Modulation of central endocannabinoid system results in gastric mucosal protection in the rat

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Abstract

Previous findings showed that inhibitors of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), degrading enzymes of anandamide (2-AEA) and 2-arachidonoylglycerol (2-AG), reduced the nonsteroidal anti-inflammatory drug-induced gastric lesions. The present study aimed to investigate: i./whether central or peripheral mechanism play a major role in the gastroprotective effect of inhibitors of FAAH, MAGL and AEA uptake, ii./ which peripheral mechanism(s) may be responsible for mucosal protective effect of FAAH, MAGL and uptake inhibitors. *Methods:* Gastric mucosal damage was induced by acidified ethanol. Gastric motility was measured in anesthetized rats. Catalepsy and the body temperature were also evaluated. Mucosal calcitonin gene-related peptide (CGRP), somatostatin concentrations and superoxide dismutase (SOD) activity were measured. The compounds were injected intraperitoneally (i.p.) or intracerebroventricularly (i.c.v.). *Results:* 1. URB 597, JZL184 (inhibitors of FAAH and MAGL) and AM 404 (inhibitor of AEA uptake) decreased the mucosal lesions significantly given either i.c.v. or i.p. 2. URB 937, the peripherally restricted FAAH inhibitor failed to exert significant action injected i.p. 3. Ethanol-induced decreased levels of mucosal CGRP and somatostatin were reversed by URB 597, JZL 184 and AM 404, the decreased SOD activity was elevated significantly by URB 597 and JZL 184. 4. Neither compounds given i.c.v. influenced gastric motility, elicited catalepsy, or hypothermia. *Conclusion:* Elevation of central endocannabinoid levels by blocking their degradation or uptake via stimulation of mucosal defensive mechanisms resulted in gastroprotective action. These findings might suggest that central endocannabinoid system may play a role in gastric mucosal defense and maintenance of mucosal integrity.

1. Introduction

The endocannabinoid system (ECS), which comprises the cannabinoid CB_1, CB_2 receptors, the endocannabinoids and their synthetic and metabolizing enzymes, is involved in the regulation of numerous physiological processes. The two main endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) may play a role in these regulatory mechanisms either as neurotransmitters or neuromodulators (Palmer et al., 2002), and dysregulation of the ECS has been demonstrated in several diseases (for review see: Pacher and Kunos, 2013).

Besides endocannabinoids other naturally occurring cannabinoids are the plant-derived phytocannabinoids. Cannabis sativa plant contains more than 80 cannabinoids (Kumar et al., 2001), among them the main active constituent of marijuana is the psychoactive Δ^9-tetrahydrocannabinol (Δ^9-THC), which acts at CB_1 and CB_2 receptors as a partial agonist. Other important natural cannabinoids of marijuana are the non-psychoactive cannabidiol, Δ^9-tetrahydrocannabivarin and cannabichromene (Kumar et al., 2001; Russo, 2011; Turner and Elsohly, 1981). Some of these plant-derived cannabinoids are used in the medical practice, such as Δ^9-THC (dronabinol) and its synthetic analogue, nabilone against chemotherapy-induced nausea and emesis, and as appetite stimulants (e.g. in AIDS patients). However, as psychoactive drugs, both of them may cause psychotomimetic action. Furthermore, combination of the non-psychoactive cannabidiol with Δ^9-THC has beneficial effect in neuropathic pain, in spasticity in multiple sclerosis, and as an adjunctive analgesic treatment in advanced cancer pain (Pertwee, 2012).

The role of ECS in the physiology and pathophysiology of gastrointestinal (GI) tract has also been extensively studied (Di Carlo and Izzo, 2003; Esposito et al., 2013; Kunos and Pacher, 2004; Massa and Monory, 2006; Vigna, 2003). High levels of AEA and 2-AG, and of the enzymes responsible for their synthesis and metabolism can be detected in the digestive tract (Marquez et al., 2009; Katayama et al., 1997; Izzo et al., 2001; Duncan et al., 2008). Similarly, cannabinoid receptors are also widely distributed in the GI tract. CB_1 receptors have been shown by immunohistochemical studies in myenteric and submucosal nerve plexuses along the alimentary tract (for review see: Izzo and Coutts, 2005). Co-localization of CB_1 receptors with the cholinergic marker, choline acetyltransferase in neural elements innervating smooth muscle, mucosal and submucosal blood vessels of rat stomach fundus, corpus and antrum has been shown (Adami et al., 2002). In the gut, in non-inflamed tissues, CB_1 receptors are mainly localized on excitatory motor neurons, interneurons and intrinsic primary afferent neurons of the enteric nervous system. In addition, epithelial cells, smooth muscles and immune cells express CB_1 receptors (Wright et al., 2005; Marquez et al., 2009; Coutts et al., 2002). CB_2 receptors, on the other hand, are mainly expressed by subepithelial immune cells (such as macrophages and plasma cells) (Wright et al., 2005), and also by enteric neurons (Duncan et al., 2008, Wright et al., 2008), while they are absent or weakly expressed in epithelial cells in humans (Wright et al., 2005; Wright et al., 2008).

The effect of cannabinoids on GI mucosal injury has been intensively studied (for recent review see: Gyires and Zádori, 2016). Activation of CB receptors by endocannabinoids or synthetic derivatives has been shown to exert mucosal protective effect against different types of experimental gastric ulcers. For example Δ^9-THC reduced mucosal damage induced by pylorus ligation in the rat (Sofia et al., 1978). Δ^9-THC also attenuated diclofenac-induced gastric mucosal lesions given either orally or intraperitoneally (i.p.) in the mouse (Kinsey and Cole, 2013; Kinsey et al., 2011). The protective action was mediated by CB_1 receptors and the dose range of the gastroprotective effect was lower than that producing classical cannabimimetic effects, such as locomotor immobility, antinociception, hypothermia and catalepsy (Kinsey and Cole, 2013; Kinsey et al., 2011). These results indicate that Δ^9-THC is able to protect the gastric mucosa at doses insufficient to cause common cannabinoid side effects. In addition, gastric lesions induced by water immersion and restraint stress in the rat were reduced by AEA as well as by the synthetic analog WIN 55,212-2 (both are given i.p.), and their gastroprotective action was mediated also by CB_1 receptors (Dembinski et al., 2006; Germano et al., 2001). The protective effect of AEA was associated with an increase in gastric mucosal blood flow and mucosal DNA synthesis, and with reduced level of pro-inflammatory interleukin-1β (IL-1β) (Dembinski et al., 2006). Involvement of CB_1 receptors in gastroprotection was further supported by the results with the selective cannabinoid CB_1 receptor agonist ACEA (arachidonyl-2-chloroethylamide), which effectively reduced the aspirin-induced gastric mucosal lesions (given i.p.) (Rutkowska and Fereniec-Goltbiewska, 2006).

While gastric acid secretion is involved in the pathomechanism of pylorus-ligation-, nonsteroidal anti-inflammatory drug- (NSAID) or stress-induced gastric ulcer models, gastric acid does not play a role in the development of mucosal injury induced by ethanol. This ulcer model originally described for demonstration of the cytoprotective effect of prostaglandins by Robert et al. (Robert et al., 1979), has been widely used method for the analysis of the mechanism of gastroprotective action. AEA, methanandamide and WIN 55,212-2 have been found to reduce the ethanol-induced gastric lesions following both peripheral (intravenous /i.v./) and central (intracerebroventricular, /i.c.v./) administration, indicating that their mucosal protective effect is not likely to be related to inhibition of gastric acid secretion, but rather to the activation of mucosal defensive processes (Shujaa et al., 2009). Since the protective effect of methanandamide injected i.v. was reversed by the i.c.v injected CB_1 receptor antagonist SR141716A, the primary site of action is likely to be central and mediated by CB_1 receptors (Shujaa et al., 2009).

CB_1 and CB_2 receptors can be activated not only directly by the natural and synthetic ligands, but also indirectly, by elevating the level of endocannabinoids in the vicinity of cannabinoid receptors, either by blocking their degradation or uptake. AEA and 2-AG levels are regulated *in vivo* by catabolic enzymes, like the intracellular fatty acid amide hydrolase (FAAH), which hydrolyzes AEA into arachidonic acid and ethanolamine (Cravatt et al., 2001), and monoacylglycerol lipase (MAGL)

(Blankman et al., 2007), which is the main contributor to 2-AG hydrolysis. In addition, biological activity of AEA is terminated by its removal from the extracellular space via cellular uptake (Fowler, 2006).

Systemic administration of 4-[*Bis*(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester (JZL 184), a selective MAGL inhibitor, increased the level of 2-AG in mouse whole brain without affecting the level of AEA (Long et al., 2009). Similarly, elevated level of 2-AG, but not that of AEA was observed in the gastric mucosa of mice following i.p. administration of JZL 184 (Kinsey and Cole, 2013; Kinsey et al., 2011; Long et al., 2009). Moreover, the FAAH inhibitor URB 597 (*N*-Cyclohexylcarbamic acid 3'-(Aminocarbonyl)-[1,1'-biphenyl]-3-yl ester) produced a profound, dose-dependent inhibition of brain FAAH activity accompanied by a significant elevation of the brain level of AEA in rats, whereas it did not change the brain content of 2-AG (Kathuria et al., 2003). Similarly, to FAAH inhibitor-treated mice, in FAAH (-/-) mice, elevated levels of AEA in the central nervous system (CNS), as well as CB_1-receptor mediated antinociception could be observed (Cravatt et al., 2001; Lichtman et al., 2004).

It has been raised that increasing endocannabinoid tissue levels may provide a functionally selective way of enhancing endocannabinoid tone only in those tissue and cells that have active synthesis and release of endocannabinoids (Ahn et al., 2009; Di Marzo, 2008). Consequently, it has been hypothesized that increasing endocannabinoid tissue levels would induce less psychoactive effects (such as catalepsy, hypothermia or hyperphagia) than the direct stimulants of CB_1 receptors (Kathuria et al., 2003), while the beneficial effects due to activation of CB_1 and/or CB_2 receptors would be retained. Accordingly, promising results have been obtained for FAAH inhibitors in preclinical models of acute, inflammatory, and neuropathic pain, as well as in anxiety, depression, nausea, hypertension, pruritus, smoking cessation, post-traumatic stress syndrome or Parkinson's disease (for reviews see: Ahn et al., 2009; Pacher and Kunos, 2013) as well as in inflammatory bowel diseases and irritable bowel syndrome (Salaga et al., 2014). Similarly, numerous studies demonstrated that inhibition or genetic deletion of MAGL exerts antiemetic, antineoplastic, anxiolytic and antinociceptive effects in rodents, and protects against brain injury, acute liver injury/inflammation and colitis (for review see: Pacher and Kunos, 2013).

Moreover, AM 404 (N-(4-hydroxyphenyl) arachidonamide), an AEA analog (Rogosch et al., 2012), inhibits the carrier-mediated transport of AEA into presynaptic neurons from the synaptic cleft (Beltramo et al., 1997). AM 404 is an active metabolite of paracetamol, and responsible for its analgesic action, at least partly (Ottani et al., 2006). In addition, AM 404 was reported to exert anxiolytic effect in the rat (given i.p.). The effect was correlated with increased level of AEA (but not that of 2-AG), in the prefrontal cortex and was prevented by the CB_1 receptor antagonist SR141716A (Bortolato et al., 2006).

The effect of FAAH and MAGL inhibitors has also been studied against experimental gastric mucosal lesions. It was demonstrated that the globally acting irreversible inhibitor URB 597, given i.p., inhibited the diclofenac-induced gastric mucosal lesions. Similarly, reduction of the mucosal lesions was observed in transgenic, FAAH (-/-) mice. URB 597 proved to be effective also in CB_2 (-/-) mice, but was ineffective in CB_1 (-/-) mice, indicating that the gastroprotective effect was mediated entirely by CB_1 receptors (Naidu et al., 2009). Another FAAH inhibitor, URB 937 (3′-carbamoyl-6-hydroxy-[1,1′-biphenyl]-3-yl cyclohexylcarbamate]), in contrast with the globally acting URB 597, inhibits FAAH only in peripheral tissues (Clapper et al., 2010) and proved to be also effective against gastric lesions induced by indomethacin in the mouse (Sasso et al., 2012). Similarly, inhibition of MAGL, the primary catabolic enzyme of 2-AG resulted in protection against gastric damage induced by diclofenac (Kinsey et al., 2011). However, no data have been found on the effect of AM 404 on gastric mucosal injury.

The aim of the present study was to clarify:

i./whether elevation of endocannabinoid level by inhibition of FAAH, MAGL enzymes and anandamide uptake protects the gastric mucosa against ethanol-induced mucosal injury, an acid independent ulcer model, appropriate for studying gastroprotective potency and efficacy,

ii./whether the primary site of gastroprotective action is central or peripheral,

iii./which peripheral mechanism may play role in centrally initiated mucosal protective effect of endocannabinoid modulators.

Here we showed that inhibitors of FAAH, MAGL and anandamide membrane uptake, URB 597, JZL 184 and AM 404, respectively exerted mucosal protective effect against ethanol given both i.p. and i.c.v. The primary site of action is likely to be central, since the i.c.v. injected CB_1 receptor inverse agonist AM 251 antagonized the gastroprotective effect of i.p. given URB 597, JZL 184 and AM 404. In addition, the peripherally restricted FAAH inhibitor, URB 937 following i.p administration failed to exert mucosal protective effect. The centrally injected URB 597, JZL 184 and AM 404 resulted in an increase of the gastric mucosal CGRP and somatostatin levels that are involved in mucosal defensive mechanisms. The results indicate that elevation of central endocannabinoid level may initiate a chain of events which results in activation of gastric mucosal defensive mechanisms, consequently, maintenance of mucosal integrity.

2. Materials and Methods

2.1. Animals

Experiments were carried out on male Wistar rats 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 efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. All procedures conformed to the Directive 2010/63/EU on European Convention for the protection of animals used for scientific purposes. The experiments were approved by the National Scientific Ethical Committee on Animal Experimentation and permitted by the government (Food Chain Safety and Animal Health Directorate of the Government Office for Pest County (PEI/001/1493-4/2015)).

2.2. In vivo studies

2.2.1. Gastric mucosal lesions induced by absolute ethanol

After 24 h food deprivation the animals were given orally 0.5 ml acidified ethanol (98 ml absolute ethanol + 2 ml concentrated HCl). One hour later the animals were sacrificed by inhalation of CO2, the stomachs were excised, opened along the greater curvature, rinsed with saline and examined for lesions. The degree of mucosal lesions was assessed in blinded manner by calculation of lesion index based on a 0-4 scoring system described previously (Gyires, 1990). 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 – [ulcer index in treated group/ulcer index in control group x 100].

Drugs were injected either i.p. or i.c.v. to the lateral ventricle as described previously (Gyires et al., 2000) in a volume of 5 ml/kg or 10 µl, 20 and 10 min before the ethanol challenge, respectively. To avoid a rapid increase of intracerebroventricular pressure, the volume was injected during 1 min period by a special apparatus. The antagonist of CB_1 receptor was injected i.c.v. either together with the i.c.v. given, or 10 min after the i.p. administered FAAH, MAGL and anandamide uptake inhibitors.

2.2.2. In vivo measurement of gastric motor activity

Gastric motility was measured in anesthetized rats with the rubber balloon method (Zádori and Gyires, 2013; Lefebvre et al.,1992). Briefly, after 24 h food deprivation male Wistar (200-300 g) rats 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 cmH_2O. 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. An equilibrium period (40-60 min) was registered before each experiment. AM 404, URB 597 and JZL 184 were given i.c.v. in a volume of 10 µl within 5 min via a CMA/100 microinjection pump. For i.c.v injection guide cannulas were implanted with stereotaxic surgery, and the following coordinates were used (relative to bregma): posterior 0.8 mm; lateral 1.6 mm; ventral 4.5 mm (Paxinos and Watson, 1986). The site of injection was verified after 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.

2.2.3. Behavior changes

2.2.3.1. Catalepsy

Experiments were carried out according to the methods of Tseng et al. (Tseng and Craft, 2001). 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.

2.2.3.2. 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.

2.3. Biochemical assays

2.3.1. Determination of gastric mucosal level of calcitonin gene-related peptide (CGRP) and somatostatin

For determination of gastric mucosal level of CGRP and somatostatin following CO_2 inhalation the stomachs of the rats were removed and put in 1 ml cold distilled water, sonicated and stored at -80 °C till the determination. CGRP and somatostatin concentrations were determined by radioimmunoassay (RIA) described previously (Németh et al., 1998). For the specific RIA the antisera (CGRP: C1012; somatostatin: 775/7) were raised in rabbit immunized with synthetic peptide conjugated to thyroglobulin

by glutaraldehyde. The RIA tracers were mono-125l-labeled peptides prepared by Németh et al (Németh et al., 1998; 2002). Synthetic peptides were used as RIA standards ranging from 0 to 1000 fmol/ml (somatostatin) and from 0 to 100 fmol/ml (CGRP). Detection limits of the assays were 2 fmol/ml (somatostatin) and 0.2 fmol/ml (CGRP). These techniques have proved to be specific, sensitive and valid for the measurement of neuropeptides in pharmacological research. Peptide concentrations were calculated as the measured amount of peptide per wet tissue weight, expressed as fmol/mg tissue.

2.3.2. Superoxide dismutase assay

Superoxide dismutase (SOD) activity was measured by using an assay kit (Cayman Europe, Tallinn, Estonia) according to the manufacturer's instructions. This kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine, and measures the activity of all three types of SOD (Cu/Zn, Mn and Fe-SOD). One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical ($O_2^{-\cdot}$). SOD activity was expressed in unit/mg tissue.

2.4. Chemicals

N-(4-Hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (AM 404) was purchased from Tocris Bioscience (Bristol, UK), cyclohexylcarbamic acid 3'-(Aminocarbonyl)-[1,1'-biphenyl]-3-yl ester (URB 597) and 4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester (JZL 184) were ordered from Abcam Biochemicals (Cambridge, UK). N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251) and cyclohexylcarbamic acid 3′-carbamoyl-6-hydroxybiphenyl-3-yl ester (URB 937) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

All drugs were dissolved in DMSO, and stock solutions were diluted with saline. Animals in the control groups received the drug solvents.

2.5. Statistical analysis

All data are presented as the means ± S.E.M. Statistical analysis of the data was evaluated by means of analysis of variance (ANOVA) followed by Newman-Keuls post hoc test. In the case of gastric motility experiments the pre- and postinjection values were compared with paired Student's t-test. Two-way repeated measures ANOVA test was employed to evaluate the behavior (catalepsy) and body temperature (hypothermia) changes. A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. The gastroprotective effect of URB 597, JZL 184 and AM 404 given centrally (i.c.v.)

Acidified ethanol given orally induced multiple hemorrhagic lesions on the gastric mucosa in control (vehicle-treated) animals (lesion index: 84.4 ± 12.6, n=5). Both the globally acting FAAH inhibitor URB 597 (2.9 - 29.5 nmol i.c.v.), the MAGL inhibitor JZL 184 (0.3 - 1.3 nmol i.c.v.) and the membrane uptake inhibitor AM 404 (2.5 - 25 nmol i.c.v.) induced a significant inhibition of the mucosal lesions induced by ethanol. All dose-response curves proved to be bell-shaped, the maximal protective effects (80% or above) were achieved at the following doses: 2.9 nmol (URB 597), 1.3 nmol (JZL 184) and 2.5 nmol (AM 404) (*Fig. 1*). AM 251 (3.6 nmol), a CB_1 receptor inverse agonist, failed to affect the ethanol-induced mucosal injury in a significant manner *per se*, however, inhibited the mucosal protective effect of both URB 597, JZL 184 and AM 404 (*Fig. 2*).

3.2. The effect of URB 597, JZL 184, AM 404 and URB937 given peripherally (i.p.) on acidified ethanol-induced gastric mucosal lesions

Both the globally acting FAAH inhibitor URB 597 and the MAGL inhibitor JZL 184 in the doses of 2.9-8.8 and 9.6-19.2 μmol/kg i.p., respectively, inhibited in a dose-dependent manner the mucosal lesions induced by ethanol. Similarly, the uptake inhibitor AM 404 (1.5-15 μmol/kg i.p.) also exerted mucosal protective effect given peripherally. In contrast, injection of the peripherally restricted FAAH-inhibitor URB 937 (in the doses of 0.8-16.92 μmol/kg i.p.) failed to result in a significant reduction of gastric mucosal lesions induced by ethanol (*Fig. 3*).

3.3. The effect of the centrally (i.c.v.) given CB_1 receptor inverse agonist AM 251 on the protective action of URB 597, JZL 184 and AM 404 given peripherally (i.p.)

URB 597 (8.8 μmol/kg i.p.), JZL 184 (19.2 μmol/kg i.p.) and AM 404 (15 μmol/kg i.p.) induced a significant inhibition of gastric mucosal injury evoked by ethanol. AM 251 (3.6 nmol) given i.c.v. 10 min after the i.p. injection of URB 597, JZL 184 and AM 404 inhibited the development of the protective effect of the all substances (*Fig. 4*).

3.4. The effect of centrally given URB 597, JZL 184 and AM 404 on the mucosal levels of somatostatin and calcitonin gene-related peptides (CGRP), and on the activity of superoxide dismutase (SOD)

Ethanol induced a profound reduction of the mucosal levels of both CGRP and somatostatin. When the rats were pretreated with either URB 597 (2.95 nmol i.c.v.), JZL 184 (1.3 nmol i.c.v.) or AM 404 (2.5 nmol i.c.v.), a significant increase of both CGRP and somatostatin levels were observed, that is, all the compounds prevented (or at least significantly reduced) the deleterious effect of ethanol on mucosal mucosal protective factors (*Fig. 5*).

The mucosal activity of SOD also showed a significant reduction following ethanol treatment. URB 597 and JZL 184 reversed the effect of ethanol on SOD activity parallel with the gastroprotective effect, whereas AM 404 failed to significantly influence it (*Fig. 6*).

3.5. The effect of URB 597, JZL 184 and AM 404 injected i.c.v. on gastric motor activity

The effect of URB 597, JZL 184 and AM 404 on gastric motor activity was studied in gastroprotective, as well as in 10 times higher doses. The results showed that the compounds URB 597 (2.95 - 29.5 nmol), JZL 184 (1.3 - 13 nmol) and AM 404 (2.5 - 25 nmol) injected i.c.v. influenced neither the mean amplitude of gastric contractions nor the gastric tone *in vivo* by using the rubber balloon technique (*Fig. 7*).

3.6. The cataleptic and hypothermic effects of i.c.v. injected URB 597, JZL 184 and AM 404

Control, saline (i.c.v.) treated rats moved away their front paws from the bar immediately. Rats treated with URB 597 and AM 404 in gastroprotective doses (2.95 nmol and 2.5 nmol i.c.v., respectively) failed to show immobility in the bar test (means of latency period of paw withdrawal are below1 sec). Similarly, neither JZL 184 (1.3 nmol) induced a significant prolongation of the latency of paw removal (mean: 1 sec) from the bar suggesting that no cataleptic effect was elicited by the drugs in their gastroprotective dose-range during the 40 min observation period.

Similarly, no differences could be observed in the body temperatures compared to the baseline, pre-treatment values in control and drug-treated groups (*Fig. 8*).

4. Discussion

The present results demonstrated that elevation of anandamide and 2-AG levels by blocking their degradation or uptake resulted in gastric mucosal protective effect against ethanol-induced lesions, given both peripherally (i.p.) and centrally (i.c.v.). Moreover, it was first shown that inhibition of AEA cellular uptake by AM 404 (i.c.v. and i.p.) also decreased the mucosal injury induced by ethanol. Since the peripherally acting FAAH inhibitor, URB 937, failed to inhibit the ethanol-induced lesions in a significant manner, it may be raised that the primary site of the gastroprotective action is likely to be central. Further evidence for the central site of mucosal protective effect was provided by the findings that the gastroprotective action of peripherally (i.p.) injected FAAH, MAGL inhibitors and uptake inhibitor of AEA was reversed by i.c.v. given CB_1 receptor antagonist/inverse agonist AM 251, indicating the prominent role of central CB_1 receptors in gastric mucosal defense. Similarly, parallel administration of AM 251 with either FAAH, MAGL or AEA uptake inhibitors i.c.v. prevented the development of the mucosal protective effect suggesting the significance of central CB_1 receptors in gastroprotection.

Pharmacological blockade of the degradation of endocannabinoids is an attractive strategy for enhancing endocannabinoid signaling. It has been supposed that elevated endocannabinoid tissue levels would result in less psychoactive effects (such as catalepsy, hypothermia or hyperphagia) than the direct stimulants of CB_1 receptors, while the beneficial effects due to activation of CB_1 and/or CB_2 receptors would be retained (Makriyannis et al., 2005; Kathuria et al., 2003).

Several data confirmed the analgesic/anti-inflammatory and mucosal protective effect of FAAH and MAGL inhibitors. For example, inhibitors of FAAH (URB 597, OL-135) and MAGL (JZL 184) enzymes induced analgesic effect in a wide range of antinociceptive tests in the dose range of 16-40 mg/kg i.p. (Kinsey et al., 2009). The irreversible FAAH inhibitor URB 537 exerted antinociceptive effect both in several acute rodent model of inflammatory pain, as well as in chronic arthritis model in mice (Kinsey et al., 2011). URB 597 combined with diclofenac resulted in synergistic analgesic interactions and parallel significant reductions in the gastric mucosal injury induced by diclofenac. The gastroprotective effects of URB 597 (10 mg/kg s.c.) could not be observed in CB_1 (-/-) mice, whereas it maintained its efficacy in CB_2 (-/-) mice, indicating a CB_1-receptor mediated mechanism of action. Similarly, diclofenac-induced ulcer formation was less pronounced in FAAH (-/-) mice than in wild-type animals (Naidu et al., 2009). The *in vivo* effect correlates with the changes of FAAH activity or AEA level in the brain: FAAH (-/-) mice (Cravatt et al., 2001), or mice and rats treated with FAAH inhibitor URB 597 (10 and 0.3 mg/kg i.p., respectively) (Kathuria et al., 2003; Kinsey et al., 2009) showed either elevated levels of AEA (Cravatt et al., 2001; Kinsey et al., 2009) or decreased FAAH activity in the CNS with a rapid onset (< 15 minutes) (Kathuria et al., 2003).

Moreover, the MAGL inhibitor JZL 184 prevented diclofenac-induced gastric hemorrhages in

the mouse (0.25-40 mg/kg i.p.) (Kinsey et al., 2011). The gastroprotective effect is in correlation with the elevated level of 2-AG, namely, JZL 184 (4 mg/kg i.p.) was shown to increase the 2-AG levels both in the stomach (Kinsey et al., 2011) and in the brain (Long et al., 2009). The protective effect of JZL 184 may be due to its inhibitory effect on the diclofenac-induced increases in gastric levels of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and granulocyte colony-stimulating factor. Furthermore, inhibition of acid secretion, increased gastric blood flow and central mechanisms may also contribute to the protective action of JZL 184 (Kinsey et al., 2011).

Moreover, para-hydroxy derivative of URB 597, the compound URB 937, is a potent inhibitor of FAAH activity in peripheral tissues, but not in the CNS, since it is extruded from the CNS and spinal cord by the membrane transporter ATP-binding cassette (Clapper et al., 2010). URB 937 induces elevation of AEA level in the periphery, in the liver of the mouse (ED_{50}: 0.2 mg/kg s.c.), and 200 times higher dose (40 mg/kg s.c.) was needed to suppress the brain FAAH activity (Clapper et al., 2010). Similarly, orally administered URB 937 inhibited FAAH activity in liver tissue (ED_{50}: 0.3 mg/kg) without affecting FAAH activity in brain tissue (Clapper et al., 2010; Sasso et al., 2012). *In vivo*, URB 937 was found to exert antinociceptive effect against mechanical, thermal hyperalgesia, and mechanical allodynia given orally (0.3-3 mg/kg) in the mouse, in the dose range that elevated the AEA level in peripheral tissue (in liver) without affecting FAAH activity in brain tissue (Clapper et al., 2010). In addition, URB 937 reduced the number and severity of gastric lesions produced by indomethacin (0.3-1 mg/kg per os) in mice (Sasso et al., 2012).

We showed that the globally acting inhibitors of FAAH and MAGL enzymes, URB 597 and JZL 184, respectively, injected peripherally (i.p.) exerted gastric mucosal protective action against the acidified ethanol-induced lesions in the doses of 2.9-8.8 μmol/kg (1-3 mg/kg), and 9.6-19.2 μmol/kg (5-10 mg/kg) respectively. However, the peripherally restricted FAAH inhibitor, URB 937 given in similar dose-range, 0.8-16.9 μmol/kg (0.3-6 mg/kg i.p.), that was shown to inhibit the indomethacin-induced lesions and to elevate the AEA level in the periphery (Sasso et al., 2012) in the mouse, failed to inhibit the ethanol-induced mucosal injury.

Based on the data of the literature and our present findings it can be concluded that elevation of endocannabinoid level in the periphery by peripherally restricted FAAH inhibitor prevents the gastric mucosal lesions induced by NSAID in the mouse, but does not affect gastric mucosal injury elicited by ethanol (in similar dose range) in the rat. The ethanol-induced gastric damage was inhibited, however, by the globally acting FAAH and MAGL inhibitors, indicating that increase of central endocannabinoid level may initiate a chain of events that result in protective action against the mucosal damaging effect of ethanol, in a CB_1 receptor-mediated fashion. The central site of gastroprotective effect of URB 597 and-JZL 184 against ethanol-induced lesions was confirmed by the findings, that after peripheral (i.p.) administration their protective effect was reversed by centrally (i.c.v.) given CB_1 receptor

antagonist/inverse agonist AM 251. Moreover, both agents were highly effective and potent following i.c.v. administration; their central gastroprotective dose range proved to be 0.3-29.5 nmol/rat (150-170 g body weight), while their effective dose range following peripheral administration was 2.9-19.5 μmol/kg (see above).

AM 404 inhibits the carrier-mediated transport of AEA into presynaptic neurons from the synaptic cleft (Rogosch et al., 2012; Beltramo et al., 1997). However, AM 404 is not a selective uptake inhibitor, since it also inhibits the cyclooxygenase (COX)-1 and COX-2 enzymes, as well as it is an agonist on $TRPV_1$ receptors, and inhibits FAAH activity (De Petrocellis et al., 2000).

Since no data have been published how inhibitors of AEA membrane uptake influence the development of experimental mucosal lesions, we examined the effect of AM 404 given both peripherally (i.p.) and centrally (i.c.v.) on ethanol-induced gastric lesions.

Our data showed that AM 404 given either i.p. or i.c.v. inhibited the ethanol-induced mucosal lesions. Since the protective effect was highly reduced by the CB_1 receptor antagonist AM 251, the cannabinoid CB_1 receptor plays a significant role in mediating this mucosal protective action. Moreover, similarly to URB 593 and JZL 184, AM 251 given i.c.v. reversed the protective effect of peripherally injected AM 404, indicating that the primary site of action is likely to be central. Further argue for the central site of the gastroprotective is that AM 404 given i.c.v. proved to be a highly potent and effective gastroprotective agent against ethanol-induced lesion; the effective dose range was 2.5-25 nmol/rat (body weight 150-170 g) i.c.v., while the effective dose range of i.p. administered AM 404 was 1.5-15 μmol/kg.

Since our previous findings demonstrated that bilateral cervical vagotomy reduced the centrally-induced gastroprotective effect of cannabinoids in a significant manner (Shujaa et al., 2009), dorsal vagal complex (DVC) and vagal nerve may play a role in conveying the centrally initiated effect of FAAH, MAGL and AEA uptake inhibitors to the periphery, to gastric mucosa. Similarly, vagal dependent mechanism of gastric mucosal protection was demonstrated for several, centrally injected neuropeptides (for reviews see: Tache et al., 1994; 2012; Gyires and Zádori, 2014) indicating the prominent role of DVC in maintaining gastric mucosal homeostasis. Furthermore, several data indicate that the centrally initiated gastroprotection correlates with increased gastric mucosal microcirculation mediated by CGRP, prostaglandin and NO in a vagal-dependent mechanism (Tache et al., 1994; Tache, 2012; Gyires and Zádori, 2014).

Our present data showed that gastric mucosal CGRP level dramatically decreased following ethanol administration, while i.c.v. injection of FAAH-, MAGL- and AEA uptake inhibitors elevated the ethanol-induced decreased level of CGRP in a significant manner. Moreover, somatostatin may also mediate gastric mucosal defense. Namely, it has been shown that somatostatin given i.p. exerts mucosal protection against ethanol-induced injury. The gastroprotective dose of somatostatin prevented the

decrease of gastric mucosal blood flow caused by ethanol, which effect is likely to be dependent on NO generation by increasing the impaired (but not the normal) mucosal blood flow and NO release (Ancha et al., 2003). Our findings suggest, in agreement with the results of Ancha et al (Ancha et al., 2003), that the gastric mucosal somatostatin level was also highly reduced following ethanol administration. The decreased somatostatin level of gastric mucosa was restored by i.c.v. administration of FAAH-, MAGL- and AEA membrane uptake inhibitors. These results indicate that both CGRP and somatostatin may play a role in conveying the centrally initiated gastroprotective effect, most probable via restoration of impaired mucosal microcirculation.

Furthermore, we also investigated whether the FAAH-, MAGL- and AEA membrane uptake inhibitors given centrally may affect the activity of free radical scavenging enzyme, SOD. Namely, it has been well documented that superoxide free radicals are involved in the development of ethanol-induced gastric mucosal lesions, probably via an interaction with cellular membranes (Szelenyi and Brune, 1988) and that the enzymatic antioxidant SOD reduced mucosal damage induced either by ethanol or aspirin (Pihan et al., 1987). On the other hand, gastroprotective agents may increase the activity of SOD in gastric mucosa, which may be responsible, at least partly, for their gastric mucosal protective action (Kim et al., 2012). Our present findings showed that ethanol, administered orally, resulted in a significant reduction of the activity of SOD, compared to the vehicle-treated control group. Both FAAH- and MAGL inhibitors given i.c.v. increased the reduced SOD enzyme activity in gastric mucosa in a significant manner, indicating that the ethanol-induced decreased free radical scavanging capacity of gastric mucosa could be reversed by elevation of central endocannabinoid level. However, AM 404, the AEA membrane uptake inhibitor failed to result in a significant elevation of mucosal SOD activity, suggesting that restoration of SOD activity in gastric mucosa is not likely to play a significant role in the mucosal protective mechanism of AM 404.

Previous data suggest that gastric mucosal protection may correlate with gastric motor activity (Guidobono et al., 1998). Therefore, we analyzed whether of FAAH-, MAGL- and AEA membrane uptake inhibitors given centrally at gastroprotective dose range may influence the gastric tone and contractions. Our results showed that neither the basal tone, nor the phasic antral contractions were influenced by the centrally injected inhibitors of the degradation or uptake of AEA or 2-AG in gastroprotective dose-range.

Direct agonists of CB_1 receptors are known to elicit multiple behavioral effects in rodents, including analgesia, hypomotility, hypothermia and catalepsy (referred as the tetrad test for cannabinoid activity) (Wiley and Martin, 2003; Kathuria et al., 2003). It has been raised that increasing endocannabinoid tissue levels would be less likely to cause psychoactive effects than would the direct stimulants of cannabinoid receptors, while the beneficial effects due to $CB_{1/2}$ receptor activation, such as analgesia, would be maintained (Makriyannis et al., 2005). For example, URB 597 and its analogue

URB 532 at doses that almost abolished FAAH activity and substantially raised brain AEA level, neither reduced body temperature nor evoked catalepsy, key symptoms of cannabinoid intoxication in the rodent (Kathuria et al., 2003) and marker for psychoactivity in humans. On the other hand, Long et al (Long et al., 2009) have shown that MAGL inhibitor JZL 184 (16 mg/kg i.p.), besides antinociceptive action, in contrast to FAAH inhibitors, induced hypothermia and hypomotility, but not catalepsy. In addition, dual FAAH/MAGL inhibitor, JZL 195, showed broad activity in the tetrad test, such as analgesia, hypomotility and catalepsy. In the catalepsy test, neither JZL 184 nor PF-3845 (FAAH inhibitor) had an effect, but JZL 195 (and JZL 184 + PF-3845) showed a pronounced activity, indicating that AEA and 2-AG signaling pathways interact to regulate specific behavioral processes (Long et al., 2009). Similarly, guineensine, a novel plant natural product was shown to inhibit the cellular uptake of anandamide and 2-AG elicited strong catalepsy, hypothermia, reduced locomotion and analgesia in BALB/c mice. The catalepsy and analgesia were blocked by the CB_1 receptor antagonist rimonabant indicating a cannabinoid CB_1-receptor mediated effect (Nicolussi et al., 2014).

Our results showed that neither URB 597, nor JZL 184 or AM 404, given i.c.v. induced cataleptic effects or hypothermia in gastroprotective doses.

In conclusion, the present findings indicate that pharmacological inhibition of either FAAH, MAGL or AEA uptake (by URB 597, JZL 184 and AM 404, respectively) decreases gastric mucosal injury induced by acidified ethanol, following central administration in a significant manner. The mucosal protective effect may be due, at least partly, to the increase of mucosal CGRP and somatostatin levels, as well as of SOD activity, that all were diminished by ethanol. The globally acting FAAH-, MAGL- and AEA uptake inhibitors decreased the ethanol-induced lesions also following peripheral administration, however, the peripherally restricted FAAH inhibitor URB 937 proved to be ineffective. Since others (Sasso, 2012) found that URB 937 was effective against indomethacin-induced lesions in the mouse, it might be raised that peripheral endocannabinoids are likely to play a role in mucosal protection against NSAID-, but not ethanol-induced gastric mucosal lesions. In the later case the central endocannabinoid system seems to play a prominent role in gastric defense.

The present results provided the first evidence for gastric mucosal protective effect of the central inhibition of FAAH-, MAGL- and AEA uptake. These data are additional evidence for the importance of brain-gut axis in gastric mucosal defense and gastric mucosal integrity. Endocannabinoid modulation has become one of the exciting new areas also in the field of gastrointestinal research as a promising way to treat different inflammatory and ulcerative disorders. Further studies are needed to clarify the involvement of central and peripheral endocannabinoids in regulation of protective mechanisms, the potential side effects and the potential therapeutic indications of endocannabinoid modulators in different types of gastrointestinal mucosal inflammatory, ulcerative disorders.

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Legends

Fig. 1.

The inhibitory effect of URB 597, JZL 184 and AM 404 on acidified ethanol-induced gastric mucosal lesions in the rat. The compounds were injected intracerebroventricularly (i.c.v.) 10 min before the ethanol challenge. The inhibitory effect is expressed in % compared to the control (vehicle-treated) group. Each column represents mean ± S.E.M. (n=5/group). Statistical analysis of the data was evaluated by means of ANOVA followed by Newman-Keuls post hoc test for multiple comparisons. **$p < 0.01$; ***$p < 0.001$ compared to the control (vehicle-treated) group.

Fig. 2.

The effect of URB 597 (2.9 nmol), JZL 184 (1.3 nmol) and AM 404 (2.5 nmol) on ethanol-induced gastric mucosal injury given either alone or in combination with AM 251 in the rat. All the compounds were given intracerebroventricularly (i.c.v.) 10 min before ethanol challenge. The inhibitory effect is expressed in % compared to the respective control (vehicle- or AM 251-treated) groups. Each column represents mean ± S.E.M. (n=5/group). Statistical analysis of the data was evaluated by means of ANOVA followed by Newman-Keuls post hoc test for multiple comparisons. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the respective control (vehicle-treated) group.

Fig. 3.

The inhibitory effect of URB 597, JZL 184, AM 404 and URB 937 on acidified ethanol-induced gastric mucosal lesions in the rat. The compounds were injected intraperitoneally (i.p.) 20 min before the ethanol challenge. The inhibitory effect is expressed in % compared to the control (vehicle-treated) group. Each column represents mean ± S.E.M. (n=5/group). Statistical analysis of the data was evaluated by means of ANOVA followed by Newman-Keuls post hoc test test for multiple comparisons. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the control (vehicle-treated) group.

Fig. 4.

The effect of AM 251 (3.6 nmol, intracerebroventricularly /i.c.v./) on the mucosal protective effect of URB 597 (8.8 μmol/kg), JZL 184 (19 μmol/kg) and AM 404 (15 μmol/kg) injected intraperitoneally (i.p.) 20 min before the ethanol challenge in the rat. AM 251 was given 10 min after the i.p. injection. The inhibition of gastric mucosal lesion is expressed in % compared to the respective control (vehicle- or AM 251-treated) group. Each column represents mean ± S.E.M. (n=5/group). Statistical analysis of the data was evaluated by means of ANOVA followed by Newman-Keuls post hoc test for multiple comparisons. **$p < 0.01$; ***$p < 0.001$ compared to the respective control (vehicle-treated) group.

Fig. 5.

The effect of URB 597 (2.9 nmol), JZL 184 (1.3 nmol) and AM 404 (2.5 nmol) on the mucosal CGRP (panel A) and somatostatin (panel B) concentration. The compounds were given intracerebroventricularly (i.c.v.) 10 min before ethanol. CGRP and somatostatin concentrations were determined by radioimmunoassay (RIA) 1 hour after ethanol challenge. Each column represents mean ± S.E.M. (n=5/group). Statistical analysis of the data was evaluated by means of ANOVA followed by Newman-Keuls post hoc test for multiple comparisons. *$p < 0.05$; ***$p < 0.001$ compared to absolute control group (no ethanol, no vehicle treatment, column 1), $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared to ethanol control group (vehicle + ethanol treatment, column 2).

Fig. 6.

The effect of URB 597 (2.9 nmol), JZL 184 (1.3 nmol) and AM 404 (2.5 nmol) on the mucosal SOD activity. The compounds were given intracerebroventricularly (i.c.v.) 10 min before ethanol. Gastric mucosal SOD activity was determined by using an assay kit 1 hour after ethanol challenge. Each column represents mean ± S.E.M. (n=5/group). Statistical analysis of the data was evaluated by means of ANOVA followed by Newman-Keuls post hoc test for multiple comparisons. **$p < 0.01$ compared to absolute control group (no ethanol, or vehicle treatment, column 1), $^{\#}p < 0.05$ compared to ethanol control group (vehicle + ethanol treatment, column 2).

Fig. 7.

The effect of URB 597 (2.9, 29.5 nmol), JZL 184 (1.3, 13 nmol) and AM 404 (2.5, 25 nmol) on the amplitudes of phasic antral contractions and gastric tone in anesthetized rats using the balloon technique. Each column represents mean ± S.E.M., n = 4–7/group. Paired Student's t-test did not indicate statistically significant difference between the pre- and postinjection values.

Fig. 8.

The effect of URB 597 (2.9 nmol), JZL 184 (1.3 nmol) and AM 404 (2.5 nmol) on catalepsy-test (Panel A) and on rectal temperature (Panel B). The compounds were given intracerebroventricularly (i.c.v.) and the duration of immobility (font paws on the bar, sec) was measured immediately prior and 10 min, 20, 30 and 40 min after the injection. The effects are expressed as means ± S.E.M. (n = 5/group). For statistical analysis of the data two-way repeated measures ANOVA was employed, and it did not indicate statistically significant difference compared to the control (vehicle-treated) group.

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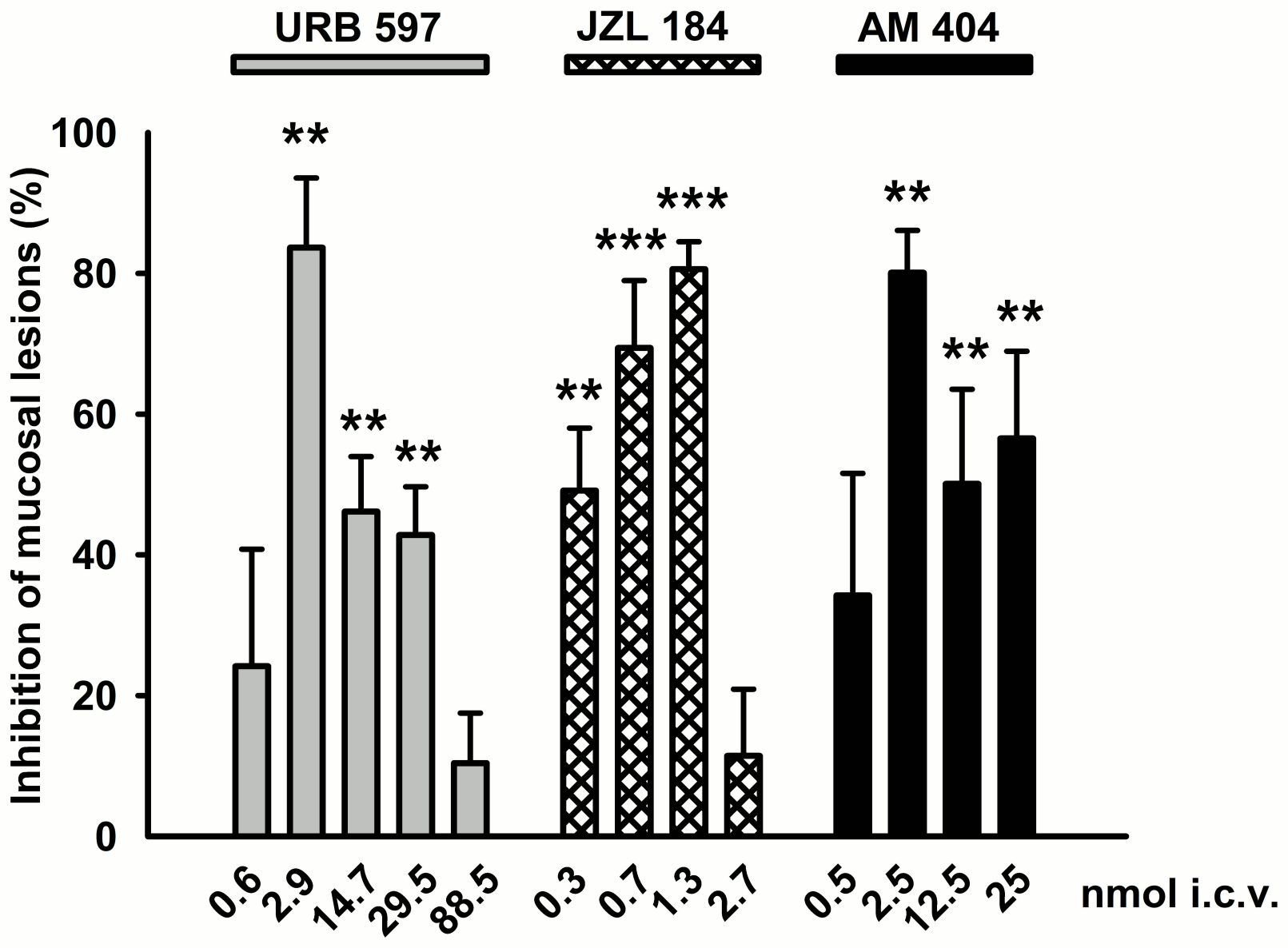
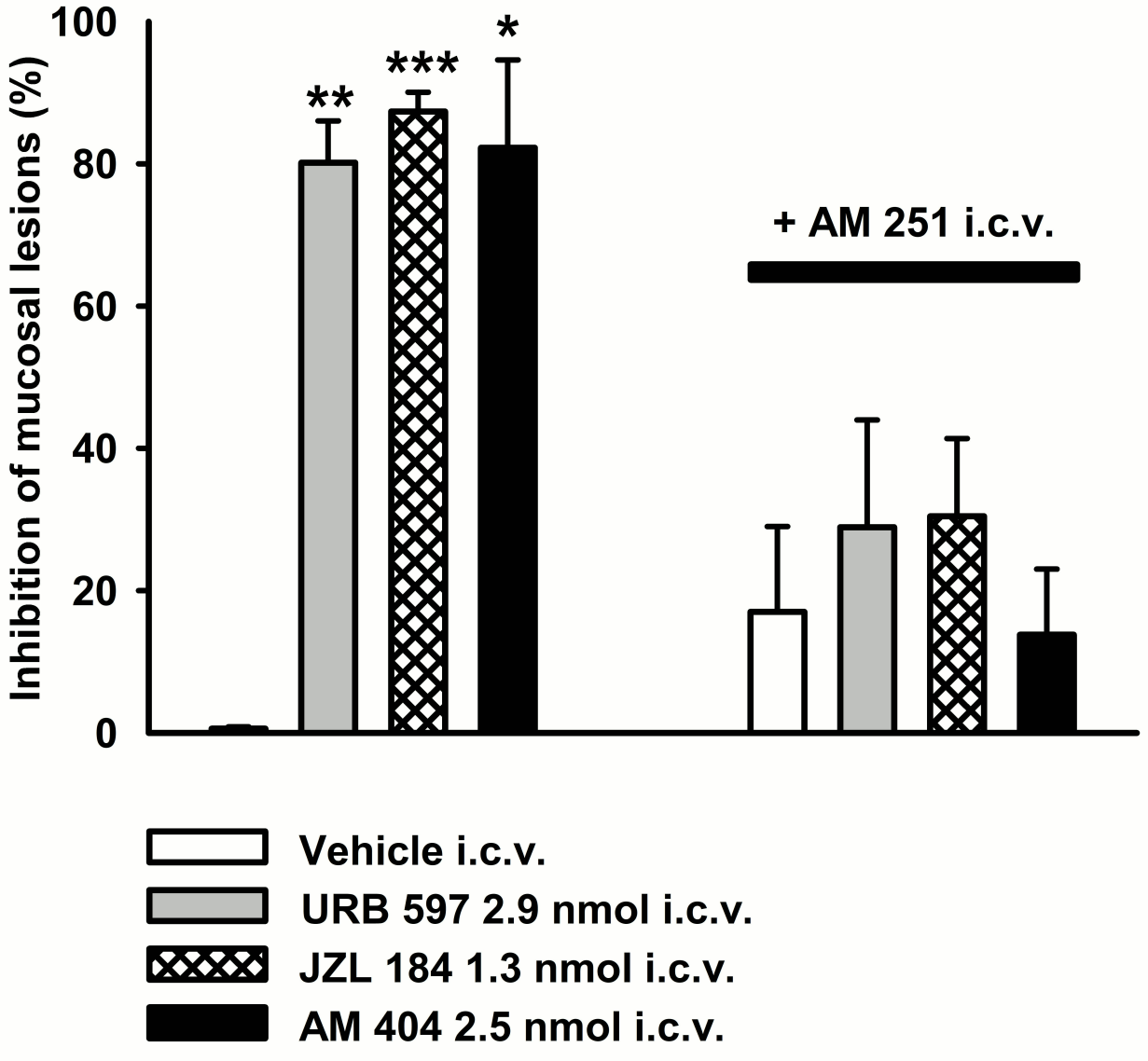
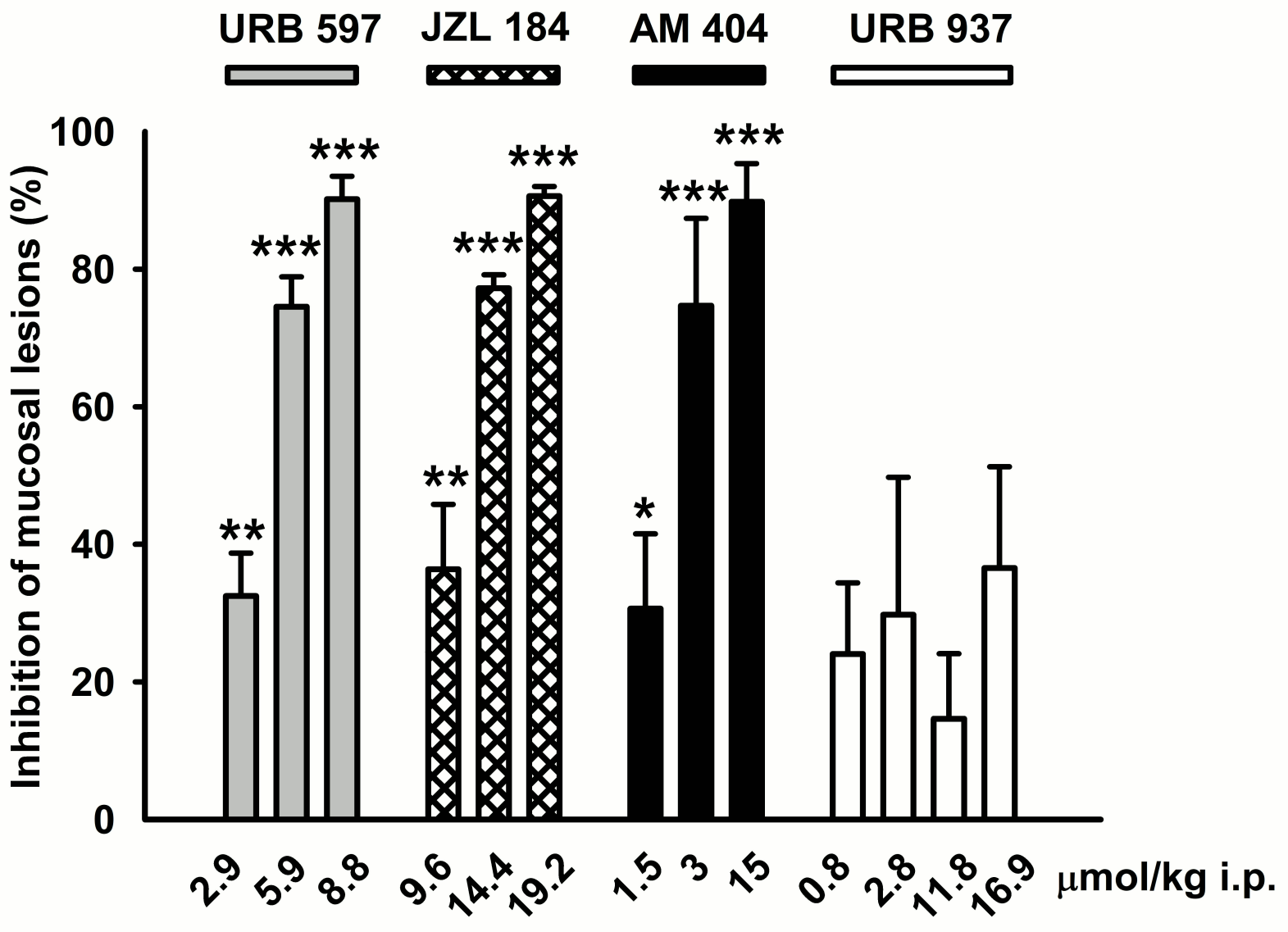
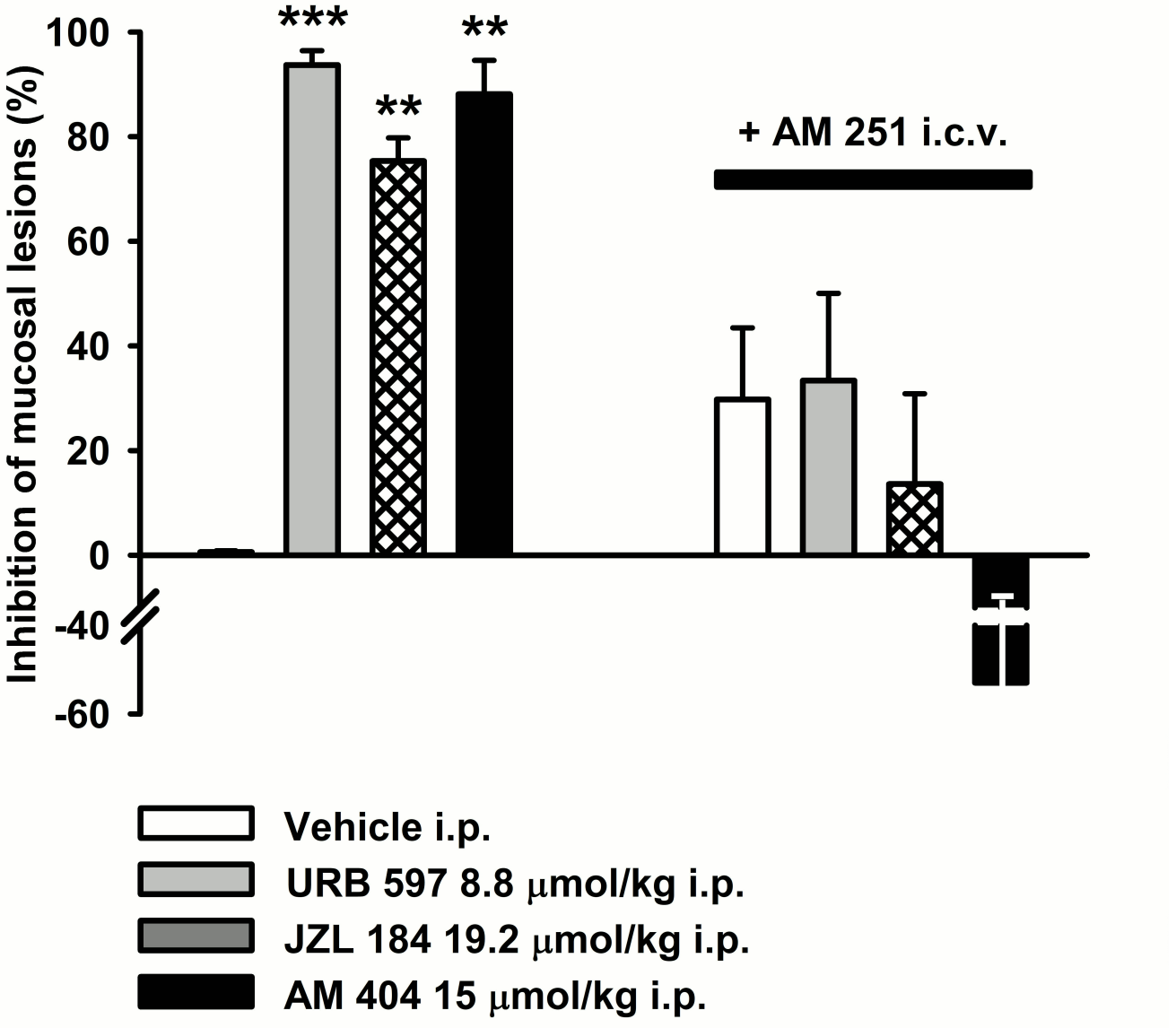
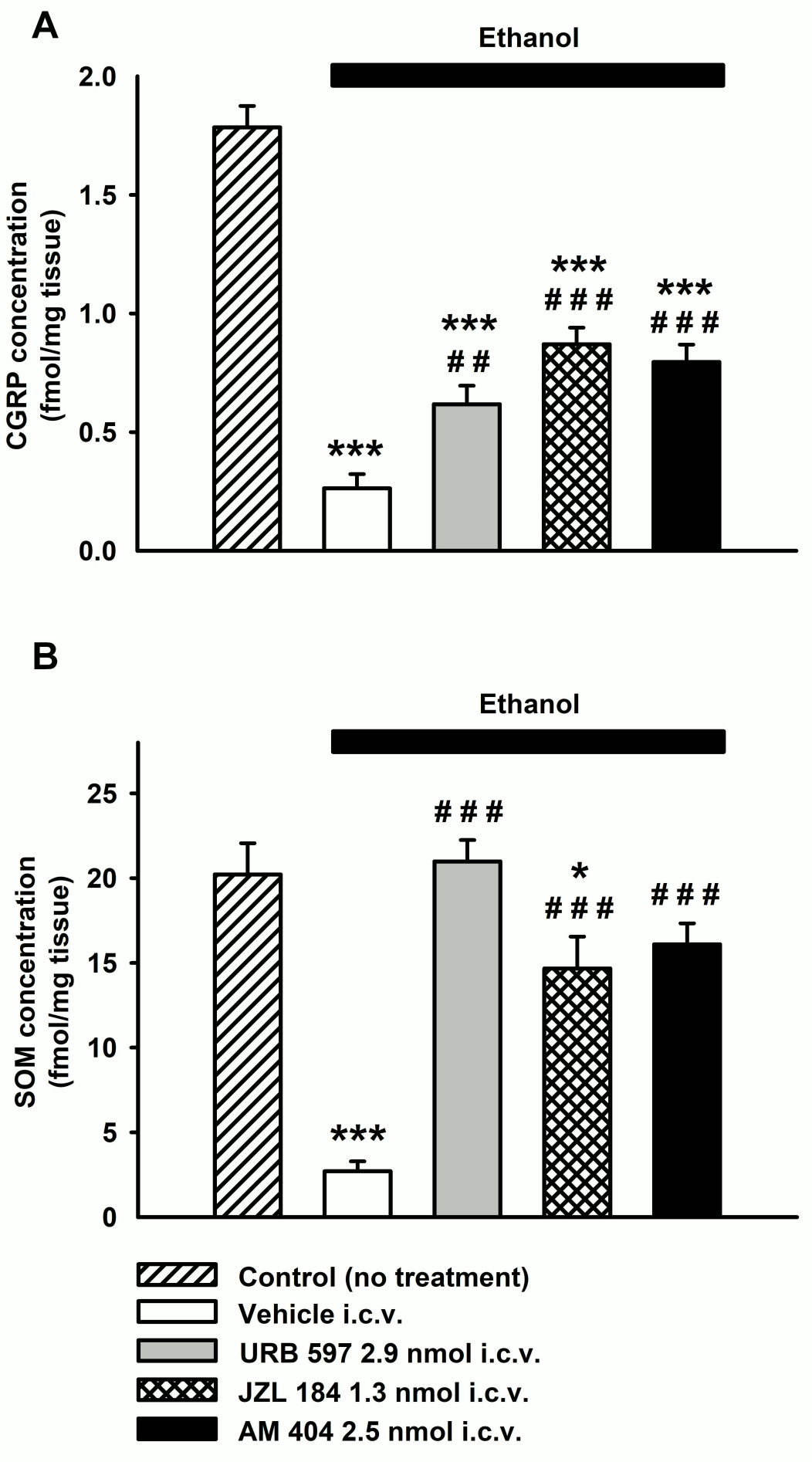
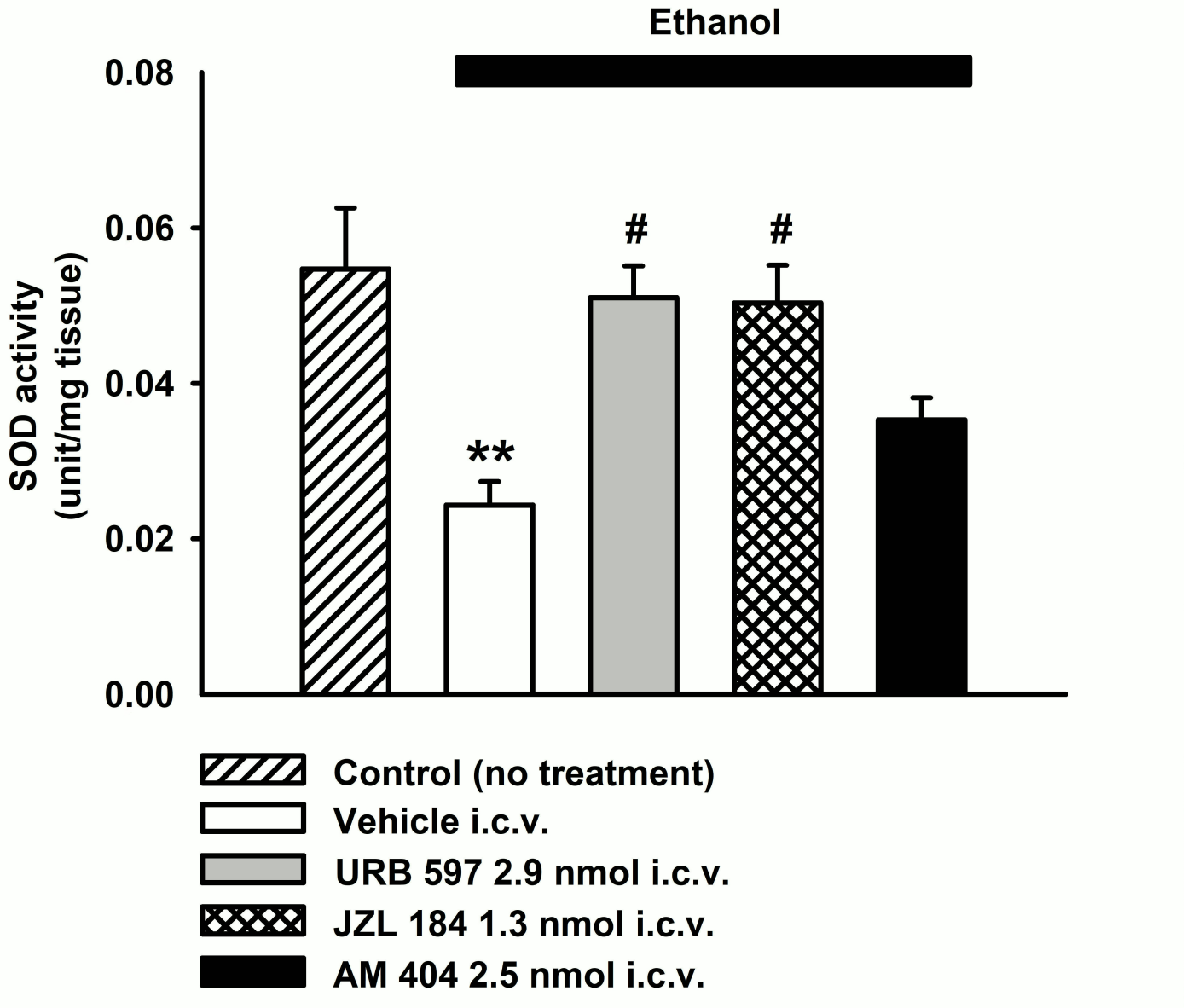

URB 597
JZL 184
AM 404
Inhibition of mucosal lesions (%)
100
80
60
40
20
0
**
**
**
**
***
***
**
**
**
0.6
2.9
14.7
29.5
88.5
0.3
0.7
1.3
2.7
0.5
2.5
12.5
25
nmol i.c.v.

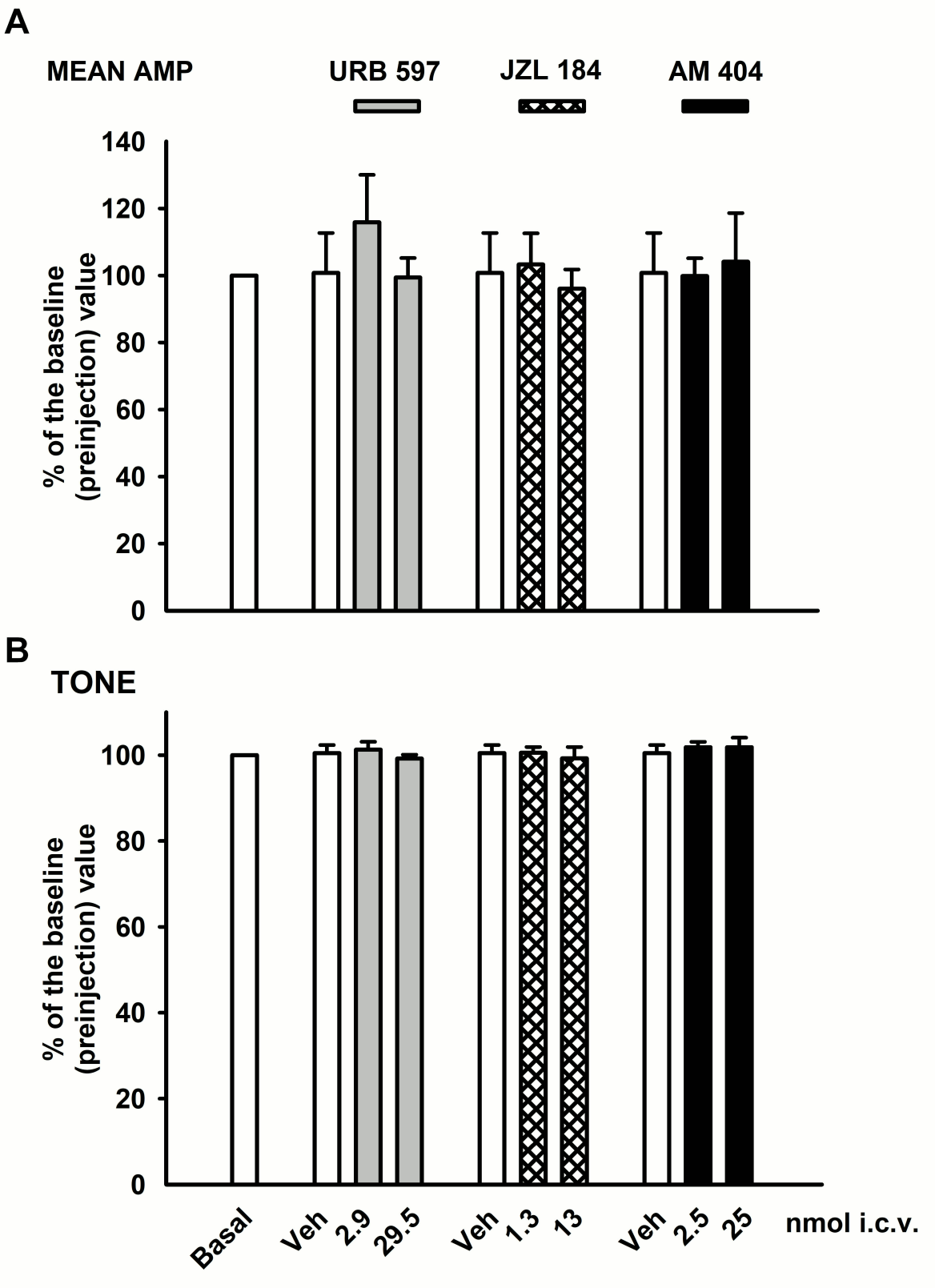

Inhibition of mucosal lesions (%)
100
80
60
40
20
0
**
***
*
+ AM 251 i.c.v.
Vehicle i.c.v.
URB 597 2.9 nmol i.c.v.
JZL 184 1.3 nmol i.c.v.
AM 404 2.5 nmol i.c.v.

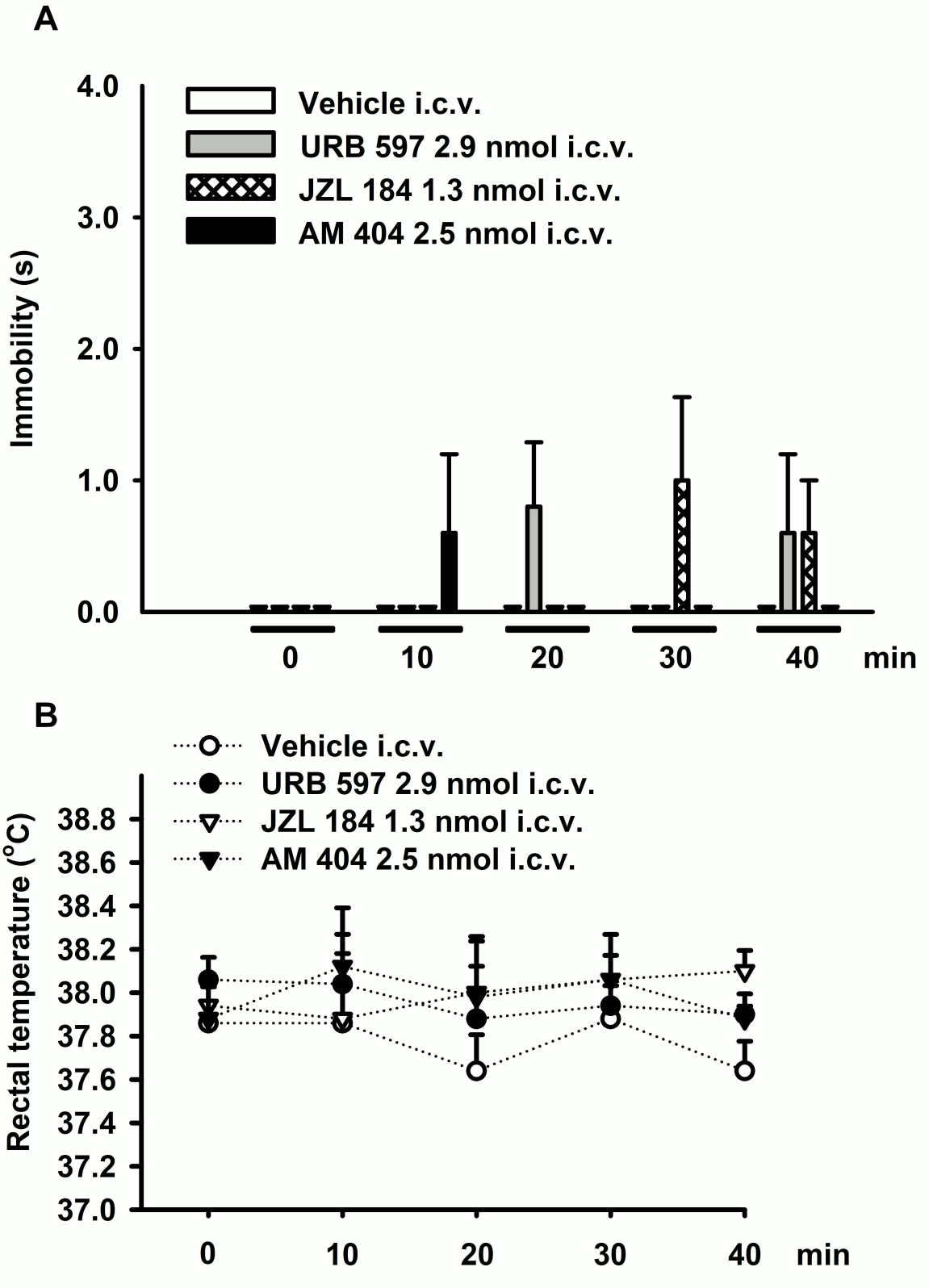

URB 597
JZL 184
AM 404
URB 937
Inhibition of mucosal lesions (%)
100
80
60
40
20
0
**
***
***
**
***
***
*
***
***
2.9
5.9
8.8
9.6
14.4
19.2
1.5
3
15
0.8
2.8
11.8
16.9
μmol/kg i.p.


Inhibition of mucosal lesions (%)
100
80
60
40
20
0
-40
-60
***
**
**
+ AM 251 i.c.v.
Vehicle i.p.
URB 597 8.8 μmol/kg i.p.
JZL 184 19.2 μmol/kg i.p.
AM 404 15 μmol/kg i.p.


A
Ethanol
CGRP concentration (fmol/mg tissue)
2.0
1.5
1.0
0.5
0.0
***
***
##
***
###
***
###
B
Ethanol
SOM concentration (fmol/mg tissue)
25
20
15
10
5
0
***
###
*
###
###
Control (no treatment)
Vehicle i.c.v.
URB 597 2.9 nmol i.c.v.
JZL 184 1.3 nmol i.c.v.
AM 404 2.5 nmol i.c.v.


Ethanol
0.08
0.06
0.04
0.02
0.00
SOD activity
(unit/mg tissue)
**
#
#
Control (no treatment)
Vehicle i.c.v.
URB 597 2.9 nmol i.c.v.
JZL 184 1.3 nmol i.c.v.
AM 404 2.5 nmol i.c.v.


A
MEAN AMP
URB 597
JZL 184
AM 404
% of the baseline (preinjection) value
140
120
100
80
60
40
20
0
B
TONE
% of the baseline (preinjection) value
100
80
60
40
20
0
Basal
Veh
2.9
29.5
Veh
1.3
13
Veh
2.5
25
nmol i.c.v.


A
Vehicle i.c.v.
URB 597 2.9 nmol i.c.v.
JZL 184 1.3 nmol i.c.v.
AM 404 2.5 nmol i.c.v.
Immobility (s)
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
min
B
Vehicle i.c.v.
URB 597 2.9 nmol i.c.v.
JZL 184 1.3 nmol i.c.v.
AM 404 2.5 nmol i.c.v.
Rectal temperature (°C)
38.8
38.6
38.4
38.2
38.0
37.8
37.6
37.4
37.2
37.0
0
10
20
30
40
min