

New mechanisms in gastric mucosal protection: the role of endocannabinoid system

Ph.D Thesis

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INTRODUCTION

Peptic ulcer disease (PUD) is endemic nowadays, affecting about 10-15% of the total population during their lifetime. In the past 20 to 30 years, the prevalence of PUD has been rapidly increasing, which can be explained by the development of preventive use of secretion inhibitors and *H.pylori* eradication therapy. However, it does not explain that it causes problems with hydrochloric acid among ~ 15% of the whole population, although hydrochloric acid production is a physiological process and about 20% of the *H. pylori* infected population develops ulcerative lesions. There is also a problem with the use of proton-pump inhibitors, so while the background of gastroduodenal ulcers is generally hyperacidism, in case of gastric ulcers, normo- or hypoacidity is often measured, and they do not represent sufficient protection against the development of increasingly commonly observed small intestinal lesions due to side effects of NSAIDs. For some diseases (e.g. stress-ulcer syndrome, which is commonly found in intensive care units in hospitals), they are strongly contraindicated because by increasing the gastric acid pH, they significantly increase the incidence of various infections and consistent aspiration pneumonia. Based on these data, it can be stated that only therapeutic mechanisms based on the reduction of aggressive factors (gastric acid neutralization, inhibition of acid secretion, *H. pylori* eradication) are not necessarily sufficient to heal the peptic ulcer, the complex mechanism of gastric mucosal defence and its therapeutic use are intensively researched topics.

Apart from peripheral factors (mucus bicarbonate-phospholipid barrier, mucosal microcirculation, prostaglandins, CGRP, SOM, etc.) in mucosal protection, the involvement of the central nervous system is also significant. The hypothalamus and the dorsal vagal complex (DVC) play an important role in the defence of the various brain structures, but the role of other structures (e.g. amygdala, nucleus accumbens) is also proven. Centrally induced gastric protection at the periphery is done through n. vagus mediation. The role of the vagus in gastrointestinal regulation is clearly demonstrated by the effects of acute vagotomy: reduced antral contractions and acid secretion, increased tone in the gastric fundus.

The endocannabinoid system

During my doctoral work, I studied the role of the endocannabinoid system in the gastric mucosal protection.

The role of the endocannabinoid system is a widely circulated and studied question in the physiological and pathophysiological processes of the gastrointestinal system. Both anandamide and 2-AG, as well as the enzymes responsible for their synthesis and degradation, just like CB₁- and CB₂-receptors, are found in the gastrointestinal tract and their several gastrointestinal effects were described, e.g. influencing acid secretion, reducing gastric motor activity, inhibition of gastric emptying. Cannabinoids have been shown to be protective in several acid-dependent and acid-independent ulcer models. They blocked acid secretion through CB₁-receptor, thus exerting a protective effect on acid-dependent ulcer models, and endogenous cannabinoid anandamide has been shown to be protective against stress ulcers (cold-restraint stress model/water immersion stress model). In both non-steroids and stress-induced ulcers, the formation of lesions is due to increased acid secretion, meaning that cannabinoids inhibit the formation of lesions by their secretion-inhibiting effect. However, few data are available with models that are independent of acid secretion. For these models, ulcers are caused by mucosal necrotizing agents (e.g. absolute alcohol, acid alcohol, 0.2 N NaOH, 25% NaCl), so that enhancement of mucosal protection, i.e. cytoprotection is required to prevent erosion. In our previous group experiments, anandamide, methanandamide and WIN 55,212-2 were investigated by i.v. and i.c.v. administration in an alcoholic ulcer model. All three compounds induced a significant inhibition of gastric mucosal injury evoked by ethanol, i.c.v. injected selective CB₁-receptor antagonist (SR141716A) inhibited the development of the protective effect of the all substances, which indicates the involvement of central CB₁-receptors in the process. During my doctoral thesis, I examined the effect of peripherally and centrally injected 2-AG on an acid-independent, alcoholic ulcer model.

Direct activation of cannabinoid receptors often involves the appearance of psychoactive side effects (mood disorders, dizziness, restlessness, somnolence). Indirect activation of receptors can be achieved by inhibition of enzymes responsible for the metabolism of endogenous cannabinoids (FAAH, MAGL, uptake inhibition), and activation of G_{q/11}-linked receptors. Indirect activation can significantly limit the number of side effects that may occur.

In the synthesis of 2-AG, phospholipase C (PLC) enzyme plays an important role. PLC stimulation is the result of activation of G_{q/11} protein coupled transmembrane receptors. In a broader sense, therefore, any neurotransmitter

that acts on a $G_{q/11}$ protein coupled receptor (noradrenaline α_1 adrenergic, acetylcholine $M_{1,3,5}$ ACh, angiotensin AT_1 , bradykinin B_2 , vasopressin V_1 receptors) can achieve the paracrine activation of cannabinoid receptors. In Turu's experiments, on Chinese hamster ovary (CHO) cells, the transactivation of AT_1 and CB_1 receptors was demonstrated. Activated AT_1 -receptor stimulates PLC activity, which catalyses PIP_2 transformation to IP_3 and DAG. DAG is the generation molecule of 2-AG, and the process takes place by the activation of the DAG-lipase (DAGL) enzyme. Overall, the activation of the AT_1 -receptor increases the in-vivo biosynthesis of 2-AG, which results in the activation of cannabinoid receptors. On the CHO cells, the AT_1 receptor-induced CB_1 -receptor activation could be inhibited by the preincubation of tetrahydropipstatin (THL), a DAGL antagonist. So, during my doctoral work, I studied whether the activation of CB_1 -receptors by AT_1 -receptors can be achieved in ethanol ulcer model.

Another way of indirect activation of cannabinoid receptors is to increase the level of endogenous cannabinoids. In our experiments, the effects of three elevating compounds, namely the FAAH inhibitor URB 597 (increasing the endogenous level of anandamide), MAGL inhibitor (2-AG degradation enzyme), JZL 184 and anandamide uptake inhibitor AM 404 were examined for gastroprotection. In acid-dependent, NSAID-induced ulcers, the protective effect of URB 597 and JZL 184 has been already demonstrated, which has been shown to reduce the number of ulcerative lesions by activating peripheral CB_1 -receptors. During my work, I studied an acid-dependent, alcoholic ulcer model whether the gastroprotection of the endogenous elevating compounds is established in case of i.c.v. injection.

Gastric mucosal protection is achieved through complex, coordinated operation of central and peripheral factors. The examination of these factors can bring us closer to detecting the precise mechanism of mucosal defence and the peripheral connection points of central nervous system regulation. Therefore, we investigated the effect of the central and peripheral receptors involved, as well as changes in the tissue levels of neuropeptides (CGRP, SOM) and antioxidant enzyme systems (CAT, SOD) that stimulate the local cytoprotective processes of mucous membranes (enhancing mucosal blood flow, ROS elimination, etc.).

Evaluating the role of gastric motility in mucosal defence is controversial among researchers. On the one hand, it seems logical that inhibition of gastric emptying enhances the degree of damage, because detrimental agents have

more time to penetrate. In this context, prokinetic metoclopramide exerted a protective effect on an acid-dependent ulcer model, which was associated with its gastric emptying stimulant effect. However, in recent years, the theory has also been demonstrated that hypermotility causing mucosal damage agents (e.g. NSAID indomethacin) increases mucosal microcirculation disturbances, which exerts abnormal pressure on the gastric wall and increases vascular permeability, enhancing inflammation and tissue damage. In this case, inhibition of hypermotility may reduce the rate of lesions. Therefore, in our experiments, the effect of endogenous cannabinoid elevating compounds was studied on gastric motor activity.

The limitation of the therapeutic use of direct cannabinoid agonists/antagonists is undesirable psychotic side effects (sedation, malaise, depression, which may even lead to an increase in suicidal tendency, as we have seen in case of Rimonabant, which since been withdrawn from market, etc.). Indirect activation of cannabinoid receptors may be indirectly activated by endogenous cannabinoid level elevating compounds, so the number of cannabimimetic side effects may be reduced. We investigated the effect of the three endogenous level elevating compounds on catalepsy and hypothermia in a maximum concentration exerting gastroprotective effect, during intracerebroventricular administration.

AIMS

During my doctoral work, I examined the following:

1. The gastroprotective effect of endogenous cannabinoids

- 1.1. Like in case of anandamide, does 2-AG also have a gastroprotective effect during central and peripheral administration?
- 1.2. Which receptors are involved in mediating the gastroprotective effect of endogenous cannabinoids?
- 1.3. Which peripheral factors mediate the effect?

2. Investigation of activation of CB₁-receptors by AT₁-receptors

- 2.1. Does the centrally administered angiotensin II have a gastroprotective effect?
- 2.2. If so, does activation by AT₁-receptors occur on CB₁-receptors?

3. Investigating gastroprotective effect of endogenous cannabinoid level elevating compounds

- 3.1. Can gastroprotection be observed by not only the direct activation of cannabinoid receptors, but by increasing their tissue level as well?
- 3.2. If so, what kind of central and peripheral receptors are involved in the process?
- 3.3. In the periphery of the gastric mucosa, which factors and mechanisms mediate the effect?
- 3.4. Do the level elevating compounds affect gastric motility?
- 3.5. Do the compounds we investigated cause catalepsy and hypothermia (central cannabimimetic effects)?

METHODS

Animals

For our experiments, we used male Wistar rats from the breeding colony of Semmelweis University, weighing between 140-170 g for the alcohol ulcer model and 200-300 g for in vivo motility measurement. The animals were kept in a 12-hour light cycled air-conditioned room under controlled conditions ($22 \pm 2^{\circ}\text{C}$, $\sim 30\%$ humidity), according to the guidelines of US National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. Prior to the experiments, the animals were fasted for 24 hours while water could be consumed on demand. The experiments were carried out in accordance with the ethical guidelines established by the Ethics Committee of Semmelweis University (license number: 22.1/606/001/2010), which are based on the guidance of the European Union (EU Directive 2010/63/EU).

Chemicals

The following compounds were used during my work: anandamide, 2-AG, URB 597, JZL 184, Ang II (Ascent Scientific Ltd.); AM 251, AM 404 (Tocris Bioscience); candesartan (Toronto Research Chemicals); URB 937, tetrahydrolipstatin - THL, capsazepine, atropine sulphate, sodium pentobarbital, urethane (Sigma Chemical Co.).

The stock solutions of the compounds were made with the following solvents: anandamide - ethanol; 2-AG - acetonitrile/ethanol; AM 251 - DMSO; AM 404, URB 597, JZL 184 - 70% DMSO; URB 937 - 20% DMSO; Ang II, THL, candesartan, capsazepine - 0.9% physiological saline.

Dilution of different concentrations was made with 0.9% physiological saline. The control animals received the appropriate solvent.

Methods of application of the compounds

Central administration was performed by intracerebroventricular (i.c.v.) injection at 10 µl volume while intravenous (i.v.), intraperitoneal (i.p.) and per os dosing regimens (0.5ml/100tg) were used peripherally.

Gastric mucosal damage induced by acidified ethanol

After 24 h food deprivation 0.5 or 0.2 ml acidified ethanol (98 ml absolute ethanol + 2 ml concentrated HCl) was given orally to the rats and mice, respectively. 60 min later the animals were sacrificed, the stomachs were excised, opened along the greater curvature, rinsed with saline and examined for lesions. Total number of mucosal lesions was assessed in blinded manner by calculating the ulcer index based on a 0–4 scoring system described previously. The ulcer index was calculated as the total number of lesions multiplied by the respective severity factor. The percentual inhibition of mucosal damage was calculated as follows: $100 - (\text{ulcer index in treated group} / \text{ulcer index in control group}) * 100$. Drugs were injected intracerebroventricularly (i.c.v.) to the lateral ventricle as described previously in a volume of 10 µl for rats, respectively, 10 min before the ethanol challenge. To avoid a rapid increase of intracerebroventricular pressure the syringe was put into a special apparatus which allowed to inject the volume during optional (1 min) period. The i.c.v. and i.p. administration of agonists occurred 10 and 20 minutes prior to the administration of alcohol. The antagonists were injected together with agonists by i.c.v administration.

Measurement of gastric motility *in vivo*

The effect of endocannabinoid modulators on gastric motility was measured in anesthetized male Wistar rats (200–300 g) with the rubber balloon method. A total of 21 rats was used for this study. Briefly, after 24 h food deprivation animals were anesthetized with urethane (1.25 g/kg i.p.), a tracheal cannula was inserted to ensure a clear airway and an intragastric balloon created from thin latex rubber connected with plastic tubing was introduced into the

stomach via mouth. The balloon was filled with 2 ml warm water (37 °C) to set the basal intragastric pressure to 10 ± 0.5 cmH₂O. The exact location of the balloon was verified after each experiment. The distal end of tubing was connected to a pressure transducer and to a PowerLab Instrument with a Chart 5 program (AdInstruments, Bella Vista, Australia) to monitor the intragastric pressure. AM 404, URB 597, JZL 184 or vehicle were given i.c.v. in a volume of 10 μ l, via previously implanted guide cannulas. The stereotaxic coordinates of these cannulas (relative to bregma) were: posterior 0.8 mm; lateral 1.6 mm; ventral 4.5mm. The site of injection was verified after each experiment. To avoid significant intracranial pressure elevation a slow infusion was applied (over 5 min) by using a CMA/100 microinjection pump, and one animal received maximum 2 injections with at least 30 min interval. The blood pressure of animals was continuously monitored during the whole procedure. An equilibrium period (40–60 min) was registered before each experiment. For analysis of gastric motor activity two parameters were determined. The gastric tone, which correlates well with fundic activity, was calculated from the bottom points of phasic pressure wave. The mean amplitude of phasic contractions, which correlates with the antral contractions superimposed on tonic pressure, was calculated from the amplitude of each contraction. Both parameters were determined from 5 min segments, before and after the injection of test compounds. Values were expressed in percentage of the basal (pre-injection) values. The values were given as a percentage of the basal value. In the evaluation, the pre-administration value was considered as the basal value and the 5-minute interval after injection of the total volume was depicted in the percentage relative to the basal value. At the end of the experiment, animals were decapitated.

Bilateral cervical vagotomy

Under pentobarbital anesthesia (35 mg/kg intravenously), the cervical section of vagal nerves was exposed and bilateral cervical vagotomy was performed. Vagotomy slowed the respiration phase and enlarged the magnitude of respiration. Sham operated control rats had their vagus similarly exposed but the vagal trunks were not sectioned. The incisions were closed and all animals were allowed 3 h recovery from operation.

Measurement of catalepsy

In order to determine the behavioral effect of endocannabinoid modulators, a total of 20 rats were randomly allocated into 4 groups, with 5 rats in each

group. Each animal was treated i.c.v. with a single dose of URB 597, JZL 184, AM 404, or with the vehicle. Experiments were carried out according to the methods of Tseng and Craft. Catalepsy was evaluated using the bar test, in which the forepaws of the rats were placed on a raised bar. Latency to withdraw both forepaws from the bar or jump onto the bar was recorded 3 times at each animal; if the rat did not respond by 60 s, the test was terminated and 60 s was recorded. If the animal moved away from the bar on three occasions, the test was ended. The compounds or vehicle were given i.c.v. and 10 min later the rat's forepaws were placed on an elevated bar. The duration of immobility (that is catalepsy, the absence of voluntary movement) was measured for 40 min at 10 min intervals right after the catalepsy test.

Measurement of hypothermia

Body temperature was determined rectally by the probe inserted 2 cm into the rectum. The temperature was recorded immediately prior to and 10 min after the i.c.v. injection of the compounds for 40 min period at 10 min intervals.

Radioimmunoassay (RIA) measurements

For determination of gastric mucosal level of CGRP and somatostatin following CO₂ inhalation the stomachs of the rats were quickly removed, placed on a cooled plate, and after determination of the lesions index they were put in 1 ml cold distilled water, sonicated and stored at -80 °C till the determination. The RIA measurements were made in Debrecen, in collaboration work (Dr. József Németh and his colleagues, University of Debrecen, Pharmacology and Pharmacotherapy Institute).

CGRP antiserum C1012 was produced against the α -CGRP bovine thyroglobulin antigen in a rabbit (Sigma). RIA measurements were carried out in a phosphate buffer of 0.02 M (pH 7.4) with a final volume of 1 ml. The assay buffer contained 0.75% (v/v) bovine serum albumin (BSA), 0.1 mol/l sodium chloride (NaCl), 0.1% EDTA and 0.05% sodium azide (NaN₃). The ¹²⁵I isotopically labeled rat Tyr- α -CGRP (23-37) (Bachem) peptide was prepared with iodine and the mono iodinated peptide was separated from the other derivatives on an HPLC column (Merck). The antiserum was used in a dilution of 1: 350,000. Rat Tyr- α -CGRP (23-37) (Bachem) peptide was used as standard for RIA in the range of 0-100 fmol/ml (the detection limit of the assay was 0.2 fmol/ml).

The **SOM** antiserum 775/7 was produced in a sheep against the SOM14 bovine thyroglobulin antigen, and it was applied during the measurement in a 1: 600,000 dilutions. The ¹²⁵I isotopically labeled Tyr (1)-somatostatin-14 (Sigma) was obtained using iodine, and the mono-iodinated peptide was separated from the other fragments on a reversed-phase HPLC column (Merck). During the RIA measurement, the SOM-14 (Sigma) peptide was used as standard in a range of 0-1000 fmol/ml (detection limit of 2 fmol/ml).

Antioxidant enzyme activity measurements

Superoxide dismutase (SOD) is a metal-containing enzyme (metalloenzyme) that catalyses the conversion of superoxide radical anion to water and hydrogen peroxide. After decapitating the animals, the stomachs were excised, and the gastric mucosa was dissected on a chilled plate, samples were immediately frozen in liquid nitrogen and stored at -80 °C until processing. For homogenization, the samples were powdered in liquid nitrogen and measured in ~ 40-80 mg homogenizing tubes. To a 1 mg sample, 10 µl of homogenizing fluid was added containing 20 mM HEPES buffer with pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose (concentrations are valid per gram of tissue). Homogenization was performed in a ball mill for 2 x 3 min at 50/sec vibration, and the samples were centrifuged for 5 minutes at 4 °C at 1500 rcf. 200-200 µl aliquots were taken from the supernatants and they were stored at -80 °C until subsequent assays. For assay, the assay kit produced by Cayman Chemical Company was used. According to the protocol, a standard line was prepared from certain dilutions of the SOD stock solution, and then, according to the pre-prepared position, duplicates were applied to the plate. Tetrazolium salt was added, and the reaction was initiated with xanthine oxidase. After 20 minutes, the absorbance of each sample was read at a wavelength of 450 nm, a standard curve for the absorbance of the stock solution was fitted with linear regression, which enabled SOD activity (U/ml) to be easily calculated.

Catalase (CAT) is a heme-containing oxidoreductase that is primarily found in peroxisomes in the mammalian body and catalyses the breakdown of hydrogen peroxide (H₂O₂) of the reactive oxygen species physiologically present or produced during pathologically induced oxidative stress. Samples were removed and homogenized as described in superoxide dismutase except for the composition of the homogenizing buffer containing the following components: 50 mM NaPO₄, pH 7.0, 1 mM EDTA (per gram of tissue). For determination, I used the assay kit manufactured by Cayman Chemical

Company. The CAT enzyme activity is calculated from the amount of formaldehyde produced as follows: $\text{CAT activity} = [\text{formaldehyde content of the sample } (\mu\text{M})/20 \text{ min}] * \text{dilution of the sample}$. For the validation of the process, it was necessary to apply positive control, which was powdered CAT extracted from lyophilized bovine liver. Standard row was made from the formaldehyde stock solution according to the protocol, then I loaded the plate and I applied the samples in duplicates. I initiated the reaction by adding 30 μl of methanol and 20 μl of H_2O_2 and after 20 minutes of incubation at room temperature, I stopped the hydrolysis with KOH and the Purpald reagent was added to the mixture. After a further 15 minutes of incubation, the absorbance of the samples at a wavelength of 540 nm can be read, and the standard curve fitted with linear regression to the absorbance of the stock solution gives the amount of formaldehyde (μM), of which the CAT activity (nmol/min/ml) of the samples can be calculated according to the above equation.

Statistical analysis

The values presented in the experimental results correspond to the averages and they were presented together with standard error of mean, S.E.M. The statistical analysis was performed using the ANOVA method (Newman-Keuls post hoc test), and Kruskal-Wallis test for nonparametric samples. In motility experiments, the pre and post-injection values were compared with Student's paired t-test. Comparison of the immobilization and temperature changes of the different groups was measured by two-sample variance analysis and Friedman probes. Significant difference was $p < 0.05$.

CONCLUSION, NEW RESULTS

The most important findings of my doctoral work, which were published as a new result:

1. The gastroprotective effect of endogenous cannabinoids

- 1.1. Like in case of anandamide, 2-AG also exerts a gastroprotective effect both during i.v. and i.c.v. administration in an ethanol-induced ulcer model.
- 1.2. Central CB_1 -receptors are involved in mediating the effects of both anandamide and 2-AG.
- 1.3. Among the peripheral effects, the enhancement of the CGRP level plays an important role in the protection induced by anandamide and 2-AG.

2. Investigation of activation of CB_1 -receptors by AT_1 -receptors

2.1. The i.c.v. injected Ang II exerts a gastroprotective effect in an ethanol-induced ulcer model.

2.2. The gastroprotective effect of Ang II is triggered by the activation of the AT₁-receptor-induced CB₁-receptor and the process can be inhibited by a DAGL inhibitor, which implies the role of 2-AG in the emergence of the effect.

2.3. Activation of the vagus and cholinergic fibres is also involved in the Ang II-induced gastroprotection, which could be inhibited by atropine and vagotomy.

3. Investigating gastroprotective effect of endogenous cannabinoid level elevating compounds

3.1. By blocking the metabolism of endogenous cannabinoids (i.e. by increasing the endogenous levels of anandamide and 2-AG), gastroprotective effects can also be exerted on an acid-independent model.

3.2. The FAAH inhibitor URB 597 and MAGL inhibitor JZL 184 exert a gastroprotective effect with anandamide and 2-AG elevation via CB₁-receptors.

3.3. In the periphery, this effect is mediated by increasing CGRP and SOM levels and SOD enzyme activity. However, the compounds did not affect the gastric motor activity and tone.

3.4. The anandamide uptake inhibitor AM 404 also had a gastroprotective effect, but its mechanism requires further studying. Central CB₁-receptors are involved in its mediating, but other receptors may also be involved. In the periphery, the increase of CGRP and SOM release is involved in the effect, but neither the effect on the gastric motor activity and tone affects it, nor the influence of SOD on the enzyme activity.

3.5. The cannabimimetic side effects (catalepsy, hypothermia) were present in none of the compounds we examined with gastroprotective dose by i.c.v. injection.

PUBLICATIONS LIST

Publications in connection with the PhD dissertation

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Gyires K, **Toth V**, Kiraly K, Barna I, Zadori ZS. (2013) Both supraspinal and spinal mechanisms may be involved in the maintenance of gastric mucosal integrity in the rat. *FASEB J* 27: 1093.21.