INTRODUCTION

Acid and pepsin play a prominent role in the development of peptic ulcer disease and the treatment with antisecretory drugs (proton pump inhibitors, histamine H₂-receptor blocking drugs) results in excellent therapeutic effect. However, there are numerous data which suggest that factors relating to mucosal resistance to acid may be equally important. E.g. stress ulcer can develop in patients in intensive care units and mortality due to gastric bleeding associated with stress ulcer can reach, even exceed 50% (1-3). Moreover, bleeding complications and fatal events have not decreased even after the introduction of proton pump inhibitors (4). It is well known that in upper gastric ulcers the acid secretion is normal or decreased, indicating that decreased mucosal resistance may be responsible for the development of mucosal damage. In distal, antral and duodenal ulcers, where typically hypersecretion is observed, acid output is in normal range in about half of the patients, referring again to the basic role of gastric mucosal defensive processes. The same questions can be raised, that was raised by Sachs et al. (5) more than 30 years ago, namely how it can be explained that while the incidence of acid-related disorders in life is about 20% of the population, everybody secretes acid throughout the life. Though several additional factors have been identified which contribute to the development of mucosal lesions in a given individual, the impaired mucosal defensive mechanisms may play a crucial role in this process. Mucosal defense can be induced also centrally. Several neuropeptides, such as TRH, amylin, adrenomedullin, enkephalin, β-endorphin, nociceptin, nocistatin, ghrelin or orexin given centrally induce gastroprotection and the dorsal vagal complex and vagal nerve may play prominent role in this centrally initiated effect. Since also cannabinoid receptors are present in the dorsal vagal complex, we aimed to study whether activation of central cannabinoid receptors result in gastric mucosal defense and whether there is an interaction between cannabinoids and endogenous opioids. Gastric mucosal damage was induced by 100% ethanol in rats. The cannabinoids were given intravenously (i.v.) or intracerebroventricularly (i.c.v.), while the antagonists were given i.c.v. or intracisternally (i.c.). Gastric lesions were evaluated macroscopically 60 min later. Anandamide, methanandamide and WIN55,212-2 reduced ethanol-induced mucosal lesions after both peripheral (0.28-5.6 µmol/kg, 0.7-5.6 µmol/kg and 0.05-0.2 µmol/kg i.v., respectively) and central (2.9-115 nmol/rat, 0.27-70 nmol/rat and 1.9-38 nmol/rat i.c.v., respectively) administration. The gastroprotective effect of anandamide and methanandamide given i.c.v. or i.v. was reversed by the CB₁ receptor antagonist SR141716A (2.16 nmol i.c.v.). Naloxone (27.5 nmol i.c.v.) also antagonized the effect of i.c.v. or i.v. injected anandamide and WIN55,212-2, but less affected that of methanandamide. The gastroprotective effect of anandamide was diminished also by endomorphin-2 antiserum. In conclusion it was first demonstrated that activation of central CB₁ receptors results in gastroprotective effect. The effect is mediated at least partly by endogenous opioids.

Key words: cannabinoid agonist, cannabinoid antagonist, naloxone, endomorphin-2 antiserum, intracerebroventricular, ethanol, damage CB₁ receptor
In contrast with the peripheral mechanisms of mucosal protection, much less has been known about the central processes, mediators and brain area(s) that may play a role in the maintenance of gastric mucosal integrity and/or stimulation of mucosal defensive mechanisms. Dorsal vagal complex (nucleus tractus solitarii and dorsal motor nucleus of vagus) and vagal nerve play a prominent role in the regulation of gastric functions, like acid secretion and motility and as it was demonstrated recently, also in gastric mucosal defense (11-15). Several neuropeptides and their receptors were identified in dorsal vagal complex (DVC), like angiotensin II, β-endorphin, bombesin, CCK, corticotropin-releasing factor (CRF), dynorphin, enkephalins, galanin, neuropeptide Y (NPY), neurotensin, somatostatin, thyreotropin releasing hormone (TRH), vasopressin, vasoactive intestinal polypeptide (VIP) (16), angiotensin II, somatostatin, endomorphin-releasing factor (17), nociceptin and nocistatin (19) or leptin (20). Neuropeptides can influence the activity of dorsal vagal complex also by neuronal projections. For instance, from the cerebral cortex and hypothalamus ghrelin-containing neurons project to the DVC (21), or similarly, orexigenic neurons project from the lateral hypothalamic area to the dorsal vagal complex (22). Neuropeptides can influence gastric acid secretion, gastrointestinal motility, and as it was demonstrated in the last decade, they can induce gastric mucosal protection given centrally. For example, in rats TRH injected intracisternally (i.c.) or directly into the dorsal motor nucleus of vagus in the dose below the threshold that stimulates acid secretion reduced mucosal lesions induced by ethanol (14). Intracisternal injection of peptide YY and adrenomedullin (13, 15) as well as i.c.v. administered amylin (23) decreased the ethanol-induced gastric mucosal lesions in the rat. Moreover, different opioid peptides (11, 12), ghrelin (24, 25), orexin (25), nociceptin (26) and nocistatin (27) also induced mucosal protection against ethanol-induced mucosal lesions following central administration.

The important role of the endocannabinoid system in the gastrointestinal (GI) tract under physiological and pathophysiological conditions has been demonstrated recently. Cannabinoid type 1 (CB1) receptors are present in neurons of the enteric nervous system and in sensory terminals of vagal and spinal neurons, moreover, CB1 receptors are also identified in the dorsal vagal complex: in nucleus tractus solitarii (NTS) (28), in dorsal motor nucleus of vagus (29) and prominently, in area postrema (29). Beside the DVC, CB1 receptors were described also in the paraventricular nuclei (PVN) of the hypothalamus (30), and projection from PVN to the dorsal vagal complex is well documented (31, 32). Cannabinoids given both peripherally and centrally affect numerous gastrointestinal functions; it was shown that cannabinoids inhibited gastric motility in the rat through activation of CB1 receptors given peripherally (33, 34). Similarly, cannabinoid agonists inhibited gastrointestinal transit in the mice after both central and peripheral administration (35). CB1 receptor agonists given i.v. decreased the gastric acid secretion induced by indirect acting secretagogues, such as 2-deoxy-D-glucose (36, 37), but did not change acid output to histamine. This latter finding indicates that CB1 receptors are not located on parietal cells, but rather on vagal pathways. Since intracerebroventricularly injected cannabinoid agonists were ineffective in preventing the pentagastrin stimulated gastric acid secretion, the CB1 receptor-mediated inhibition of gastric acid secretion in the rat may be located mainly peripherally (38).

Cannabinoids have been shown to decrease the formation of experimental gastric ulcers as well. Tetrahydrocannabinol, for example, reduced mucosal damage induced by pylorus ligation (39) and Cannabis sativa was effective against restraint-induced gastric ulcerations (40). Furthermore, anandamide reduced the gastric ulceration induced by water immersion and restraint stress (41) and WIN55,212-2 produced anti-ulcer effect in the cold/restraint stress model (42). Moreover, the selective cannabinoid CB1 receptor agonist, ACEA (arachidonyl-2-chloroethylamide) significantly reduced gastric ulcer formation induced by aspirin (43). These ulcer models are acid dependent models; consequently, the gastric mucosal protective effect of cannabinoids may be related to their antisecretory effect. Since cytoprotective (gastroprotective) effect, originally described by Andre Robert (44) was demonstrated in chemically or physically induced acute gastric ulcer models and the protective effect was unrelated to inhibition of acid secretion, the question was raised whether cannabinoids can inhibit gastric mucosal lesions in acid-independent ulcer models as well.

Therefore the aims of the present study were to determine: i) whether cannabinoids can influence the formation of gastric mucosal lesions induced by ethanol, which is an acid-independent ulcer model; ii) whether central components are involved in the gastroprotective effect of cannabinoids, and finally; iii) whether interaction between cannabinoid and opioid system can be demonstrated in the gastroprotection as well.

MATERIALS AND METHODS

Animals

Experiments were carried out on male Wistar rats (Charles River) weighing 150-170 g received from the breeding colony of Semmelweis University. The animals were kept in a 12-hour light/dark cycle and under condition of controlled temperature. They were maintained on standard rat laboratory chow and tap water ad libitum. All procedures conformed to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. The study was approved by the Animal Ethics Committee of Semmelweis University, Budapest (permission number: 1810/003/2004).

Gastric mucosal damage induced by acidified ethanol

After twenty four hours food deprivation the animals were given orally 0.5 ml acidified ethanol (98% ethanol in 200 mmol/l HCl). One hour later the animals were killed by overdose of ether, 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 calculation of lesion index based on a 0-4 scoring system described previously (45). The lesion index was calculated as the total number of lesions multiplied by the respective severity factor. The percentile inhibition of mucosal damage was calculated as follows:

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\text{percentile inhibition} = \frac{\text{lesion index in control group} - \text{lesion index in treated group}}{\text{lesion index in control group}} \times 100
\]

Drugs were injected either intravenously (i.v.), intracerebroventricularly (i.c.v.) or intracisternally (i.c.) in a volume of 5 ml/kg, 10 µl and 5 µl, respectively. The i.v. injection to the lateral ventricle was performed according to Noble et al. (46) in conscious rats, the intracisternal injection was carried out as described previously (11) based on the method of Ueda et al. (47). The cannabinoid receptor agonists (anandamide, methanandamide, WIN55,212-2 and ACEA) were injected 10 min before the ethanol challenge. The different antagonists (the cannabinoid CB1 receptor antagonist SR141716A, the non-selective opioid receptor antagonist naloxone, the δ-opioid receptor selective naltrindole and the κ-opioid receptor selective norbinaltorphimine (norBNI)) were injected i.c.v., the endomorphin-2 antiserum i.c. 10 min before the administration of the cannabinoid receptor agonists.
Materials

Anandamide, methanandamide, arachidonyl-2-chloroethylamide (ACEA) and (R(+))-[2,3-Dihydro-5-methyl-1-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylethanolamine mesylate (WIN 55 212-2) were purchased from Tocris Bioscience. N-(piperidine-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A) (NIDA) was a generous gift of T. Freund. Naloxone, naltrindole and norbinaltorphimine (NorBNI) were purchased from Sigma. The property of antiserum to endomorphin-2 was described previously (48, 49). The antiserum was used at a 20-fold final dilution. The same dilution of non-reactive rabbit serum was used as control. Anandamide, methanandamide and ACEA were dissolved in ethanol, and stock solutions were diluted with saline. WIN55,212-2 and SR141716A were dissolved in DMSO and then diluted with saline. All the other drugs were dissolved in saline. Animals in the control groups received the drug solvents.

RESULTS

The effect of anandamide, methanandamide and WIN55,212-2 given i.v. and i.c.v. on gastric mucosal damage induced by ethanol

As Fig. 1. demonstrates, anandamide (0.28-5.6 µmol/kg), methanandamide (0.7-5.6 µmol/kg) and WIN55,212-2 (0.05-0.2 µmol/kg) inhibited the ethanol-induced gastric mucosal lesions in a dose-dependent manner given i.v. Similarly, gastroprotective effect was induced by anandamide (2.9-115 nmol/rat), methanandamide (0.27-70 nmol/rat) and WIN55,212-2 (1.9-38 nmol/rat) when they were injected i.c.v. WIN55,212-2 in the dose of 1.9 nmol/rat induced still a very pronounced (80%) inhibition of gastric mucosal lesions, experiments are in progress to determine the threshold dose (Fig. 2).

The effect of SR141716A given i.c.v on the gastroprotective effect of anandamide and methanandamide given either i.c.v. or i.v.

Pretreatment with the selective CB1 receptor antagonist SR141716A (2.16 nmol/rat i.c.v.) reversed the gastroprotective

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Fig. 1. The inhibitory effect of different non-selective cannabinoid receptor agonists (anandamide, methanandamide and WIN55,212-2) on gastric mucosal injury induced by ethanol in the rat. The compounds were injected intravenously (i.v.) 15 min before the ethanol challenge. Each column represents mean±S.E.M., n=5. *p<0.05, **p<0.01, ***p<0.001 compared with the respective control groups (ANOVA, Newman–Keuls post hoc test).

Fig. 2. The inhibitory effect of different non-selective cannabinoid receptor agonists (anandamide, methanandamide and WIN55,212-2) on gastric mucosal injury induced by ethanol in the rat. The compounds were injected intracerebroventricularly (i.c.v.) 10 min before the ethanol challenge. Each column represents mean±S.E.M., n=5. *p<0.05, **p<0.01, ***p<0.001 compared with the respective control groups (ANOVA, Newman–Keuls post hoc test).

Fig. 3. The effect of the CB1 cannabinoid receptor antagonist SR141716A (2.16 nmol/rat i.c.v.) on the gastroprotective effect of anandamide (115 nmol/rat i.c.v.) and methanandamide (70 nmol/rat i.c.v.) on gastric mucosal injury induced by ethanol in the rat. Each column represents mean±S.E.M., n=5. ***p<0.001 compared with vehicle-treated group (column 1); ##p<0.01, ###p<0.001 compared with vehicle + CB receptor agonist (anandamide or methanandamide)-treated group (column 2) (ANOVA, Newman–Keuls post hoc test).
effect of both anandamide (115 nmol/rat i.c.v.) and methanandamide (70 nmol/rat i.c.v.) (Fig. 3). Similarly, centrally injected SR141716A also antagonized the gastroprotective effect of methanandamide administered peripherally (5.6 µmol/kg i.v.) (Fig. 4).

The effect of ACEA on gastric mucosal damage given i.c.v.

Since the previous results suggested the potential involvement of central CB1 receptors in the gastroprotective effect of cannabinoids, the effect of selective CB1 receptor agonist ACEA was studied against ethanol-induced ulcer formation. It was found that ACEA in the doses of 0.13-1.37 nmol/rat induced 60-80% inhibition of the mucosal lesions (Fig. 5).

The effect of naloxone given i.c.v. on the gastroprotective effect of anandamide, methanandamide and WIN55,212-2 injected either i.c.v or i.v.

The gastroprotective effect of centrally (i.c.v.) injected anandamide (115 nmol/rat) and WIN55,212-2 (38 nmol/rat) was reversed by naloxone (27.5 nmol/rat i.c.v.), however the mucosal protective effect of methanandamide (70 nmol/rat i.c.v.) was less affected (Fig. 6). Similar results were obtained when the effect of centrally injected naloxone was examined on the protective effect of anandamide (5.6 µmol/kg), methanandamide (5.6 µmol/kg) and WIN55,212-2 (0.2 µmol/kg) given intravenously; naloxone antagonized the mucosal protective effect of anandamide and WIN55,212-2,
and decreased the protective effect of methanandamide in a significant manner. However, methanandamide exerted gastroprotective effect also following the pretreatment with naloxone (Fig. 7).

The effect of naltrindole and norbinaltorphimine given i.c.v. on the gastroprotective effect of anandamide injected i.c.v.

Both the δ-opioid receptor antagonist naltrindole (5 nmol/rat i.c.v.) and the κ-opioid receptor antagonist norbinaltorphimine (norBNI) (14 nmol/rat i.c.v.) counteracted the mucosal protective effect of anandamide (115 nmol/rat i.c.v.) (Fig. 8).

DISCUSSION

The present results demonstrate first that the endocannabinoid anandamide, its stable analogue methanandamide and the synthetic, non-selective cannabinoid derivative WIN55,212-2
inhibit the gastric mucosal lesions induced by ethanol given both centrally (i.c.v.) and peripherally (i.v.). Since the centrally induced protective effect of the cannabinoid agonists was reversed by centrally injected SR141716A, a selective cannabinoid CB1 receptor antagonist, it can be concluded that central CB1 receptors may mediate the gastric mucosal protective effect. Moreover, centrally injected SR141716A was also able to reverse the mucosal protection induced by the agonists given peripherally, confirming the primary role of centrally located CB1 receptors in the gastroprotective effect of cannabinoids. This assumption is further supported by the findings that the effective dose range of cannabinoids (anandamide, methanandamide) against ethanol-induced lesions given i.c.v. are much lower than that injected i.v.

There are only few data in the literature on the role of central cannabinoid receptors in gastrointestinal functions. E.g. WIN55,212-2 given centrally was more effective in inhibition of intestinal motility, when administrated peripherally, which may suggest central site of action. However, SR141716A given intracerebroventricularly failed to antagonize the effect of WIN55,212-2 injected intraperitoneally, indicating the primary role of peripheral CB1 receptors in the inhibition of upper intestinal motility (50). Others showed that WIN55,212-2 (2-239 nmol/mouse) and cannabinol (24-4027 nmol/mouse) decreased, while the CB1 antagonist SR141716A (2-539 nmol/mouse) increased transit in mice and the ED50 values were lower when administered i.c.v., than when administered i.p., suggesting the involvement of a central CB1 receptors in the action. However, since hexamethonium failed to affect the action of cannabinoid agonists on intestinal transit, the role of peripheral components in the effect has also been raised (35). Adami et al. (38) found that the synthetic cannabinoid receptor agonists WIN55,212-2 (50 and 100 µg/kg) and HU-210 (25, 50 and 100 µg/kg) were ineffective either on the basal secretion or on the pentagastrin-stimulated acid output after i.c.v. administration, but in contrast, intravenously both WIN55,212-2 (100 and 1000 µg/kg) and HU-210 (10-100 µg/kg) significantly inhibited pentagastrin-induced acid secretion, and SR141716A antagonized this effect, indicating that CB1 receptors mediating inhibition of gastric acid secretion in the rat are mainly peripherally located. Moreover, it was found that anandamide and WIN55,212-2, when administered peripherally to partially satiated animals, elicited significant and prolonged hyperphagia. In contrast, central injections of these cannabinoids had no effect on feeding, except at the highest dose (10 µg), which resulted already in motor impairment (51). They concluded that cannabinoid agents can affect food intake predominantly by engaging peripheral CB1 receptors localized to capsaicin-sensitive sensory terminals.

Several lines of evidence suggest that opioid and cannabinoid receptors can functionally interact. Very recent data show that the antihyperalgesic action of anandamide against carrageenan-induced hyperalgesia was reversed by the opioid receptor antagonist naloxone, indicating that its antinociceptive effect may involve at least partly the opioid system (52). Another recent paper showed that AM404, an endocannabinoid transport inhibitor, potentiated antinociception induced by cholestasis, which is associated with increased activity of the endogenous opioid system in gastric mucosal defense is the dorsal vagal complex, from where endorphin-containing fibers project to the NTS and also endomorphin-1 and endomorphin-2 has been found in this area (18), iii) endomorphin-2 antiserum given intracisternally decreased the mucosal protective effect of anandamide that was given, i.c.v. The above data on colocalisation of ligands and receptors of cannabinoid and opioid system may serve a basis for a potential interaction between these two systems. It may be hypothesised that activation of CB1 receptors in DVC (or hypothalamus) directly or indirectly through the release of endogenous opioids (or by both mechanisms) initiates a chain of events which results in gastric protection against mucosal injury. Previous studies suggested that gastroprotection can be induced by low level of central vagal stimulation, and the consequent release of NO, PG and CGRP (64, 65). Experiments are in progress on the role of vagal nerve in the gastroprotective effect of cannabinoids.

In conclusion, it was first demonstrated that cannabinoids induce gastric mucosal protection against ethanol-induced lesions by activation of central CB1 receptors. The gastroprotective effect may be mediated at least partly by endogenous opioids, since naloxone as well as endomorphin-2 antiserum decreased the
protective effect anandamide. Further experiments are needed to clarify the mechanism of the gastroprotective action of cannabinoids in the periphery.

Acknowledgements: The authors wish to thank Mrs. I. Szalai and Mrs. I. Wachtl for their technical assistance.

The work was supported by ETT 529/2006 from the Scientific Health Council and National Office for Research and Technology (NKTH), Hungary, provided via Szentagothai Knowledge Centre.

Conflict of interests: None declared.

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Received: October 15, 2009
Accepted: December 11, 2009

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