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Published in:
Acta Physiologica

DOI:
10.1111/apha.13731

Publication date:
2022

Document version:
Accepted manuscript

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Article type : Regular Paper

Endothelial Mineralocorticoid Receptor Ablation Confers Protection Towards Endothelial Dysfunction in Experimental Diabetes in Mice

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Short title: Endothelial function in experimental diabetes

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Abstract

Aim: With diabetes comes significant risk of macrovascular and microvascular complications. Circulating aldosterone levels increase in patients with diabetes. Aldosterone can directly affect vascular function via activation of the mineralocorticoid receptor (MR). We hypothesized that aldosterone via endothelial MR impairs endothelial function in a murine model of experimental diabetes.

Method: Endothelial cell-specific mineralocorticoid receptor knockout $MR^{flox/flox}$;Tie2-Cre mice (ECMR-KO) and wild-type FVB littermates were subjected to an experimental type-1 diabetic model by low dose streptozotocin injections (55mg/kg/day) for five consecutive days. After 10 weeks of diabetes, second-order mesenteric resistance arteries were perfused *ex vivo* to evaluate vessel contractility and endothelial function. The effect of *ex vivo* incubation with aldosterone with and without the antagonist, spironolactone was determined.

Results: Diabetic ECMR-KO and wild-type mice had similar, elevated, plasma aldosterone concentration while only diabetic wild-type mice displayed elevated urine albumin excretion and cardiac and kidney hypertrophy at 10 weeks. There were no differences in contraction (Emax and EC_{50}) to thromboxane receptor agonist (U46619) and elevated K^+ between groups. Wild-type diabetic mice showed impaired acetylcholine (ACh)-dependent relaxation, while diabetic ECMR-KO mice had intact ACh-mediated relaxation. Aldosterone incubation *ex vivo* impaired ACh mediated relaxation and rendered responses similar to diabetic WT arteries. Direct, *ex vivo* aldosterone effects were absent in ECMR-KO animals. *Ex vivo* inhibitory effects of aldosterone on endothelial relaxation in arteries from WT were abolished by spironolactone.

Conclusion: These findings show that endothelial cell mineralocorticoid receptor activation accounts for diabetes-induced systemic endothelial dysfunction in experimental diabetes and may explain the cardiovascular protection by MR antagonists in diabetes.

Keywords: Aldosterone, Mineralocorticoid Receptor, Vascular complications

Introduction

Diabetes mellitus (DM) is a multi-factorial chronic disease triggered by genetic and environmental factors.[1] The World Health Organization (WHO) reports that prevalence of diabetes has quadrupled since 1980.[2] According to the International Diabetes Federation, this number is estimated to increase, to affect nearly 700 million individuals by year 2045.[3] With diabetes comes a significant risk of both macrovascular and microvascular pathologies, such as ischemic heart disease, retinopathy, nephropathy, and atherosclerotic diseases. Furthermore, the relative risk of cardiovascular disease increases by two- to four-fold in diabetic patients[4] and higher rates of co-morbidity.[5-7] Endothelial cells regulate vascular function by secreting vasoactive paracrine substances, e.g. nitric oxide (NO), endothelins and prostanoids.[8] Diabetes can promote endothelial dysfunction[9] in part by impairing endothelium-dependent nitric oxide (NO) production[9] via endothelial NO synthase (eNOS) shown in both type-1 and type-2 diabetes and by increasing reactive oxygen species (ROS)-generating enzymes, including nicotinamide adenine dinucleotide phosphate oxidase (NOX) in a db/db mouse model of diabetes type-2.[10] However, the molecular mechanisms that contribute to diabetes-associated vascular complications remain to be delineated in detail.

Aldosterone has been suggested as a potential contributor to vascular dysfunction in diabetes[11] and aldosterone concentrations increase in diabetic patients.[12] Aldosterone exerts vascular effects through acute, non-genomic pathways and by acute or chronic, genomic pathways involving transcriptional mechanisms.[13] The direct effect of aldosterone on blood vessels varies greatly among different vascular beds, with vessel calibers and can go in opposite directions: Contractile responses, but also dilatory responses depending on co-morbidities, to aldosterone have been described on carotic, aortic, mesenteric, kidney, and cerebral blood vessels.[13-15] Thus both relaxing and pro-contractile responses have been observed; time of exposure is a factor and the vascular response towards aldosterone is determined by the functional status of the endothelium.[16] Circulating aldosterone exerts its effect on blood vessels in part by activating the MR present in endothelial cells (EC)[17] and vascular smooth muscle cells (VSMC)[18] or via MR-independent actions.[19]

Detrimental effects of aldosterone have been reported in animal studies where activation of the endothelial cell-specific MR (ECMR) mediated endothelial dysfunction, oxidative stress and attenuated the bioavailability of NO.[20,21] Furthermore, in an experimental model of diabetes,

induced with a single dose of STZ, MR blockade using eplerenone enhanced nitric oxide (NO) bioavailability and improved endothelial vascular function in rats.[22] However, it was not clear if this was driven through endothelial MR blockade. If the beneficial vascular effect of MR blockade in diabetes occurs primarily by blocking the receptor in the endothelium, such therapy could be refined or targeted, to confer better protection from diabetic injury. Based on the increased circulating aldosterone concentrations found in diabetic patients, the benefit of MR blockade on vascular function, and the finding that the ECMR mediates endothelial dysfunction, we hypothesized that aldosterone via the endothelial MR evokes endothelial dysfunction in diabetes. To test the hypothesis, we employed the model of experimental type-1 diabetes induced by streptozotocin and applied it in 10-week experiments to FVB mice with and without endothelial-specific deletion of the MR.

Results

Characteristics of the mouse during 10 weeks of experimental diabetes

Animals that reached and maintained a fasting blood glucose higher than 15 mmol L-1 were considered diabetic and were included in the study (n=17). A fraction of the STZ-injected mice was thus excluded as they failed to reach and maintain a sufficiently high fasting blood glucose (See consort diagram in figure S1). There were no sex-differences in fasting blood glucose in groups or between groups following 10 weeks of experimental diabetes (WT M 23.9±1.2 mmol L-1 vs. WT F 20.7±1.1 mmol L-1 and KO M 22.1±1.3 mmol L-1 vs KO F 19.5±1.0 mmol L-1) (Figure 1A). Furthermore, no change in body weight gain was observed following induction of diabetes for 10 weeks (Figure 1B). Diabetic WT mice had significantly larger heart (HW/BW) and kidney (KW/BW) to body weight ratios than diabetic ECMR-KO mice and controls (Figure 1C and D). Experimental diabetes increased the urinary albumin/creatinine ratio in WT, but not in ECMR-KO mice (Figure 1E). No differences appeared to be present in heart weight and urinary albumin excretion between sexes (Figure S2). Experimental diabetes increased plasma aldosterone concentrations to a similar degree in both diabetic WT mice and diabetic ECMR-KO mice compared to their respective controls (Figure 1F).

Diabetes does not affect primary constrictor responses in mesenteric vessels

Mesenteric vessels (average baseline vessel diameter 186.5±5.6μm) that contracted more than 20 % to K^+-depolarization were included (Figure S1). Concentration-response contraction to the thromboxane A2 analog U46619 was similar in all groups despite genotype or diabetes, with no difference in EC_{50} or maximum contraction (E_{max}) (Figure S3A). Furthermore, there was no difference in contraction to U46619 between groups when comparing total integrated contraction (Figure S3B). Experimental diabetes did not affect the constrictor response to K^+, as there were no differences between vasoconstriction (either Emax or EC_{50}) in diabetic WT and ECMR-KO or non-diabetic WT and ECMR KO (Figure S3C).

Endothelial dysfunction develops after 10 weeks of experimental diabetes

Acetylcholine-induced dilatation in vessels isolated from WT mice with diabetes and pre-constricted with U46619, was significantly impaired compared to arteries from WT controls (Figure 2A). Endothelial dysfunction was not observed in ECMR-KO mice following diabetes (Figure 2A).

Experimental diabetes significantly reduced endothelial-dependent dilatation in WT mice compared to ECMR-KO ($p<0.013$) (Figure 2A). Likewise, maximal dilatation (E_{max}) was significantly reduced in WT STZ compared to all other groups (Table 1). No differences in the affinity towards ACh was observed at 10^{-8}M ACh between the groups. Significant changes were seen between ECMR-KO control mice at 10^{-7}M ACh, in comparison to the WT STZ ($P=0.0099$), while only a trend is observed when comparing the WT control vs. ECMR-KO control at 10^{-7}M ACh ($P=0.1866$). When comparing the total integrated response to ACh, diabetic WT mice had significantly lower vasodilatation compared to ECMR-KO controls ($p<0.025$) (Figure 2B). Secondary dilatation following K^+-induced depolarization depends on NO-production in isolated mesenteric resistance vessels.[23] Diabetic WT mice had a significantly lower secondary dilatation following K^+-induced depolarization, compared to non-diabetic WT mice confirming endothelial dysfunction following prolonged experimental diabetes (Figure 2C). Importantly, arteries from diabetic ECMR-KO mice showed intact secondary dilatation similar to WT mice and ECMR-KO mice without diabetes. This response was significantly higher than the response in vessels from diabetic WT (Figure 2C). The vascular smooth muscle responsiveness to nitric oxide was tested using the NO-donor, SNP. SNP caused a concentration-dependent dilatation and there was no difference in the endothelial-independent dilatation was found between groups (Figure S2D). Furthermore, no sex differences were found when analyzing these data between males and females in all groups (Figure S4).

Aldosterone-mediated endothelial dysfunction can be prevented by mineralocorticoid receptor antagonism with spironolactone in experimental diabetes

Incubation of mesenteric resistance vessels with aldosterone (10^{-9} mol L-1, 1 hour) *ex vivo* decreased significantly endothelial-dependent dilatation to ACh in WT controls compared to ECMR-KO (both control and diabetic) (Figure 3A). Aldosterone abolished the difference between WT groups as diabetic WT mice already had decreased dilatation to ACh at baseline. Incubation with aldosterone did not affect potency but decreased E_{max} in WT mice compared to ECMR-KO mice (Table 1) and significantly decreased the total dilatation in WT mice compared to ECMR-KO (Figure 3B). Aldosterone incubation significantly decreased the secondary dilatation following K^+ in WT controls. This aldosterone-mediated decrease in secondary dilation was not observed in WT animals with experimental diabetes as the secondary dilatation was already low (Figure 3C). Importantly, knockout of the ECMR protected against the aldosterone-mediated decrease in secondary dilatation (Figure 3C). This was exemplified when the secondary dilatory response to

incubation with aldosterone in each vessel was corrected for the secondary dilatory response at baseline (Figure 3D). No obvious differences were found between sexes when comparing male vs female in each group (Figure S5).

To investigate the hypothesis pharmacologically, vessels incubated with the MR antagonist spironolactone (10^{-7} mol L-1) with aldosterone (Figure 4). Co-incubation with aldosterone and spironolactone *ex vivo* did not restore the decreased dilatation in 10w diabetic WT mice (Figure 4A). Co-incubation of vessels from WT controls with the MR antagonist and aldosterone counteracted the direct detrimental effects of aldosterone on ACh-mediated relaxation (Figure 4A). There were no differences in potency or in maximal vasorelaxation (Table 1). The difference between diabetic WT compared to diabetic KO was also observed in the total integrated response to ACh in the presence of both aldosterone and spironolactone (Figure 4B). With aldosterone and spironolactone co-incubation, the secondary dilatation following depolarization-induced constriction in WT controls and WT STZ did not differ, nor was it any different when compared to diabetic and control ECMR-KO mice (Figure 4C). When comparing to aldosterone incubation alone, co-incubation with spironolactone restored the endothelium-dependent secondary dilatation in both diabetic and non-diabetic WT animals (Figure 4D vs Figure 3). In addition, no differences were found when comparing male vs female in all groups following co-incubation of aldosterone and spironolactone (Figure S6).

Discussion

Diabetes is a common condition that may lead to late vascular complications. The underlying molecular mechanisms driving endothelial dysfunction remain unclear. With this study we have shown that after 10 weeks of STZ-induced type-1 diabetes in the FVB mouse strain, that: *i)* Endothelial dysfunction with albuminuria and impaired acetylcholine-induced relaxation are manifest. *ii)* Plasma aldosterone is elevated. iii) The endothelial mineralocorticoid receptor, ECMR confers detrimental impaired endothelial NO-mediated relaxation, while direct NO-mediated vascular smooth muscle relaxation was intact in mesenteric resistance vessels during experimental diabetes *iv)* and ECMR deletion confers protection from cardiac and renal hypertrophy in experimental diabetes. *v)* incubation of vessels *ex vivo* with aldosterone mimics MR-dependent *in vivo* impairment of endothelial function. *vi)* Short term incubation with an MR receptor antagonist counteracted the effects of aldosterone incubation *ex vivo* in WT but did not correct diabetes-induced endothelial dysfunction. In conclusion, aldosterone-MR signaling drives diabetes-dependent endothelial dysfunction via the endothelial mineralocorticoid receptor.

Sex differences in cardiovascular disease, vascular tone and blood flow have been reported (reviewed in detail by[24]). We did not in a previous study find sex differences under basal conditions between WT and ECMR-KO mice.[25] Based on these findings and animal experimental ethics considerations, we chose to include both sexes in the experiment. ECMR-KO-mediated protection from diabetes did not differ by sex, both with respect to changes in vessel function, albuminuria and heart weight as outlined in supplemental figures 2 and 4-6. Even in groups with only 3-5 animals of either sex there were no tendencies towards sex differences underlying these alterations. This suggests that endothelial MR is equally detrimental in each sex in diabetes and conversely, in perspective, pharmacological intervention by MR blockers would be relevant in both sexes. Our findings contrast with previous studies in mice on western diet where ECMR deletion prevented endothelial dysfunction in female mice only.[20,26] This difference was found in obese mice whereas the present STZ-treatment showed similar weight development as in wildtype mice. The difference could result from the presence of hyperlipidemia. In the present study the FVB strain background was chosen because of a larger sensitivity towards diabetes compared with other strains as the BL6 strain that is often used.[27,28]

It is well established that endothelial dysfunction in diabetes results from a decrease in NO, excess production of vasoconstrictors and involvement of both ROS and inflammation.[8]

Endothelial dysfunction occurs early in the pathogenesis of diabetic vasculopathy and is involved in progression to late diabetic complications.[29] In our study, endothelial dysfunction was documented by impaired acetylcholine-driven relaxation and via decreased secondary dilatation following depolarization-induced constriction. The response was not caused by different sensitivity in vascular smooth muscle towards NO in diabetes, because the responses to an NO-donor were similar across groups and sex. Both mechanisms depend on endothelial NO formation.[23,30,31] Importantly, functional deletion of ECMR protected against diabetes-associated endothelial dysfunction. In line with our data, western diet also confers endothelial dysfunction partly due to ECMR-dependent reduced NO bioavailability.[21] A potential shift in receptor-affinity in the lower doses of ACh-administration could be present, but we did not observe a statistically significant difference. Acute *ex vivo* incubation of arteries from WT mice with aldosterone mimicked the diabetes-associated *in vivo* endothelial dysfunction in WT mice. Aldosterone incubation did not further exaggerate existing endothelial dysfunction in mesenteric vessels from diabetic WT mice.

Aldosterone has been shown to exert its vascular effect by MR present both in EC and VSMC from non-diabetic humans and animals.[17,18] Chronic aldosterone reduced endothelial NO synthase (eNOS)-derived NO formation and increased ROS generation in cultured ECs and decreased NO bioavailability.[32,33] A suggested mechanism for this is MR-mediated eNOS uncoupling and phosphorylation of the NADPH oxidase.[34] Chronic elevations in aldosterone decreases NO release from EC, increases ROS production in both EC and VSMC,[14] increases expression of NOx and decreases NO production through diminished phosphorylation of eNOS at SER1177.

We find elevated plasma aldosterone levels in both diabetic WT and diabetic ECMR-KO mice. These observations are in line with findings from man showing increased aldosterone secretion in diabetes.[12] In addition, it has been suggested that aldosterone confers insulin resistance and impairs glucose homeostasis in patients with primary aldosteronism[35] and a positive correlation between aldosterone and insulin resistance has been demonstrated in healthy subjects.[36] Thus, there are potentially multiple benefits of inhibition of mineralocorticoid receptor in diabetic patients. Selective activation of the MR by aldosterone is conferred by co-localized 11βHSD-2, an enzyme that cleaves and inactivates glucocorticoids.[37] The presence of both MR and 11βHSD-2 at mRNA level but also protein level has previously been shown in human coronary arteries and aortic EC.[38,39]

At mRNA levels, both MR and 11βHSD-2 have also been reported in mouse aorta,[37,40] and MR have been reported in mouse mesenteric arteries.[15]

Our data showed that ECMR contributed to diabetes-associated cardiac and renal hypertrophy. Interestingly, cardiac hypertrophy was attenuated by lower plasma aldosterone and by MR antagonists in mice with Ang-II-induced hypertension, despite unchanged, elevated, AngII concentration and hypertension[41] suggesting a significant independent aldosterone-MR-driven effect in mice. We have previously shown that ECMR-KO mice do not have elevated blood pressure at baseline or following AngII infusion compared to WT.[25] Furthermore, STZ-diabetic control mice on a FVB background have no change in blood pressure compared to healthy controls in the 5th week of diabetes by 24h invasive measurements in freely moving unstressed mice.[42] Due to the uncompensated diabetes with hyperglycemia, glucosuria and 4-6 times increased diuresis on the FVB background,[42] mice are likely to exhibit extracellular volume contraction which would fit with reactive stimulation of aldosterone secretion and the observed elevated level in plasma. It is therefore less likely that blood pressure is increased in the present ECMR-KO mice as also observed previously in mouse STZ-diabetes models.[43,44] Type-1 DM has been reported to increase BP in FVB mice at 12 weeks measured by unblinded tail-cuff manometry,[45] but not at 4 weeks measured in catheterized mice.[44] The endothelial dysfunction and cardiac hypertrophy by diabetes could be related to elevated glucose or aldosterone *per se* while hypertension is less likely. On a different timescale, development of hypertension over time is a common complication among patients with diabetes.[46]

ECMR-KO protected against increased urinary albumin excretion despite similar level of hyperglycemia. With significant albuminuria, the diabetic WT mice most likely developed a more severe kidney injury along the 10 weeks of experimental diabetes as compared to ECMR-KO mice. It is not clear why this protection is seen. In fact, this could be due to direct actions of MR deletion in the glomerular capillaries or as a result of a general reduction in ECMR-dependent endothelial dysfunction throughout the body. The development of generalized endothelial dysfunction is supported by our findings on vessel contractility in mesenteric resistance vessels. Of interest, kidney hypertrophy was mitigated by ECMR. Normally, the hypertrophy is coupled to the excess glucose transport but with similar plasma glucose this could not account for the difference.

Spironolactone inhibited the detrimental acute effect of aldosterone on vessel dilation and the direct effect of aldosterone was not present in ECMR-KO mice. Spironolactone is less

specific than other MR antagonists which would be a limitation of our study. However, we performed targeted deletion of the receptor in endothelial cells to overcome these well-known challenges with confounding causes, when interpreting pharmacological findings. The vascular response to aldosterone is complex as acute aldosterone administration can also mediate vasoconstriction[47,48] or vasodilation.[39,49] In fact, acute aldosterone administration for only a few minutes dilates both conduit and resistance vessels by increasing NO production through an aldosterone-MR- phosphoinositide 3-kinase-eNOS pathway[39,49] while other studies fund vasoconstriction likely due to different status of the endothelium[14] or different vessel types and caliber. Accordingly, spironolactone co-incubation with aldosterone did not ameliorate established endothelial dysfunction in arteries from 10-week diabetic WT mice. Long-term effect of diabetes and the ensuing vasculopathy likely involves more persistent mechanisms and irreversible injury to the endothelium. Chronically elevated aldosterone mediates hypertrophy of VSMCs and inflammation,[50] which could also account for the vascular complications seen in diabetes. This would also suggest that aldosterone inhibition would have a more pronounced effect, if initiated earlier in patients and animals. The newly developed MR antagonist finerenone improved cardiovascular events in patients with type 2 diabetes clearly highlighting the advantage of MR blockade on vascular function.[51]

It is concluded that in a murine model of chronic type-1 diabetes, aldosterone drives the development of endothelial dysfunction via the ECMR. Selective blockade of the ECMR may confer protection from endothelial dysfunction and albuminuria in diabetes.

Perspectives

Based on the present study, treatment targeted at selective endothelial cell blockade of the MR could provide several desirable cardiovascular and renal treatment outcomes without the adverse effects of hyperkalemia and gynecomastia in diabetic patients. The present study provides proof-of-concept that aldosterone directly impairs endothelial function and support that aldosterone receptor inhibition is efficient therapy for diabetes and its early vascular complications. The vascular effects could account for the beneficial long-term effects on "hard" endpoints like mortality[52,53] and explain why spironolactone improved endothelial function in diabetic and non-diabetic patients.[6,54] Thus, the present *ex vivo* data suggest a direct detrimental action of MR activation in endothelial cells in diabetes independent of plasma K^+ changes and blood pressure. Future studies should investigate

whether direct, endothelial cell-targeted MR blockade can better endothelial function and vascular complications in patients with diabetes.

Methods

Please see the Data supplement for full experimental details.

Endothelial-specific mineralocorticoid receptor knockout $MR^{flox/flox}$;Tie2-Cre mice maintained on a FVB background (ECMR-KO) and their WT littermates (ages 7-12 weeks) were made diabetic by streptozotocin injections (STZ, 55 mg/kg/day, i.p.) for five consecutive days (275 mg/kg per animal). Mice were left for 10 weeks to develop experimental diabetes and were then euthanized by cervical dislocation followed by decapitation. All animals were sacrificed between 8AM and 9AM and both spot urine and plasma samples and isolation of blood vessels were made in this time frame. *Ex vivo* experiments on isolated blood vessels were performed between 10AM-5PM. Both spot urine and plasma were taken for further analysis. Plasma aldosterone was measured using an aldosterone ELISA kit (LDN, MSE-5200). U-Creatinine and U-albumin was measured using standardized analysis at the Department of Clinical Biochemistry and Pharmacology at Odense University Hospital. To characterize and determine the effect of diabetes and the mineralocorticoid receptor in the endothelium, second order mesenteric arteries from WT and ECMR-KO mice were isolated and perfused *ex vivo* for pharmacological studies of vascular function with both the thromboxane A2 analog, U46619, acetylcholine (ACh) and high K^+ in the presence or absence of aldosterone (10^{-9} mol L-1) and/or spironolactone (10^{-7} mol L-1).

Statistics

Data are normally distributed and are expressed as mean ± SEM. Blood glucose measurements, change in body weight and artery diameters were compared using two-way analysis of variance (ANOVA) followed by Bonferronis' post-hoc test for reduction for multiple comparisons. Comparisons of different groups with only one parameter, E_{max} and EC_{50} were done using unpaired one-way ANOVA followed by Bonferronis' post hoc test for reduction for multiple comparisons. $P<0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, USA).

Acknowledgement

The authors would like to thank Kenneth Andersen, Camilla Enggaard, Kristoffer Rosenstand, Mie Rytz and Gitte Kitlen (University of Southern Denmark, Odense, Denmark) for expert technical assistance. Professors Stefan Berger and Günther Schütz (German Cancer Research Center, Heidelberg, Germany) are thanked for generously providing floxed *Nr3c2* mice and Professor Rong Wang (University of California, San Francisco, US) is thanked for generously sharing the *Tie2*-Cre mice.

Funding

The project was funded by the Novo Nordisk Foundation, the Carlsberg Foundation, the Independent Research Fund Denmark, The Danish Heart Association, The Faculty of Health Sciences, University of Southern Denmark and The Institute of Molecular Medicine, University of Southern Denmark.

Disclosure

PBLH is employed by AstraZeneca, but AstraZeneca did not have any additional role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author contributions

KSL, BLJ, PBLH and HD conception and design of research; KSL performed experiments; KSL analyzed data; KSL, BLJ, PBLH and HD interpreted results of experiments; KSL and HD prepared figures; KSL and HD drafted the manuscript. KSL, BLJ, PBLH and HD edited and revised manuscript; KSL, BLJ, PBLH and HD approved the final version of the manuscript.

Tables:

	Control		**+Aldosterone**		**+Spironolactone**	
	EC_{50}	**E_{max}**	**EC_{50}**	**E_{max}**	**EC_{50}**	**E_{max}**
WT STZ	6.28±0.29	16.88±5.80*	6.16±0.41	11.70±9.39*	6.78±0.56	33.31±5.07

WT	6.11±0.25	35.32±4.65	6.25±0.37	17.40±4.92#	6.25±0.29	35.60±4.17
KO STZ	6.22±0.25	36.74±2.92	6.12±0.36	42.16±3.62	6.67±0.19	43.10±3.04
KO	6.47±0.45	38.56±6.28	6.49±0.49	40.74±5.12	6.54±0.22	30.00±3.98

Table 1: List of EC_{50} and E_{max} for Ach-induced dilatation in pre-constricted vessels. EC_{50} and E_{max} are listed for the four different groups (WT STZ, WT, KO STZ, and KO) during basal (Control), *ex vivo* administration of aldosterone (+Aldosterone), and *ex vivo* administration of spironolactone on top of aldosterone (+Spironolactone). Data are represented as mean±SEM. $P<0.05$ is considered significant. *represents statistically significant difference of WT STZ to the WT, KO STZ and KO groups (Control: P=0.0134; +Aldosterone: P=0.0018). #represents statistically significant difference of WT to the KO STZ and KO groups (+Aldosterone: P=0.0175). Unpaired one-way ANOVA followed by Bonferronis' post hoc test for reduction for multiple comparisons.**References**

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Figure legends:

Figure 1: Characteristics of the FVB-Streptozotozin (STZ)-induced model of experimental type-1 diabetes. A) Consecutive plasma glucose concentration measurements in diabetic versus non-diabetic WT and ECMR-KO mice (n=31). B) Change in body weight in diabetic versus non-diabetic WT and ECMR-KO. C-F show values from termination at day 70. C) Heart weight to body

weight ratio (HW/BW). D) Kidney weight to body weight ratio (KW/BW). E) Urinary albumin-to-creatinine ratio (n=20). F) Plasma aldosterone levels in diabetic and control groups (n=28). Data are represented as mean±SEM. P<0.05 is considered statistically significant. * P<0.05, † P<0.01, and ‡ P<0.001. Repeated measurements 2-way ANOVA followed by Bonferronis' post hoc test of simple effects within rows.

Figure 2: Diagrams show relaxation *ex vivo* of isolated second order mesenteric arteries isolated from WT and ECMR-KO mice following experimental diabetes. A) Concentration-response curve to the endothelium-dependent dilatator acetylcholine (ACh). P-value for main effects: P<0.0001. B) Area under curve (AUC) of dilatation response towards ACh. C) Secondary, spontaneous, dilatation following vasoconstriction with high bath K^+ concentration. Data are represented as mean±SEM, n= STZ WT(8), WT(7), STZ KO(9), KO(7). P<0.05 is considered statistically significant. * P<0.05, † P<0.01, and ‡ P<0.001 when comparing WT STZ with WT, KO STZ and KO. Repeated measurement 2-way ANOVA followed by Bonferronis' post hoc test of simple effects within rows.

Figure 3: Effect of aldosterone addition *ex vivo* on relaxation in second order mesenteric arteries harvested from WT and ECMR-KO mice following experimental diabetes. A) Concentration-response curve to ACh after incubation with aldosterone (10^{-9}mol L-1) *ex vivo* for one hour. P-value for main effects: P<0.0001. B) Area under the curve (AUC) for dilatation to ACh in the presence of aldosterone. C) Secondary dilatation after 1-hour incubation with aldosterone. D) Change in secondary dilatation after aldosterone incubation compared to basal conditions. Data are represented as mean±SEM, n= STZ WT(8), WT(7), STZ KO(9), KO(7). Repeated measure 2-way ANOVA followed by Bonferronis' post hoc test of simple effects within rows. P<0.05 is considered statistically significant. * P<0.05, † P<0.01, and ‡ P<0.001 when comparing WT groups with KO groups.

Figure 4: Effect of co-incubation of spironolactone and aldosterone ex vivo on constriction in second order mesenteric arteries harvested from WT and ECMR-KO following experimental diabetes. A) Concentration-response curve to ACh with presence of both aldosterone (10^{-9}mol L-1) and spironolactone (10^{-7}mol L-1) in diabetic and non-diabetic groups. P-value for main effects: P<0.0001. B) Area under the curve (AUC) to ACh following incubation with both aldosterone and spironolactone. C) Secondary dilatation with presence of both aldosterone and spironolactone. D) Change in secondary dilatation after incubation with aldosterone and spironolactone compared to aldosterone alone. Data are represented as mean±SEM, n=STZ WT(8), WT(7), STZ KO(9), KO(7).

Repeated measurement 2-way ANOVA followed by Bonferronis' post hoc test of simple effects within rows. $P<0.05$ is considered statistically significant. * $P<0.05$, † $P<0.01$, and ‡ $P<0.001$ when comparing WT STZ with WT, KO STZ and KO.

Figure 1

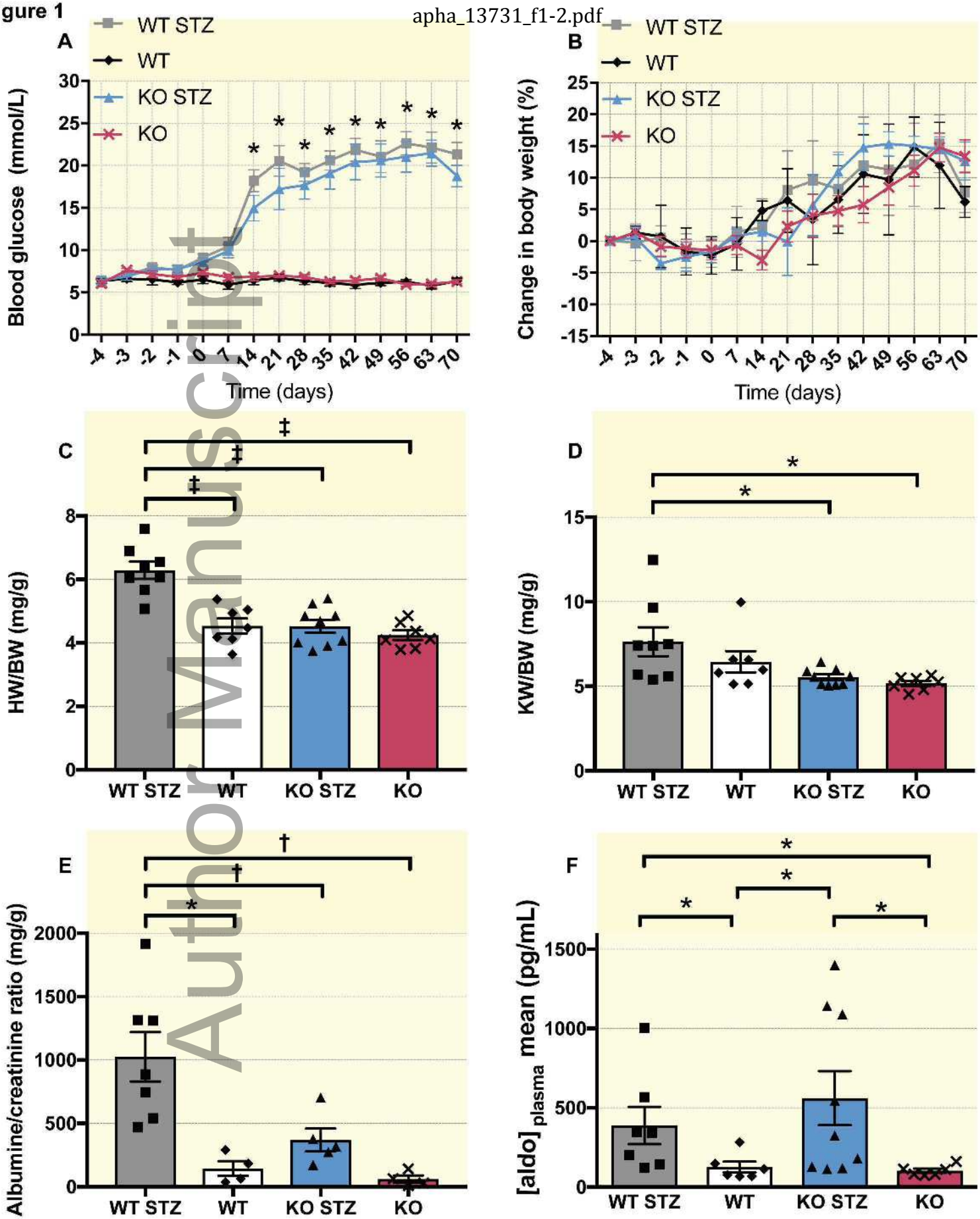

Figure 2

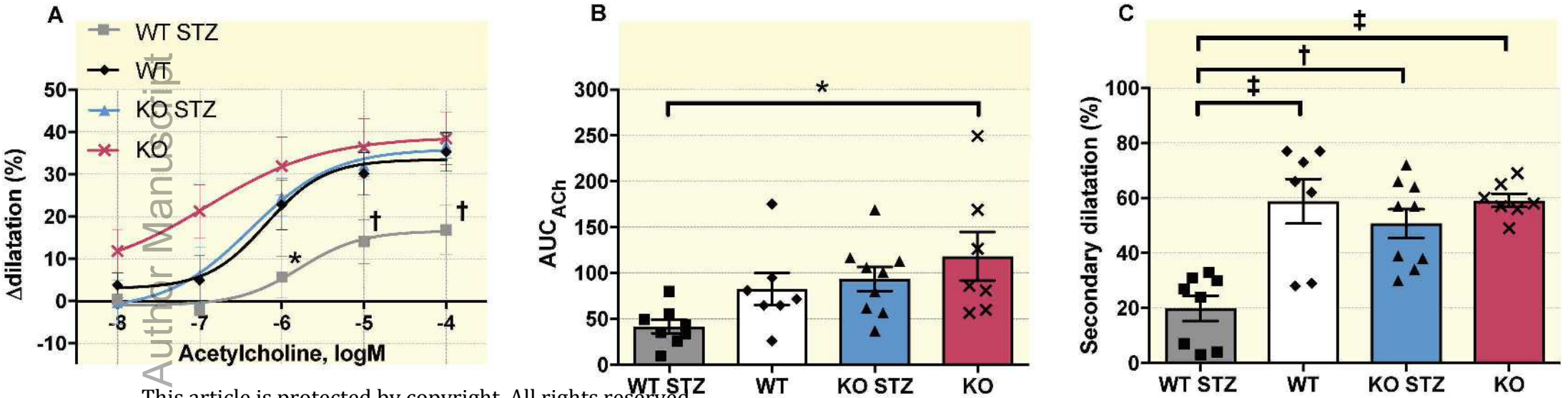

Figure 3

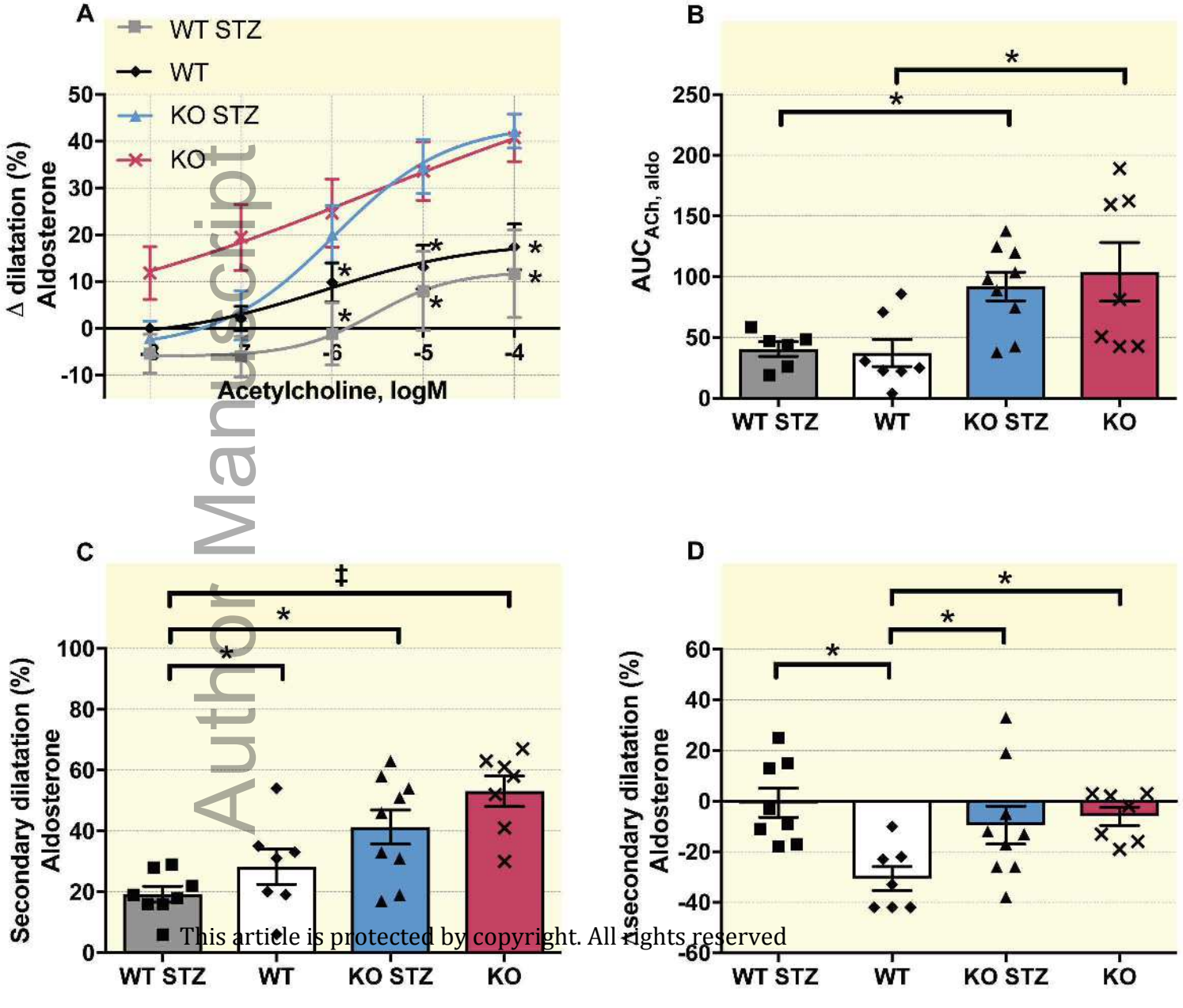

Figure 4