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Local gastric RAAS inhibition improves gastric microvascular perfusion in dogs

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Abstract

During circulatory shock, gastrointestinal microcirculation is impaired, especially via activation of the renin-angiotensin-aldosterone system. Therefore, inhibition of the renin-angiotensin-aldosterone system might be beneficial in maintaining splanchnic microcirculation. The aim of this study was to analyze whether locally applied losartan influences gastric mucosal perfusion (μ flow, μ velo) and oxygenation (μ HbO₂) without systemic hemodynamic changes. In repetitive experiments six anesthetized dogs received 30 mg losartan topically on the oral and gastric mucosa during normovolemia and hemorrhage (–20% blood volume). Microcirculatory variables were measured with reflectance spectrometry, laser Doppler flowmetry and incident dark field imaging. Transpulmonary thermodilution and pulse contour analysis were used to measure systemic hemodynamic variables. Gastric barrier function was assessed via differential absorption of inert sugars. During normovolemia, losartan increased gastric μ flow from 99 ± 6 aU to 147 ± 17 aU and μ velo from 17 ± 1 aU to 19 ± 1 aU. During hemorrhage, losartan did not improve μ flow. μ velo decreased from 17 ± 1 aU to 14 ± 1 aU in the control group. Application of losartan did not significantly alter μ velo (16 ± 1 aU) compared to the control group and to baseline levels (17 ± 1 aU). No effects of topical losartan on macrohemodynamic variables or microcirculatory oxygenation were detected. Gastric microcirculatory perfusion is at least partly regulated by local angiotensin receptors. Topical application of losartan improves local perfusion via vasodilation without significant effects on systemic hemodynamics. During mild hemorrhage losartan had minor effects on regional perfusion, probably because of a pronounced upstream vasoconstriction.

Key Words

- ▶ gastric microcirculation
- ▶ hemorrhagic shock
- ▶ losartan
- ▶ angiotensin 2

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Introduction

Adequate splanchnic perfusion and oxygenation is vital to maintain the intestinal mucosal barrier function. During circulatory shock the microcirculatory perfusion is critically impaired, resulting in oxygen extraction deficit and regional ischemia. Concerning gastrointestinal microcirculation, this leads to impaired barrier function

and has been shown to enable translocation of bacteria and toxins from the intraluminal to the intravascular compartment (Deitch *et al.* 2004). In shock, circulating blood volume is redistributed in favor of more vital organs at the expense of the gut (Jakob & Takala 2000). In contrast to sepsis, circulatory shock causes splanchnic

hypoperfusion with no initial change in splanchnic oxygen consumption (Ackland *et al.* 2000). Procedures that recruit the gastrointestinal microcirculation, thereby counteracting the splanchnic vasoconstriction, may improve regional organ function. Splanchnic circulation is impaired early in development of hypoperfusion, but shows a delayed recovery beyond restoration of normal systemic hemodynamic variables (Price *et al.* 1966).

The renin-angiotensin-aldosterone system (RAAS) plays a major role in regulating blood pressure and vascular response to injury (Kurz & Zehr 1978). In particular renin via angiotensin reduces mesenteric perfusion due to high affinity and expression of the angiotensin receptor in the splanchnic region (Gunther *et al.* 1980) and increased regional angiotensin production (Yilmaz *et al.* 1999). In this context, the RAAS plays a more important role than the sympathetic-mediated mesenteric vasoconstriction following major trauma. Splanchnic hypoperfusion is not consistently abolished by sympathetic ablation via thoracic epidural anesthesia (Väisänen *et al.* 1998, Meissner *et al.* 1999, Siniscalchi *et al.* 2015), whereas pharmacologic RAAS inhibition or nephrectomy blunted mesenteric vasoconstriction (Bailey *et al.* 1987, Aneman *et al.* 2000). Especially angiotensin 2 is believed to play a crucial role in mediating splanchnic vasoconstriction in hemorrhagic shock (Ceppa *et al.* 2003). Angiotensin 2 induces its biological effects in particular via two receptor subtypes, i.e. angiotensin 2 receptor 1 (AT₁R) and angiotensin 2 receptor 2 (AT₂R). The stabilizing effects on hemodynamics during conditions of compromised circulation, like vasoconstriction, release of aldosterone and retention of salt and water are mainly mediated by interaction of angiotensin 2 with AT₁Rs. Likewise many of the pathologic, proinflammatory or proatherogenic effects of angiotensin 2 are thought to be mediated via AT₁Rs (Weir 2007). Effects of the AT₂R are less understood. Mainly AT₂R activation mediates opposing effects of the AT₁R-like vasodilation and natriuresis (Carey 2017). The AT₁R is widely distributed throughout the body. Therefore, inhibition of RAAS, precisely blocking the AT₁R pathway is thought to be a promising concept to attenuate the disproportionate decrease in splanchnic mucosal perfusion during circulatory shock and might even preserve gastrointestinal organ function. A constant-rate infusion of enalapril improves mesenteric blood flow during resuscitation from hemorrhagic shock in dogs (Wall *et al.* 2003). Systemic RAAS inhibition might even show deleterious effects on mucosal oxygenation. In a previous study we were able to demonstrate that intravenous application of captopril in otherwise healthy dogs

strongly reduced gastric mucosal oxygenation (Vollmer *et al.* 2013a). In addition, systemic RAAS inhibition in the critically ill patient is limited due to considerably adverse side effects. Most prominent is a distinct reduction in blood pressure (Francis *et al.* 2004), which by itself might negatively affect gastrointestinal perfusion and oxygenation. To avoid systemic adverse side effects, topical drug application was established in a variety of different organs, like inhaled prostacyclin in pulmonary hypertension (Olschewski *et al.* 2000). Recently we were able to show that topical application of vasodilatory agents like the nitric oxide (NO)-donor nitroglycerin or iloprost improved μHbO_2 during hemorrhagic shock in dogs (Truse *et al.* 2017). Thus, as splanchnic perfusion is mainly regulated by the RAAS during hemorrhage, topical application of losartan, a specific antagonist to the AT₁R, might be a promising concept to improve gastric perfusion at the microvascular level with markedly reduced systemic adverse side effects. The combination of different vasoactive agents (NO donor, RAAS inhibitor) to improve gastrointestinal microcirculation may reduce side effects of one drug and result in synergistic effects.

Taken together, the aim of this study was to evaluate the effect of topical RAAS inhibition with losartan during physiological conditions and hemorrhage on oral and gastric mucosal perfusion and oxygenation. As reduction of systemic blood pressure can directly impair splanchnic perfusion, the effect of topical AT₁R inhibition on systemic hemodynamic variables was analyzed in parallel.

Materials and methods

Animals

The data were derived from repetitive experiments on six dogs (female foxhounds, weighing 28–36 kg) treated in accordance with the NIH guidelines for animal care. Experiments were performed with approval of the Local Animal Care and Use Committee (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, Recklinghausen, Germany; ref. 84-02.04.2012.A152).

A slightly modified, well-established canine model of hemorrhagic shock was used as published previously (Schwartges *et al.* 2010). The anatomic conditions in dogs enable the nontraumatic placement of the measuring probe to the gastric mucosa without laparotomy. Additionally, larger blood sample volumes can be collected without hemodynamic side effects. Repetitive experiments are

possible which reduce the number of experiments and hence the number of laboratory animals. There is no need to kill the animals at the end of the experiment. Prior to the experiments, access to food was withheld for 12 h with water *ad libitum* to ensure complete gastric depletion and to avoid changes in mucosal perfusion and oxygenation due to digestive activity and to allow the undisturbed coupling of the measuring probe to the mucosa. Each dog underwent each experimental protocol in a randomized order and served as its own control. The experiments were performed at least 3 weeks apart to prevent carryover effects. The experiments were performed under general anesthesia (induction of anesthesia with 4 mg/kg propofol, maintenance with sevoflurane, end-tidal concentration of 3.0% (1.5 minimum alveolar concentration for dogs)). The dogs were mechanically ventilated ($\text{FiO}_2=0.3$, tidal volume=12.5 ml/kg, a normal tidal volume for dogs; Dyson 2012) after endotracheal intubation with the respiratory frequency adjusted to achieve normocapnia (end-expiratory carbon dioxide (etCO_2)=35 mmHg), verified by continuous capnography (Capnomac Ultima, Datex Instrumentarium, Helsinki, Finland). During baseline conditions, the dogs were placed on their right side and covered with isolating blankets to maintain the body temperature at 37.5°C (continuous arterial measurement). Throughout the experiments, no additional fluid replacement was carried out to avoid volume effects that could influence tissue perfusion and oxygenation. However, after withdrawal of each blood sample, normal saline was infused at three times the sampling volume to maintain blood volume.

Measurements

Systemic hemodynamic and oxygenation variables

The aorta was catheterized via the left carotid artery for continuous measurement of mean arterial pressure (MAP, Gould-Statham pressure transducers P23ID, Elk Grove, IL, USA) and intermittent arterial blood gas analysis (Rapidlab 860, Bayer AG, Germany). Cardiac output (CO) was determined via transpulmonary thermodilution (PiCCO 4.2 non US, PULSION Medical Systems, Munich, Germany) at the end of each intervention. Arterial oxygen content and systemic oxygen delivery (DO_2) were calculated subsequently. Heart rate (HR) was continuously measured by electrocardiography (Powerlab, ADInstruments, Castle Hill, Australia).

Gastric and oral mucosal oxygenation and perfusion

Microcirculatory oxygenation (μHbO_2) and perfusion (μflow) of the gastric and oral mucosa were continuously

assessed by tissue reflectance spectrophotometry and laser Doppler flowmetry (O2C, LEA Medizintechnik, Gießen, Germany), as described previously (Krug 2006). Briefly, white light (450–1000 nm) and laser light (820 nm, 30 mW) were transmitted to the tissue of interest via a micro-light guide and the reflected light was analyzed. The wavelength-dependent absorption and overall absorption of the applied white light can be used to calculate the percentage of oxygenated hemoglobin (μHbO_2). Due to the Doppler effect, magnitude and frequency distribution of changes in wavelength are proportional to the number of blood cells multiplied by the measured mean velocity (μvelo) of these cells. This product is proportional to flow (μflow) and expressed in arbitrary perfusion units (aU). Hence, this method allows assessment and comparison of oxygenation and perfusion of the examined region at the same time. Since light is totally absorbed in vessels with a diameter $>100\mu\text{m}$ only microvascular oxygenation of nutritive vessels of the mucosa is measured. The biggest fraction of the blood volume is stored in venous vessels; therefore, mainly postcapillary oxygenation is measured which represents the critical partial pressure of oxygen (pO_2) for ischemia. One flexible light guide probe was placed in the mouth facing the buccal side of the oral mucosa and a second probe was introduced into the stomach via an orogastric tube. Online evaluation of the signal quality throughout the experiments allowed verification of the correct position of the probe tip. The μHbO_2 and μflow values reported are the means of the last 5 min (150 spectra, 2 s each) of the respective intervention under steady-state conditions. The nontraumatic access to the gastric mucosa allowed the determination of mucosal microcirculation in the absence of surgical stress. This is particularly desirable with respect to the marked alterations that surgical stress exerts on splanchnic circulation. In this situation reflectance spectrophotometry reliably detects even clinically asymptomatic reductions in μHbO_2 (Fournell *et al.* 2003) and highly correlates with the morphologic severity and extent of gastric mucosal tissue injury (Sato *et al.* 1986).

Oral mucosal microcirculation – videomicroscopy

Microcirculatory perfusion of the oral, buccal mucosa was consecutively measured by incident dark field (IDF) imaging (CytoCam, Braedius Medical, Huizen, Netherlands) as described elsewhere (Aykut *et al.* 2015). Illumination was provided by light-emitting diodes at a wavelength of 530 nm, the isobestic point for deoxy- and oxyhemoglobin and directed toward the oral mucosa. The reflected and scattered light was filtered and pictures

red blood cells in capillaries. All videos were obtained by the same operator and stored anonymized for blinded analysis. The microcirculation was measured according to the round table guidelines as published by [De Backer *et al.* \(2007\)](#). To assess perfusion, a semiquantitative scoring method, the microcirculatory flow index (MFI) was used to characterize microcirculatory flow as 'no flow', 'intermittent flow', 'sluggish flow' and 'continuous flow' ([De Backer *et al.* 2007](#)). The total vessel density (TVD), including perfused and non-perfused microvessels, and perfused vessel density (PVD), including perfused microvessels only, was analyzed using dedicated software (MicroCirculation Analysis software, Braedius Medical, Huizen, Netherlands) ([Carsetti *et al.* 2015](#)). The ratio PVD/TVD was used to express the proportion of perfused vessels (PPV). Only vessels with a diameter smaller than 20 μm were included in the analysis, so the PVD represents the functional capillary density, considered to be the main determinant of microcirculatory blood supply ([Bezemer *et al.* 2012](#)).

Intestinal barrier function

The disaccharide lactulose (16.7 mg/kg, Bifital, Abbott Germany, Ludwigshafen, Germany) and the monosaccharides rhamnose (6.7 mg/kg, L(+)-rhamnose monohydrate, Carl Roth, Karlsruhe, Germany), mannitol (6.7 mg/kg, Mannit-Solution 15%, Serag-Wiessner, Naila, Germany) and xylose (66.7 mg/kg, D(+)-xylose, Carl Roth, Karlsruhe, Germany) were infused into the stomach via an orogastric tube prior to the induction of hemorrhagic shock. Lactulose is a parameter of paracellular permeability, thereby indicating passive absorption of larger molecules. Rhamnose, mannitol and D-xylose are passively absorbed mainly via transcellular permeation with negligible affinity for the monosaccharide transport system, indicating the degree of absorption of small molecules. These saccharides are not further metabolized. Plasma levels can therefore be used to assess altered intestinal barrier function under pathologic conditions ([Marsilio *et al.* 1998](#)).

Blood samples were collected under baseline conditions and during hemorrhagic shock. The collected samples were prepared as previously described ([Brilhaus *et al.* 2016](#)). Briefly, blood samples were stored in small tubes (Vacutainer K2E EDTA 18.0 mg, Plymouth, UK) and plasma was separated via centrifugation at 0°C and 3000g for 15 min (Rotina 420R, Hettich Zentrifugen, Mülheim a.d. R., Germany). Cold extraction solvent mixture containing 10 μM ribitol (Adonitol, Carl Roth, Karlsruhe, Germany) as internal standard, acetone (Aceton, Carl

Roth) and isopropanol (2-Propanol, Carl Roth) at a ratio of 2:1 were freshly prepared before use and 0.4 mL were added to each sample containing 30 μL plasma. Samples were shaken for 5 min at 4°C prior to centrifugation at 20,800g for 2 min. The liquid supernatant was collected, degassed with a gentle stream of nitrogen (Stickstoff verdichtet, Linde AG, Pullach, Germany) and stored at -80°C for later analysis. After resuspension, aliquots of the extracts were dried using a speed vacuum concentrator, measured by gas chromatography-mass spectrometry (GC-MS) and analyzed using an appropriate software as described elsewhere ([Brilhaus *et al.* 2016](#)). Results are presented as relative amount/ μL plasma.

Induction of hemorrhagic shock

Hemorrhagic shock was induced by removing 20% of the estimated total blood volume via a large bore intravenous cannula in a peripheral vein and the arterial catheter (i.e. 16 mL/kg of whole blood over five min). According to Advanced Trauma Life Support this model represents a class II shock ([Kortbeek *et al.* 2008](#)). This reversible and non-lethal shock model allows the investigation of either protective or harmful effects of various interventions. Heparinized shed blood was stored and later retransfused using an infusion set with a 200 μm filter.

Experimental protocol

After instrumentation, 30 min were allowed to establish steady-state conditions and baseline values were recorded before the animals were randomized to the respective protocol ([Fig. 1](#)). Steady-state conditions were defined as stability of hemodynamic variables as well as ventilation parameters. Subsequently, saccharides were administered as described earlier, followed by further 30 min to establish stable measurement in the gastric region.

The investigators were blinded concerning the applied drug during the experiment and later analysis.

Losartan (L)

To study the effects of losartan, after 90-min stable baseline conditions the animals received a gastric and oral bolus of 30 mg losartan solved in 1 mL NaCl 0.9% and all variables were recorded for the next 90 min ([Fig. 1](#)). Losartan was applied next to the site of the microcirculation measurement. The gastric bolus reached the stomach via a gastric tube fixed to the O2C probe, whereas the bolus to the oral mucosa was applied around the measuring point via a perforated silicon tube.

Figure 1

Experimental protocol: Application of normal saline (C) or losartan (L) during physiological conditions or during hemorrhagic shock with additional application of normal saline (CH) or iloprost (LH), ↓ bolus application to the oral and gastric mucosa.

Control experiment (C)

As time control experiment, only the vehicle (NaCl 0.9%) was applied via the same route of administration as described for losartan and all variables were recorded for 90 min.

Losartan + hemorrhagic shock (LH)

To study the effect of AT_1R blockade on hemorrhage and retransfusion, losartan was injected 30 min after induction of hemorrhagic shock. Hemorrhage was maintained for further 30 min followed by retransfusion of the shed blood with an additional observation period of 60 min.

Control experiment, hemorrhagic shock (CH)

To study the effects of hemorrhage alone, only the vehicle was injected during hemorrhage followed by retransfusion as described above.

Blood samples were taken every 30 min for blood gas analysis.

Statistical analysis

Data for analysis were obtained during the last 5 min of baseline and intervention periods under steady-state conditions. All data are presented as absolute values of mean $\pm$ s.e.m. for six dogs. Differences within the groups and between the groups were tested using a two-way analysis of variance for repeated measurements (ANOVA) and a Bonferroni test as *post hoc* analysis (GraphPad Prism version 6.05 for Windows, GraphPad Software). An *a priori* power analysis (G*Power Version 3.1.9.2) revealed a power of 0.85 for detection of differences between the different groups with $n=6$ in 4 groups, repeated measurements, $\alpha<0.05$ and η^2 of 0.5 (calculated from previous experiments).

Results

During physiological conditions, losartan transiently increased gastric μ flow from 99 ± 6 aU to 147 ± 17 aU ($P<0.05$) 10 min after application. This effect was

Figure 2

Gastric μ flow and μ velo. Gastric microcirculatory flow (μ flow) and gastric microcirculatory velocity (μ velo) in anesthetized dogs during normovolemia (A and B) and during hemorrhagic shock (C and D) with topical application ($\downarrow$ L) of vehicle (C, CH) or losartan (L, LH). Data are presented as mean $\pm$ s.e.m. for $n=6$ dogs; # $P<0.05$ vs baseline, * $P<0.05$ vs respective control group (vs C for L, vs CH for LH), two-way ANOVA for repeated measurements followed by Bonferroni *post hoc* test.

detectable until 15 min after application. Thirty minutes after application of losartan no differences were found between intervention and control group values (Fig. 2A). In parallel to gastric microcirculatory perfusion, losartan enhanced gastric μ veto from 17 ± 1 aU to 19 ± 1 aU ($P < 0.05$, Fig. 2B). This effect lasted for 30 min. Control group values remained stable during the experiment. In contrast to gastric perfusion, losartan had no effect on gastric oxygenation (Table 1). Local AT_1R blockade with losartan had no significant effect on oral microvascular perfusion or oxygenation. A transient decrease in oral μ flow compared to baseline values was observed 15 min after application of losartan, however, without differences to the control group (Table 1). The oral vascular density parameters TVD and PVD and the perfusion parameters MFI and PPV remained unchanged during physiological conditions and after losartan treatment (Table 1). Topical application of losartan had no effect on systemic hemodynamic variables (Fig. 3A and B). MAP, DO_2 , CO and SVR remained stable throughout the experiment (Table 2).

A detailed presentation of micro- and macrovascular parameters and further metabolic and respiratory variables is given in Tables 1 and 2.

Gastric μ veto was significantly decreased during hemorrhage from 17 ± 1 aU to 14 ± 1 aU (CH, $P < 0.05$). Topical treatment with losartan did not improve μ veto compared to baseline values and to the control group (Fig. 2D 4). Induction of a mild hemorrhagic shock had no significant effect on gastric μ flow (decrease from 120 ± 14 aU to 87 ± 17 aU, $P = 0.12$) (Fig. 2C 4). After application of losartan, gastric microcirculatory perfusion tended to be higher compared to the control group, but this increase was not significant ($P = 0.09$). In hemorrhagic shock, gastric mucosal oxygenation was reduced in both groups from $83 \pm 2\%$ to $52 \pm 2\%$ (CH, $P < 0.05$) and from $80 \pm 2\%$ to $48 \pm 6\%$ (LH, $P < 0.05$). After administration of losartan, local gastric μ HbO₂ remained depressed during hemorrhage ($56 \pm 7\%$) similar to the control group ($57 \pm 6\%$) (Table 1).

Hemorrhage led to a pronounced reduction in oral perfusion. Oral μ flow decreased from 179 ± 26 aU to 70 ± 21 aU (LH, $P < 0.05$) without differences to the control group (decrease from 167 ± 28 aU to 89 ± 29 aU, $P < 0.05$). Application of losartan did not increase oral μ flow (80 ± 21 aU vs 99 ± 33 aU in the control group, $P > 0.05$). Likewise, oral μ veto decreased from 29 ± 4 to 19 ± 3 aU (LH, $P < 0.05$) and from 29 ± 4 to 20 ± 4 aU (CH, $P < 0.05$) without differences between the groups and was not restored by losartan (19 ± 3 aU vs 21 ± 4 in the control group, $P > 0.05$)

(Table 1). Similar to gastric mucosal oxygenation, losartan had no effect on oral μ HbO₂ during hemorrhagic shock. Oral buccal capillary density and oral perfusion measured with IDF were strongly reduced during hemorrhage. Topical application of losartan did not improve capillary density (TVD), overall capillary perfusion (PVD, PPV) or flow quality (MFI) (Table 1).

During hemorrhage DO_2 decreased to a similar degree in all groups: from 13 ± 1 to 7 ± 1 ml/kg/min (CH, $P < 0.05$) and from 12 ± 1 to 6 ± 1 ml/kg/min (LH, $P < 0.05$). The decrease in DO_2 was accompanied by a similar decrease in CO (Fig. 3C). After retransfusion DO_2 was restored similarly to 13 ± 1 ml/kg/min (CH) and 13 ± 2 ml/kg/min (LH) (Table 2). After application of losartan, no differences in hemodynamic variables were found compared to the control group. Particularly local application of losartan had no acute effects on MAP (Fig. 3D). Directly after administration of losartan, MAP was not different compared to the control group (52 ± 2 mmHg vs. 53 ± 2 mmHg, $P > 0.05$). Likewise, no differences were observed 90 min after losartan application (LH: 64 ± 1 mmHg vs CH: 66 ± 2 mmHg, $P > 0.05$).

Intestinal barrier function

Plasma levels of rhamnose, lactulose and mannitol were below the detection limit of the GC-MS. Xylose plasma levels show a high variability among all groups without a detectable increase during hemorrhage (Fig. 4).

A detailed presentation of micro- and macrovascular parameters and further metabolic and respiratory variables is given in Tables 1 and 2.

Discussion

This investigation was performed to test the hypothesis that local blockade of AT_1R via topical application of losartan influences oral and gastric microcirculation and intestinal barrier function during hemorrhagic shock without systemic side effects. We could show that losartan significantly increased gastric μ flow during physiological conditions. This went along with an increased gastric μ veto compared to the control group. During mild hemorrhage topical treatment with losartan prevented the shock-induced decrease in gastric μ veto but did not significantly increase gastric μ flow. Effects on microcirculatory perfusion were not accompanied by changes in systemic hemodynamic variables, which remained stable throughout the experiment.

Table 1 Microcirculatory variables.

Parameter (hh:mm)	Group	00:30	01:00	01:30	01:40	01:45	02:00	02:30	03:00
μHbO_2 oral (%)	C	83 ± 2	84 ± 1	83 ± 1	82 ± 1	78 ± 1	82 ± 2	85 ± 1	84 ± 2
	L	86 ± 2	85 ± 1	85 ± 1	82 ± 1	82 ± 1	84 ± 1	86 ± 1	87 ± 1
	CH	83 ± 2	83 ± 3	44# ± 3	46# ± 3	44# ± 4	51# ± 4	86 ± 3	93# ± 1
	LH	87 ± 1	85 ± 2	48# ± 6	46# ± 6	44# ± 4	53# ± 2	88 ± 3	95# ± 1
μHbO_2 gastric (%)	C	81 ± 2	81 ± 2	81 ± 2	81 ± 3	81 ± 2	80 ± 3	81 ± 3	81 ± 3
	L	81 ± 2	82 ± 1	81 ± 2	83 ± 2	81 ± 4	84 ± 1	82 ± 1	85 ± 2
	CH	83 ± 2	82 ± 2	52# ± 2	57# ± 6	55# ± 5	59# ± 4	81 ± 2	85 ± 2
	LH	80 ± 2	81 ± 3	48# ± 6	56# ± 7	54# ± 6	59# ± 7	79 ± 2	83 ± 1
μflow oral (aU)	C	190 ± 41	194 ± 41	198 ± 41	205 ± 39	182 ± 37	209 ± 42	215 ± 40	229# ± 34
	L	159 ± 29	156 ± 27	162 ± 27	164 ± 24	136# ± 17	172 ± 24	175 ± 23	181 ± 23
	CH	167 ± 28	163 ± 31	89# ± 29	99# ± 33	92# ± 29	114# ± 34	215# ± 50	299# ± 47
	LH	179 ± 26	172 ± 26	70# ± 21	80# ± 21	70# ± 22	89# ± 20	226# ± 22	272# ± 20
μvelo oral (aU)	C	28 ± 4	30 ± 5	30 ± 5	23 ± 2	28 ± 6	29 ± 5	30 ± 7	32 ± 6
	L	31 ± 3	30 ± 6	28 ± 5	29 ± 5	22 ± 3	23 ± 3	25 ± 2	30 ± 6
	CH	29 ± 4	27 ± 4	20# ± 4	21 ± 4	18# ± 2	19 ± 3	28 ± 3	41# ± 4
	LH	29 ± 4	31 ± 3	19# ± 3	19# ± 3	15# ± 1	16# ± 1	33 ± 4	37 ± 3
TVD (mm/mm ²)	C	13 ± 1	13 ± 2	13 ± 1	14 ± 1	14 ± 1	15 ± 1	14 ± 1	13 ± 1
	L	15 ± 2	15 ± 1	14 ± 1	13 ± 1	13 ± 1	13 ± 1	13 ± 0	13 ± 1
	CH	13 ± 1	13 ± 1	10# ± 1	10 ± 1	12 ± 1	12 ± 1	14 ± 1	15 ± 1
	LH	15 ± 1	14 ± 0	11# ± 1	10# ± 1	10# ± 1	10# ± 1	15 ± 1	14 ± 1
PVD (mm/mm ²)	C	10 ± 2	11 ± 2	11 ± 1	10 ± 1	13 ± 1	13 ± 1	11 ± 1	10 ± 1
	L	12 ± 2	12 ± 1	10 ± 1	10 ± 1	10 ± 1	10 ± 1	9 ± 1	11 ± 1
	CH	10 ± 1	9 ± 1	6# ± 1	6 ± 1	8 ± 0	8 ± 0	11 ± 1	12 ± 1
	LH	11 ± 1	11 ± 0	7# ± 2	7# ± 1	7# ± 1	7# ± 1	12 ± 1	10 ± 1
PPV (%)	C	70 ± 11	76 ± 9	85 ± 4	69 ± 8	84 ± 2	84 ± 2	79 ± 5	72 ± 9
	L	77 ± 7	79 ± 4	74 ± 6	76 ± 7	70 ± 7	70 ± 7	72 ± 5	78 ± 7
	CH	73 ± 4	67 ± 7	57 ± 6	61 ± 9	69 ± 4	69 ± 4	79 ± 4	85 ± 4
	LH	77 ± 5	78 ± 3	59 ± 13	65 ± 7	64 ± 7	64 ± 7	80 ± 3	73 ± 7
MFI	C	2.9 ± 0.0	2.8 ± 0.0	2.8 ± 0.0	2.9 ± 0.0	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
	L	2.9 ± 0.1	2.8 ± 0.1	2.9 ± 0.0	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	2.9 ± 0.0
	CH	2.9 ± 0.0	2.9 ± 0.1	2# ± 0.2	2# ± 0.2	2# ± 0.1	2# ± 0.1	2.9 ± 0.1	2.9 ± 0.1
	LH	2.9 ± 0.0	2.8 ± 0.0	2# ± 0.0	2# ± 0.0	2# ± 0.1	2# ± 0.1	2.9 ± 0.1	2.9 ± 0.0

Microcirculatory variables of the experimental groups – gastric and oral mucosal hemoglobin oxygenation (μHbO_2); for the oral mucosa: microcirculatory perfusion (μflow , μvelo), total vessel density (TVD), perfused vessel density (PVD), proportion of perfused vessels (PPV) and microvascular flow index (MFI). Data are presented as absolute values, mean $\pm$ S.E.M., $n = 6$, # $p < 0.05$ vs baseline. Two-way ANOVA for repeated measurements followed by Bonferroni *post hoc* test.

Figure 3

Cardiac output and mean arterial pressure. Cardiac output (CO) and mean arterial pressure (MAP) in anesthetized dogs during otherwise physiologic conditions (A and B) and during hemorrhagic shock (C and D) with topical administration ($\pm$ L) of vehicle (C, CH) or losartan (L, LH). Data are presented as mean $\pm$ s.e.m. for $n = 6$ dogs; $\#P < 0.05$ vs baseline, two-way ANOVA for repeated measurements followed by Bonferroni *post hoc* test.

In our investigation, 30mg losartan each were administered locally to the oral and gastric mucosa; this dose is within the range of daily clinical routine (Konstam *et al.* 2009). Gastric microvascular perfusion increased directly after topical application of losartan during physiological conditions. As peak plasma concentrations of losartan appear at 60–90min after oral application (Lo *et al.* 1995), this early-onset short-lasting modulation of gastric perfusion most likely indicates blockade of locoregional AT₁R. AT₁R are known to be highly expressed especially in the splanchnic vasculature (Reilly & Bulkley 1993). Thus, vasotone regulation was probably modified via AT₁R at the level of the microcirculation rather than via systemic effects on major upstream vessels. Growing evidence supports the existence of local RAAS in various tissues and new bioactive angiotensin metabolites, like Ang-(1-7) (Ferrario & Chappell 2004). Inside the stomach wall, angiotensin 1 is metabolized predominantly to Ang-(1-7) and to a minor part to angiotensin 2 (Olszanecki *et al.* 2009). Ang-(1-7) exerts vasoactive and tissue protective properties via Mas-receptor activation (Pawlik *et al.* 2016). In the context of local gastric AT₁R inhibition, this alternative pathway may have a relevant effect on local microcirculation. Beside the reduced activity of angiotensin 2, treatment with AT₁R inhibitors improved endothelial function (Hornig *et al.* 2001) and NO-mediated vasodilation via enhanced expression of endothelial NO synthase (Thai *et al.* 2003). The effect of losartan on gastric perfusion is diminished during hemorrhagic shock.

Especially during conditions of compromised perfusion and intensified sympathetic activity like hemorrhage, one must assume angiotensin 2 blockade to gain more influence on vascular tone. Our results do therefore not agree with other studies, which demonstrate increased jejunal mucosal blood flow during hypovolemia in pigs after systemic angiotensin 2 inhibition (Aneman *et al.* 2000, Laesser *et al.* 2000). However, these studies are focusing on intestinal perfusion with the predominant role of the superior mesenteric artery rather than gastric perfusion via the coeliac trunk. Furthermore, we used a therapeutic treatment regime with losartan applied topically during hemorrhagic shock, whereas Laesser *et al.* and Aneman *et al.* used a pretreatment protocol of systemic candesartan administration prior to a pronounced hypovolemia (Aneman *et al.* 2000, Laesser *et al.* 2000). As resistance arterioles are the predominant determinant of microvascular resistance (Ceppa *et al.* 2003), a pronounced vasoconstriction upstream the mucosal capillary bed might disguise a potential losartan-mediated local vasodilation in the capillary bed. As losartan acts as a competitive antagonist, the dose chosen in this experiment might be insufficient in the context of increased angiotensin 2 levels during hemorrhagic shock. Furthermore, during conditions of hemorrhage, regulation of splanchnic blood flow is complex with a special involvement of vasopressin (Francis *et al.* 2004) which is known to diminish mesenteric blood flow in the upper gastrointestinal tract (Hiltebrand *et al.* 2007).

Table 2 Systemic hemodynamic and metabolic variables.

Parameter (hh:mm)	Group	00:30	01:00	01:30	01:40	01:45	02:00	02:30	03:00
HR (1/min)	C	117 ± 3	115 ± 3	114 ± 3	114 ± 3	114 ± 3	113 ± 3	112 ± 3	112 ± 4
	L	113 ± 3	115 ± 3	113 ± 3	112 ± 3	113 ± 3	112 ± 3	112 ± 4	111 ± 4
	CH	114 ± 3	116 ± 3	114 ± 4	116 ± 4	117 ± 4	117 ± 4	103* ± 5	105* ± 5
	LH	113 ± 4	113 ± 3	113 ± 4	116 ± 4	117 ± 4	117 ± 4	103* ± 4	104* ± 4
DO ₂ (mL/kg/min)	C	12 ± 1	12 ± 1	12 ± 1			12 ± 1	12 ± 1	12 ± 1
	L	12 ± 1	12 ± 1	12 ± 1			12 ± 1	12 ± 1	12 ± 1
	CH	13 ± 1	12 ± 1	7* ± 1			8* ± 1	12 ± 1	13 ± 1
	LH	12 ± 1	12 ± 1	6* ± 1			7* ± 1	12 ± 1	13 ± 2
SVR (mmHg·min/L)	C	26 ± 2	27 ± 2	27 ± 2			27 ± 2	28 ± 2	28 ± 2
	L	25 ± 3	27 ± 2	27 ± 2			27 ± 2	27 ± 3	27 ± 2
	CH	24 ± 2	26 ± 2	34* ± 4			34* ± 2	33* ± 3	25 ± 2
	LH	24 ± 2	26 ± 2	37* ± 2			37* ± 3	31* ± 2	26 ± 3
P _a O ₂ (mmHg)	C	136 ± 5	138 ± 6	140 ± 4			142* ± 6	141* ± 4	141* ± 4
	L	139 ± 5	141 ± 5	141 ± 5			141 ± 5	143 ± 4	143* ± 5
	CH	139 ± 5	144 ± 4	130* ± 4			136 ± 4	151* ± 6	149* ± 6
	LH	134 ± 5	139* ± 5	126* ± 5			132 ± 4	145* ± 4	144* ± 3
P _a CO ₂ (mmHg)	C	36 ± 1	37 ± 1	38 ± 1			38* ± 1	38 ± 1	38* ± 1
	L	37 ± 1	37 ± 1	37 ± 1			38 ± 1	38 ± 1	38 ± 2
	CH	37 ± 1	37 ± 1	42* ± 1			42* ± 1	37 ± 1	37 ± 1
	LH	38 ± 1	37 ± 1	43* ± 1			42* ± 1	37 ± 1	38 ± 1
etCO ₂ (mmHg)	C	34.1 ± 0.2	34.7* ± 0.1	34.7 ± 0.2	34.7 ± 0.2	34.5 ± 0.3	34.8* ± 0.1	34.7 ± 0.2	34.6 ± 0.2
	L	34.7 ± 0.1	34.4 ± 0.1	34.6 ± 0.2	34.8 ± 0.3	35.1 ± 0.3	35.3 ± 0.2	34.2 ± 0.1	34.4 ± 0.2
	CH	34.5 ± 0.2	34.7 ± 0.1	34.5 ± 0.3	34.5 ± 0.2	34.4 ± 0.3	34.3* ± 0.2	34.5 ± 0.3	34.6 ± 0.2
	LH	34.4 ± 0.2	34.5 ± 0.2	34.5 ± 0.2	35.0 ± 0.2	34.7 ± 0.3	34.7 ± 0.2	34.1 ± 0.2	34.3 ± 0.2
S _a O ₂ (%)	C	98.1 ± 0.3	98.0 ± 0.3	98.1 ± 0.2			98.2 ± 0.3	98.2 ± 0.2	98.2 ± 0.2
	L	98.1 ± 0.3	98.3 ± 0.2	97.4 ± 0.3			97.6 ± 0.3	98.4 ± 0.2	98.4 ± 0.2
	CH	98.1 ± 0.3	98.2 ± 0.3	98.2* ± 0.2			98.2* ± 0.2	98.2* ± 0.2	98.2* ± 0.3
	LH	97.9 ± 0.3	98.1 ± 0.3	97.1* ± 0.4			97.4* ± 0.3	98.3* ± 0.2	98.2* ± 0.2
Hct (%)	C	35.0 ± 1.6	34.5 ± 1.7	34.5 ± 1.9			34.3 ± 1.8	34.2 ± 1.9	34.2 ± 1.9
	L	34.2 ± 1.7	33.5 ± 1.7	33.7 ± 1.7			33.5 ± 1.9	33.7 ± 1.7	33.5 ± 1.7
	CH	34.7 ± 1.7	34.2 ± 1.7	34.0 ± 1.5			33.7* ± 1.6	33.3* ± 1.4	33.7* ± 1.5
	LH	34.3 ± 1.4	34.2 ± 1.6	33.8 ± 1.4			33.2* ± 1.3	33.3* ± 1.6	33.5 ± 1.6
Hb (g/dL)	C	11.4 ± 0.6	11.3 ± 0.6	11.3 ± 0.6			11.3 ± 0.6	11.3 ± 0.7	11.3 ± 0.6
	L	11.4 ± 0.6	11.3 ± 0.6	11.2 ± 0.5			11.1 ± 0.5	11.0 ± 0.5	10.9 ± 0.6
	CH	11.2 ± 0.6	11.2 ± 0.6	11.1 ± 0.6			11.1* ± 0.6	11.1* ± 0.6	11.0* ± 0.6
	LH	11.3 ± 0.5	11.2 ± 0.5	11.2 ± 0.5			11.0* ± 0.4	10.9* ± 0.5	11.0* ± 0.5
pH	C	7.39 ± 0.01	7.38 ± 0.01	7.38* ± 0.01			7.37* ± 0.01	7.37* ± 0.01	7.37* ± 0.01
	L	7.38* ± 0.01	7.37 ± 0.01	7.37 ± 0.01			7.37 ± 0.01	7.37 ± 0.01	7.37 ± 0.02
	CH	7.38 ± 0.01	7.38 ± 0.01	7.31* ± 0.01			7.31* ± 0.01	7.36* ± 0.01	7.36* ± 0.01
	LH	7.37 ± 0.01	7.37 ± 0.01	7.30* ± 0.01			7.31* ± 0.02	7.36* ± 0.01	7.36* ± 0.01
HCO ₃ (mmol/L)	C	21.3 ± 0.3	21.3 ± 0.4	21.3 ± 0.3			21.3 ± 0.3	21.3 ± 0.4	21.3 ± 0.3
	L	21.1 ± 0.3	21.0 ± 0.2	21.0 ± 0.3			21.1 ± 0.3	21.2 ± 0.4	21.0 ± 0.3
	CH	21.2 ± 0.4	20.8 ± 0.4	20.4* ± 0.3			20.4* ± 0.3	20.5* ± 0.3	20.6* ± 0.2
	LH	21.1 ± 0.1	20.9 ± 0.2	20.4* ± 0.2			20.4* ± 0.2	20.4* ± 0.3	20.9 ± 0.2
Lactate (mmol/L)	C	0.7 ± 0.1	0.9 ± 0.1	1.0* ± 0.1			1.0* ± 0.1	1.0* ± 0.1	1.0* ± 0.1
	L	0.8 ± 0.1	1.0 ± 0.1	1.0* ± 0.1			1.1* ± 0.1	1.1* ± 0.1	1.1* ± 0.1
	CH	0.8 ± 0.1	1.0* ± 0.2	1.4* ± 0.2			1.3* ± 0.2	1.1* ± 0.1	1.1* ± 0.2
	LH	0.8 ± 0.1	0.9* ± 0.1	1.3* ± 0.2			1.3* ± 0.1	1.1* ± 0.1	1.1* ± 0.1

Systemic hemodynamic and metabolic variables of the experimental groups – heart rate (HR), systemic oxygen delivery (DO₂), systemic vascular resistance (SVR), arterial oxygen partial pressure (P_aO₂), carbon dioxide partial pressure (P_aCO₂), end-expiratory carbon dioxide (etCO₂), hematocrit (Hct), arterial oxygen saturation (S_aO₂), hemoglobin concentration (Hb), pH, bicarbonate (HCO₃) and lactate plasma levels. Data are presented as absolute values, mean ± s.e.m., n = 6, #P < 0.05 vs baseline, *P < 0.05 vs respective control group during physiological conditions (vs C for L) and hemorrhagic shock (vs CH for LH), two-way ANOVA for repeated measurements followed by Bonferroni *post hoc* test.

Figure 4

Xylose plasma levels. Xylose plasma levels during baseline conditions, after application of the saccharide solution and during the next 60 min. Data are presented as mean $\pm$ s.e.m. for $n = 6$ dogs; * $P < 0.05$ vs. baseline, two-way ANOVA for repeated measurements followed by Bonferroni *post hoc* test.

Therefore, altered microcirculatory perfusion by vasopressin or other vasoactive mediators might outweigh potential beneficial effects of local RAAS inhibition.

As topical treatment with losartan had no significant effects on systemic hemodynamic variables, especially DO_2 , one would expect that an increased microvascular perfusion results in increased local oxygenation. However, our data reveal that in the presence of losartan, μHbO_2 did neither increase during normovolemia nor in hemorrhagic shock. Since the gastrointestinal microcirculation is composed of series- and parallel-coupled circuits (Ceppa *et al.* 2003), angiotensin 2 inhibition might open shunts, thereby bypassing the oxygenated blood and impede a net increase in postcapillary microcirculatory oxygenation. Despite modulation of oxygen supply, altered oxygen utilization might account for our findings. Angiotensin 2 is known to impair mitochondrial respiratory activity (Inoue *et al.* 2012). Treatment with AT_1R antagonists could improve mitochondrial function in rat kidneys (de Cavanagh *et al.* 2003) and murine skeletal muscle (Takada *et al.* 2013). As these studies investigated the long-term treatment in a chronic murine and rat model, these results cannot be easily transferred to our model of healthy dogs. Therefore, it remains speculative whether a losartan-induced rise in mitochondrial respiratory activity with increased oxygen consumption superposed the raised oxygen supply.

AT_1R antagonists are well known to induce gastric mucosal protective effects. Continuous subcutaneous application of candesartan for 14 days increased blood flow

to the stomach and reduced the number of gastric ulcers in rats submitted to cold-restraint stress (Bregonzio *et al.* 2003). Intestinal mucosal barrier function was evaluated by measuring the permeability of saccharide molecules (Marsilio *et al.* 1998). In the present study plasma levels for mannitol, rhamnose and lactulose were below the limit of detection, probably indicating only minor damage to the mucosal barrier. Xylose plasma levels showed no consistent increase during hemorrhage. This is unexpected as D-xylose plasma levels are known to indicate damage to the intestinal mucosal barrier (Li *et al.* 2008). A rather large and variable passive oral reflux of the saccharide solution through the inserted orogastric tube might substantially reduce the effective applied saccharide dose. Intestinal content with high osmolality is known to increase intestinal permeability by itself even in the absence of pathologic factor (Pappenheimer & Reiss 1987). Therefore, these technical limitations do not allow a safe interpretation of the measured values.

In the present study, a single application of losartan to the gastric mucosa caused a prompt but short-lasting increase in gastric mucosal blood flow. Distribution in the entire stomach with a consecutive decrease in local mucosal drug concentration most likely causes the short duration of the observed effect. A sustained topical substitution of losartan might cause different effects. As losartan has a rather low *per os* bioavailability and up to 29% are directly excreted with the stool (Christ *et al.* 1994), topical treatment might even be suitable for further aboral parts of the intestine. However, the use of a higher dose is limited most likely due to adverse systemic side effects.

Conflicting evidence exists concerning the impact of elevated systemic angiotensin 2 levels during circulatory shock. Angiotensin 2 was successfully used as a rescue approach in catecholamine-refractory shock (Thomas & Nielsen 1991). On the other hand, RAAS blockade showed organ protective effects (Laesser *et al.* 2004). But treatment with orally administered enalapril worsened the severity of hypotension in resuscitated porcine septic shock (Corrêa *et al.* 2014). Considering these conflicting results, systemic RAAS inhibition might promote negative effects on microcirculation and organ function via reduction of the perfusion pressure, especially during pathologic conditions like septic or hemorrhagic shock.

Topical application of losartan increased μflow and μvelo of gastric mucosal microcirculation during physiologic conditions. In contrast, although mild hemorrhage reduced oral regional perfusion (overall microcirculatory perfusion (μflow) and velocity (μvelo)) and even revealed a

pronounced reduction of the oral microvascular capillary density (TVD, PVD), no effect of a treatment with losartan could be observed. Oral microcirculation is used as an indicator of microcirculation of other regions of the gastrointestinal tract (Verdant *et al.* 2009). However, we have previously shown different reaction patterns of oral and gastric oxygenation and perfusion during hemorrhage (Vollmer *et al.* 2013b, Truse *et al.* 2017). Thus, the present study provides further evidence that oral microcirculation is not an indicator of gastrointestinal microcirculation and organ function *per se*.

Our study has several limitations. The sample size of $n=6$ animals per group seems to be rather small compared to usual sample sizes in non-repetitive experiments, for example, using rats. However, the cross-over design allows sufficient power for small sample sizes, as each animal serves as its own control. However, the study was not powered to detect subtle, clinically irrelevant differences. The impact of hemorrhage is moderate, as this is a non-lethal animal model. Thus, application of AT₁R antagonists might have more impact on splanchnic perfusion in more severe levels of hemorrhagic shock. Nevertheless, the small sample size was sufficient to detect significant differences. In this animal model even mild improvement of microvascular flow or oxygenation seems relevant for barrier function (Truse *et al.* 2017, Vollmer *et al.* 2017). Hemorrhagic shock did not result in an increase in HR. This could, at least partially be explained by sevoflurane-induced depression of the baroreflex (Umehara *et al.* 2006). This phenomenon has been observed in this animal model under hemorrhagic shock before and might be related to the rather mild shock (Vollmer *et al.* 2013b, Truse *et al.* 2017). No relevant changes in hemoglobin and hematocrit were observed during acute hemorrhage. The decrease of Hct in hypovolemia is mainly caused by a delayed shifting of interstitial fluid into the vascular compartment, thereby increasing plasma volume (Cordts *et al.* 1992). Therefore, Hct measurements do not represent the blood volume during acute hemorrhage by all means. With respect to the drug, different dosing regimens of losartan, especially long-term or continuous application may also have different effects; although the dose used in this study is based on sound pharmacological data, no data are available for dogs, so far. The mechanism of action of losartan and direct effects on gastric mucosal cell integrity was not further studied, as this chronic, non-lethal animal model did not allow sample taking for histologic analysis. To elucidate selective effects on splanchnic microcirculation and tissue integrity, further studies in small rodents are planned, to allow

the measurement of coeliac trunk and mesenteric artery blood flow via microflow probes and enable histological analysis of the gastric and intestinal mucosa.

Taking together, topical treatment of the gastric mucosa with losartan improves mucosal microcirculatory perfusion in anesthetized dogs, without significant effects on systemic hemodynamic variables. Thus, this demonstrates the regional role of AT₁R and that modification of vascular tone via AT₁R is possible at the level of the microcirculation. These results may allow to generate hypothesis on how local modulation of microcirculatory perfusion with AT₁R antagonists could improve future treatment of human patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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