–6 h) and CB_2 expression was determined using immunoblot assays. The protein from mouse brain extract and human monocytic cell line (THP-1) lysate was used as appropriate positive controls for CB_1 and CB_2 receptors, respectively.

Flow cytometry

The surface expression of CB_2 receptors was determined using flow cytometry technique. For surface staining, cells were incubated with CB_2 antibody (rabbit-polyclonal, Cayman, (5–20 μg ml^{-1})) for 1 h at RT. THP-1 monocytes were used as a positive control. After blocking with 1%BSA (W/V) in Hank's buffered salt solution (HBSS) buffer (Invitrogen), the cells were incubated with CB_2 antibody (rabbit-polyclonal, Cayman, (5–20 μg ml^{-1})) for 1 h at RT. After washing in HBSS, the cells were incubated with secondary anti-rabbit FITC-conjugate (1:100 dilution; Pierce Biotechnology) for 1 h. After washing two times with HBSS, the cells were analysed by FACS Calibur flow cytometer (BD, CA, USA) as described (Rajesh *et al*., 2007; Mukhopadhyay *et al*., 2007a, b). To rule out the potential cross-reaction with immunoglobulin receptor (Fc_γII, CD32) while determining the surface expression of CB_2 receptors in THP-1 monocytes, cells were blocked with/without CD32 antibody (5 μg ml^{-1}; BD Biosciences) for 15 min followed by incubation with CB_2 antibody (Cayman) as described above. After washing, cells were incubated with secondary antibody-antirabbit FITC (1:100 dilution, Pierce Biotechnology). Later, the analysis was performed in FACS Calibur System (Becton Dickinson, CA, USA) and the data were exported to and analysed using FlowJo version 8.5 (Ashland, OR, USA).

Detection of apoptosis by flow cytometry

Human coronary artery smooth muscle cells were grown in 12-well tissue-culture plates and then treated with/without TNF-α and CB_2 receptor agonists/antagonists for 36 h. In separate experiments, smooth muscle cells were also treated with TNF-α and with/without CB_1 antagonist for 36 h and apoptosis was determined by flow cytometry using the assay as described previously (Mukhopadhyay *et al*., 2007a, b).

Ras activation assay

Ras activation in HCASMCs was determined using commercially available kit (Pierce). In brief, Ras activation assay involved the use of GTP-fusion protein containing Ras-binding domain (RBD) of Raf1, which specifically binds to and pulls down active Ras. The pulled-down active Ras was detected by western blot analysis using anti-Ras antibody.

Migration assay

The migration assays were performed in a modified, 24-well Boyden chamber as described earlier (Rajesh *et al*., 2006). In brief, 8 μm cell culture inserts (BD Biosciences) coated with 0.2% gelatin (Sigma) were placed on the bottom chamber with/without 50 ng ml^{-1} TNF-α. HCASMCs (3×10^4 cells) were suspended in 150 μl in growth-factor free medium containing 1% FBS. Later, the cells that were treated with CB_2 agonists ± CB_2 antagonists for 90 min at 37 °C in 5% CO_2 incubator. Later, the cell suspension was added to the upper chamber. After 8 h of incubation at 37 °C, the non-migrated cells on the upper surface of the filter were removed by gentle swabbing with cotton-tipped applicators. The cells that had migrated to the lower side of the chamber were fixed with 100% methanol for 15 min at RT. After complete drying, the inserts containing migrated cells were stained with 0.5% Giemsa solution (Sigma). Three to four fields per insert were counted using × 10 objective of Olympus IX81 microscope. The assays were performed in duplicates and the experiments were repeated three times.

Proliferation assay

Human coronary artery smooth muscle cells (5×10^3) were suspended in 150 μl of growth factor free smooth muscle cell (SMC) medium containing 1% FBS and were plated on to 96-well plates and were allowed to adhere for 1 h. Later, the cells were treated with TNF-α (50 ng ml^{-1}) ± CB_2 agonists (4 μM)/antagonists (1 μM) and were allowed to proliferate for 24 h. After which, 100 μl of BrdU labelling solution (10 μM final concentration) was added to each well and incubated additionally for 12 h. At the end of incubation, the proliferation was determined by the extent of BrdU incorporated using an ELISA kit (Roche GmbH). Each treatment was performed in triplicates and the entire set of experiments was repeated three times.

Western blot analysis of signalling proteins in HCASMCs

The cells were grown in 100 mm dishes and after treatment with TNF-α ± CB_2 agonists/antagonists, the cell lysates were prepared using RIPA lysis buffer (Pierce). A measure of 25 μg protein was resolved in 12% SDS-PAGE and was transferred onto nitrocellulose membranes (Invitrogen) and the blots were probed with antihuman rabbit p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), ERK1/2, phosphor-ERK1/2, SAPK/JNK 1/2, phospho-SAPK/JNK (Thr183/Tyr185), Akt or pAkt (Ser 473) at 1:1000 dilution, respectively (Cell Signaling Technology, MA, USA) and the blots were developed using antirabbit HRP with a chemiluminescence detection kit (Super Signal -West Pico Substrate, Pierce). Protein content in the cell lysates was measured by Lowry protein assay (BioRad).

Statistical analysis

All the values were represented as mean ± s.d. Statistical significance of the data was assessed by one-way ANOVA with Tukey's *post hoc* test (GraphPad-Prism4, CA, USA). $P<0.05$ was considered significant.

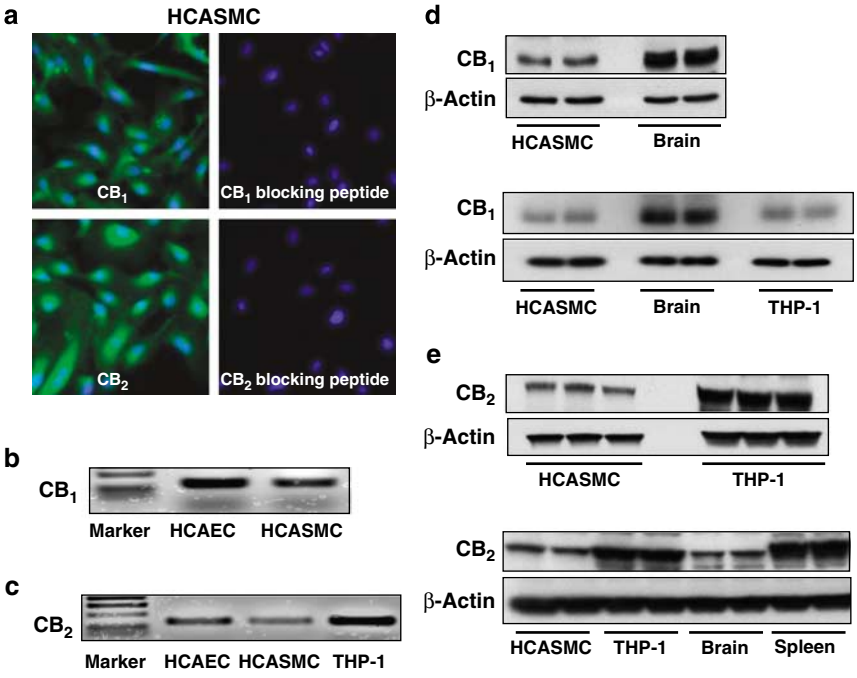

Figure 1 CB_1 and CB_2 receptors expression in HCASMCs. (**a**) Expression of CB_1 and CB_2 receptors in HCASMCs demonstrated by immunofluorescence staining. CB_1 and CB_2 expression in the vascular smooth muscle cells was detected using rabbit-polyclonal anti-CB_1 and -CB_2 antibodies (Cayman Chemicals) and using secondary antibody goat-anti-rabbit FITC (Pierce), the nucleus was contrastained with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Invitrogen). (**b**, **c**) RT-PCR analysis of CB1/2 receptor expression in human endothelial and smooth muscle cells. (**d**, **e**) Western immunoblot demonstrating expression of CB_1 and CB_2 receptors in HCASMCs. Here, representative images from three independent experiments with identical results are shown.

Results

CB_2 receptors are expressed in HCASMCs

As shown in Figures 1 and 2, CB_1 and CB_2 receptors are expressed in cultured human vascular smooth muscle cells, at basal conditions as demonstrated by immunofluorescence assays (Figure 1a), conventional RT-PCR (Figures 1b and c), real-time PCR (Figure 2c), western blot (Figures 1d, e and 2b) and flow cytometry (Figure 2a) assays, respectively. Protein extracts from mouse brain, spleen and THP-1 monocytes lysate or THP-1 monocytes were also used as appropriate positive controls for the detection of CB_1 and/or CB_2 receptors, respectively (Figures 1c, d, e and 2b–d). Preabsorbing either the CB_1 or CB_2, with the corresponding blocking peptides supplied with the primary antibodies, abrogated the detection of CB_1 and CB_2 in HCASMCs by immunofluorescence (Figures 1a and b) and western blot assays (data not shown).

We further studied the surface expression of CB_2 using flow cytometry. As shown in Figure 2a, CB_2 receptors are expressed in HCASMCs and TNFα pretreatment further augmented their expression by ~30%. The mean intensities are provided in the respective panels. We used THP-1 monocyte cell line as a positive control for surface expression of CB_2. To rule out the potential non-specific binding of CB_2 antibody with immunoglobulin receptor (Fc_γII, CD32) while determining the surface expression of CB_2 receptors in THP-1 monocytes, CD32 antibody (5 μg ml^{-1}; BD Biosciences) was used for blocking. As shown in Figures 2b and c, indeed, CD32 blockade decreased CB_2 receptor binding (353 mean intensity vs 96.5) emphasizing the importance of Fc receptor binding in these sorts of experiments.

TNF-α upregulates CB_2 expression in HCASMCs

Human coronary artery smooth muscle cells were pretreated with TNF-α (50 ng ml^{-1}) for different time points as indicated in Figures 2f and g and then the CB_2 expression was determined by western blot and quantitative real

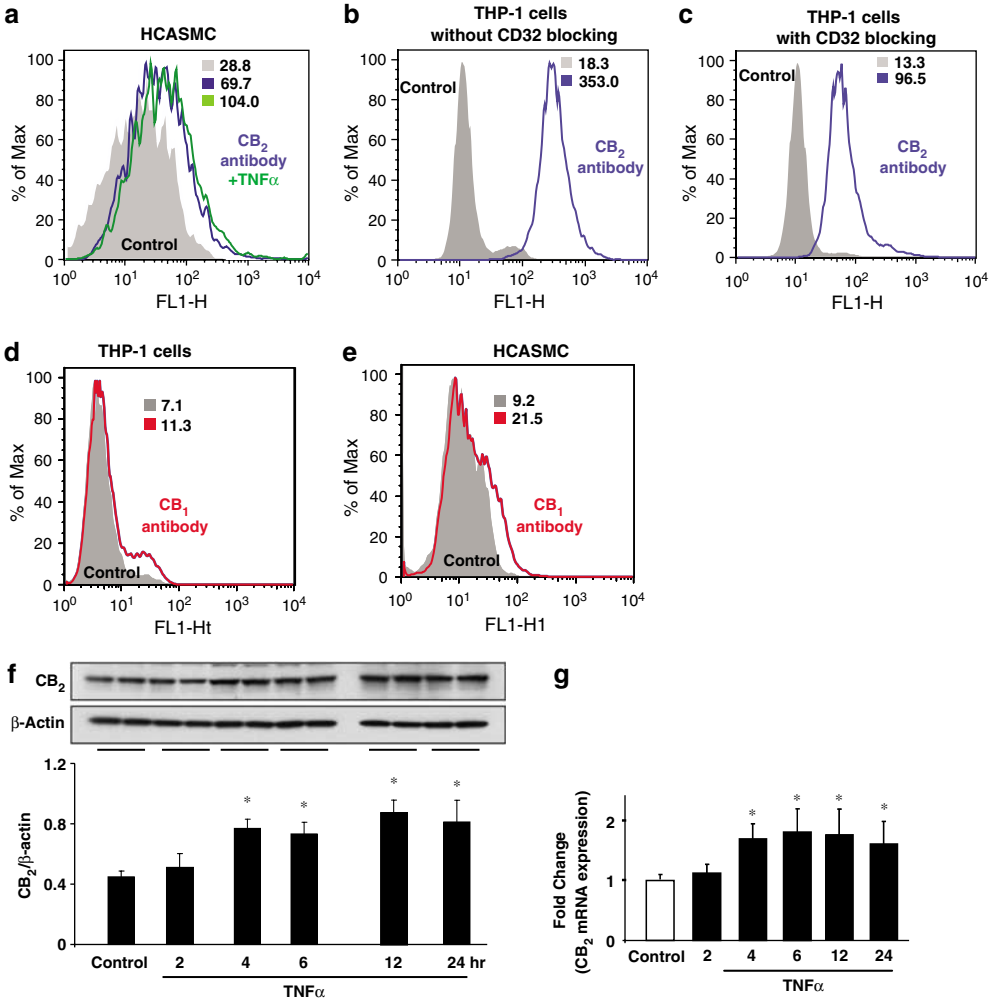

Figure 2 CB_2 receptor expression in HCASMCs and effect of TNF-α. Cells were treated with TNF-α (50 ng ml^{-1}) for 6 h or indicated time intervals and flow cytometry (**a–e**), western blot (**f**) or quantitative real-time PCR (**g**) analyses were performed for CB_2 and/or CB_1 expression in HCASMCs and THP-1 monocytes, respectively. Panels a–c show surface expression of CB_2 receptors in HCASMCs and THP1-monocytes (blue traces) and the effect of TNF-α on CB_2 expression in HCASMCs (green trace, panel **a**). Panels **d** and **e** show surface expression of CB_1 receptors in HCASMCs and THP1-monocytes (red traces). Panels f and g denote CB_2 receptor expression in HCASMCs and the effect of TNF-α by western blot and real-time PCR, respectively. Data presented are representative of 5–7 independent experiments. *$P<0.05$ vs control.

time-PCR and FACS assays. Results revealed that TNF-α treatment resulted in a time-dependent increase (up to ~1.8 fold vs control, depending on the method used) in the CB_2 receptor expression in HCASMCs (Figures 2f and g), respectively.

CB_2 agonists/antagonists did not induce apoptosis in HCASMC

As shown in Figure 3, treatment of vascular smooth muscle cells with TNF-α (50 ng ml^{-1}) alone for 36 h induced a moderate increase (~2.5–3.5%) in early apoptotic (Annexin-V positive) but not late apoptotic/necrotic (Annexin-V and Sytox Green positive) cells, which was not significantly affected by either cannabinoid receptor agonists or antagonist (Figures 3a and b).

CB_2 agonists inhibit TNF-α-induced HCASMCs proliferation

TNF-α-significantly stimulated the proliferation in vascular smooth muscle cells (~3.5-fold increase vs untreated control cells; Figure 4a). Pretreatment of the cells with either JWH-133 or HU-308 (0.5–4 μM) dose-dependently inhibited proliferation of vascular smooth muscle cells (Figure 4a), which was attenuated by CB_2 antagonists SR2/AM630 (1 μM; Figure 4b) but not by the CB_1 antagonist, SR1 (Figure 4b). The CB_2 agonists/antagonists or the CB_1 antagonist SR1 alone did not affect the basal proliferation of smooth muscle cells (Figure 4b).

CB_2 agonists attenuate TNF-α-induced HCASMCs migration

Tumour necrosis factor-α profoundly stimulated the migration of vascular smooth muscle cells (~3.7-fold increase vs

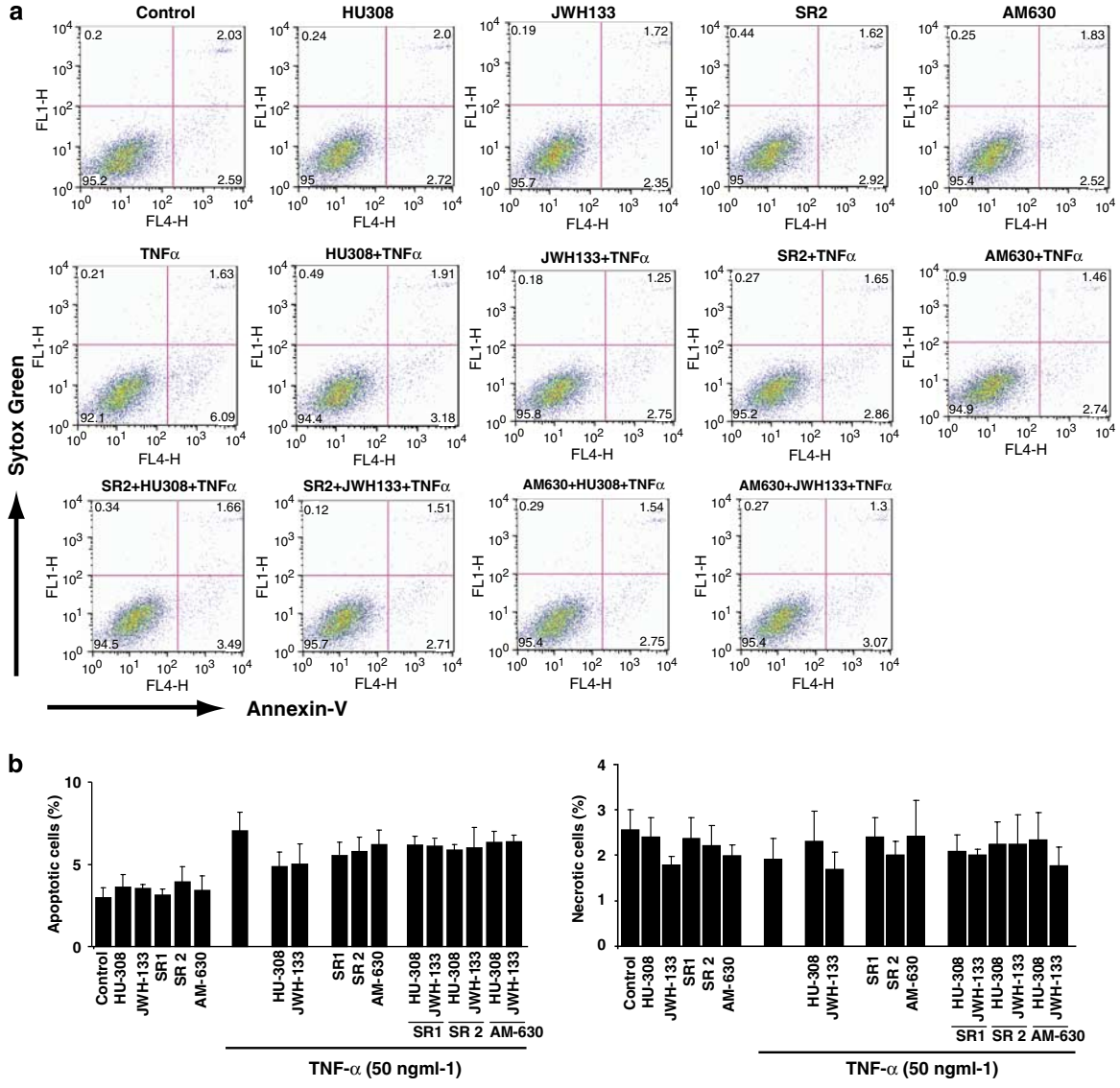

Figure 3 Effect of TNF-α and/or CB_2 agonists/antagonists on cell death in HCASMCs. Smooth muscle cells were grown in 12-well tissue-culture plates and then treated with agonists/antagonists, with or without TNF-α (50 ng ml^{-1}) for 36 h. Agonists and antagonists were used at 4 and 1 μM, respectively. Later, apoptosis/necrosis was measured by FACS calibur flow cytometer (BD Biosciences, CA, USA) using Sytox Green and Annexin-V respectively. Panel (**a**) depicts representative FACS plots for the treatments indicated. Panel (**b**) shows the percentage of early apoptotic and late apoptotic\necrotic cells as indicated.

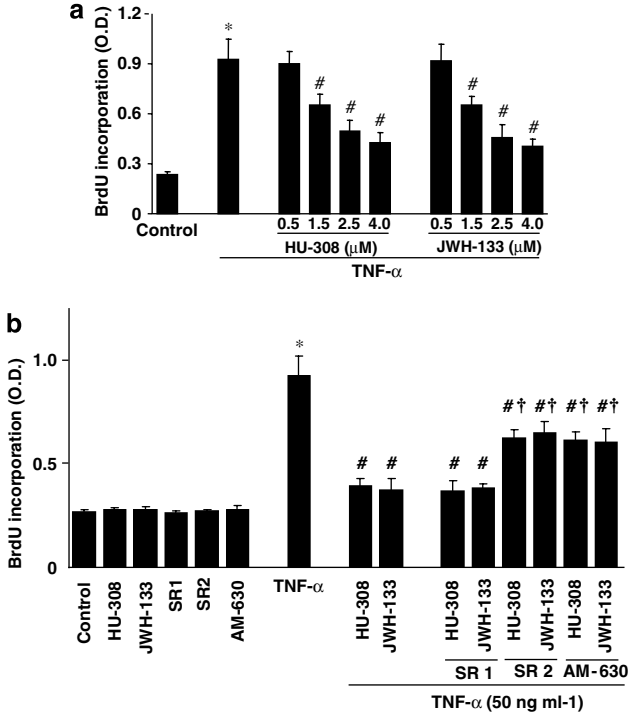

Figure 4 Effect of CB_2 agonists on TNF-α-induced human vascular smooth muscle cell proliferation. Panel (**a**) shows HCASMCs were treated as indicated and proliferation was determined by measuring the extent of BrdU incorporation using ELISA kit by absorbance at 450 nm. $^*P<0.05$ vs control; $^{\#}P<0.05$ vs TNF-α; $n=6$. The concentration of TNF-α used in these experiments was 50 ng ml^{-1}. In panel (**b**) HCASMCs were treated as shown, and proliferation was determined by ELISA. The CB_2 agonists were used at concentration of 4 μM, while the antagonists were used at 1 μM, respectively. $^*P<0.05$ vs control; $^{\#}P<0.05$ vs TNF-α; $^{\dagger}P<0.05$ vs TNF-α + HU-308/JWH-133, $n=6$.

unstimulated control cells; Figure 5). HU-308/JWH-133 (4 μM) treatment (Figures 5a and b) abrogated TNF-α-induced migration. In turn, this was attenuated by AM630/SR2 but not by SR1 (Figures 5a and b). CB_2 agonists/antagonists or CB_1 antagonist SR1 alone did not affect the basal migration of smooth muscle cells (Figure 5b).

CB_2 agonists attenuate TNF-α-induced Akt activation in HCASMCs

As shown in Figure 5c, TNF-α (50 ng ml^{-1}) treatment for 6 h significantly (~2.4. fold vs control) induced Akt activation, which was attenuated by the CB_2 agonists HU-308 and JWH-133, whose actions were attenuated by AM630.

CB_2 agonists inhibit TNF-α-induced Ras activation

Tumour necrosis factor-α has been shown to induce Ras activation resulting in proliferation and migration of human vascular smooth muscle cells, and Ras activation has also been shown to be involved in the pathogenesis of atherosclerosis (Mehrhof *et al*., 2005; Cai, 2006; Gerthoffer, 2007). Since CB_2 agonists inhibited smooth muscle cells proliferation and migration, we tested the effect of HU-308 and JWH-133 on TNF-α-induced Ras activation in HCASMCs. We observed that TNF-α treatment led to marked Ras activation (~3.0-fold increase vs control; Figures 6a and b). Treatment of the cells with HU-308/JWH-133 (4 μM) significantly decreased Ras activation by TNF-α, a decrease that could be attenuated by AM630.

CB_2 agonists mitigate TNF-α-induced MAPK pathway in HCASMCs

Tumour necrosis factor-α has been shown to induce MAPK kinase activation and to stimulate proliferation and migration of human vascular smooth muscle cells (Huang *et al*., 2004; Mehrhof *et al*., 2005; Cai, 2006; Gerthoffer, 2007). Since CB_2 agonists inhibited proliferation and migration of HCASMCs, we studied the effects of CB_2 activation on these signalling pathways. As shown in Figures 6a and b, TNF-α treatment of HCASMCs resulted in robust activation of p38 MAPK, ERK 1/2 and SAPK/JNK demonstrated by western blot assays with specific activation state antibodies. Pretreatment of cells with HU-308/JWH-133 blunted activation of MAPK pathway and these effects were attenuated by AM 630.

Discussion

In this study, we show, for the first time using multiple techniques that human coronary smooth muscle cells express CB_2 receptor. Furthermore, we demonstrate that two selective CB_2 receptor agonists attenuate TNF-α-triggered proliferation and migration of human coronary smooth muscle cells and the activation of various interrelated signalling pathways (Ras, p38 MAPK, ERK 1/2, SAPK/JNK and Akt).

It was previously held that CB_2 receptors are mainly expressed by immune and haematopoietic cells (Klein, 2005; Mackie, 2006; Pacher *et al*., 2006a). Intriguingly, recent studies have provided evidence on the presence of CB_2 receptors in brain (Van Sickle *et al*., 2005), myocardium (Mukhopadhyay *et al*., 2007a), endothelial cells (Blazquez *et al*., 2003; Zoratti *et al*., 2003; Golech *et al*., 2004; Mestre *et al*., 2006) and cardiomyoblasts (Shmist *et al*., 2006; Mukhopadhyay *et al*., 2007a). Here, we report expression of CB_2 and/or CB_1 receptors in human coronary smooth muscle cells under basal cell culture conditions by using immunofluorescence staining, western blotting, RT-PCR, real-time PCR and flow cytometry (Figures 1 and 2). Interestingly, the expression of CB_2 receptors could be enhanced by the pro-inflammatory cytokine TNF-α both at mRNA and at protein levels in vascular smooth muscle cells. An analogous phenomenon has been observed during the microglia activation (Walter *et al*., 2003), and several recent studies have demonstrated markedly increased CB_2 receptor expression in various inflammatory and other cell types under various pathological conditions (Zhang *et al*., 2003; Maresz *et al*., 2005; Mendez-Sanchez *et al*., 2007).

Previous studies have documented that TNF-α can contribute to migration and proliferation of vascular smooth muscle cells (Warner and Libby, 1989; Goetze *et al*., 1999; Selzman *et al*., 1999; Wang *et al*., 2005; Gerthoffer, 2007). Smooth muscle migration and proliferation play important

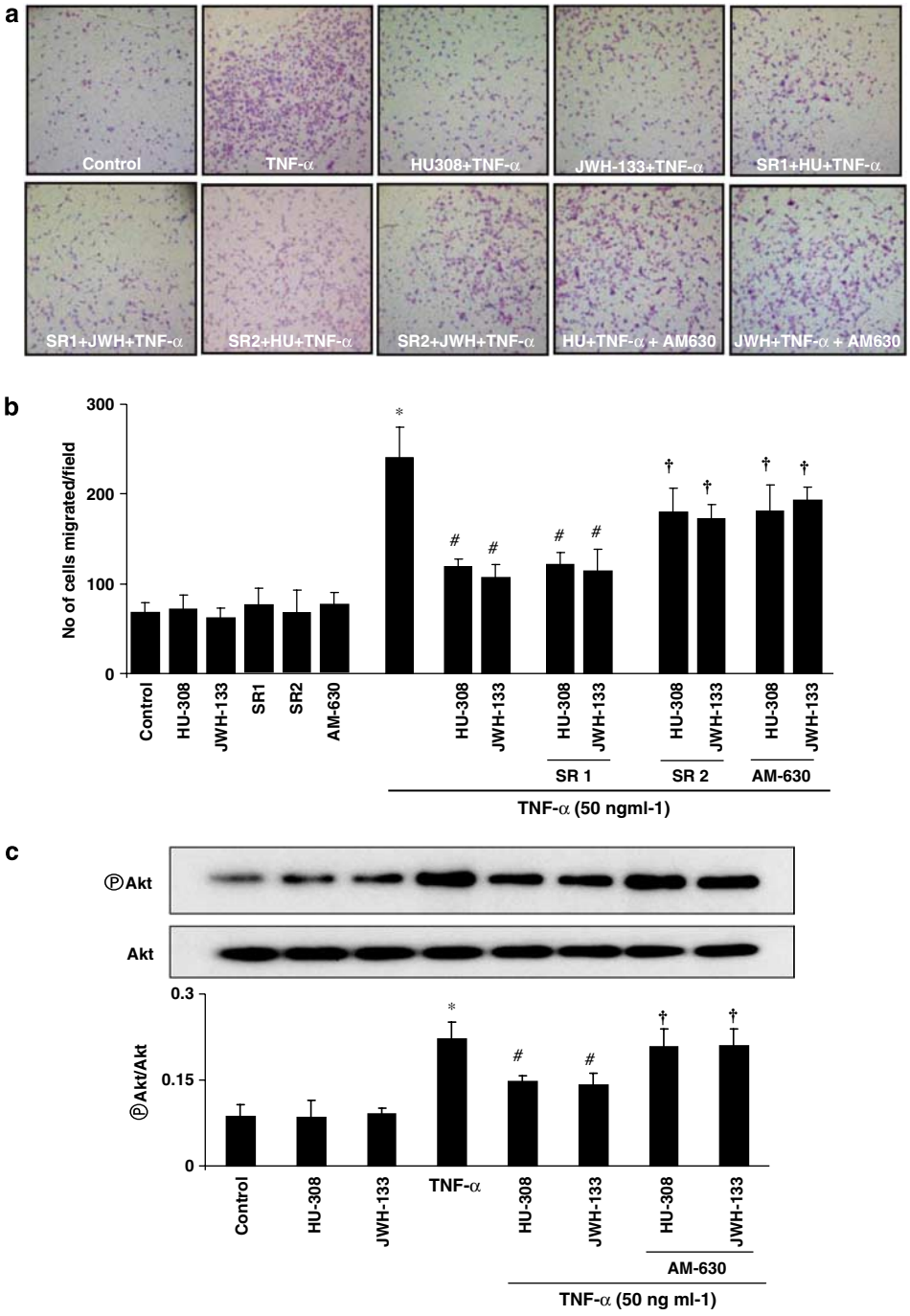

Figure 5 Effect of CB_2 agonists on TNF-α-induced human vascular smooth muscle cell migration and Akt activation. Panel (**a**) indicates the migration of smooth muscle cells as per the treatments indicated. Panel (**b**) shows quantification data for the migrated cells. $^*P<0.05$ vs controls; $^{\#}P<0.05$ vs TNF-α; $^{\dagger}P<0.05$ vs TNF-α ± HU-308/JWH-133 $n=4$. Panel (**c**) shows that cells were grown in 100 mm dishes and treated as indicated. In brief, cells were either treated with TNF-α (50 ng ml^{-1}) alone for 6 h or TNF-α + CB_2 agonists (4 μM)/antagonists (1 μM). Later, cell lysates were subjected to western blot analysis for the determination of Akt activation. $^*P<0.05$ vs controls; $^{\#}P<0.05$ vs TNF-α; $^{\dagger}P<0.05$ vs TNF-α ± HU-308/JWH-133 $n=3$.

roles in the development of atherosclerosis and vascular remodelling that occurs during restenosis in patients who undergo balloon angioplasty (Tanaka *et al*., 1996; Zimmerman *et al*., 2003; Cai, 2006; Tedgui and Mallat, 2006). Numerous studies have demonstrated that endocannabinoids and their synthetic analogues may differentially modulate cell proliferation in various cell types through CB_2 receptor-dependent and -independent mechanisms (McAllister *et al*., 2005; Blazquez *et al*., 2006; Ofek *et al*., 2006; Lombard *et al*., 2007; Maresz *et al*., 2007; Wilkinson and Williamson, 2007) (reviewed in Lopez-Rodriguez *et al*., 2005). Therefore, we hypothesized that CB_2 receptors in vascular smooth muscle cells may play an important role in modulating TNF-α-induced cell migration and proliferation.

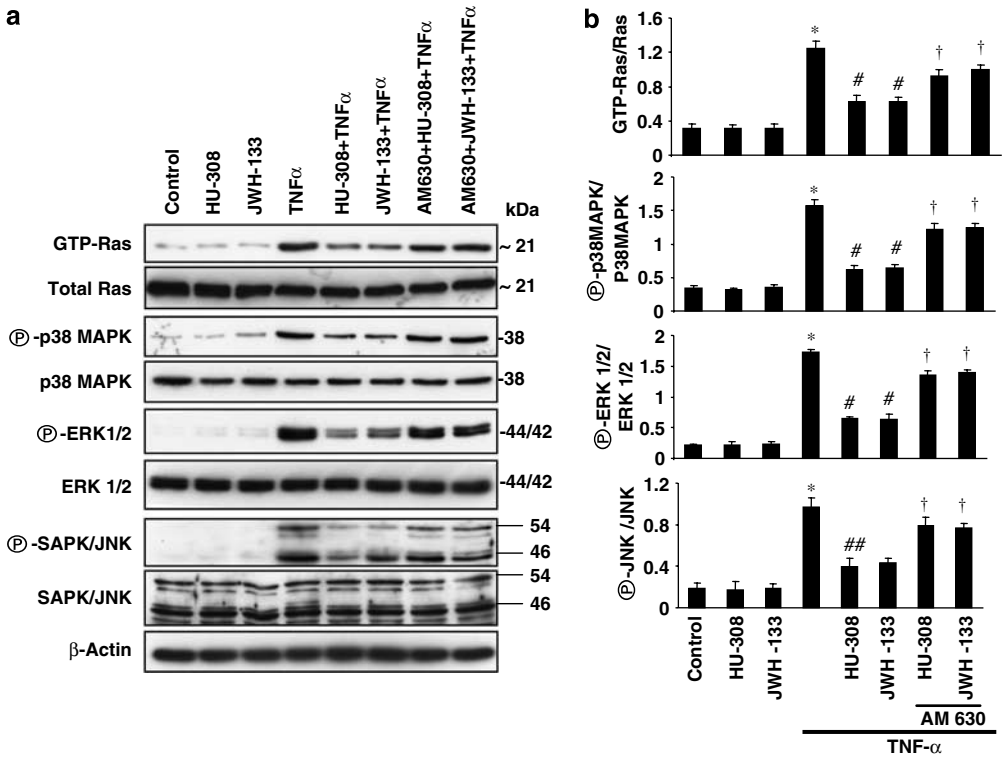

Figure 6 Effect of CB_2 agonists on TNF-α-induced Ras-MAPK pathway activation in human vascular smooth muscle cells. Panel (**a**) shows cells were grown in 100 mm dishes and were treated as indicated. In brief, cells were either treated with TNF-α (50 ng ml^{-1}) alone for 6 h or TNF-α + CB_2 agonists (4 μM)/antagonists (1 μM). To determine Ras activation, 500 μg of protein was used for pull-down assays. For the rest of the signalling proteins, 25 μg, were resolved in 12% SDS-PAGE and blots were probed with antibodies as indicated. The representative blots from three identical experiments are also shown and panel (**b**) represents the densitometry quantification of protein expression. $^{*}P<0.05$ vs controls; $^{\#}P<0.05$ vs TNF-α; $^{\dagger}P<0.05$ vs TNF-α ± HU-308/JWH-133 $n=3$.

Indeed, as shown in Figures 4 and 5, CB_2 receptor stimulation with HU-308 and JWH-133 dose-dependently attenuated TNF-α-induced but not basal cell proliferation (measured by BrdU incorporation) as well as cell migration in a CB_2-dependent fashion.

Next, to determine if CB_2 agonists also influenced the TNF-α-induced signalling cascade leading to cell proliferation, additional experiments were conducted. Previous studies have suggested that TNF-α stimulates p21 GTP-loading and activates the MAPK cascade, SAPK/JNK leading to upregulation of nuclear regulatory factors. These ultimately upregulate cyclin-dependent kinases stimulating cell proliferation (Huang *et al*., 2004). Akt has also been shown to play an important role in the orchestrating cell migration and proliferation in various cell types including smooth muscle (Hsu *et al*., 2001; Shiojima and Walsh, 2002; Lee *et al*., 2007; Wang *et al*., 2007).

Our data also support a role for TNF-α in inducing cell migration and proliferation in normal human vascular smooth muscle. We now propose that CB_2 receptor agonists may suppress the TNF-α-TNFR complex-triggered signalling cascade ultimately involved in cell proliferation and migration. Since vascular smooth muscle proliferation and migration triggered by inflammatory stimuli are crucial events in the pathogenesis and progression of atherosclerosis and restenosis our results suggest that selective CB_2 receptor agonists may offer a novel approach in the treatment of these pathologies. Thus, the attenuation of TNF signalling, coupled with the absence of psychoactive effects associated with CB_2 receptor stimulation, makes this a particularly encouraging therapeutic approach. Furthermore, implantable stents could also be coated with releasable CB_2 agonists, and perhaps other cannabinergic ligands which decrease cell proliferation by other mechanisms, in order to decrease vascular smooth muscle proliferation/migration and thereby the chance of the consequent restenosis in the surgically reconstructed vessel.

Potential limitations of our study and currently available CB_2 antibodies

In our current report, we have utilized a CB_2 antibody that was previously reported to detect CB_2 in numerous studies (for example, Blazquez *et al*., 2003; Casanova *et al*., 2003; Steffens *et al*., 2005 to list a few). Most probably, this antibody detects CB_2 receptor in tissues/cells because: (a) it gives stronger signal in positive control THP-1 monocytes, both by western blot and flow cytometry techniques (Figures 1e, 2b and c); (b) it also gives much stronger signal in THP-1 monocytes and spleen extracts by western blot than in brain extract, where the CB_2 expression is low; (c) the signal could be abolished with its blocking peptide; (d) the receptor expression and changes in the pattern observed with the antibody were also confirmed with RT-PCR and real-time PCR (Figures 1c, 2f and G). However, so far we have not been able to find any antibody, including the one used in the

present study, which does not give signal in CB_2-knockout mice by western blot. Similar observations have been reported by other investigators in the field (various personal communications, meeting: 'CB_2 Cannabinoid Receptors: A New Vista', May 31–June 2, 2007, Banff, Canada). The possible reason(s) for the above-mentioned phenomenon are varied and should be evaluated in further studies in order to design better and more specific antibodies. However, great care should be taken using the currently available CB_2 antibodies to draw any conclusions based on using a single method (especially immunohistochemistry) without the proper, concurrent positive and negative controls. Furthermore, some of the commonly used positive controls should be re-evaluated. For example, we find surprisingly low level of CB_2 receptor expression in normal mouse spleen by immunohistochemistry and flow cytometry (the latter from freshly isolated cells, where cells are not activated), consistently with a note in the manufacturer's product information 'this antibody is not sensitive enough to detect CB_2 from spleen homogenates'. However, using the same antibody, we see strong CB_2 band from the spleen extract by western blot. In contrast, we find high-levels of CB_2 receptors in human THP-1 monocytes by flow cytometry. Importantly, our recent observations also indicate that CD32 blockade should be performed in such experiments to avoid interactions with Fc receptors in cells that express this and related immunoglobulin binding proteins. Thus, we propose that human THP-1 monocytes could be more appropriate positive control for CB_2 receptor detection (at least for flow cytometry experiments) than spleen homogenate.

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Conflict of interest

The authors state no conflict of interest.

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