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Peripheral corticotropin-releasing factor receptor type 2 activation increases colonic blood flow through nitric oxide pathway in rats

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Abstract

Background—Corticotropin-releasing factor (CRF) peptides exert profound effects on the secretomotor function of the gastrointestinal tract. Nevertheless, despite the presence of CRF peptides and receptors in colonic tissue, their influence on colonic blood flow (CBF) is unknown.

Aim—To determine the effect and mechanism of members of the CRF peptide family on CBF in isoflurane-anesthetized rats.

Methods—Proximal CBF was measured with laser Doppler flowmetry simultaneously with mean arterial blood pressure (MABP) measurement. Rats were injected with intravenous human/rat CRF (CRF_1>CRF_2 affinity), mouse urocortin 2 (mUcn2, selective CRF_2 agonist) or sauvagine (SVG, CRF_2>CRF_1 affinity) at 1 – 30 μg/kg. The nitric oxide (NO) synthase inhibitor, L-NAME (3 mg/kg, iv), the cyclooxygenase inhibitor, indomethacin (Indo, 5 mg/kg, ip) or selective CRF_2 antagonist, astressin$_2$-B (Ast_2B, 50 μg/kg, iv) was given before SVG injection (10 μg/kg, iv).

Results—SVG and mUcn2 dose-dependently increased CBF while decreasing MABP and colonic vascular resistance (CVR). CRF had no effect on CBF, but increased CVR. The hyperemic effect of SVG was inhibited by L-NAME but not by Indo, whereas hypotension was partially reduced by L-NAME. Sensory denervation had no effect on SVG-induced changes. Ast_2B inhibited SVG-induced hyperemia and decreased CVR, and partially reduced the hypotension.

Conclusions—Peripheral CRF_2 activation induces colonic hyperemia through NO synthesis, without involving prostaglandin synthesis or sensory nerve activation, suggesting a direct action

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on the endothelium and myenteric neurons. Members of the CRF peptide family may protect the colonic mucosal via the activation of the CRF_2 receptor.

Keywords

colonic blood flow; vascular resistance; corticotropin-releasing factor; mouse urocortin 2; sauvagine; astressin$_2$-B; nitric oxide; indomethacin

INTRODUCTION

The gastrointestinal tract receives up to 25% of the total cardiac output of which nearly 80% flows through the mucosal and submucosal layers (1), implying that the high metabolic need of mucosal cells combined with the constant exposure to luminal noxious agents emphasize the importance of the regulation of the enteric microcirculation to physiology. The mucosal microcirculatory defense concept is well established in the upper gastrointestinal tract, where the mucosa is regularly exposed to luminal acid (2-4). Similarly, in the lower intestine, blood flow helps prevent mucosal oxidative stress, increased mucosal permeability, and bacterial translocation (5).

Gastrointestinal blood flow is regulated by sensory afferent nerve-mediated reflexes (6) in series or in parallel with other mediators including nitric oxide (NO), calcitonin gene related peptide (CGRP), and prostaglandins (PGs) (2;7-9). An emerging signaling pathways implicated in the gut response to noxious stimuli includes the action of corticotropin releasing factor- (CRF) related peptides, which mediate and modify stress-induced endocrine, behavioral, autonomic and immune responses (10). The mammalian CRF peptide family consists of 4 distinct paralogs, including CRF and urocortins (Ucn 1, 2 and 3) (10-12). In non-mammalian vertebrates, CRF peptide orthologs include frog sauvagine (13) and fish urotensin-I (14). These peptides exert their effects by activating two class B1 G-protein coupled receptors, the CRF receptor 1 (CRF_1) (15) or CRF_2 (16-19). CRF is a non-selective peptide with affinity $CRF_1 > CRF_2$ whereas urocortin (Ucn1), also non-selective peptide, has equivalent and high affinity to CRF_1 and CRF_2 receptors. Urocortin 2 (Ucn 2) and 3 (Ucn 3) in contrast are selective agonists to CRF_2 receptors (20). Amphibian sauvagine is a non-selective CRF peptide with higher affinity to CRF_2 than CRF_1 (21).

CRF signaling, in addition to its involvement in stress-related gastrointestinal secretomotor and pain responses (22-24), also affects hemodynamics when injected centrally or peripherally. In sheep with experimental heart failure, intravenous (iv) Ucn 1 improves hemodynamics by increasing cardiac output and decreasing vascular resistance (25). Intracisternal CRF affects hepatic microcirculation via CRF_2 receptor activation in rats (26). Topical Ucn 2 increases coronary blood flow via CRF_2 receptor activation (27). Ucn 2 injected iv to healthy humans, increases cardiac out put, heart rate and left ventricular ejection while decreasing systemic vascular resistance (28). CRF, sauvagine and urotensin increase mesenteric blood flow when injected centrally (29). The presence of CRF receptors in colonic tissue both in humans (30;31) and rodents (22;32;33) predicts their possible role in the modulation of colonic mucosal blood flow, an enteric defense factor. Yet, the effect of peripheral CRF signaling on colonic mucosal blood flow is thus far unknown.

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Here, we investigated the effects of iv injections of members of the CRF peptide family: CRF (CRF_1>CRF_2 affinity), mouse urocortin 2 (mUcn2, selective CRF_2 agonist), and sauvagine (SVG, CRF_2>CRF_1 affinity) on colonic blood flow, mean arterial pressure and vascular resistance in the anesthetized rat. Colonic CRF_2 activation increased colonic blood flow primarily through local NO pathway without involving PGs or extrinsic afferent neurons.

Methods

Animals

Male Sprague-Dawley rats weighing 200-250 g (Harlan, San Diego, CA) were used. All studies were performed with approval of the Veterans Affairs Greater Los Angeles Healthcare System Institutional Animal Care and Use Committee (VA IACUC).

Chemicals

CRF, mUcn2, SVG and astressin$_2$-B (Ast_2B) were synthesized (34) and provided by Dr. Rivier (Salk Institute, Peptide Laboratories). N^G-nitro-L-arginine methyl ester (L-NAME), indomethacin (Indo), capsaicin, HEPES, and other chemicals were obtained from Sigma Chemical (St. Louis, MO). Krebs solution contained (in mM) 136 NaCl, 2.6 KCl, 1.8 $CaCl_2$, and 10 HEPES at pH 7.4. Each solution was prewarmed to 37 °C using a water bath, with temperature maintained during the experiment with a heating pad. For stock solutions, capsaicin was dissolved in 10% Tween©-80, 10% ethanol, and 80% saline, and the solvent was used as a vehicle. For iv injection, agonists or antagonists were freshly dissolved in saline containing 0.1 % bovine serum albumin (BSA). Saline containing BSA was used as a vehicle control.

Measurement of colonic blood flow

Under isoflurane anesthesia (1.5-2.0 %) using a rodent anesthesia inhalation system (Summit Medical Systems, Bend, OR), rats were placed supine on a heating block system with warmed recirculating water (Summit Medical) to maintain body temperature at 36-37 °C, as monitored by a rectal thermistor. Prewarmed saline was infused via the right femoral vein at 1.08 ml/hr using a Harvard infusion pump (Harvard Apparatus, Holliston MA); blood pressure was monitored via a catheter placed in the left femoral artery using a pressure transducer (Kent Scientific, Torrington, CT). Colonic blood flow was measured with laser Doppler flowmetry by modified method as previously described (3).

The cecum and proximal colon were exposed via a midline incision. The cecum and mid-colon, 3-cm caudal from the cecum, were loosely ligated with nylon suture, and the proximal colon segment was filled with 1 ml saline prewarmed at 37 °C. A ~ 2-cm incision was made in the anterior wall of the segment using a thermal cautery (Geiger Medical Technologies, Inc., Monarch Beach, CA) to expose the colonic mucosa. Luminal contents and feces were gently removed, followed by rinsing the lumen with warmed saline. A concave stainless steel disk (16-mm diameter and 1-2 mm deep) with a 3-mm central aperture was fixed watertight on the mucosal surface with a silicone plastic adherent (Silly Putty, Binney & Smith, Easton, PA). The serosal surface of the colon was supported with a

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laser-Doppler flow probe (described below). A thin plastic cover slip was fixed to the disk with the silicone adherent to permit closed superfusion with solutions at a rate of 0.25 ml/min by means of a Harvard infusion pump.

For the measurement of colonic blood flow, a right-angle probe (R-type, Transonic, Ithaca, NY) was surrounded with a silicone plastic adherent and attached on the colonic serosa just below the chambered mucosa. Blood flow was measured as the voltage output of the laser-Doppler instrument (model BLF21, Transonic) and was expressed relative to the stable level (the basal level) ~ 60 min after the superfusion started. Blood flow and blood pressure were continuously recorded with a strip chart recorder.

Animals had free access to water and food before the experiments, since our preliminary studies showed that, regardless of the surgical stress, colonic blood flow was unstable over time and the effects of CRF peptides on blood flow irreproducible following an overnight fast, presumably due to excessive stress.

Experimental protocol

Blood flow was stabilized during a continuous perfusion of pH 7.4 Krebs buffer, after which the time was set as t = 0 min. Because of the laparotomy and surgical stress, the stabilization of colonic blood flow usually took > 1 hr. The colonic mucosa was continuously superfused at 0.25 ml/min with pH 7.4 Krebs buffer throughout the experiments as in our previous duodenal superfusion (35;36),. Colonic blood flow (CBF) and mean arterial blood pressure (MABP) were simultaneously measured every 5 min and expressed as % basal and mmHg, respectively. Colonic vascular resistance (CVR) was calculated as an equation; CVR (% basal) = MABP (% basal at t = 0)/ CBF (% basal) × 100 as in a previous study (37).

To examine the dose-dependent effect of the CRF peptide family on the proximal colonic blood flow, after 10-min baseline measurement (t = 0 to 10 min), 1 – 30 μg/kg of CRF, mUcn2 or SVG in 0.1 ml BSA-saline solution was iv injected every 10 min followed by gentle flushing iv line with 0.1 ml BSA-saline.

To investigate the involvement of NO or PGs in the effects of SVG, the animals were given the NO synthase (NOS) inhibitor L-NAME (3 mg/kg, iv at t = 10 min) or pretreated, 1 hr before the experiments, with indomethacin (Indo (5 mg/kg, ip)) an inhibitor of cyclo-oxygenase and production of prostaglandins (PGs) that are known to affect intestinal blood flow (2;7-9), followed by SVG (10 μg/kg) iv injection at t = 20 min. Some animals were pretreated with the high dose of capsaicin (Cap-t, 125 mg/kg, sc) or vehicle (veh-t) in order to denervate sensory nerves 10-14 days prior to the experiments as previously described (3).

To confirm the receptor specificity of SVG, a selective CRF_2 receptor antagonist Ast_2B (50 μg/kg, iv) (34) was injected 10 min before SVG injection. The doses of CRF ligands and Ast_2B were adopted from our prior studies (38;39). SVG was used in these studies as a pharmacological tool based on its higher potency, than Ucn2, in inducing CRF2 mediated vagal efferent activity (40) and in attenuating visceral pain responses in rats (unpublished data).

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Statistics

All data from six rats in each group are expressed as means ± SEM. Comparisons between groups were made by one-way ANOVA followed by Fischer's least significant difference test. *P* values of 0.05 were taken as significant.

Results

Dose-dependent effect of CRF peptides on CBF, MABP and CVR

CBF and MABP were stable during perfusion with pH 7.4 Krebs solution in control group for ~ 1 hr (**Fig. 1A-C**). CRF iv injection somewhat decreased CBF with stable MABP, significantly increasing CVR at the 3 and 10 μg/kg doses. In contrast, mUcn2 or SVG dose-dependently increased CBF (**Fig. 1A**) with a decrease in MABP (**Fig. 1B**), decreasing CVR (**Fig. 1C**). The effects of SVG on MABP and CVR were more potent than those of mUcn2 at 3 and 10 μg/kg ($p < 0.05$). **Fig. 1D** and **1E** depict dose-response curve of the peak response in CBF and CVR to CRF peptides. The maximum response to mUcn2 and SVG was achieved at the 10 μg/kg dose.

Effects of L-NAME and Indo on SVG-induced colonic hyperemia

Based on the dose-dependent effect and potency on CBF as above, we chose SVG iv injection at the 10 μg/kg dose for the following experiments to examine the mechanism involved in the blood flow response in rat colon.

SVG iv injection (10 μg/kg) increased CBF (**Fig. 2A**) with a decrease in MABP (**Fig. 2B**), markedly decreasing CVR (**Fig. 2C**). L-NAME iv injection (3 mg/kg) rapidly increased MABP with no effect on CBF (t = 10 to 20 min). L-NAME pretreatment abolished SVG-induced hyperemia and reduced SVG-induced hypotension, preventing the SVG-induced CVR decrease. Alternatively, SVG injection after L-NAME treatment decreased CBF and reversed L-NAME-induced hypertension to hypotension. In contrast, Indo pretreatment (5 mg/kg, ip) had no effect on SVG-induced hypotension, but partially enhanced hyperemia, resulting in more decrease in CVR.

Capsaicin-sensitive afferent nerves in SVG-induced hyperemia

In vehicle-treated rats, SVG (10 μg/kg, iv) increased CBF (**Fig. 3A**) accompanied by hypotension (**Fig. 3B**) and decreased CVR (**Fig. 3C**). Capsaicin pretreatment (Cap-t, 125 mg/kg, sc) had no effect on CBF, MABP or CVR during the basal period (t = 0 to 10 min). Cap-t had no effect on SVG-induced changes in CBF (**Fig. 3A**), MABP (**Fig. 3B**) or CVR (**Fig. 3C**). This result suggests that the activation of sensory nerves is not involved in SVG-induced colonic vasodilation.

Effect of CRF_2 receptor antagonist on SVG-induced changes

Since SVG is a preferable CRF_2 receptor agonist, we confirmed whether SVG-induced changes were mediated via CRF_2 receptor activation. The selective CRF_2 receptor antagonist Ast_2B (50 μg/kg, iv) was injected prior to SVG injection. Ast_2B injection had no effect on CBF, MABP or CVR (t = 10 to 20 min). Ast_2B injection abolished SVG-induced

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hyperemia (**Fig. 4A**) and inhibited the associated hypotension (**Fig. 4B**), strongly inhibiting the CVR decrease (**Fig. 4C**). This result suggests that SVG-induced colonic hyperemia is selectively mediated via CRF_2 receptor activation.

Discussion

We demonstrate that, in isoflurane-anesthetized rats, peripheral CRF_2 receptor activation increases colonic blood flow through a NO-dependent mechanism but not involving PG synthesis or sensory nerve activation. Similarly, iv injection of CRF_2 agonists potently induced hypotension in part through NO. L-NAME abolished the iv SVG-induced decrease in CVR while only modestly reducing SVG-induced hypotension. The data suggest that the CRF_2-mediated hypotension is in part due to the peripheral vasodilatation via NO, but may also involve cardiac effects on the systemic hemodynamics as previously reported (27).

The regulation of intestinal blood flow involves capsaicin-sensitive afferent C-fiber-mediated reflex and several locally produced enteric neuromodulators and transmitters including PGs, CGRP and NO_(2;6-9). The present study provides the first evidence that CRF peptides induce colonic hyperemia in anesthetized rats through CRF_2 receptor activation, demonstrated by the robust blood flow response following iv administration of the selective CRF_2 agonist mUcn2, (20) and SVG, a peptide with affinity $CRF_2 > CRF_1$ (21) in contrast to the effect of native CRF with higher affinity to CRF_1 (21) which exerted little to no effect. This is further confirmed by the blockade of the effects of SVG by the selective CRF_2 receptor antagonist, Ast_2B. Given that the peripherally administered CRF peptides do not cross the blood brain barrier (29;41;42), the likely site of action of the observed colonic blood flow is decidedly peripheral. The effect of mUcn2 and SVG was dose-dependent up to the 10 μg/kg dose, but became less robust at 30 μg/kg, possibly due to action on CRF1 or due to a CRF2-CRF1 receptor cross talk where lower doses of centrally injected mUcn2 showed no effect on colonic motor response but when given at a 5-fold higher doses than CRF, it stimulated colonic motor response through a CRF1 activation (38;43).

Interestingly, the effect of SVG was similar to those of mUcn2, with regard to blood flow, although SVG more strongly reduced MABP. Although SVG binds to CRF_1, albeit at a lesser affinity than to CRF_2, its more pronounced hypotensive effect cannot be due to its potential effect on CRF_1, because CRF, with its higher affinity to CRF_1 does not affect any of the hemodynamic endpoints measured. In dogs injected intracerebroventriculalry (icv) with either SVG or CRF, only SVG increased mesenteric blood flow (29). SVG injected subcutaneously (sc) is also more potent than CRF (sc) in inhibiting food intake in rats (44). Nonetheless, the maximal effects of SVG (10 μg/kg), on colonic blood flow, systemic blood pressure, and vascular resistance were all prevented by pretreatment with the selective CRF_2 antagonist Ast_2B suggesting that the effects of mUcn2 or SVG on colonic blood flow and vascular resistance are mediated through CRF_2.

Our data further show that CRF_2-mediated colonic hyperemia involves NO but not PG or afferent nerve fibers as shown by the blockade of the response by pretreatment with L-NAME but not by Indo or capsaicin. Although NO and PG affect acid-induced gastric mucosal hyperemia (45;46) and the gastric microcirculation (47), PG has no effect on the

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CRF_2-mediated colonic hyperemia. Similarly, CRF_2-mediated colonic hyperemia does not involve capsaicin-sensitive afferent fibers. Taken together, these data suggest that CRF_2 receptor ligands induce colonic hyperemia most likely through a direct release of NO, similar to our prior report that CRF_2 receptors are present in the colonic vascular endothelium (33) and co-localize with neuronal NOS in enteric neurons (22). Endothelial and neuronal NO induces capsaicin-induced gastric mucosal hyperemia (48;49) reinforcing the notion that CRF_2-mediated endothelial and neuronal NO release in the colon be directly vasodilatory.

An intriguing finding in the present study is the anomalous increase of CRF_2-mediated colonic mucosal blood flow and the decrease of MABP and vascular resistance following pretreatment with Indo, which cannot be explained from the data we present in this study, but may be inferred from the variable vascular responses reported to EP receptor activation. For instance, in mouse kidney, low-dose PGE_2 vasoconstricts through EP1 and EP3 receptors but at a higher dose vasodilates through activation of EP2 and EP4 (50). Similarly, PGs administered centrally and systemically exert differing effects dependent on the receptor types involved (51). Alternatively Indo may exert differential effects depending on the physiological state of the tissue. For instance, in hypertensive but not in normotensive dogs, Indo increases renal blood flow (52). Whether the presence of CRF_2 activation in rats constitutes a different state in which Indo enhances the CRF_2-mediated blood flow response needs further studies. Taken together it is likely that Indo, by preventing or reducing PG synthesis, reduces EP1 or EP3-mediated vasoconstriction, thereby enhancing the CRF_2-mediates NO-dependent vasodilatation.

In conclusion, peripheral CRF_2 receptor activation induces colonic hyperemia through NO but not through the PG pathway or through capsaicin-sensitive afferent fibers. The data suggest that members of the CRF peptide family may protect the mucosa via the activation of the CRF_2 receptors in the rat lower intestine. Overall, the protective effects of CRF_2 against ischemia/reperfusion-induced cardiac myocyte injury [see (53)], its anti-angiogenic effect in the inflamed colon (54) and our finding of colonic mucosal hyperemia strongly suggest that peripheral CRF_2 signaling is a key component of the hemodynamic regulatory system in normal and in disease states. Of note is that the effect of CRF peptides on mesenteric blood flow could be different in awake *vs* anesthetized state, as intestinal motility *per-se* can influences blood flow (55). However, in awake rats CRF2 activation by Ucn 2 in awake rodents does not have effect on the basal colonic transit. Thus, the increased mucosal blood flow due to Ucn2 or SVG is unlikely to be due to increased intestinal motility. As such, while the anesthetized rat model is a limitation in the current study and extrapolation to awake state mucosal blood flow needs caution, the model has the advantage to study the direct effect of CRF peptides on mucosal blood flow without the confounding factor of the awake-state intestinal motility.

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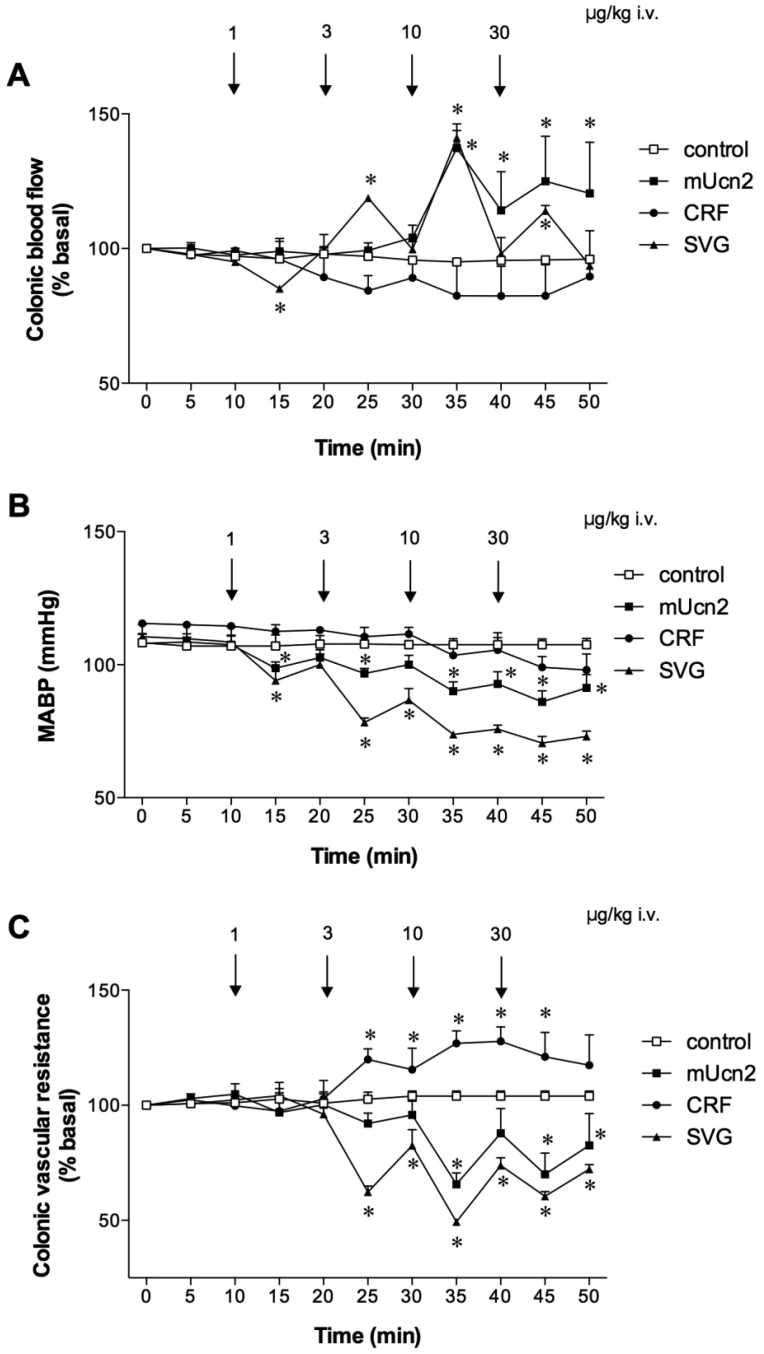

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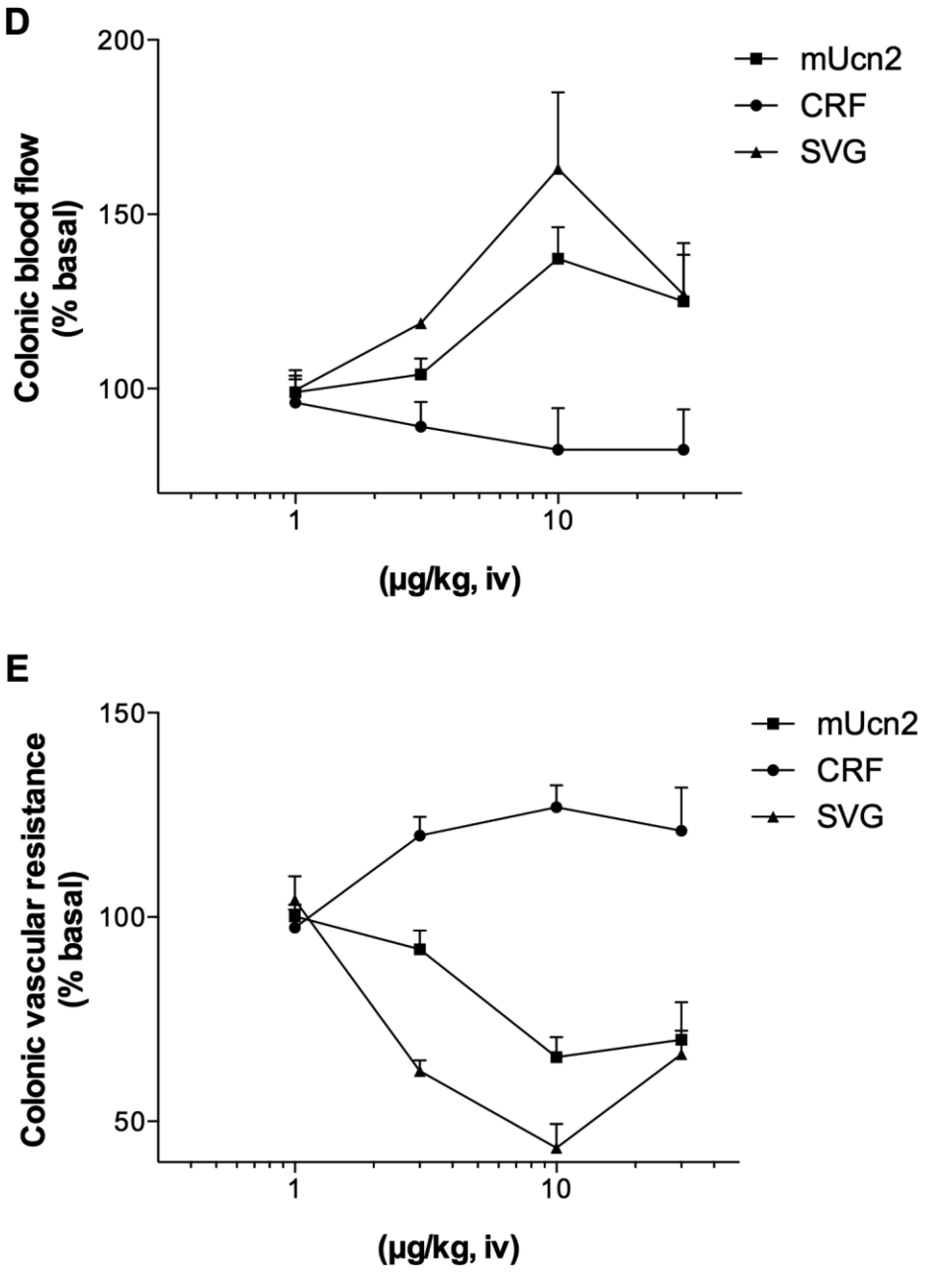

Fig. 1. Effects of mouse urocortin 2 (mUcn2), corticotropin-releasing factor (CRF) and sauvagine (SVG) on blood flow and vascular resistance in rat proximal colon

Colonic blood flow was measured with laser-Doppler flowmetry with colonic vascular resistance calculated from blood flow and mean arterial blood pressure (MABP) monitored simultaneously under isoflurane anesthesia. SVG and mUcn2 (1-30 μg/kg, iv) dose-dependently increased colonic blood flow (**A**) and decreased MABP (**B**), decreasing vascular resistance (**C**), whereas CRF increased vascular resistance. Each datum is expressed as mean ± SEM (n = 6). *p < 0.05 vs. control group. Dose-response effects of mUcn2, CRF and SVG on blood flow (**D**) and vascular resistance (**E**) are shown, where the

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peak values after the drug iv injection were plotted. Each datum is expressed as mean ± SEM (n = 6).

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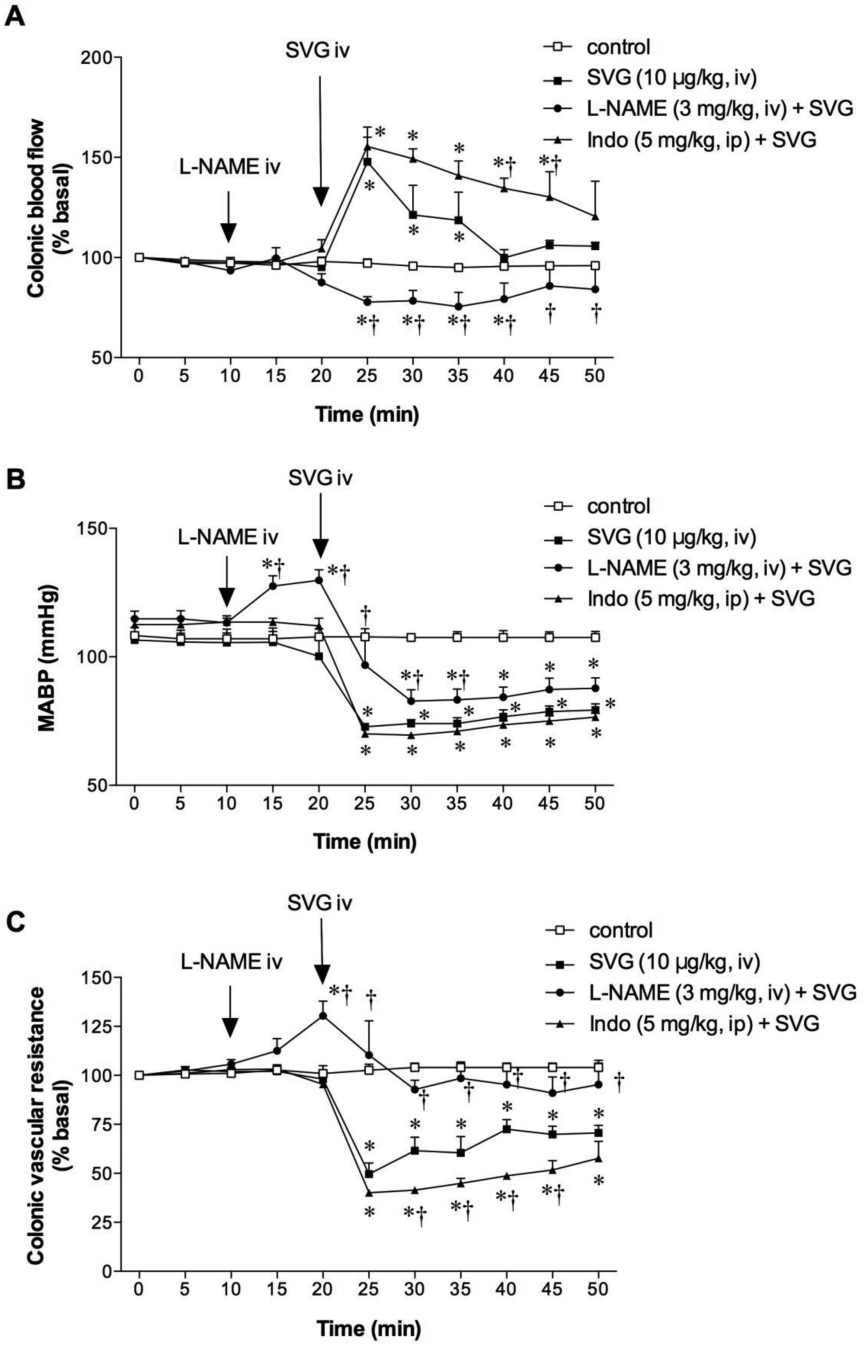

Fig. 2. Effects of L-NAME and indomethacin (Indo) pretreatment on sauvagine (SVG)-induced hyperemia in rat proximal colon

SVG (10 μg/kg, iv) increased colonic blood flow (**A**) accompanied by a decrease in MABP (**B**), decreasing vascular resistance (**C**). L-NAME (3 mg/kg, iv) increased vascular resistance and inhibited SVG-induced hyperemia and vascular dilatation. Indo pretreatment (5 mg/kg, sc) sustained SVG-induced hyperemia and enhanced vascular resistance decrease. Each datum is expressed as mean ± SEM (n = 6). *$p < 0.05$ vs. control, $^{\dagger}p < 0.05$ vs. SVG iv alone.

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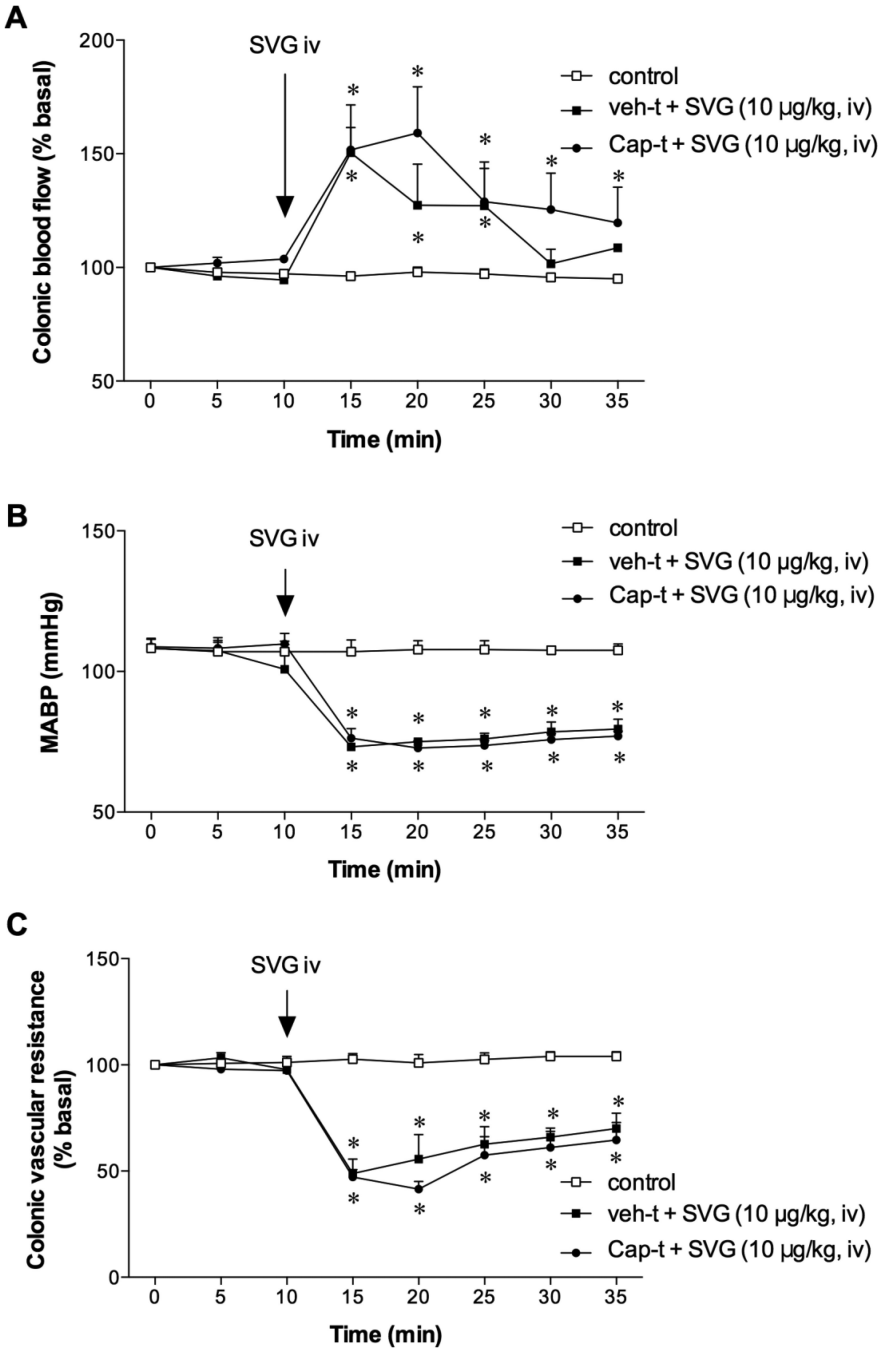

Fig. 3. Effect of afferent denervation on sauvagine (SVG)-induced hyperemia in rat proximal colon

Capsaicin treatment (125 mg/kg, sc; Cap-t) had no effect on SVG-induced hyperemia (**A**), hypotension (**B**) or vascular dilatation (**C**). Each datum is expressed as mean ± SEM (n = 6). *$p < 0.05$ vs. control.

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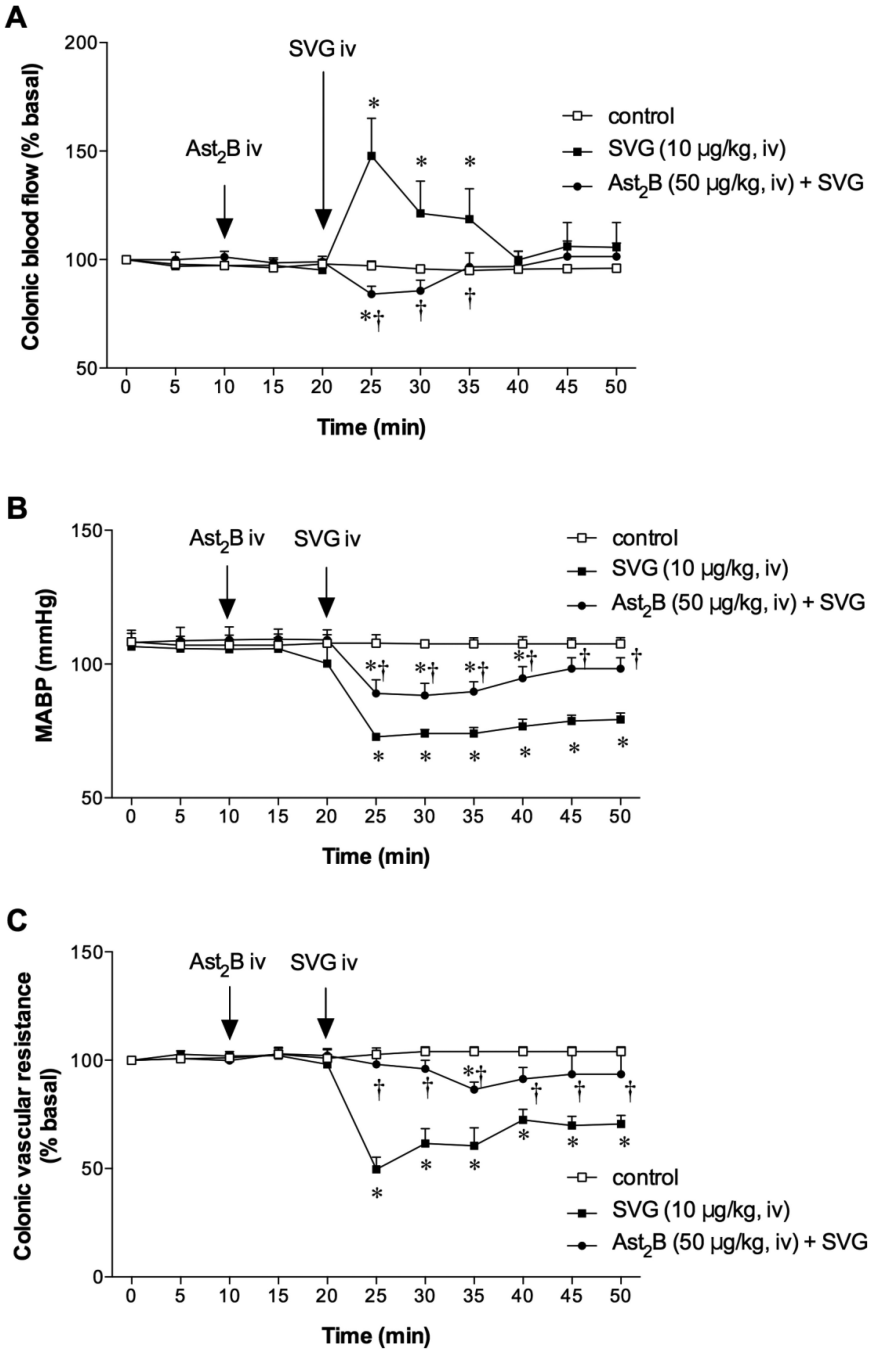

Fig. 4. Effect of CRF_2 receptor antagonist on sauvagine (SVG)-induced hyperemia in rat proximal colon

Astressin$_2$-B (Ast_2B, 50 µg/kg, iv) abolished SVG-induced hyperemia (**A**) and vascular dilatation (**C**), and inhibited hypotension (**B**). Each datum is expressed as mean ± SEM (n = 6). *$p < 0.05$ vs. control, $^{\dagger}p < 0.05$ vs. SVG iv alone.