MMP-065

New Jersey Department of Health
Medicinal Marijuana Program
PO 360
Trenton, NJ 08625-0360

MEDICINAL MARIJUANA PETITION
(N.J.A.C. 8:64-5.1 et seq.)

INSTRUCTIONS

This petition form is to be used only for requesting approval of an additional medical condition or treatment thereof as a "debilitating medical condition" pursuant to the New Jersey Compassionate Use Medical Marijuana Act, N.J.S.A. 24:6I-3. Only one condition or treatment may be identified per petition form. For additional conditions or treatments, a separate petition form must be submitted.

NOTE: This Petition form tracks the requirements of N.J.A.C. 8:64-5.3. Note that if a petition does not contain all information required by N.J.A.C. 8:64-5.3, the Department will deny the petition and return it to petitioner without further review. For that reason the Department strongly encourages use of the Petition form.

*This completed petition **must** be postmarked **August 1 through August 31, 2016** and sent by **certified mail** to:*

New Jersey Department of Health
Office of Commissioner - Medicinal Marijuana Program
Attention: Michele Stark
369 South Warren Street
Trenton, NJ 08608

Please complete each section of this petition. If there are any supportive documents attached to this petition, you should reference those documents in the text of the petition. If you need additional space for any item, please use a separate piece of paper, number the item accordingly, and attach it to the petition.

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1. **Petitioner Information**

Name: [redacted]
Street Address: [redacted]
City, State, Zip [redacted]
Telephone Nu[redacted]
Email Address: [redacted]

2. **Identify the medical condition or treatment thereof proposed. Please be specific. Do not submit broad categories (such as "mental illness").**

Chronic Acute Pancreatitis

3. **Do you wish to address the Medical Marijuana Review Panel regarding your petition?**

- ☐ Yes, in Person
- ☒ Yes, by Telephone
- ☐ No

4. **Do you request that your personally identifiable information or health information remain confidential?**

- ☒ Yes Yes
- ☒ No

If you answer "Yes" to Question 4, your name, address, phone number, and email, as well as any medical or health information specific to you, will be redacted from the petition before forwarding to the panel for review.

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OFFICE OF THE CHIEF OF STAFF

5. **Describe the extent to which the condition is generally accepted by the medical community and other experts as a valid, existing medical condition.**

Acute Pancreatitis is an inflamatory Condition of the Pancreas. It is very painful and at times deadly.

6. **If one or more treatments of the condition, rather than the condition itself, are alleged to be the cause of the patient's suffering, describe the extent to which the treatments causing suffering are generally accepted by the medical community and other experts as valid treatments for the condition.**

Treatments such as The Whipple procedure and a Pancreasetomy Can Cause Complications

7. **Describe the extent to which the condition itself and/or the treatments thereof cause severe suffering, such as severe and/or chronic pain, severe nausea and/or vomiting or otherwise severely impair the patient's ability to carry on activities of daily living.**

Debilitating Chronic Pain
Nausea
Vomiting
loss of Apetite
loss of Weight

8. **Describe the availability of conventional medical therapies other than those that cause suffering to alleviate suffering caused by the condition and/or the treatment thereof.**

Narcotics for Pain
Enzymes to help to digest food
The Whipple Procedure and Pancreasectomy, Both Complicated Procedures to help treat.

9. **Describe the extent to which evidence that is generally accepted among the medical community and other experts supports a finding that the use of marijuana alleviates suffering caused by the condition and/or the treatment thereof.** *[**Note:** You may attach articles published in peer-reviewed scientific journals reporting the results of research on the effects of marijuana on the medical condition or treatment of the condition and supporting why the medical condition should be added to the list of debilitating medical conditions.]*

Due to Restrictions on Marijuana not Many Studies are found with humans. But test on Animals show that it Can help Maintain the Remaining pancreas. It Can also Enclosed are studies along with a note stating that a Surgeon in the field that has Seen patients who have benefited other patients in other States.

10. **Attach letters of support from physicians or other licensed health care professionals knowledgeable about the condition. List below the number of letters attached and identify the authors.**

1. Dr. Beth Schnope

I certify, under penalty of perjury, that I am 18 years of age or older; that the information provided in this petition is true and accurate to the best of my knowledge; and that the attached documents are authentic.

Signature of Petitioner [redacted]	Date 8/30/16

College of Physicians & Surgeons of Columbia University | *New York, N.Y. 10032*

BETH A. SCHROPE, MD, PHD, FACS
161 FORT WASHINGTON AVE., 810
NEW YORK, NY 10032
ASSOCIATE PROFESSOR, DIVISION OF GI/ENDOCRINE SURGERY
DEPARTMENT OF SURGERY

Phone: (212) 305-9441
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August 24, 2016

To Whom It May Concern:

[redacted] is currently under my care for treatment of chronic pancreatitis (due to a condition of her pancreatic duct known as pancreas divisum).

Unfortunately this is a frustrating, unrelenting and often escalating chronic disease. Treatment options are limited to managing the symptoms, which most often are pain and inability to eat. Pain exacerbations are often prompted by dietary indiscretion but can also be provoked by increased stress. [redacted] has tried numerous treatment options including various pain medication strategies, as well as pancreatic enzyme supplementation, without relief and indeed with escalation of symptoms over the past few months that I have known her.

We are planning a surgical procedure for [redacted] in the near future. Until that time and indeed even after successful surgery I anticipate that pain management will be a challenge. I have seen anecdotal success in a few of my patients with this diagnosis with the use of marijuana. It reduces their use and dosages of opiate pain medications, and likely makes it more feasible to withdraw safely from these medications after surgery.

Please do not hesitate to contact me for any questions or concerns.

Sincerely,

Beth A. Schrope, MD, PhD
Associate Professor of Surgery
Director, Pancreatic Autologous Islet Cell Transplantation Program
Columbia University College of Physicians and Surgeons

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Cannabinoids Ameliorate Pain and Reduce Disease Pathology in Cerulein-Induced Acute Pancreatitis

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Abstract

Background & Aims—The functional involvement of the endocannabinoid system in modulation of pancreatic inflammation, such as acute pancreatitis, has not been studied to date. Moreover, the therapeutic potential of cannabinoids in pancreatitis has not been addressed.

Methods—We quantified endocannabinoid levels and expression of cannabinoid receptors 1 and 2 (CB1 and CB2) in pancreas biopsies from patients and mice with acute pancreatitis. Functional studies were performed in mice using pharmacological interventions. Histological examination, serological, and molecular analyses (lipase, myeloperoxidase, cytokines, and chemokines) were performed to assess disease pathology and inflammation. Pain resulting from pancreatitis was studied as abdominal hypersensitivity to punctate von Frey stimuli. Behavioral analyses in the open-field, light-dark, and catalepsy tests were performed to judge cannabinoid-induced central side effects.

Results—Patients with acute pancreatitis showed an up-regulation of cannabinoid receptors and elevated levels of endocannabinoids in the pancreas. HU210, a synthetic agonist at CB1 and CB2, abolished abdominal pain associated with pancreatitis and also reduced inflammation and decreased tissue pathology in mice without producing central, adverse effects. Antagonists at CB1- and CB2-receptors were effective in reversing HU210-induced antinociception, whereas a combination of CB1- and CB2-antagonists was required to block the anti-inflammatory effects of HU210 in pancreatitis.

Conclusions—In humans, acute pancreatitis is associated with up-regulation of ligands as well as receptors of the endocannabinoid system in the pancreas. Furthermore, our results suggest a therapeutic potential for cannabinoids in abolishing pain associated with acute pancreatitis and in partially reducing inflammation and disease pathology in the absence of adverse side effects.

Acute pancreatitis (AP) is a potentially lethal disorder involving inflammation, cell death, and complex neuroimmune interactions. Pain management in acute pancreatitis represents a major clinical challenge and influences the clinical outcome of the disease.[1,2] Understanding pathophysiological mechanisms underlying morbidity and pain associated with pancreatitis is therefore a prerequisite toward the design of novel therapeutic approaches. Only very recently, aided by the establishment of disease models, focus has been placed on understanding

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C.W.M. and T.L. contributed equally to this work.

nociceptive mechanisms in pancreatitis.[1–5] For example, abdominal hyperalgesia observed upon cerulein-induced murine pancreatitis was recently found to closely represent the pain syndrome seen in human disease.[3–5] Involvement of peripheral nociceptive neurons[5] as well as central neurons in the spinal cord[6] and rostroventral medulla[3] has been demonstrated in pain associated with pancreatitis. In addition to mediating and modulating pancreatic pain, neural innervation of the pancreas plays an important role in edema, inflammatory responses, and vascular processes in pancreatitis and significantly affects disease initiation and progression.[6]

Recently, the endocannabinoid system has been identified as a major regulator of physiological and pathological processes, such as pain, inflammation, cell growth, cell death, and as a regulator of diverse gastrointestinal functions, such as intestinal motility and secretion.[7] Although cannabinoid-induced analgesia was initially primarily attributed to the activation of cannabinoid receptor 1 (CB1) in the nervous system, later studies demonstrated a contribution of cannabinoid receptor 2 (CB2), localized peripherally on immune cells as well as in the nervous system.[7–9] A complex interplay between endogenously released cannabinoids, such as anandamide or 2-arachidonoylglycerol, and their receptors both on inflammatory cells and neurons is involved in modulation of inflammatory pain.[7] However, the significance of the endocannabinoid system and of exogenously applied cannabinoids has not been adequately addressed in the context of visceral inflammatory pain. In this article, we demonstrate the in vivo significance and therapeutic potential of cannabinoids in inflammation and pain associated with pancreatitis using human specimens and mouse models as test systems.

Materials and Methods

Patients and Tissue Sampling

Tissue samples were collected from patients following pancreatic resections for acute pancreatitis (n = 19). Normal pancreatic tissue samples were obtained through an organ donor procurement program whenever there was no suitable recipient for pancreas transplantation (n = 10). Pancreatic tissues were immediately snap frozen at −80°C or formalin fixed and paraffin embedded. Local ethical committees approved the use of human tissue for the analysis (University of Heidelberg, Germany and the University of Berne, Switzerland), and written informed consent was obtained.

Measurement of Endocannabinoids

For endocannabinoid measurements, pancreatic tissue specimens from acute pancreatitis were dissected, and necrotic parts were removed prior to analysis. Levels of anandamide (AEA), 1-arachidonoylglycerol (1-AG), and 2-arachidonoylglycerol (2-AG) in frozen human pancreas samples were determined by liquid chromatography/mass spectrometry as described previously.[10]

Cerulein-Model for Pancreatitis and Pharmacological Treatment

All animal procedures were performed according to local ethical guidelines. Cerulein (50 μg/kg/injection in saline (Takus, Pharmacia, Karlsruhe, Germany) or saline (control) was administered intraperitoneally 10 times at hourly intervals to age- and gender-matched C57BL/6J mice. The synthetic cannabinoid, HU210 (0.05 mg/kg in saline), the CB1-specific antagonist (AM251; 3 mg/kg intraperitoneally), and the CB2-specific antagonist (AM630; 1 mg/kg) (all from Tocris Bioscience, Ellisville, MD) were administered subcutaneously[11] either alone or in combinations (information to follow) at 30 minutes before and 4 hours after the first injection of cerulein or saline. AM251 and AM630 were dissolved in one drop of Tween-20 (Roth, Karlsruhe, Germany) in 3 mL 2.5% di-methylsulfoxide in saline,[11] and control animals were given the vehicle only. In total, 10 groups of animals were used: (1) intraperitoneal saline

(control, n = 8); (2) intraperitoneal saline + subcutaneous HU210 (n = 6); (3) intraperitoneal cerulein (n = 8); (4) intraperitoneal cerulein + subcutaneous HU210 (n = 8); (5) intraperitoneal cerulein + subcutaneous HU210 + subcutaneous AM251 (n = 8); (6) cerulein + subcutaneous HU210 + subcutaneous AM630 (n = 6); (7) cerulein + subcutaneous HU210 + subcutaneous AM251 + subcutaneous AM630 (n = 8); (8) cerulein + subcutaneous AM251 (n = 8); (9) cerulein + subcutaneous AM630 (n = 8); and (10) cerulein + subcutaneous AM251 + subcutaneous AM630 (n = 8).

Behavioral Testing

Behavioral measurements were carried out in awake, unrestrained mice in a blinded manner using standardized methods.[12] Frequency of abdominal nocifensive responses (licking of the abdomen, abdominal and/or whole-body withdrawal) to graded punctate abdominal pressure was analyzed using von Frey filaments (0.008*g* to 0.6*g*). Withdrawal frequency was calculated as the mean number of withdrawals out of 10 applications of the respective filament at 10-second intervals. As described previously, potential central side effects of HU210 were evaluated in the open-field test,[12] light/dark test,[12] and catalepsy tests.[13]

Immunohistochemical Analysis and Immunoblotting

Immunohistochemistry on paraffin sections of human pancreas was performed using rabbit anti-CB1 and anti-CB2 antibodies (Cayman Chemical, Ann Arbor, MI) as described previously.[14] Specificity controls included preadsorption of the primary antibody (1 μg) with the corresponding blocking peptide (1 μg) for 1 hour at 37°C. Immunoblotting of normal human pancreas and acute pancreatitis tissue lysates was performed using rabbit anti-CB1 and anti-CB2 antibodies (2.5 μg/mL and 2.0 μg/mL, respectively) ± corresponding blocking peptide preincubated for 1 hour at 37°C,– as described previously.[14]

Histology Scoring, Serum Lipase, Pancreatic Myeloperoxidase, Interleukin-6, and KC1 Levels

Following behavioral analyses, mice were anesthetized, and mixed arteriovenous blood and pancreata were collected. The wet weight/dry weight ratio of mouse pancreata was determined as described previously.[15,16] Whole blood glucose levels were determined using an Accucheckcomfort device (Roche, Mannheim, Germany). Serum lipase levels were measured in the central clinical laboratories (Heidelberg University) according to locally defined guidelines. Histological grading was performed on hematoxylin-eosin–stained paraffin sections (5 μm) by a clinical pathologist unaware of the experimental design using modified criteria, which have been described in detail previously.[6] Total histological score (0 –3) was expressed as the sum of edema, neutrophil infiltration, and tissue necrosis scores. Myeloperoxidase (MPO) and interleukin-6 (IL-6) levels were quantified using a mouse MPO-ELISA kit or a mouse IL-6 ELISA kit on tissue homogenates according to the manufacturers' instructions (HyCult Biotechnology, Uden, the Netherlands or BD Biosciences, San Diego, CA). Total RNA was isolated using an RNeasy mini kit and replacing RLT buffer with Trizol (Qiagen, Hilden, Germany). Quantitative real-time reverse-transcription polymerase chain reaction was performed as described previously.[14] Mouse KC1 mRNA was amplified by using the primers sense: 5′-TAG TAG AAG GGT GTT GTG CGA AA-3′and antisense: 5′-CGA GCG AGA CGA GAC CAG GAG-3′.

Statistical Analysis

Results are expressed as mean ± SEM. The Mann–Whitney *U* test and analysis of variance for random measures followed by Bonferroni's post hoc test were used to evaluate statistical significance. Significance was established at $P < .05$.

Results

Regulation of CB1, CB2, and Endocannabinoids in Human Acute Pancreatitis

Immunohistochemical analyses of normal human pancreata revealed weak immunoreactivity for CB1 and CB2 in pancreatic acini (*arrowheads*), nerves (*insets*), blood vessels, and duct cells (*arrows*) (Figure 1*A* and *G*). Vital pancreatic parenchyma in acute pancreatitis specimens displayed a striking increase in immunostaining of CB1 in ducts and a moderate increase in nerves (*insets*) and acini as compared to normal pancreas (Figure 1*B*), whereas acinar and ductal CB2 appeared to be increased to a lesser extent than CB1 (Figure 1*H*). Acinar cells in the vicinity of severe necrosis in human acute pancreatitis specimens revealed intense cytoplasmic CB1 and CB2 immunoreactivity (Figure 1*C* and I; magnified in respective insets). Furthermore, acinar cell necrosis in cerulein-induced acute pancreatitis showed a strong immunoreactivity for both CB1 and CB2 (*dotted arrows*, Figure 1*F* and *L*).

To evaluate significance of the cannabinoid system in acute pancreatitis, we used the well-established mouse model of cerulein-induced acute pancreatitis.[6] Importantly, following cerulein-induced pancreatitis, the mouse pancreas demonstrated similar changes in CB1 and CB2 immunoreactivity (Figure 1*D–F* and *J–L*), as described in the preceding paragraph for the human disease. Following preadsorption with the corresponding blocking peptide, antibodies against CB1 or CB2 failed to show any appreciable staining on human or mouse pancreas (Supplementary Figure 1). (See supplementary figure online at www.gastrojournal.org.)

Western blot analysis on human pancreas using the anti-CB1 antibody revealed a prominent up-regulation of a 125-kDa band (which was abolished in preadsorption controls) in acute pancreatitis (Figure 1*M*), thereby corroborating our observations from immunohistochemistry experiments. Furthermore, Western blotting using the anti-CB2 antibody confirmed a weak-to-moderate up-regulation of approximately 28 kDa and 36 kDa bands corresponding to CB2 (which were abolished in preadsorption controls) in acute pancreatitis (Figure 1*N*).

We then addressed whether levels of endocannabinoids are up-regulated in pancreatitis concomitant with changes in expression of cannabinoid receptors. Several endocannabinoids, such as anandamide (AEA), 1- and 2-arachidonoylglycerol (1-AG, 2-AG) were detectable in human pancreas tissues, whereas oleoylethanolamide was not detectable (Figure 1*O*). Interestingly, anandamide was increased 6.6-fold in biopsy material from patients with acute pancreatitis over normal human pancreas ($P = .002$). In contrast, levels of 1-AG and 2-AG remained unchanged (Figure 1*O*; $P = .93$).

Functional Significance of the Endocannabinoid System in Acute Pancreatitis

To evaluate the potential functional relevance of increased endocannabinoid release and up-regulation of cannabinoid receptors in human disease, we performed experiments with CB1/CB2 antagonists in the cerulein model of acute pancreatitis in mice. Consistent with previous reports,[6,17] treatment of mice with cerulein led to a steep increase in serum levels of lipase, tissue MPO, IL-6, and KC1 mRNA expression (Figure 2*A* and *B*), pancreatic wet weight/dry weight ratio (Figure 2*A*), and to characteristic histological changes associated with acute pancreatitis (Figure 1*E* and *K*). AM251 and AM630 were used as specific antagonists to block CB1 and CB2, respectively. Neither disease pathology nor serological and inflammatory markers (serum lipase, pancreatic tissue MPO and IL-6 levels, wet/dry weight ratio) were affected by treatment with CB receptor antagonists (Figure 2*A* and *B*). However, with combined application of AM251 and AM630, there was a trend for a further increase in serum lipase levels and pancreatic MPO levels, which did not reach statistical significance (Figure 2*A*).

Surprisingly, administration of AM630 alone or in combination with AM251 reduced KC1 mRNA levels ($P < .05$; Figure 2*B*).

Because disease pathology was not significantly worsened by application of CB1/CB2 antagonists, we addressed effects on pancreatitis-induced pain. Abdominal pain is a cardinal feature of acute pancreatitis. Here, we assessed pancreatitis-induced pain by studying nocifensive reactions to acute, punctuate stimuli applied to the abdomen of mice. Mice with acute pancreatitis demonstrated a significant increase in the response frequency to forces of 0.008*g*, 0.02*g*, 0.04*g*, or 0.07*g* ($P < .01$, respectively; Figure 3*A*). These results show that acute pancreatitis is accompanied by marked mechanical hyperalgesia and allodynia. When cannabinoid receptor antagonists were administered to animals with acute pancreatitis, the frequency of withdrawal was increased in response to forces of 0.008*g*, 0.07*g*, and 0.16*g* for AM251 alone ($P < .05$; Figure 3*B*), 0.07g and 0.16g for AM630 alone ($P < .01$; Figure 3*C*), or 0.008*g*, 0.07*g*, and 0.16*g* for AM251 in combination with AM630 ($P < .01$; Figure 3*D*). These results suggest that the endocannabinoid system tonically inhibits pain associated with pancreatitis.

Cannabinoid Treatment Reduces Inflammation and Disease Pathology in Acute Pancreatitis

We then addressed whether an augmentation of the endocannabinoid system via exogenously applied cannabinoids could be beneficial in acute pancreatitis. Cannabinoid receptors were activated by treatment with HU210, a synthetic agonist at CB1 and CB2.[11] Intraperitoneal application of HU210 led to a reduction of the levels of various serological and inflammatory markers, such as serum lipase ($P < .015$; Figure 4*A*), pancreatic MPO ($P = .001$; Figure 4*A*), pancreatic IL-6 levels ($P = .02$; Figure 4*B*), as well as pancreatic KC1 mRNA ($P = .08$; Figure 4*B*). Furthermore, HU210 significantly reduced the pancreatic edema as judged by pancreas wet weight/dry weight ratio ($P = .007$; Figure 4*A*). Pancreatitis-associated tissue histopathology was ameliorated with HU210, with a 40% reduction in overall histology score of edema, neutrophil infiltration, and acinar cell necrosis ($P < .05$; data not shown). The incidence of acinar cell necrosis was lower in HU210-treated mice (0% to 0.5% overall necrosis) than in the saline-treated group with acute pancreatitis (2% overall necrosis). To assess whether pancreatitis or cannabinoids impact upon glucose metabolism, we assessed serum glucose levels in mice with acute pancreatitis. Surprisingly, glucose levels were significantly lower in animals with cerulein-treated mice compared with saline-treated mice ($P = .0002$, data not shown). However, glucose levels remained unaffected by HU210 treatment in these mice ($P = .38$; data not shown).

The Anti-Inflammatory Effects of HU210 in Pancreatitis Are Mediated by CB1 and CB2

We then performed pharmacological interventions in order to study mechanisms mediating the beneficial effects of HU210 on inflammation associated with pancreatitis. Treatment with either AM251 or AM630 did not significantly alter the anti-inflammatory effects of HU210, as judged by serum levels of lipase, pancreatic MPO, IL-6, and KC1 mRNA as well as the pancreatic wet/dry weight ratio (Figure 4*A* and *B*). In contrast, a combination of AM251 and AM630 fully reversed the beneficial effects of HU210 on pancreatitis-induced inflammatory changes as judged by increased serum lipase, pancreatic MPO, and IL-6 levels ($P < .05$, respectively; Figure 4*A* and *B*). Both AM251 and AM630 worsened the pancreatitis histology score by 20% and 24%, respectively, when compared with animals receiving HU210 alone.

CB1 and CB2 Mediate Cannabinoid-Induced Analgesia in Pancreatitis

One of the most important aims of this study was to test whether treatment with exogenous cannabinoids can alleviate pain associated with pancreatitis. After cerulein administration, mice treated with HU210 developed hyperalgesia and allodynia to abdominal pressure to a much lesser extent as compared to mice with mock treatment. At all pressures tested (from

0.008*g* to 0.6*g*), withdrawal latencies were significantly decreased in the presence of HU210 (Figure 5*A*; $P < .001$). Administration of HU210 to control mice in the absence of pancreatitis did not affect nociceptive responses (Figure 5*B*; $P > .05$).

Furthermore, we observed that administration of either AM251 or AM630 concurrently with HU210 significantly reduced the beneficial effects of HU210 on pancreatitis-associated abdominal hypersensitivity (Figure 5*C* and *D*; $P < .05$). Similar effects were seen with a combination of AM251 and AM630 together with HU210 (Figure 5*E*; $P < .01$). These data indicate that both CB1 and CB2 are required for the antinociceptive effects of HU210 in pancreatitis.

Absence of Central Side Effects on CB Receptor Antagonist and HU210 Treatment in Acute Pancreatitis

Side effects such as sedation, psychotropic effects, and motor dysfunction can limit the use of cannabinoids to alleviate pain. It was therefore important to ensure that these parameters do not confound our analysis of the antinociceptive effects of HU210 and were not influenced by CB receptor antagonist treatment. Mice with acute pancreatitis did not demonstrate any overall impairment of locomotor functions following HU210 administration, as judged via vertical and horizontal activity in the open field test compared with mice receiving saline injections (Figure 6*A*). Furthermore, time spent in the center of the open field (Figure 6*A*) and grooming (Figure 6*B*) remained unchanged upon HU210 treatment, suggesting that, at the dose used here, HU210 did not affect anxiety levels in mice, which was further confirmed in the light-dark test (Figure 6*B*). Similarly, combined administration of HU210 with antagonists at CB1 or CB2 did not have any effect on motor performance or affective behavior (Figure 6*A* and *B*). Furthermore, administration of AM251 and AM630 did not significantly alter behavior in these tests (Supplementary Figure 2*A* and *B*) (See supplementary figures online at www.gastrojournal.org.). Catalepsy is another classical behavior associated with cannabinoids, which is believed to be mediated by receptors in the central nervous system.[13] In the ring-catalepsy test, we observed that the dose of HU210 used in this study failed to induce freezing behavior in mice (Figure 6*C*). Taken together, these results show that the dose of HU210, which abrogated pancreatitis-induced abdominal pain in our mouse model, did not produce side effects such as catalepsy, inhibition of activity, impaired ambulation, or psychotropic effects.

Discussion

The most important finding of this study is that activation of cannabinoid receptors is beneficial against abdominal pain as well as disease pathology of cerulein-induced acute pancreatitis. Pain management is a key cornerstone in the conventional therapy of acute pancreatitis and still represents a major clinical challenge. In-sufficient pain control results in high respiratory rates, reduced lung function, and inadequate ingestion, factors that significantly delay recovery and may worsen the course of the disease.[1,2] The use of opioids as a standard therapy regimen is effective but frequently results in severe and difficult-to-handle side effects, such as nausea, vomiting, decreased gastrointestinal motility, and adynamia, which overlap with the symptoms of pancreatitis.[2] Nonsteroidal analgesics lack these side effects but do not provide adequate pain relief. We demonstrate here that a cannabinoid completely blocked abdominal pain in a state of cerulein-induced acute pancreatitis. Importantly, this beneficial effect was elicited following systemic delivery of HU210 at a low dose lacking central side effects, suggesting that HU210 abolished pancreatitis-induced abdominal pain via peripheral mechanisms. Consistent with this appraisal, we could demonstrate that cannabinoid-induced analgesia was not accompanied by effects such as hindrance of activity, motor dysfunction, sedation, or

catalepsy, which are typically associated with activation of cannabinoid receptors in the central nervous system.

Because lower-abdominal tactile hypersensitivity after pancreatic inflammation essentially represents secondary hyperalgesia and allodynia (phenomena that require sensitization of central synapses), the antinociceptive effects we observed likely occur as a result of cannabinoid-induced decrease in nociceptor excitability and consequently, reduced central sensitization. Consistent with peripheral antinociceptive effects of cannabinoids, CB1 localized on peripheral nociceptive endings has been shown to negatively modulate excitability of nociceptors and inhibit release of peptides,[18] thereby countering neurogenic inflammation. Interestingly, neurogenic inflammation has been postulated to be a key mechanism in the pathophysiology of acute pancreatitis.[19] In addition to CB1, we also observed a CB2-component to the antinociceptive effects of HU210 in cerulein-induced pancreatitis. Although a functional contribution of CB2 expressed on nerves is possible, the anti-inflammatory effects of CB2 could also indirectly account for its antinociceptive role. Our anatomical data from human specimens reveal the presence and up-regulation of both CB1 and CB2 in neural, as well as non-neural cells in the pancreas. Future studies are therefore required to pinpoint the precise loci and mechanisms of cannabinoid-induced anti-nociception in acute pancreatitis.

Another key feature of acute pancreatitis is local inflammation resulting from acinar cell injury, which, if significant and unchecked, can lead to a systemic inflammatory response syndrome, thereby initiating a potentially fatal stage of the disease.[20] The probable benefits of anti-inflammatory therapies in acute pancreatitis have been hypothesized previously.[20] In this study, we found that a low dose of HU210, which is devoid of central side effects, does exert moderate anti-inflammatory effects in the pancreas and partially reduces cerulein-induced disease pathology. This finding is consistent with a recent study[11] demonstrating that HU210 exerts local anti-inflammatory effects in a model of experimental colitis in mice, suggesting that cannabinoids are generally beneficial in visceral inflammatory disorders. Whereas CB1 receptors were found to play a key role in the study by Massa et al, we observed that the moderate anti-inflammatory effects achieved via low-dose HU210 in acute pancreatitis largely require both CB1 and CB2 activation. Another interesting aspect of the present study is the finding that anandamide as well as cannabinoid receptors are up-regulated in human pancreatitis. We observed that blockade of CB1 and CB2 led to a significant worsening of pancreatitis-induced pain but produced only a nonsignificant trend toward exacerbation of cerulein-induced disease pathology. These data suggest that in our experimental conditions, an induction of the endocannabinoid system during acute pancreatitis primarily represents an endogenous protective mechanism against pancreatic pain.

Recently, Matsuda et al[21] reported prolonged survival in rats upon AM251 treatment in a model of tauro-cholate-induced necrotizing pancreatitis. Because effects of cannabinoid agonists were not reported in the study by Matsuda et al and cerulein-induced pancreatitis in mice is far less severe, non-necrotizing, and does not lead to animal death, it is difficult to directly compare the outcome of the study by Matsuda et al[21] with the results described in this paper. Our results are more in line with a recent study reporting a protective role for the endogenous cannabinoid system against colonic inflammation in a mouse model of experimental colitis.[9,11,22] Consistent with the above, we now show that acute pancreatitis, a visceral inflammatory disease in humans, is associated with an activation of the endocannabinoid system. Because management of visceral inflammatory diseases should ideally include antinociceptive[23] as well as anti-inflammatory components, our results lay a basis for testing the therapeutic value of cannabinoids as supplements to conventional analgesic therapy.

Acknowledgements

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Abbreviations used in this paper

1-/2-AG
1-/2-arachidonoylglycerol

AEA
anandamide

AP
acute pancreatitis

CB1
cannabinoid receptor 1

CB2
cannabinoid receptor 2

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Appendix

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at zdoi: 10.1053/j.gastro.2007.02.035.

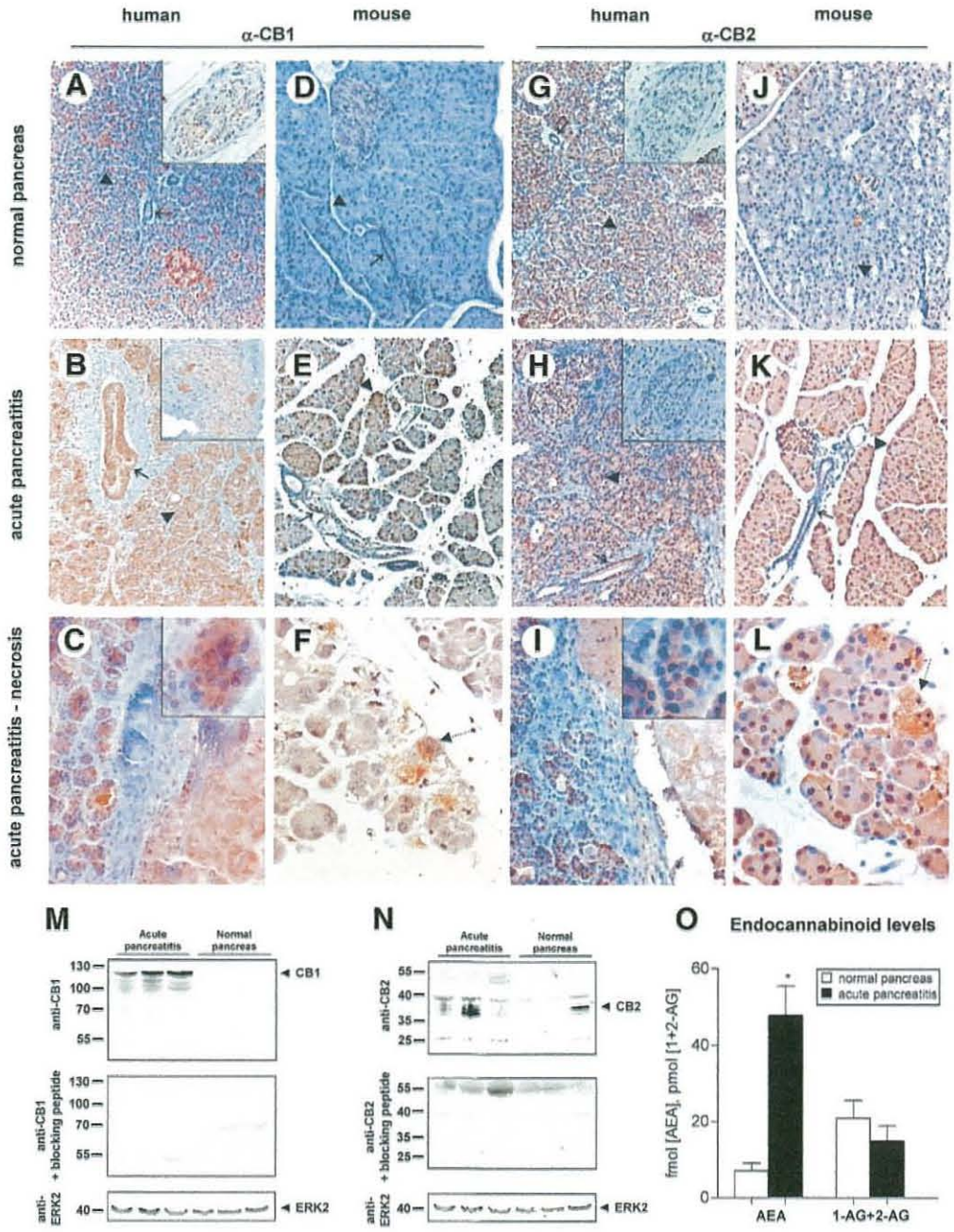

Figure 1.

Induction of the endocannabinoid system in acute pancreatitis. Immunohistochemical detection of CB1 and CB2 is shown in human pancreas (*A–C* and *G–I*) or mouse pancreas (*D–F* and *J–L*) derived either from control subjects (*A*, *D*, *G*, and J) or subjects with acute pancreatitis (*B* and *C*, *E* and *F*, *H* and *I*, *K* and *L*). (*C*, *F*, *I* and *L*) Areas of necrosis within human and mouse acute pancreatitis are indicated by *dotted lines*. In normal human pancreas (*A*) CB1-immunoreactivity in acinar cells (▶; magnified in *inset C*), ducts (→), and nerves (magnified in insets), was weak but strong in acute pancreatitis (*B*, *C*). (*D–F*) Similarly, pancreas demonstrated increased immunoreactivity for CB1 after induction of acute pancreatitis with cerulein (*E*, *F*) over normal mouse pancreas (*D*). Intense CB1 immunostaining is observed on

acinar cell necrosis (*dotted arrow; F*). (*G–I*) Moderate staining for CB2 is seen in normal human pancreas (*G*) with a slight increase in acute pancreatitis (*H, I*) in acinar cells (▶; magnified in *inset I*), ducts (→), and nerves (magnified in *insets*). Upon induction of acute pancreatitis in mice, there is a pronounced increase in CB2 immunoreactivity over expression levels in normal mouse pancreas (*J–L*). This is particularly present within acinar cell necrosis (*dotted arrow; L*). Original magnification: 40x (*A* and *B*, *D* and *E*, *G* and *H*, *J* and *K*) or 80x (*F* and *L; insets* in *A–C* and *G–I*) objective. (*M, N*) Immunoblot analysis of pancreas samples derived from acute pancreatitis (*lanes* 1–3) and control human donors (*lanes* 4–6) with antibodies recognizing CB1 (*M*) or CB2 (*N*). (▶) indicates anti-CB1 (at approximately 128 kDa) and anti-CB2-immunoreactive bands (at 38 and 26 kDa), which were abolished by preadsorption with the respective blocking peptide (*lower lanes*). Anti-ERK2 was used as an equal loading control. (*O*) In humans, pancreatic concentrations of the endocannabinoid anandamide (AEA) are higher in acute pancreatitis (*black bars*) than in normal pancreas (*white bars;* $P < .05$), whereas levels of combined 1- plus 2-arachidonoylglycerol (1 - AG + 2 - AG) are unchanged.

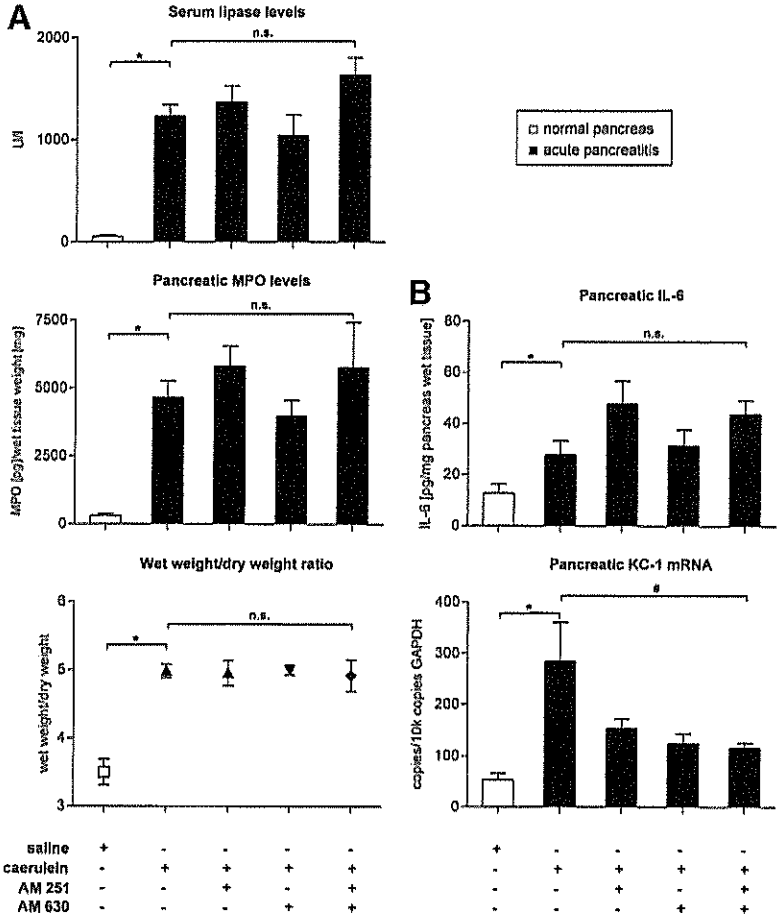

Figure 2.

Levels of serological, molecular, and inflammatory markers in acute pancreatitis and effects of CB1/CB2 antagonists. (*A*) Levels of serum lipase, pancreatic MPO, and the pancreatic wet weight/dry weight ratio are increased in mice following cerulein-induced pancreatitis (*black bars*) over control animals (*white bars*; * $P < .05$). (*B*) Pancreatic levels of IL-6 protein and KC1 mRNA (normalized to GAPDH expression levels) rise significantly following induction of acute pancreatitis. Treatment with cannabinoid receptor antagonists AM251 or AM630 either alone or in combination did not change levels of these parameters, except pancreatic KC1 mRNA expression ($P < .05$). * and $^{\#}$ represent $P < .05$ as compared with the saline group or the cerulein group, respectively. (*) Mann–Whitney *U* test. ($^{\#}$) Analysis of variance followed by post hoc Bonferroni's multiple comparison test. n.s. = statistically not significant.

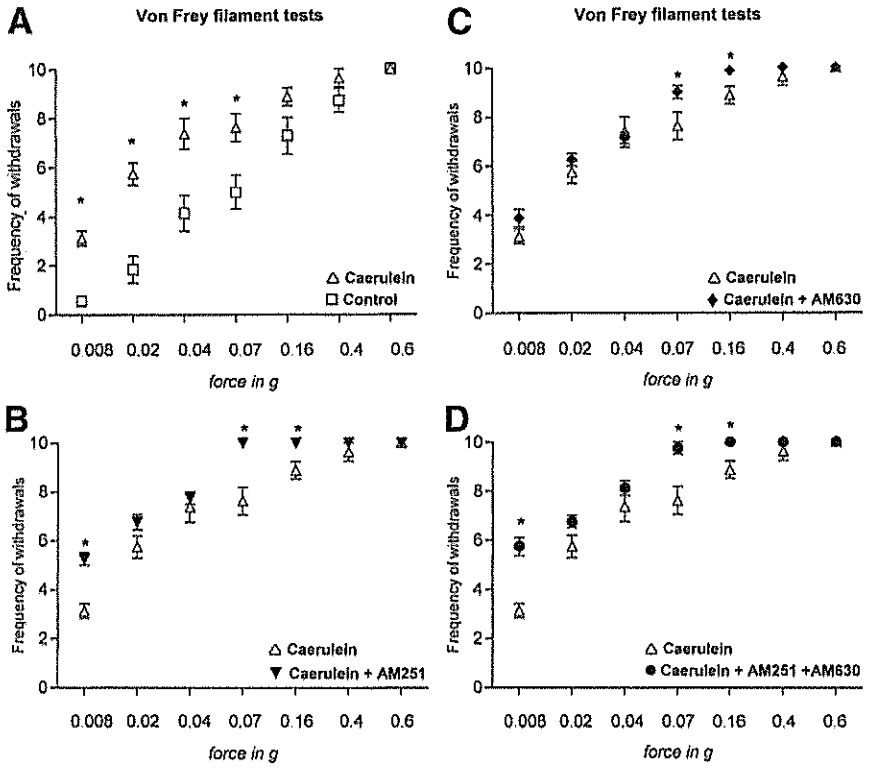

Figure 3.

Analysis of abdominal pain thresholds to graded pressure applied via von Frey filaments in saline- or cerulein-treated mice. Y-axes show frequency of withdrawals to von Frey filaments over 10 applications. (*A*) Cerulein-induced pancreatitis leads to hyperalgesia (increased response frequency to a force of 0.02*g* to 0.16*g*) and allodynia (increased response frequency to 0.008*g*) over saline-injected mice (control). (*B–D*) AM251, AM630, and a combination of both increase response thresholds significantly at some filament forces. Data are shown as mean ± SEM. $*P < .05$; analysis of variance with post hoc Bonferroni's multiple comparison test.

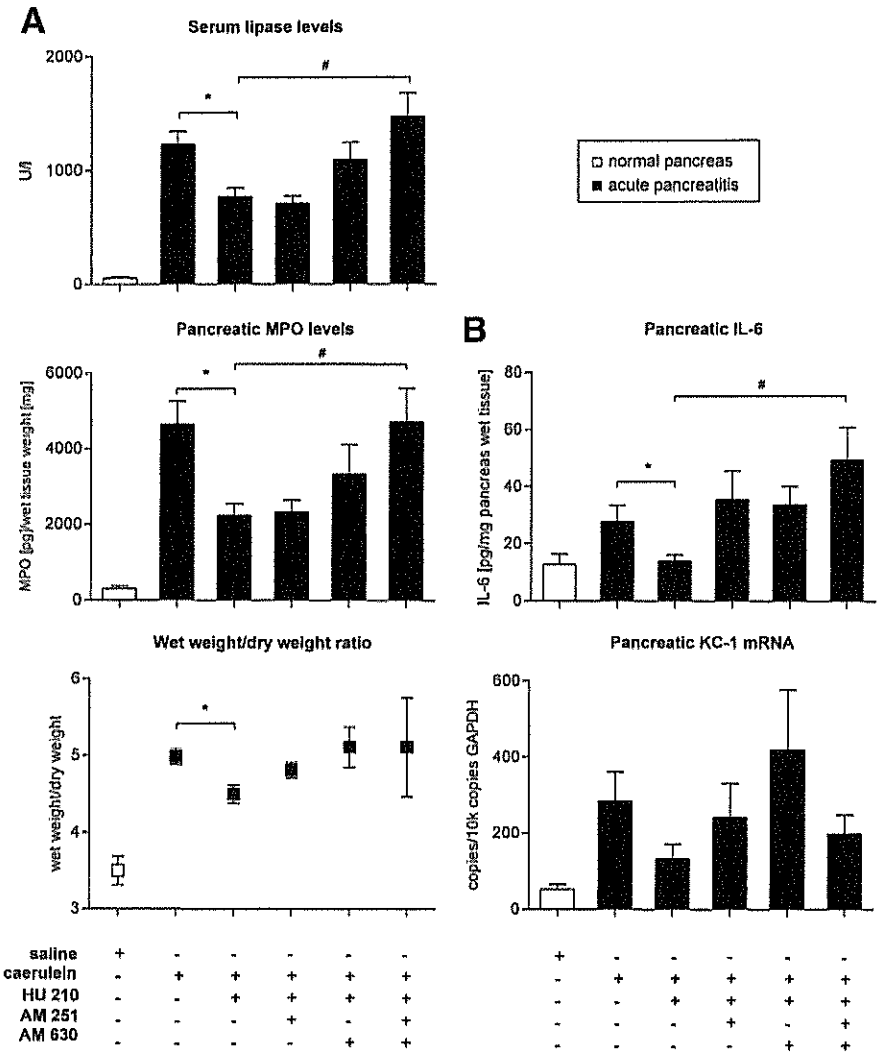

Figure 4.

Nature and receptor mechanisms of effects of a cannabinoid agonist (HU210) on serological, molecular, and inflammatory markers in acute pancreatitis. Subcutaneous treatment with HU210 (0.05 mg/kg intraperitoneally) led to decreased serum lipase ($P = .015$), pancreatic MPO levels, and pancreatic IL-6 levels ($P = .001$ and $P = .02$, respectively; *A*, *B*), which was reversed completely by a combination of AM251 and AM630 ($^{\#}P < .05$). Pancreatic edema (as judged by wet weight/dry weight ratio) was significantly reduced by HU210 ($P = .007$) with a tendency toward reversal by CB receptor antagonists ($P > .05$). Data are shown as mean ± SEM. $^{*,\#}P < .05$; Mann–Whitney *U* test. (*) analysis of variance with post hoc Bonferroni's multiple comparison test ($^{\#}$).

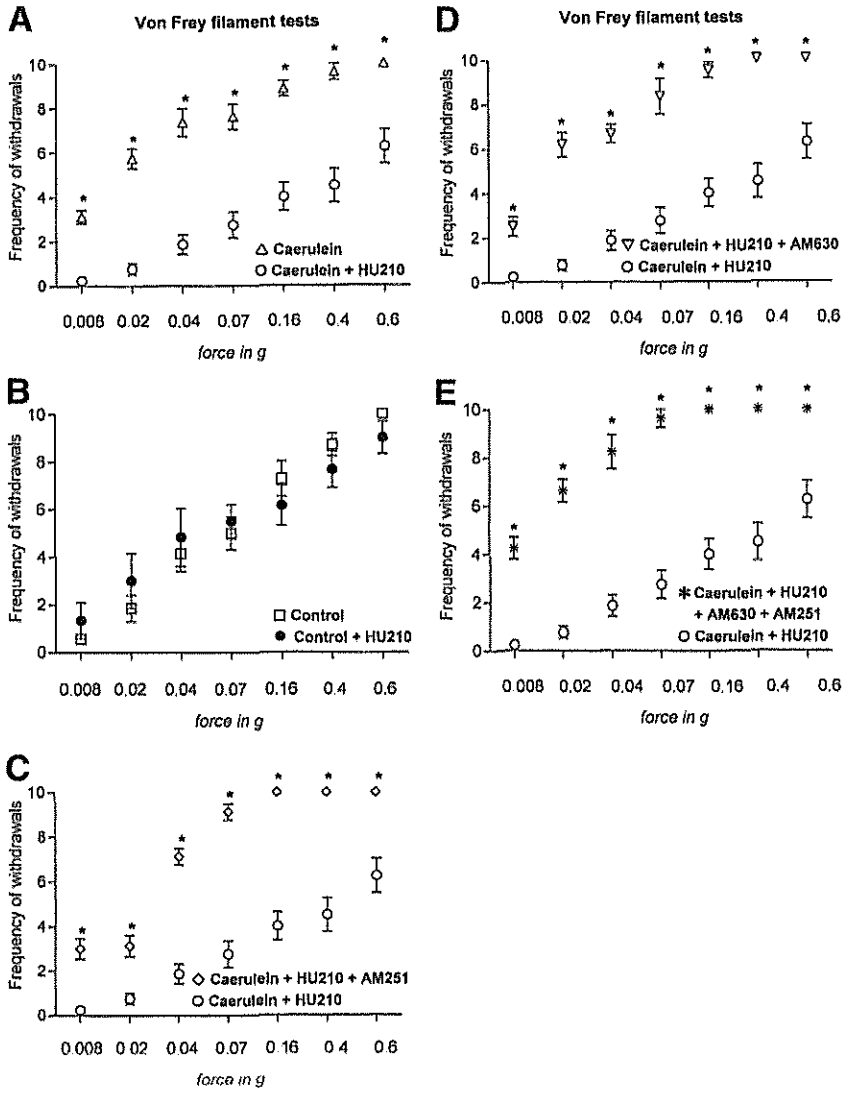

Figure 5.

Nature and receptor mechanisms of effects of a cannabinoid agonist (HU210) on abdominal pain thresholds to graded pressure applied via von Frey filaments in cerulein- or saline-treated mice. Y-axes show frequency of withdrawals to von Frey filaments over 10 applications. (*A*) HU210 (0.05 mg/kg intraperitoneally) completely reversed cerulein-induced hyperalgesia (increased response frequency to a force of 0.02*g* to 0.16*g*) and allodynia (increased response frequency to 0.008*g*) and produced analgesia. (*B*) In control mice (saline-injected), HU210 treatment did not affect nociceptive responses by itself. (*C–E*) Pretreatment with AM251, with AM630, or a combination of both blocked HU210-induced analgesia in cerulein-injected mice. Data are shown as mean ± SEM. $^*P < .05$; analysis of variance with post-hoc Bonferroni's multiple comparison test.

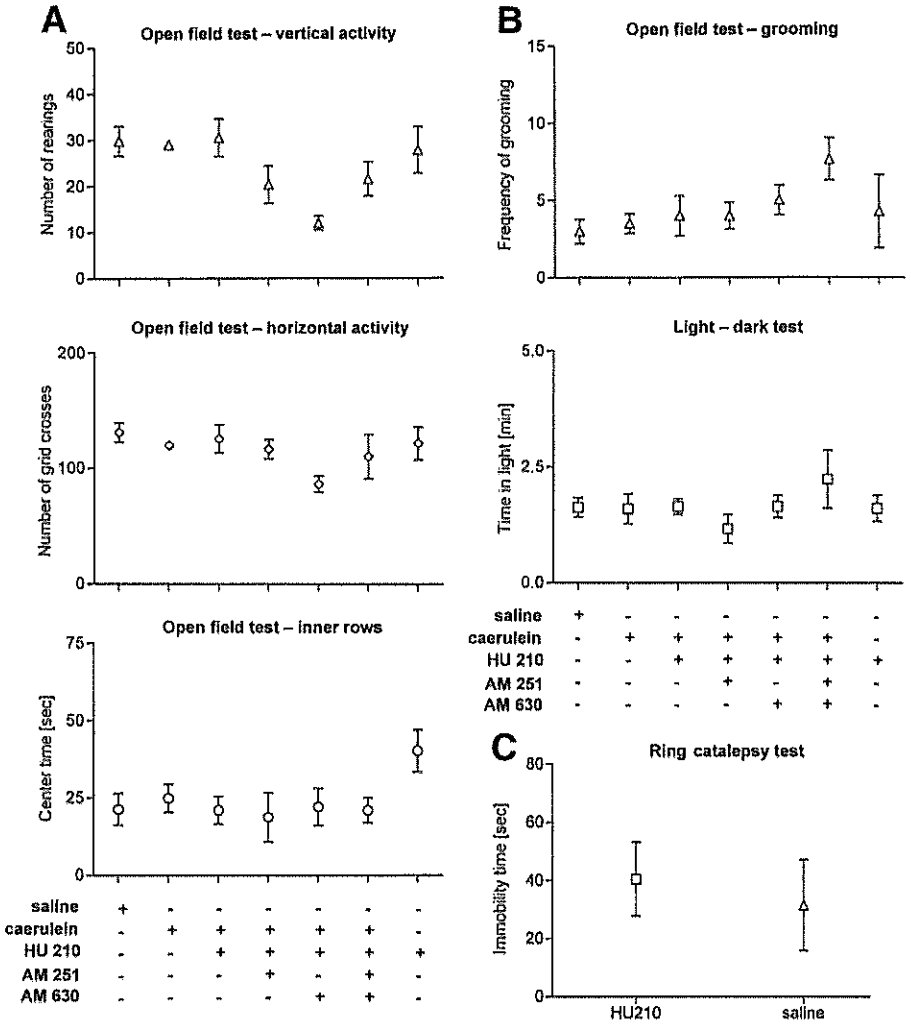

Figure 6.

Effects of HU210 (0.05 mg/kg intraperitoneally) on locomotor activity, anxiety, and grooming in mice with acute pancreatitis. (*A*) In the open-field test, the numbers of rearings (vertical activity, *A*), of grid crosses (horizontal activity, *A*), and of time in inner rows (center time, *A*) is not affected by HU210. (*B*) HU210 treatment does not affect the frequency of grooming in the open-field test and time spent in the dark chamber in the light-dark test for evaluation of anxiety. Co-administration of AM251 or AM630 has no significant effects in any of the previously mentioned tests. (*C*) HU210 (0.05 mg/kg) did not induce freezing behavior in the ring catalepsy test.

Cannabinoids Reduce Markers of Inflammation and Fibrosis in Pancreatic Stellate Cells

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Abstract

Background: While cannabinoids have been shown to ameliorate liver fibrosis, their effects in chronic pancreatitis and on pancreatic stellate cells (PSC) are unknown.

Methodology/Principal Findings: The activity of the endocannabinoid system was evaluated in human chronic pancreatitis (CP) tissues. In vitro, effects of blockade and activation of cannabinoid receptors on pancreatic stellate cells were characterized. In CP, 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 and led to changes in PSC phenotype accompanied by a reduction of intracellular stress fibres.

Conclusions/Significance: Augmentation of the endocannabinoid system via exogenously administered cannabinoid receptor agonists specifically induces a functionally and metabolically quiescent pancreatic stellate cell phenotype and may thus constitute an option to treat inflammation and fibrosis in chronic pancreatitis.

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Introduction

The management of chronic pancreatitis still remains a clinical challenge, with no definite medical cure and only symptomatic treatment available for this disease[1–3]. In some cases, surgical resection of the inflammatory mass (usually localized in the pancreatic head) may permanently relieve symptoms[4]. Histologically, areas of fibrosis (deposits of extracellular matrix (ECM) proteins) are found which may contain clusters of mononuclear cell infiltration[5–7]. Enlarged nerves may be invaded by mononuclear cells, potentially leading to neural damage, which may in part explain the severe pain syndrome[8,9]. As a consequence, endocrine and exocrine functions of the pancreas are progressively lost, ultimately resulting in a scarred pancreas without its physiological functions.

In the past years, pancreatic stellate cells (activated myofibroblasts; PSC) have been identified as major determinants of pancreatic fibrosis: they have been shown to be the major source of extracellular matrix production[10,11] and to stringently control the balance of ECM secretion and digestion by producing matrix metalloproteinases and their corresponding inhibitors[12]. PSC also modulate the local immune reaction by production and secretion of cytokines and chemokines as well as by their phagocytic activity[13–17]. However, the pathobiology of pancreatic fibrogenesis/inflammation and the interplay between stellate cells[18–20], immune cells and nerves is poorly understood, and currently no potentially curative medical treatment is available.

Comparable with liver cirrhosis, prevention of loss of functional pancreatic parenchyma by controlling and resolving the overt scarring reaction to an inflammatory stimulus may constitute a therapeutic approach. Although a number of substances have been identified so far which were initially promising in ameliorating or even reversing the disease, none of these was clinically proven to exert such beneficial properties[21–23].

Besides the well-known central-nervous analgesic properties of exogenously administered cannabinoids, the endocannabinoid system (ECS) and its changes in pathological states have recently attracted considerable attention[24,25]. Particularly, cannabinoids' immune-modulatory function and their influence on lymphocytes constitute a basis for their use in a wide variety of inflammatory diseases[26–30]. Besides these well-studied effects, (endo-)cannabinoids have recently been shown to influence liver fibrogenesis through various mechanisms. Siegmund and co-workers[31,32] have shown that the endocannabinoid anandamide induces necrosis in hepatic stellate cells independent of CB1 and CB2 receptors. In contrast to these results, Julien et al.[33] have found in experimentally induced liver cirrhosis that an activation of the CB2 receptor on hepatic stellate cells leads to apoptosis and attenuated liver fibrosis progression. Teixeira-Clerk and co-authors[34] have proposed CB1 antagonism as a new strategy to treat liver fibrosis. Altogether, these results point towards a potential use of cannabinoids as substances to ameliorate or even revert liver fibrogenesis. However, the exact mechanism of how (and particularly in which disease stage) either CB receptor activation or antagonism may be useful in attenuating chronic liver cirrhosis remains to be determined.

In chronic pancreatitis, activity of the endocannabinoid system and effects of exogenously administered cannabinoids have not been analyzed so far. In this study, we evaluated levels of endocannabinoids and their receptors as well as the potential function of cannabinoid activation and antagonism by synthetic cannabinoid derivatives and their respective antagonists in human chronic pancreatitis tissues and CP-derived PSC.

Materials and Methods

Patients and tissue collection

Pancreas tissues were obtained intraoperatively from patients undergoing resection for chronic pancreatitis (21 men, 19 women). Normal pancreas tissue samples were collected within the organ donor program at Heidelberg University hospital whenever there was no suitable recipient for organ transplantation (n = 20). All patients were informed, and written consent was obtained. The studies were approved by the Ethics Committee of the University of Heidelberg (Germany).

Immunohistochemistry of human pancreatic tissues

CB1- and CB2-receptors were localized in the human pancreas using immunohistochemistry. Rabbit anti human-CB1 and anti human-CB2 antibodies (Cayman Chemical, Ann Arbor, MI, USA) were used at a dilution of 1:150 and 1:300, respectively. Specificity was checked by performing pre-adsorption of the primary antibody with the corresponding blocking peptide for 1 hour at 37°C.

Endocannabinoid level measurements

Levels of anandamide (AEA), 1-arachidonoylglycerol (1-AG) and 2-arachidonoylglycerol (2-AG) in frozen human pancreas samples (normal pancreas: n = 6; chronic pancreatitis: n = 6) were determined by liquid chromatography/mass spectrometry as described previously [35].

Reagents

Ham's F12 medium, DMEM, trypsin-EDTA and penicillin-streptomycin were purchased from Invitrogen (Karlsruhe, Germany); amphotericin B 250 µg/ml was purchased from PAA Laboratories (Pasching, Germany); fetal calf serum (FCS) was purchased from PAN Biotech (Aidenbach, Germany); the enhanced chemoluminescence (ECL) immunoblotting detection reagents were obtained from Amersham Biosciences (Buckinghamshire, UK); Mini EDTA-Free Protease inhibitor was purchased from Roche Molecular Biochemicals (Basel, Switzerland); and the BCA protein assay was from Pierce Chemical Co. (Rockford, IL, USA) and the MTT test reagent was purchased from Sigma Aldrich (Taufkirchen, Germany).

Isolation of human pancreatic stellate cells

Human PSC isolation and culture were performed as described by Bachem et al.[11] using the outgrowth method. For our experiments, cell populations between passage 3 and 5 were used. A 1:1 (vol/vol) mixture of low glucose (1000 mg/L) DMEM with Ham's F12 medium supplemented with 20% FCS, L-glutamine (2 mM), penicillin/streptomycin, and amphotericin B was the standard growth medium, whereas for pharmacology experiments, 1% FCS was used.

Immunocytochemistry

Immunocytochemistry was performed as described previously[36]. Cells were seeded in 20% FCS. Anti-CB1-receptor and anti-CB2-receptor antibodies were diluted 1:300 in Antibody Diluent (DakoCytomation, Hamburg, Germany). Specificity controls included pre-adsorption of the primary antibody with the corresponding blocking peptide (1:1) for 1 hour at 37°C.

Cannabinoid treatment and proliferation assays

PSC were plated on the bottom of 24-well plates at densities of 40,000/well in 500 µl DMEM/Ham's F12 (1/1, v/v) in the presence of 20% FCS for 24 hours. Subsequently, the medium was changed to DMEM/Ham's F12 with 1% FCS. After overnight incubation, drugs were added at concentrations of 1.25, 2.5 and 5 µM. WIN55,212-2 (WIN; Tocris Cookson Ltd., Avonmouth, UK) was dissolved in DMSO and further diluted in ethanol (EtOH). Specific CB1-receptor and CB2-receptor antagonists (AM251 and AM630, respectively) were dissolved in EtOH. Controls included DMSO/EtOH at a dilution of 1:1000. After 48 hours of incubation, proliferation was determined using MTT tests, as described previously[37]. These experiments were repeated five times, whereas experiments for determination of WIN-specificity (pre-incubation with AM251 and/or AM630 before addition of WIN) were repeated twice. To exclude that potential anti-proliferative effects of cannabinoids influenced evaluation of cytokine and ECM protein secretion, treatment was begun when the cells reached 100% confluency.

Apoptosis assays

Cells were grown to 70–80% confluency in medium containing 20% FCS before the FCS concentration was reduced to 1%. After 24 hours of incubation, drugs were added for another 24 hours, as described for the proliferation assays. Single cell suspensions were obtained using trypsin-EDTA. Subsequently, the Guava Nexin™ kit (Guava Technologies, Hayward, CA, USA) was used to determine the number of early and late apoptotic cells according to the manufacturer's instructions.

LDH analysis

Lactate dehydrogenase (LDH) in cell culture supernatants was measured in the central clinical laboratories at the University of Heidelberg using an ADVIA2400 machine (Siemens, Erlangen, Germany).

Invasion assay

Matrigel™-coated invasion chambers (BioCoat Matrigel Invasion Chamber, BD Biosciences, Heidelberg, Germany) were rehydrated for 2 hours in serum-free medium at 37°C and 5%

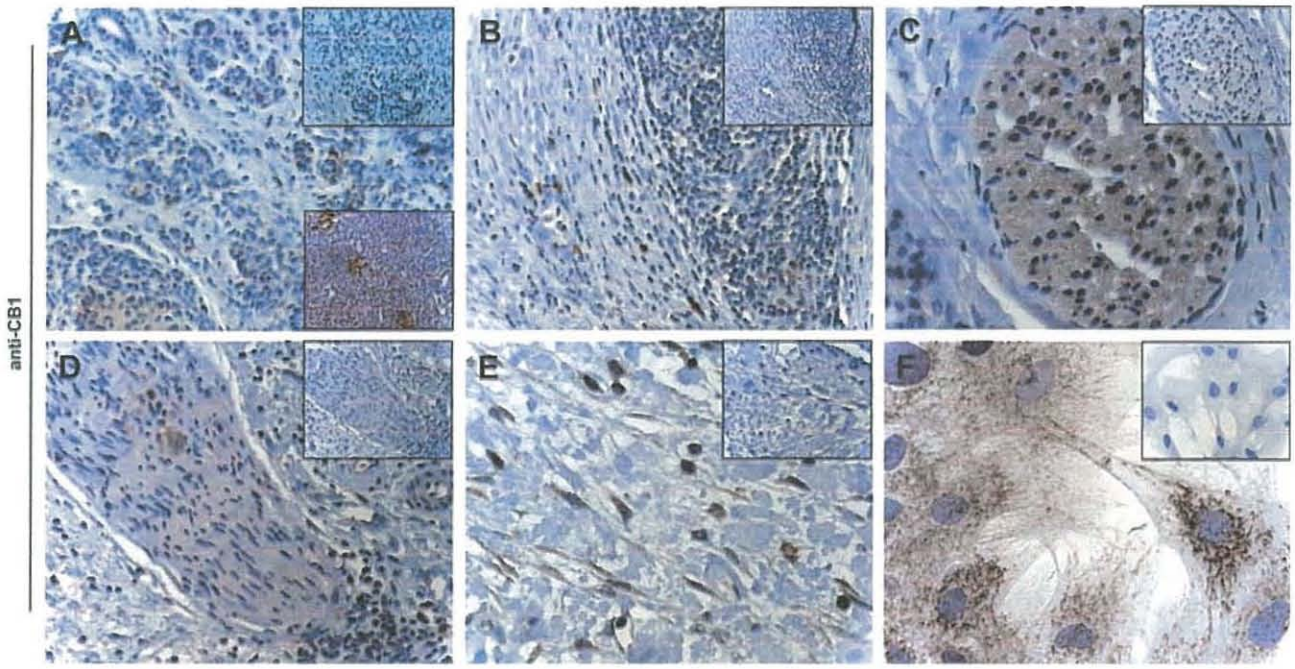

Figure 1. Cannabinoid receptor 1 in human chronic pancreatitis tissues. CB1 immunohistochemistry: staining of tubular complexes (A) but immunonegativity of infiltrating mononuclear cells (B); various staining intensities of intrapancreatic nerves (C&D); pancreatic stellate cells in areas of fibrosis (E) or isolated and cultured in vitro (F). Original magnification: x20 (A, B, D), x40 (C), x80 (F). Insets: negative controls. Lower insets (A): CB1 in normal pancreas.
doi:10.1371/journal.pone.0001701.g001

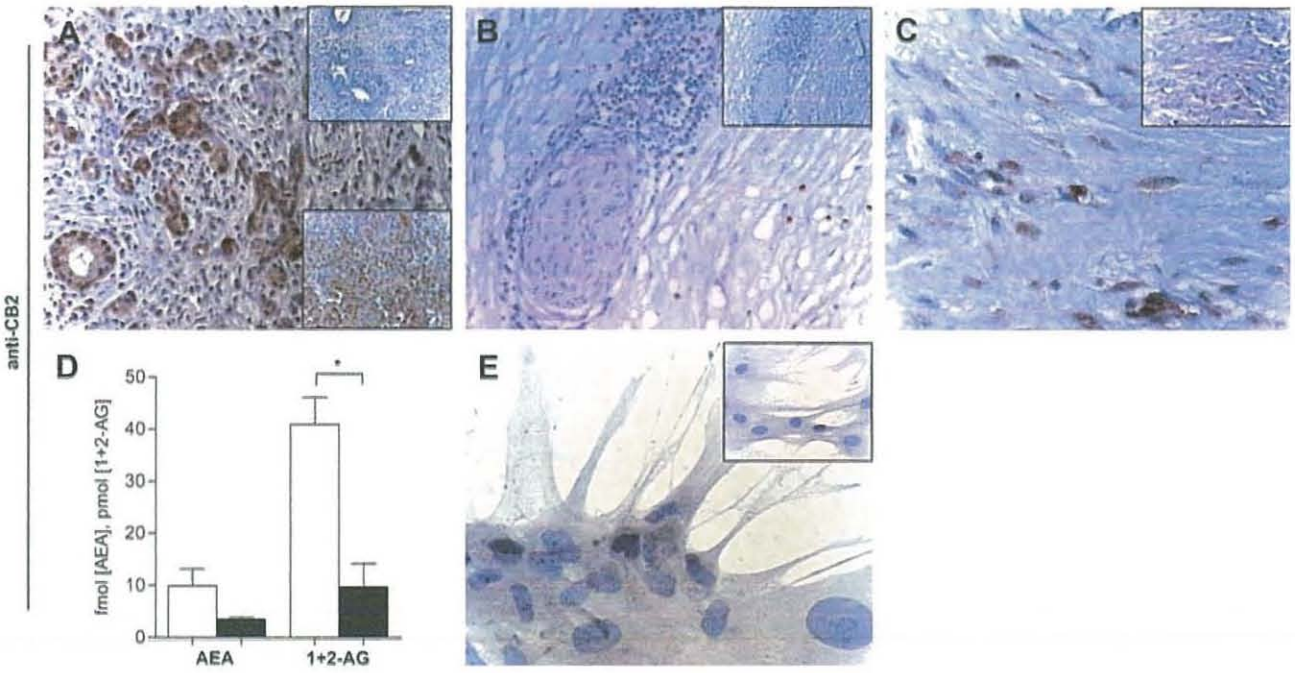

Figure 2. Cannabinoid receptor 2 and endocannabinoid levels in chronic pancreatitis. Using an anti-CB2 antibody, tubular complexes were strongly stained (A). Invading mononuclear cells were also immunopositive (B), whereas intrapancreatic nerves were mostly unstained or only faintly positive (B). Pancreatic stellate cells were immunopositive for CB2 (C). The endocannabinoids anandamide (AEA) and 1+2-arachidonoylglycerol (1+2-AG) were lower in chronic pancreatitis (AEA: $p = 0.14$ and 1+2-AG: $p = 0.0066$; D). Cultured pancreatic stellate cells were faintly CB2-immunopositive (E). Original magnification: x20 (A, B), x40 (C), x80 (F). Insets: negative controls. Lower insets (A): CB2 in normal pancreas.
doi:10.1371/journal.pone.0001701.g002

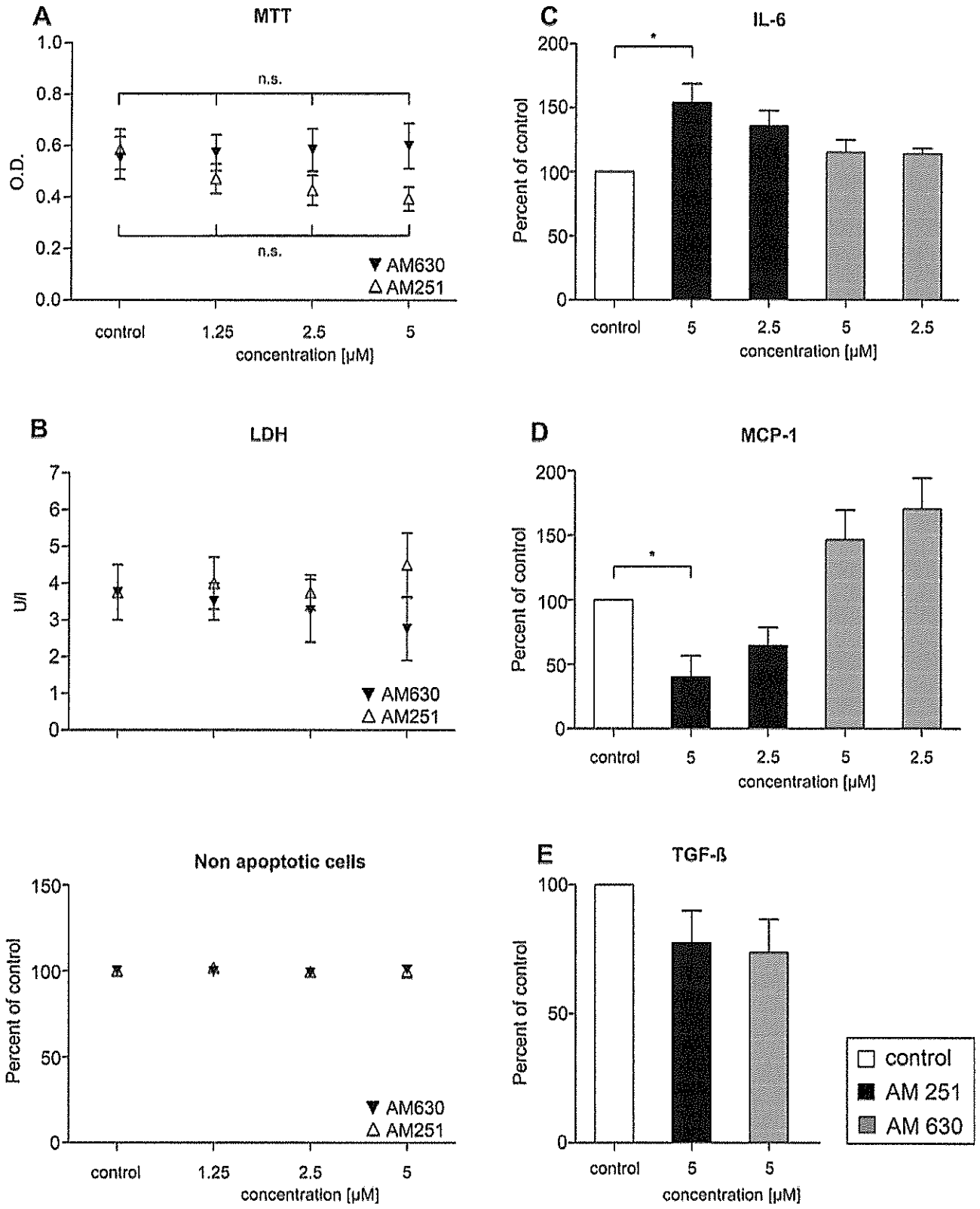

Figure 3. Effects of cannabinoid receptor antagonism on pancreatic stellate cells. (A) MTT assays after 48 hours' incubation of PSC with graded concentrations of CB1- and CB2-receptor antagonists AM251 and AM630 (p = n.s. for concentrations of 1.25, 2.5 and 5 µM). (B) LDH in cell culture supernatants and the fraction of apoptotic cells as judged by the GuavaNexin™ test. AM251 significantly increased IL-6 (C) but decreased MCP-1 secretion (D); AM630 had no effect on IL-6 (C) but induced a tendency towards increased MCP-1 levels (D). CB1-/2-receptor antagonism did not affect TGFbeta (E). Data are shown as mean±SEM (*, $p<0.05$).
doi:10.1371/journal.pone.0001701.g003

analysis of the very low number of apoptotic cells (both in control and antagonist groups) showed that there was no difference in the number of early and late stage apoptotic cells, as well (not shown).

To further assess functional consequences of endocannabinoid blockade in PSC, production of cytokines IL-6 and TGFbeta, chemokine MCP-1 and ECM proteins collagen1 and fibronectin was analyzed. While AM251 significantly induced IL-6 secretion at a concentration of 5 μM ($p<0.05$, ANOVA; Figure 3C), an inverse effect was observed regarding MCP-1, with AM251 decreasing its levels in cell culture supernatants ($p<0.05$, ANOVA; Figure 3D). AM630 did not influence IL-6 levels; however, there was a trend toward increased MCP-1 secretion (Figure 3C&D).

In a next step, the profibrogenic cytokine TGFbeta, which stimulates the synthesis and secretion of fibronectin, collagen1 and matrix metalloproteinases, was analyzed, revealing that neither AM251 nor AM630 significantly altered secreted TGFbeta levels (Figure 3E). Furthermore, AM215 had no effects on fibronectin and collagen 1 secretion (cell culture supernatants of cells grown to 100% confluency before initiation of treatment; immunoblots from three pooled experiments; Figure 4). Blockade of the CB2-receptor by AM630 did also not change collagen 1 and fibronectin production (Figure 4). However, AM630 induced an increase of alphaSMA protein levels (pooled cell lysates from three independently performed experiments; Figure 4).

Evaluation of invasiveness as another key feature of pancreatic stellate cells was carried out using Matrigel™-coated cell culture inserts, as previously described[38]. Although there was a tendency toward an increased number of invaded cells treated with AM251 ($p=$ n.s.; mean increase compared with control: +47%; Figure 5A), MMP-2 levels in cell culture supernatants were not different from the control treatment group (Figure 5B). However, there were distinct changes in stellate cell morphology after incubation with the CB1-receptor antagonist AM251, as judged by actin cytoskeleton staining (Figure 5C&D; control PSC: inserts), whereas AM630-treated PSC closely resembled the control cell phenotype (Figure 5E&F). Treatment of PSC with AM251 induced a thinner, more stretched shape in the cells, with an increased length but an overall smaller size (Figure 5 C&D). Additionally, we observed a marked loss of intracellular fibres upon incubation with AM251. These results are supported by a recent publication which reports on an association between loss of actin polymerization and increased invasiveness [40].

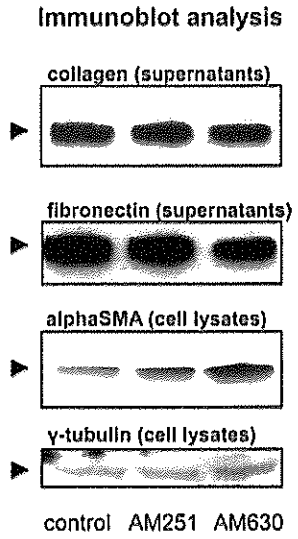

Figure 4. Cannabinoid receptor blockade alters synthesis of ECM proteins. As assessed by immunoblot analysis of cell culture supernatants, AM251 and AM630 (at 2.5 μM) did not affect collagen and fibronection secretion. In PSC cell lysates, alphaSMA levels remain unchanged following CB1-receptor antagonism whereas AM630 induced increased alphaSMA protein levels. White bars: control; black bars: AM251; grey bars: AM630. Data are shown as mean±SEM.
doi:10.1371/journal.pone.0001701.g004

Cannabinoid receptor activation reduces markers of inflammation and fibrosis in PSC

In an approach to reduce the overt activity of stellate cells present in chronic pancreatitis through augmentation of the down-regulated endocannabinoid system, we treated PSC with the synthetic cannabinoid receptor agonist WIN55,212-2 (WIN; all results from three independently performed experiments). Cannabinoid receptor selectivity of the effects of WIN on PSC was evaluated by pre-incubation with the respective antagonists at the cannabinoid-1 and -2 receptors and with a combination of both. As shown by MTT assays, WIN dose-dependently decreased proliferation of PSC ($p<0.05$ for concentrations of 2.5 μM and 5 μM, respectively, ANOVA), which was partially reversible by pre-incubation with a combination of the CB1- and CB-2-receptor antagonists AM251 and AM630 (Figure 6A–D), but not by the respective receptor antagonists alone (Figure 6B&D). To evaluate whether this effect was due to induction of apoptosis or necrosis, lactate dehydrogenase was analyzed in cell culture supernatants and a GuavaNexin™ test was performed. These experiments revealed that the growth-inhibitory effects of WIN were not due to necrotic cell death, as shown by unchanged LDH levels following WIN treatment with/without pre-incubation with the CB1/2-receptor antagonists (Figure 6E). Similarly, we observed no induction of apoptosis (Figure 6E), either at an early or a late stage (not shown).

To assess the effects of WIN treatment of PSC on the secretion of cytokines IL-6, MCP-1 and TGFbeta, cell culture supernatants were subjected to ELISA. These experiments demonstrated that IL-6 and MCP-1 secretion were significantly reduced by WIN ($p=0.001$ and $p=0.0002$, respectively; Figure 7A). Preincubation with a combination of AM251 and AM630 (5 μM, respectively) partially reversed this effect (Figure 7A). In contrast, there was a trend toward increased TGFbeta levels (increase by 42%; Figure 7B) which was nearly absent when cells had been pre-treated with a combination of AM251 and AM630 (increase by only 8%; Figure 7B). Immunoblot analysis of cell culture supernatants showed significantly reduced fibronectin and collagen 1 levels after WIN treatment (supernatants of PSC grown to 100% confluency before initiation of treatment; Figure 7B). Pre-incubation with AM251 and/or AM630 (5 μM) revealed that the suppressive effect of WIN on fibronectin and collagen 1 production was partially reversible only when using both antagonists concomitantly (Figure 7B; pooled cell culture supernatanty from three independently performed experiments). Furthermore, WIN induced a reduction in alphaSMA levels which was also partially reversed by pre-incubation with a combination of both CB-receptor antagonists (Figure 7B; equal loading: gamma-tubulin).

Cannabinoids decrease invasiveness of PSC and downregulate MMP-2

As with CB receptor antagonists alone, invasiveness of PSC following treatment with WIN (+/− pre-incubation with AM251 and/or AM630) was analyzed using Matrigel™ invasion chambers (results from four independent experiments which were performed in duplicates). WIN significantly reduced the number of invaded cells ($p=0.0081$; Figure 8A), whereas pre-incubation with AM251 or a

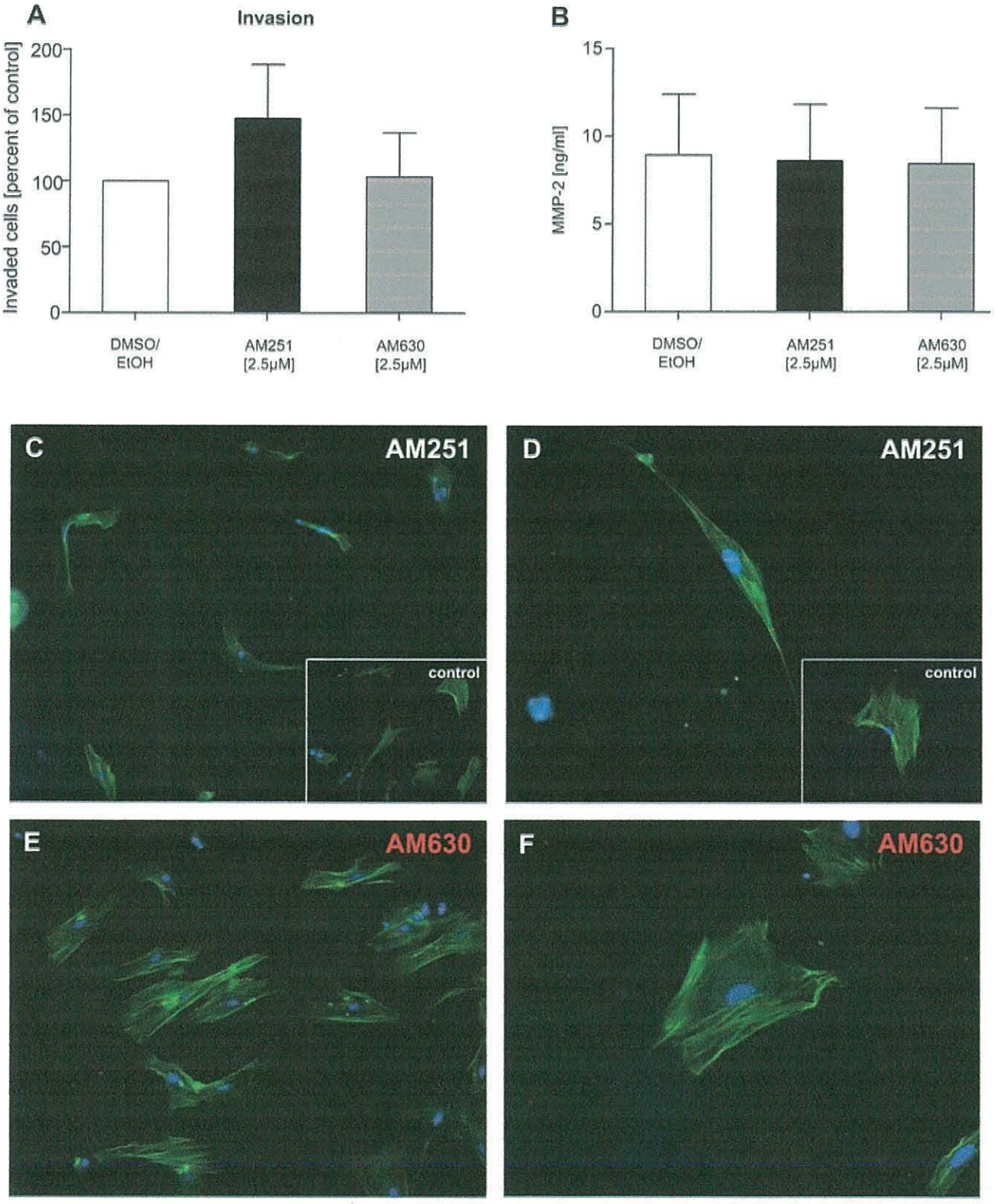

Figure 5. AM251 induces changes in PSC phenotype. (A) Invasion assays demonstrated a trend toward increased invasiveness induced by AM251. (B) MMP-2 levels were unchanged by antagonist treatment. (C&D) AM251-treated pancreatic stellate cells were smaller and thinner, with a more stretched shape, an increased length, and a loss of intracellular fibres (control PSC: insets and Figure 8C&D). Cells treated with AM630 (E&F) closely resembled the control PSC (see insets C&D and Figure 8C&D). Original magnification: x 40 (C&E), x 80 (D&F). Nuclear stain: DAPI. White bars: control; black bars: AM251; grey bars: AM630. Data are shown as mean±SEM.
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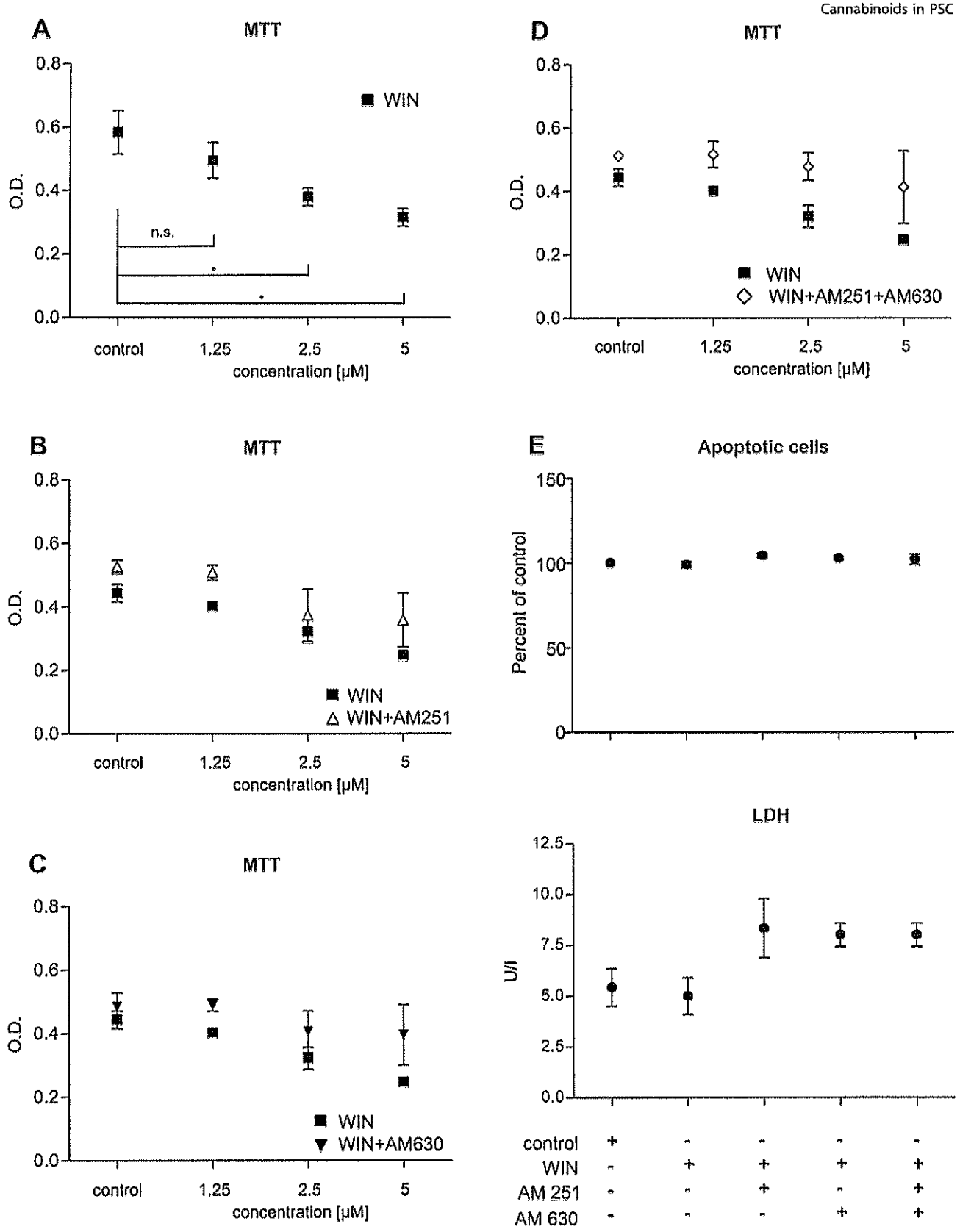

Figure 6. Cannabinoid receptor activation on PSC reduces growth, independent of apoptosis or necrosis. (A) MTT tests of WIN-treated PSC revealed dose-dependent inhibition of growth ($p<0.05$ at concentrations of 2.5 and 5 μM). (B&C) While AM251 and AM630 alone were not effective, pre-treatment with a combination of AM251 and AM630 blocked WIN-induced growth inhibition (D). The reduction in proliferated cells was neither due to necrosis (unchanged LDH levels) nor to apoptosis (constant low number of apoptotic cells in treated versus control PSC; E). Data are shown as mean±SEM (* $p<0.05$).
doi:10.1371/journal.pone.0001701.g006

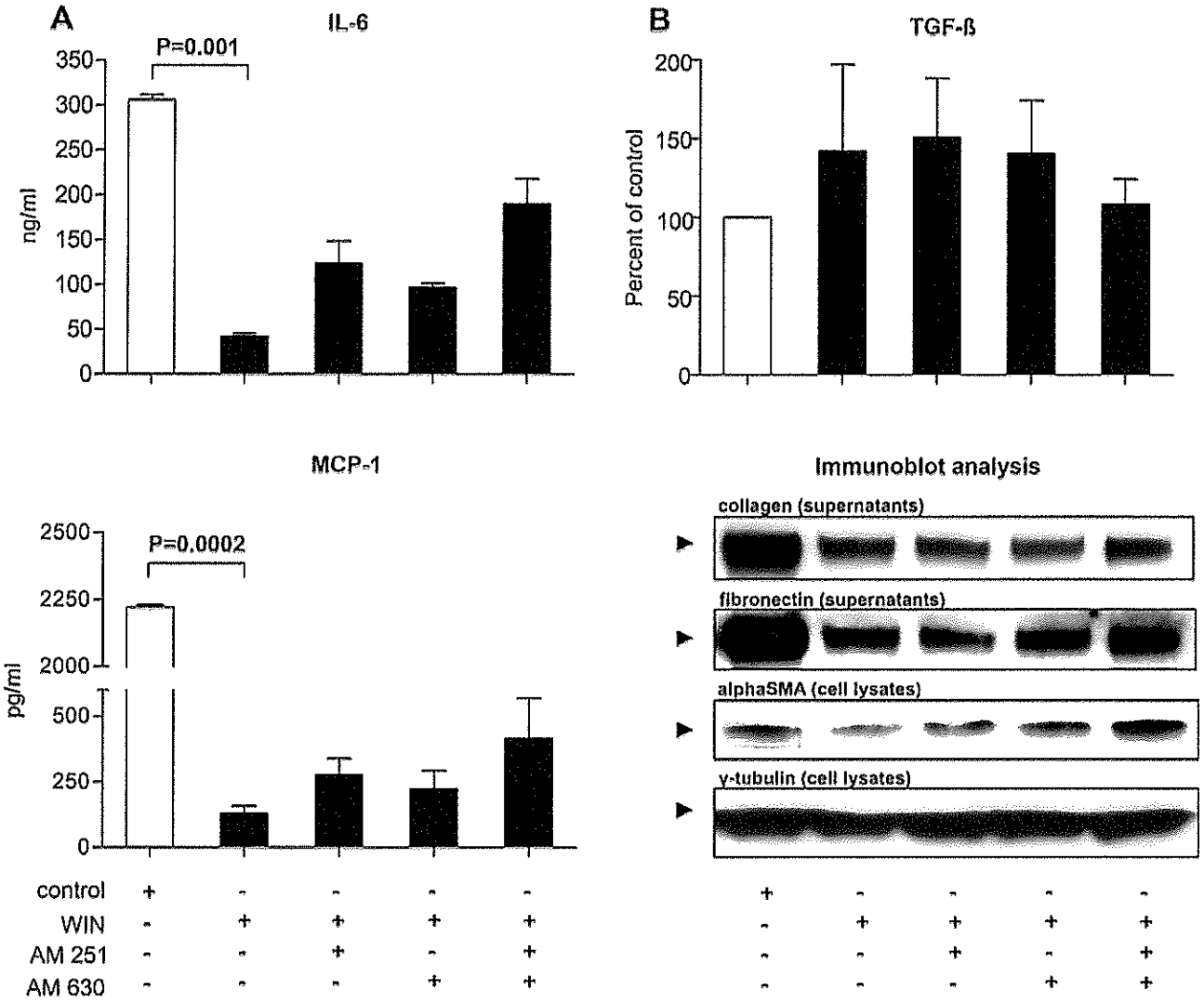

Figure 7. WIN de-activates pancreatic stellate cells. (A) IL-6 and MCP-1 secretion were significantly reduced by WIN ($p=0.001$ and $p=0.0002$, respectively), independent of TGFbeta (B; unchanged TGFbeta levels). The reduction in IL-6 and MCP-1 levels was partially reversed by a combination of the CB1-receptor and CB2-receptor antagonists AM251 and AM630 (A). While control PSC secreted significant amounts of fibronectin and collagen 1 (as seen by an intense signal at 220 and 190 kDa, respectively), treatment with WIN reduced the signal at the respective molecular weights (B; immunoblots of cell culture supernatants, pooled from three independent experiments). AlphaSMA levels were also suppressed by WIN (B; immunoblot of PSC cell lysates; gamma-tubulin: equal loading control). A combination of both antagonists AM251 and AM630 partially reversed the suppressive effects elicited by incubation with WIN (B, immunoblots of fibronectin, collagen 1 and alphaSMA). White bars: control; black bars: WIN55,212-2±AM251/AM630. Data are shown as mean±SEM.
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combination of AM251 and AM630 partially reversed this effect (5 μM, respectively; Figure 8A). Pre-treatment with AM630 showed no differences from treatment with WIN alone (Figure 8A).

To evaluate whether the WIN-induced reduction in invasiveness was due to decreased levels of matrix metalloproteinase 2, cell culture supernatants were analyzed by ELISA after WIN treatment (+/− pre-incubation with AM251/AM630, 10 μM). We observed a significant decrease of MMP-2 levels after treatment with WIN ($p=0.026$; Figure 8B) which was specifically reversible using a combination of both antagonists (to >50% of control cell culture supernatants).

Analysis of the PSC morphology after treatment with WIN (as shown by actin cytoskeleton immunofluorescence) demonstrated distinct changes: whereas cell morphology was unchanged in control PSC (Figure 8C&D), WIN induced a more round-shaped phenotype, with a reduction in cellular extensions (Figure 8E&F). We also observed a loss of intracellular fibres, accompanied by a disorganization of these fibres and a generally smaller PSC phenotype. Furthermore, the number of cell-cell contacts tended to be lower after treatment with WIN (Figure 8E).

Discussion

In the present study, we show that activation of the endocannabinoid system in chronic pancreatitis-derived stellate cells specifically induced a more quiescent phenotype, accompanied by suppression of pro-inflammatory cytokines and extracellular matrix proteins as well as a decrease in invasiveness of PSC. The loss of functional pancreatic parenchyma, which is substituted by a fibrotic scar with massive infiltration of lymphocytes, is

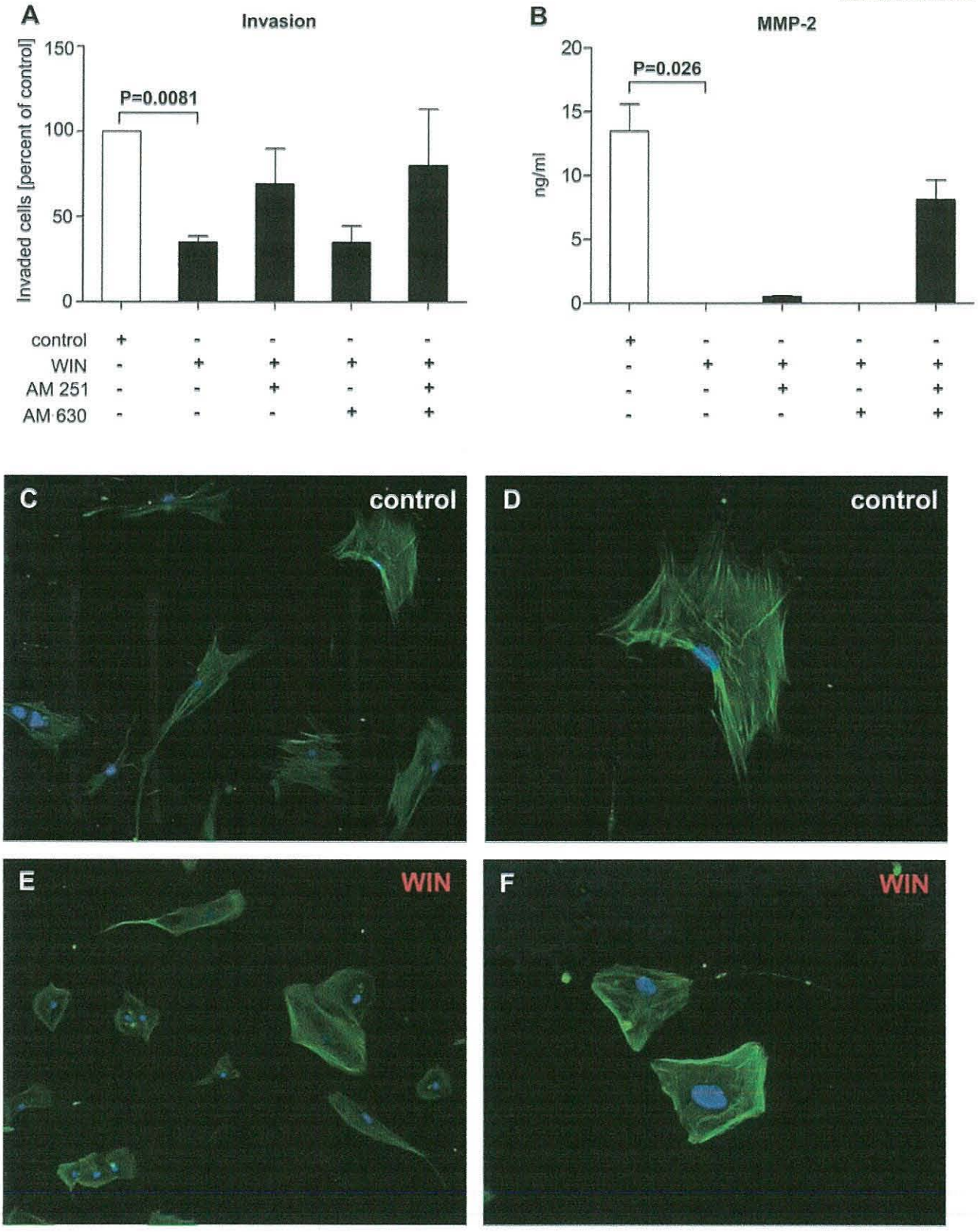

Figure 8. Cannabinoid receptor activation reduces invasiveness. A significant reduction in the number of invaded cells was seen after treatment with WIN ($p = 0.0081$; A). A near-complete reversal of the reduced invasiveness was seen when the cells were pre-incubated with AM251 or a combination of AM251 and AM630 (A). This was not found with AM630 alone (A). WIN induced a significant decrease in MMP-2 levels ($p = 0.026$; B) which was partially reversible by a combination of AM251 and AM630 (B). Actin cytoskeleton staining with phalloidin demonstrated that control PSC showed the normal cellular structure (C&D) but that WIN induced a more round and smaller PSC phenotype (E&F). Original magnification: x 40 (C&E), x 80 (D&F). Nuclear stain: DAPI. White bars: control; black bars: WIN55,212-2±AM251/AM630. Data are shown as mean±SEM.
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characteristic of chronic pancreatitis pathobiology. So far, there is no treatment available for controlling the excessive activation of pancreatic stellate cells, which mainly produce the extracellular matrix and which activate themselves in an autocrine loop by secreting pro-fibrogenic molecules such as TGFbeta and by producing pro-inflammatory cytokines.

The most important finding of this study is that cannabinoid receptor activation induces a quiescent phenotype of chronic pancreatitis-derived PSC by downregulating production of extracellular matrix proteins and inflammatory cytokines. This effect was accompanied by marked changes in PSC appearance toward a less mesenchymal-like phenotype. Since migration of PSC to sites of damage can induce either re-differentiation of PSC and tissue repair or support activation and fibrosis, we evaluated the invasive potential as a factor contributing to PSC motility[12,13,41,42]. We observed that suppression of PSC invasiveness by unselective CB receptor activation was accompanied by decreased MMP-2 levels. This could be particularly important not only in terms of invasiveness but also for influencing the distorted balance of matrix synthesis and degradation, since it is known that increased levels of MMP-2 (not only in chronic pancreatitis) facilitate the deposition of pathological fibrillar collagen[12,43]. Furthermore, it has been suggested for both pancreatic and hepatic stellate cells that MMP-2 may have effects on proliferation. Thus, cannabinoid-induced suppression of MMP-2 could be a mediator of the induction of a more quiescent phenotype, leading to reduced collagen synthesis.

Furthermore, there are a number of regulators of PSC activation: 1) paracrine factors such as cytokines (IL-1, MCP-1), growth factors (TGFbeta and PDGF) and endothelium-derived substances (endothelin-1) which are released by recruited inflammatory cells; and 2) factors secreted by destroyed parenchymal cells, such as acinar, endothelial and ductal cells [14,15,44–46]. PSC can also perpetuate their activation by producing autocrine mediators such as IL-6, TGFbeta, PDGF or the most recently discovered periostin [38]. Once activated, PSC recruit and stimulate leukocytes via MCP-1 and IL-8. In our experimental setup, we chose to analyze MCP-1 levels upon blockade or augmentation of the endocannabinoid system with synthetic cannabinoids. Here, significantly reduced levels of MCP-1 and IL-6 were observed, suggesting interference of cannabinoids in the autocrine loop, with the effect of de-sensitization of excessively stimulated pancreatic stellate cells. These results are supported by the observation that the endocannabinoid system is suppressed in chronic pancreatitis, which is particularly important since chronic pancreatitis—in contrast to liver cirrhosis—is usually associated with a severe pain syndrome. Thus, down-regulation of pain-inhibitory endocannabinoids could participate in pain generation in chronic pancreatitis. In concordance with the contradictory results of cannabinoid receptor activation and antagonism in hepatic stellate cells, blockade of the remaining ECS activity exerted at least some beneficial effects, such as suppression of the chemokine MCP-1 which may be explained by increased endocannabinoid secretion upon blocking the receptors. However, we also observed that cannabinoid receptor-1 antagonism induced production of the pro-inflammatory cytokine IL-6 and induced a more motile PSC phenotype, as judged by a trend toward increased invasiveness and a longitudinally stretched appearance. For liver cirrhosis it has recently been shown that CB2-receptor activation mediated anti-fibrosis by apoptosis induction and growth inhibition of hepatic stellate cells [33]. In our study, antagonism of cannabinoid receptor-2 elicited no such effects.

Because drugs for the treatment of chronic pancreatitis should ideally exert anti-fibrotic and anti-inflammatory properties, their bimodal effects rather contradict a therapeutic use of CB-receptor antagonists and promote the hypothesis that (re-)activation of the (endo-)cannabinoid system in chronic pancreatitis may be beneficial for suppressing disease progress. This holds particularly true since the suppressive effects induced by the cannabinoid receptor agonist WIN55212,2 were reversible by pre-incubation with a combination of CB1- and CB2 receptor antagonists AM251 and AM630. Though CB1 receptors constitute the "main primary antinociceptive targets for systemically- or peripherally-applied cannabinoids in vivo" [47], activation of central CB1 receptors also dose-dependently induces side-effects such as hindrance of activity, motor dysfunction, sedation or catalepsy [47–49]. Thus, a major challenge for the clinical use of cannabinoids, i.e. as an anti-fibrogenic therapy, will be the reduction of these cognitive, affective and motor function side-effects [47,50]. An alternative to selective, peripheral cannabinoid receptor agonists might be the manipulation of endocannabinoid metabolism by blockade of endocannabinoid degradation or uptake [50,51]. This approach could be particularly advantageous in chronic pancreatitis where the endocannabinoid system is locally suppressed and where this pathological state could thus be reversed. However, future studies are needed to pinpoint the precise mechanisms of cannabinoid-induced de-activation of pancreatic stellate cells and particularly its effects in vivo: 1) in a disease-prevention model, 2) in an approach to slow down the destruction of functional pancreatic parenchyma, and 3) in their potency to reverse fibrosis.

In conclusion, we show that the endocannabinoid system is downregulated in chronic pancreatitis and that its augmentation via exogenously administered cannabinoids specifically reduces activation of pancreatic stellate cells. These experiments lay a basis for testing the value of synthetic cannabinoids in the treatment of chronic pancreatitis.

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Author Contributions

Conceived and designed the experiments: CM ME HF JK. Performed the experiments: CM MM ME DS FB PP SB TG. Analyzed the data: CM MM ME DS FB PP SB NG TG HF JK. Contributed reagents/materials/analysis tools: CM. Wrote the paper: CM MM ME JK. Other: Contributed to writing the paper: NG HF.

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Cannabinoid HU210 Protects Isolated Rat Stomach against Impairment Caused by Serum of Rats with Experimental Acute Pancreatitis

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Abstract

Acute pancreatitis (AP), especially severe acute pancreatitis often causes extra-pancreatic complications, such as acute gastrointestinal mucosal lesion (AGML) which is accompanied by a considerably high mortality, yet the pathogenesis of AP-induced AGML is still not fully understood. In this report, we investigated the alterations of serum components and gastric endocrine and exocrine functions in rats with experimental acute pancreatitis, and studied the possible contributions of these alterations in the pathogenesis of AGML. In addition, we explored the intervention effects of cannabinoid receptor agonist HU210 and antagonist AM251 on isolated and serum-perfused rat stomach. Our results showed that the AGML occurred after 5 h of AP replication, and the body homeostasis was disturbed in AP rat, with increased levels of pancreatic enzymes, lipopolysaccharide (LPS), proinflammtory cytokines and chemokines in the blood, and an imbalance of the gastric secretion function. Perfusing the isolated rat stomach with the AP rat serum caused morphological changes in the stomach, accompanied with a significant increment of pepsin and [H^+] release, and increased gastrin and decreased somatostatin secretion. HU210 reversed the AP-serum-induced rat pathological alterations, including the reversal of transformation of the gastric morphology to certain degree. The results from this study prove that the inflammatory responses and the imbalance of the gastric secretion during the development of AP are responsible for the pathogenesis of AGML, and suggest the therapeutic potential of HU210 for AGML associated with acute pancreatitis.

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Introduction

Acute pancreatitis (AP), especially severe AP, is a potentially lethal inflammatory disease of pancreas which often leads to extra-pancreatic complications, even multiple systemic organ dysfunctions. It has been reported that 52% of patients with acute pancreatitis develop acute gastrointestinal mucosal lesion (AGML) or stress ulcer [1], [2]. Although the endoscopic observation shows that the majority of subjects merely have multiple shallow erosions in the gastrointestinal tract, the optimal pharmacological intervention continues to be a matter of debate, and the pathogenesis of AGML remains unclear.

Some investigators report that the stressful condition with acute pancreatitis causes the diminished blood supply or hypoperfusion in the gastric mucosa, and the counter-diffusion of gastric hydrogen ion (H^+) is an important factor for AGML as well [3], [4]. Other investigations discovered that the serum and ascitic fluid from AP patients and experimental animals contained a large amount of toxic substances, such as pancreatic enzymes, endotoxins, inflammatory mediators [5], [6], which may contribute to the multiple organ dysfunctions in acute pancreatitis [7], [8].

For centuries, Cannabis plant and its extracts have been used to alleviate symptoms of gastrointestinal inflammatory diseases. It has been established that D^9-tetrahydrocannabinol, the major psychoactive component of Cannabis, exerts its primary cellular actions though two G protein-coupled receptors, cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors [9]–[11]. Since then,

these two receptors have been recognized as the major regulators of physiological and pathological processes [12]. Cannabinoids can reduce gastrointestinal secretion [13], and the activation of CB1 receptor exhibits protective role against stress-induced AGML [14], [15], but the mechanisms of their action remain elusive.

The aim of the present work was to explore, by both in vivo and in vitro experiments, the changes in the serum components, the alterations of gastric endocrine and exocrine functions in rat AP model, and the possible contributions of these alterations in the pathogenesis of AGML. Also probed were the interventional effects of CB1 by using its agonist HU210 and antagonist AM251, in an effort to better elucidate the pathophysiological mechanisms of AP-associated AGML and the antiulcer potentials of these cannabinoid agents.

Materials and Methods

Animals

Male Sprague–Dawley rats (220–250 g) were obtained from the Experimental Animal Center of Fudan University, Shanghai, China. Prior to the experiments, all animals were housed for 1 week under standard conditions with free access to water and laboratory chow. All experimental procedures below were in agreement with international guidelines for the care and use of laboratory animals and were approved by the Animal Ethics Committee of Tongji University, Shanghai, China.

Induction of Acute Pancreatitis in Rats

The rats were allocated randomly into two groups: AP and sham-operation group with 24 animals in each group. The rats were fasted overnight with only water allowed before surgery. AP model was induced by the method developed by Aho et al [16]. Briefly, the rats got laparotomy (~3 cm abdominal-midline incision) following the standard aseptic procedure and under general anesthesia with intraperitoneal injection of 20% ethyl carbamate at 10 mL/kg. The biliopancreatic duct was temporarily occluded at the liver hilum with a fine soft microvascular clamp to prevent reflux of the infused material to the liver. A retrograde injection of 3% sodium deoxycholate into the biliopancreatic duct was then performed (0.1 mL/100 g bodyweight). The clamp was removed after the injection. Sham-operation was performed accordingly without the sodium deoxycholate injection, and the surgery was concluded with abdominal stratified closing. On the fifth hour after the surgery, the blood was collected from the abdominal aorta puncture under anaesthetization. All the samples of blood were centrifuged and the supernatant fluid (serum) was collected, aliquoted, and stored at −20°C for subsequent applications. The pancreas was removed, divided into two parts, and one part was put into trizol immediately and store at −20°C for genechip analysis, as the other part was fixed with 10% paraformaldehyde. The stomach was also removed, opened along the large curve, and fixed with 10% paraformaldehyde for ensuing pathological examination.

Histological Evaluation

Histological evaluation was performed on rat pancreas and stomach that were fixed in 10% paraformaldehyde and embedded in paraffin. Thereafter, 5 μm thickness sections were sliced on a Leica RM2126 microtome (Leica, Shanghai, China) and stained with haematoxylin (0.5%) and eosin (0.5%), followed by observation under a Motic BA300 microscope (Motic China Group Co. Ltd., Xiamen, China). Histological Scoring was appraised on pancreatic sections using a modified criterion from Nathan JD, et al [17]. The evaluation was made in ten randomly chosen microscopic fields of each animal's slides, and repeated in three rats /group in a blinded manner. And the total histological score (0–9) was expressed as the sum of edema (0–3), inflammatory cell infiltration (0 –3), and tissue necrosis (0–3).

Microarray Hybridization Assay

Microarray analysis was used to identify transcription profiles of some inflammatory indexes in the pancreas from rat with acute pancreatitis. Array hybridizations were carried out using three biological replicates of RNA samples extracted from the pancreas of AP and control rats. Probe preparation, chip hybridization, and primary data analysis were performed by Capital Bio Corporation (a firm licensed and authorized by Affymetrix to operate in Beijing, China). Arrays were scanned using the Genechip Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA). Quantitative analysis was performed using Affymetric MicroArray Suite 5.0-Specific Terms (Statistical Algorithms) GCOS (Affymetrix GeneChip Operating Software) Version 1.4. The differentially expressed genes were identified using SAM (Significant Analysis of Microarray) software, and selected on the basis of their fold changes (>2-fold) as compared to the control specimens.

Immunohistochemistry Analysis

Immunohistochemistry staining on paraffin sections of rat stomach and pancreas were performed using rabbit polyclonal anti-CB1 and anti-CB2 antibodies (Cat. no: ALX-210-314 for anti-CB1 and Cat. no: ALX-210-315 for anti-CB2, Enzo, Plymouth Meeting, PA, USA) as described previously [18]. The slides with sections of rat stomach and pancreas were incubated overnight at 4°C with anti-CB1 or anti-CB2 antibodies, and the biotin-labeled goat anti-rabbit IgG working fluid (Cat. no: SP0023; Biosynthesis Biotechnology Co. Ltd., Beijing, China) was then applied onto each slide and incubated at 37°C for 15 minutes, followed by incubation with a HRP-labeled streptavidin working solution at 37°C for 15 minutes, and slides were rinsed thoroughly. Finally, the slides were DAB-stained and nuclear re-stained with hematoxylin. The slides of the negative control were processed through the identical steps, but the primary antibody was replaced with PBS. Image analysis was accomplished using digital Motic Med 6.0 image analysis system (Motic; China Group Co. Ltd., Xiamen, China).

Western Blotting for Measuring CB1 and CB2 Expression

CB1 and CB2 protein expression in the pancreas and stomach were evaluated by western blotting. As described previously [19], after incubation with the primary antibodies in a 1:250 dilution individually (rabbit polyclonal anti-CB1 and anti-CB2 antibodies, Cat. no: ALX-210-314 for anti-CB1 and Cat. no: ALX-210-315 for anti-CB2, Enzo, Plymouth Meeting, PA, USA), the blotted nitrocellulose membranes (Whatman, Dassel, Germany) were rinsed thoroughly, and the appropriate secondary antibody conjugated to horseradish peroxidase was incubated for 1 hr at room temperature. For internal reference, polyclonal rabbit anti-

mouse β-actin antibody (1:2,000 dilution) (Abmart, Shanghai, China) was used. Finally, antibody binding was detected by exposure to ECL western blotting detection reagents (Cat. no: SC-2048, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and recorded on film.

Preparation of Isolated- vascularly Perfused Rat Stomach

Rat was anesthetized and the isolated, vascularly perfused rat stomach was prepared as described previously [20]. Briefly, the abdomen was opened with a midline incision under sterile condition. After ligation of the abdominal aorta just above the branching of the celiac artery, a cannula was inserted into the celiac artery via an incision placed on the aorta. Two milliliters of saline solution containing 600 U of heparin were then injected into the gastric artery via the arterial cannula. Subsequently, a warm (37°C) modified Krebs-Ringer solution bubbled with a mixture of 95% O_2 and 5% CO_2 was introduced. The venous effluent was collected via a portal vein cannula. A polyethylene tube for gastric lumen perfusate was inserted into the esophagus and the tip positioned in the luminal portion of the stomach. Afterward, the pyloroduodenal junction was exposed, and another polyethylene tube was introduced into the stomach via an incision on the duodenum, and then fixed by a ligature around the pylorus. The perfused rat stomach was isolated and placed in a warm (37°C) small chamber with Krebs-Ringer solution.

Treatment of the Isolated Rat Stomach

The isolated stomach was vascularly perfused with modified Krebs-Ringer solution for 30 min equilibration before the formal experiments. The perfusion was then carried out sequentially with three fluids and each fluid for 20 minutes, totaling 60 minutes. The control group got: 1) Krebs-Ringer solution, 2) serum from normal control rats, 3) Krebs-Ringer solution. The AP group got: 1) Krebs-Ringer solution, 2) serum from AP rats, 3) Krebs-Ringer solution. The group of AP+HU got: 1) Krebs-Ringer solution+HU210 (10^{-7}M), 2) AP serum+HU210 (10^{-7}M), 3) Krebs-Ringer solution. And the group of AP+AM got: 1) Krebs-Ringer solution+AM251 (10^{-7}M), 2) AP serum+AM251(10^{-7}M), 3) Krebs-Ringer solution. The gastric lumen of the isolated stomach was perfused with normal saline (pH 7.0). All perfusion fluids ran at a constant rate of 1 ml/min by using micro-infusion pumps. Meanwhile, the solutions and the isolated organs were kept at 37°C by thermostatically controlled units throughout the experiment. The samples from venous effluent or from gastric lumen effluent were collected, at the end of every 20 minutes, into chilled test tubes that were immediately stored at –80°C for subsequent measuring experiments.

Amylase and Lipopolysaccharide Levels

The assays of amylase and lipopolysaccharide (LPS) levels in the serum from AP or control rats were performed based on the manufacturer recommended procedures (Cat. No: C016 for amylase assay kit, Jiancheng Technology, Nanjing, China; and Cat. No: CE32545 for LPS assay kit, Chinese Horseshoe Crab Reagent Co. Ltd., Xiamen, China).

Assays for Inflammatory Mediators

The levels of interleukin-6 (IL-6) and cytokine-induced neutrophil chemoattractant 1 (CINC1/KC) in the serum of rat and in the venous effluent from the isolated rat stomach were quantified using the rat IL-6 and KC ELISA kits based on the manufacturer recommended protocols (Cat. no: F01731D for rat interleukin 6 ELISA kits; Cat. no: F01723D for rat KC ELISA kits. H-Y Biological Co. Ltd., Shanghai, China). The optical density was determined at 490 nm for absorbance in an enzyme-linked immunoabsorbent assay instrument (Microplate Reader, Model Elx800; BioTek, Winooski, VT, USA). Each specimen was measured three times and the measurement was repeated in 6 rat samples of each group. The data in the venous effluent from the isolated rat stomach was presented with the difference of IL-6 or KC level between the perfusion and the effluent, being considered as the release of IL-6 and KC from the rat stomach.

Gastrin and Somatostatin Levels in Animal Specimens

Gastrin and somatostatin levels in the animal serum and in the isolated stomach venous effluent were measured using commercially-available gastrin and somatostatin radioimmunoassay Kits (Gastrin Kit: Cat. No. G01PJB, North Institute of Biologic Technology, Beijing, China. Somatostatin Kit: Cat. No. S111013, Second Military Medical University, Shanghai, China). Measurement procedures were based on the manufacturers' recommendations as described before [21]. As same as above, each specimen was measured three times and the measurement was repeated in 6 rat samples of each group. The data in the venous effluent from the isolated rat stomach was presented with the differences of gastrin or somatostatin level between the perfusion and the effluent, being considered as the release of gastrin and somatostatin from the rat stomach.

Pepsin and H^+ Levels in Animal Specimens

The assays of pepsin level in the rat gastric juice and in the gastric lumen effluent from the isolated rat stomach were performed using the manufacturer recommended protocols (Cat. No. A081-1, Jiancheng Technology, Nanjing, China), as $[H^+]$ in these samples were measured by delta 320 pH-meter (Mettler-Toledo Inc. Zurich, Switzerland) and the readings were then converted to $[H^+]$.

Solutions and Chemicals

HU210 [(6aR)-trans-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl -6H-dibenzo[b,d]pyran-9-methanol], AM251 [(N-(Piperidin-1-yl)-5-(4-iodophenyl)-1- (2,4-dichlorophenyl)- 4-methyl-1H-pyrazole-3-carboxamide)], were purchased from Tocris (Tocris-Bioscience, Ellisville, MO, USA). Both chemicals were dissolved in a solvent consisted of ethanol, Tween80 and normal saline (NS), volume ratio 1:1:18, to concentration 10^{-2}M, at 37°C using an ultrasonicator, and then were further diluted in NS to 10^{-5} M, and again to 10^{-7}M with perfusion fluid just before use under the conditions that were determined in pilot study [22]. The modified Krebs-Ringer solution was composed of 117.5 mM NaCl, 4.7 mM KCl, 2.4 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.05% of bovine serum albumin, and 4% dextran. Other agents, if sources were not mentioned above, were purchased from Sigma (Shanghai, China).

Data Analysis

All data are expressed as mean ± SEM. Student's t-test or single factor analysis of variance (ANOVA) was performed using the SPSS 13.0 software (SPSS Co. Ltd., Shanghai, China). P-values of <0.05 were considered statistically significant.

Results

Results from Experiment *In Vivo*

Pathological changes in the pancreas of AP rats.

Under light microscopy, it was evident that after treatment with sodium taurocholate, rats developed severe acute pancreatitis with obvious edema, vacuolization and serious necroses in the acinar cells of the pancreatic tissues. And the histological scores in AP rats were much higher than those of the control rats (Fig. 1). Combined with the increased level of amylase activity in the serum of AP rats, the results demonstrated that the AP model replication in rats was successful.

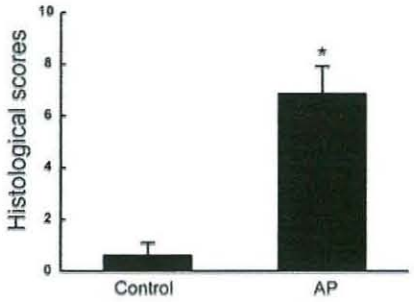

Figure 1. Histological scores for pancreas sections of the control and AP rats.
After the induction of acute pancreatitis, rats were sacrificed and organs were harvested. Using the harvested pancreas, histological slides were prepared, stained, examined under microscopy, and scored, as described in MATERIALS AND METHODS. The data are expressed as mean ± SEM (n=6), *P<0.01 vs control group.
http://dx.doi.org/10.1371/journal.pone.0052921.g001

Pathological changes in the stomach of AP rats.

In the stomach of the rats with acute pancreatitis, severe pathological changes emerged, exhibiting mucosal edema, erosion and hemorrhages as demonstrated by both macrography (Fig. 2A and 2B) and microscopical examinations (Fig. 2D); and these injuries congregated mainly in the gastric antrum.

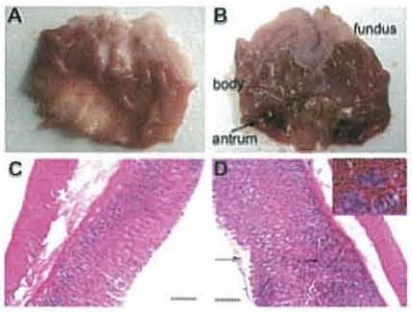

Figure 2. Morphological changes of the isolated stomach from rats with or without experimental acute pancreatitis.
(A) Stomach from a control rat. (B) Stomach from an AP rat, showing severe edema and hemorrhages on the gastric antrum (arrowheads). (C) A representative tissue section of the stomach of a control rat, and (D) A representative tissue section of the stomach of an AP rat (hematoxylin and eosin staining, with original magnification ×100; the scale bar=100 μm). The hemorrhages and mucosal erosions were observed and marked with arrowheads.
http://dx.doi.org/10.1371/journal.pone.0052921.g002

GeneChip analysis.

As shown in Fig. 3A, the scatter plots represented genes with two-fold and higher expression were in the upper (red) boundary, while genes with two-fold and lower expression in the lower (green) boundary; and the changed genes closely linked to the acute pancreatitis were shown in the clustering patterns (Fig. 3B). It was obvious that in the expression profile, the genes with significantly differential expressions (≥2-fold, P<0.05) are mainly those which were related with the pancreatic digestive enzymes, inflammatory mediators and the signal transduction pathways, which were singled out and listed with their Gene Name and Genebank ID in Table 1.

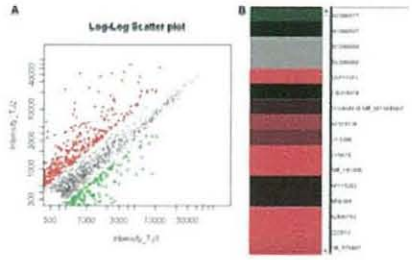

Figure 3. The analyzed expression profile of selected genes in AP rats using a genechip software.
(A) The Scatter Plots illustrate the the relative gene expression in pancreas of AP and control rats. Red dots represent genes that were upregulated at least 2-folds (≥2× value of the control, P<0.05), as green dots represent genes downregulated at least 2-folds (≤0.5× value of the control, P<0.05), depicted with the upper and lower boundaries, respectively. (B) The clustering patterns illlustrate the 15 chosen genes (with their Genebank ID) that are closely linked to acute pancreatitis. Red bars symbolize the genes that were upregulated 2-folds or more (P<0.05) and green bars the genes that were downregulated 2-folds or more (P<0.05). Each sample was triplicated and the upregulated genes, alongwith their Gene Name and Genebank ID were singled out and listed in Table 1.
http://dx.doi.org/10.1371/journal.pone.0052921.g003

Table 1. The differentially expressed genes in the pancreas of the rats with acute pancreatitis.
http://dx.doi.org/10.1371/journal.pone.0052921.t001

Changes of IL-6, KC and LPS levels in AP serum.

Both IL-6 and KC levels in the serum of AP rats displayed significant increases as compared to those of control rats, with upsurges of 145% and 186%, respectively (P<0.05; Fig. 4). A similar but more prominent increase was seen in the LPS level in the serum of AP rats, with an upsurge as much as 231 times of that of the control group (P<0.01; Fig. 4A).

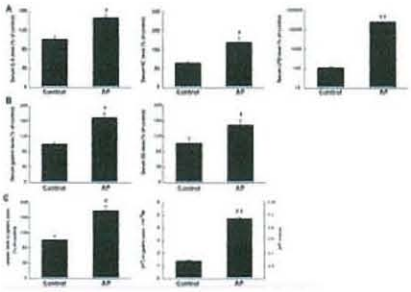

Figure 4. Changes of the components in serum and in gastric juice of rats with experimental acute pancreatitis.
(A) IL-6, KC and LPS levels in rat serum. (B) Gastrin and somatostatin levels in rat serum. (C) Pepsin levels and [H^+] in rat gastric juice. Each specimen was measured three times and data are expressed as mean ± SEM (n=8). *P<0.05 vs control, **P<0.01 vs control.
http://dx.doi.org/10.1371/journal.pone.0052921.g004

Changes of gastrin and somatostatin levels in the serum of AP rats.

In the serum of AP rats, gastrin and somatostatin levels increased significantly as compared to those of control rats, with upsurges of 169% and 147%, respectively (in both cases, P<0.05; Fig. 4B).

Changes of pepsin levels and [H^+] in gastric juice of AP rats.

To evaluate the changes of gastric exocrine function, assays for pepsin level and [H^+] were performed by using the gastric juice of AP and control rats. Both pepsin level and [H^+] in the gastric juice showed a distinct increase in AP rats as compared to those of control rats, with upsurges of 177% and 347%, respectively (Fig. 4C).

Expression of CB1 and CB2 receptors in rat pancreas and stomach.

The expression characteristics of CB1 and CB2 receptors in rat pancreas and stomach were investigated. The results demonstrated that the specimens from animals in control group presented only weak immunohistological staining for CB1 and CB2 receptors in the pancreas, whereas specimens from AP rats had exhibited increased expressions of CB1 and CB2 receptors. Mainly, the strong positive signs of brown dyeing clustered in the pancreatic acini (Fig. 5 A arrowheads). The up-regulations of CB1

and CB2 receptors in the pancreatic tissues of AP rats were further demonstrated by western blot analysis and presented in Fig.5 B. The similar expression characteristics of CB1 and CB2 receptors were also found in the stomach of the AP rats, as demonstrated by both immunohistological staining and western blot assay (Fig. 5 C and 5 D). The strong positive signs of brown dyeing were mainly in the gastric mucosa (Fig. 5 C, arrowheads).

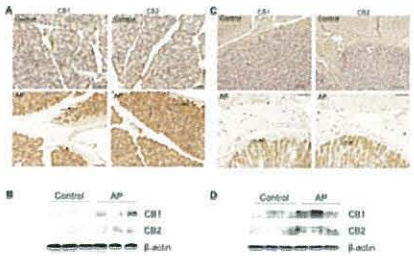

Figure 5. Expression of CB1 and CB2 receptors in rat pancreas and stomach by immunohistochemistry and western blot analyses.
(A) Immunohistochemical detection of CB1 and CB2 receptors in rat pancreatic tissue sections, with the arrowheads showing the specific CB1/CB2 staining. (B) Western blot staining of CB1 and CB2 receptors in rat pancreatic tissue lysates. (C) Immunohistochemical detection of CB1 and CB2 receptors in rat stomach tissue sections, with the arrowheads showing the specific CB1/CB2 staining. (D) Western blot staining of CB1 and CB2 receptors in rat stomach tissue lysates. Note that the pancreatic acini and gastric mucosa exhibit increased immunological activity for CB1 and CB2 receptors after the induction of acute pancreatitis. (Original magnification: ×200, and scale bar=50 μm).
http://dx.doi.org/10.1371/journal.pone.0052921.g005

Results from Experiment *In Vitro*

Effect of cannabinoids on gastric pathological changes and on gastrin and somatostatin release.

To investigate the effect of CB1 receptor agonist HU210 on the endocrine function of the isolated rat stomach stimulated with AP rat serum, we examined the alterations of gastrin and somatostatin levels in the venous effluent of the stomach, with or without intervention of CB1 receptor agonist HU210 and antagonist AM251. The results showed that compared to the control group, the rat stomach treated with AP serum provoked an increased gastrin release ($P<0.05$), but a decreased somatostatin release ($P<0.05$), HU210 reversed the gastrin and somatostatin changes induced by serum of AP rats ($P<0.05$), while AM251 did not exhibit detectable impact on the release of the two hormones (Fig. 6).

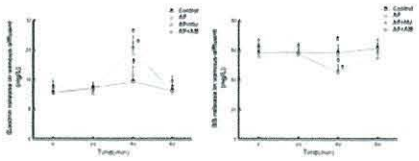

Figure 6. Effects of HU210 and AM251 on gastrin and somatostatin (SS) release from the isolated rat stomach.
As described in MATERIALS AND METHODS, the levels of gastrin and somatostatin were measured in the gastric venous effluent of rats during 60 min perfusion with or without the administration of HU210 or AM251. Each specimen was measured three times and data are expressed as mean ± SEM (n=6). *P<0.05 vs control, #P<0.05 vs those in AP group.
http://dx.doi.org/10.1371/journal.pone.0052921.g006

Effects of cannabinoids on pepsin activity and [H^+] in the gastric lumen effluent.

The effects of the agents HU210 and AM251 on pepsin activity and [H^+] in the gastric lumen effluent of the isolated rat stomach were presented in Fig. 7. Compared to the counterparts of the control group, AP serum stimulated the pepsin secretion and acid output in the isolated rat stomach ($P<0.05$). The intervention of CB1/2 receptor agonist HU210 attenuated the AP serum-induced changes of pepsin secretion and acid output ($P<0.05$), while the receptor antagonist AM251 failed to exhibit obvious effect on these two parameters.

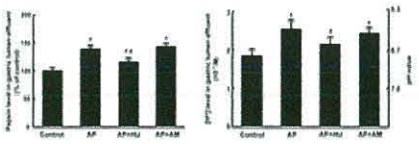

Figure 7. Effects of HU210 and AM251 on pepsin and acid output from the isolated rat stomach.
The levels of pepsin and [H^+] were measured in the rat gastric lumen effluent with or without the administration of HU210 or AM251. Each specimen was measured three times and data are expressed as mean ± SEM (n=6). *P<0.05 vs control, #P<0.05 vs those in AP group.
http://dx.doi.org/10.1371/journal.pone.0052921.g007

Effects of cannabinoids on the levels of IL-6 and KC in the gastric venous effluent of rats.

After the rats received the treatment of the AP serum, IL-6 and KC levels significantly elevated in the venous effluent from the isolated rat stomach; HU210 reversed the IL-6 and KC changes induced by serum of AP rats ($P<0.05$), while AM251 had no detectable impact on the levels of the cytokine and chemokine in the venous effluent (Fig. 8).

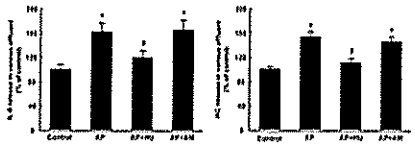

Figure 8. Effects of HU210 and AM251 on the releases of IL-6 and KC from the isolated rat stomach.
The levels of IL-6 and KC were measured in the rat gastric venous effluent as described in MATERIALS AND METHODS, Each specimen was measured three times and data are expressed as mean ± SEM (n=6). *P<0.05 vs control, #P<0.05 vs those in AP group.
http://dx.doi.org/10.1371/journal.pone.0052921.g008

Discussion

In clinic, the patients with acute pancreatitis, especially with severe acute pancreatitis, often suffer AGML or stress ulcer, a common complication of AP. The causative factors for stomach injury include, but not limited to, the stress from the inflammatory stimulation which can induce the activation of the locus ceruleus-norepinephrine/sympathetic-adrenal medulla system and the hypothalamus-pituitary-adrenal cortex system. The secretive increases of catecholamines and glucocorticoid hormones are the most important factors of body stress, for these components provoke gastric acid hyper-secretion and blood-flow shifting that cause gastrointestinal mucosal ischemia. Importantly, the ischemia sequentially downgrades the ability of the gastric mucosa to dispose of back-diffusing acid, resulting in a decrease of intramural pH and activation of protease, and subsequent ulceration [3], [4], [23]. Other mechanisms, including oxygen-derived free radicals and some uncertain factors, also play roles in the gastrointestinal injury related with acute pancreatitis.

Previous investigations have found that AP plasma and AP-related ascitic fluid contain a large amount of toxic substances which are harmful to the body [5], [6], [8], causing the damage of the liver, kidney, lung and circulatory system, and gastrointestinal dysfunction, etc. [24]–[28]. Our prior study discovered that the pancreatic acinar cells suffered calcium overload and reduced vitality, as being incubated with AP serum or ascitic fluid [8]. In this study, we first induced experimentally AP in rats and proved the induction of AP animal model was successful by demonstrating the pathological change of pancreatic morphology and the increase of pancreatic enzyme in rat serum after the induction. Upon affirmation of the model, we continued to establish a gene expression profile to illustrate the altered gene expression of pancreatic enzymes and inflammatory mediators, in an attempt to trace the underline genes that played most critical roles in the pathogenesis of AGML associated to AP. And the results from AP and control rats profiled using gene chip analysis were consistent with those of biochemical assays. In addition, we tested if there were beneficial effects of cannabinoid antagonists and/or agonists in the animals with experimental acute pancreatitis.

Based on the aforementioned results, we addressed the question whether gastric secretion, both the endocrine or exocrine functions, would be altered in AP rats. It is known that gastrin stimulates acid output and pepsin secretion, as somatostatin counteracts the effects of gastrin. When gastrin or somatostatin secretion fails to maintain a basic equilibrium, the surplus pepsin and acid release disproportionally, resulting in damages and dysfunctions of the stomach during acute pancreatitis. As demonstrated in this report, we found a significantly raised gastrin level in serum, and elevated pepsin and acid levels in the gastric juice of AP rats, which confirmed that the endocrine and exocrine functions of the stomach were disturbed in the AP model.

Moreover, the circulating activated proteolytic enzymes, vasoactive proteins and endotoxin specific to the pathogenesis of acute pancreatitis may be responsible for AGML as well. Therefore, we explored the effects of the serum from AP rats on the isolated and perfused rat stomach such that the organ could ignore the systemic stress and impacts. The isolated rat stomach stimulated by serum of AP rat not only showed the eye-visible mucosal injury, but also presented a series of biochemical abnormalities, including higher levels of gastrin, cytokine IL-6, chemokine KC, and lower level of somatostatin in the gastric venous effluent, as well as raised pepsin and acid output in the gastric lumen effluent. It is reasonable to infer that there is an imbalance between the aggressive factor and the protective factor of the gastric mucosa during acute pancreatitis. In particular, the increased gastrin, gastric acid output and pepsin jointly play important roles in the pathogenesis of AGML, aggravating the damage of the stomach and triggering vicious cycles during acute pancreatitis.

During the last decade, a number of publications have shown the anti-inflammatory effects of cannabinoids [29]–[32]. Several studies have shown that cannabinoids inhibit gastric acid secretion and reduce the inflammatory cytokines and other mediator in the plasma of animals with AP [33], [34]. Our results not only confirm these earlier discoveries, but also demonstrate that a chemical HU210, presumably a cannabinoid receptor agonist, serve functions in the same way as cannabinoids in reducing the inflammatory cytokines and other mediators, hence ameliorate the symptoms of AP-associated AGML. Interestingly, the results of this study demonstrate that HU210 can attenuate the gastric endocrine and exocrine changes in the isolated rat stomach irritated by AP serum, reverse the abnormally inflated levels of gastrin, gastric acid and pepsin and muffle the effect of these damaging factors. On the other side, HU210 raises the level of somatostatin which inhibits secretion of gastrin and gastric acid, hence exerts protective action on the gastric mucosa.

The outcomes of the study provide harmonic coherence of gene-chip analysis and biochemical assay data using samples from the animal model, suggesting a novel mechanism that the onset of AGML is, at least partly, due to the gastrin, and gastric acid / somatostain imbalance triggered by the toxins in the AP serum; and cannabinoid agonist HU210 restores the equilibrium, hence the

protection. The findings support that HU210 is beneficial for treating acute pancreatitis because of its anti-inflammation role and the preventing effect on the AGML related with acute pancreatitis. The results that the CB1 receptor antagonist AM251 fails to play any role in the AP induced gastric damage support our postulation, confirming the positive roles of CB1/2 receptors.

In a prospective experiment to investigate if the proton pump inhibitors (PPIs) can protect animals with experimental acute pancreatitis, we administered omeprazole (OME, i.p., 40 mg/kg weight), a representative PPI agent, to a group of rats at the same time when AP induction was performed. The preliminary results showed that OME increased the survival rate of AP rats (data not shown). However, it may need multicenter study to elucidate if PPIs are beneficial as a therapeutic option in acute pancreatitis of humans.

Taking all above, the results from our experimental investigation reveal that the inflammatory responses and the disturbances of the gastric secretion, both the endocrine and exocrine functions, are the outcomes of acute pancreatitis, and they in turn contribute to the pathogenesis of AGML. Furthermore, the results suggest that cannabinoid HU210, the CB1/2 receptor agonist, has the therapeutic potential for AGML in acute pancreatitis by attenuating inflammation and restoring gastrin/somatostatin equilibrium, and then decreasing the secretion of gastric acid and pepsin. Therefore, our experimental results suggest a novel mechanism in the onset of AGML and new therapeutic values of cannabinoids as supplement of anti-inflammatory therapy in acute pancreatitis.

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Author Contributions

Conceived and designed the experiments: YYL CJC. Performed the experiments: MHC YYL JX YJF XHL KL TH. Analyzed the data: MHC YYL. Contributed reagents/materials/analysis tools: YYL MHC. Wrote the paper: MHC YYL CJC.

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