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THEMED ISSUE: CANNABINOIDS

RESEARCH PAPER

CB_1 cannabinoid receptors promote oxidative/nitrosative stress, inflammation and cell death in a murine nephropathy model

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Background and purpose: Accumulating recent evidence suggests that cannabinoid-1 (CB_1) receptor activation may promote inflammation and cell death and its pharmacological inhibition is associated with anti-inflammatory and tissue-protective effects in various preclinical disease models, as well as in humans.

Experimental approach: In this study, using molecular biology and biochemistry methods, we have investigated the effects of genetic deletion or pharmacological inhibition of CB_1 receptors on inflammation, oxidative/nitrosative stress and cell death pathways associated with a clinically relevant model of nephropathy, induced by an important chemotherapeutic drug cisplatin.

Results: Cisplatin significantly increased endocannabinoid anandamide content, activation of p38 and JNK mitogen-activated protein kinases (MAPKs), apoptotic and poly (ADP-ribose)polymerase-dependent cell death, enhanced inflammation (leucocyte infiltration, tumour necrosis factor-α and interleukin-1β) and promoted oxidative/nitrosative stress [increased expressions of superoxide-generating enzymes (NOX2(gp91phox), NOX4), inducible nitric oxide synthase and tissue 4-hydroxynonenal and nitrotyrosine levels] in the kidneys of mice, accompanied by marked histopathological damage and impaired renal function (elevated creatinine and serum blood urea nitrogen) 3 days following its administration. Both genetic deletion and pharmacological inhibition of CB_1 receptors with AM281 or SR141716 markedly attenuated the cisplatin-induced renal dysfunction and interrelated oxidative/nitrosative stress, p38 and JNK MAPK activation, cell death and inflammatory response in the kidney.

Conclusions and implications: The endocannabinoid system through CB_1 receptors promotes cisplatin-induced tissue injury by amplifying MAPK activation, cell death and interrelated inflammation and oxidative/nitrosative stress. These results also suggest that inhibition of CB_1 receptors may exert beneficial effects in renal (and most likely other) diseases associated with enhanced inflammation, oxidative/nitrosative stress and cell death.

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Keywords: nephropathy; endocannabinoids; cannabinoid receptors

Abbreviations: 2-AG, 2-arachidonoylglycerol; 4-HNE, 4-hydroxynonenal; AEA, anandamide; BUN, blood urea nitrogen; $CB_1^{-/-}$ mice, CB_1 knockout mice; CB_1 receptor, cannabinoid-1 receptor; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; SR141716, rimonabant, a CB_1 antagonist (inverse agonist); TNF-α, tumour necrosis factor-α

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Introduction

Cisplatin is a potent and widely used chemotherapy drug (a platinum compound) for the treatment of a range of solid tumours and other malignancies (Ries and Klastersky, 1986; Schrier, 2002). Even though the precise mechanism of the anticancer activity of cisplatin is not completely understood, it is widely held that it binds to DNA, leading to the formation of inter- and intrastrand cross-links, resulting in defective DNA templates and arrest of DNA synthesis and replication, particularly in rapidly dividing cancer cells (Wang and Lippard, 2005). Unfortunately, the major limitation of its clinical application is the development of dose-dependent nephrotoxicity in about one-third of patients. This prevents the use of high doses to take full advantage of the therapeutic efficacy (Ries and Klastersky, 1986; Schrier, 2002). Regrettably, efficient treatment to decrease this devastating complication of cisplatin chemotherapy is not available.

The mechanism of cisplatin-induced nephrotoxicity is complex and involves numerous interconnected processes (Pabla and Dong, 2008), such as formation of reactive oxygen (Matsushima *et al*., 1998; Davis *et al*., 2001) and nitrogen species (ROS and RNS) (Chirino *et al*., 2004; 2008), DNA damage (Ries and Klastersky, 1986) and activation of apoptotic and poly (ADP-ribose) polymerase (PARP)-dependent cell death pathways (Ries and Klastersky, 1986; Racz *et al*., 2002; Mukhopadhyay *et al*., 2010a; Pan *et al*., 2009a). Numerous recent studies highlight the importance of inflammatory mechanisms in the pathogenesis and progression of cisplatin-induced nephropathy, particularly the recruitment of inflammatory cells (e.g. leucocytes and macrophages), which may amplify the drug-induced tubular injury by further increasing ROS and RNS generation and production of a variety of pro-inflammatory mediators [e.g. cytokines: tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)], eventually leading to activation of cell death pathways (Ramesh and Reeves, 2002; Yamate *et al*., 2002; Faubel *et al*., 2007; Zhang *et al*., 2007; Mukhopadhyay *et al*., 2010a).

Cannabinoid-1 (CB_1) receptor antagonists exert potent anti-inflammatory and cytoprotective effects in multiple preclinical disease models ranging from hepatic steatosis (Gary-bobo *et al*., 2007), ischaemia–reperfusion injury (Berger *et al*., 2004; Muthian *et al*., 2004; Sommer *et al*., 2006; Lim *et al*., 2009; Zhang *et al*., 2009), to endotoxin shock (Kadoi and Goto, 2006; Villanueva *et al*., 2009), atherosclerosis (Pacher and Hasko, 2008; Dol-gleizes *et al*., 2009; Pacher, 2009; Sugamura *et al*., 2009), cardiomyopathy (Mukhopadhyay *et al*., 2007; 2010b, Pacher *et al*., 2008) and in *in vitro* models of inflammation (Malfitano *et al*., 2008; Schafer *et al*., 2008; Han *et al*., 2009). More importantly, rimonabant (SR141716) also attenuates multiple inflammatory markers (e.g. TNF-α, C-reactive protein, etc.), plasma leptin and insulin levels, and increases 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). Furthermore, a functional endocannabinoid system was reported in the kidney (Deutsch *et al*., 1997; Janiak *et al*., 2007), and blockade of cannabinoid CB_1 receptors improved renal function, metabolic profile and increased survival of obese Zucker rats (Janiak *et al*., 2007).

In this study we investigated the interplay of the CB_1 receptors with oxidative/nitrosative stress, inflammation and cell death pathways using a well-established mouse model of cisplatin-induced nephropathy. These results may have important implications not only for the prevention of the cisplatin-induced nephrotoxicity, but also for the therapy of other kidney diseases.

Methods

Animals and drug treatment

All animal experiments conformed to National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism (NIAAA; Bethesda, MD, USA). Six- to 8-week-old male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). CB_1 knockout mice ($CB_1^{-/-}$) and their wild-type littermates ($CB_1^{+/+}$) were as described previously (Osei-Hyiaman *et al*., 2005) and had been backcrossed to a C57BL/6J background. All animals were kept in a temperature-controlled environment with a 12 h light–dark cycle and were allowed free access to food and water at all times, and were cared for in accordance with National Institutes of Health (NIH) guidelines. Mice were killed 72 h following a single injection of cisplatin (cisdiammineplatinum(II) dichloride 25 $mg \cdot kg^{-1}$ i.p.; Sigma, St. Louis, MO, USA). The selective CB_1 receptor antagonist SR141716 (obtained from NIDA) or AM281 (Tocris, Ellisville, MI, USA) were dissolved as described previously (Mukhopadhyay *et al*., 2007), and administered at 10 $mg \cdot kg^{-1}$, i.p. daily, starting 2 h before the cisplatin administration. 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).

Renal function monitoring

Once the animals had been killed, blood was immediately collected, and serum levels of creatinine and blood urea nitrogen (BUN) were measured using kits from Drew Scientific and a Prochem-V chemical analyser (TX, USA).

Western blot analysis

Antibodies for phosho-p38 mitogen-activated protein kinase (MAPK), total p38 MAPK, phospho-JNK and total JNK were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies for β-actin were obtained from Cayman (Ann Arbor, MI, USA). Antibodies for CB_1 were as previously described (Mukhopadhyay *et al*., 2007; 2010b). The kidney protein samples were mixed in Laemmli loading buffer, boiled for 8 min, and then subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary antibody (1:1000 dilution) for 16 h. Membranes were washed with PBS-T and incubated with a secondary antibody (1:1000 dilution) for 2 h. Protein bands were visualized by chemiluminescence reaction using SuperSignal West Pico Substrate (Fisher Scientific, Pittsburgh, PA, USA).

Endocannabinoid measurements
For measuring endocannabinoid levels, mice were killed and their kidneys were removed and extracted. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels were determined by liquid chromatography/mass spectrometry from kidney tissues (Batkai *et al*., 2007). Values are expressed as fmol or pmol·mg^{-1} wet tissue.

Histological examination
Following fixation of the kidneys with 10% formalin, renal tissues were sectioned and stained with periodic acid-Schiff (PAS) reagents for histological examination. Tubular damage in PAS-stained sections was examined under the microscope (200× magnification) and scored based on the percentage of cortical tubules showing epithelial necrosis: 0 = normal; 1 ≤ 10%; 2 = 10–25%; 3 = 26–75%; 4 ≥ 75%. Tubular necrosis was defined as the loss of the proximal tubular brush border, blebbing of apical membranes, tubular epithelial cell detachment from the basement membrane or intraluminal aggregation of cells and proteins as described (Mukhopadhyay *et al*., 2010a; Pan *et al*., 2009a,b). The morphometric examination was performed in a blinded manner by two independent investigators.

Detection of apoptosis by terminal deoxynucleotidyl transferase-mediated uridine triphosphate (dUTP) nick-end labelling (TUNEL), renal DNA fragmentation and caspase-3/7 activity assays
Apoptosis was assessed by TUNEL, and the number of apoptotic cells, as defined by chromatin condensation or nuclear fragmentation (apoptotic bodies), was counted. Apoptosis was detected in the kidneys by TUNEL assay according to the instructions of the manufacturer of the kit (Roche Diagnostics, Indianapolis, IN, USA) as described previously (Mukhopadhyay *et al*., 2010a). The morphometric examination was performed by two independent, blinded investigators. The number of apoptotic cells in each section was calculated by counting the number of TUNEL-positive apoptotic cells in 10, 200× fields per slide (Mukhopadhyay *et al*., 2010a).

Caspase-3/7 activity of the lysate was measured using Apo-One Homogenous caspase-3/7 Assay Kit (Promega Corp., Madison, WI, USA). An aliquot of caspase reagent was added to each well, mixed on a plate shaker for 1 h at room temperature with light protection, and the fluorescence was measured.

The DNA fragmentation assay is based on measuring the amount of mono- and oligonucleosomes in the cytoplasmic fraction of tissue extracts using a commercially available kit (Roche, GmbH) according to manufacturer's instructions, as described previously (Pan *et al*., 2009a; Mukhopadhyay *et al*., 2009).

Renal PARP activity and nitrotyrosine (NT) content
Poly (ADP-ribose) polymerase activity was determined by an assay kit according to manufacturer's instructions (Trevigen, Gaithersburg, MD, USA) (Pan *et al*., 2009a; Mukhopadhyay *et al*., 2009). NT was measured by the NT ELISA kit from Hycult Biotechnology (Cell Sciences, Canton, MA, USA) from tissue homogenates as described previously (Mukhopadhyay *et al*., 2009). Levels were presented as fold change compared with vehicle-treated control sample.

Renal myeloperoxidase activity assay
Myeloperoxidase (EC1.11.1.7) was measured by an InnoZyme™ Myeloperoxidase Activity Kit (EMD, Gibbstown, NJ, USA) according to manufacturer's instruction. Myeloperoxidase activities were expressed as fold change compared with the vehicle-treated control sample (Mukhopadhyay *et al*., 2010a).

Renal 4-hydroxynonenal (4-HNE) content
4-HNE in the kidney tissues was determined using the kit (Cell Biolabs, San Diego, CA, USA). In brief, BSA or myocardial tissue extracts (10 μg·mL^{-1}) were adsorbed on to a 96-well plate for 12 h at 4°C. 4-HNE adducts present in the sample or standard were probed with anti-HNE antibody, followed by an horseradish peroxidase-conjugated secondary antibody. The HNE protein adducts content in an unknown sample was determined by comparing with a standard curve as described previously (Mukhopadhyay *et al*., 2010a).

Real-time PCR analyses
Total RNA was isolated from kidney homogenate using Trizol reagents (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. The isolated RNA was treated with RNase-free DNase (Ambion, Austin, TX, USA) to remove traces of genomic DNA contamination. One microgram of total RNA was reverse-transcribed to cDNA using the Super-Script II (Invitrogen). The target gene expression was quantified with Power Syber Green PCR Master Mix using ABI 7500 Realtime PCR Instrument. Each amplified sample in all wells was analysed for homogeneity using dissociation curve analysis. After denaturation at 95°C for 2 min, 40 cycles were performed at 95°C for 10 s, 60°C for 30 s. Relative quantification was calculated using the comparative CT method [2-Ct method: Ct = Ct sample – Ct (Mukhopadhyay *et al*., 2010a,b)]. Lower CT values and lower CT reflect a relatively higher amount of gene transcript. Statistical analyses were carried out for at least 6–15 replicate experimental samples in each set.

Primers used:

TNF-α: 5′-AAGCCTGTAGCCCACGTCGTA-3′ and 5′-AGG TACAACCCATCGGCTGG-3′

IL-1β: 5′-AAAAAAGCCTCGTGCTGTCG-3′ and 5′-GTCG TTGCTTGGTTCTCCTTG-3′

Inducible nitric oxide synthase (iNOS): 5′-ATTCACAGCT CATCCGGTACG-3′ and 5′-GGATCTTGACCATCAGCTTGC-3′

gp91phox: 5′-GACCATTGCAAGTGAACACCC-3′ and 5′-AA ATGAAGTGGACTCCACGCG-3′

NOX4: 5′-TCATTTGGCTGTCCCTAAACG-3′ and 5′-AAGGA TGAGGCTGCAGTTGAG-3′

Actin: 5′-TGCACCACCAACTGCTTAG-3′ and 5′-GGATGC AGGGATGATGTTC-3′.

Statistical analysis
Results are reported as mean ± SEM. Statistical significance between two measurements was determined by Student's two-

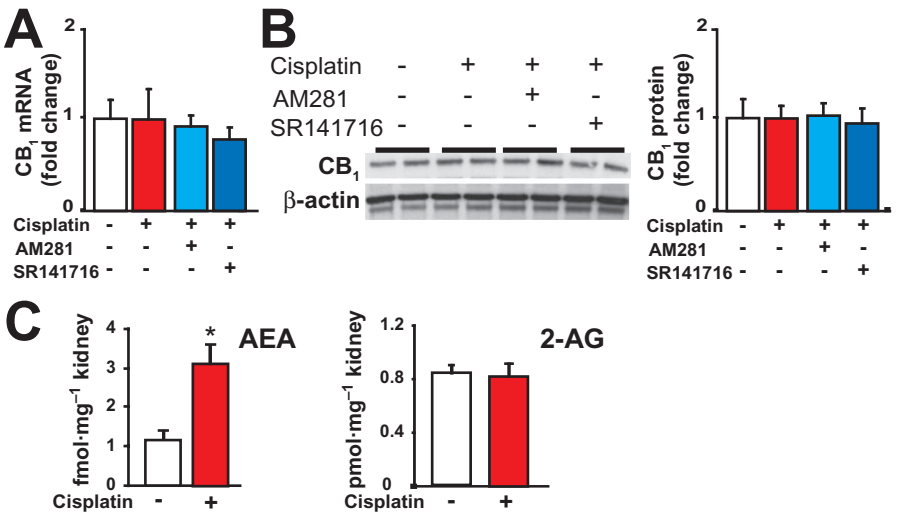


Figure 1 Effect of cisplatin on cannabinoid-1 (CB_1) receptor expression and endocannabinoid levels in the kidneys of mice. (A) and (B) show that neither cisplatin nor the treatments indicated influenced CB_1 mRNA or protein expression in the kidney 72 h after its administration to mice. (C) depicts cisplatin-induced increased renal anandamide (AEA) but not 2-arachidonoylglycerol (2-AG) levels. Results are mean ± SEM of 6–8 experiments per group. *$P < 0.05$ versus vehicle.

tailed unpaired *t*-test (and among groups it was determined by ANOVA followed by *post hoc* Student-Newman-Keuls) by using GraphPad Prism 4.3 software (San Diego, CA, USA). Probability values of $P < 0.05$ were considered significant.

Results

CB_1 receptors were expressed in the kidneys and cisplatin increased endocannabinoid AEA levels

CB_1 receptors were expressed in the normal kidneys, and their levels were not altered by cisplatin or by the treatments indicated (Figure 1A,B). Endocannabinoids AEA and 2-AG were detectable in the kidneys of mice, and cisplatin increased tissue levels of AEA but not 2-AG (Figure 1C).

Pharmacological inhibition or genetic deletion of CB_1 receptors attenuates the cisplatin-induced renal dysfunction in mice

Levels of BUN and creatinine were measured at 72 h after cisplatin or vehicle administration in the serum of either SR141716- or AM281-treated and untreated mice or in $CB_1^{-/-}$ or $CB_1^{+/+}$ mice treated with cisplatin or vehicle. As shown in Figure 2, cisplatin administration induced severe renal dysfunction, which was attenuated by CB_1 antagonists AM281 and SR141716 (Figure 2A) or in $CB_1^{-/-}$ mice (Figure 2B) compared with $CB_1^{+/+}$ littermates. AM281/SR141716 alone had no effects on BUN and creatinine levels as compared with the vehicle-treated group.

Pharmacological inhibition or genetic deletion of CB_1 receptors attenuated the cisplatin-induced histopathological damage in murine kidneys

Cisplatin induced profound histopathological renal injury 72 h after its administration to mice as evidenced by protein

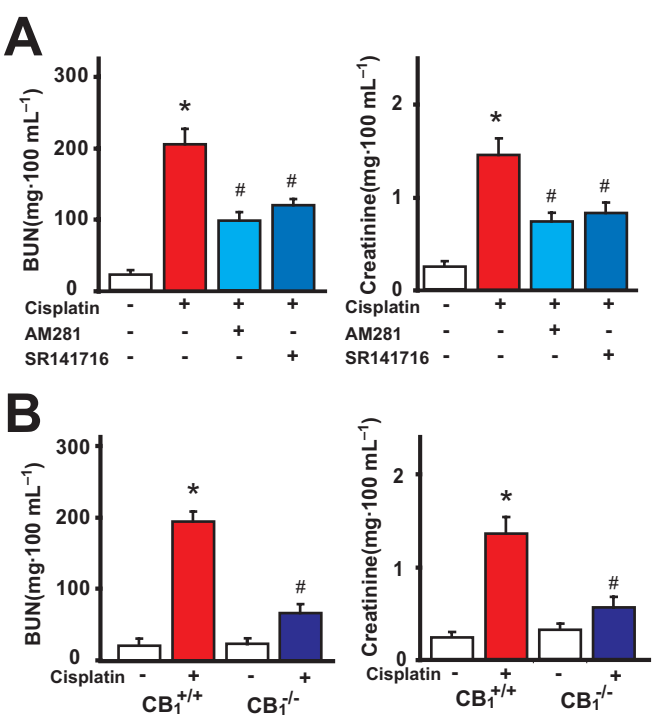


Figure 2 Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB_1) receptors attenuated the cisplatin-induced renal dysfunction in mice. The cisplatin-induced profound renal dysfunction 72 h after its administration to mice, indicated by increased serum levels of blood urea nitrogen (BUN) and creatinine (A and B), was attenuated by treatment with AM281 or SR141716, and also in CB_1 knockout ($CB_1^{-/-}$) mice compared with $CB_1^{+/+}$ littermates. Results are mean ± SEM of 7–10 experiments per group. *$P < 0.01$ versus vehicle in C57BL/6 mice (A) or versus $CB_1^{+/+}$ mice treated with vehicle (B); #$P < 0.01$ versus cisplatin in C57BL/6 or $CB_1^{+/+}$ mice (A and B respectively). AM281 or SR141716 alone had no effects on BUN and creatinine levels as compared with the vehicle-treated group ($n = 5$, not shown).

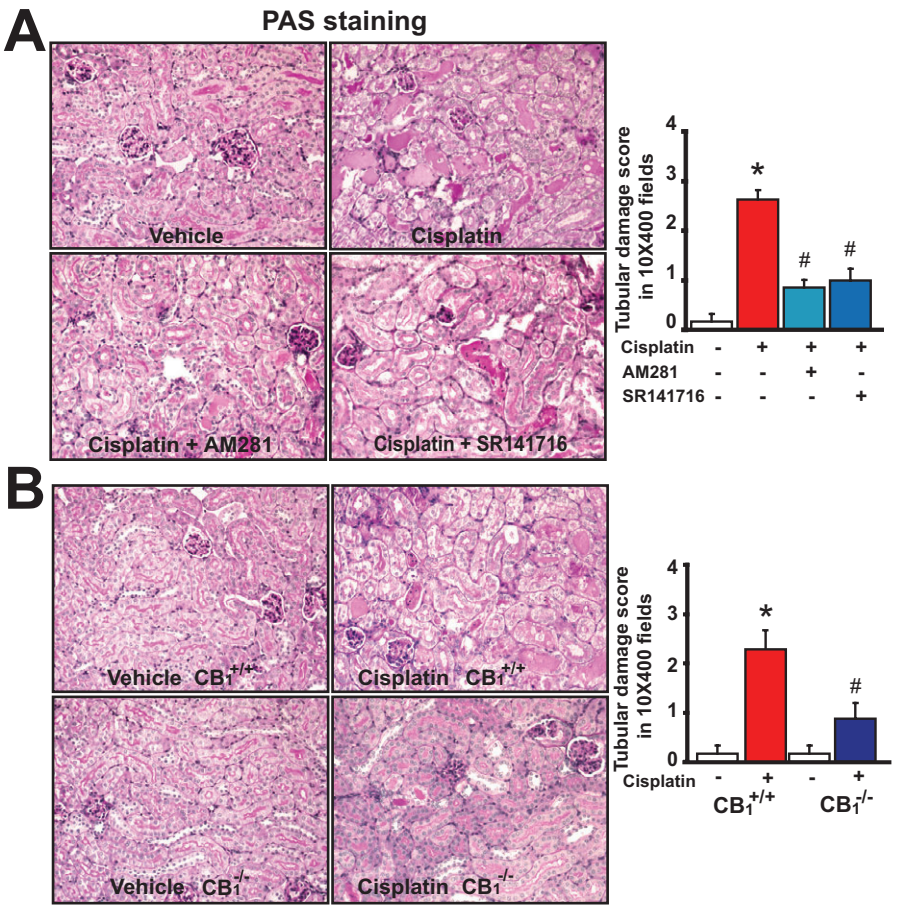


Figure 3 Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB_1) receptors attenuated the cisplatin-induced renal histopathological damage. The cisplatin-induced profound histopathological renal injury 72 h after its administration to mice [characterized by protein casts, vacuolation and desquamation of epithelial cells in the renal tubules visualized using periodic acid-Schiff (PAS) staining] was attenuated by treatment with AM281 or SR141716 (A), and also in CB_1 knockout ($CB_1^{-/-}$) mice compared with $CB_1^{+/+}$ littermates (B). Results are mean ± SEM of eight experiments per group. *$P < 0.01$ versus vehicle in C57BL/6 mice (A) or versus $CB_1^{+/+}$ mice treated with vehicle (B); #$P < 0.01$ versus cisplatin in C57BL/6 or $CB_1^{+/+}$ mice (A and B respectively).

casts, vacuolation and desquamation of epithelial cells in the renal tubules using PAS staining (Figure 3). These histopathological changes were attenuated by treatment with AM281 or SR141716 (Figure 3A). Likewise less damage was seen in $CB_1^{-/-}$ mice compared with $CB_1^{+/+}$ littermates (Figure 3B).

Pharmacological inhibition or genetic deletion of CB_1 receptors attenuated the cisplatin-induced cell death in murine kidneys

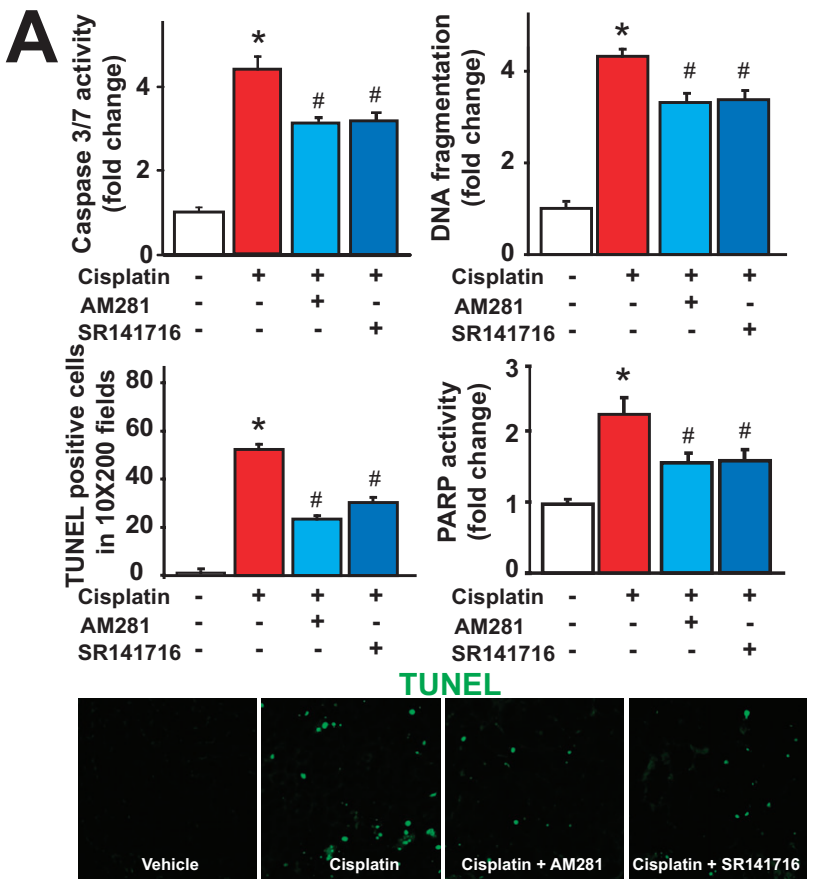
Cell death in the kidneys was evaluated by caspase-3/7 activity, DNA fragmentation, TUNEL and PARP activity assays. Cisplatin increased all markers of cell death in the kidneys, which were attenuated by treatment with AM281 or SR141716 (Figure 4A). Likewise, kidneys of cisplatin-treated $CB_1^{-/-}$ mice showed considerably less cell death compared with those from their $CB_1^{+/+}$ littermates (Figure 4B).

Pharmacological inhibition or genetic deletion of CB_1 receptors attenuated the cisplatin-induced increased leucocyte infiltration and inflammatory response in murine kidneys

Cisplatin significantly increased renal myeloperoxidase activity (Figure 5A,B; an indicator of leucocyte infiltration) and expression of TNF-α and IL-1β mRNA (Figure 5C,D), indicating an enhanced inflammatory response. All of these markers of inflammation were attenuated by AM281 or SR141716 treatment (Figure 5A,C), as well as by genetic deletion of CB_1 receptors (Figure 5B,D).

Pharmacological inhibition or genetic deletion of CB_1 receptors attenuated markers of cisplatin-induced increased oxidative/nitrosative stress

Cisplatin induced significant elevations of renal 4-HNE and NT levels (markers of oxidative and nitrosative damage), which were attenuated by AM281 or SR141716 treatment (Figure 6A,C). Likewise, in $CB_1^{-/-}$ mice the markers of oxidative/nitrosative stress were attenuated compared with $CB_1^{+/+}$ littermates (Figure 6B,C). Interestingly, NT was localized in damaged renal tubular cells in cisplatin-treated mice (Figure 6C) supporting the important role of peroxynitrite in cell death processes (Liaudet *et al.*, 2009; Mukhopadhyay *et al.*, 2010a). NT was undetectable in kidneys of normal mice by immunohistochemistry (Figure 6C).


A
Caspase 3/7 activity (fold change)
DNA fragmentation (fold change)
TUNEL positive cells in 10X200 fields
PARP activity (fold change)
Cisplatin
AM281
SR141716
TUNEL
Vehicle
Cisplatin
Cisplatin + AM281
Cisplatin + SR141716

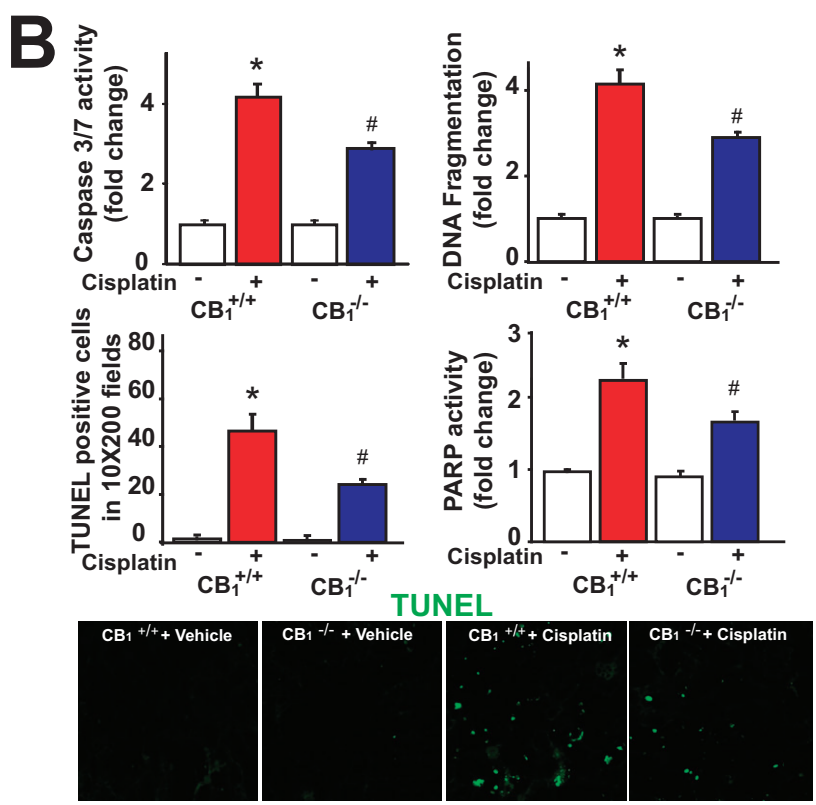

B
Caspase 3/7 activity (fold change)
DNA Fragmentation (fold change)
TUNEL positive cells in 10X200 fields
PARP activity (fold change)
Cisplatin
CB1+/+
CB1-/-
TUNEL
CB1 +/+ + Vehicle
CB1 -/- + Vehicle
CB1 +/+ + Cisplatin
CB1 -/- + Cisplatin

Figure 4 Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB_1) receptors attenuated the cisplatin-induced renal cell death. All markers of cell death [evaluated by caspase-3/7 activity, DNA fragmentation, terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick-end labelling (TUNEL) and poly (ADP-ribose) polymerase (PARP) activity assays] in the kidneys were significantly increased by cisplatin and were attenuated by AM281 or SR141716 treatment (A), and also in CB_1 knockout ($CB_1^{-/-}$) mice compared with $CB_1^{+/+}$ littermates (B). Results are mean ± SEM of 6–8 experiments per group. *$P < 0.01$ versus vehicle in C57BL/6 mice (A) or versus $CB_1^{+/+}$ mice treated with vehicle (B); #$P < 0.05$ versus cisplatin in C57BL/6 or $CB_1^{+/+}$ mice (A and B respectively). Representative images at the bottom of each panel show cisplatin-induced increased number of TUNEL-positive cells (green staining) in kidney sections, which was attenuated with AM281 or SR141716 (A), and also in $CB_1^{-/-}$ mice compared with $CB_1^{+/+}$ littermates (B).

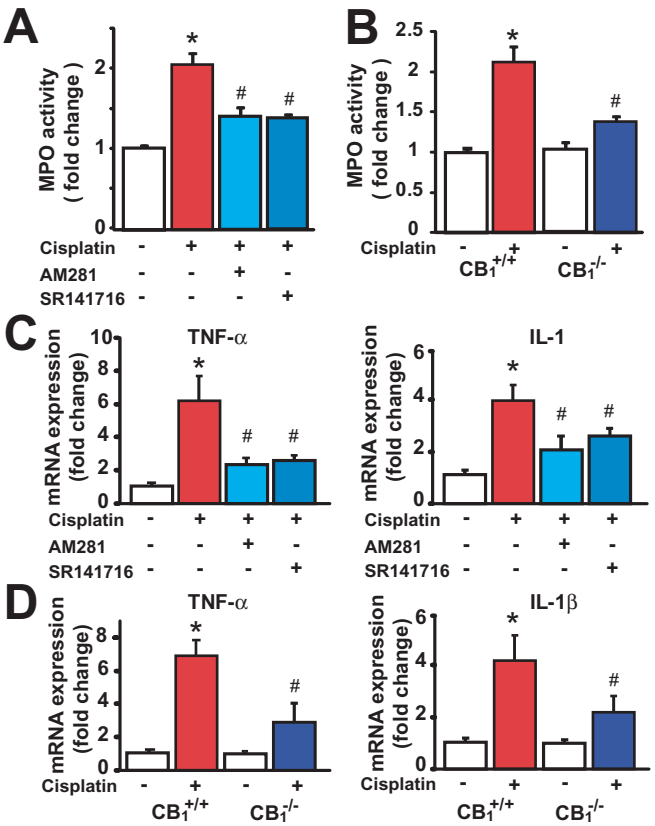


Figure 5 Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB_1) receptors attenuated the cisplatin-induced increased myeloperoxidase (MPO) activity and expression of mRNA of tumour necrosis factor (TNF)-α and interleukin (IL)-1β in kidneys of mice. Cisplatin significantly increased renal MPO activity (an indicator of leucocyte infiltration; A and B) and mRNA expression of TNF-α and IL-1β mRNA (C and D), indicating an enhanced inflammatory response. These increases were attenuated by treatment with AM281 or SR141716 (A and C), and also in CB_1 knockout ($CB_1^{-/-}$) mice compared with $CB_1^{+/+}$ littermates (B and D). Results are mean ± SEM of 6–8 per group. *$P < 0.01$ versus vehicle in C57BL/6 mice (A and C) or versus $CB_1^{+/+}$ mice treated with vehicle (B and D); #$P < 0.05$ versus cisplatin in C57BL/6 or $CB_1^{+/+}$ mice (A/C and B/D respectively).

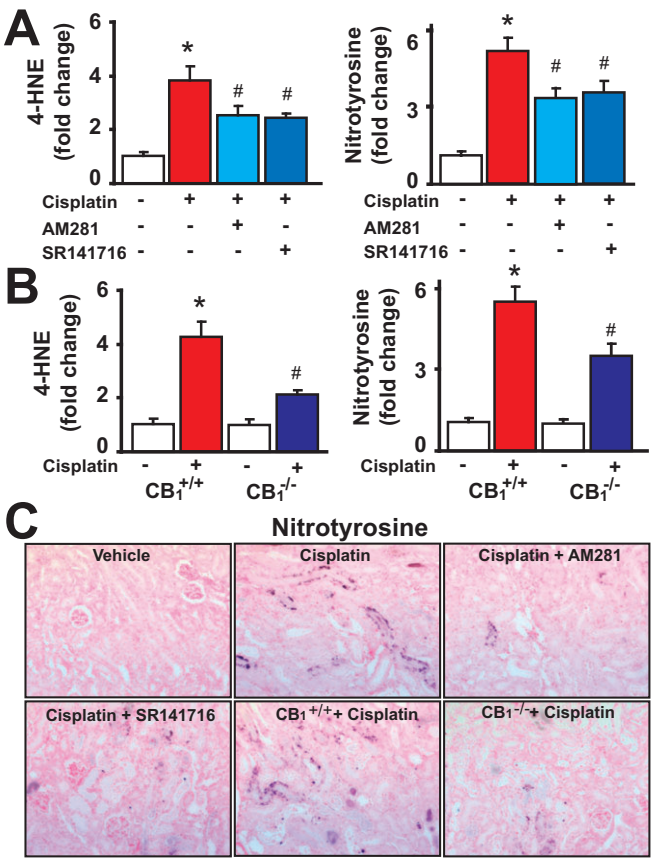


Figure 6 Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB_1) receptors attenuated the cisplatin-induced increased oxidative and nitrosative stress. Cisplatin induced marked elevations of renal 4-hydroxynonenal (4-HNE) and nitrotyrosine levels, which were attenuated by treatment with AM281 or SR141716 (A), and also in CB_1 knockout ($CB_1^{-/-}$) mice compared with $CB_1^{+/+}$ littermates (B). Results are mean ± SEM of 8–10 experiments per group. *$P < 0.01$ versus in C57BL/6 mice (A) or versus $CB_1^{+/+}$ mice treated with vehicle (B); #$P < 0.05$ versus cisplatin in C57BL/6 or $CB_1^{+/+}$ mice (A and B respectively). (C) Representative images of cisplatin-induced increased nitrotyrosine accumulation in damaged tubular cells (dark staining) in kidney sections, which was attenuated with AM281 or SR141716, and also in $CB_1^{-/-}$ mice compared with $CB_1^{+/+}$ littermates.

Pharmacological inhibition or genetic deletion of CB_1 receptors attenuated the cisplatin-induced increased expression of ROS-generating NADPH oxidase enzymes [NOX4(RENOX), NOX2(gp91phox)] and the iNOS

Cisplatin induced marked increases in mRNA levels of renal NOX4, NOX2 and iNOS 72 h after its administration to mice (Figure 7). These increases were attenuated by AM281 or SR141716 treatment (Figure 7A,C). Similarly, in $CB_1^{-/-}$ mice the increase in the mRNA expression for these enzymes was reduced compared with $CB_1^{+/+}$ littermates (Figure 7B,D).

Pharmacological inhibition or genetic deletion of CB_1 receptors attenuated the cisplatin-induced increased activation of p38 and JNK MAPKs

Cisplatin induced marked phosphorylation of renal p38 MAPK and JNK 72 h following its administration to mice. The CB_1 antagonists AM281 or SR141716 (Figure 8A) significantly attenuated these increases. Likewise these increases were reduced in $CB_1^{-/-}$ mice compared with their $CB_1^{+/+}$ littermates (Figure 8B).

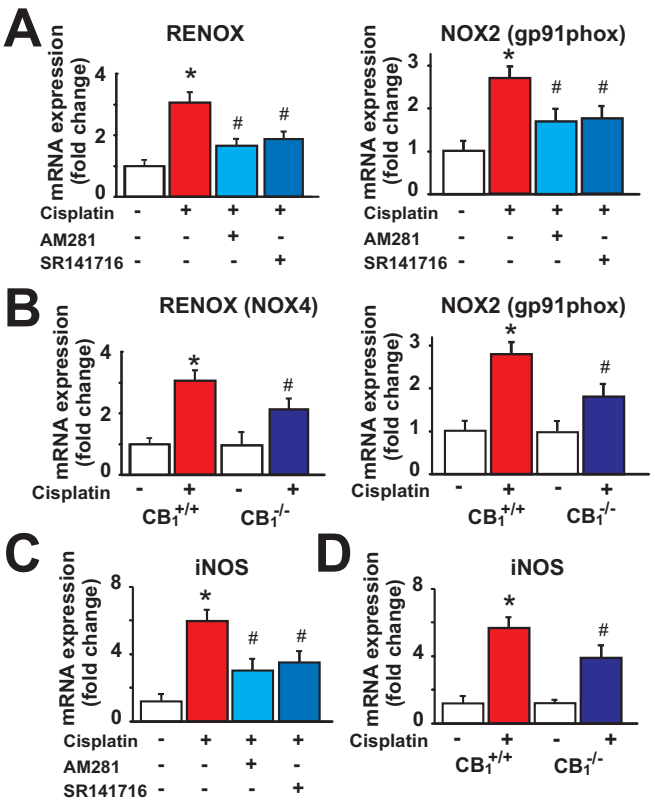


Figure 7 Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB_1) receptors attenuated the cisplatin-induced increased expression of reactive oxygen species-generating NADPH oxidase enzymes [NOX4(RENOX), NOX2(gp91phox)] and the inducible nitric oxide synthase (iNOS) in kidneys of mice. Cisplatin induced marked increases in expression of renal NOX4, NOX2 and iNOS mRNA 72 h following its administration to mice (A–D). These increases were attenuated by AM281 or SR141716 treatment (A and C), and also in CB_1 knockout ($CB_1^{-/-}$) mice compared with $CB_1^{+/+}$ littermates (B and D). Results are mean ± SEM of 9–10 experiments per group. *$P < 0.01$ versus vehicle in C57BL/6 mice (A and C) or versus $CB_1^{+/+}$ mice treated with vehicle (B and D); #$P < 0.05$ versus cisplatin in C57BL/6 or $CB_1^{+/+}$ mice (A/C and B/D respectively).

Discussion

In this study, we have explored the interplay of oxidative/nitrosative stress, inflammation and cell death pathways with CB_1 receptors in a clinically relevant model of cisplatin-induced nephropathy by using pharmacological antagonists of the cannabinoid CB_1 receptor (AM281 and SR141716) as well as CB_1 receptor knockout mice. We demonstrated that genetic deletion of CB_1 receptors or its pharmacological inhibition with AM281 or SR141716 attenuates cisplatin-induced increased p38 and JNK MAPK activation, oxidative/nitrosative stress, cell death and interrelated inflammatory cell infiltration in the kidney, and the consequent release of reactive oxidants and pro-inflammatory mediators, leading to decreased cell death of renal tubular cells and marked improvement in cisplatin-induced compromised renal function. These findings suggest that CB_1 cannabinoid receptor activation by endocannabinoids promotes cisplatin-induced tissue injury by amplifying MAPK activation, cell death and the associated inflammation and oxidative/nitrosative stress. Therefore, CB_1 inhibition may exert beneficial effects in kidney (and most likely other) diseases associated with inflammation, oxidative/nitrosative stress and cell death. These findings are in good agreement with numerous recent reports demonstrating anti-inflammatory and cytoprotective effects of CB_1 antagonists in various preclinical disease models, as well as in humans with metabolic syndrome as described above (Pacher *et al*., 2006; Di Marzo, 2008; Engeli, 2008; Mach *et al*., 2008; Pertwee, 2009).

There is increasing recognition that in various pathological conditions CB_1 receptor activation by endocannabinoids may promote activation of signalling pathways (e.g. p38 and JNK MAPKs) leading to cell death (Pertwee, 2002; Di Marzo, 2008; Dalton *et al*., 2009). Indeed increased tissue and/or serum endocannabinoid levels during reperfusion injury positively correlate with tissue damage and cell death in experimental models of hepatic ischaemia–reperfusion (Batkai *et al*., 2007; Pacher and Gao, 2008; Pacher and Hasko, 2008; Ishii *et al*., 2009) and stroke (Berger *et al*., 2004; Muthian *et al*., 2004; Sommer *et al*., 2006; Zhang *et al*., 2009). Under many of these experimental conditions CB_2 receptor activation on inflammatory, endothelial and perhaps some parenchymal cells exerts anti-inflammatory and cytoprotective effects (Batkai *et al*., 2007; Rajesh *et al*., 2007a,b), while CB_1 activation may promote inflammation and tissue injury. Support for this comes from the observation that in these models CB_1 antagonists consistently exert beneficial effects on inflammation and the subsequent oxidative/nitrosative stress-cell death cascade (Pacher and Hasko, 2008; Caraceni *et al*., 2009; Zhang *et al*., 2009). One can envisage that the overactivated endocannabinoid system during reperfusion, and most likely in other forms of tissue injury [as we describe in the case of cisplatin-induced nephropathy and have recently demonstrated in doxorubicin-induced cardiomyopathy models (Mukhopadhyay *et al*., 2010b)] may promote cell death through the activation of CB_1 receptors in certain cell types, and the consequent inflammatory cell infiltration and oxidative stress, while it may serve as an endogenous mechanism to limit early inflammatory response through the activation of CB_2 receptors (Pacher and Hasko, 2008). Therefore, the beneficial or detrimental effects of endocannabinoids may largely depend on the tissue and injury type (e.g. role of the inflammatory component for example, expression of $CB_{1/2}$ receptors, etc.), as well as on the stage of the disease progression. Indeed, CB_2 receptor activation limits inflammation and interrelated oxidative/nitrosative stress-cell death associated with cisplatin-induced nephropathy. However, the protective effect of a CB_2 agonist is lost when it is administered after the development of the initial inflammatory response (Mukhopadhyay *et al*., 2010a). Other examples of such opposing regulation of inflammatory and/or fibrotic pathways by $CB_{1/2}$ cannabinoid receptors are atherosclerosis (Mach *et al*., 2008; Pacher and Steffens, 2009) and liver fibrosis (Julien *et al*., 2005; Teixeira-clerc *et al*., 2006; Lotersztajn *et al*., 2008), where CB_2 activation (Steffens *et al*., 2005) and/or CB_1 inactivation (Dol-gleizes *et al*., 2009; Sugamura *et al*., 2009) appears to limit vascular inflammation and/or fibrosis and interrelated disease progression. In clinical trials the CB_1 antagonist/inverse agonist rimonabant (SR141716) also attenuated multiple inflammatory markers (e.g. TNF-α, C-reactive

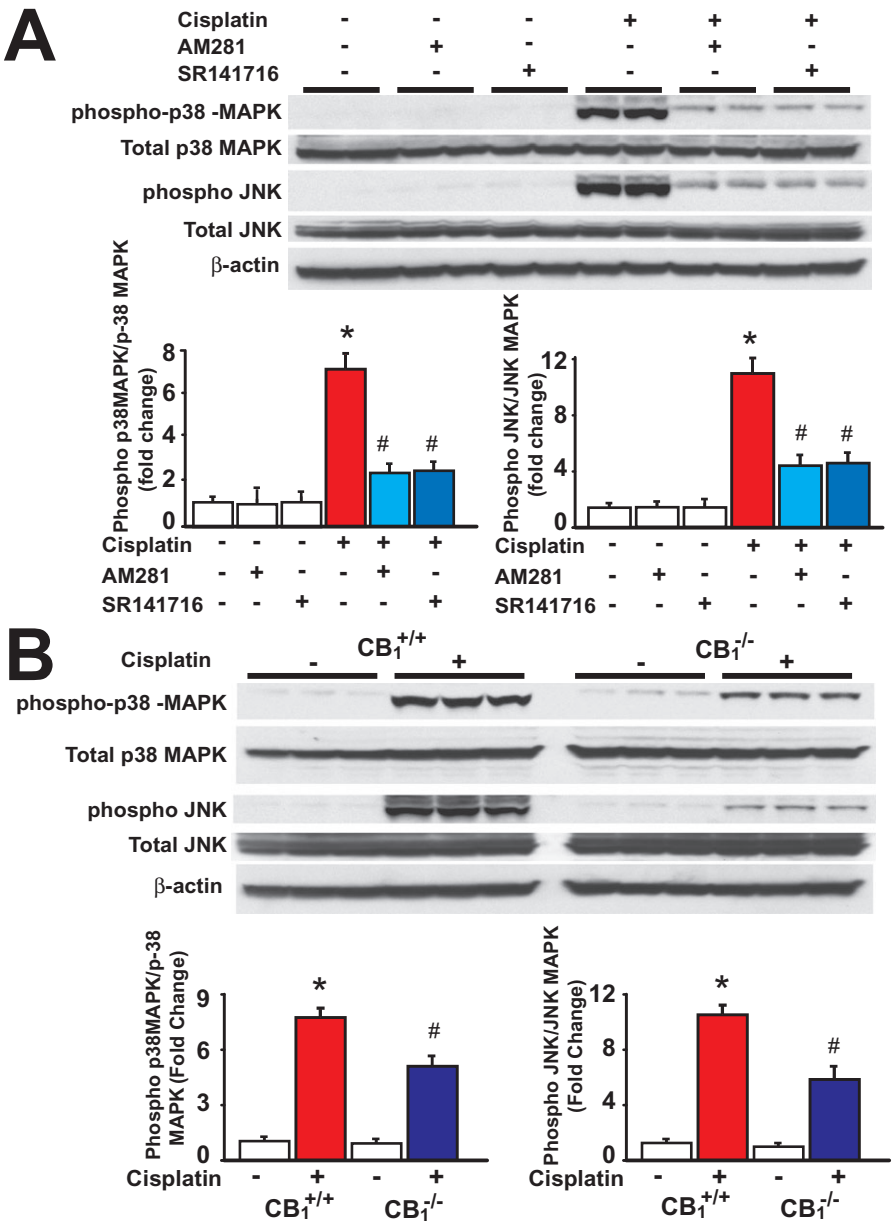


Figure 8 Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB_1) receptors attenuated the cisplatin-induced increased activation of p38 and JNK mitogen-activated protein kinases (MAPKs). The cisplatin-induced marked phosphorylation of renal p38 MAPK and JNK was attenuated by AM281 or SR141716 treatments (A), and also in CB_1 knockout ($CB_1^{-/-}$) mice compared with $CB_1^{+/+}$ littermates (B). Results are mean ± SEM of 4–6 experiments per group. *$P < 0.01$ versus vehicle in C57BL/6 mice (A) or versus $CB_1^{+/+}$ mice treated with vehicle (B); #$P < 0.05$ versus cisplatin in C57BL/6 or $CB_1^{+/+}$ mice (A and B respectively).

protein, etc.), plasma leptin and insulin levels and increased plasma adiponectin in obese patients with metabolic syndrome and/or type 2 diabetes (Engeli, 2008; Pacher *et al*., 2008; Pacher and Steffens, 2009). Furthermore, chronic rimonabant treatment restores plasma levels of the anti-inflammatory hormone adiponectin, reduces the elevated plasma/serum levels of TNF-α (Gary-bobo *et al*., 2007), reduces RANTES (regulated on activation, normal T cell expressed and secreted) and MCP-1(Schafer *et al*., 2008) in obese Zucker fa/fa rats and decreases NF-κB activation and consequent iNOS expression in mitogen-stimulated human peripheral blood mononuclear cells (Malfitano *et al*., 2008). In the context of cisplatin-induced nephrotoxicity, the above-mentioned effect of rimonabant on TNF-α, NF-κB activation and increased expression on iNOS in stimulated human peripheral blood mononuclear cells and on LPS-stimulated macrophage inflammatory responses may be particularly relevant, as pro-inflammatory cytokines such as TNF-α and iNOS-derived oxidative/nitrosative stress are crucial mediators in the pathogenesis of cisplatin-induced kidney injury (Ramesh and Reeves, 2002; Zhang *et al*., 2007; Chirino *et al*., 2008).

Indeed, cisplatin-induced oxidative (Matsushima *et al*., 1998; Davis *et al*., 2001) and nitrosative (Chirino *et al*., 2004;

2008) stress, subsequent inflammation (Yamate *et al*., 2002; Faubel *et al*., 2007) and the associated activation of various cell death pathways [e.g. p38 JNK MAPKs, PARP (Racz *et al*., 2002)] play an important role in the pathogenesis of renal dysfunction. The most likely sources of increased ROS generation by cisplatin in the kidney, in particular superoxide, are the superoxide-generating enzymes NAD(P)H oxidase NOX4(RENOX) and phagocyte NAD(P)H oxidase (NOX2) (Mukhopadhyay *et al*., 2010a; Pan *et al*., 2009a). It is well known that inflammatory cells upon activation produce a plethora of various ROS and RNS [e.g. superoxide, iNOS-derived nitric oxide (NO) and consequently peroxynitrite via diffusion-limited reaction of superoxide with NO], which contribute to tissue injury via complex interrelated mechanisms comprising of increased lipid peroxidation, changes in pro-inflammatory gene expressions in both inflammatory and parenchymal cells, secretion of pro-inflammatory mediators (e.g. cytokines, chemokines), oxidation/nitration of key regulatory proteins involved in cell metabolism, signalling processes implicated in proliferation, survival and/or death, eventually leading to the activation of various mitochondrial-dependent or independent cell death pathways culminating in organ dysfunction and failure (Pacher *et al*., 2007; Szabo *et al*., 2007). Indeed, increasing evidence suggests that the reactive nitrogen species peroxynitrite and/or the consequent protein nitration may be involved in the modulation of various cell survival and death pathways (Liaudet *et al*., 2009), as well as in certain physiological processes (Ferdinandy, 2006), in addition to promoting tissue injury (Ferdinandy and Schulz, 2003; Pacher *et al*., 2007). Cisplatin-induced ROS generation might also favour augmented expression of iNOS through the activation of NF-κB, which further increases the generation of NO and RNS amplifying nitrosative stress.

In agreement with previous reports, we found that a single dose of cisplatin induced marked histopathological damage, increased inflammatory cell infiltration and impaired renal function. It also lead to marked up-regulation of TNF-α and IL-1β mRNA in the kidneys, consistent with the important role of TNF-α (Ramesh and Reeves, 2002; Zhang *et al*., 2007) in cisplatin-induced nephrotoxicity. Interestingly, cisplatin-induced kidney injury largely depends on TNF-α, as TNF-α-deficient mice and TNF-α antibody-treated wild-type mice display resistance to cisplatin-induced kidney toxicity (Ramesh and Reeves, 2002; Zhang *et al*., 2007). Further supporting the importance of iNOS-derived increased nitrosative stress in cisplatin-induced renal injury (Chirino *et al*., 2008), we also found significantly increased iNOS expression in the kidneys of cisplatin-treated mice. In fact, nitrosative stress and/or peroxynitrite, and the activation of interconnected effector downstream pathways such as PARP, have importantly been implicated in the development of cisplatin-induced cell demise and subsequent nephropathy (Racz *et al*., 2002; Chirino *et al*., 2004; 2008). This notion is also supported by our current observations that cisplatin treatment increased renal NT formation, DNA fragmentation and PARP activity.

Remarkably, the cisplatin-induced pathological alterations were markedly attenuated by CB_1 antagonists or in $CB_1^{-/-}$ mice compared with their wild-type littermates. CB_1 antagonists AM281 and SR141716 not only attenuated the cisplatin-induced increased inflammatory response (chemokine secretion, inflammatory cell infiltration, TNF-α and IL-1β levels), but also reduced the expression of ROS-generating enzymes, NOX4 and NOX2, and the renal oxidative stress. In agreement with these results, a recent study has elegantly demonstrated a pivotal role of CB_1 receptors in the generation of ROS by macrophages (Han *et al*., 2009). In addition, $CB_1^{-/-}$ mice were largely resistant to cisplatin-induced nephropathy, further supporting an important role for the endocannabinoid system and CB_1 receptors in the development of the above-mentioned pathological processes and consequent nephropathy. CB_1 genetic deletion or pharmacological inhibition was also associated with decreased cisplatin-induced iNOS overexpression and NT formation [the marker of peroxynitrite generation and more broadly nitrosative stress (Pacher *et al*., 2007)] in the kidneys, and consequent cell death (both apoptotic and necrotic) and renal dysfunction. Genetic deletion of CB_1 receptors (Mukhopadhyay *et al*., 2010b) or pharmacological inhibition (P. Mukhopadhyay and P. Pacher, unpublished) also attenuates the doxorubicin [another chemotherapeutic drug known for its cardiotoxicity (Mukhopadhyay *et al*., 2009)]-induced increased myocardial oxidative/nitrosative stress and cardiac dysfunction (Mukhopadhyay *et al*., 2007; 2010b).

Our results are in good agreement with the emerging anti-inflammatory and cytoprotective effects of CB_1 pharmacological inhibition or genetic deletion observed in the numerous preclinical and clinical reports discussed above. These effects may involve attenuation of the inflammatory cell infiltration, TNF-α production, NF-κB activation and consequent increased expression of iNOS in peripheral blood mononuclear cells and/or parenchymal cells, just to name a few. Because increased oxidative/nitrosative stress and inflammation is known to trigger increased endocannabinoid production or impair endocannabinoid inactivation (Di Marzo, 2008; Liu *et al*., 2008; Pacher and Hasko, 2008), it is likely that endocannabinoids contribute to cisplatin-induced nephrotoxicity by promoting oxidative/nitrosative stress, inflammation and cell death through the activation of CB_1 receptors. The enhanced cisplatin-induced p38 and JNK MAPK activation may also be a consequence, at least in part, of the dysregulated endocannabinoid production, as this signalling pathway (gaining increasing recognition as a part of CB_1 receptor activation) is attenuated in $CB_1^{-/-}$ mice or in mice treated with CB_1 antagonists. In agreement with these results, we have recently demonstrated that CB_1 receptors promote oxidative/nitrosative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human primary cardiomyocytes through activation of p38 and JNK MAPKs (Mukhopadhyay *et al*., 2010b).

In summary, the endocannabinoid system through CB_1 receptors promotes cisplatin-induced tissue injury by amplifying MAPK activation, cell death and interrelated inflammation and oxidative/nitrosative stress. Thus, pharmacological inhibition of CB_1 receptors may exert beneficial effects against cisplatin-induced nephrotoxicity, which is particularly exciting as recent studies have demonstrated multiple beneficial effects of CB_1 antagonists in various cancer types (Bifulco and Pisanti, 2009; Pisanti and Bifulco, 2009; Santoro *et al*., 2009) and suggested that endocannabinoid overactivity might be involved in renal complications of human visceral obesity

(Bordicchia *et al*., 2009). These results may also have important implications for the treatment of kidney or other diseases associated with enhanced inflammation, oxidative/nitrosative stress and cell death.

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Conflict of interest

No conflict of interest to disclose.

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