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CB₁ 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 (CB₁) 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ 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; CB₁−/− mice, CB₁ knockout mice; CB₁ 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 CB₁ 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; Schriber, 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 intranuclear 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; Schriber, 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 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; Fauvel et al., 2007; Zhang et al., 2007; Mukhopadhyay et al., 2010a).

Cannabinoid-1 (CB1) 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 et al., 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 vitro models of inflammation (Malffitano 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 cannabinoind CB1 receptors improved renal function, meta- bolic profile and increased survival of obese Zucker rats (Janiak et al., 2007).

In this study we investigated the interplay of the CB1 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). CB1 knockout mice (CB1−/−) and their wild-type littermates (CB1+/+) were as previously described (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 (cis-diammineplatinum(II) dichloride 25 mg·kg−1 i.p.; Sigma, St. Louis, MO, USA). The selective CB1 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·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 phospho-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 CB1 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, bleeding 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 (Trevisgen, 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:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5′ → 3′)</th>
<th>Reverse Primer (5′ → 3′)</th>
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</thead>
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<td>5′-AGGAGCATTCAATGGTTCGTTGGC-3′</td>
</tr>
<tr>
<td>IL-8</td>
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<td>5′-GTCGTTGCTCTCTGC-3′</td>
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<tr>
<td>iNOS</td>
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<td>5′-GGATGCATTGTGCCTGTG-3′</td>
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<td>NFκB</td>
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<td>5′-GACATTGATGAAACCGC-3′</td>
</tr>
<tr>
<td>NOX4</td>
<td>5′-TATCGGCTCTGCTGCTGCTGCTG-3′</td>
<td>5′-GGGATGATGATGATGATG-3′</td>
</tr>
</tbody>
</table>

Statistical analysis
Results are reported as mean ± SEM. Statistical significance between two measurements was determined by Student’s two-
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. AM281 or SR141716 alone had no effects on BUN and creatinine levels as compared with the vehicle-treated group ($n = 5$, not shown).

![Figure 1](image1.png)  
**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 $\pm$ SEM of 6–8 experiments per group. $^{*}P < 0.05$ versus vehicle.

![Figure 2](image2.png)  
**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 $\pm$ 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).
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 CB1−/− mice compared with CB1+/+ littermates (Figure 3B).

Pharmacological inhibition or genetic deletion of CB1 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 CB1 knockout (CB1−/−) mice compared with CB1+/+ littermates (B). Results are mean ± SEM of eight experiments per group. *P < 0.01 versus vehicle in C57BL/6 mice (A) or versus CB1+/+ mice treated with vehicle (B); #P < 0.01 versus cisplatin in C57BL/6 or CB1−/− mice (A and B respectively).

Pharmacological inhibition or genetic deletion of CB1 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 CB1−/− mice showed considerably less cell death compared with those from their CB1+/+ littermates (Figure 4B).

Pharmacological inhibition or genetic deletion of CB1 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 CB1 receptors (Figure 5B,D).

Pharmacological inhibition or genetic deletion of CB1 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 CB1−/− mice the markers of oxidative/nitrosative stress were attenuated compared with CB1+/+ 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)
  - Cisplatin: -
  - AM281: +
  - SR141716: -

- **DNA fragmentation** (fold change)
  - Cisplatin: -
  - AM281: +
  - SR141716: -

- **TUNEL positive cells in 10X200 fields**
  - Cisplatin: -
  - AM281: +
  - SR141716: -

- **PARP activity** (fold change)
  - Cisplatin: -
  - AM281: +
  - SR141716: -

**B**

- **Caspase 3/7 activity** (fold change)
  - Cisplatin: CB\(^{+/+}\)
  - CB\(^{-/-}\)

- **DNA Fragmentation** (fold change)
  - Cisplatin: CB\(^{+/+}\)
  - CB\(^{-/-}\)

- **TUNEL positive cells in 10X200 fields**
  - Cisplatin: CB\(^{+/+}\)
  - CB\(^{-/-}\)

- **PARP activity** (fold change)
  - Cisplatin: CB\(^{+/+}\)
  - CB\(^{-/-}\)

Images of TUNEL staining are shown for different conditions.
Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB1) 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 CB1 knockout (CB1−/−) mice compared with CB1+/+ littermates (B). Results are mean ± SEM of 6–8 experiments per group. *P < 0.01 versus vehicle in C57BL/6 mice (A) or versus CB1−/− mice treated with vehicle (B); #P < 0.05 versus cisplatin in C57BL/6 or CB1−/− 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 CB1−/− mice compared with CB1+/+ littermates (B).

Pharmacological inhibition or genetic deletion of CB1 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 CB1−/− mice the increase in the mRNA expression for these enzymes was reduced compared with CB1+/+ littermates (Figure 7B,D).

Pharmacological inhibition or genetic deletion of CB1 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 CB1 antagonists AM281 or SR141716 (Figure 8A) significantly attenuated these increases. Likewise these increases were reduced in CB1−/− mice compared with their CB1+/+ littermates (Figure 8B).
CB₁ antagonists for nephropathy

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Therefore, CB₁ 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 CB₁ 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 CB₁ 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 CB₁ 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 CB₁ activation may promote inflammation and tissue injury. Support for this comes from the observation that in these models CB₁ 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 CB₁ 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 CB₁ receptors. 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 CB₁ receptors, etc.), as well as on the stage of the disease progression. Indeed, CB₂ receptor activation limits inflammation and interrelated oxidative/nitrosative stress-cell death associated with cisplatin-induced nephropathy. However, the protective effect of a CB₁ 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 CB₁ and 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 CB₁ activation (Steffens et al., 2005) and/or CB₂ inactivation (Dol-glezies et al., 2009; Sugamura et al., 2009) appears to limit vascular inflammation and/or fibrosis and interrelated disease progression. In clinical trials the CB₁ antagonist/ inverse agonist rimonabant (SR141716) also attenuated multiple inflammatory markers (e.g. TNF-α, C-reactive protein, NOX2 (gp91phox)RENOX (NOX4))

Discussion

In this study, we have explored the interplay of oxidative/nitrosative stress, inflammation and cell death pathways with CB₁ receptors in a clinically relevant model of cisplatin-induced nephropathy by using pharmacological antagonists of the cannabinoid CB₁ receptor (AM281 and SR141716) as well as CB₁ receptor knockout mice. We demonstrated that genetic deletion of CB₁ 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 CB₁ cannabinoid receptor activation by endocannabinoids promotes cisplatin-induced tissue injury by amplifying MAPK activation, cell death and the associated inflammation and oxidative/nitrosative stress.

Figure 7 Pharmacological inhibition or genetic deletion of cannabinoid-1 (CB₁) receptors attenuated the cisplatin-induced increase in 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 CB₁ knockout (CB₁⁻⁻) mice compared with CB₁⁺⁺ 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 CB₁⁻⁻ mice treated with vehicle (B and D); #P < 0.05 versus cisplatin in C57BL/6 or CB₁⁻⁻ mice (A/C and B/D 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;
induced cell demise and subsequent nephropathy (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 (Laudet et al., 2009), as well as in certain physiological processes (Ferdinandy, 2006), in addition to promoting tissue injury (Ferdiandny and Schulz, 2003; Pacher et al., 2007). Cisplatin-induced ROS generation might also favour augmented expression of iNOS through the activation of NF-kB, 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 led 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-treatment 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 CB1 antagonists or in CB1−/− mice compared with their wild-type littermates. CB1 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 CB1 receptors in the generation of ROS by macrophages (Han et al., 2009). In addition, CB1−/− mice were largely resistant to cisplatin-induced nephropathy, further supporting an important role for the endocannabinoid system and CB1 receptors in the development of the above-mentioned pathological processes and consequent nephropathy. CB1 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 CB1 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 CB1 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, 2008b), it is likely that endocannabinoids contribute to cisplatin-induced nephrotoxicity by promoting oxidative/nitrosative stress, inflammation and cell death through the activation of CB1 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 CB1 receptor activation) is attenuated in CB1−/− mice or in mice treated with CB1 antagonists. In agreement with these results, we have recently demonstrated that CB1 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 CB1 receptors promotes cisplatin-induced tissue injury by amplifying MAPK activation, cell death and interrelated inflammation and oxidative/nitrosative stress. Thus, pharmacological inhibition of CB1 receptors may exert beneficial effects against cisplatin-induced nephrotoxicity, which is particularly exciting as recent studies have demonstrated multiple beneficial effects of CB1 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.

**References**


