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Angiotensin II-induced activation of central AT_1 receptors exerts endocannabinoid-mediated gastroprotective effect in rats

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[†] For the memory of our co-author, András Z. Rónai, an outstanding peptide pharmacologist

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Abstract

The aim of the present study was to analyze whether angiotensin II via the endocannabinoid system can induce gastric mucosal protection, since transactivation of cannabinoid CB_1 receptors by angiotensin AT_1 receptor in CHO cells was described. Experimental ulcer was induced by acidified ethanol given orally in male Wistar rats, CB_1(+/+) wild type and CB_1(-/-) knock out mice. The compounds were administered intracerebroventricularly. It was found, that 1./ Angiotensin II inhibited the ethanol-induced gastric lesions (11.9-191 pmol); *the effect of angiotensin II (191 pmol)* was inhibited by the CB_1 receptor inverse agonist AM 251 (1.8 nmol) and the inhibitor of diacylglycerol lipase (DAGL), tetrahydrolipstatin (0.2 nmol). 2./ Angiotensin II exerted gastroprotection in wild type, but not in CB_1(-/-) mice. 3./ The gastroprotective effect of angiotensin II (191 pmol) was reduced by atropine (1 mg/kg i.v.) and bilateral cervical vagotomy. In conclusion, stimulation of central angiotensin AT_1 receptors via activation of cannabinoid CB_1 receptors induces gastroprotection in a DAGL-dependent and vagus-mediated mechanism.

Keywords: angiotensin II, endocannabinoids, gastroprotection, rat, CB_1 KO mice

Abbreviations

Ang II: Angiotensin II; AT_1 receptor: angiotensin AT_1 receptor; 2-AG: 2-arachidonoylglycerol; CHO: Chinese hamster ovary; CGRP: calcitonin gene-related peptide; DAG: diacylglycerol; DAG lipase: diacylglycerol lipase; THL: tetrahydrolipstatin

1. Introduction

Cannabinoid receptors are a class of cell membrane receptors under the G protein-coupled receptor (GPCR) superfamily (Matsuda et al., 1990). To date, two cannabinoid receptors have been identified by molecular cloning, CB_1 and CB_2 receptors (Howlett et al., 2002; Matsuda et al., 1990; Munro et al., 1993), however the existence of additional CB receptors have also been proposed (Brown, 2007; Pacher et al., 2005; Ryberg et al., 2007).

CB_1 receptors are expressed by central and peripheral neurons, while CB_2 receptors are mainly expressed by immune cells. The major and best characterized endocannabinoids are arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG). *The endocannabinoids* are synthesized on demand by neurons and brain tissue in response to increased intracellular calcium concentrations or during calcium signal generation. Anandamide is a partial or full agonist of CB_1 receptors, depending on the tissue and biological response measured. Although it also binds to CB_2 receptors, it has very low efficacy and may act as an antagonist (Gonsiorek et al., 2000). 2-AG acts as a full agonist and is the true natural ligand for both the CB_1 and the CB_2 receptors (Sugiura et al., 2006).

Anandamide is produced from the membrane phospholipid N-arachidonoyl by a phospholipase C dependent process, while 2-AG is generated by diacylglycerol lipases (DAGLs) from diacylglycerol (DAG), which is produced during the signaling of Ca^{2+}-mobilizing hormones, such as angiotensin II (Ang II) (Gyombolai et al., 2012).

Mounting evidence suggest that cannabinoid receptors and their ligands are involved in numerous physiological and pathophysiological processes. In the central nervous system endocannabinoids can be produced postsynaptically, and act on presynaptic cannabinoid receptors as retrograde transmitters after they are translocated across the plasma membrane (Freund et al., 2003). CB_1 receptors located presynaptically in several brain regions act as

inhibitory retrograde signaling messengers at glutamatergic and GABAergic synapses, modulating the release of several neurotransmitters (Marsicano and Lutz, 1999; Piomelli, 2003). Thus, the endocannabinoid system, through its neuromodulating activity, could be involved in several physiological functions, including memory processing, pain perception, locomotion and inflammation; additionally, its dysregulation could underlie several pathological conditions known to accompanying psychiatric disorders (Di Marzo, 2008). In human therapy CB_1 receptor agonists (e.g. dronabinol) are already used against chemotherapy-induced nausea and vomiting, whereas CB_1 receptor antagonist rimonabant was introduced in the therapy of obesity (Pacher et al., 2006), however, it was withdrawn from the market due to increased risk of suicide and depression (Steinberg and Cannon, 2007). Nevertheless, specific targeting of peripheral CB_1 receptors (Kunos et al., 2009) and/or identification of the population, which is genetically resistant to the central side effects of rimonabant is still under consideration (Lazary et al., 2011). Furthermore, modulating CB_1 receptor activity has therapeutic potential in a wide range of pathological conditions including mood and anxiety disorders, movement disorders, neuropathic pain, and multiple sclerosis, as well as cancer, cardiovascular diseases, obesity/metabolic syndrome and musculoskeletal disorders (Pacher et al., 2006).

Increasing number of evidence suggest that CB_1 receptors may play a role in the modulation of gastrointestinal functions. Cannabinoids were shown to inhibit gastrointestinal motility (Izzo et al., 1999; Krowicki et al., 1999), gastric acid secretion (Adami et al., 2002) and development of gastric mucosal lesions both in acid-dependent (Germano et al., 2001; Rutkowska and Fereniec-Goltbiewska, 2006) and acid-independent ulcer models (Shujaa et al., 2009). Accordingly, CB_1 receptors were identified in neurons of the enteric nervous system and in sensory terminals of vagal and spinal neurons, moreover, CB_1 receptors are also identified in the dorsal vagal complex: in the nucleus of the solitary tract (NTS)

(Partosoedarso et al., 2003), in the dorsal motor nucleus of vagus (DMNV) (Mackie, 2005) and prominently, in the area postrema (Mackie, 2005).

Paracrine transactivation of CB_1 receptors by co-expressed type 1 angiotensin receptors (AT_1) in Chinese hamster ovary (CHO) cells was observed recently (Turu et al., 2007). AT_1 receptor is a Gq-protein-coupled receptor. Activation of Gq/11 protein-coupled receptors results in activation of phospholipase C, which produces inositol-trisphosphate and diacylglycerol (DAG) from phosphatidylinositol (4,5)-bisphosphate. From DAG 2-AG is generated by diacylglycerol lipases (DAGLs). Accordingly, inhibition of DAGL in CHO cells interfered with the activation of CB_1 receptors (Turu et al., 2007). The inhibitory effect of DAGL-inhibitor on these processes suggests that these actions are mediated mainly by 2-AG (Bisogno et al., 2005; Makara et al., 2005; Turu et al., 2009).

Since our recent findings showed that anandamide and synthetic cannabinoid analogues given centrally induced gastric mucosal protection by CB_1 receptor-mediated mechanism in the rat (Shujaa et al., 2009), the present study was designed to examine whether Ang II injected centrally can induce gastric mucosal defense in the rat and mouse via activation of the endocannabinoid system.

Here we show, that i.c.v. injection of both anandamide, 2-AG and Ang II induced gastric mucosal protection in the rat. The protective action of Ang II was likely to be mediated via activation of AT_1 and CB_1 receptors. The protective effect was decreased by tetrahydrolipstatin, an inhibitor of DAGL, the principle enzyme responsible for synthesis of 2-AG. Moreover, Ang II induced gastroprotective effect also in wild type mice, however, it failed to exert mucosal protective effect in CB_1 receptor deficient mice. Consequently, these data are the first in vivo evidence on the interaction between central AT_1 receptors and the endocannabinoid system in a DAGL-dependent mechanism.

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 and on CB1 receptor knockout (-/-, CB_1R KO) and wild type (+/+, WT) C57BL/6J mice (21-25 g), kindly provided by Professor Andreas Zimmer, University of Bonn (Zimmer et al., 1999).

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 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: 22.1/606/001/2010).

2.2. Experimental procedures

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 (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:

$$100 - \left[\frac{\text{ulcer index in treated group}}{\text{ulcer index in control group}} \times 100 \right]$$

Drugs were injected intracerebroventricularly (i.c.v.) to the lateral ventricle as described previously (Gyires et al., 2000) in a volume of 10 μl and 5 μl for rats and mice, 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 antagonists (the CB_1 receptor inverse agonist AM 251, the AT_1 receptor antagonist candesartan and the DAGL inhibitor THL) were injected together with the agonists.

The doses of the antagonists were selected partly on the basis of our preliminary experiments, partly on the basis of the literature (for AM 251: Bakkali-Kassemi et al., 2011; for candesartan: Fujisawa et al., 2011; Nishimura et al., 1998; for tetrahydrolipstatin: Gregg et al., 2012). *The doses of agonists were selected on the basis of dose-response curves: 115 and 26.4 nmol for anandamide and 2-AG, respectively, and 191 pmol for Ang II.* Atropine was given intravenously (i.v.) 15 min before the i.c.v. injection of Ang II.

Bilateral cervical vagotomy

Under pentobarbital anaesthesia (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 hours recovery from operation.

Determination of gastric mucosal CGRP level

For determination of gastric mucosal level of calcitonin gene-related peptide (CGRP) the rats were anesthetized with ether, the stomachs were removed and gastric mucosa was separated on a cooled plate. It was weighed and put in 1 ml cold distilled water, sonicated and stored at -80 ºC till the determination.

CGRP concentration was determined by radioimmunoassay (RIA) described previously (Németh et al., 1998). For the specific RIA the antiserum (C1012) was raised in rabbit immunized with synthetic peptide conjugated to thyroglobulin by glutaraldehyde. The RIA tracer was mono-^{125}l-labelled peptide prepared by Németh et al. (2002). Synthetic peptide was used as RIA standard ranging from 0 to 100 fmol/ml. The detection limit of the assay was 0.2 fmol/ml. This technique has proved to be specific, sensitive and valid for the measurement of neuropeptides in pharmacological research. CGRP concentration was calculated as the measured amount of peptide per wet tissue weight, expressed as fmol/mg.

2.3. Materials

Arachidonylethanolamide (anandamide), 2-arachidonoylglycerol (2-AG) and angiotensin II (Ang II) (human) were ordered from Ascent Scientific Ltd. (Bristol, UK). N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251) were purchased from Tocris Bioscience (Bristol, UK),while *N*-Formyl-L-leucine(1*S*)-1-[[(2*S*,3*S*)-3-hexyl-4-oxo-2-oxetanyl] methyl] dodecyl ester (tetrahydrolipstatin), atropine sulphate and pentobarbital sodium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Candesartan was ordered from Toronto Research Chemicals (Toronto, Canada).

The stock solutions of anandamide (ethanol), 2-AG (acetonitrile) and AM-251 (DMSO) were diluted with saline. All the other drugs were dissolved in saline. Animals in the control groups received the drug solvents.

2.4. 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 test for multiple comparisons. A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. The effect of anandamide and 2-AG on ethanol-induced gastric mucosal damage and gastric mucosal level of CGRP.

Both anandamide and 2-AG in the doses of 2.9-115 and 3.3-26.4 nmol i.c.v., respectively, inhibited the development of ethanol-induced gastric mucosal lesions in a dose-dependent manner (Fig. 1), however, at higher dose (52.8 nmol) the protective effect of 2-AG was diminished. AM 251 (1.8 nmol i.c.v.), an inverse agonist of the CB_1 cannabinoid receptor, failed to affect the ethanol-induced gastric mucosal lesions, but reversed the gastroprotective effect of both anandamide (115 nmol i.c.v.) and 2-AG (6.6 nmol i.c.v.) suggesting the involvement of central CB_1 cannabinoid receptors in the mucosal defensive processes (Fig. 2).

The mucosal CGRP content decreased from 1.34 ± 0.1 fmol/mg to 0.25 ± 0.1 fmol/mg ($p<0.001$) values 60 min after oral administration of ethanol. I.c.v. administration of the vehicles failed to affect the mucosal CGRP content. Anandamide (58 nmol i.c.v.) and 2-AG (26.4 nmol i.c.v.) given 10 min before the ethanol administration restored the ethanol-induced decrease of mucosal CGRP level (1.20 ± 0.1 fmol/mg and 1.15 ± 0.1 fmol/mg, respectively) (Fig. 3).

3.2. The effect of Ang II and its combination with candesartan, AM 251 and THL on ethanol-induced gastric mucosal damage in the rat.

Ang II inhibited the ethanol-induced gastric mucosal lesions in a dose-dependent manner in the doses of 11.9-191 pmol i.c.v., however at higher dose (956 pmol) the mucosal protective effect was diminished (Fig. 4). *I.c.v. injection of the AT_1 receptor antagonist candesartan inhibited the gastroprotective effect of Ang II (191 pmol) in a dose dependent manner (5.2 and 31.7 nmol) (Fig. 5A).* Moreover, the inverse agonist of CB_1 cannabinoid receptor, AM 251 (1.8 nmol, Fig. 5B) and the DAGL inhibitor THL (0.2 nmol, Fig. 5C) also interfered with the gastroprotective effect of AngII (191 pmol). None of the antagonists affected the ethanol-induced gastric mucosal lesions in a significant manner per se.

3.3. The effect of Ang II on cannabinoid CB_1(+/+) wild type and CB_1(-/-) knock out mice.

Ang II (191 pmol i.c.v.) inhibited the ethanol-induced mucosal lesions in wild type mice (ulcer indices: 107.8 ± 7.3 vs. 29.2 ± 4.7). However, Ang II in the same dose failed to elicit mucosal protective effect in CB_1 receptor deficient mice (ulcer indices: 97.8 ±2.4 vs. 85.7 ± 7.9, Fig. 6). Fig. 7 illustrates representative macroscopic pictures of the protective effect of Ang II in wild type and in CB_1 receptor deficient mice.

3.4. The effect of bilateral cervical vagotomy and atropine on the gastroprotective action of Ang II in the rat.

In sham operated rats Ang II (191 pmol) exerted a highly significant inhibition of ethanol-induced mucosal damage. Bilateral cervical vagotomy resulted in a slight, but not significant aggravation of gastric mucosal lesions compared to sham operated rats and reduced in a significant manner the Ang II-mediated gastric mucosal protective effect (Fig. 8A). Atropine (1 mg/kg i.v.) failed to affect the ethanol-induced mucosal lesions, however, given 15 min before the i.c.v. administration of Ang II reduced in a significant manner the mucosal protective effect of Ang II, though did not abolish it (Fig. 8B).

4. Discussion

G protein-coupled receptors mediate the effects of several neurotransmitters and neuromodulators on the target cells by stimulating G protein dependent and independent intracellular signaling pathways (DeWire et al., 2007; Hunyady and Catt, 2006; Lefkowitz, 2004). Activation of Gq/11 protein-coupled receptors results in phospholipase C activation, which produces inositol-trisphosphate and DAG from phosphatidylinositol (4,5)-bisphosphate, and DAG can be converted to 2-AG by DAGL (Basavarajappa, 2007; Sugiura et al., 2006).

It was recently shown that activation of the Gq/11-coupled angiotensin AT_1 receptor by angiotensin II resulted in activation of cannabinoid CB_1 receptors via a DAGL-dependent mechanism in CHO cells (Turu et al., 2007). Namely, it was observed that inhibition of DAGL in these cells led to inhibition of the constitutive activity of CB_1 receptors, and similar results were obtained in HEK-293 cells (Turu et al., 2007, 2009). The transactivation of cannabinoid CB1 receptors by Ang II is supported by the finding that showed an inhibitory effect of AM 251, inverse agonist of CB_1 receptors, on the hypertensive effect of Ang II injected directly into the hypothalamic paraventricular nucleus of anaesthetized rats

(Gyombolai et al., 2012). Vice versa, AT_1 receptor expression may be directly regulated by CB_1 receptors, namely in cultured vascular smooth muscle cells inhibition of CB_1 receptor by rimonabant and AM 251 led to down-regulation of AT_1 receptor expression (Tiyerili et al., 2010).

Cannabinoids are involved in numerous physiological and pathophysiological processes, and affect also gastrointestinal functions. For example they inhibit GI transit and motility in rats (Landi et al., 2002), decrease the intragastric pressure and the pyloric contractility by activating CB_1 receptors (Krowicki et al., 1999), tonically inhibit colonic propulsion (Pinto et al., 2002) and decrease GI transit in mice (Izzo et al., 2000). CB_1 receptor agonists decreased the gastric acid secretion induced by pentagastrin given intravenously (Adami et al., 2002), but i.c.v. injection of the synthetic non-selective agonist WIN 55,212-2 was ineffective in preventing the pentagastrin stimulated gastric acid secretion (Adami et al., 2004). Cannabinoids have been shown to decrease the formation of experimental gastric ulcers as well. Tetrahydrocannabinol, for example, reduced mucosal damage induced by pylorus ligation (Sofia et al., 1978) and Cannabis sativa was effective against restraint-induced gastric ulcerations (De Souza et al., 1978). Furthermore, anandamide reduced the gastric ulceration induced by water immersion and restraint stress (Dembinski et al., 2006) and WIN55,212-2 produced anti-ulcer effect in the cold/restraint stress model (Germano et al., 2001). Moreover, the selective CB_1 receptor agonist ACEA (arachidonyl-2-chloroethylamide) significantly reduced gastric ulcer formation induced by aspirin (Rutkowska and Fereniec-Goltbiewska, 2006).

The ulcer models used in the above experiments are gastric acid-dependent models, consequently, the inhibition of the mucosal lesions can be due either to inhibition of gastric acid secretion, or stimulation of gastric mucosal defense (or both). Moreover, since cannabinoids have been given peripherally, no conclusion could be drawn on the site of

action, whether it is central or peripheral (or both), because cannabinoids are lipophilic compounds and easily pass the blood-brain barrier. We showed recently that anandamide and synthetic cannabinoids given i.c.v. exerted gastroprotective effect against the acid-independent ethanol ulcer model. Moreover, the mucosal protective effect of intravenously injected methanandamide was reversed by the centrally injected CB_1 receptor antagonist SR 141716A, indicating the role of central CB1 receptors in the gastroprotection (Shujaa et al., 2009). *As our present results indicate, the gastroprotective effect of centrally administered anandamide and 2-AG may be due - at least partly - to elevation of CGRP in gastric mucosa, which was dramatically decreased following ethanol challenge. There is evidence that CGRP-containing capsaicin-sensitive primary afferent nerves participate in the control of gastric mucosal integrity by inducing mucosal vasodilation and increased mucosal microcirculation mediated partly by NO release (Holzer et al., 1990, 1991).*

Since paracrine transactivation of the CB_1 receptors by angiotensin AT_1 receptors has recently been observed (Turu et al., 2009), we aimed to test if activation of central AT_1 receptors by Ang II results in gastric mucosal protection through a cannabinoid-dependent mechanism. It was found that Ang II injected i.c.v. induced a dose-dependent inhibiton of ethanol-induced mucosal lesions in picomolar dose range. In higher dose range, however, the gastroprotective effect was reduced. *Similar bell-shaped dose-response curve was observed following central administration of clonidine (Gyires et al., 2000), nociceptin (Zádori et al., 2008) and substance P (Brancati et al., 2013) in the ethanol-ulcer model. Though the precise mechanism of the bell shaped dose-response curves has not been known, it might be speculated that in high doses the selectivity of the compounds to the receptor responsible for the gastroprotective effect is lost and the compound may bind to additional receptors. Activation of these receptors may counteract the primary action manifesting in reduced effect.*

Or it also might be raised that administration of higher doses results in formation of biologically active metabolites that could diminish the effect of the parent compound.

The protective effect was reversed by candesartan indicating that the effect was mediated by AT_1 receptors. AM 251, an inverse agonist of CB_1 receptors, also decreased the Ang II-induced mucosal protective effect suggesting the AT_1 receptor-initiated activation of the endocannabinoid system. Moreover, we aimed to clarify, whether a DAGL-dependent mechanism plays a role in the effect of Ang II, similarly to that observed in CHO cells. It was found that inhibition of DAGL by THL antagonized the gastroprotective effect of Ang II, which implies that the mucosal protection *induced by Ang II* is likely to be mediated by 2-AG.

A convincing evidence for the role of CB_1 receptors in Ang II-induced gastric mucosal protection was obtained by the experiment performed in genetically engineered mice: Ang II exerted gastric mucosal protective effect in wild type mice, but not in CB_1 receptor deficient mice.

Ang II was initially described as a hormone of peripheral origin, the active end product of the renin-angiotensin system. The discovery of brain Ang II receptors located in neurons inside the blood brain barrier confirmed the existence of an endogenous brain Ang II system. Classical actions of Ang II in the brain include the regulation of hormone formation and release, the control of sympathoadrenal systems (both centrally and peripherally) the regulation of water and sodium intake. However, AT_1 receptors are localized not only in areas related to the regulation of autonomic and endocrine control, but also in many other areas of the brain responsible for emotional, sensory and motor functions. For example central Ang II through AT_1 receptors *may be* a specific brain modulatory factor in the control of the reaction to stress (Mayorov, 2011; Saavedra et al., 2011). Stress can affect not only the hormonal and cardiovascular system, but also the gastric mucosal integrity and can initiate gastric mucosal damage. A potent, non-peptide AT_1 receptor antagonist with a good penetration to the brain

given peripherally inhibited the development of stress-induced gastric ulcers (Armando et al., 2003). Similarly, in spontaneously hypertensive rats 2 weeks treatment with AT_1 receptor antagonist candesartan (given subcutaneously) resulted in inhibition of stress-induced ulcerations, by protection of gastric mucosal blood flow, decreased sympathoadrenal activation and prevention of the stress-induced inflammatory response of the gastric mucosa (Bregonzio et al, 2003; Saaverda and Benicky, 2007). Moreover, telmisartan and in a lesser extent candesartan were shown to inhibit indomethacin- and cold restraint-stress induced gastric ulcer formation, gastric acid secretion and the oxidative stress of gastric mucosa, and partial peroxisome proliferator-activated receptor gamma agonistic properties were supposed to play a role in the anti-ulcer action (Morsy et al., 2009). In addition the central, anti-stress effect may also contribute to the gastroprotective effect of the angiotensin II AT_1 receptor blocking drugs.

However, data of the literature suggest that not only the blockade but activation of central AT_1 receptor can also elicit gastric mucosal protection, *e.g.* microinjection of Ang II into the PVN inhibited mucosal injury in gastric ischemia-reperfusion induced ulcer model in a dose-dependent manner. The underlying mechanism is the increase of gastric blood flow, which effect turned to be mediated by central AT_1 receptors (Zhang et al., 2008). Moreover, reversal of the elevated mucosal level of NF-kappaB during gastric ischemia-reperfusion by Ang II may contribute to the Ang II-induced protection against injury (Zhang et al., 2008). Accordingly, also our present findings suggest that activation of central AT_1 receptors may elicit gastric mucosal protection against an acid independent, ethanol-ulcer model via the endocannabinoid system both in rats and mice.

The precise mechanism and site of centrally initiated gastroprotective action has been intensively analyzed. The dorsal vagal complex is likely to have a prominent role in conveying the centrally initiated effect to the periphery. It was shown that both vagotomy and

atropine decreased the gastroprotective effects of centrally injected thyrotropin releasing hormone (TRH), adrenomedullin, peptide YY, opioid peptides, clonidine (for reviews see Gyires, 2005; Tache 2012), nociceptin, nocistatin and cannabinoids (Shujaa et al., 2009; Zádori et al., 2008). Pharmacological and biochemical studies have shown that activation of vagal cholinergic pathways stimulates gastric prostaglandin and NO release and the "efferent function" of capsaicin sensitive primary afferent fibers containing CGRP (Kato et al., 1994; Saperas et al., 1995; Yoneda and Tache, 1993). Also nucleus of the solitary tract (NTS) was hypothesized to be involved in centrally initiated gastroprotective effect of clonidine and opioid peptides (Gyires et al., 2000).

As the present results show bilateral cervical vagotomy reduced in a significant manner the gastroprotective effect of centrally given Ang II indicating that vagal nerve is likely to be involved in conveying the centrally initiated effect to the periphery. Since atropine also significantly inhibited (but not abolished) the mucosal protective effect of Ang II, the involvement of cholinergic muscarinic receptors in the protective effect is suggested. This assumption is supported by the results of Lenkei et al. (1998) who found high AT_1 messenger RNA expression in the hypothalamic periventricular nucleus, in the paraventricular nucleus, in the NTS and in the area postrema. Others found that Ang II receptors have been detected at all levels of the vagal sensory and motor system (Diz et al., 2002). The role of the dorsal vagal complex in AT_1 receptor-mediated actions was suggested by the findings, that microinjections of ANG-(1-12) (0.06 mM) into the NTS decreased the mean arterial pressure and heart rate by activation of AT_1 receptors, and bilateral vagotomy abolished this effect (Chitravanshi and Sapru, 2011). Moreover, microinjection of Ang II into the dorsal motor nucleus of vagus resulted in increased vagal outflow to the heart and GI tract (Diz et al., 1984). In addition, microinjection of low (femtomolar) doses of Ang II into the dorsal medial region of the NTS produced a transient fall in blood pressure and heart rate in anesthetized rats (Fow et al., 1994;

Mosqueda-Garcia et al., 1990; Rettig et al., 1986), however, higher doses evoked pressor responses (Casto and Phillips, 1986; Luoh and Chan, 1998; Michelini and Bonagamba, 1990) creating a bisphasic dose-response curve. Interestingly, the mucosal protective effect of i.c.v. injected Ang II proved to be also biphasic, in low (picomolar) doses it induced a pronounced gastroprotective action, however, the effect was decreased when higher doses were given.

Both AT_1-and CB_1-receptors were identified in the NTS, in the dorsal motor nucleus of vagus and in the paraventricular nuclei (Castelli et al., 2007; Partosoedarso et al., 2003; Seagard et al., 2004). On the basis of this findings it may be speculated that AT_1 receptor-induced activation of CB_1 receptors might take place in the dorsal vagal complex, however, whether there is an overlap in expression of CB_1 and AT_1 receptors in this brain area remains to be clarified. Moreover, since both vagotomy and atropine reduced, but did not abolish the protective effect of Ang II, besides vagal nerve additional mechanism - for example the sympathetic nervous system - may also play a role in conveying the centrally initiated action to the gastric mucosa.

In summary, here we showed that Ang II exerted gastroprotective effect injected i.c.v., via activating the endocannabinoid system, since both CB_1 cannabinoid receptor *antagonist* and inhibitor of DAGL (responsible for the synthesis of 2-AG) decreased the effect of Ang II. In addition, Ang II induced mucosal protective effect in wild type, but not in CB_1 cannabinoid receptor deficient mice. The centrally initiated protective effect is likely to be vagal dependent. *Our results support the hypothesis on the central neuronal control of GI functions, which is based on a complex array of interconnecting brain structures and neuro-chemical systems and characterised by redundancy seen not only in the manner as certain brain areas participate in regulation of GI functions, but also in the extent to which certain neurotransmitters, neuropeptides modify these functions.*

However, further studies are needed to clarify several questions. *First of all, how Ang*

II can induce activation of endocannabinoid (2-AG) system in vivo. Whether CB1 and AT1 receptors are co-localized and the transactivation of CB1 receptors by AT1 receptors is valid also under in vivo condition and play a role in Ang II - cannabinoid interaction, remains to be determined. Or other mechanisms may also be raised: whether Ang II through activation of AT1 receptor might increase the synthesis/release of 2-AG. Furthermore, how can it be explained that antiulcer effect can be induced both by stimulation (ischemia-reperfusion- and ethanol-induced lesions) and inhibition (stress-ulceration, indomethacin-induced lesions) of AT1 receptors? What is the role of central and peripheral AT1 receptors in gastric mucosal protection/damage? Analysis of these issues may help to clarify the potential role of renin-angiotensin system in gastric mucosal integrity.

Acknowledgement

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Figure captions

Fig. 1.

The inhibitory effect of intracerebroventricularly (i.c.v.) injected anandamide (2.9 - 115 nmol/rat) and 2-arachidonoylglycerol (2-AG, 3.3 - 52.8 nmol/rat) on gastric mucosal injury induced by ethanol. Drugs were injected 10 min before the ethanol challenge. Each column represents mean ± S.E.M., the number of animals was 5 per group. *P<0.05; ***P<0.001 (ANOVA, Newman-Keuls post hoc test, compared with the respective control group).

Fig. 2.

The effect of AM 251 (1.8 nmol/rat i.c.v.) on the gastroprotective effect of anandamide (115 nmol/rat i.c.v., Fig. 2A) and 2-arachidonoylglycerol (2-AG, 6.6 nmol/rat i.c.v., Fig. 2B). AM 251 was injected together with the agonists 10 min before the ethanol challenge. Each column represents mean ± S.E.M., n=5 per group. **P<0.01; ***P<0.001 compared with the vehicle-treated group (column 1); $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 compared with the agonist-treated group (column 2) (ANOVA, Newman-Keuls post hoc test).

Fig. 3.

The effect of anandamide (58 nmol/rat) and 2-arachidonoylglycerol (2-AG, 26.4 nmol/rat) on gastric mucosal CGRP content measured 1 h after ethanol administration. Each column represents mean ± S.E.M., the number of animals was 5 per group. ***P<0.001 compared with the control group (column 1); $^{\#\#}$P<0.01 compared with the vehicle-treated group (column 3).

Fig. 4.

The effect of intracerebroventricularly (i.c.v.) injected angiotensin II (2.9 - 956 pmol/rat) on gastric mucosal injury induced by ethanol. Angiotensin II was injected 10 min before the ethanol challenge. Each column represents mean ± S.E.M., the number of animals was 5 per group. **P<0.01; ***P<0.001 (ANOVA, Newman-Keuls post hoc test, compared with the respective control group).

Fig. 5.

The effect of candesartan (*5.2 and* 31.7 nmol/rat i.c.v., Fig. 5A), AM 251 (1.8 nmol/rat i.c.v., Fig. 5B) and tetrahydrolipstatin (THL, 0.2 nmol/rat i.c.v., Fig. 5C) on the gastroprotective effect of angiotensin II (191 pmol/rat i.c.v.). Antagonists were injected together with angiotensin II 10 min before the ethanol challenge. Each column represents mean ± S.E.M., n=5 per group. **P<0.01; ***P<0.001 compared with the vehicle-treated group (column 1); $^{\#}$P<0.05; $^{\#\#}$P<0.01 compared with the angiotensin II-treated group (column 2) (ANOVA, Newman-Keuls post hoc test).

Fig. 6.

The gastroprotective effect of intracerebroventricularly (i.c.v.) injected angiotensin II (191 pmol/rat) on gastric mucosal injury induced by ethanol in wild type (WT) and CB_1 receptor deficient (CB_1(-/-)) mice. Angiotensin II was injected 10 min before the ethanol challenge. Each column represents mean ± S.E.M., the number of animals was 6 per group. ***P<0.001 compared with the vehicle-treated wild type mice (column 1); $^{\#\#\#}$P<0.001 compared with angiotensin II-treated wild type mice (column 2) (ANOVA, Newman-Keuls post hoc test).

Fig. 7.

Macroscopic picture of the effect of intracerebroventricularly (i.c.v.) injected angiotensin II (191 pmol/rat) on gastric mucosal injury induced by ethanol in wild type and CB_1 receptor deficient mice. Angiotensin II was injected 10 min before the ethanol challenge.

Fig. 8.

The effect of bilateral cervical vagotomy (Fig. 8A) and atropine (1 mg/kg i.v.) (Fig. 8B) on the gastroprotective action of Ang II (191 pmol/rat i.c.v.). Each column represents mean ± S.E.M., the number of animals was 5 per group. **P<0.01; ***P<0.001 compared with the vehicle-treated group (column 1); $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 compared with the agonist-treated group (column 2); $^{++}$P<0.01 compared with vagotomized/atropine-treated group (column 3) (ANOVA, Newman-Keuls post hoc test).

Highlights:

- Ang II (i.c.v.) induces gastroprotection via a DAGL-dependent mechanism in rats.
- Ang II (i.c.v.) fails to induce gastroprotection in CB1 (-/-) mice.

Figure1

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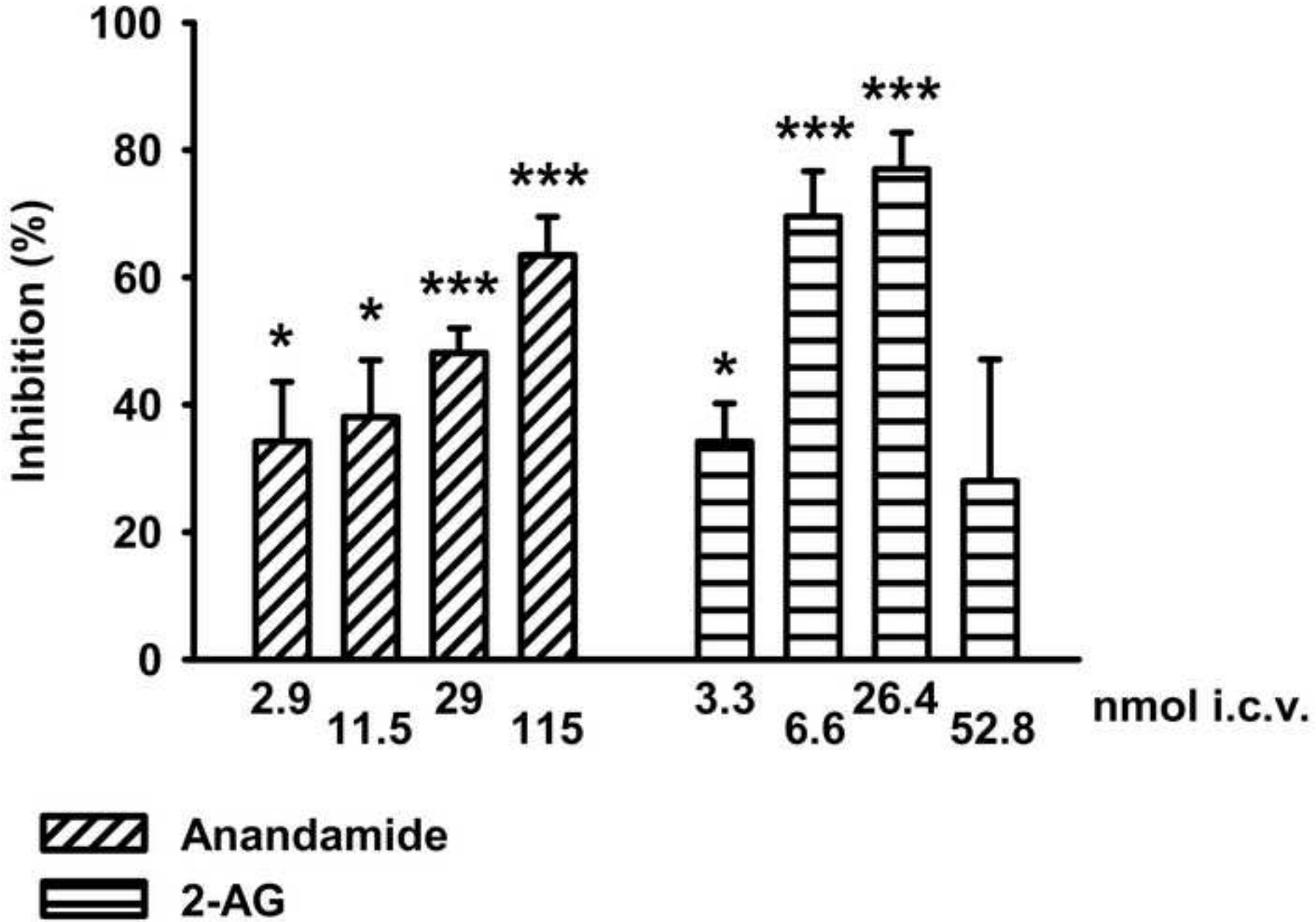

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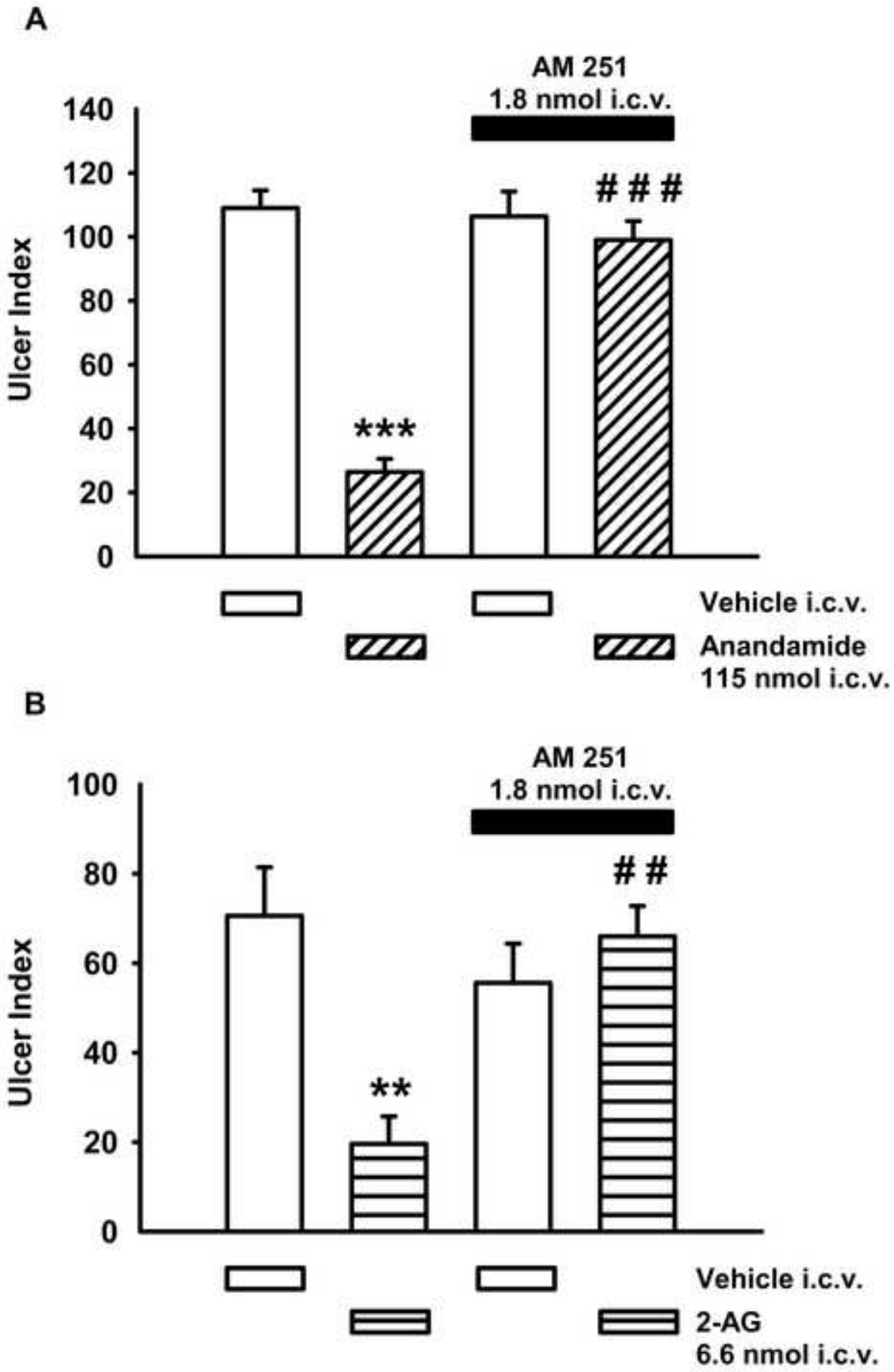

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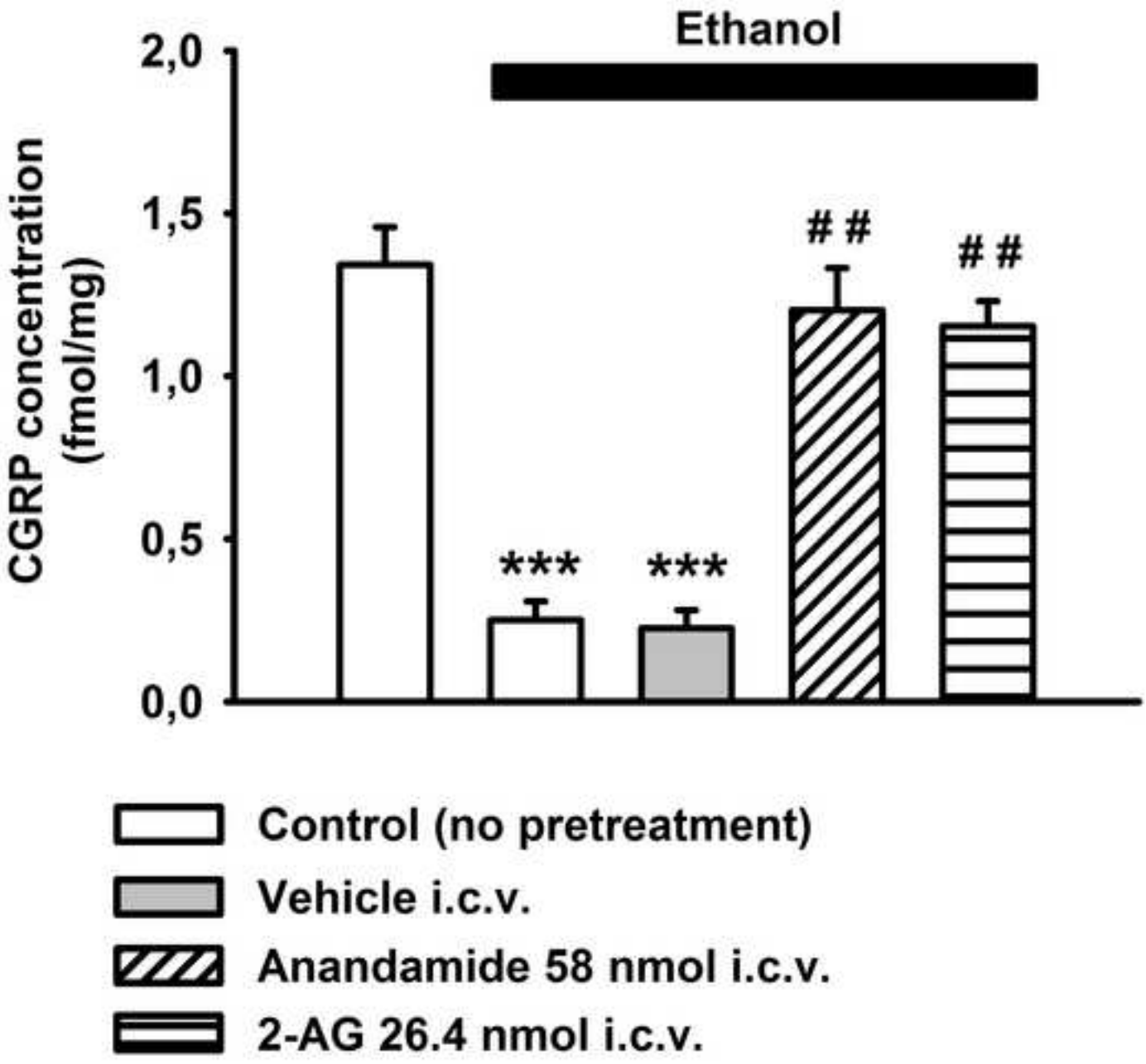

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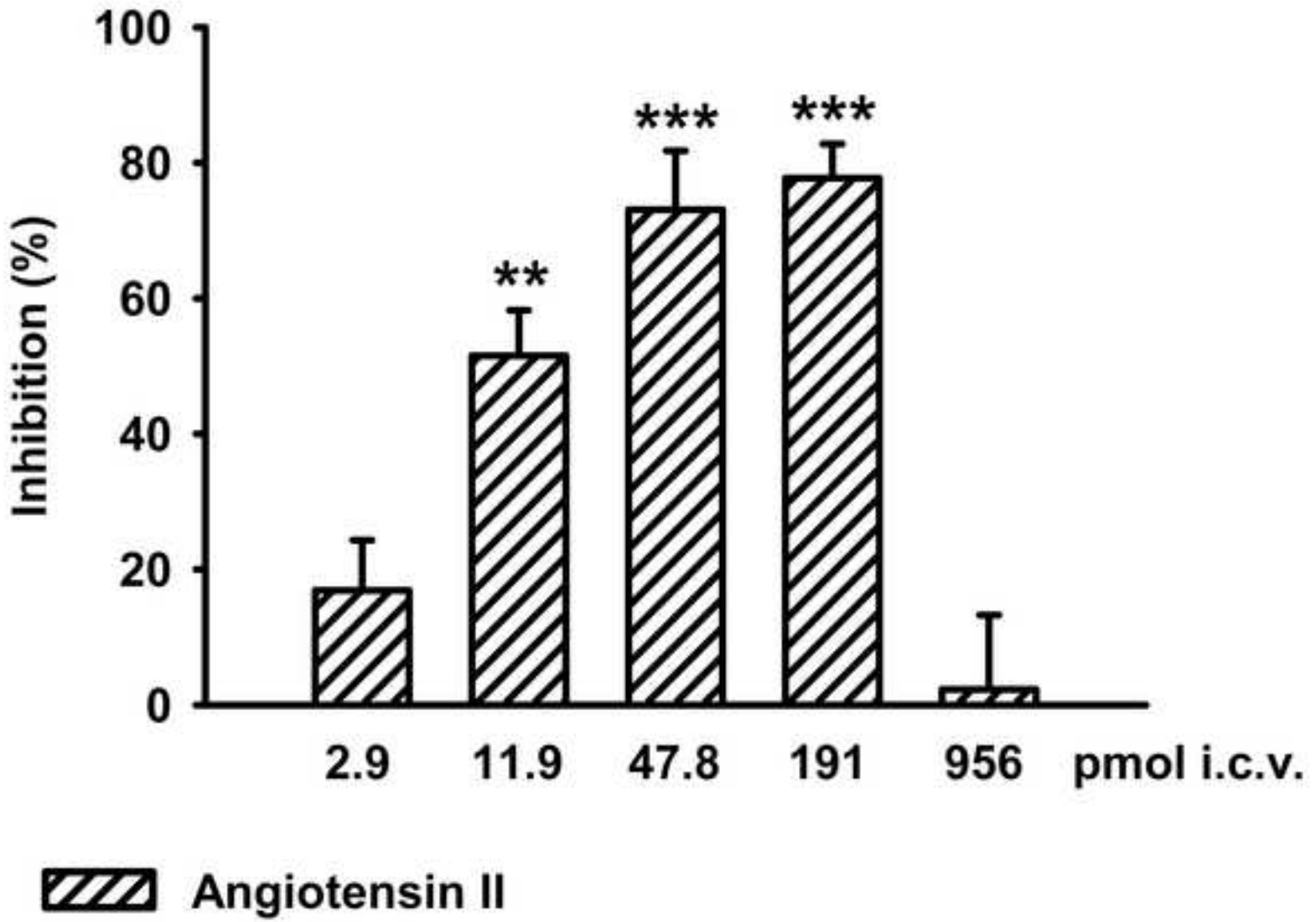

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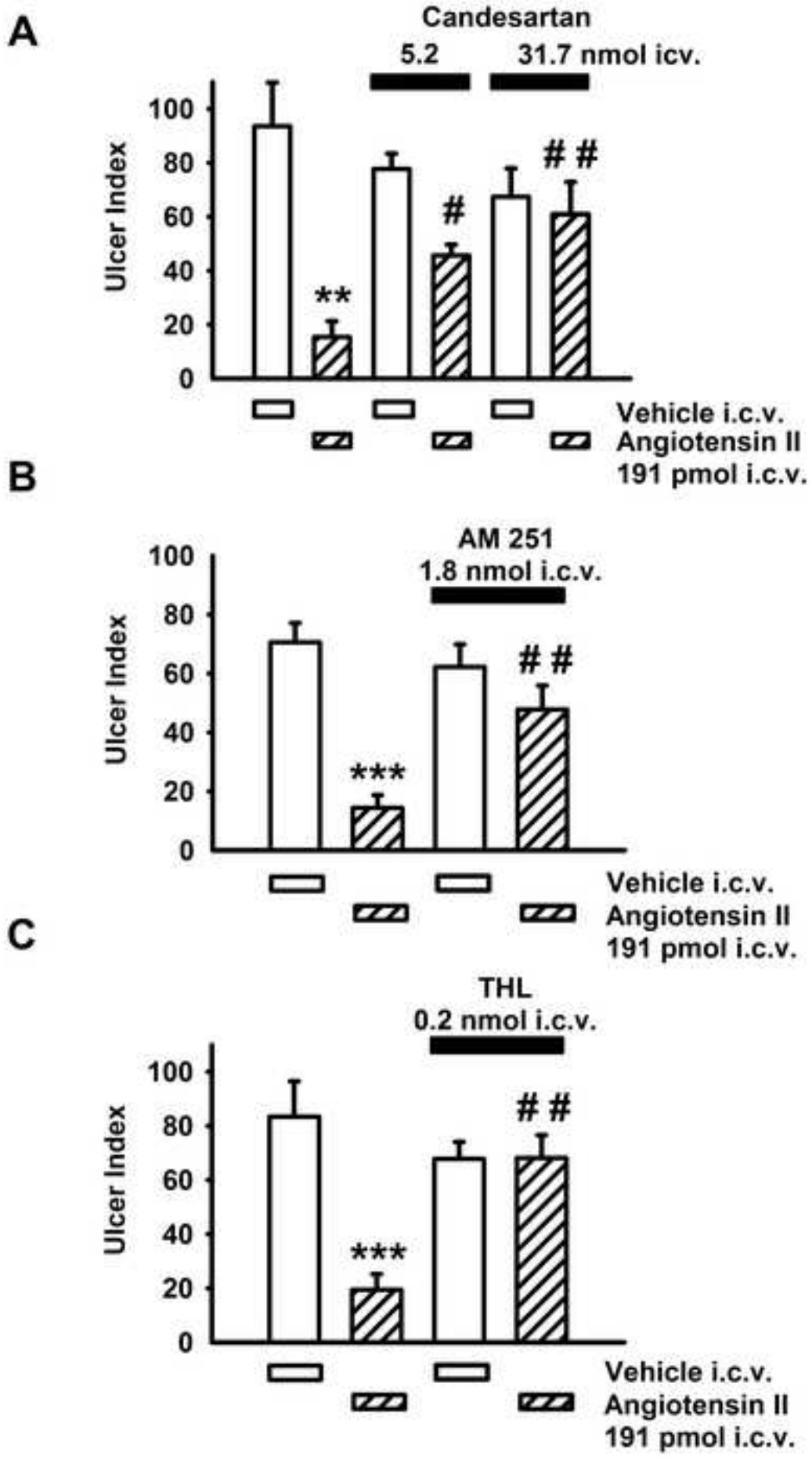

Figure6

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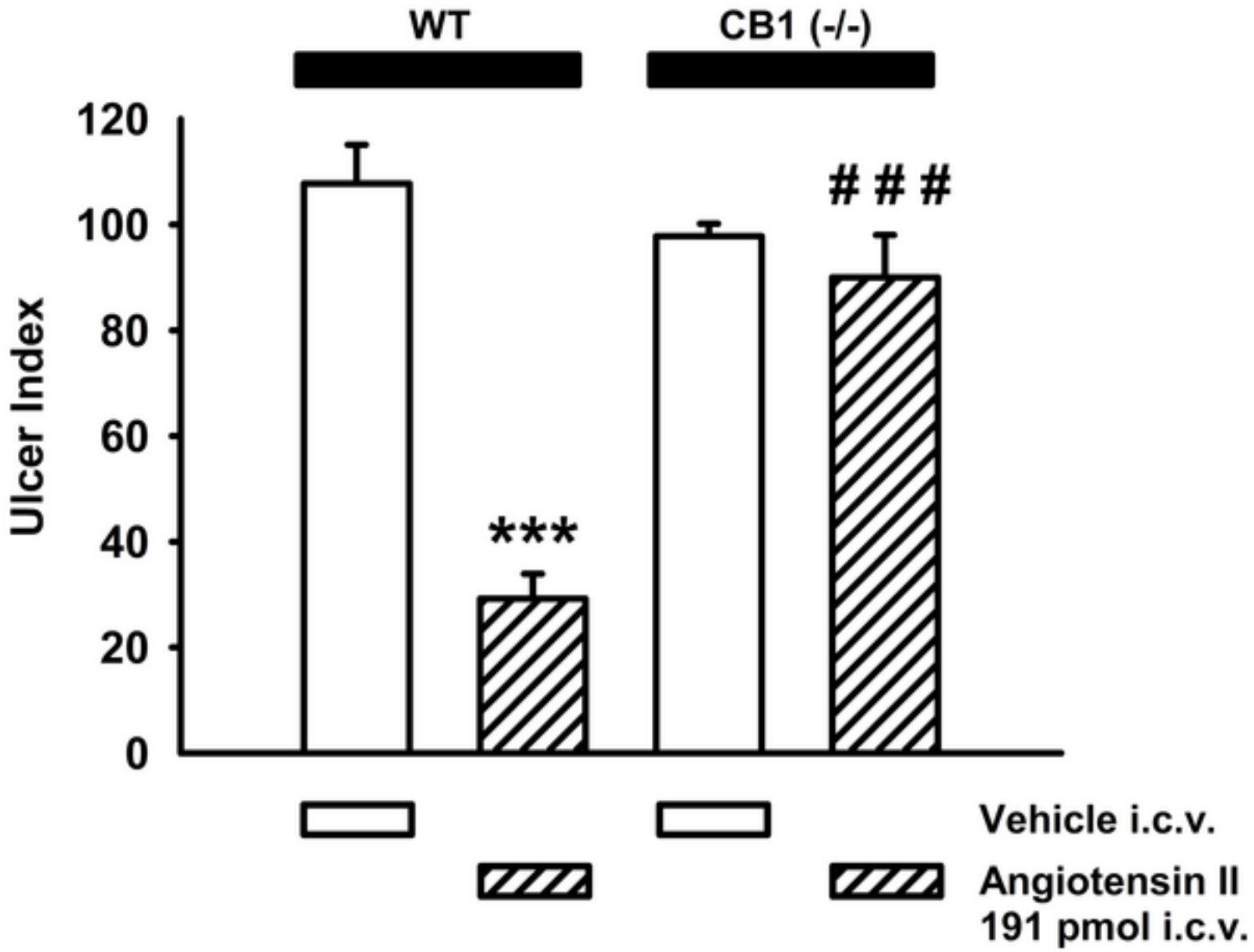

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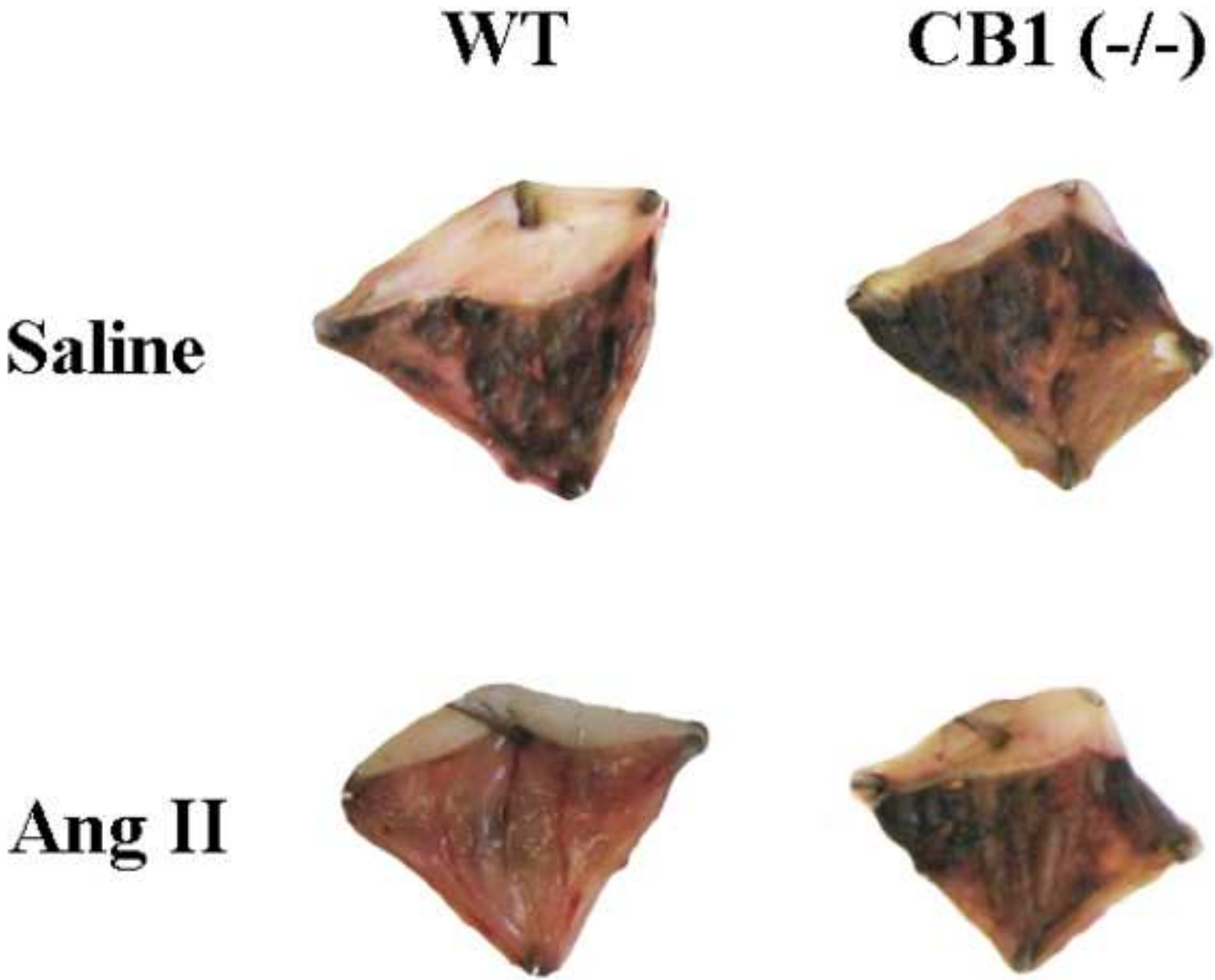

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