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Research Paper

Distinct roles of angiotensin receptors in autonomic dysreflexia following high-level spinal cord injury in mice

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ABSTRACT

Autonomic dysreflexia (AD), a syndrome caused by loss of supraspinal control over sympathetic activity and amplified vascular reflex upon sensory stimuli below injury level, is a major health problem in high-level spinal cord injury (SCI). After supraspinal sympathetic control of the vasculature below the lesion is lost, the renin-angiotensin system (RAS) is thought to be involved in AD by regulating blood pressure and vascular reactivity. In this study, we aimed to assess the role of different RAS receptors during AD following SCI. Therefore, we induced AD by colorectal distention (CRD) in wild-type mice and mice deficient in the RAS components angiotensin (Ang) II type 1a receptor (AT1a) (*Agtr1a*$^{-/-}$) and Ang-(1–7) receptor Mas (*Mas*$^{-/-}$) four weeks after complete transection of spinal cord at thoracic level 4 (T4). Systemic blood pressure measurements and wire myography technique were performed to assess hemodynamics and the reactivity of peripheral arteries, respectively. CRD increased mean arterial blood pressure (MAP) and decreased heart rate (HR) in all three animal groups. However, we found less increases in MAP in *Mas*$^{-/-}$ mice compared to control mice after CRD, whereas AT1a deficiency did not affect the hemodynamic response. We found that the reactivity of wild-type and *Mas*$^{-/-}$ mesenteric arteries, which are innervated from ganglia distal but close to thoracic level T4, was diminished in response to Ang II in AD after T4-SCI, but this difference was not observed in the absence of AT1a receptors. CRD did not influence the reactivity of femoral arteries which are innervated from ganglia more distal to thoracic level T4, in response to Ang II in AD. In conclusion, we identified a specific role of the Ang-(1–7) receptor Mas in regulating the systemic blood pressure increase in AD in T4-SCI mice. Furthermore, AT1a signaling is not involved in this hemodynamic response, but underlies increased vascular reactivity in mesenteric arteries in response to Ang II, where it may contribute to adaptive changes in regional blood flow.

1. Introduction

Due to the loss of tonic inhibitory and excitatory drive to the sympathetic preganglionic neurons, high-level spinal cord injury (SCI) causes a number of cardiovascular problems, among which autonomic dysreflexia (AD) is common and potentially life-threatening by resulting in stroke, haemorrhage, pulmonary embolism or cardiac arrest (Hou and Rabchevsky, 2014; Krassioukov et al., 2003). AD is triggered by both noxious and non-noxious somatic or visceral stimuli (e.g. bladder or bowel distension) (Krassioukov et al., 2009), which can lead to excessive activation of a vasoconstrictor reflex. Importantly, this post-injury increased sensitivity of the visceral vascular bed to vasoactive substances has been proposed to contribute as key component of AD (Brock et al., 2006). Patients with quadriplegia and cervical SCI showed enhanced pressor response to norepinephrine (NE) and angiotensin II (Ang II) (Innes and Kosterlitz, 1954; Krum et al., 1992). Studies

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on arteries from rat SCI models by myography revealed elevated pressor responses of the arteries to phenylephrine (PE) in cats and rats (Innes and Kosterlitz, 1954; West et al., 2016). However, some studies failed to detect increased vasoconstriction (Hou and Rabchevsky, 2014; Osborn et al., 1989). On the other hand, there might be regional heterogeneity within the body since hyper-responsiveness to Ang II has been found in tail arteries, but not in mesenteric arteries of rats (Al Dera and Brock, 2014).

When supraspinal sympathetic control of the vasculature below the lesion is lost, activation of renin-angiotensin system (RAS) is believed to play a key role in AD, by e.g. modulating vasoconstriction in both acute and chronic high thoracic or cervical SCI (Frankel et al., 1972; Hou and Rabchevsky, 2014). Renin released from juxtaglomerular cells in kidneys is elevated in SCI patients (Groothuis et al., 2010; Mathias et al., 1975) to metabolize angiotensinogen in the plasma into angiotensin I (Ang I), which is then converted into Ang II by the angiotensin converting enzyme (ACE). Ang II is a hormonal vasoconstrictor acting via the G-protein-coupled receptors (GPCR) Ang II type 1a (AT1a) and 1b (AT1b) in rodents, which also facilitate peripheral norepinephrine (NE) and adrenal aldosterone release. The ACE inhibitor captopril has been reported to lower systemic blood pressure during AD in patients (Esmail et al., 2002). However the circulating levels of renin and Ang II do not change during experimentally induced AD, indicating that RAS may not directly contribute to AD, but through facilitating effects of Ang II on NE-mediated sympathetic nerve activity (Al Dera and Brock, 2014).

In recent years, a second axis of the RAS has been uncovered with mostly opposite functions to the classical RAS in cardiovascular physiology, based on the enzyme ACE2, a homologue of ACE. This enzyme has been shown to metabolize Ang II to Ang-(1–7), which induces signaling through activation of the GPCR Mas (Santos et al., 2013; Santos et al., 2003). Mas is protective in the cardiovascular system by lowering blood pressure and suppressing inflammation and oxidative stress (da Silveira et al., 2010; Passos-Silva et al., 2013; Rabelo et al., 2008). Ang-(1–7)/Mas has been reported to produce vascular relaxation and act antagonistic to the AT1 receptor (Kostenis et al., 2005). However, the relative relevance of these two RAS axis in the cardiovascular consequences of high thoracic or cervical SCI has not been studied yet.

In this study, we tested the hypothesis that AT1 and Mas receptors play important roles in regulating the hemodynamic response and regional peripheral vascular reactivity in AD following SCI. As AT1a deficient mice have decreased peripheral vascular tonus (Schleifenbaum et al., 2014), we tested the hypothesis that these mice will have attenuated AD, whereas mice which lack Mas, which is involved in vasodilation in certain vascular beds (Botelho-Santos et al., 2012), would have more severe AD.

2. Methods

2.1. Mice

Experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were previously approved by the local Animal Care and Use Committee from Berlin LAGeSo (G0132/14). All mice were housed in groups of 4–6 animals in cages with nesting material, mouse lodges and open access to water and food, at 23 °C with a 12 h/12 h circadian cycle. Experiments were performed using wild-type (WT, *Mas*$^{+/+}$) vs. *Mas*$^{-/-}$ mice both on C57Bl/6 N background (Walther et al., 1998), and WT C57Bl/6 J (*Agtr1a*$^{+/+}$) vs. AT1a receptor deficient mice (B6.129P2-*Agtr1a*^{tm1Unc}/J from the Jackson Laboratory) (*Agtr1a*$^{-/-}$) and WT C57Bl/6N (*Agtr1b*$^{+/+}$) vs. AT1b receptor deficient mice (*Agtr1b*$^{-/-}$) (Oliverio et al., 1998) weighing 20 to 25 g, and at about 3 months of age.

2.2. Spinal cord injury (SCI)

All operative interventions were done under intraperitoneal Ketamine (10 mg/kg) - Xylazine (100 mg/kg) anaesthesia in combination with Isoflurane (1.5–1.8%) inhalation. A dorsal midline incision was made in the superficial muscle overlying the C7-T3 vertebrae. The dura was opened at the T2-T3 intervertebral gap and the spinal cord was completely transected using microscissors. Complete transection was confirmed by pulling a needle twice between the rostral and caudal spinal cord stumps. Gelfoam was placed above spinal cord to achieve hemostasis. The muscle and skin were closed with absorbable sutures (Vicryl, 4–0, Ethicon GmbH). Animals received warmed saline (1 ml, s.c.), recovered and were kept in the heated cages (30 °C). For analgesia mice were treated with Carprofen (4 mg/kg, s.c.) directly after operation and next day with 12 h interval if necessary longer. The bladder was manually emptied three times daily for the whole duration of the experiment, as the bladder function did not recover.

2.3. Monitoring of mean arterial blood pressure and heart rate

Four weeks post-SCI, mice were anesthetised with Isoflurane (1.5–1.8%) and the left femoral artery was cannulated to allow for continuous monitoring of mean arterial blood pressure (MAP) and heart rate (HR) and drug administration as described previously (Todiras et al., 2017). Briefly, sterile heparanized saline-filled catheters (polyethylene tubing of 0.010 in OD × 0.005 in. ID connected to PE-50, Clay Adams) were inserted with aid of a surgical microscopy into the left femoral artery and vein. The free ends of the catheters were tunnelled subcutaneously, exteriorized and sutured at the dorsal surface of the neck. After recovery for 4 h, the arterial catheter was connected to a pressure transducer (PowerLab, ADI Instruments) and mean arterial pressure was recorded under basal conditions. Data was analysed using PowerLab software.

2.4. Induction of AD by colorectal distention

Changes in mean MAP and HR in response to an increase in intracolonic pressure were determined in conscious mice 4 weeks after SCI. To initiate spinal viscero-sympathetic reflexes, a balloon tipped catheter (Swan-Ganz monitoring catheter model116F4; Edwards Life Sciences) was inserted for approximately 1 cm into the rectum and secured to the tail with tape. Mice usually did not show any signs of distress to the presence of the catheter within the distal portion of the large intestine. Mice were allowed to adjust for 10–15 min to stabilize cardiovascular parameters. Colorectal distention (CRD) was induced by inflation of the balloon with 250 μl of air during 1 min and balloon was kept inflated for 1 min. Parameters were continuously measured before (baseline), during, and after balloon catheter inflation (ca 30 min). Mice were regarded as dysreflexic if CRD produced a rise in MAP and a decrease in HR (Krenz and Weaver, 1998; Maiorov et al., 1998). For each mouse, the difference between baseline MAP and CRD-induced MAP change was calculated for each trial and then averaged over the two trials (the one with the highest values was chosen).

2.5. Bolus injections of PE and Ang II

One hour after AD testing when hemodynamics had normalized cardiovascular reflexes were stimulated by bolus injection of pressor doses of phenylephrine (PE, 10 μg/kg, Sigma, St. Louis, MO) and Ang II (100 ng/kg body weight)(Cardoso et al., 2010). The injections of these drugs were performed through 50 μl Hamilton syringe connected to polyethylene tubing on the venous catheter as described previously (de Moura et al., 2010; Todiras et al., 2017). These substances were dissolved in 0.9% saline solution and prepared freshly each time. There was a recovery period of 30 min for MAP and HR to return to baseline values before administering the next drug.

2.6. Measurement of vascular reactivity by wire myography

The second branches of mesenteric arteries and right and left femoral arteries were isolated from mice sacrificed under general anaesthesia with Rompun/Ketamin, 10 mg/Kg and 100 mg/kg pro bodyweight, respectively. The vessels were then quickly transferred to cold (4 °C), oxygenated (95% O_2/5% CO_2) physiological salt solution (PSS) containing (in mmol/L): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose and 1.6 $CaCl_2$. After cleaning the connective tissue and perivascular adipose tissue under dissecting microscope with scissors without damaging the adventitia, the arteries were dissected into 2 mm rings. Each ring was positioned between two stainless steel wires (diameter 0.0394 mm) in a 5-mL organ bath of a Small Vessel Myograph (DMT 610 M, Danish Myo Technology, Aarhus, Denmark) (Fesus et al., 2007). The organ bath was filled with PSS. The bath solution (pH 7.4) was continuously oxygenated (95% O_2/5% CO_2), and kept at 37 °C. The rings were placed under a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg (DMT Normalization module by CHART software) (Fesus et al., 2007). The software Chart5 (AD Instruments Ltd. Spechbach, Germany) was used for data acquisition and display. The rings were pre-contracted and equilibrated for 30 min until a stable resting tension was acquired. Thereafter, the rings were constricted with isotonic external 60 mM potassium chloride (KCl) to ensure viability and allow normalization of developed force. The composition of isotonic external 60 mM KCl (in mmol/L) was 63.7 NaCl, 60 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose and 1.6 $CaCl_2$. Following PSS wash, Ang II was added in a cumulative manner (from 100 nM to 100 μM). Tension is expressed as a percentage of the steady-state tension (100%) obtained by 60 mM KCl. Endothelial integrity and functionality were confirmed by the relaxant response to acetylcholine (ACh 1 μM).

2.7. RNA sequencing of murine mesenteric arteries

For this analysis separate groups of mice were operated. Four weeks post-T4-SCI or sham-OP mice were sacrificed and the mesenteric arterial arcade, excluding the superior mesenteric artery, was dissected, cleaned of fat, and snap frozen. For RNA isolation with Trizol and FastPrep beads, the manufacturer's instructions were followed. Reverse transcription was performed with total RNA digested by DNase I (Roche). Total RNA was stored in a 1.5 ml Safe Lock LoBind Tubes. RNA samples were quantified on a spectrophotometer (NanoDrop ND-1000; Thermo Scientific) and quality-analysed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US). RNA sequencing was performed at the Scientific Genomics Platform located at the Max Delbrück Center for Molecular Medicine (MDC). mRNA-Seq libraries were generated from 3 sham and 3 T4-SCI mesenteric artery samples using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) as per the manufacturer's protocol. Data was de-multiplexed to generate the sequencing reads in FastQ format files. The final list of differentially expressed genes was used to compute the enrichment of biological pathways separately for the upregulated and downregulated genes using DAVID (Huang Da et al., 2009).

2.8. Real-time qPCR

To evaluate the expression levels of selected genes by RT-PCR, 1 μg of DNA-free total RNA isolated from murine mesenteric arteries was used for first strand cDNA synthesis with using oligo(dT) and random primers. Quantitative polymerase chain reaction (qPCR) analysis was performed using Fast SYBR Green PCR Master Mix (Applied Biosystem) and QuantStudio 5 real-time PCR system (Thermo Fischer). Each cDNA sample was tested in triplicate, and the expression level of each gene was normalized to the hypoxanthine-guanine phosphoribosyltransferase (Hprt) level. Following primers were used: *Agtr1a* forward: 5′-CCATTGTCCACCCGATGAAG-3′, *Agtr1a* reverse: 5′-TGCAGGTGACT TTGGCCAC-3′, *Agtr1b* forward: 5′-TGGCTTGGCTAGTTTGCCG-3′*Agtr1b* reverse: 5′-ACCCAGTCCAATGGGGAGT-3, *Hprt* forward: 5′- GTA ATG ATC AGT CAA CGG GGG AC - 3′, *Hprt* reverse: 5′- CCA GCA AGC TTG CAA CCT TAA CCA - 3′. The fold change was determined using the $2^{-\Delta\Delta C(t)}$ method (Livak and Schmittgen, 2001).

2.9. Statistical analyses

Results are presented as mean ± SEM. Data were analysed statistically using the GraphPad Prism 7 software (GraphPad Software, CA, USA) and SPSS (IMB Analytics). To determine genotype-related differences in MAP or HR change upon CRD in vivo, we first conducted two-way ANOVAs, incorporating the following variables: genetic deficiency (knockout vs wild type), deficiency type (*Agtr1a* vs *Mas*). To determine expression changes of *Agtr1a* and *Agtr1b*, two-way ANOVA was used, incorporating the following variables: deficiency type (*Agtr1a* vs *Mas*) and surgery type (sham vs SCI). To determine SCI-related differences in Ang II and PE response in vivo, we first subjected the values to three-way ANOVA, incorporating the following variables: genetic deficiency (knockout vs wild type), deficiency type (*Agtr1a* vs *Mas*), and surgery (sham vs SCI). Significant interactions were analysed by simple effects tests by fixing one interaction partner. Individual differences between knockouts and their respective wild type controls were determined by unpaired Student's t-tests with Bonferroni post hoc procedure.

For mesenteric and femoral arteries data analysis, curve fittings were done by Prism 7 software using non-linear regression. Statistical significance was determined by three-way ANOVA as described above or repeated-measures two-way ANOVA, followed by Bonferroni post hoc test. Significance for all tests was assumed when $p < 0.05$.

3. Results

3.1. Mas deficiency is hemodynamically protective against AD

Mean arterial blood pressure (MAP) and heart rate (HR) were measured at baseline and during AD, i.e. at 4 weeks after T4- SCI. Two-way ANOVA for basal MAP revealed significant interaction between genetic deficiency (WT, KO) and deficiency type (*Agtr1a, Mas*) ($F_{1,27} = 15.52$, $p < 0.001$) and a significant main effect for genetic deficiency ($F_{1,27} = 10.17$, $p = 0.004$). At baseline, MAP in the *Agtra*$^{-/-}$ mice was lower compared to WT control mice (*Agtr1a*$^{+/+}$)

Table 1
Baseline values of MAP and HR prior to maximal increase during induction of autonomic dysreflexia (AD) by colorectal distention (CRD). ΔMAP/ΔHR; change in MAP and HR. Significance of differences (Student's *t*-tests with Bonferroni post hoc procedure.

	Basal MAP	Δ MAP	Basal HR	Δ HR
Agtr1a$^{-/-}$	75.1 ± 4.4***	16.3 ± 1.8	635.8 ± 28.11	−89.6 ± 20.8**
Agtr1a$^{+/+}$	100.3 ± 2.3	15.6 ± 2.2	672.6 ± 74.46	−105.7 ± 89.7
Mas$^{-/-}$	88.2 ± 2.1	5.4 ± 1.0*	633.4 ± 33.90**	−72.00 ± 20.3
Mas$^{+/+}$	88.8 ± 3.0	11.0 ± 2.1	746.0 ± 12.19	−102.9 ± 21.2

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ vs. its control strain group.

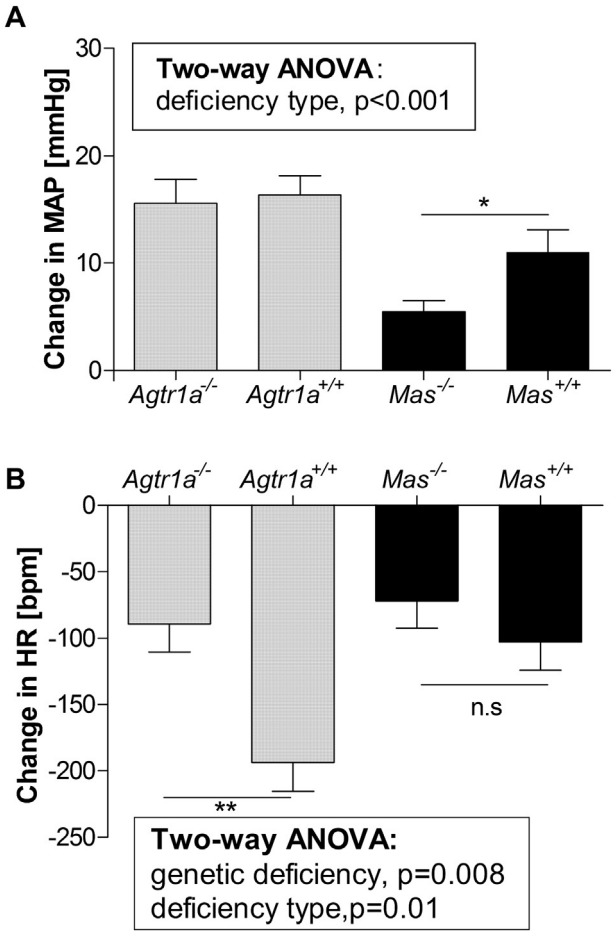

Fig. 1. Changes in MAP and HR during autonomic dysreflexia (AD) induced by colorectal distention (CRD): (A) maximal change in MAP, significance of differences by Student's t-test: *$P < 0.05$ and (B) maximal change in HR Global ANOVA analysis appears in the boxes in respective figures. Significance of differences by Student's t-test with Bonferroni procedure: **$P < 0.01$. *Agtr1a*$^{-/-}$ SCI (n = 9), *Agtr1a*$^{+/+}$ SCI (n = 6), *Mas*$^{-/-}$ SCI (n = 9), *Mas*$^{+/+}$ SCI (n = 8).

($p = 0.0008$, Student's t-test) (Table 1). For basal HR, there was no significant interaction between genetic deficiency and deficiency type and main effects of genotype and gene deficiency (all F values < 4.23, $p > 0.05$). There were no differences in HR between *Agtr1a*$^{-/-}$ mice and control mice ($p = 0.6287$, Student's t-test) (Table 1). Basal HR was lower in *Mas*$^{-/-}$ compared to control mice ($p = 0.0094$, Student's t-test) (Table 1).

As expected, CRD caused elevations in MAP and decreases in HR in all four animal groups. For change in MAP upon CRD, there was no significant two-way interaction between genetic deficiency and deficiency type ($F_{1,27} = 2.73$, $p = 0.110$), but a significant effect was observed in the *Mas* deficiency group ($F_{1,27} = 21.17$, $p < 0.001$). This indicated that *Mas* animals (WT, KO) are generally less responsive to CRD relative to the *Agtr1a* groups. *Mas*$^{-/-}$ mice had significantly smaller MAP rise compared to *Mas*$^{+/+}$ ($p = 0.0258$, Student's t-test) (Table 1, Fig. 1A). For change in HR during CRD an ANOVA showed that the main effect of genetic deficiency and deficiency type were statistically significant (both F values > 7.66, $p < 0.010$), but no significant interaction of genetic deficiency and deficiency type ($F_{1,26} = 2.06$, $p = 0.163$) (Fig. 1B). *Agtr1a*$^{+/+}$ mice had significantly higher HR drop compared to *Agtr1a*$^{-/-}$ ($p = 0.0074$, Student's t-test).

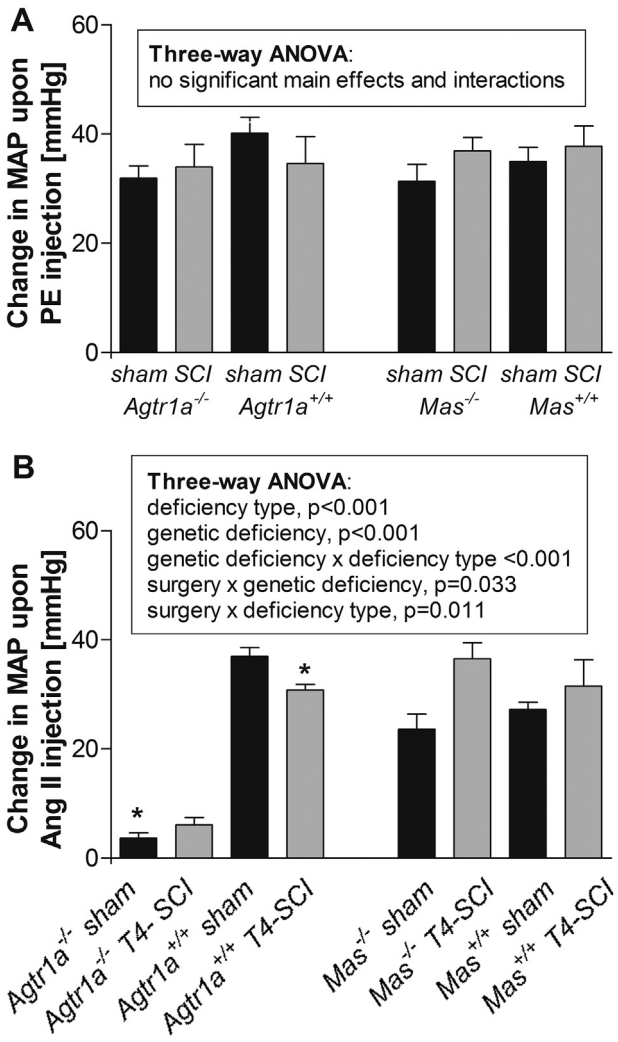

Fig. 2. Changes in MAP in response to either phenylephrine (PE) or angiotensin II (Ang II: (A) maximal change in MAP after the bolus injection of PE (10 µg/kg/KG, i.v.) and (B) maximal change in MAP after the bolus injection of Ang II (100 ng/kg/KG, i.v.). Global ANOVA analysis appears in boxes in respective figures. Significance of differences by Student's t-tests with Bonferroni procedure: *$P < 0.05$. *Agtr1a*$^{-/-}$ SCI (n = 9) and sham (n = 8) *Agtr1a*$^{+/+}$ SCI (n = 5) and sham (n = 6), *Mas*$^{-/-}$ SCI (n = 10) and sham (n = 6) and, *Mas*$^{+/+}$ SCI (n = 8) and sham (n = 6).

3.2. No significant change in response to PE bolus injection following SCI

We found no significant differences in MAP increases caused by phenylephrine (PE, 10 µg/kg/KG i.v) bolus injection between T4-SCI or sham-operated mice irrespective of the genotypes, i.e. in the absence or presence of AT1a or Mas receptors (Fig. 2A, three-way ANOVA, all F values < 1.84, $p > 0.05$).

3.3. Increased MAP response to Ang II bolus injection following SCI

To evaluate the functionality of peripheral AT1 receptors, we tested the blood pressure response to i.v. administered Ang II (100 ng/kg body weight) in conscious mice. As expected, bolus injection of Ang II caused small increases in MAP in *Agtr1a*$^{-/-}$ mice with or without T4-SCI (Fig. 2B). Interestingly, *Agtr1a*$^{+/+}$ sham operated mice had higher MAP increases in response to Ang II than *Agtr1a*$^{+/+}$ SCI mice. In contrast, *Mas*$^{+/+}$ showed no difference and in *Mas*$^{-/-}$ mice with SCI the MAP response to bolus Ang II injection exceeded the one observed in sham-operated *Mas*$^{-/-}$ mice (Fig. 2B). Three-way ANOVA revealed

significant effect of genetic deficiency, deficiency type and interaction of genetic deficiency and deficiency type (all F values < 56.95, $p < 0.001$). Surgery and genetic deficiency as well as surgery and deficiency type interaction were also significant (all F values < 7.02, $p < 0.033$) on Ang II response. There was no three-way interaction and effect of a surgery type (both F values < 2.95, $p > 0.092$). Simple main effects of surgery at KO and at WT level were also not significant (both F values < 3.54, $p > 0.069$), meaning Ang II response was not altered by SCI in KO group and not in WT group. Simple main effect of surgery at *Agtr1a* level ($F_{1,26} = 0.27$, $p = 0.610$) and at *Mas* level ($F_{1,28} = 6.25$, $p = 0.019$) indicated that there was no SCI-dependent effect in *Agtr1a* mice, but in the *Mas* group. Simple main effects of deficiency type at KO level ($F_{1,31} = 98.71$, $p < 0.001$) and at WT level ($F_{1,23} = 1.74$, $p = 0.20$) showed significant difference in Ang II response between $Agtr1a^{-/-}$ and $Mas^{-/-}$ groups, but not between $Agtr1a^{+/+}$ and $Mas^{+/+}$ groups. $Agtr1a^{-/-}$ sham and SCI groups did not differ significantly ($p = 0.1608$), whereas $Agtr1a^{+/+}$ sham and SCI groups did ($p = 0.0150$). $Mas^{-/-}$ sham and SCI mice had significantly different reactions to Ang II ($p = 0.0110$), whereas $Mas^{+/+}$ sham and SCI groups had no differences ($p = 0.4729$). Thus, we could not find a general effect of SCI on Ang II response, since *Mas* group showed increased responsiveness to Ang II following SCI, whereas $Agtr1a^{+/+}$ mice had reduced responsiveness.

3.4. Unchanged reactivity to PE or ACh in mesenteric arteries from SCI mice

We measured the reactivity of isolated mesenteric arteries from T4-SCI and sham-operated mice using organ bath technique. SCI did not influence the vasocontraction response to PE in the absence or presence of AT1a or Mas receptors (Fig. 3). Relaxation induced by acetylcholine (ACh) (1 μM) in PE-pre-constricted arteries also did not differ between vessels from SCI and sham-operated mice irrespective of the genotype, i.e. $Agtr1a^{+/+}$, $Agtr1a^{-/-}$, $Mas^{+/+}$ or $Mas^{-/-}$.

3.5. Decreased reactivity to Ang II in mesenteric arteries from SCI mice

We next studied whether SCI changes the reactivity of mesenteric arteries to Ang II. Vessels were isolated from T4-SCI *and* sham operated $Agtr1a^{+/+}$, $Agtr1a^{-/-}$, $Mas^{+/+}$,and $Mas^{-/-}$ mice (Fig. 4A–C original traces). We found T4-SCI decreased the reactivity of $Agtr1a^{+/+}$, $Mas^{+/+}$ and $Mas^{-/-}$ mesenteric arteries in response to Ang II (Fig. 4D, F and G), where as, no change was observed after T4-SCI in $Agtr1a^{-/-}$ mesenteric arteries (Fig. 4E).

For comparison, we also studied femoral arteries from T4-SCI *and* sham-operated $Agtr1a^{+/+}$, $Agtr1a^{-/-}$, $Mas^{+/+}$ and $Mas^{-/-}$ mice. Our results show that T4-SCI did not affect the reactivity of femoral arteries in response to Ang II in all genotypes (Fig. 5), which may indicate that most probably femoral arteries exhibit contractions by AT1b but not AT1a receptors in response to Ang II (Zhou et al., 2003). Consistent with this data, we found $Agtr1b^{-/-}$ femoral arteries did not produce any contraction in response to different doses of Ang II (Fig. 6A). However, in control experiments, we found that contractions of $Agtr1b^{-/-}$ mesenteric arteries in response to Ang II were only partly reduced (Fig. 6B). Together, the data indicate that Ang II contraction of femoral arteries are mediated by AT1b receptors whereas Ang II contractions of mesenteric arteries are mediated by both AT1a and AT1b receptors.

3.6. Unchanged gene expression of AT1a or AT1b receptors in mesenteric arteries from T4-SCI mice

Since SCI decreased the reactivity of $Agtr1a^{+/+}$, $Mas^{+/+}$ and $Mas^{-/-}$ mesenteric arteries in response to Ang II and this blunted response was not observed in $Agtr1a^{-/-}$ mesenteric arteries, we hypothesised that changes in arterial AT1a and/or AT1b expression may underlie the effect. We therefore performed real-time qPCR experiments to analyse

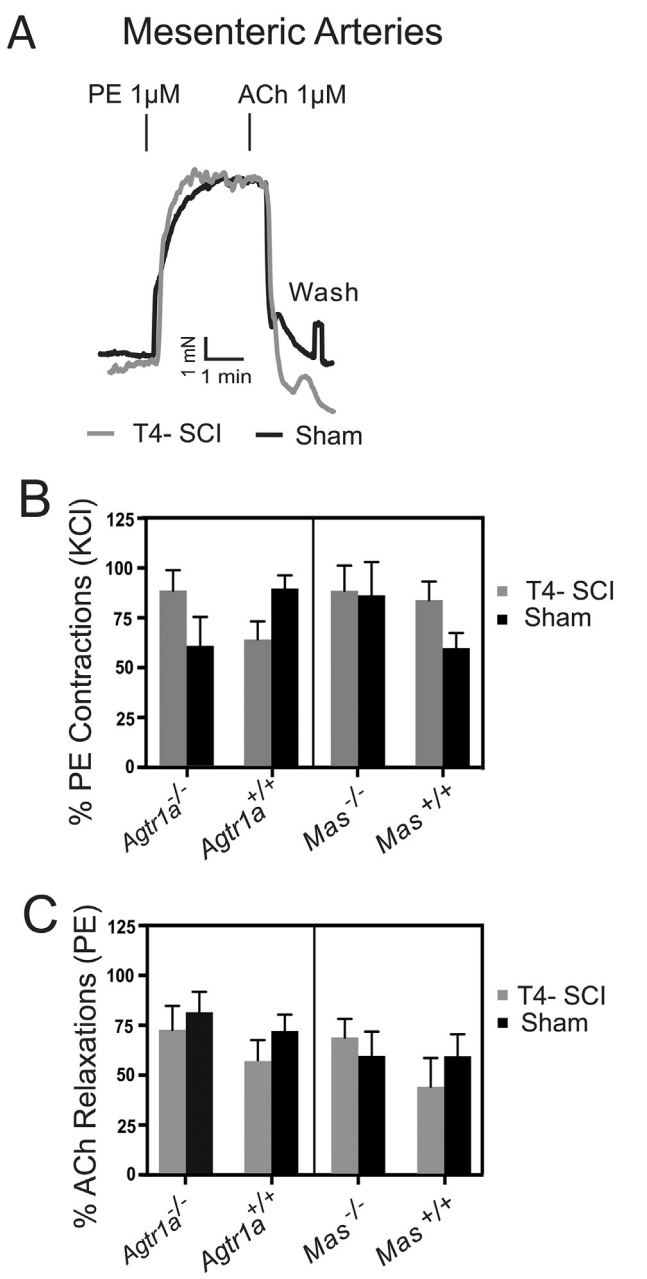

Fig. 3. Reactivity of mesenteric arteries to phenylephrine (PE) and acetylcholine (ACh). (A) Contractions induced by PE (1 μM) and relaxations induced by ACh (1 μM) in mesenteric artery rings from sham-operated or SCI mice (original recordings). (B) Summary data for PE induced contractions $P < 0.05$, using three-way ANOVA. (C) Summary data for ACh induced relaxations. $P < 0.05$, using three-way ANOVA. Samples were from $Agtr1a^{+/+}$ SCI (n = 8 rings, n = 3) or sham-operated (n = 6 rings, n = 3), $Agtr1a^{-/-}$ SCI (n = 10 rings, n = 5) or sham-operated (n = 8 rings, n = 3), $Mas^{+/+}$ SCI (n = 8 rings, n = 5) or sham-operated (n = 7 rings, n = 4) and $Mas^{-/-}$ SCI (n = 7 rings, n = 4) or its sham-operated (n = 6 rings, n = 4).

mRNA expression in mesenteric arteries isolated from T4-SCI and sham-operated $Agtr1a^{+/+}$ and $Mas^{+/+}$ mice (Fig. 7). For *Agtr1a* as well as *Agtr1b* expression change no significant differences were found (two-way ANOVA all F values < 4.20, $p > 0.05$). These data show that downstream mechanisms of AT1a activation are likely involved in decreased reactivity of mesenteric arteries in response to Ang II in AD following T4-SCI.

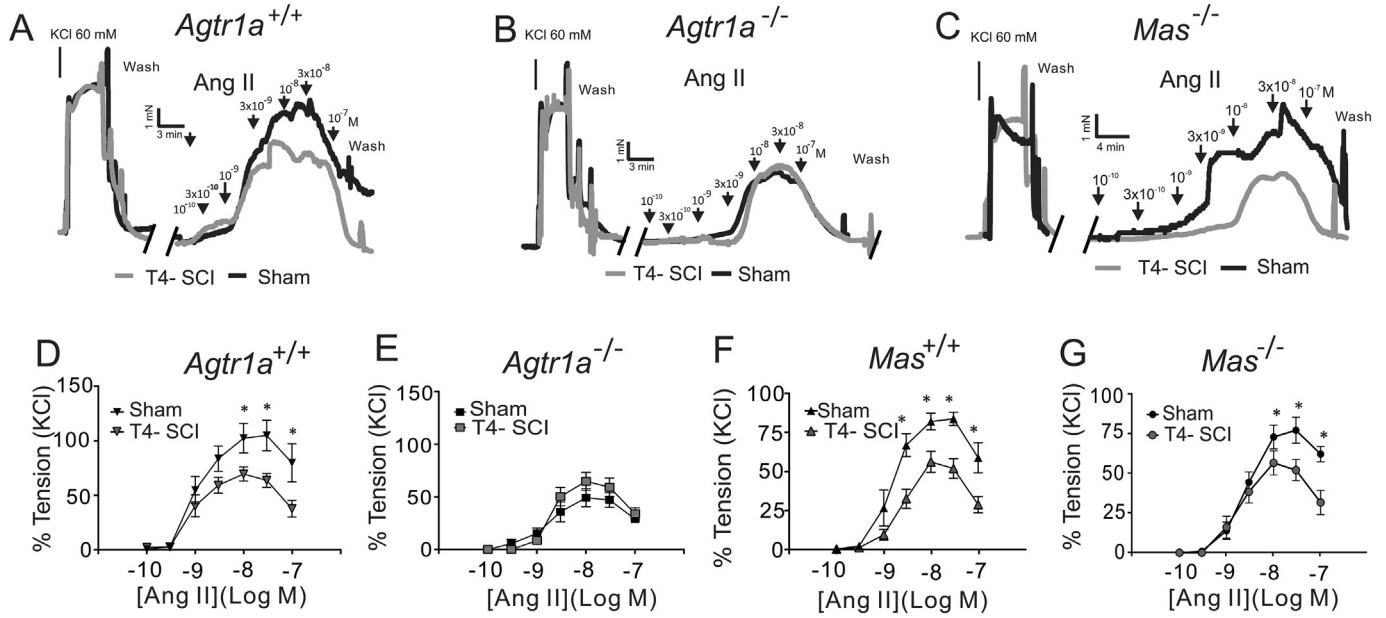

Fig. 4. Reactivity of Agtr1a$^{-/-}$ and Mas$^{-/-}$ and control mesenteric arteries to Ang II after T4-SCI. Original recordings of contractions induced by Ang II (100 nM-100 μM) in mesenteric artery rings isolated from sham-operated or T4-SCI mice of Agtr1a$^{+/+}$ (A), Agtr1a$^{-/-}$ (B) and Mas$^{-/-}$ (C) genotypes. Summary data for Ang II induced contractions of arterial rings from sham-operated (n = 6 rings, n = 3) or SCI Agtr1a$^{+/+}$ mice (D) (n = 8 rings, n = 3), Agtr1a$^{-/-}$ mice sham-operated (n = 8 rings, n = 3) or SCI (n = 10 rings, n = 5) (E) as well as Mas$^{+/+}$ sham-operated (n = 7 rings, n = 4) or SCI (n = 8 rings, n = 5) (F) and Mas$^{-/-}$ sham-operated (n = 6 rings, n = 4) or SCI (n = 7 rings, n = 4) (G). *$P < 0.05$ using repeated-measures two-way ANOVA, followed by Bonferroni post hoc procedure.

3.7. RNA sequencing of mesenteric arteries post T4-SCI

To reveal possible AT1a downstream signaling candidates, we performed RNAseq experiments with mesenteric arteries isolated from intact (n = 5) and T4-SCI mice four weeks post-surgery (n = 5). We found 80 genes which were ≥ 1.5 fold significantly regulated after T4-SCI, 20 of them were up-regulated (Supplementary Table 1). Analysis of the biological processes in which these genes are involved using over-representation analysis of biological processes by DAVID revealed developmental and immune process as well as vasculature developmental processes being enriched (Supplementary Table 2).

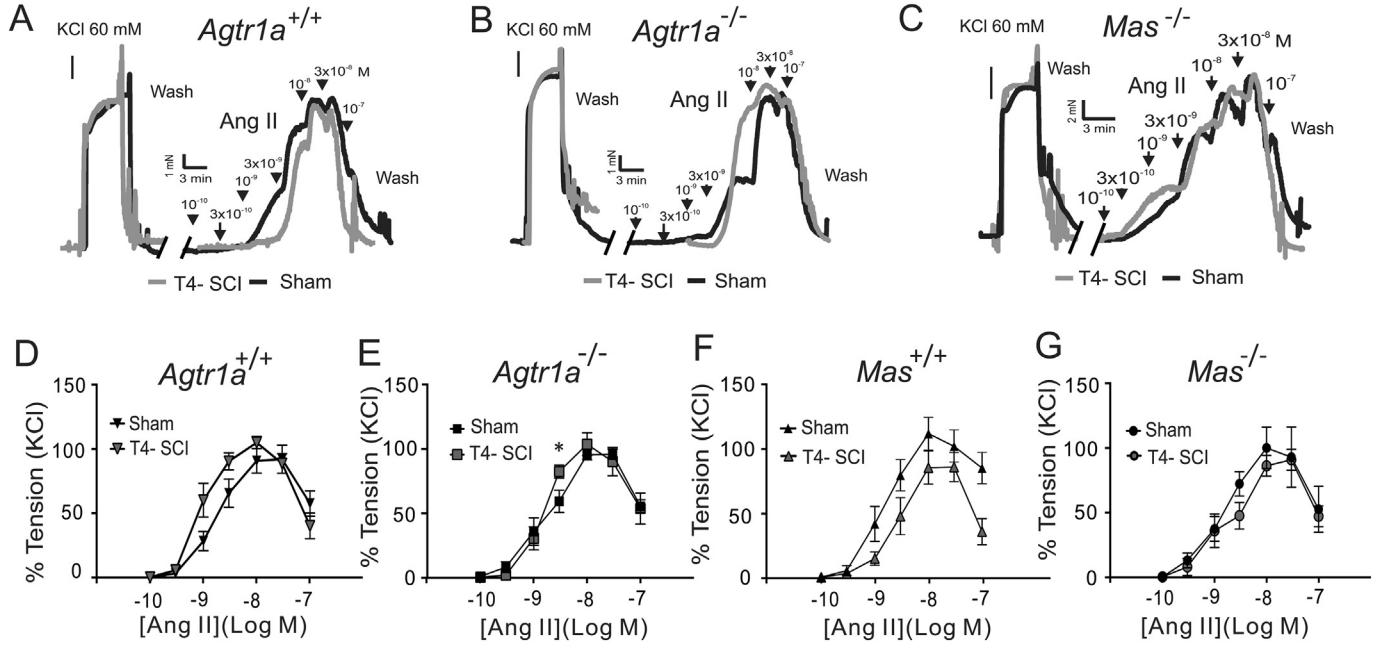

Fig. 5. Reactivity of Agtr1a$^{-/-}$ and Mas$^{-/-}$ and control femoral arteries to Ang II after T4-SCI. (A-C) Original recordings of contractions induced by different concentrations of Ang II (100 nM-100 μM) in femoral arterial rings isolated from sham-operated or SCI mice of Agtr1a$^{+/+}$ (A), Agtr1a$^{-/-}$ (B) and Mas$^{-/-}$ (C) genotypes. (D-G) Summary data for Ang II induced contractions of arterial rings from sham-operated (n = 6 rings, n = 3) or SCI Agtr1a$^{+/+}$ mice (D) (n = 7 rings, n = 3), Agtr1a$^{-/-}$ mice sham-operated (n = 8 rings, n = 3) or SCI (n = 8 rings, n=) (E) as well as Mas$^{+/+}$ sham-operated (n = 6 rings, n = 5) or SCI (n = 7 rings, n = 4) (F) and Mas$^{-/-}$ sham-operated (n = 7 rings, n = 3) or SCI (n = 6 rings, n = 3) (G). *$P < 0.05$ using repeated-measures two-way ANOVA, followed by Bonferroni post hoc procedure.

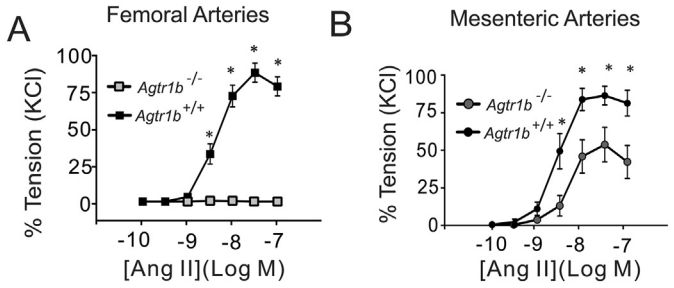

Fig. 6. Reactivity of *Agtr1b*$^{-/-}$ and control arteries to Ang II. (A) Summary data for Ang II induced contractions of femoral arterial rings from *Agtr1b*$^{-/-}$ (n = 6 rings, n = 4) or *Agtr1b*$^{+/+}$ mice (n = 6 rings, n = 4). (B) Summary data for contractions of mesenteric arterial rings from *Agtr1b*$^{-/-}$ (n = 11 rings, n = 4) or *Agtr1b*$^{+/+}$ mice (n = 11 rings, n = 4). *$P < 0.05$ using repeated-measures two-way ANOVA, followed by Bonferroni post hoc procedure.

4. Discussion

The main finding of this study is that AT1 and Mas receptors play differential roles in AD in T4-SCI mice. Although we found that CRD increased MAP and decreased HR in all three animal groups, we observed reduced increases in MAP in the Mas knockout mice compared to control mice in AD, whereas in AT1a deficient mice the central hemodynamic response was not affected. We found that the reactivity of wild-type and *Mas*$^{-/-}$ mesenteric arteries was diminished in response to Ang II after T4-SCI, although AT1a expression at the mRNA level remained unchanged in response to SCI. However, this effect in mesenteric arteries was not observed in the absence of AT1a receptors. In contrast, T4-SCI did not influence the response to Ang II in femoral arteries, i.e. distal to thoracic level T4. We conclude that Mas contributes to increased systemic blood pressure in AD following T4-SCI. Furthermore, AT1a signaling is not involved in this hemodynamic response, but contributes to increased vascular reactivity in mesenteric arteries in response to Ang II, i.e. near thoracic level T4. We suggest that this response may contribute to decreased visceral regional blood flow in high-level SCI.

4.1. Autonomic dysreflexia following T4- SCI

AD is a symptom complex characterized by a sudden exaggerated increase in blood pressure accompanied by bradycardia in response to a stimulus originating below the level of the SCI (Krassioukov and Claydon, 2006). As expected, we observed that all three groups of T4-SCI mice showed elevated MAP and decreased HR following CRD. After high-level SCI, when the supraspinal control of sympathetic spinal cord neurons is lost, the vascular tonus distal to the level of injury is maintained, most likely due to intact postganglionic neurons to induce a sympathetic reflex (Gondim et al., 2004; Yeoh et al., 2004). Sympathetic activation causes massive vasoconstriction within the arterial system leading to arterial hypertension. After the brain perceiving the hypertensive crisis throughout cervical baroreceptors, it generates inhibitory impulses, which cannot be transmitted below the level of injury to regulate vascular tone. On the other hand, there is a role of vasomotor centers in the medulla oblongata to lower the arterial blood pressure by decreasing HR (Ross et al., 1984). We tested the contribution of Mas and AT1a receptors in this hemodynamic response.

4.2. Attenuated AD in Mas deficient mice following T4-SCI

Mas$^{-/-}$ mice exhibit increased vascular resistance (Botelho-Santos et al., 2012) and enhanced Ang II–mediated vasoconstriction in a number of vascular beds (Kostenis et al., 2005). In contrast, *Agtr1a*$^{-/-}$ mice showed less vascular tonus and reduced vasoconstriction (Schleifenbaum et al., 2014). We found an attenuated CRD-induced MAP increase in *Mas*$^{-/-}$ mice compared to control *Mas*$^{+/+}$ mice. On the other hand, we detected a normal CRD-response in *Agtr1a*$^{-/-}$ mice. Central mechanisms of AD include injury-induced elevation of nerve growth factor caused sprouting of pelvic primary afferents (unmyelinated C-fibers and thinly myelinated Aδ-fibers terminating in the laminae I, II, and V, lamina X above the central canal, as well as the lateral gray matter) and sprouting of propriospinal interneurons (Gelber et al., 2008; Weaver et al., 1997), which transmit information to sympathetic preganglionic neurons. Since Mas is highly expressed in the brain and spinal cord (Metzger et al., 1995; Nemoto et al., 2014), we speculate that these plastic changes in the spinal cord may be affected by the absence of Mas. However, future studies are required to validate this possible scenario.

4.3. α-AR sensitivity and endothelial function following T4-SCI

Peripheral vasculature below the level of SCI is reported to become hypersensitive to α-adrenergic receptor (α-AR) stimulants. In animal experiments, decentralization of postganglionic sympathetic neurons by cutting their preganglionic inputs results in hypersensitivity response to norepinephrine (Hou and Rabchevsky, 2014). Some authors have questioned the concept of α-AR hypersensitivity and suggested apparent enhanced sympathetic response may be caused by periodic episodes of sympathetic nerve hyperactivity rather than denervation-related hypersensitivity (Osborn et al., 1989). Moreover, analysis of α-AR hyper-responsiveness (i.e., the pressor response) in vivo is complicated by the fact that able-bodied subjects and uninjured control animals have an intact baroreflex (Hou and Rabchevsky, 2014). Importantly, it has been reported that high thoracic SCI in rats is associated with MAP changes differently affected by the dose of administered PE: the MAP increase was comparable to that observed in uninjured controls at lower PE doses but augmented in animals with SCI at higher PE doses (Landrum et al., 1998). The in vitro myograph experiment showed higher sensitivity of mesenteric arteries to PE in a rat model of SCI (Alan et al., 2010), but the maximal contractions in response to PE were unchanged (Brock et al., 2006; West et al., 2016). We observed no differences in the MAP change to PE bolus injection in the

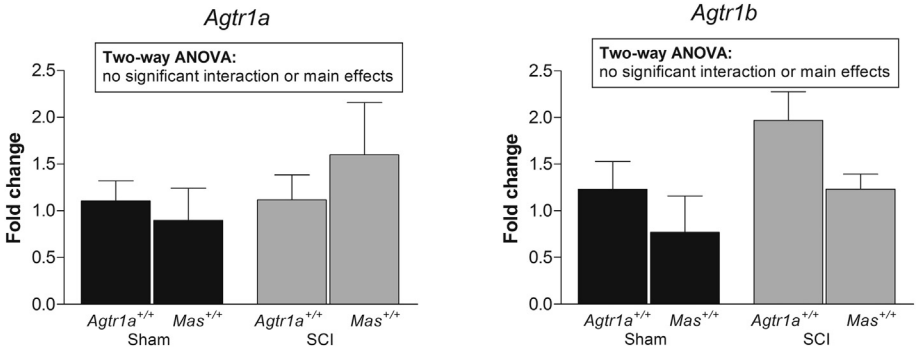

Fig. 7. mRNA expression of *Agtr1a* (A) and *Agtr1b* (B) during AD induced by CRD. qPCR for *Agtr1a* and *Agtr1b* was performed on WT *Agtr1a*$^{+/+}$ and *Mas*$^{+/+}$ sham-operated (n = 8) and T4-SCI operated mice (n = 8) 4 weeks post-operation. Global ANOVA analysis appears in the upper box in the figures. One-way ANOVAs revealed no significant differences between the groups, $p < 0.05$.

SCI mice compared to sham-operated mice. In our experiments, the mean of MAP change to PE bolus injection and the response of mesenteric arteries to 1 μM PE in the T4-SCI 4-week post-injury mouse model was not changed. This variable response to PE means that other concentrations, i.e. dose-response experiments, might be required to demonstrate variability in α-AR hypersensitivity.

One potential mechanism of α–AR hypersensitivity is endothelial dysfunction because endothelium-derived dilators control the extent of vasoconstriction in response to α-AR contractions (Angus et al., 1986; Dora et al., 2000). However, in our experiments, SCI did not change the reactivity of mesenteric arteries to acetylcholine (ACh), which produces endothelial dependent relaxation. Although *Mas*$^{-/-}$ mice can exhibit endothelial dysfunction with reduced NO production in certain vessels (Rabelo et al., 2008; Xu et al., 2008), ACh-dependent relaxation is not changed in AD. Thus, we believe that although the endothelial dysfunction seems to be a plausible explanation for α–AR hypersensitivity, it is not a major player in AD. This conclusion is supported by findings of Alan et al. (Alan et al., 2010), who observed hyper-responsiveness to PE in mesenteric arteries from SCI rats compared to controls without changes in vasodilator responses to ACh.

4.4. Reactivity of arteries to Ang II following T4-SCI

Mesenteric arteries are normally controlled by preganglionic neurons that project from T4 to T13. Femoral arteries are controlled by preganglionic neurons that project from L2 to L4. It is also known that T4-SCI results in a severity-dependent decline in the number of Fluorogold positive neurons in the rostral ventrolateral medulla (RVLM) (Squair et al., 2017), where it impacts on cardiovascular function and primary composition of C1 adrenaline-synthesizing neurons (Card et al., 2006). After SCI, the sympathetic preganglionic neurons controlling the vasculature abruptly fail to receive signals projected from adrenergic neurons within the RVLM. This process initiates rapid sprouting of remaining sympathetic preganglionic terminals in ganglia to restore transmission to postganglionic neurons, which in the following may be reconnected with the cord below the lesion and can participate at least in simple spinal reflexes and sympathetic neuroeffector transmission on smooth muscle tissues, including the vasculature (McLachlan and Brock, 2006). Functionally inappropriate reconnections may cause uncontrollable sympathetic outflow and increased nerve-evoked contractions in the presence of Ang II (Maiorov et al., 1997; Yeoh et al., 2004), which cannot be reduced by the AT1-receptor antagonist losartan (Al Dera and Brock, 2014). However, Ang II causes vasoconstriction via AT1 and has been reported to augment vasocontraction through its facilitating effect on norepinephrine (NE)-mediated sympathetic nerve activity (Reid, 1992). We found SCI decreases the reactivity to Ang II in mesenteric arteries, but not in femoral arteries. We also found that this effect is absent in mesenteric arteries from AT1a$^{-/-}$ mice, which suggests that it requires AT1a receptor signaling. In rodents, the presence of two pharmacologically identical AT1 receptor subtypes have been described, namely AT1a and AT1b, which exhibit different patterns of expression in vasculature. Previous studies have implicated that AT1a is primarily responsible for the regulation of systemic blood pressure and cardiac function (Ryan et al., 2004), but the regional blood flow mechanisms differ between peripheral systemic and splanchnic vascular beds (van Esch et al., 2010). In line with a previous report, AT1b receptors account for most of the total AT1 mRNA in mouse femoral artery (Zhou et al., 2003) and mediate Ang II contractions by AT1b receptors in this vessel (Fig. 6) (Zhou et al., 2003). This may explain why we did not observe post-SCI decreased reactivity of femoral arteries to Ang II, because our data obtained on *Agtr1a*$^{-/-}$ mesenteric arteries indicate that this effect relies on functional AT1a receptors. Of note, it has been found that AT1b receptors contribute to the regulation of resting blood pressure particularly when AT1a receptors are absent (Oliverio et al., 1997). Based on our data it is tempting to speculate that the AT1b receptor remains the dominant receptor for vascular Ang II signaling in rodents' mesenteric arteries after the sensitivity of AT1a is diminished in SCI. We are not able to detect changes in AT1a expression at the mRNA level in the vasculature after T4-SCI, although a tendency of increased AT1b expression in mesenteric arteries was observed after SCI. It is therefore likely that reduced sensitivity of AT1a receptors leads to a diminished response of the vasculature to Ang II post SCI. In order to find the genes involved in the diminished sensitivity of AT1a in SCI we performed RNAseq analysis of mesenteric arteries. We found dysregulated mRNAs for proteins important for vasorelaxation (e.g., Bdkrb2 and arginase 1) and vascular remodelling (e.g., Hbegf and Hey2), but alterations in these processes would also affect the response to PE and are therefore probably not responsible for the hyporesponsiveness to Ang II. We hypothesize that still elusive signaling pathways in mesenteric arteries downstream of the AT1a receptor are altered after SCI on a non-genomic level.

4.4.1. Conclusions and perspective

The major findings of our study are two-fold. First, deficiency of Mas in SCI mice attenuates the hemodynamic response in AD following T4-SCI in mice. Second, T4-SCI decreases the reactivity of mouse mesenteric arteries to Ang II via AT1a signaling. Although humans do not possess multiple AT1 receptor subtypes, our findings highlight the role of specific functions of individual RAS receptors in AD caused by SCI. Our study indicates that downstream mechanisms of AT1 receptors underly increased vascular reactivity in mesenteric arteries in response to Ang II, which may contribute to adaptive changes in regional blood flow in SCI. Our data suggest that MAS-and the AT1 receptor could represent novel targets for pharmacological interventions in hypertension, particularly in AD of SCI patients.

Disclosures

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2018.10.003.

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