Activation of Cannabinoid Receptor 2
reduces inflammation in acute experimental
pancreatitis via intra-acinar activation of p38
and MK2-dependent mechanisms

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Abstract

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The endocannabinoid system has been shown to mediate beneficial effects on gastrointestinal inflammation via Cannabinoid receptor 1 (CB₁) and 2 (CB₂). These receptors have also been reported to activate the MAP-kinases p38 and c-jun N-terminal kinase (JNK), which are involved in early acinar events leading to acute pancreatitis and induction of pro-inflammatory cytokines. Our aim was to examine the role of cannabinoid receptor activation in an experimental model of acute pancreatitis and the potential involvement of MAP-kinases. Cerulein-pancreatitis was induced in wild-type, CB₁⁻/⁻ and MK2⁻/⁻ mice pre-treated with selective cannabinoid receptor agonists or antagonists. Severity of pancreatitis was determined by serum amylase and IL-6 levels, intracellular activation of pancreatic trypsinogen, lung myeloperoxidase activity, pancreatic edema and histological examinations. Pancreatic lysates were investigated by western blotting using phospho-specific antibodies against p38 and JNK. QPCR-data, western blotting experiments and immunohistochemistry clearly show that CB₁ and CB₂ are expressed in mouse pancreatic acini. During acute pancreatitis, an up-regulation especially of CB₂ on apoptotic cells occurred. The unselective CB₁/CB₂-agonist HU210 ameliorated pancreatitis in wild-type and CB₁⁻/⁻ mice, indicating that this effect is mediated by CB₂. Furthermore, blockade of CB₂, not CB₁, with selective antagonists engraved pathology. Stimulation with a selective CB₂-agonist attenuated acute pancreatitis and an increased activation of p38 was observed in the acini. Using MK2⁻/⁻ mice, it could be demonstrated that this attenuation is dependent on MK2. Hence, using the MK2⁻/⁻ mouse model we reveal a novel CB₂-activated and MAP-kinase dependent pathway that modulates cytokine expression and reduces pancreatic injury and affiliated complications.

Introduction
Acute pancreatitis has been the subject of investigations for many years, and cytokines have become a new focus in the investigation of the pathophysiology of pancreatitis. Although the exact underlying mechanisms only begin to emerge, cytokines play a crucial role in the process of pancreatitis. Since Multiple Organ Dysfunction Syndrome (MODS) is the primary cause for morbidity and mortality in acute pancreatitis (6, 64), there is a great need to understand the pathophysiological processes leading to this fatal complication. Here, TNF-α and IL-6 as pro-inflammatory cytokines play a predominant role (5, 6, 23). As a consequence of an inflammatory cascade triggered by induction of pro-inflammatory cytokines in acinar cells (39, 45), neutrophil granulocytes immigrate into lung tissue (5, 49). This constitutes a key event in genesis of Acute Respiratory Distress Syndrome (ARDS) (1, 24), the most fatal complication of acute pancreatitis (55, 64). It is not clear by which pathway cytokines trigger the cascade of inflammation after onset of pancreatitis. However, the Mitogen-Activated-Protein-Kinases (MAPK) p38 and JNK are known to play a role in onset of pancreatitis and their activation results in activation of several pro-inflammatory transcription factors (54). We have previously shown that MK2, a downstream target of p38, is an essential element involved in regulation of cytokine gene-expression and that genetic disruption of the MK2 gene protects against cerulein-induced pancreatitis (62). But p38 induced MK2 activation also conducts protective measures via the heat shock protein 27 (HSP27) and consecutive stabilization of the actin cytoskeleton (20, 35, 52, 53). P38 and JNK have been described to become activated by CB₁ and CB₂ in cells of hippocampus in mice (11, 13). Recently, a study showed that CB₁ and CB₂ are also expressed on pancreatic cells and that the relatively non-selective CB₁/CB₂-agonist HU210 ameliorates acute experimental pancreatitis (42). Anti-inflammatory effects of cannabinoids and components of the ECS have also been described in other models.
Recently, a study showed the potential of a CB$_2$-agonist for treating colitis in mice (60). The respective roles of CB$_1$ and CB$_2$ in acute pancreatitis have not been investigated yet.

The endocannabinoid system (ECS) consists of endocannabinoids (eCB), cannabinoid receptors and eCB synthesizing and eCB degrading enzymes. Amongst eCB, anandamide (AEA), 2-acylglycerol (2-AG) and noladin ether (NE) act as agonists whereas virodhamine seems to have additional antagonist activities (19, 47). Presently, CB$_1$ and CB$_2$ are named as cannabinoid receptors and additional receptors like the GPR55 receptor and the GPR119 receptor were implicated to be novel cannabinoid receptors since eCB bind to these receptors (9, 44, 51). In addition, eCB bind to other non-cannabinoid receptors like the transient receptor potential vanilloid 1 (TRPV1) (57, 65). The in vivo biosynthesis of eCB occurs through pathways involving various phospholipases like phospholipase A (PLA), phospholipase C (PLC), N-acylphosphatidylethanolamide-phospholipase D (NAPE-PLD) for AEA (15, 17, 27) and phosphatidic acid phsophohydrolase, diacylglycerol lipase (DAGL), phosphoinositide-specific PLC (PI-PLC) and lyso-PLC for 2-AG (7, 8, 10, 14, 58). They are released to the extracellular space and bind to cannabinoid-receptors. Termination of eCB action requires intracellular degradation. Cellular re-uptake of eCB occurs through a putative membrane transporter (endocannabinoid membrane transporter, EMT) (3, 4, 26, 50) or diffusion (21, 22, 31). Finally, intracellular degradation of eCB results from enzymatic hydrolysis by the enzymes fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase (MAGL) (12, 18, 32). The ECS is involved in the regulation of various gastrointestinal (GI) functions. Physiological involvement of the ECS within the GI tract was suggested for the regulation of motility, inflammation, secretion and sensation (16, 28-30, 41, 56). The ECS seems not only to be involved under physiological conditions but also under
pathological conditions (16, 25, 40). In addition, GI functions can be altered by pharmacological targeting of the structures of the ECS (2, 59). In vivo studies furthermore suggest influences of the ECS on cell growth and cell death (37, 48).

The aim of our study was to identify whether CB$_1$ or CB$_2$-activation protects against acute experimental pancreatitis and to furthermore identify possible intracellular mechanisms involved. Additionally we were interested whether MAP-kinase and MK2 dependent pathways are activated in the protective effects following cannabinoid receptor activation.

**Material and methods**

**Animals**

C57bl mice, CB1-/- mice and MK2-/- mice weighing 20 to 30 g were used in this study. MK2-/- animals were generated on a C57bl/6J background as described previously (34). During the experiments animals were housed and maintained under controlled environmental conditions. All animal studies were in accordance with national and international guidelines as outlined in the Guide for the care and use of animal laboratory animals and performed according to protocols approved by the institutional animal care office and the government of Bavaria.

**Induction of acute pancreatitis**

Wild-type mice, CB1-/- mice and MK2-/- mice received six hourly intra-peritoneal injections containing 50 μg/kg bw of cerulein. The respective control groups received comparable injections of 0.9% saline at hourly intervals, respectively. One hour after the final cerulein or saline injection animals were sacrificed by decapitation under isoflurane anesthesia, blood was collected and preparation of pancreas and lung followed.
Quantification of cerulein-induced injuries

Blood and pancreatic tissue were processed as described previously (61). Serum α-amylase activity was measured using a colorimetric assay according to manufacturer’s instructions (Phadebas Amylase Test, 1994, Pharmacia AB, Uppsala, Sweden). Results are expressed in U/l. The extent of pancreatic edema was determined by measuring tissue water content. The difference between wet and dry tissue weights was calculated and expressed as percentage of tissue wet weight. As an additional indicator for pancreatic edema, ratios of pancreatic and whole body weight were compared.

Quantification of pancreatic trypsin activity

Pancreas samples were homogenized as described previously (61) in ice-cold 3-[N-Morpholino] propane sulfonic acid (MOPS) buffer, pH 6.5 (250 mM sucrose, 5 mM MOPS, 1 mM magnesium sulphate) using a teflon glass homogenizer. The resulting homogenate was centrifuged and supernatant was used for the assay. Trypsin activity was measured fluorometrically using Boc-Glu-Ala-Arg-AMC-HCL (BACHEM, Biochemika GmbH, Heidelberg, Germany) as substrate and activity was calculated from the slope using a standard curve generated with purified trypsin. Protein content was measured using the Bio-Rad® protein assay and trypsin activation expressed as fmol/mg protein.

Histological studies and evaluation of pancreatic morphology

One portion of the pancreas was fixed in freshly prepared 4% formaldehyde (Merck KGaA, Darmstadt, Germany) containing phosphate-buffered saline (PBS) (pH 7.4) and stored at 4°C. The tissue was embedded in paraffin, cut in sections, and processed for H&E staining by standard procedures for histological studies. Multiple randomly chosen microscopic fields from each sample were examined based on
necrosis, vacuolization, presence of inflammatory cells and edema as described previously (35).

**Quantification of lung myeloperoxidase activity**

Activity of myeloperoxidase in lung tissue was measured as a marker for neutrophil immigration. Tissue samples were homogenized in an Elmer potter at 2,400 rpm. An aliquot of this homogenate was taken for protein determination; the remaining lysate was centrifuged at 10,000 g and 4°C for 10 min. Pellets were re-suspended in extraction buffer and snap frozen and thawed four times. Samples were subsequently sonicated twice for 10 s and centrifuged for 5 min at 10,000 g and 4°C. The supernatant was used for MPO measurement. The reaction mixture consisted of 1 ml KH₂PO₄ buffer (pH 6.0), 10 µl o-dianisidine, 10 µl H₂O₂ and 50-200 µl of the supernatant. The supernatant was added to the mixture after 1 min of monitoring the absorbance at 460 nm, and absorbance was monitored for additional 4 min. Activity was calculated from the slope and expressed in mU/mg protein.

**Determination of IL-6 serum levels**

Il-6 levels were measured using a commercially available ELISA kit for mouse IL-6 (Quantikine® Mouse IL-6 Kit, R&D Systems, Minneapolis, USA) according to the manufacturer’s protocol. After decapitation, blood was collected and centrifuged at 10,000 rpm at 4°C for 10min. Serum was transferred into new vials and frozen at -80°C until measurement. Each sample was measured in duplicates with a microplate reader and expressed as means ± S.E.M.

**RNA isolation and RT PCR analysis**

Total cellular RNA was extracted from pancreatic sections. Approximately 50 mg of pancreatic tissue was homogenized with 1 ml TRIzol® (Invitrogen, Carlsbad, CA) with a Polytron® homogenizer. After centrifugation, phase separation was achieved
with chloroform. The aqueous phase was transferred to a new microfuge tube. RNA was precipitated by adding 0.5 ml isopropylalcohol per 1 ml TRIzol® and a 10 min centrifugation period at 4°C and 12,000 g. The pellet was washed with ethanol. RNA was further purified utilizing the RNA Easy kit (Quiagen, Basel, Switzerland) following the manufacturer’s instructions. 2-4 μg of total RNA were subjected to first strand cDNA synthesis in 20 μl reaction mixture containing Superscript™ First Strand reverse transcriptase (Invitrogen, Carlsbad, CA). Primer sequences were designed as following (5' to 3'):

CB1:      GTACCATCACCACAGACCTCCTC/GGATTCAGAATCATGAAGCACTCA
CB2:       CCCTACCTGTAATCCCAGCA / GCAGTCTGTGCTCTCAACCA
murine β-actin:  CATTGCTGACAGGATGCAGAA / GCTGATCCACATCTGCTGGAA

For preparation of the master mix, the HotStar Taq reagent kit (Invitrogen, Karlsruhe, Germany) with a total reaction volume of 20 μl containing 8 μl of a 1:5 diluted DNA was used. Primers were the ones described above. Buffer and magnesium chloride were used as supplied with the enzyme and dNTPs from peqlab (Helsingborg, Sweden). Identity of PCR products was confirmed by melting point analysis and agarose gel electrophoresis. All assays were performed with the Corbett Research Rotor-Gene RG-3000 machine using SYBR Green detection (Molecular Probes, Leiden, Netherlands).

**Western blotting**

Pancreatic tissues weighing 50-100 µg were homogenised in 1 ml Tris-buffer (pH 7.4) using an Elmer Potter homogenizer. After centrifugation for 5 min at 4°C and 14,000 rpm, supernatant was kept for 30 min on ice to allow lysis to take place. After centrifugation for another 30 min at 4°C and 14,000 rpm, 5 μl of supernatant was used to measure protein content (Bradford). The lysates were mixed with Tris- and SDS- buffer and mercapto-ethanol to gain a protein content of 2 mg/ml and boiled at 95°C for 5 min. Primary antibodies used in this study included anti-CB₂ (Cayman
Chemicals, Ann Arbor, Michigan, USA), p38 (Santa Cruz Biotechnology, Santa Cruz, USA), phospho-p38, SAPK/JNK and anti-phospho-SAPK/JNK (all three from Cell Signaling Technology, Frankfurt am Main, Germany) in a 1:1,000 dilution. After washing of the membranes, the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Freiburg, Germany) was applied in a 1:5,000 dilution and incubated for 1 h at room temperature. Finally, antibody binding was detected using an enhanced chemiluminescent detection system (ECL, Amersham Pharmacia Biotech, Freiburg, Germany) and recorded on film. For comparison of MAPK-activation, only unsaturated blots were scanned and the signal was quantified using the software ImageJ 1.41 (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). For better comparability, signals were referred as percentage of the corresponding saline-control animal of the same experiment and blot. Diagrams showing quantified western blot signals include data of all test animals.

Pre-treatment with CB1 and CB2-agonists/antagonists

30 min before induction of acute pancreatitis animals were pre-treated via intraperitoneal injections with the non-selective CB1/CB2-agonist HU210 (50ng/g bw; dissolved with 1.2% dimethylsulfoxid (DMSO) in 0.9% NaCl; from Tocris Cookson Inc., USA.), with the selective CB2-agonist JWH133 (5µg/g bw) (dissolved in Tocrisolve™ from Tocris Bioscience, Ellisville, Missouri, USA), the selective CB1-antagonist AM281 (1mg/kg bw, dissolved with 1.2% dimethylsulfoxid (DMSO) in 0.9% NaCl) or the selective CB2-antagonist AM630 (1µg/g bw) (from Tocris Bioscience, dissolved in 1.2% dimethylsulfoxid (DMSO) in 0.9% NaCl). For all experiments respective solvent controls were performed and neither DMSO nor Tocrisolve had significant effects on the observed parameters. In experiments with antagonist and agonist co-treatment, the respective antagonist was administered 30
min before the agonist. Control animals were pre-treated with 0.9% NaCl (10 μl/g bw). The concentrations used in the experiments were analogue as previous published and being effective in other experimental systems ((33, 40, 42, 60)).

**Statistical analysis**

Results shown represent mean ± SEM values obtained from multiple determinations in 3 or more separate experiments. In every experiment, there were at least 3-4 animals in each treatment group. Effect of pre-treatment with cannabinoid agonists/antagonists on acute pancreatitis is referred to cerulein-only induced rises as follows:

\[
\frac{[(x - \text{group}) - (\text{NaCl - group})] - [(\text{cerulein - group}) - (\text{NaCl - group})]}{[(\text{cerulein - group}) - (\text{NaCl - group})]} \times 100
\]

Statistical analysis was carried out using the Student t-test for independent samples. All statistical computations were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Significance was assumed for p<0.05.

**Immunohistochemistry**

Paraffin embedded sections of pancreatic tissue were cut in 3 μm thick slices and placed on slides. All examinations were performed in duplicates. 2 slices for analysis and 2 as negative controls were cut from each probe. After removal of paraffin and rehydration, tissue was treated for 30 min in citrate buffer (pH 6.0) at 120°C for antigen retrieval and endogenous peroxidase was blocked by administration of 0.3% \( \text{H}_2\text{O}_2 \) (in Methanol). After incubation with \( \text{CB}_1 \)- or \( \text{CB}_2 \)- antibodies (both from Cayman Chemicals, Ann Arbor, Michigan, USA, 1:100, 4°C over night) treatment with Super Enhancer, Polymer HRP Chromogen and DAB followed as described by the manufacturer (Polymer-HRP IHC kit, DCS Innovative Diagnostik-Systeme, Hamburg, Germany). Negative controls were not incubated with primary antibodies. Visualization was achieved by incubation for 5 min with liquid 3,3’-diaminobenzidine
(DAB) and counterstaining by administration of 1:10 hematoxylin solution for 5 min. Two researchers independently examined the preparations using an Olympus BX41 microscope (magnifications: 200/400, software: Olympus Cell^A) for intensity of staining and calculation of percentage and type of positive cells.

### Results

**Expression of CB\textsubscript{1} and CB\textsubscript{2} in pancreas**

Expression of CB\textsubscript{1} and CB\textsubscript{2} encoding mRNA was detected by RT-PCR in all investigated pancreatic probes derived from C57/BL6 mice. Also, CB\textsubscript{1} mRNA was detected in brain with strong signal and in spleen and heart with weak signals (Fig.1A, top panel). CB\textsubscript{2} mRNA was also detected with weak signals in brain, spleen and heart (Fig.1A, middle panel). CB1-/- mice lacked mRNA for CB\textsubscript{1} (Fig.1B) but CB\textsubscript{2}-expression was detected via western blot with strong signal in pancreatitis tissue (Fig.1C). Immunohistochemical studies further showed expression of CB\textsubscript{1} and CB\textsubscript{2} within pancreatic tissue in acinar and duct cells (Fig.1D, upper row) as described previously (42). While CB\textsubscript{1} was found with an equal or only slightly elevated signal in pancreatitis compared to non-pancreatitis tissue (Fig.1D, middle column), expression of CB\textsubscript{2} was lower in non-pancreatitis but was drastically up-regulated on apoptotic cells found during acute pancreatitis (Fig.1D, right column).

**The non-selective cannabinoid agonist HU210 ameliorates pancreatitis in wild-type and CB1-/- mice**

As described by Michalski et. al, pre-treatment of wild-type C57/BL6 mice with the non-selective CB\textsubscript{1}/CB\textsubscript{2}-agonist HU210 ameliorated cerulein induced acute pancreatitis and lead to reductions of trypsin activity in pancreas, MPO activity in lung tissue and IL\textsubscript{6} serum levels. Amylase activity in serum remained unchanged by the pre-treatment in our hands (Fig.1E-H,rows 3&5). Using mice deficient of CB\textsubscript{1}, a
similar pattern was observed. Cerulein injections lead to pancreatitis with comparable severity as in wild-type mice (Fig 1, row 3&4 of E-H). No measured parameters were significantly elevated compared to wild-type mice, indicating that physiologic CB1-activation does not lead to protection against pancreatitis since then an engraved pancreatitis would be expected in mice lacking CB1. Noteworthy, the inflammatory parameters MPO activity in lung tissue and IL-6 levels in serum were reduced compared to wild-type mice. MPO activity was reduced significantly by -71% (p<0.01) (Fig.1F) whereas the reduction of the IL-6 Levels did not reach statistically significant levels (-22%) (Fig.1G). Interestingly, HU210 still remained most of its protectiveness on acute pancreatitis in absence of CB1. MPO activity (-172%; p=0.01), trypsin activity (-68%; p=0.08) and IL-6 levels (-53%; p=0.19) were still reduced by HU210 pre-treatment (Fig.1E-G, rows 4&6) showing that a large proportion of the protective effects of HU210 are mediated via a CB1-independent mechanism.

Blockade of endogenous CB2-activation worsened pathology and increased JNK-activation

We then pre-treated animals with (a) a selective CB1-antagonist (AM281) or (b) a selective CB2-antagonist (AM630) prior to induction of pancreatitis. While AM281 pre-treatment had no effect on CB1-expression in non-pancreatitis or pancreatitis tissue (Fig.2A), pre-treatment with AM630 led to a slight up-regulation of CB2 on acinar cells in non-pancreatitis tissue (Fig.2B, left column). In AM630 pre-treated pancreatitis animals, an engraved pathology was observed, as there were more apoptotic cells visible which presented with a strong up-regulation of CB2 (Fig.2B, right picture on lower row). We performed western blots for JNK-phosphorylation, a signal pathway well known for its induction during pancreatitis and also its role in apoptosis. While there was no effect seen by selective CB1-antagonisation (Fig.2C), selective blockade of CB2 with AM630 lead to a drastically intensified JNK-phosphorylation
(Fig.2D) with an increase of 54% (p<0.05) in non-pancreatitis and 204% (p<0.01) in pancreatitis animals (Quantification data of western blot signal, not shown). We then evaluated the effect of CB2-blockade on severity of acute pancreatitis. Mice receiving AM630 prior to induction of pancreatitis had a more severe pathology compared to non-pre-treated animals. Especially, systemic inflammation was engraved while the pancreatitis specific parameters only increased insignificantly or remained unchanged. The rise in IL-6-levels and MPO-activity after pancreatitis induction more than doubled to 219% (p<0.05) and 241% respectively (p<0.01) (Fig.2H/I). Intra-acinar activation of trypsinogen increased by 28% but was not statistically significant (Fig.2E) as was pancreas/body weight ratio (Fig.2G). Serum α-amylase levels were nearly unchanged (Fig.2F).

Acute experimental pancreatitis was ameliorated by pre-treatment with a selective CB2-agonist

Pre-treatment with the CB2-selective agonist JWH133 led to comparable reductions of pancreatitis parameters as seen by HU210 pre-treatment. Most pronounced, intra-acinar activation of trypsinogen was reduced by 92% (Fig.3A) and IL-6 serum levels by 69% (p<0.05 for both) (Fig.3D). The mean MPO activity remained on control animal level in animals pre-treated with JWH133 (-106% compared to cerulein induced rise in not pre-treated animals) but this was statistically not significant (Fig.3E). The activity of serum α-amylase was reduced insignificantly by 8% (Fig.3B) and pancreatic edema by 20% (Fig.3C). On the other hand, pretreatment with JWH133 markedly reduced the morphologic changes seen after cerulein treatment (Fig. 3F). Interestingly, when looking at the intracellular signaling pathways, pre-treatment with the CB2-agonist had an impressive impact on p38-phosphorylation: In control animals without pancreatitis p38-phosphorylation rose by
255% (p<0.05). This increase in activation was still detected after induction of pancreatitis with an increase of 177% (p<0.05) (Fig.3G/H).

Protective effects of JWH133 could be reversed by co-administration of the CB$_2$-selective antagonist AM630

To further specify that effects seen after JWH133 treatment were mediated via CB$_2$, we administered the selective CB$_2$-antagonist, AM630, 30 min prior to injection of JWH133. AM630 blocked all protective effects of JWH133 confirming that JWH133 acts via CB$_2$. Some parameters elevated compared to non-pretreated pancreatitis animals. Trypsin activity increased by 105% (p<0.01) (Fig.4A), serum IL-6 level by 107% (p<0.05) (Fig.4D), pancreatic edema by 34% (p<0.05) (Fig.4C), and activity of MPO increased by 267% (n.s.) (Fig.4E). Serum activity of α-amylase was unchanged (-6%; n.s.) (Fig.4B).

Knockout of MK2 abolished the protective effects of CB$_2$-activation on pancreatitis and systemic inflammation

Following the observation that increased p38-phosphorylation correlated with the protective effects of CB$_2$-activation, we performed experiments in mice lacking MK2, a kinase known to be activated by p38 and to mediate many of its effects on pancreatitis. In our previous studies we reported protective effects of an MK2 knockout (36, 62) in acute pancreatitis induced by cerulein injections. Using the same MK2-/- germline, these effects were not that strong as previously reported by us. Activation of trypsinogen was elevated by 83% but was not statistically significant, serum levels of α-amylase increased by 116% (p<0.01) and IL-6 levels by 75% (p=0.06). Activation of lung MPO increased by 32% (n.s.) and pancreatic edema increased by 56% (p<0.05) (Fig.5A-E). Interestingly, CB$_2$-stimulation with JWH133 had different effects in MK2-/- mice as compared to wild-type mice. In animals missing the MK2 gene, the JWH133 induced attenuation of acute pancreatitis and...
systemic inflammation seen in wild-type mice was abolished and some parameters were even elevated instead of decreased by JWH133 pre-treatment as seen in Figure 5. Trypsinogen activation was not prevented by CB$_2$-activation and trypsin activity in JWH133 pre-treated MK2-/- was 58% higher than in wild-type mice (p<0.01) (Fig.5A). Pancreatic edema, IL-6 levels and MPO activity were also significantly elevated in MK2-/- compared to wild-type mice and JWH133 pre-treatment even led to increases instead of decreases of these parameters. Compared to wild-type mice with same treatment, IL-6 levels were raised by 224% (Fig.5D), MPO activity by 61% (Fig.5E) and pancreatic edema by 20% (Fig.5C) (all p<0.05). Amylase activity in MK2-/- was nearly unchanged by JWH133 pre-treatment (Fig.5B) as seen in wild-type mice (5%, n.s.). Concerning p38, knockout of its downstream kinase MK2 had no effect on CB$_2$-agonist induced p38-activation: Non-pancreatitis animals had an increase of phosphorylation by 78% (n.s.) and pancreatitis animals had an increase of 199% (p<0.01) (not shown). Parallel to the observed increase of inflammation after JWH133 pre-treatment, an increase of JNK-phosphorylation was detectable (Fig.5F, top). JWH133 pre-treated pancreatitis MK2-/- mice corresponded with an increase of JNK-phosphorylation by 137% (p<0.05) instead of 2% (n.s.) in wild-type animals (Fig.5F, bottom). In direct comparison, JNK-phosphorylation was elevated by 159% in MK2-/- compared to wild-type (p<0.01) (Fig.5G).

**Discussion**

The ECS may be involved in initiation, augmentation or protection of acute pancreatitis since the ECS is involved in the regulations of functions of the GI tract that may influence pancreatic homeostasis. The ECS is involved in GI inflammation and secretion, functions that are involved in the pathophysiology of acute pancreatitis.
and additionally motility effects of the ECS may influence acute pancreatitis by altering pancreatic duct and sphincter of oddi motility. During pancreatitis in humans, an up-regulation of the most prominent eCB, anandamide, in pancreatic tissue was reported (42) and suggests a role of anandamide and the ECS in the pathophysiology of acute pancreatitis. A previous study showed that experimental pancreatitis and pancreatitis associated pain were reduced by the non-selective CB₁/CB₂-agonist HU210. Furthermore, cannabinoid-receptors have been shown to play a role in the modulation of secretion of pancreatic enzymes by acinar cells (38), an effect potentially involved in the early phase of acute pancreatitis. Recently, a study showed that another non-selective CB₁/CB₂-agonist WIN55,212 had inhibitory effects on synthesis and secretion of pro-inflammatory cytokines by rat acinar cells in vitro (46). Since this effect was antagonized by AM630, a role of CB₂ was suggested. So far, no studies have been performed using selective agonists and antagonists or transgenic mice with genetically manipulated cannabinoid-receptors to characterize the cannabinoid receptor subtype responsible for protective effects of cannabinoids in experimental pancreatitis in vivo. Furthermore, the intra-cellular mechanisms mediating these protective effects of cannabinoids on inflammation are unknown.

Receptor responsible for the protective effect of cannabinoids on pancreatitis

Our initial observation showing a protective effect of HU210 on pancreatitis also in CB₁-/- mice, together with the finding that the selective CB₂-antagonist AM630 but not the selective CB₁-antagonist AM281 led to increased apoptosis and JNK-activation during pancreatitis, dismantles previous indistinct reports of cannabinoid agonist induced amelioration of pancreatitis. Also, injections of cerulein did not lead to a more severe pancreatitis in CB₁-/- mice than in wild-type mice, excluding a distinctive role of CB₁ in the cannabinoid-induced amelioration of acute pancreatitis. Noteworthy, MPO-activity was even significantly lower in CB₁-/- than in wild-type
pancreatitis mice, indicating that endogenous CB\textsubscript{1}-activation even leads to a higher influx of neutrophile granulocytes into the lung.

In addition, the CB\textsubscript{2}-selective agonist JWH133 improved pancreatitis in a similar fashion as did HU210. This effect could be completely abolished by previous administration of AM630, which furthermore shows that effects of JWH133 are mediated by CB\textsubscript{2}. In line with our findings, also in Michalski et al. as well as in Petrella et al. studies, decreased effects of the used non-selective cannabinoid agonists were reported after antagonisation by AM630. Our finding that some parameters in the AM630 and JWH133 pre-treated animals elevated compared to non-pretreated pancreatitis animals can be explained by additional blockade of eCB at CB\textsubscript{2}. The main effect of CB\textsubscript{2}-activation seems to be the inhibition of secretion of pro-inflammatory cytokines like IL-6 by acinar-cells (see also (46)) which is concordant with the suggested role of CB\textsubscript{2} as a physiologic brake of inflammation (63). CB\textsubscript{2}-stimulation in our hands reduced serum IL-6 levels and efficiently prevented infiltration of neutrophil granulocytes into lung tissue thus prevented a key event in pathogenesis of ARDS, the most fatal complication of acute pancreatitis.

Possible mechanisms of CB\textsubscript{2}-mediated amelioration of acute pancreatitis

Following CB\textsubscript{2}-antagonisation, a higher proportion of apoptotic cells was observed in pancreatitis tissue correlating with an increased intra-acinar activation of the pro-apoptotic kinase JNK. Apoptotic cells also showed a drastic up-regulation of CB\textsubscript{2}. Thus, CB\textsubscript{2}-stimulation inhibits apoptosis and intra-acinar JNK activation during pancreatitis. One possible way as how CB\textsubscript{2}-activation prevents JNK-activation and apoptosis might be the inhibition of pro-inflammatory cytokines like IL-6, which is an activator of JNK and thereby can act pro-apoptotic. Interestingly, experiments with MK2/- mice suggest a connection between MK2 and JNK-activation: In presence of
MK2, activation of CB$_2$ causes inhibition of JNK and in consequence attenuation of acute pancreatitis. In absence of MK2, activation of CB$_2$ had opposite effects and led to increased activation of JNK and increased IL-6 and MPO levels. Thus, using MK2/-/- mice, we were able to show that inhibition of JNK through CB$_2$-activation is MK2-dependent, and that an alternative MK2-independent pathway of CB$_2$-induced JNK-activation exists, which leads to increased JNK-activation in absence of MK2-dependent inhibition. This may occur inside the acinar cell or may be indirectly mediated by increased serum IL-6 levels since IL-6 is an activator of JNK.

In contrast, following CB$_2$-activation, increased p38-phosphorylation was clearly seen in non-pancreatitis and in pancreatitis animals, in both, wild-type and MK2/-/- mice. At this point, both, inhibition of JNK and activation of p38 seem possibly involved in mediating the protective effects of CB$_2$-activation during acute pancreatitis. At a closer look, JNK-activation correlated closely with elevated IL-6 and MPO levels, not with the more pancreatitis specific parameters such as trypsinogen activation and serum α-amylase levels. Pre-treatment with JWH133 in MK2/-/- mice also led to an increased JNK-activation but activities of trypsinogen and α-amylase remained unchanged. This makes a causative relation between intra-acinar JNK-activation and digestive enzyme activation unlikely. In contrast, IL-6 and MPO levels in JWH133 pre-treated MK2/-/- mice correlated closely with the observed increased JNK-activation and were elevated. JNK-activation after induction of pancreatitis therefore seems to be a parallel phenomenon to digestive enzyme activation, which may be a result of elevated levels of pro-inflammatory cytokines, which again increase inflammation by activating pro-inflammatory transcription factors. In contrast, p38-activation correlated with the severity of acute pancreatitis more closely than JNK-activation: Animals with an increased activation of p38 showed milder pathology with decreased intra-acinar activation of trypsinogen. Increased p38-
phosphorylation was seen in wild-type and MK2-/- mice. The effects on trypsinogen activation, pancreatic edema, IL-6 and MPO levels were distinctly changed in absence of MK2. This indicates that protective effects of p38 on pancreatitis and systemic inflammation are mediated by MK2. Recently, Michalski et al. reported another possible mechanism by which activation of cannabinoid receptors can reduce inflammation in pancreatic tissue (43). In tissues of chronic pancreatitis, cannabinoid receptors were detected predominantly in areas with inflammatory changes, stellate cells and nerves. Levels of endocannabinoids were decreased compared with normal pancreas. Cannabinoid-receptor-1 antagonism effectuated a small PSC phenotype and a trend toward increased invasiveness. Activation of cannabinoid receptors, however, induced de-activation of PSC and dose-dependently inhibited growth and decreased IL-6 and MCP-1 secretion as well as fibronectin, collagen1 and alphaSMA levels. De-activation of PSC was partially reversible using a combination of cannabinoid-receptor-1 and -2 antagonists. Concomitantly, cannabinoid receptor activation specifically decreased invasiveness of PSC, MMP-2 secretion (43). The authors hypothesize that this may thus constitute an option to treat inflammation and fibrosis in chronic pancreatitis.

Summary
Our study confirms previous findings showing CB1 and CB2-expression on acinar and tubular cells of the exocrine pancreas and furthermore confirms that in pancreatitis, an up-regulation of CB2 in acinar cells occurs which is most abundant on apoptotic cells. With the use of CB1-/- mice, a selective CB2-agonist and selective antagonists, we show for the first time that CB2 is crucially involved in mechanisms ameliorating acute pancreatitis and that activation of CB2 protects against experimental acute pancreatitis. Furthermore, we show that during acute pancreatitis
CB$_2$-activation by endogenous ligands leads to an amelioration of experimental pancreatitis, which can be prevented by selective antagonisation. Downstream of CB$_2$-activation, p38, JNK and additionally MK2 are involved in these protective mechanisms. We describe an MK2-dependent pathway mediating the CB$_2$-induced reduction of trypsinogen activation, IL-6 and MPO levels and intra-acinar JNK-Inhibition. Selective CB$_2$-agonists are not associated with central side effects as are CB$_1$-agonists, and could thus be useful future drugs for the treatment of pancreatitis in humans. Our study for the first time shows that CB$_2$-active drugs ameliorate acute pancreatitis and that p38, JNK and MK2 are involved in these mechanisms. In respect of the potential of CB$_2$-drugs for future use, the effects of CB$_2$-agonists on acute pancreatitis should be further developed for human use.

**Abbreviations used in this paper**

CB$_1$ Cannabinoid Receptor 1

CB$_2$ Cannabinoid Receptor 2

ECS endocannabinoid-system

eCB endocannabinoid

MPO myeloperoxidase

JNK c-jun n-terminal Kinase

MK2 MAPKAPK-2: Mitogen-Activated-Protein-Kinase-Activated-Protein-Kinase 2

MK2-/- MK2 knockout mice

p38 Mitogen-Activated-Protein-Kinase p38

SAPK Stress-Activated-Protein-Kinase
Figure legends

Fig. 1: Contribution of CB₁ and CB₂ to cannabinoid-mediated amelioration of acute pancreatitis

Expression of CB₁ and CB₂ was examined at mRNA levels by RT-PCR (A and B), and at protein levels by western blotting (C) and immunohistochemical stainings of paraffin embedded pancreatic tissue (D) as described in “Material and Methods”. RT-PCR (A): genomic DNA was used as positive (row 1) and water as negative control (row 11). Messenger-RNA encoding for CB₁ (upper panel) and CB₂ (middle panel) was clearly detectable in pancreatic probes of six different mice. CB₁ was furthermore detected with a strong signal in brain and with weak signal in spleen and heart while CB₂ was detected in spleen as well as in heart and brain but with weaker signals (row 2-4 of upper and middle panels). The lower panel shows a representative example for actin as a loading control. (B): RT-PCR for CB₁ mRNA from wild-type and CB1-/- mice: CB₁ mRNA was detected in pancreas from wild-type but not in pancreas from CB1-/- mice and also in colon, liver and kidneys of wild-type mice. (C) Western blotting: CB₂ was detectable with strong signals in pancreatitis tissue of wild-type and CB1-/- mice and also with a weaker signal in brain and spleen of wild-type mice. (D) Distribution of CB₁ and CB₂ was examined via immunohistochemical stainings of healthy pancreas (D, upper row) and pancreatitis tissue (D lower row). CB₁ and CB₂ were both detected in healthy tissue (middle and right picture on upper row) on tubular cells and acinar cells. While there was a stronger signal for CB₁ in healthy tissue (middle picture on upper row), after induction of pancreatitis, CB₁ expression remained on base level (middle picture on lower row) in contrast to CB₂, which was particularly up-regulated in apoptotic cells (right picture on lower row). Original magnification: 400x. (≥ area with high receptor density; * = apoptotic cell). (E-H) The effect of the non-selective CB₁/CB₂-agonist HU210 on parameters of acute pancreatitis was studied in wild-type and CB1-/- mice. Pancreatitis was induced via i.p. injections of cerulein and pancreatitis parameters
determined as described in Material and Methods. In wild-type mice, HU210 had an ameliorating effect on parameters of acute experimental pancreatitis (E-H, column 3 and 5). Activity of trypsin in pancreas homogenisat (E) and MPO in lung tissue (F) were significantly reduced in wild-type C57/BL6 mice. While IL-6 values in serum showed an insignificantly reduction (G), activity of amylase in serum remained almost unchanged (H). In mice lacking CB1, injections of cerulein also led to induction of acute pancreatitis with comparable severity to pancreatitis in wild-type mice. While most parameters were not significantly changed, MPO values were reduced in CB1/- (F). HU210 pre-treatment still improved acute pancreatitis in absence of CB1, indicating that the main effects of HU210 are mediated by CB2. As seen in wild-type mice, MPO activity was reduced significantly, trypsin activity and IL-6 values insignificantly and amylase activity remained unchanged (E-H). Figures represent data from at least three independent experiments, all samples were treated equally.

Fig. 2: Blockade of CB2 but not CB1 leads to engraved inflammation

HE and immunohistological stainings for CB1 and CB2 in animals pre-treated with a selective CB1 (A) or CB2-antagonist (B). As seen in Fig.1, CB1 was expressed on a constant level in healthy and pancreatitis tissue whereas CB2 was up-regulated in pancreatitis tissue especially on apoptotic cells (top row pictures of A&B). While CB1-expression remained unchanged by antagonisation with AM281 (bottom row, left picture of A), there was a slight up-regulation of CB2 seen in healthy tissue after antagonisation with AM630 (bottom row, left picture of B). Noteworthy, there was an increase of apoptotic acinar cells in pancreatitis animals pre-treated with AM630 and CB2 was strongly up-regulated especially on these cells (bottom row, right picture of B. (→= area with high receptor density; * = apoptotic cell). (C/D) Effekt of CB1 (C) or CB2-blockade (D) on JNK-phosphorylation in pancreatic lysates: Activation of JNK signaling pathway was investigated by western blotting of pancreatic lysates with phypo-specific antibodies (upper panels) and pan JNK antibodies (lower panels). Induction of pancreatitis with cerulein (row 3-8) lead to strong increases of JNK-phosphorylation compared to non-panreatitis animals (row 1&2). While pre-treatment with the CB1-selective antagonist AM281 had no effect on JNK-phosphorylation (C), pre-treatment with the CB2-selective antagonist AM630 lead to a mild increase of JNK-phosphorylation in non-pancreatitis animals (D, row 1&2), and to a drastic up-regulation in pancreatitis animals (D, row 3-8). (E-I) Effect of CB2-blockade on
biochemical parameters of acute pancreatitis: Degree of acute pancreatitis was determined by biochemical parameters for activation of trypsinogen in pancreatic acini (E), serum amylase activity (F), pancreatic edema (G), serum IL-6 levels (H) and activation of lung MPO (I) as described in “Material and Methods”. While IL-6 and MPO values were drastically and significantly increased (H/I), trypsin activity (E) and pancreatic edema (G) were insignificantly elevated while amylase activity was remained unchanged (F). Pooled data of at least 3 independent experiments are shown as mean +/- SEM (*p<0.05; #p<0.01).

**Fig. 3: The selective CB₂-agonist JWH133 ameliorates acute pancreatitis**

Effect of pre-treatment with the selective CB₂-agonist JWH133 on acute pancreatitis. Degree of acute pancreatitis was determined by biochemical parameters for activation of trypsinogen in pancreatic acini (A), serum amylase activity (B), pancreatic edema (C), serum IL-6 levels (D) and activation of lung MPO (E) as described in “Material and Methods”. Pre-treatment with the CB₂-selective agonist JWH133 led to decreased or abolished rises of pancreatitis parameters after cerulein injections. Activity of trypsinogen in pancreas was reduced by 92% (p<0.05) (A) and IL-6 values in serum by 69% (p<0.05) (D). Mean MPO activity was reduced by -106% (n.s.). Amylase activity in serum (B)(-8%; n.s.) and pancreas/body weight ratio (-20%; n.s.) (C) were unchanged or slightly reduced. Pooled data of at least 3 independent experiments are shown as mean +/- SEM (*p<0.05; #p<0.01). (F) Effect of JWH133 pre-treatment on pancreatic tissue was examined via histological stainings (HE). No effect was seen on normal pancreas without pancreatitis (1st & 2nd picture from left). In animals receiving cerulein only (3rd picture from left), typical signs of pancreatitis could be detected with signs of edema with a general disaggregation of pancreatic tissue and an immigration of immune cells and especially neutrophil granulocytes. In animals receiving JWH133 prior to cerulein injections (4th picture from left) the inflammatory signs observed in the cerulein-only group where ameliorated. Edema was reduced and most strikingly, far fewer granulocytes were visible in pancreatic tissue. (G/H) Activation of p38 signaling pathway was investigated by western blotting of pancreatic lysates with phospo-specific antibodies (G, upper panel) and pan p38 antibodies (G, lower panel). Pre-treatment with JWH133 led to increased p38-phosphorylation. In non-pancreatitis animals an intense increase was detectable. After induction of pancreatitis the effect was still detectable. A representative western
Degree of acute pancreatitis was determined by biochemical parameters for activation of trypsinogen in pancreatic acini (A), serum amylase activity (B), pancreatic edema (C), serum IL-6 levels (D) and activation of lung MPO (E) as described in “Material and Methods”. Left 3 columns show control groups without cerulein treatment, right 3 columns pancreatitis animals treated with cerulein.

Previous administration of AM630 efficiently prevented all protective effects of JWH133 pre-treatment on parameters for pancreatitis. Pooled data of at least 3 independent experiments are shown as mean +/- SEM. (*p<0.05; #p<0.01).

Fig. 4: The protective effect of JWH133 can be abolished by pre-treatment with a selective CB2-antagonist

Fig. 5: Protective effect of CB2-stimulation is dependent on presence of MK2

Pancreatitis was induced in wild-type and MK2-/- mice pre-treated with JWH133. Degree of acute pancreatitis was determined by biochemical parameters for activation of trypsinogen in pancreatic acini (A), serum amylase activity (B), pancreatic edema (C), serum IL-6 levels (D) and activation of lung MPO (E) as described in material and methods. Left columns show values in wild-type, right columns corresponding values in MK2-/- mice. Cerulein injections led to induction of mild acute pancreatitis in MK2-/- mice with increases of all measured parameters. The attenuation of all parameters by JWH133 pre-treatment in wild-type mice is completely abolished in MK2-/- mice. General inflammatory parameters reacted nearly opposite in MK2-/- as in wild-type and were increased instead of decreased. Pooled data of at least 3 independent experiments are shown as mean +/- SEM. (*p<0.05; #p<0.01). (F/G) Effect of CB2-stimulation on JNK-activation in wild-type and MK2-/- mice: Activation of JNK signalling pathway was investigated by western blotting of pancreatic lysates with phospo-specific antibodies (F, 1st & 3rd panels) and pan JNK antibodies (F, 2nd & 4th panels). In contrast to wild-type mice (2 lower panels), in MK2-/- mice (2 upper panels), pre-treatment with JWH133 led to an increased JNK-activation in pancreatitis animals corresponding to increased rises of biochemical parameters. In non-pancreatitis animals, no effect on JNK-activation was observed. A representative western blot of at least 3 independent experiments is shown in the left panel (F), the quantitated data of all test animals are shown as
mean +/- SEM in the right panel (G). The left columns show value in wild-type, right columns values in MK2-/- mice (*p<0.05; #p<0.01).

Appendix

See attached files

Disclosure Statement

Data are part of the medical thesis of Thomas Michler (http://edoc.ub.uni-muenchen.de/11993/). The authors have no conflicts of interest to declare.

References


Fig. 1

A) α-Amylase activity in serum

B) MPO activity in lung

C) IL-6 in serum

D) HE

E) Trypsin activity in pancreas

F) MPO activity in lung

G) IL-6 in serum

H) α-Amylase activity in serum
**Fig. 2**

**A**

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**E**

E: Trypsin activity in pancreas

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**F**

F: α-Amylase activity in serum

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**G**

G: Pancreas/body weight ratio

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**H**

H: IL-6 in serum

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**I**

I: MPO activity in lung

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Fig. 3

A. Trypsin activity in pancreas

B. α-Amylase activity in serum

C. Pancreas/body weight ratio

D. IL-6 in serum

E. MPO activity in lung

F. NaCl, JWH133, Cerulein, JWH133+Cerulein

G. Phospho-p38 and p38

H. Quantification of western blot signal
Fig. 4

A. Trypsin activity in pancreas
- AM630 - - + - - +
- JWH133 - + + - + +

B. α-Amylase activity in serum
- AM630 - - + - - +
- JWH133 - + + - + +

C. Pancreas/body weight ratio
- AM630 - - + - - +
- JWH133 - + + - + +

D. IL-6 in serum
- AM630 - - + - - +
- JWH133 - + + - + +

E. MPO activity in lung
- AM630 - - + - - +
- JWH133 - + + - + +

Legend:
- non-pancreatitis
- pancreatitis
Fig. 5

A. Activated trypsin in pancreas

B. α-Amylase activity in serum

C. Pancreas/body weight ratio

D. IL-6 in serum

E. MPO activity in lung

F. Quantification of western blot signal

G. Phospho-JNK

Wild-type

MK2 -/-