The Cannabinoid 1 Receptor Antagonist, AM251, Prolongs the Survival of Rats with Severe Acute Pancreatitis

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The Cannabinoid 1 Receptor Antagonist, AM251, Prolongs the Survival of Rats with Severe Acute Pancreatitis. Tohoku J. Exp. Med., 2005, 207 (2), 99-107 —— It has recently been recognized that anandamide (arachidonylethanolamide), which is an endogeneous-cannabinoid (endocannabinoid), mediates septic shock. Cannabinoid means a mind-active material in cannabis (marijuana). Anandamide is mainly produced by macrophages. Cannabinoid 1 (CB1) receptor, which is one of the cannabinoid receptors, is also known to mediate hypotensive shock. The role of endocannabinoids in the progression of acute pancreatitis is unclear. The aims of this study are to clarify their relationship and to find a new therapeutic strategy by regulating the endocannabinoid signaling in acute pancreatitis. Male Wistar rats were injected with caerulein intravenously to induce mild edematous pancreatitis or injected with 5% sodium taurocholate to the bilio-pancreatic duct to induce severe necrotizing pancreatitis. The animals in the latter group were also injected with a CB1 receptor antagonist, AM251, or vehicle solution to see if the inhibition of endocannabinoids improves their survival. Plasma anandamide level was measured by the liquid chromatography/tandem mass spectrometry method. In both models of acute pancreatitis, the plasma anandamide levels were increased, and the levels were significantly higher in rats with severe necrotizing pancreatitis than those in rats with mild edematous pancreatitis. The mean arterial pressure and survival rate were significantly improved by the treatment with AM251, despite that the local inflammatory changes in the pancreas and various parameters (white blood cells, hematocrit, serum amylase, and serum interleukin-6) were similar. This is the first report to show that endocannabinoids are involved in the deterioration of acute pancreatitis and that the down-regulation of endocannabinoid signaling may be a new therapeutic strategy for severe acute pancreatitis.

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Severe acute pancreatitis is one of the intractable pancreatic diseases, in which the mortality rate extends to 20%, yet there are still many unclear points about the deterioration mechanism. However, it recently became clear that several mediators such as cytokines, reactive oxygen species, and platelet-activating factor (PAF), participate in the deterioration of acute pancreatitis (Nonaka et al. 1992; Yotsumoto et al. 1994; Rongione et al. 1997) and new therapeutic strategies for these have been developed. Despite the evidence, it is difficult to conclude that adequate results are provided (Kingsnorth et al. 1995; Dumot et al. 2001; Schulz et al. 2001). Therefore, discovery of a new factor participating in the deterioration of acute pancreatitis and development of a new therapy are desired.

In 1964, tetrahydrocannabinol (THC) was isolated and identified as a mind-active material in cannabis (marijuana) (Mechoulam and Gaoni 1965), which has many similar structures, termed cannabinoids. The cDNA of a cannabinoid 1 (CB1) receptor for cannabinoids was cloned from the brain (Matsuda et al. 1990). Subsequently arachidonic acid derivatives, anandamide (arachidonylethanolamide, AEA) (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al. 1995), were discovered as candidates of endogenous ligands of the receptor. Anandamide is mainly produced by macrophages, and 2-AG is by platelets.

The CB1 receptor, which is one of the cannabinoid receptors, is expressed in the central nervous system, endothelial cells and the smooth muscle cells of blood vessels. The stimulation of CB1 receptor brings about hypotension, tachycardia, and disturbance of consciousness (Lake et al. 1997). It has also been established that endocannabinoids are one of the mediators in septic shock (Varga et al. 1998). In addition, it was reported that the endotoxin-absorption therapy (PMX-DHP, direct hemoperfusion with polymixin-B immobilized fiber) absorbs endocannabinoids as well as endotoxins and that it is effective even in some cases of septic shock when endotoxins are not high level. This suggests that absorption/removal of endocannabinoids may improve shock (Wang et al. 2000, 2001).

Severe acute pancreatitis frequently causes circulatory insufficiency and disturbance of consciousness. Although endocannabinoids may participate in the deterioration of acute pancreatitis, the relationship between acute pancreatitis and endocannabinoids is not clarified. The primary aim of this study is to clarify the role of endocannabinoids in the deterioration of acute pancreatitis through animal experiments.

**Materials and Methods**

**Animals**

Male Wistar rats weighing 200-250 g were maintained at 23°C in a 12 h: 12 h light-dark cycle and allowed free access to water and standard laboratory chow. Twelve hours prior to the start of the experiments, the animals were deprived of food but allowed access to water. This study was conducted with the consent of the Ethics Committee for the Use of Experimental Animals of Tohoku University School of Medicine.

**Plasma anandamide level and immunohistochemical examination of the CB1 receptor in acute pancreatitis**

**Study design.** Rats were randomly divided into three groups: (a) Normal rat control (Normal) ($n = 4$); (b) Caerulein-induced pancreatitis (CE-AP) ($n = 16$); and (c) 5% sodium taurocholate induced pancreatitis (TCA-AP) ($n = 16$).

Caerulein pancreatitis rats were used as the mild edematous acute pancreatitis model and 5% sodium taurocholate pancreatitis rats were used as the severe necrotizing pancreatitis model.

Under diethylether (Wako Pure Chemical, Osaka) anesthesia, CE-AP was induced by a bolus injection of caerulein (Sigma, St. Louis, MO, USA, 100 μg/kg body weight) into the right jugular vein. TCA-AP was induced by the method of Aho et al. (1980). In brief, under diethylether anesthesia and laparotomy, 5% sodium taurocholate (TCA; Sigma, 1 ml/kg body weight) was injected into the biliopancreatic duct at a rate of 0.2 ml/min with a microinfusion pump (MINIPULS 3; Gilson Medical Electronics, Villiers le Bel, France).

Under diethylether anesthesia, blood was collected after 1, 6, 12, and 24 hours, and the plasma anandamide was measured in each group. The plasma was separated by 3,000 rpm, 10 min centrifusion, and stored at −80°C. In all rats, the pancreas and kidney were resected at 12
hours after the induction of pancreatitis.

Histological examination. The pancreas and the kidney were fixed by immersion in 5% paraformaldehyde, dehydrated and then embedded in paraffin wax. Sections of 3 μm thickness were cut, dewaxed, and stained with haematoxylin and eosin for histological examination. A pathologist blind to the experimental design performed histological assessment.

Plasma anandamide level. The plasma anandamide level was measured by liquid chromatography/tandem mass spectrometry (LC/MS/MS). Internal standard material was added in the measurement samples, and the samples were stuck to a cartridge (OASIS HLB, Waters, Milford, AR, USA). After washing with distilled water and hexan, the samples were extracted with ethyl acetate. Then, the samples were concentrated and dried at 40°C under nitrogen current, and then dissolved in 80% methanol again. Finally, the fraction containing anandamide was collected using ultrafiltration (ULTRAFREE-MC, Millipore, Billerica, MA, USA, 10,000 rpm, 3 min, 4°C), and measured on API3000 (Applied Biosystems/MDS, Ontario, Canada).

Immunohistochemical localization of CB1 receptors. The organs were embedded in OCT compound (Tissue-Tek) (Sakura Finetek, Torrance, CA, USA) at −80°C. Sections of 5 μm thickness were cut using cryostat (CM3000, LEICA, Wetzlar, Germany) and were fixed in acetone.

1) Enzyme antibody method: The sections were pre-treated in phosphate buffered saline (PBS), 0.1% sodium azide and 0.3% H₂O₂/PBS and blocked in 10% bovine serum albumin (BSA) (Wako Pure Chemical). Anti-CB1 receptor antibody (rabbit, Cheyman chemical, Ann Arbor, USA) or CD31 antibody (mouse, BD Biosciences, Franklin Lakes, USA) were added as a primary antibody, and peroxidase-conjugated second antibody (Jackson ImmunoResearch, West Grove, USA) was used. Coloring reaction was made with DAB (3, 3-diaminobenzidine, Wako Pure Chemical) with haematoxylin nucleus counter staining.

2) Fluorescence antibody method (double staining): Some of the sections were blocked in 10% BSA and prepared with anti-CB1 receptor rabbit antibody and anti-CD31 mouse antibody mixture. After washing in PBS, FITC-conjugated anti-rabbit antibody and Texas Red-conjugated anti-mouse antibody (Jackson Immuno Research) mixture were added as the second antibodies. CB1 receptor positive cells (Green) and CD31 positive cells (Red) were observed.

Treatment with a CB1 receptor antagonist

Study design. In the severe pancreatitis model (TCA-AP), the effects of CB1 receptor antagonist were analyzed. Just after inducing severe pancreatitis by TCA injection and 6 hours later, a CB1 receptor antagonist, AM251 (Tocris Cookson Inc., Ballwin, USA, 3 mg/kg per once) (Gatlay et al. 1996), dissolved in the mixture of ethanol, vegetable oil, and saline (1 : 1 : 18), was injected into the right jugular vein (AM251 [+], n = 6). As a control, animals group were induced severe pancreatitis but were injected only vehicle (AM251 [−], n = 6).

Histological examination of pancreas. The pancreas was resected in all rats at 12 hours after the induction of TCA-AP for histological examination.

Measurement of white blood cells, hematocrit, serum amylase, serum interleukin (IL)-6, and mean arterial pressure. Blood samples were drawn at 12 hours after the induction of TCA-AP. The number of white blood cells, hematocrit, serum amylase, and serum IL-6 were measured. The concentration of serum IL-6 was measured with Rat IL-6 ELISA kits (TFB, Tokyo). Each assay was performed in duplicate with 100 μl of supernatant using 96-well plates. The detection limit of these assays was 10 pg/ml. For measurement of mean arterial pressure (MAP), 24G catheter was inserted into the right femoral artery after anesthesia by Nembutal (Dainippon Pharmaceutical, Osaka, Japan; 1 ml/kg body weight, subcutaneous injection), and was connected to the DMS-760 (Nishiyama Production, Osaka).

Survival rate. We analyzed the AM251 (+) group (n = 10) and the AM251 (−) group (n = 10), treated as described above. After the second injection of AM251, rats were allowed free access to water and food, and the survival time was observed for 72 hours after the induction of pancreatitis.

Statistical analysis

Results are expressed as means ± s.e.m. One-way Analysis of variance (ANOVA) and Fisher protected least significant difference post hoc tests were used to determine the significance for plasma anandamide and serum IL-6 levels. Differences for other studies were evaluated with a two-tailed unpaired Student’s t-test. Survival rate was evaluated by the log rank method. A p value of less than 0.05 was accepted as significant.
RESULTS

Plasma anandamide level and immunohistochemical examination of the CB1 receptor in acute pancreatitis

At 12 hours after induction of pancreatitis, the remarkable edematous swelling and slight inflammatory cell permeation were recognized in the CE-AP rats, while there was broad necrosis of acinar cells in the TCA-AP rats (Fig. 1).

In CE-AP rats, there was a slight increase of plasma anandamide but the difference was not statistically significant from that of normal rats. On the other hand, significant increase was observed in TCA-AP rats after the induction of pancreatitis (Fig. 2).

The enzyme antibody method was used in the renal artery of the normal rat and the TCA-AP rat. CB1 receptors were expressed in the endothelium and the smooth muscle of the blood vessel and CD31 was strongly stained only in the endothelium of the blood vessel. There was no recognizable difference between the CB1 staining in normal rats and in the TCA-AP rats (Fig. 3). The fluorescence antibody method revealed that the CB1 receptor was localized in the smooth muscle of the renal artery and the pulmonary artery of the TCA-AP rats. Co-localization of CB1

Fig. 1. Histological examination of pancreas; H-E (Hematoxylin and eosin) staining.
The remarkable edematous swelling and slight inflammatory cell permeation in the CE-AP rat, and broad necrosis of acinar cells in the TCA-AP rat were recognized.

Fig. 2. Plasma anandamide level; Mean ± s.e.m. (n = 4), * p < 0.05 vs CE-AP group.
There was no significant difference between normal rats and CE-AP rats, but there was significant increase in TCA-AP rats at 6, 12, and 24 hours after the induction of pancreatitis.
In the kidney of the normal and the TCA-AP rats, CB1 receptor was stained in the endothelium and the smooth muscle of the blood vessel and CD31 was strongly stained in the endothelium of the blood vessel. There seems to be no remarkable differences between the staining of normal rat and that of the TCA-AP rat.

CB1 receptor was strongly expressed in the smooth muscle of the arteries in the kidney and the lung of the TCA-AP rats. Similarly, CB1 receptors were also partially recognized in the endothelium of the blood vessel. Co-localization of CB1 receptor and CD31 were stained in yellow.
receptors and CD31 were stained in yellow, suggesting that CB1 receptors are also partially recognized in the endothelium of the blood vessel (Fig. 4).

**Effects of a CB1 receptor antagonist**

In order to clarify the role of endocannabinoids in the deterioration of acute pancreatitis, a CB1 receptor antagonist, AM251, was administered in TCA-AP rats. Massive areas of necrosis were observed in both the AM251 (+) group and the AM251 (−) group, accompanied by infiltration with inflammatory cells and hemorrhages compatible with severe acute pancreatitis. The histological findings of local inflammation in the pancreas were not different regardless of the injection of AM251 (Fig. 5).

Serum amylase, white blood cells, hematocrit and serum IL-6 were measured. All parameters showed remarkable increase, but neither recognized significant difference between AM251 (+) group and AM251 (−) group (Table 1).

Although the blood examination and local inflammatory changes in the pancreas were not different regardless of the injection of AM251, the MAP and the survival rate was significantly improved by the injection of AM251. At six hours after the induction of pancreatitis, the MAP of the AM251 (+) group was significantly higher than that of the AM251 (−) group. After the second injection, the MAP elevated immediately in both groups, but this was thought to be a volume effect. Briefly, in the AM251 (+) group, the MAP was improved significantly compared with the

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**Table 1. Comparison of parameters in AM251 (+) group and AM251 (−) group; Mean ± s.e.m. (n = 6)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>TCA-AP</th>
<th>AM251 (−)</th>
<th>AM251 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum amylase (IU/l)</td>
<td>5,710 ± 560</td>
<td>9,945 ± 419</td>
<td>9,942 ± 448</td>
<td></td>
</tr>
<tr>
<td>WBC (μl)</td>
<td>9,900 ± 3,010</td>
<td>11,833 ± 2,246</td>
<td>8,375 ± 1,644</td>
<td></td>
</tr>
<tr>
<td>Ht. (%)</td>
<td>39.9 ± 3.49</td>
<td>57.8 ± 7.2</td>
<td>57.6 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>81.0 ± 22.0</td>
<td>177.1 ± 174.2</td>
<td>197.9 ± 160.2</td>
<td></td>
</tr>
</tbody>
</table>

Serum amylase, white blood cells, hematocrit, and serum IL-6 were measured. Both parameters showed high value, but neither recognized significant difference.
AM251 (−) group (Fig. 6).

The survival was observed for 72 hours after the induction of pancreatitis. The survival of rats in AM251 (+) group was significantly higher than that of AM251 (−) group (AM251 [+]: 60% [6/10], AM251 [−]: 10% [1/10]; p < 0.05) (Fig. 7).

**DISCUSSION**

Cannabinoid is the principal ingredient of cannabis (marijuana). The CB1 receptor is mainly expressed in the central nervous system, the endothelial cells and the smooth muscle cells of the blood vessel. Cannabinoid also shows mind nervous system action and vasodilatation (Lake et al. 1997). Also, the CB2 receptor is located in blood cells and immunity system cells, which are used in the immune system control (Facci et al. 1995). Cannabinoids stimulate/excite the nervous system, promote coagulation, and activate immune system whereas they create hypotension and have inhibitory effects on the nervous system, coagulation system, and immune system (Wang et al. 2000). In addition, anandamide, one of the endocannabinoids, is produced by macrophages and is a mediator of septic shock (Varga et al. 1998).

Even though severe acute pancreatitis can produce circulatory insufficiency and disturbance of consciousness, a role of endocannabinoids in the pathogenesis of acute pancreatitis is not clarified. We measured the plasma anandamide level by LC/MS/MS method in normal rats, the CE-AP rats (mild pancreatitis model), and the TCA-AP rats (severe pancreatitis model).

Anandamide is an endogenous neurotransmitter which binds to the cannabinoid receptors and is proven to have a pharmacological action similar to THC, which is an active principle of cannabis (Felder et al. 1993). In this study, the plasma anandamide level was significantly elevated after the induction of severe acute pancreatitis. Severe pancreatitis model showed significantly high level when compared with the mild pancreatitis model. In the cases of sepsis, it has been proven that macrophages produce anandamide and participate in shock by LPS stimulation (Varga et al. 1998). In severe acute pancreatitis it bacteremia occurs by bacterial translocation (Runkel et al. 1991). However it is rare that complication of bacteremia is observed by 6 hours after the induc-
tion of acute pancreatitis, as reported in our previous study (Iwasaki et al. 1994). Since the plasma anandamide level was elevated six hours after the induction of acute pancreatitis in this study, anandamide production mechanism other than the production by endotoxin stimulation from macrophages should be further investigated.

The CB1 receptor antagonist AM251 is an analog of SR141716A (Gatlay et al. 1996). Injection of AM251 showed remarkable improvement in MAP and survival rate, but there was no clear change in the blood exam and histological findings of the pancreas. Endocannabinoids are known as materials inducing hypotension. It is thought that there are three pathways for this mechanism. First, nitrogen oxide (NO) is released by activating the CB1 receptor in the blood vessel. Second, endocannabinoids are taken into the cells through transporter to be used for PGI2 production. Third, calcitonin-gene related peptide (CGRP), which is known to cause hypotension, is produced by activating capsaicin receptor on nerve cells (Hogestatt and Zygmunt 2002). In a rat ischemic model, it was reported that injection of SR141716A, which is one of the CB1 receptor antagonists, brought about improvement of blood pressure and extension of survival time (Wagner et al. 1997). The mechanism of improvement in survival rate in this study is considered to be the inhibition of hypotension by blocking the CB1 receptor.

In summary, the rat experimental model of severe acute pancreatitis showed a significantly elevated level of plasma anandamide compared to the mild pancreatitis. The blood examination and local inflammatory changes in the pancreas were not different regardless of the injection of the CB1 receptor antagonist, AM251, after the induction of severe acute pancreatitis, but improvement of mean arterial pressure and survival rate were observed.

Conclusion

This is the first report to provide evidence that endocannabinoids are involved in the deterioration of acute pancreatitis. Using plasma anandamide level as an index of severity of acute pancreatitis a new therapeutic strategy of severe acute pancreatitis by down-regulation of endocannabinoid signaling is expected in the future.

References


Nonaka, A., Manabe, T., Kyogoku, T., Tamura, K. & Tobe, T.