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Policlinico – Via Giustiniani, 2 – 35128 Padova

ADMIRE COST ANNUAL MEETING 2014

Padua, Italy

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Abstract Book

Welcome to Padua for the first meeting of the ADMIRE COST Action. ADMIRE is an international network of expert clinicians and scientists from over 20 countries researching the role of aldosterone and mineralocorticoid receptors in the physiology and pathophysiology of cardiovascular, renal and metabolic disease diagnosis and treatment. ADMIRE fill an unmet need in the doctoral and postodctoral training in the field and.our main focus is on 1) Developing novel diagnostic tools and targeted personalised therapies 2) Networking and training of young researchers to ensure the sustainability of quality basic and clinical research in the field.

We hope that you will make new contacts, expand existing collaborations and meet old friends in Padua. The meeting will be a platform for innovation and discovery of the role of ALDO/MR in disease via a multidisciplinary approach of molecular and structural biology, proteomics, genomics, animal studies and clinical trials.

We would like to thank our invited speakers Professor Paolo Bernardi, University of Padua, Italy and Professor Bernard Rossier, Université de Lausanne, Switzerland and all of those who have submitted abstrtacts for poster and oral presentaions. We would also like to thank our chairs, reviewers, judges and local organising committee. A special thanks to the Asclepio Ensemble who entertained delegates with a classical recital to finish the conference.

We hope to see you in Zermatt, October 7th-11th 2015 for the second ADMIRE meeting in collaboration with the ENaC meeting!

***Conference Convener**:*
Gian Paolo Rossi, University of Padua

Conference Chairs:
Gian Paolo Rossi, University of Padua;
Frederic Jaisser, ADMIRE Management Commitee Chair, Cordeliers Research Centre, Paris Descartes
Brian Harvey, ADMIRE Management Commitee Vice-Chair, Royal College of Surgeons in Ireland.

Local Organising Committee
Decio Armanini
Franco Fallo
Franco Mantero
Gian Paolo Rossi

ORAL PRESENTATIONS (in alphabetical order)

Characterization of three potential GR dimerization and/or DNA binding deficient mutant rats

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Institute of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland

We are interesting in the implication of GR (Nr3c1) in edematous disorders, like liver cirrhosis, nephrotic syndrome or cardiac failure. The rat might present a more appropriate physiological model compared to human than the mouse. Our project aims to understand the role of glucocorticoid-dependent signalling in the control of sodium and water balance. Hereby, we generated potential GR dimerization and/or DNA binding mutants using Transcription Activator-Like Effector Nucleases (TALENs) technology (Ponce de León V., et.al. PLoS One. 2014). Overall, we observed targeted genetic modification in 17% of the offspring, indicating high TALEN efficiency in rat zygotes. Three founders with deletions of 7-309 bp were selected and further analyzed, since these may present potential in-frame mutations of the glucocorticoid receptor dimerization and/or DNA-binding domain. All three founders transmitted their deletion to their offsprings and heterozygous mutants (GRΔ) rats are viable and fertile and rats with an in-frame 18bp deletion and two out of frame deletions of 7bp and 309bp were detected. The transcript expression and protein of GR was analyzed by qRT-PCR and Western blot. Homozygous rats with an 7bp or 309bp deletion die soon while homozygous rats for an 18bp deletion were never identified after birth. Surprisingly, heterozygous rats, with a 18bp deletion, exhibit higher GR protein expression levels in kidney compared to wild-type rats. Further analysis using isolated fibroblasts analyses and will reveal further whether the mutant receptor lack dimerization and/or DNA binding domain and whether the heterozygous (18bpΔ) exhibit a Cushing syndrome.

This work was supported by the Marie Curie co-funding International Fellowship Programme on Integrative Kidney Physiology and Pathophysiology (IKPP) and the Swiss Science Foundation in the National Center of Competence in Research (NCCR: Kidney.CH: Control of Homeostasis) programme to EH.

MR blockade protects against diet induced obesity, adipocyte dysfuntion and cardiac inflammation in mice, through browning of the adipose organ and modulation of autophagy

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ABSTRACT

Obesity is a key factor in the development of insulin resistance (IR), cardiovascular disease, hypertension, type 2 diabetes etc. Given the near epidemic incidence of obesity in western society there is a clear need for effective treatment options. Mineralocorticoid receptor (MR) blockade has shown significant promise in transgenic mouse models of obesity in limiting IR and adipocyte dysfunction, a disease that is independent of classical MR actions (renal). Female 10-week-old C57bl6 mice were fed with normal chow or a high fat

(HF) diet for 12 weeks. Mice fed HF diet were concomitantly treated for 12 weeks with drospirenone (DRSP, 6 mg/Kg/day), a potent MR antagonist with antiadipogenic activity, or spironolactone (SPIRO, 20 mg/kg/day). Mice fed HF diet showed a significant increase in total body weight, fat mass, mean adipocyte size, expression of white adipose tissue (WAT) marker genes and showed impaired glucose tolerance after intraperitoneal plasma glucose tolerance test. DRSP and SPIRO prevented weight gain and white fat mass expansion induced by HF diet in parametrial, perivescical, and inguinal depots without affecting interscapular fat pad weight. Magnetic Resonance Imaging (MRI) confirmed that MR antagonists blocked the HF diet-driven expansion of abdomino-pelvic (parametrial and perivescical) fat volume. High levels of MR mRNA were detected in all depots of adipose tissue. HF fed mice showed no increase in heart or kidney weight and tissue fibrosis. Cardiac macrophage recruitment and osteopontin staining was increased in hearts of HF fed mice and reversed by both MR antagonists. Moreover, both DRSP and SPIRO prevented the impaired glucose tolerance in mice fed HF diet, and countered HF diet-induced up-regulation of WAT markers transcripts and adipocyte hypertrophy. Importantly, MR antagonists increased uncoupling protein 1 (UCP-1) positive brown-like adipocyte content in WAT, and improved metabolic activity of adipose tissue, as indicated by PET/CT imaging. In keeping with this, MR antagonism significantly increased expression of brown-like adipocyte marker genes such PRDM16, CIDEA, beta-3 adrenergic receptor (ADRB3) and UCP-1 in all WAT depots analysed. In exploring the mechanism, we demonstrated that MR antagonism induced brown adipose tissue (BAT) markers, and reduced the autophagic rate, a key remodelling process in adipocyte differentiation, in WAT depots *in vivo* as well as in primary cultured adipocytes. We conclude that adipocyte MR regulates BAT-like remodeling of WAT through modulation of autophagy. MR blockade therefore has promise as a novel therapeutic option for the prevention of metabolic dysfunctions and the cardiac consequences of obesity.

Role of GR and MR in the glucocorticoid effects in epidermal keratinocytes: *In Vivo* and *In Vitro* approaches

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Synthetic glucocorticoids (GCs) are effectively and largely used in therapy for skin inflammatory diseases, however, chronic treatments or high doses produce common undesirable side effects, such as skin atrophy. The biological and therapeutical effects of endogenous and synthetic GCs are mediated by binding to the glucocorticoid receptor (GR), a ligand-dependent transcription factor of the nuclear hormone receptor superfamily. The mineralocorticoid receptor (MR) also belongs to this superfamily and can bind both mineralocorticoids and GCs with similar affinities. GR and MR regulate gene expression through binding to identical DNA sequences located at regulatory regions of their target genes, making difficult to discriminate the specific actions of each receptor.

We hypothesize that both GR and MR may contribute to the unwanted side-effects elicited by topical GC treatment on skin, including epidermal thinning, which constitute a major drawback for the prolonged used of GCs in the clinic. To clarify which receptor is mediating GC effects, and to unequivocally elucidate the role of each of GR and MR on keratinocyte function, we performed experiments using GR- and MR-keratinocyte-specific knock-out mice and cell lines (GR epidermal KO or GR^{EKO}, and MR epidermal KO or MIR^{EKO}, respectively; Sevilla et al., 2013; our unpublished results).

Either constitutive GR or MR keratinocyte-specific inactivation caused epidermal hyperplasia in adult mice to a similar extent, as assessed *in vivo* by hematoxylin/eosin staining and BrdU incorporation. However,

topical application of dexamethasone (Dex, 8 g/mouse, 48h) inhibited epidermal proliferation in control and MIR^{EKO} but not in GR^{EKO} mice, indicating that short-term Dex effects are GR- nut not MR-mediated *in vivo*. The observed anti-proliferative effects were consistent with the induction of *Tsc22d3*/*Gilz* by GR-Dex in control and MIR^{EKO} but not in GR^{EKO} keratinocyte cell lines. These findings were also in agreement with the Dex-induced recruitment of GR but not MR to *Gilz* regulatory sequences in keratinocytes, as assessed by ChIP-QPCR assays.

Collectively, our data indicate that GR is essential for Dex-induced *Gilz* transcription and keratinocyte growth inhibition. However, MR also has a partial contribution to *Gilz* expression and Dex antiproliferative effects in mouse keratinocytes, likely mediated through GR/MR heterodimers.

Spironolactone effects on human aortic endothelial ion channel expression profile in chronic kidney disease.

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Patients with chronic kidney disease (CKD) have a markedly increased incidence of cardiovascular events and cardiovascular disease (CVD) mortality compared with the age-matched general population. The high concentration of circulating uremic toxins in CDK patients may trigger vascular inflammatory responses, thereby inducing endothelial dysfunction, which is associated with CVD development and progression. In addition, plasma aldosterone levels are increased in CKD, and aldosterone has been found to increase vascular inflammation and fibrosis. The aim of our study was to analyze the influence of CKD in the expression of endothelial ion channels. Furthermore, we explored its potential modification by mineralocorticoid receptor (MR) antagonism, which would consequently affect endothelial dysfunction. To that end we used human aortic endothelial cells (HAEC) cultured in medium supplemented with pooled sera from either healthy or uremic patients undergoing dialysis, as well as both groups in the presence of spironolactone. HAEC were found to express MR under the culture conditions used. To obtain a global portrait of ion channel expression in HAEC in the four groups tested, we performed high-throughput real-time polymerase chain reaction of 92 ion channel genes using a custom-designed Taqman low-density array card. We have obtained a profile of ion channel gene expression altered by uremic serum and the effect of spironolactone both on basal and altered expression of ion channel subunits.

ALTERED VASCULAR REACTIVITY IN MICE OVEREXPRESSING ADIPOCYTE MINERALOCORTICOID RECEPTORS - ROLE OF OXIDATIVE STRESS AND RHO KINASE.

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Aldosterone (aldo) plays a role in obesity and cardiovascular diseases, such as hypertension. We previously demonstrated that adipocyte-derived factors regulate vascular function and vascular smooth muscle cells signaling. Moreover, adipocytes express aldosterone synthase (CYP11B2) and produce aldo. The mineralocorticoid receptor (MR), which is responsible for aldo signaling, is also found in these cells, but its role in regulating adipose tissue interactions with the vasculature is unknown. In this study, we investigated mechanisms whether MR activation in adipocytes regulates vascular reactivity. Conditional transgenic mice that overexpress MR in an adipocyte-specific manner were studied. Vascular reactivity of resistance mesenteric arteries to acetylcholine (Ach), sodium nitroprusside and phenylephrine (Phe), in the absence or presence of fat conditioned medium (Fcm) from control and adipocyte overexpressing MR (MROE) mice, was performed by myography. In basal conditions, endothelial dysfunction was not observed in MROE or control (Ctr) mice. However, exposure of arteries from control mice to Fcm from MROE mice induces endothelial dysfunction (Ach 10^{-6}M: 77.5±9.6% no Fcm vs. 49.8±7.5% Fcm, $p<0.05$), an effect blocked by N-acetyl-cysteine (an antioxidant) (Ach 10^{-6}M: 82.2±6.6%). Resistance arteries from MROE mice had decreased Phe-induced contraction, compared to control mice (Phe 10^{-5}M: 2.7±0.2 mN/mm Ctr vs. 1.7±0.2 mN/mm MROE, $p<0.05$). Rho Kinase activity, which regulates vascular contraction, is decreased in arteries and adipo tissue from MROE (mesenteric arteries, Ctr: 100±16.2% vs. MROE: 31.1±6.1%, arbitrary units, $p<0.01$; adipose tissue, Ctr: 100±12.6% vs. MROE: 51.3±9.3%, arbitrary units, $p<0.01$). In conclusion, MR in adipocytes may play an important role in the regulation of vascular function, through redox-sensitive pathways and activation of Rho kinase. Our study identifies novel mechanisms linking vascular/adipose tissue biology and aldo/MR activation, which may be particularly important in vascular dysfunction associated with hypertension and hyperaldosteronism.

GENETIC SPECTRUM AND CLINICAL CORRELATES OF SOMATIC MUTATIONS IN ALDOSTERONE-PRODUCING ADENOMA

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Primary aldosteronism is the most common form of secondary hypertension. Somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D* have been described in aldosterone-producing adenomas (APA). Our aim was to investigate the prevalence of somatic mutations in these genes in unselected patients with APA (n=474), collected through the European Network for the Study of Adrenal Tumors (ENS@T). Correlations

with clinical and biochemical parameters were first analyzed in a subset of 199 patients from a single center and then replicated in two additional centers. Somatic heterozygous *KCNJ5* mutations were present in 38% (180 of 474) of APA whereas *ATP1A1* mutations were found in 5.3% (25 of 474) and *ATP2B3* mutations in 1.7% (8 of 474) of APA. Previously reported somatic *CACNA1D* mutations as well as 10 novel *CACNA1D* mutations were identified in 44 out of 474 (9.3%) APA. There was no difference in the cellular composition of APA nor in *CYP11B2*, *CYP11B1*, *KCNJ5*, *CACNA1D* or *ATP1A1* gene expression in APA across genotypes. Patients with *KCNJ5* mutations were more frequently female, diagnosed younger and with higher minimal plasma potassium concentrations compared to *CACNA1D* mutation carriers or non-carriers. *CACNA1D* mutations were associated with smaller adenomas. These associations were largely dependent on the population structure of the different centers. In conclusion, recurrent somatic mutations were identified in 54% of APA. Young women with APA are more likely to be *KCNJ5* mutation carriers; identification of specific characteristics or surrogate biomarkers of mutation status may lead to targeted treatment options.

Hypotonicity triggers rapid HuR nuclear export, increases Mineralocorticoid Receptor mRNA stability and potentiates renal Aldosterone responsiveness.

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The Mineralocorticoid Receptor (MR, NR3C2) mediates sodium-retaining action of aldosterone. MR is highly expressed in the distal nephron where the renal corticopapillary gradient generates strong variations in extracellular fluid tonicity. This osmotic gradient, pivotal for the regulation of ion and water transport, is clearly modulated during renal development and seems to be affected in various kidney diseases. However, mechanisms regulating MR expression remain sparse.
Using the differentiated KC3AC1 cortical collecting duct cell line, we have demonstrated that renal MR abundance is tightly regulated by variations of the extracellular tonicity since hypertonicity (500 mOsm/L) leads to a severe reduction in MR expression while hypotonicity (150 mOsm/L) drastically increases MR expression by 3-4 fold (1). Recently, we elucidated the posttranscriptional mechanisms governing MR expression under hypertonicity. We found that hypertonicity increases expression of the RNA-Binding Protein Tis11b, a mRNA-destabilizing protein belonging to the tristetraprolin/ZFP36 family. Importantly, endogenous Tis11b physically interacts with the AU-rich motifs of the 3'-untranslated region (3'-UTR) of MR transcript in KC3AC1 cells, leading to hypertonicity-elicited MR repression as demonstrated by Tis11b knockdown and *in vivo* experiments. Moreover, hypertonicity impaired MR signaling by blunting aldosterone-stimulated target gene expression (Gilz and ENaC) in the Na^+-transporting KC3AC1 cells (2).

As a mirror image, we now demonstrate that renal MR abundance is significantly increased under hypotonicity by transcription-independent mechanisms. This led us to examine whether HuR, another RNA-Binding Protein is involved in the stabilization of MR mRNA. HuR is known to stabilize target transcripts by binding to their 3'-UTR on AU, UU or CU rich motifs. Interestingly, we demonstrate that hypotonicity

induces a very rapid (5 min) and fully reversible nuclear export of HuR in the renal KC3AC1 cells, as monitored and quantified by high throughput microscopy. This is consistent with previous reports showing that HuR activity mostly relies on its subcellular localization. RNA interference strategy enabled us to unambiguously demonstrate that HuR protein drastically increases MR expression under hypotonicity. Finally, this posttranscriptional control of MR expression has major functional consequences on mineralocorticoid signaling pathway, since hypotonic conditions not only increase MR expression but also potentiate aldosterone responsiveness as revealed by the enhancement of some MR target gene expression.

Collectively, our results constitute the starting point for a better understanding of molecular mechanisms controlling MR expression and action under variations of extracellular tonicity. Such new regulatory processes might be involved during the developmental variations of renal MR expression. Our findings have also important pathophysiological relevance in a context of kidney diseases, hypertension or mineralocorticoid resistance.

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Hypertonicity compromises renal mineralocorticoid receptor signaling through Tis11b-mediated posttranscriptional control. **J Am Soc Nephrol, 2014, April; (25): 1-9**

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INTERLEUKIN-33/ST2 SYSTEM ATTENUATES ALDOSTERONE-INDUCED ADIPOGENESIS AND INFLAMMATION.

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Obesity is a major risk factor for developing heart failure (HF). Interleukin-33 (IL-33) and the transmembrane isoform (ST2L) exert anti-inflammatory and protective effects in several tissues, whereas circulating soluble ST2 (sST2) has emerged as a prognostic biomarker in patients with myocardial infarction and HF. Aldosterone is an adipocyte-derived factor that regulates cardiovascular function. Both sST2 and Aldosterone are increased in early stages of HF, such as obesity. Moreover, a correlation between sST2 and plasma Aldosterone was described in post-myocardial infarction patients. However, the potential interactions between these systems in adipose tissue have not been investigated.

Rats fed a high fat diet for 6 weeks presented increased sST2 expression, diminished IL-33/ST2L activity and enhanced levels of differentiation and inflammation in adipose tissue as compared to controls. A similar pattern was observed in adipose tissue from C57BL/6 Aldosterone-treated mice. In both models, adipose sST2 positively correlated with Aldosterone plasma levels. Treatment of 3T3-L1 cells with IL-33 or ST2L delayed adipocyte differentiation, diminished lipid accumulation and decreased inflammation. Aldosterone

decreased IL-33 and increased sST2 expressions in differentiated adipocytes via its mineralocorticoid receptor. Aldosterone-induced adipocyte differentiation and inflammation were blocked by IL-33 or ST2L treatment, whereas co-incubation with sST2 did not exert any effects.

The crosstalk between IL-33/ST2 and Aldosterone could be relevant in the cardiovascular consequences of obesity. Therefore, IL-33 and ST2L could emerge as potential therapeutic targets in the regulation of the adipose tissue in response to Aldosterone.

Aldosterone-induced activation of protein kinase D2 in the distal nephron and its impact on renal health and disease

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Hypertension is a key risk factor for both renal and cardiovascular disease and is also indicative of impaired electrolyte homeostasis in the body. Renal Na^+ homeostasis can be affected in several ways for example, by increasing aldosterone synthesis and activation of the mineralocorticoid receptor (MR). Hypertension can also occur due to a dysregulation of aldosterone signalling which can often result in chronic pathologies of the kidney such as nephropathy and renal fibrosis. Aldosterone signalling is transduced via hormone binding to MR in segments of the distal nephron including the connecting tubule and the cortical collecting duct (CCD). Aldosterone is one of the key regulators of sodium conservation through its actions on the epithelial sodium channel (ENaC). Protein kinases such as protein kinase D2 (PKD2) can modify ENaC activity by effecting sub-cellular trafficking of the channel subunits. Here we investigated PKD2 activation by aldosterone and its effects on ENaC in the distal nephron. Aldosterone can modulate ENaC activity at the levels of transcription, protein stability and subcellular trafficking (1). We investigated the activation of PKD2 by aldosterone and its effects on ENaC in the rat distal nephron *in vivo* and in a murine cortical collecting duct (M1-CCD) cell line. ENaC current was measured as the amiloride-inhibitable transepithelial short-circuit current (I_{ENaC}). Intracellular accumulation and rapid activation of PKD2 (<10min) was induced by aldosterone (10nM) treatment. Expression of PKD2 under basal conditions was found to localise at the apical membrane and in the sub-apical cytosolic space. Longer treatment with aldosterone (30min) induced sub-cellular redistribution of PKD2 into the trans-Golgi network. PKD2 knock-down using shRNA in M1 cells resulted in an elevated basal I_{SC} from 1.9 ± 0.2μA/cm^2 in wild-type cells to 9.3 ± 1.4μA/cm^2 in PKD2 knock-down M1 cells (n=10, p=0.002). PKD2 knock-down also resulted in an increased amiloride-sensitive I_{ENaC} from 1.3 ± 0.3μA/cm^2 in wild-type cells to 6.0 ± 1.0μA/cm^2 in PKD2 knock-down M1 cells (n=11, p=0.0001). Long term treatment (24h) of wild-type M1 cells with aldosterone increased the basal I_{SC} from 1.9 ± 0.2μA/cm^2 to 4.6 ± 0.7 μA/cm^2 (n=7, p=0.008). The ENaC current showed an increase from 1.3 ± 0.3 μA/cm^2 in wild-type M1 cells to 3.3 ± 0.5 μA/cm^2 when treated for 24h with aldosterone (n=8, p=0.001). Student's t-tests were performed alongside a one way ANOVA. The effect of aldosterone on both the basal I_{SC} and I_{ENaC} is abolished with the knock-down of PKD2. The abundance of ENaCγ in the apical membrane showed an increase following knock-down of PKD2 in M1 cells. Studies in rats fed on a low Na^+ diet showed increased expression of ENaCγ at the apical membrane accompanied by a subcellular redistribution of PKD2 from the apical membrane to the trans-Golgi network. The use of animals was subject to local ethical approval in compliance with national legislation. This work was supported by COST ACTION ADMIRE BM1301, Doctrid and EMIDS.

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Deficiency of the T-type calcium channel Ca_v 3.1 attenuates plasma aldosterone level in angiotensin II-hypertensive mice.

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Background: Angiotensin and aldosterone are mediators in the development of hypertension. Voltage-gated T-type calcium channels are suggested to affect adrenal aldosterone secretion. Clinical studies show that T-type calcium channel blockers lower aldosterone levels. Combined T- and L-type channel blockers lowers blood pressure to a greater extent that L-type blockers in hypertension. It was hypothesized that angiotensin II infusion leeds to an attenuated blood pressure increase and aldosterone response in mice deficient of T-type Ca_v 3.1 channels.

Methods: Mean arterial pressure was measured continuosly in conscious, freely moving Wt and Ca_v 3.1 KO mice with chronically indwelling catheters in response to a 1-week infusion of angiotensin II (30 ng/kg/min) or vehicle. Plasma concentrations of renin and aldosterone were measured with radioimmunoassays.

Results: Angiotensin II significantly increased mean arterial pressure from 101±2 mmHg to 119±4 mmHg in Ca_v 3.1 KO and 106±3 mmHg to 118.5±4 mmHg in Wt mice, with no differences between genotypes. The increase in mean arterial pressure after angiotensin II infusion was significantly attenuated in female compared with male mice. Heart rate in the Ca_v 3.1 KO mice infused with angiotensin II was significantly lower compared to its respective control. Aldosterone plasma level was significantly lower in the Ca_v 3.1 KO mice (106.9±31.1pg/ml) compared to the Wt mice (269.2±53.1pg/ml) after angiotensin II infusion. In the vehicle group; a tendency towards a lower aldosterone level was also observed in the Ca_v 3.1 KO mice (65.2±19 pg/ml) compared to the Wt mice (190.7±79 pg/ml). Plasma renin concentrationen was significantly lower after angiotensin II infusion in both groups (Ca_v 3.1 240±60.6 10^{-5} GU/mL to 47.5±30.4 10^{-5} GU/mL and Wt 204±49.1 10^{-5} GU/mL to 72±38.2 10^{-5} GU/mL) with no significantly difference between the groups. In summary, global deletion of T-type calcium channel Ca_v 3.1 subtype attenuates aldosterone plasma increase and heart rate with no effect on mean arteriel pressure in angiotensin II-induced hypertension.

Conclusion: Aldosterone is of minor importance for Ang II-induced hypertension and adrenal Ca_v 3.1 channels contribute to stimulus-secretion coupling for aldosterone. T-type calcium channel

Comprehensive assessment of cardiac systolic, diastolic performance assessed by cardiac MRI in response to dobutamine challenge, and cardiac strains and torsion by MR Tagging in vivo in mice with atherosclerosis

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Background: ApoE/LDLR-/- mice represent a reliable model of atherosclerosis, but it is not known whether atherosclerosis progression in apoE/LDLR-/- mice induce symptomatic ischemic heart-like disease. The aim of this study was to characterize alterations in global and regional cardiac performance including cardiac reserve in apoE/LDLR-/- mice with advanced atherosclerosis as well as changes in coronary endothelial function

Methods: In vivo LV performance was assessed by cardiac MRI, cardiac strains and torsion was assessed by MR Tagging, whereas LV reserve was assessed by MRI after dobutamine challenge (0.5 and 2 mg/kg, i.p.). Presence of coronary atherosclerosis was confirmed using OMSB and ORO staining. Endothelium-dependent responses were analyzed in isolated murine hearts.

Results: ApoE/LDLR-/- hearts displayed advanced coronary atherosclerosis. In isolated hearts of ApoE/LDLR-/- mice endothelial NO-dependent vasodilation induced by Bk was preserved, while coronary flow increase in response to exogenous NO donors (SNP, SNAP) was significantly potentiated. At the same time, PGI2 release induced by Ach was increased, while basal COX-2 derived PGI2 production was upregulated. In apoE/LDLR-/- mice basal and regional in vivo cardiac performance in regard to circumferential and radial strains as well as cardiac reserve assessed by dobutamine challenge were well preserved. However, atherosclerotic mice exhibited more frequent appearance of systolic stretch and post systolic index and decreased rotation peak at the LV base as well as lower untwisting rate and un-rotating rate.

Conclusions: Despite severe coronary atherosclerosis in 6-month-old apoE/LDLR-/- mice, systolic and diastolic cardiac function, as well as chronotropic and inotropic reserve were all well preserved and only subtle diastolic alterations in cardiac performance of ischemic background were uncovered with cardiac MR tagging. The mild phenotype of ischemic heart disease in apoE/LDLR-/- mice is most likely due to robust compensatory mechanisms of coronary circulation including increased vascular responsiveness to NO and increased generation of PGI2.

A novel mixed glucocorticoid/mineralocorticoid receptor selective modulator reduces obesity and adipose tissue inflammation

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Background: Excessive glucocorticoid exposure is associated with obesity and related disorders, as is evidenced by the extreme case of Cushing's disease. Accordingly, antagonism of the glucocorticoid receptor (GR) by means of mifepristone (RU486) markedly lowers obesity in men and mice, yet mineralocorticoid receptor (MR) mediated effects may also play a role. In this study we tested the efficacy of CORT 118335 (C118335), a selective modulator type drug (exerting both agonism and antagonism) at GR, with antagonistic effects via MR.

Methods and results: 10-week old C57BL/6J mice were fed a high-fat diet for 3 weeks and treated with C118335, RU486 or vehicle. Both RU486 and C118335 reduced body weight gain and fat mass. C118335, but not RU486, reduced white adipose tissue (-41%, P<0,05) weight and macrophage infiltration. Both RU486 and C118335 improved glucose tolerance, tested via ivGTT, and lowered basal plasma glucose levels. Thymus weight was reduced by RU486 (-48%) and C118335 (-46%), whereas spleen weight was only reduced by C118335 (-44%), indicating tissue specific GR antagonism or agonism.

Conclusion: Both C118335 and RU486 reduce diet-induced obesity development and improve glucose metabolism. C118335 reduces white adipose tissue inflammation, which may be due to GR agonism and/or MR antagonism. Selective modulation of the GR combined with MR may be a promising target for combating obesity and related disorders.

Aldosterone antagonism prevents salt-induced monocyte adhesion during vascular inflammation

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Inflammatory processes are hallmarked by binding of monocytes to adhesion molecules at the surface of endothelial cells and subsequent transmigration into the subendothelial matrix. There is evidence that synergistic effects of salt (NaCl) and aldosterone support vascular inflammation which is characterized by the recruitment of monocytes and associated with endothelial dysfunction hence contributing to the development of atherosclerosis. An important target of inflammatory processes is the endothelial glycocalyx (eGC), a negatively charged mesh of proteoglycans, covering the surface of endothelial cells. Recently, it was shown that the eGC functions as a Na^+ buffer barrier with protective effects on the endothelium. Since high Na^+/aldosterone leads to a collapse of the eGC, we tested the hypothesis that under such conditions the binding of monocytes to the endothelial surface is altered. Therefore, human endothelial cells (EA.hy 926) were grown for 24 hours in low (130 mM) and high (150 mM) Na^+ concentrations in the presence of aldosterone (1 nM), TNFα (0.57 nM) and with/without the aldosterone antagonist spironolactone (100 nM). To test the adhesion of monocytes to the endothelial surface, fluorescently labeled monocytes (anti-CD14) were seeded onto confluent endothelial monolayers for 5h and were quantified afterwards. To test the barrier function of the eGC, the adhesion forces between immobilized monocytes and the eGC were quantified with a specialized Atomic Force Microscope (CellHesion). Changes in the conformation of the eGC, in response to low and high Na^+ in the presence of aldosterone, were detected by using quantum dot (QD)-mediated immunofluorescence staining of heparan sulfates (HS) on the endothelial cell surface.

We found that synergistic effects of high Na^+/aldosterone significantly increased the number of adherent monocytes on the endothelial cell surface by 49% while the adhesion forces between monocyte and eGC were significantly decreased by 26%. In addition, high Na^+/aldosterone diminished the number of detectable HS, the major component of the eGC, by 34%, indicating either shedding or conformational changes of the eGC. All effects could be prevented by treatment with the aldosterone receptor antagonist spironolactone.
The data provide evidence for a two-step mechanism of monocyte adhesion. In a first step, monocytes bind to the eGC and in a second step to receptors at the surface of the endothelial cell. Treatment with high Na^+/aldosterone collapses the eGC leading to an impeded binding of the monocytes to the eGC. The collapsed eGC in turn facilitates the access of the monocytes to the plasma membrane and binding to adhesion molecules (VCAM). We conclude that the eGC is involved in the adhesion process of monocytes and serves as an important protective barrier. High Na^+/aldosterone enhance the process of vascular inflammation. Since aldosterone receptor antagonism has protective effects on the eGC and attenuates the adhesion of monocytes this could be a useful strategy in the prevention of Na^+/aldosterone-mediated inflammatory processes of the vasculature.

POSTER PRESENTATIONS (in alphabetical order)

The Mineralocorticoid Receptor Expressed in Vascular Smooth Muscle is Mandatory in the Acute Nephrotoxicity Induced by Cyclosporine A

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Introduction: Cyclosporine A-induced Nephrotoxicity (CIN) is a major adverse event during the pharmacological therapy after kidney allographs transplantation. Although its pathophysiology remains unclear, it has been demonstrated that the pharmacological antagonism of Mineralocorticoid Receptor (MR) prevents the CIN in rats by modulating the expression of vasoactive factors. Our previous studies have shown that MR is expressed in endothelial and vascular smooth muscle cells (SMC), and modulates vasoconstrictor tone. Our hypothesis is that the activation of vascular MR is involved in acute CIN.

Methods: Wild type (WT) female mice (8-10 weeks old) and mice with MR specifically deleted from vascular SMC (SMC-MR-KO) or Endothelial cells (Endo-MR-KO) were treated with vehicle (VH) or Cyclosporine-A (CsA, 100mg/kg/d), for 2 days.

Results: CsA induced body weight loss in WT, SMC-MR-KO and Endo-MR-KO mice ($p<0.05$ *vs.* VH, ANOVA). CsA decreased renal function and induced tubular vacuolization in kidney of WT mice (Table). SMC-MR-KO, but not Endo-MR-KO mice, prevented impairment of renal function and injury (Table). Additionally, SMC-MR-KO prevented the increase protein expression of NGAL (Neutrophil Gelatinase-

Associated Lipocalin), a kidney damage marker, in proximal tubules. CsA induced an augmented phosphorylation for Myosin Light Chain-2 (pThr^{18} and pSer^{19}, ≥1.5 fold, p<0.05) and for Myosin Light Chain Kinase (pSer^{1760}, ≥1.5 fold, p<0.05) in aortas of WT and Endo-MR-KO mice; this effect is fully prevented in SMC-MR KO mice. Finally, the specific inactivation of MR in vascular SMC prevented the increment in renal vascular resistance in response to 2ng of Angiotensin II after treatment with CsA (p<0.05).

	Wild Type		**SMC-MR-KO**	
	Vehicle (n=12)	**CsA** (n=16)	**Vehicle** (n=10)	**CsA** (n=12)
% of Body Weight at Day 2	104,3 ± 2,3	95,1 ± 1,6***	104,4 ± 2,7	97,7 ± 1,3***
Plasma Urea (mmol/L)	8,1 ± 0,9	21,2 ± 5,1*	6,4 ± 0,5	11,5 ± 1,4
Plasmatic Creatinine (µmol/L)	10,4 ± 0,6	33,7 ± 9,5*	9,3 ± 0,6	12,7 ± 0,6
Tubular Vacuolization Score (arbitrary units)	0,7 ± 0,2	2,1 ± 0,4*	0,8 ± 0,3	1,1 ± 0,2

Data are expressed as average values±SEM *P<0.05 versus value of control group; ***P<0.01 versus value of control group.

Conclusions: We show that MR in vascular SMC, but not in endothelial cells, has a crucial role in the pathogenesis of acute CIN. This study provides new insights into the role of vascular MR in renal hemodynamics. The therapeutic implications of these findings are currently being tested in an ongoing clinical trial testing the safety of MR antagonism with Eplerenone in renal transplant recipients treated with Cyclosporine.

Mineralocorticoid receptor antagonism with BR-4628 or deficiency in smooth muscle cells protects against renal ischemia/reperfusion

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Introduction: The main cause of acute kidney injury (AKI) is renal ischemia/reperfusion (IR). AKI has been linked with chronic kidney disease progression and cardiac alterations. The development of an effective therapy to treat patients undergoing an AKI episode is important. Previous studies from our laboratory have shown that mineralocorticoid receptor (MR) antagonism with spironolactone prevents tubular injury and renal dysfunction induced by IR. Despite their benefits in renal diseases, the current MR antagonists have strong limitations like hyperkalemia, thus motivating the search of novel antagonists with safer profile such as the non-steroidal MR antagonists. Moreover whether the blockade of vascular MR is responsible for the protection conferred by MR antagonists in IR remains unexplored.

Objective: To test the efficacy of the non-steroidal MR antagonist BR-4628 against renal IR and evaluate the specific contribution of vascular MR in ischemic kidney injury.

Methods: 20 Wistar rats were divided in 4 groups: sham-operated (S), bilateral renal ischemia (IR) for 25 min and IR plus treatment with BR-4628 (10 mg/kg) either 3 days before IR (BR10-pre) or 3 hours after IR (BR10-post). All rats were studied 24 h after reperfusion. To evaluate the contribution of vascular MR we generated two knockout (KO) mouse models. To allow MR inactivation in endothelial cells (MR^{endoKO} mice), floxed MR mice (MR$^{fl/fl}$) were crossed with mice expressing the Cre recombinase under the Tie2cre promoter. To allow MR inactivation in vascular smooth muscle cells (MR^{SMCKO} mice), MR$^{fl/fl}$ mice were

crossed with mice expressing the inducible Cre recombinase under the SMA promoter (MR^{SMCKO}). In these mice, sham surgery or bilateral renal IR for 20 min was performed in control and KO mice and the animals were studied 24 h after reperfusion.

Results: Rats with IR developed renal dysfunction as evidenced by increased plasma creatinine (48.3±6.7 µmol/L) as compared to sham (17.4±3.1 µmol/L). In contrast, in the rats treated with the BR-4628 either before or 3h after IR, the increase in plasma creatinine was prevented (BR10-pre: 23.5±1.4 and BR10-post: 27.8±1.8 µmol/L). The percentage of tubules with histological alterations was 32.1±5.6% in the IR rats whereas in the BR10-pre was only 8.2±3.2% and 10.3±2.1% in the BR10-post rats. Renal injury was also evidenced by an increase in Hsp72, NGAL and Kim-1 and oxidized proteins. Moreover, renal IR was associated with a sulfenic acid modification in ET_B receptor. These alterations were absent in the BR10-pre and BR10-post IR groups. The benefit of BR-4628 in renal IR was prevented by selective ET_B receptor antagonism.

In WT mice, IR also induced renal dysfunction (plasma creatinine raised from 8.9±0.3 in sham to 33.8±4.8 mol/L in IR) and tubular injury. The MR^{endoKO} mice displayed similar alterations induced by IR as WT mice. In contrast, after renal IR, the MR^{SMCKO} mice presented normal renal function (plasma creatinine was 9.6±0.7 and 14.0±1.9 µmol/L in sham and IR, respectively) and absence of histological alterations. We are now testing the effect of BR-4628 or MR deficiency in preventing the chronic alterations induced by IR.

Conclusion: We show for the first time that the non-steroidal BR-4628 MR antagonist is useful to prevent or treat renal injury induced by IR. Moreover we provide evidence that the MR expressed in the SMC is essential for the development of renal injury induced by IR. Our data supports the use of non-steroidal MR antagonists as a novel therapeutic approach to prevent acute and chronic consequences of renal IR.

Role of the Mineralocorticoid Receptor in Mouse Skin Development

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The Mineralocorticoid Receptor (MR) is a transcription factor that plays a key role in ion and water homeostasis. Structurally and functionally it has a high similarity with the glucocorticoid (GC) receptor (GR). Both are steroid ligand-dependent intracellular receptors that belong to the nuclear hormone receptor superfamily (1).

MR can bind the natural ligand aldosterone (Aldo) and GCs with similar affinities. Given that endogenous GC plasma levels exceed those of Aldo by 100-fold, one mechanism preventing continuous MR occupation by GCs is the enzyme 11 β-hydroxysteroid dehydrogenase type 2 (11βHSD2). 11βHSD2 converts active GCs into inactive metabolites, unable to bind MR, thus favoring the Aldo-MR signaling pathway. It is known that GCs exert a critical role in skin function through GR (2). However, no much is known regarding MR in skin biology.

We have analyzed MR expression during mouse skin development at the mRNA and protein level. Our data show a transient peak of MR expression around embryonic (E) day 16.5 (E16.5), which decreases thereafter. Since epidermal barrier formation (EBF) starts at E16.5, the observed peak suggests a role for MR in this process.

To evaluate the consequences of MR inactivation in developing skin, we generated MR knock-out mice using a strategy based upon generalized Cre-mediated recombination of $MR^{loxP/loxP}$ mice. As previously described, $MR^{-/-}$ mice died early after birth (3), therefore, we examined $MR^{+/+}$, $MR^{+/-}$ and $MR^{-/-}$ mouse skin at different embryonic and early postnatal stages. Histopathological analysis of skin samples revealed no major defects

of $MR^{-/-}$ mice but rather subtle differences in the expression of keratinocyte-specific markers. Overall, our data suggest that, despite MR inactivation did not alter GR expression, GR may functionally compensate for MR loss during mouse skin development.

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Role of the β-ENaC subunit (*Scnn1b*) in sodium and potassium homeostasis.

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Abstract

The epithelium sodium channel (ENaC) consists of three subunits (α, β and γENaC) that are expressed at the apical side of e.g. in tight epithelia in the kidney, and is thus limiting for sodium reabsorption. Mutations in all three ENaC subunits are causative for two human diseases, namely the Liddle's syndrome (Warnock DG et al., 1998), a form of severe hypertension and the pseudohypoaldosteronism type 1 (PHA-1) (Schild L et al., 1996), a salt-losing syndrome. The constitutive knockout of each of the three ENaC subunits in mice revealed the importance for survival, and these knockouts die soon after birth (Hummler, et al., 1996, Barker et al., 1998. McDonald et al., 1999). To study the importance of βENaC in the adult kidney, we used nephron-specific β-ENaC knockout mice that are doxycycline-inducible. Following doxycycline-induction of 28 days old mice, β-ENaC deficiency rapidly causes weight loss, hyperkalemia, hyponatremia, and higher plasma aldosterone levels; they develop a classical severe PHA-I phenotype. NCC is downregulated on the mRNA transcript and protein expression, as well as on the phosphorylation level (T53). We are next interested to rescue this phenotype, and following high Na^+/ low K^+ diet, high percentage of β-ENaC-deficient mice survive.

Our data clearly show an important role of β-ENaC subunit in sodium and potassium balance. This work was supported by Leducq Fondation and National Center of Competence in Research (NCCR) kidney.CH.

Role of Lipocalin 2 (LCN2) in Cardiovascular Remodeling Induced by Aldosterone

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Neutrophil Gelatinase Associated Lipocalin or Lipocalin 2 (LCN2) is a circulating protein, member of the lipocalin family, which binds MMP9 and modulates its stability and activity. We have recently shown that LCN2 is a primary target of aldosterone/mineralocorticoid receptor (MR) in endothelial cells, vascular smooth muscle cells and cardiomyocytes (Latouche et al. 2012). We hypothesized that LCN2 could be a mediator of aldosterone/MR profibrotic and proinflammatory effects in the cardiovascular system. Wild type (WT) and LCN2 Knock Out (KO) mice were subjected to an uni-nephrectomy aldosterone salt challenge (NAS, 200µg/kg/day of aldo, 1% NaCl in tap water) for 4 weeks. Blood pressure (SBP) was measured by tail cuff method. Cardiovascular fibrosis and inflammation were analyzed by RT-PCR, western blot, immunohistochemistry and ELISA. There was no difference in SBP between transgenic mice compared to WT mice in basal condition. With NAS challenge, SBP was increased only in WT mice (SBP; CT 107±3, CT NAS 133±5, KO 109±3, KO NAS 115±3 mmHg). Quantification of pro collagen I N-terminal peptide (PINP) in plasma showed an increase of PINP due to NAS treatment in WT that was prevented by LCN2 inactivation (CT 83±15, CT NAS 129±10, KO 70±19, KO NAS 59±12 µg/l). In myocardium, NAS treatment increased collagen type I and perivascular fibrosis in WT whereas KO were resistant to fibrosis (Collagen Volume Fraction; CT 19±3, CT NAS 28±2, KO 20±3, KO NAS 20±3 %). Our results show that LCN2 plays a key role in aldosterone/MR-mediated coronary vessel fibrosis, but not in cardiac interstitial fibrosis and vascular dysfunction. We are now analyzing 1) the specificity of aldo/MR versus other pro-fibrotic challenges (AngII, catecholamines) as well as 2) the role of inflammation in the effects mediated by Lcn2. Our preliminary experiments indicated that LCN2 do not participate in the chronic effects of AngII (hypertension, cardiac fibrosis) underlying a specific role of LCN2 in the CV mineralocorticoid effects. The role of LCN2 in inflammation will be analyzed using Bone Marrow Transfer experiments between WT and LCN2 KO mice upon mineralocorticoid challenge. The mechanistic role of LCN2 on extracellular matrix remodeling and inflammation will be tested ex vivo in cardiac fibroblast and inflammatory cells (macrophage/dendritric cells) using recombinant LCN2.

Nephron-specific MR-deficient mice develop a severe renal PHA1 phenotype

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In the aldosterone-sensitive distal nephron, sodium balance, fluid homeostasis and blood pressure are regulated by transepithelial sodium transport. This fine tuning is achieved by aldosterone, which binds and activates the mineralocorticoid receptor (MR). Loss-of-function mutations of MR in the distal nephron lead to the renal type 1 pseudohypoaldosteronism (PHA1), a rare genetic disease of aldosterone resistance.

Using a doxycycline inducible system[1], nephron-specific MR knockout mice ($MR^{Pax8/LC1}$) were generated. mRNA and protein expression of MR are significantly decreased in whole kidney. Moreover, MR protein expression is absent in all microdissected nephron segments. Following induction of MR-deficiency in the nephron and under standard diet, 4 week-old $MR^{Pax8/LC1}$ mice stop to gain weight and thus exhibit a significant weight loss compared to control mice. Interestingly, despite the failure to thrive, $MR^{Pax8/LC1}$ mice survive. However, they develop a severe renal PHA1 phenotype with higher urinary Na^+ and lower urinary K^+ levels, resulting in hyponatremia and hyperkalemia. Upon sodium-deficient diet, mice continue to rapidly lose weight and plasma aldosterone levels further increase.

To test whether the PHA1 phenotype can be rescued in these mice, we currently apply a specific rescue (high sodium and low potassium diet). Preliminary results show that experimental mice continue to grow as control

mice. Moreover, urinary Na^+ and K^+ excretion values are normal, suggesting that the electrolyte homeostasis is maintained. In summary, the mineralocorticoid receptor is crucial for sodium and potassium homeostasis in the adult kidney and MR deficiency in the kidney can be rescued by high sodium and low potassium diet.

This work is supported by the Swiss Science Foundation, National Center of Competence in Research (NCCR: Kidney.CH: Control of Homeostasis).

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Identification of signaling pathways involved in the potassium dependent regulation of Sodium Chloride Co-transporter (NCC)

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Abstract

Potassium (K^+) homeostasis is essential for vital functions of heart, nervous and muscular system. Upon high K^+ diet, potassium balance in the body is accomplished through rapid renal potassium excretion. The Sodium Chloride Co-transporter (NCC), expressed in the distal convoluted tubule (DCT), plays an essential role in renal electrolyte balance. NCC phosphorylation at conserved serine and threonine residues by SPAK/OSR and WNK1/4 kinases are important for its membrane abundance and consequently for its transport function. Recently, evidence has been provided that acute potassium challenge in mice rapidly dephosphorylates NCC through an aldosterone independent mechanism, thereby compromising its transport function. Potassium delivery through food or direct systemic infusion decreases NCC phosphorylation in a similar manner. Latest studies in mice suggest that the effect of potassium on NCC dephosphorylation might be modulated by accompanying anions. However, the nature of the signals that promote dephosphorylation of NCC in the kidney are not known. Therefore, it is essential to identify potassium dependent novel regulators of NCC to better understand the link between potassium homeostasis and rapid NCC dephosphorylation. Within this project, we aim to identify the signaling pathways that regulate/s NCC dephosphorylation in a potassium dependent manner. To do so, we have generated a DCT derived cell line expressing NCC. Excitingly, stably expressed NCC is phosphorylated at conserved serine and threonine residues which are further increased in response to low chloride shock. Additionally, we have established the presence of NCC in the membrane by cell surface biotinylation assay. However, only subpopulations of cells within this DCT clone are expressing NCC due to the negative selection of the NCC expressing cells. In order to overcome the problems associated

with stably expressing NCC in DCT cells, we are currently generating cells with conditional expression of NCC.

Role of Mineralocorticoid Receptor in the regulation of autophagy and differentiation of adipose tissue and skeletal muscle.

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Autophagy is an intracellular degradation system that exerts different functions depending on the target organ. It is known that macroautophagy plays a pivotal role in cell differentiation. In particular autophagy has been shown to be a critical determinant of adipose tissue differentiation and function. Interestingly, defective autophagy in adipose tissue decreases white adipose mass and enhances insulin sensitivity in mice. We recently investigated the role of MR in the modulation of the autophagic rate in the adipose organ: we analysed responses to the MR antagonists spironolactone (SPIRO) and drospirenone (DRSP) in female mice fed a high fat (HF) diet for 90 days. MR antagonism reduced autophagy rate and induced brown adipose tissue (BAT) markers in WAT depots and in cultured adipocytes, providing evidence that MR modulates autophagic rate and, in turn, regulates brown-like remodelling of white adipose tissue.Interestingly, a critical role of autophagy has been recently shown also in skeletal muscle development. Genetic ablation of Atg7 in myogenic Myf5+ progenitors inhibits macroautophagy in skeletal muscle, with impaired muscle differentiation, reduced muscle mass and glucose intolerance., suggesting a tissue specific impact of autophagy on metabolic profile, Therefore we investigated a possible involvement of MR in regulating macroautophagy in skeletal muscle cells. We first characterised the ontogenesis of MR in a murine myoblast cell line (C2C12) by Real Time analysis in order to evaluate the expression of MR during myotube differentiation *in vitro.* We observed a 2,5 fold-increase in basal MR mRNA expression in skeletal myoblasts compared with myotubes after 72h of differentiation. To evaluate the effects of MR activity on C2C12 cells we treated C2C12 myoblasts and myotubes with aldosterone (Aldo, 10^{-8} mol/L) for 24h and observed a marked increase in LC3 mRNA expression suggesting an increased autophagic rate. This effect was reverted by treatment with MR antagonist spironolactone (Spiro, 10^{-5} mol/L). However, protein levels of LC3 were not affected by any treatment, suggesting that longer treatments could be necessary to reveal an effect. Moreover we observed that Aldo significantly reduced in Heavy Chain Myosin (MHC) mRNA levels, suggesting that MR activity represses muscle cell differentiation. Such effect was also MR dependent, given that spiro was able to revert such effect.
These data suggest a potential role for MR in modulating skeletal muscle differentiation and autophagic rate.

The diuretic torasemide does not prevent aldosterone - mediated mineralocorticoid receptor activation in cardiomyocytes

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Background: Aldosterone binds to the mineralocorticoid receptor (MR) and exerts pleiotropic effects beyond enhancing renal sodium reabsorption. Excessive mineralocorticoid signaling is deleterious during the evolution of cardiac failure, as evidenced by the benefits provided by adding MR antagonists (MRA) to standard care in humans. In animal models of cardiovascular diseases, MRA reduce cardiac fibrosis.

Interestingly diuretics such as torasemide also appear efficient to improve cardiovascular morbidity and mortality, through several mechanisms.

Objective: To show, as it has been suggested, that torasemide could block aldosterone binding to the MR.

Methods and Results: To evaluate whether torasemide (tora) acts as a MRA in cardiomyocytes, we compared its effects with a classic MRA such as spironolactone (spiro). We monitored ligand-induced nuclear translocation of MR-GFP and MR transactivation activity in the cardiac-like cell line H9C2 using a reporter gene assay and known endogenous aldosterone-regulated cardiac genes. Spironolactone slows down aldosterone-induced MR nuclear translocation. On the contrary, torasemide did not modify MR nuclear translocation. Aldosterone-induced MR transactivation activity was reduced by spiro, not by tora (% luciferase activity; control 100±16, aldosterone 246±13.8, aldosterone + spi 10^{-6}M 103±6.1, aldosterone + tora 10^{-6}M 229±11.7%)
Spironolactone 10^{-6}M blocked the induction by aldosterone 10^{-8}M of endogenous MR-responsive genes: Sgk-1, PAI-1, Orosomucoid-1, Rgs-2, Serpina-3 and Tenascin-X, while torasemide 10^{-6}M was ineffective (For example mRNA relative expression of PAI-1: control 1±0.1, aldosterone 3±0.3, aldosterone + spi 1.6±0.1, aldosterone + tora 2.7±0.2).

Conclusion: These results show that torasemide is not a MR antagonist; its association with MRA in heart failure may however be beneficial, through actions on complementary pathways.

Vascular Smooth Muscle Mineralocorticoid Receptor Contributes to Coronary and Left Ventricular Dysfunction After Myocardial Infarction.

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Abstract

Aims: Because mineralocorticoid receptor (MR) antagonists have shown efficacy in slowing down the progression of heart failure after myocardial infarction (MI), there is interest to elucidate the cell-specific involvement of MR. Indeed, the role of MR in vascular smooth muscle cells (VSMC) in heart failure, especially its impact on coronary circulation, has never been investigated.

Methods and Results: Two months after MI, mice lacking the MR specifically in VSMC (MI-MR^{SMKO}) and mice treated with the MR antagonist finerenone (MI-fine) had better coronary function than control (MI-CTL), as assessed by acetylcholine-induced relaxation of isolated arteries (relaxation %: MI-CTL: 36±5, MI-MR^{SMKO}: 54±3, MI-fine: 76±4; $P<0.05$). Furthermore, MRI showed that the coronary reserve was increased (ml/mg/min: MI-CTL: 1.1±0.5, MI-MR^{SMKO}: 4.6±1.6, $P<0.05$; MI-fine: 3.6±0.7, $P<0.01$). Incubation with the NAPDH-oxidase inhibitor apocynin of coronary arteries improved acetylcholine-induced relaxation in MI-CTL to a higher extent than in MI-MR^{SMKO} and MI-fine mice, suggesting that MR antagonism reduces oxidative stress-mediated endothelial dysfunction. Indeed, incubation of coronary arteries from non-infarcted animals with 10^{-9}M angiotensin II induced oxidative stress and impaired acetylcholine-induced relaxation

in CTL mice, but not in MR^{SMKO} or in 4 weeks finerenone-treated mice. These improvements in coronary function were accompanied in MI-MR^{SMKO} mice by reduced LV fibrosis and improved LV function.
Conclusion: After MI, VSMC-specific MR invalidation benefits LV dysfunction, likely through improvement of coronary reserve and of coronary endothelial function, demonstrating for the first time the deleterious role of smooth muscle MR activation in heart failure. Furthermore, systemic MR blockade by finerenone confers additional functional improvements.

Mineralocorticoid Receptor overexpression in mice cardiomyocyte reduces in vivo coronary reserve and induces diastolic dysfunction

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The mineralocorticoid receptor (MR) is implicated in myocardial and vascular dysfunction both in animals and humans. To evaluate whether this is associated with a modification of myocardial perfusion, we studied *in vivo* coronary reserve (CR) using MRI, in the mouse model of conditional cardiomyocyte overexpression of the human MR (MR-Cardio). CR is reported as the difference between basal and maximal perfusion obtained under stimulation of A2a adenosinergic receptor by ATL307 inducing vasodilatation.

CR is strongly decreased in MR-Cardio mice (Ctl vs. MR-Cardio: 3.5±0.4 vs. 0.72±0.5 $ml.min^{-1}.g^{-1}$, $p<0.01$). This decrease appears aldosterone dependent, as far as it is corrected by a 4 weeks treatment by FAD286, an inhibitor of aldosynthase which does not affect glucocorticoid levels (Ctl vs. MR-Cardio+FAD286: 2.4±0.5 vs. 2.9±1 $ml.min^{-1}.g^{-1}$, NS). We propose that oxidative stress influences CR in MR-Cardio mice, because SOD antioxidant activity is decreased in those mice and restored by FAD286. Otherwise, we found no difference between groups in heart capillary density, fibrosis or ventricular weights that could influence CR.

On the other hand, heart muscular function was assessed by invasive pressure-volume loops that showed a decrease in left ventricular end diastolic pressure (LVEDP) and in LVEDPVR (LVEDP Volume Relationship) in MR-Cardio mice, respectively concerning defects in diastolic filling and compliance (Ctl vs. MR-Cardio: LVEDP=5.43±0.72 vs. 9.31±1.31, $p<0.05$; LVEDPVR=2.34±0.48 vs. 5.08±0.74, $p<0.01$). Of interest, we also observed diastolic dysfunction in mice from another strain constitutively overexpressing the aldosynthase gene in cardiomyocyte.

These features of MR-Cardio mice could lead to a reduced aptitude for intensive exercise. Spontaneous locomotor activity remained similar between control and MR-Cardio mice. However, during an intensive treadmill running, 74% of MR-Cardio mouse died during exercise against 24% of MR-Cardio mice treated by FAD 286. ECG telemetry during exercise is under experimentation.

Our results suggest that MR overexpression in cardiomyocyte, reported in several human cardiac pathologies, is involved in CR impairment and diastolic dysfunction. Aldosterone by itself may be directly involved.

Is the small G protein Rac1/PAK signaling pathway linked to mineralocorticoid receptor in renal ischemia-reperfusion?

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Mineralocorticoid receptor (MR) antagonism with Spironolactone prevents the renal injuries induced by bilateral kidney ischemia reperfusion (IR) in the rat[1]. The underlying mechanisms are still poorly understood and could involve vasomotor alterations targeted by MR antagonism. Our laboratory has recently shown that MR deletion in smooth muscle cells (SMC) improves renal functions 24h after IR(unpublished data), suggesting a major role of MR in this cell type. Previous studies from Fujita and coworkers established a link between MR and the small GTPase Rac1 *in vivo*[2] and we previously demonstrated that Rac1 regulates SMC contraction[3]. Moreover, Rac1 inhibition limits ischemic injuries in brain and heart[4,5]. Our project is to determine whether the vascular Rac1/PAK signaling has a role in MR-dependent deleterious effects and/or beneficial effects of MR antagonists in renal IR injury.

The isolated perfused kidney approach was set up to study *ex-vivo* renal arteries motricity after IR. Our preliminary results show that the response to the adrenergic agonist phenylephrine (10^{-7} to 10^{-5} M) is increased 24h after reperfusion (n=2-3). This is associated with a 50% decrease in acetylcholine (10^{-5} M) and sodium nitroprussiate (10^{-4} M) relaxation (n=2-3). Nitric-oxide dependent impaired relaxation is consistent with the endothelial dysfunction caused by ischemia in different vascular beds, including kidney[6]. Ongoing biochemical analyzes suggest that Rac1 effector PAK1 could be downregulated in aorta and kidney of IR mice, compared to the control mice. We hypothesize that MR-mediated effects in IR are related to Rac1/PAK signaling pathway. To address this question, Rac1 will be inhibited *in vivo* using the Rac1 inhibitor NSC23766 prior to ischemia and renal functions will be assess 24h after reperfusion. Transgenic mice with a specific deletion of Rac1 in SMC will also be submitted to IR and the renal phenotype will be compared to wild-type IR mice.

Our preliminary data suggest vasomotor alterations in the kidney 24h after IR. Bibliography and Western blotting data suggest that Rac1/PAK could contribute to the renal injuries. Further studies are currently ongoing in order to understand the underlying mechanisms.

[1]Barrera-Chimal 2013 (Kidney Int.); [2]Shibata 2011 (J Clin Invest.); [3]André 2014 (J Am Heart Assoc.); [4]Liao 2014 (Neuropharm.); [5]Shan 2010 (Free Radic Biol Med.); [6]Conger 1995 (J Clin Invest.).

SHORT-TERM BLOOD PRESSURE VARIABILITY IN PRIMARY ALDOSTERONISM

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Introduction: Primary aldosteronism (PA) is associated with an altered pulsatile secretion of aldosterone, which may be involved in the pathogenesis of arterial hypertension and cardiovascular damage. Short-term blood pressure variability, obtained from the 24-hour ambulatory blood pressure monitoring (ABPM), is an independent predictor of cardiovascular disease. The aim of our study was to evaluate the blood pressure

variability indices in patients affected by PA, compared with essential hypertensive patients and normotensive controls. Methods: We enrolled 29 patients with PA (age 56.3±13.2, 15M 14F), 12 with aldosterone producing adenoma (APA) and 17 with idiopathic hyperaldosteronism (IHA), not taking therapy with aldosterone antagonists. We calculated from the ABPM the following indices of blood pressure variability: Weighted Standard Deviation (WSD) and Average Real Variability (ARV), for both systolic (SBP) and diastolic blood pressure (DBP). We compared the data with those of 30 hypertensive patients (in treatment with antihypertensive medications) and 30 normotensive, matched for sex, age and BMI. Results: Average renin and aldosterone of patients with PA were respectively 2.6±2.1 mU/L and 25.3±13.0 ng/dL. Both systolic and diastolic ARV, WSD and BP were not significantly different between patient with PA (ARV sis 9.3±2.1, ARV dias 7.4±2.4, WSD sis 12.1±3.1, WSD dias 9.3±2.9, SBP 135.4±15.1, DBP 83.7±8.7) and control hypertensive patients (ARV sis 8.5±1.8, ARV dias 6.9±1.3, WSD sis 11.6±3.1, WSD dias 9.1±2.5, SBP 135.9±10.1, DBP 83.5±9.2; P=ns for all indices). The indices of blood pressure variability were significantly higher in patients with PH and hypertensive controls compared to normotensive controls ($P<0.05$ for all indices). Blood pressure variability was similar between patients with APA and IHA (P=ns). Conclusion: Short-term pressure variability is significantly increased in hypertensive patients, in a similar manner between patients with PA and with essential hypertension. The role of aldosterone in the short-term regulation of blood pressure has yet to be clarified.

Analysis of differentially-expressed microRNAs and their potential targets in normal adrenal tissue and aldosterone-producing adenoma

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Up to 15% of hypertension cases are caused by primary aldosteronism (PA), which results from the secretion of inappropriately high levels of the steroid hormone aldosterone. Almost half of these cases are due to the presence of unilateral aldosterone-producing adenoma (APA). Normal synthesis of aldosterone occurs in the adrenal zona glomerulosa, with the final stages catalysed by the *CYP11B2* gene product, aldosterone synthase. Our previous studies showed aldosterone secretion and *CYP11B2* gene expression were subject to regulation by microRNAs (miRNAs). These are small, single-stranded RNAs 20–25 nucleotides in size that target particular messenger RNAs (mRNAs) and negatively regulate gene expression at the post-transcriptional level. We have now extended our studies to investigate whether microRNA has a role in the aetiology and pathophysiology of APA.

We used the Ingenuity Pathway Analysis (IPA) and DIANA micro-T software packages to analyse miRNA microarray data generated from human APA and normal adrenal (NA) tissue samples (each n=4). This enabled us to identify miRNAs that are differentially expressed between the two tissues and to predict their putative mRNA targets. Of the 723 miRNAs analysed, 78 were present above the threshold detection level in one or both tissues; 31 of these were differentially expressed between NA and APA ($p<0.05$).

IPA analysis confirmed Endocrine System Disorders as being one of the top 3 disease states associated with these 31 miRNAs, alongside Cancer and Cardiovascular. Interestingly, four miRNAs that are each significantly downregulated in APA relative to NA were predicted by IPA to target the mRNA of 3-hydroxy-3-methylglutaryl-CoA-reductase (HMGCR). This gene represents a rate-limiting step in cholesterol production and so may be an important determinant of steroid biosynthesis. Five differentially-expressed miRNAs are also predicted by DIANA micro-T to target *CYP11B2* mRNA and may therefore have a direct regulatory influence on aldosterone production.

In conclusion, miRNA data generated by microarray analysis was subjected to bioinformatic analysis in order to identify putative mRNA targets related to functions that may be disrupted in cases of APA, including cell proliferation, apoptosis, angiogenesis and steroidogenesis. These findings will direct future *in vitro* research studies designed to increase our understanding of the aetiology of APA and identify prospective biomarkers for its diagnosis and treatment.

Phosphorylation of Mineralocorticoid Receptor at residue S839 impairs aldosterone-dependent gene transactivation coupling in a dominant negative manner

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The **mineralocorticoid receptor (MR)** is a member of the nuclear receptor superfamily of transcription factors that transduces the biological effects of the corticosteroid hormone aldosterone, playing a key role in the regulation of transepithelial sodium reabsorption, extracellular volume and blood pressure. In addition, MR shows pleiotropic effects in non-epithelial tissues, affecting processes as diverse as cardiovascular homeostasis, adipocyte differentiation and function or stress responses in the hypothalamus. Antagonism of MR in humans is useful in the treatment of severe cardiac failure and some forms of hypertension. Aldosterone interaction with MR ligand binding domain (LBD) is responsible for receptor nuclear translocation, dimerization and gene transactivation. It has recently been demonstrated that phosphorylation of S843, a residue near the aldosterone binding pocket, inactivates human MR, presumably by reducing affinity for the hormone. In this work we examined the mechanisms involved in MR modulation by S843p. To that end we used mouse MR phosphomimetic mutant S839D (equivalent to S843 in human) or non-phosphorylatable mutant S839A cotransfected with wild type MR in different proportions in COS-7 cells. MR-S839D is inactive and significantly decreases wild type MR activity when both forms are coexpressed. Assuming that MR dimerization is not affected by S839D and it follows a binomial distribution, our results are consistent with a dominant negative role of MR-S839D in the dimer. Surprisingly, aldosterone is able to induce nuclear translocation of MR-S839D, although at a slower rate than wild type receptors. Therefore aldosterone is still able to bind to MR-S839D but it is inefficient for gene transactivation. Structure modeling of MR LBD and docking experiments show that S839D mutation or S839p produce the same effect, namely a small decrease in steady-state agonist docking energy. Taken together, these results suggest that the effect of S839p cannot be fully explained by decreased aldosterone binding affinity and may imply a defect in transactivation coupling.

The channel activating protease CAP2/Tmprss4 is not required for ENaC-mediated sodium homeostasis *in vivo.*

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Channel-activating proteases (CAPs), like e.g. CAP1 (also Prss8 or prostasin), CAP2 (Tmprss4) and CAP3 (ST-14 or matriptase), are membrane-bound serine proteases, that have been identified as activators of the epithelial sodium channel ENaC in the *Xenopus* oocyte expression system (Vallet *et al.* 1997, Vuagniaux *et al.* 2000, 2002). We recently demonstrated an involvement of CAP1 in ENaC-mediated sodium transport *in* vivo in lung and colon (Planès *et al.* 2005, Malsure *et al.* 2014).

In this study, we were interested whether CAP2 is equally an *in vivo* regulator of ENaC-mediated sodium currents. We generated constitutive knock-out mice for CAP2 using Cre-loxP-mediated recombination system that deletes exons 8 and 9, including the histidine and the aspartate of the catalytic triad. We confirmed that the protein is absent. The mice are viable, healthy and fertile, and do not exhibit a visible phenotype. We analysed ENaC activity by measuring plasma and urinary electrolytes, and rectal potential difference under regular and sodium-deficient diet. Neither in the colon nor in the kidney, we could not see altered ENaC expression and ENaC-mediated sodium transport. In conclusion, CAP2 knock-out mice do not show differences in the ENaC expression or activity, demonstrating that CAP2 is not required for ENaC-mediated sodium transport *in vivo*.

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Reactive oxygen species and prostanoids mediate aldosterone production from adipocytes in hypertension. Role of G protein-coupled receptor kinase 2

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Background and Aim. Preliminary studies indicate that 1) Adipocytes are a source of aldosterone in response to Angiotensin II (AngII); 2) Reactive oxygen species (ROS) mediate AngII-induced aldosterone production in adrenal cells. 3) Hypertension is associated with a reciprocal relationship between reactive oxygen species (ROS) from NADPH oxidase and/or mitochondria and cyclooxygenase-2 (COX-2)/microsomal prostaglandin E synthase type 1 (mPGES-1) which participates in vascular damage. 4) G protein-coupled receptor kinase 2 (GRK2) plays a role in hypertension-associated vascular injury and regulates adiposity. We will evaluate the role of ROS and COX-2/mPGES-1-derived prostanoids in aldosterone release from adipocytes and the involvement of GRK2 in this process.

Methods. Studies were performed in visceral adipose tissue and/or mature adipocytes isolated from the following animal models: 1) untreated and Angiotensin (AngII)-infused mice in the presence and in the absence of the COX-2 inhibitor celecoxib and the antioxidants apocynin or mito-TEMPO; 2) COX-$2^{-/-}$, mPGES-$1^{-/-}$ and GRK$2^{+/-}$ and their corresponding wild-type mice with or without AngII; 3) Normotensive (WKY) and spontaneously hypertensive rats (SHR) treated with celecoxib. 3T3-L1 and SW872 adipocytes stimulated with AngII were also used.

Results. (1) AngII infusion increased GRK2, COX-2, Nox1, Nox4 and aldosterone synthase (Cyp11b2) mRNA levels, NADPH oxidase activity and H_2O_2, PGE_2 and aldosterone production in visceral AT and/or mature adipocytes. Most of these effects were prevented by celecoxib, apocynin and mito-TEMPO treatments

and by COX-2, mPGES-1 or GRK2 deletion. (2) COX-2, Nox1 and Nox4 expression, NADPH oxidase activity, Cyp11b2 expression and aldosterone production were higher in visceral AT from SHR than WKY. Cyp11b2 expression, aldosterone production and NADPH Oxidase activity were decreased by celecoxib treatment. (3) In the 3T3-L1 and/or SW872 adipocytes, AngII increased Cyp11b2 mRNA levels and aldosterone production that were abolished by celecoxib, apocynin and mito-TEMPO.

Conclusions. A crosstalk between oxidative stress and prostanoids is responsible for aldosterone production from adipocytes in hypertensive animals, at least in part, trough GRK2. The excess of these mediators from adipose tissue might contribute to the cardiovascular damage observed in hypertension.

Multimodal assessment of endothelial dysfunction in *ex vivo* vessels from ApoE/LDLR-/- mice

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Besides kidney, the epithelial sodium channel (ENaC) is also found in vascular endothelial and smooth muscle cells (ECs; VSMCs). A positive correlation between aldosterone-induced endothelial ENaC (EnNaC) membrane insertion and the mechanical properties of the ECs was found: The more EnNaC, the stiffer the cell cortex, a region 50-150 nm beneath the plasma membrane. A stiff cortex in turn results in a decreased secretion of nitric oxide (NO) which is the hallmark for endothelial dysfunction. This process might promote the progression of atherosclerosis, hypertension and other diseases. In the present work a combined method was established to investigate the mechanical properties of ECs from *ex vivo* mouse aortae with an Atomic Force Microscope (AFM) and additionally the biochemical composition with Raman spectroscopy (RS) within the same aortic area. Aortae of either wildtype (WT) or ApoE/LDLR-/- mice were used, whereas the latter represents a reliable model of atherosclerosis and severe endothelial dysfunction. *Ex vivo* patches of dissected aortic rings were fixed onto glass-dishes coated with Cell-Tak and studied with a confocal Raman Imaging system (WITec alpha 300). The stiffness of living ECs was measured with soft cantilevers which are gradually lowered onto the cell and indent the cell membrane upon contact. The resulting deflection of the flexible cantilever is measured via reflection of a laser beam from its backside. Taking the cantilever's spring constant and the cantilever's sensitivity into account the data are transformed into force vs. distance curves (FDCs) of single cells. Cortical stiffness values of 16 ECs (each EC 6 curves) in an area of 12µm x 12µm were determined. Additionally, "digital pulsed force mode" (dPFM) was used to perform a map of the very same area to obtain topography, stiffness values and adhesion. For RS, the scattered light of a laser can be detected which is shifted in energy because of interactions between light and matter (Raman scattering). The shift intensity can be plotted against the frequency, resulting in a specific Raman spectrum of the biochemical compound. The first Raman map was performed at the highest signal and the second map 2 µm up or down. We obtained Raman maps of 15µm x 15µm (60 x 60 pixels), with the same center then during AFM measurements. To study whether EnNaC is involved in the development of atherosclerosis, the specific EnNaC-blocker benzamil was applied. Since EnNaC mediates cortical stiffness and thus endothelial dysfunction, differences in the biochemical composition and the mechanical stiffness of ECs between ApoE/LDLR(-/-) and WT ECs were expected. The same experiment can be done with aldosterone receptor antagonists. Taken together, a combined approach of AFM and RS seems useful to identify biochemical and mechanical properties in *ex vivo* artery to study various pathways leading to endothelial dysfunction and atherosclerosis including EnNaC.

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A novel combined Glucocorticoid-Mineralocorticoid Receptor antagonist inhibits adipocyte differentiation and fat mass expansion in mice fed a high-fat diet

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We have previously shown that antagonism of the mineralocorticoid receptor (MR) results in a potent antiadipogenic activity, both in vitro and in vivo. Recent data provide evidence that the glucocorticoid receptor (GR) has a more important role than the MR in promoting the early steps of adipogenesis in human preadipocytes.

In this study responses to a novel combined GR/MR antagonist were investigated in a model of diet-induced obesity. Female 10-week-old C57bl6 mice were fed with normal chow or a high fat diet (HFD) for 9 weeks. Mice fed a HFD were concomitantly treated for 9 weeks with the selective GR antagonist (CORT-108297, 80mg/kg/day), the novel combined GR/MR antagonist (CORT-118335, 80mg/kg/day) or spironolactone (SPIRO, 20 mg/Kg/day). Mice fed a HFD showed a significant increase in total body weight, white fat mass, mean adipocyte size, expression of white adipose tissue (WAT) markers and showed impaired glucose tolerance. Treatment with both CORT-118335 and CORT-108297 dramatically prevented the HFD-induced weight gain, and the effects of CORT-118335 were more pronounced. Such effects were not due to reduced food intake since caloric efficiency was significantly lower in CORT-118335 and CORT-108297-treated mice, when compared to the HFD group. Both compounds markedly improved glucose tolerance, decreased leptin and increased adiponectin levels compared to the HFD group. Again, these effects were more pronounced with CORT-118335. Furthermore, both compounds were able to reduce mean adipocyte size, a valuable marker of adipocyte function, compared to the HFD.

When tested in vitro, both compounds markedly reduced 3T3-L1 preadipocyte differentiation under steroid-deprived conditions. We then aimed to dissect the mixed GR/MR antagonist activity of CORT-118335 in this model. Interestingly, only the pro-adipogenic effects of aldosterone were antagonized by CORT-118335, whereas the GR-mediated effects of dexamethasone were not affected. This suggests that CORT-118335 mostly acts as an antagonist of MR, rather than GR, in murine cultured preadipocytes. In conclusion, this pilot study shows that both MR and GR play a relevant role in murine adipogenesis, and their reciprocal interplay is crucial for adipocyte differentiation.

Transcriptional control of ICAM-1 in human coronary artery endothelial cells by Mineralocorticoid Receptor (MR): implications for the protective effects of MR antagonists in cardiovascular diseases

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In clinical trials, mineralocorticoid receptor (MR) antagonists decrease cardiovascular ischemia and mortality suggesting a beneficial role of MR inhibition in the vasculature. We have shown that human coronary and umbilical endothelial cells (HUVEC) express functional MR. In endothelial cells MR activation by aldosterone promoted transcription of ICAM-1. Most importantly cell adhesion assays demonstrated that aldosterone promotes leukocyte adhesion to ECs, an effect that was inhibited by spironolactone and ICAM-1 blocking antibody. These data support that MR activation in human endothelial cells promotes ICAM-1-mediated leukocyte-EC adhesion, an important step in early atherosclerosis lesion formation. To further explore the mechanisms for MR-mediated EC activation, we now demonstrate that MR activation up-regulates VCAM-1 and E-selectin mRNA expression (2 and 3.5 fold respectively), whereas P-selectin is not regulated by MR. We also further explored the mechanism of MR-mediated regulation of endothelial ICAM-1 expression. In transient transfection experiments performed in HUVEC, we have shown that aldosterone is able to activate (2 fold) a 3 Kb promoter region upstream the transcription start site of human ICAM-1 gene. Co-incubation with spironolactone was able to inhibit the effect of aldosterone, confirming the presence of elements responsive to signaling pathway(s) activated by MR. In order to localize and characterize MR responsive cis-element(s) and the corresponding transcription factor(s) binding to this regulatory region, five 5'-deletion constructs of ICAM-1 promoter were subcloned in a vector upstream of the luciferase gene. Data of transcriptional activity showed the presence of regulatory element(s) required for ICAM-1 expression via MR in the promoter region between nt-872 and -1141. Bioinformatics analysis of this region revealed the presence of four different highly conserved regulatory elements: three SP1 binding sites, one NF-κB binding site, one AP1 binding site and one GRE/MRE. The role of these transcription factors in MR-mediated regulation of ICAM-1 expression was investigated using c-JUN and IKBα dominant negative constructs. Blocking of either c-Jun or NF-κB pathway resulted in a marked reduction of the aldosterone effect on ICAM-1 promoter activity, suggesting the involvement of these transcription factors. These studies explore the molecular mechanism for the pro-inflammatory effects of MR activation in the vasculature that may contribute to explain the protective effects of MR antagonists in clinical trials. Moreover to better understand the crosstalk between MR activation and ICAM1 in the development of atherosclerosis lesions, we developed a mouse model double KO for ApoE and ICAM1, where we are currently studying the pro-atherogenic effects of aldosterone.

COMPARISON OF V-PYRRO/NO, AN LIVER-SPECIFIC NO-DONOR AND METFORMIN EFFECTS ON LIVER STEATOSIS IN MICE FED HIGH FAT DIET

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases occurred simultaneously with obesity, insulin resistance, type 2 diabetes, and dyslipidemia. Even though various approaches have been proposed, there is an unmet medical need for novel NAFLD-specific treatments.

The aim of this study was to assess the effects of novel liver-selective NO donor, V-PYRRO/NO in comparison with metformin on hepatic steatosis in mice.

Six-week old C57BL/6J male mice were fed for 15 weeks the control or high fat diet (60 kcal% fat) and additionally treated for the last 5 weeks of experiment with V-PYRRO/NO and metformin. Biochemical, histopathological and chromatographic analysis were performed. The same feeding and treating approach was used in 6-month old C57BL/6J mice for portal vein blood flow and liver perfusion with MRI technique.

V-PYRRO/NO and metformin significantly blunted mice body weight increase induced by high fat diet, reduced total fat content with simultaneous reduction of TAG, DAG and CER fraction in the liver and reversed HF-induced decrease in UFA/SFA ratio. V-PYRRO/NO substantially improved postprandial glucose tolerance, while the effect of metformin was more pronounced on HOMA IR index. In addition, V-PYRRO/NO increased liver total NOx and nitrate concentrations and improved portal vein blood flow and liver perfusion in treated mice. Altogether we demonstrated that anti-steatotic mechanism of V-PYRRO/NO is related to NO release, differ from that of metformin and involved improved postprandial glucose tolerance and inhibition of *de novo* fatty acid synthesis by Akt up-regulation and ACC phosphorylation. In turn, major mechanism of metformin action involved increased expression of proteins implicated in mitochondrial biogenesis and metabolism (PGC-1α, PPARα, COX IV, cytochrome c, HADHSC).

In conclusion, V-PYRRO/NO acts as a liver-specific NO donor prodrug affording anti-steatotic effects and may represent an efficient novel approach to prevent liver steatosis with subsequent development of insulin resistance then metformin. Moreover, since mechanisms of anti-steatotic action of V-PYRRO/NO and metformin are complementary we suggest a possible additive value in combined treatment.

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MRI - based assessment of endothelial function and vascular permeability in mice models of endothelial dysfunction

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Abstract:

Purpose: The aim of the study was to develop a comprehensive method, for the *in vivo* assessment of endothelial dysfunction, using 3D techniques based on the IntraGate sequence and relying on the evaluation of endothelium-dependent vasodilation and measurements of changes its permeability and validation of the developed method, by other conventional *ex vivo* endothelial dysfunction assessment methods, in animal models of endothelial dysfunction (apoE/LDLR-/- mice, high-fat diet (HFD) mice and control group treated with L-NAME).

Methods: *MRI*: MRI was performed on Bruker BioSpec 9.4T system (Ettlingen, Germany). Brachiocephalic artery (BCA) and left carotid artery (LCA) were imaged using MRI before and every 10 min for 40 min after acetylcholine administration (ACh (Sigma-Aldrich): 50 µl, 16,6 mg/kg i.p., MRI: cine IntraGate™ FLASH 3D). Blood vessel cross-sections after ACh administration were determined. Endothelial permeability was assessed by detection of T_1 relaxation time changes around vessel lumen and the number of pixels (Npx50), for which T_1 has changed about 50%, 30 min after contrast agent administration (CA: Galbumin, BioPal, Worcester, MA - 25 mg/ml, 4.5 µl/g i.v., MRI: 3D IG-FLASH - based VFA - Variable Flip Angle technique). *Animals:* 4-months old C57BL/6J mice (ACh: n=4, Galb/Pearm: n=4), 5-months old C57BL/6J mice (ACh: n=4, Galb/Pearm: n=8) fed for 4 months a HFD60, 4-months old C57BL/6J mice (ACh: n=4) treated with L-NAME (1 week, 100 mg/kg in drinking water) and ApoE/LDLr-/- (ACh: n=3, 12-months old; Galb/Pearm: n=3, 7-months old). *Data analysis:* ImageJ 1.46r (NIH, USA) and scripts in Matlab (MathWorks, Natick, USA). *Statistical analysis:* STATISTICA 10 (Stat Soft inc., USA).
*Vascular permeability in vitro**: In vitro* study was performed on pressure myograph system (DMT, Denmark) with cannulated LCA under flow condition – wall shear stress ~ 8 dyn/cm2, flow ~ 1500 µl/min. *Animals:* 6-months old C57BL/6J mice (n=2), 6-months old ApoE/LDLr-/- (n=2). *Procedure*: 1) incubation with albumin binding fluorescein isothiocyanate (FITC-albumin, Sigma-Aldrich: 200 µl, 400 µg/ml) flowing through the vessel for 90 min, 2) administration of thrombin (BIOMED-LUBLIN: 4 U/ml) in 45 min of incubation. *Parameters*: change of FITC-albumin concentration in the fluid from the chamber in which vessel was submerged (every 15 min for 90 min).
Results: 25 min after ACh administration, the vasodilation of blood vessels in C57BL/6J mice and its paradoxical vasoconstriction in HFD60 and ApoE/LDLr-/- mice was detected. Additionally, vasoconstriction response was higher in LCA. In mice treated with L-NAME ACh did not induced vasodilation.

Group	**C57BL/6J**	**C57/HFD60**	**ApoE/LDLr-/-**	**C57/L-NAME**
BCA Area change[%]	26.69±13.35	-28.67±8.36	-31.36±10.02	1.81±3.32
LCA Area change[%]	24.18±8.52	-54.33±14.56	-50.00±4.95	-3.91±4.99

In HFD60 and ApoE/LDLr-/- mice shortening of the T_1 around BCA was observed as opposed to C57BL/6J mice, where shortening of T1 was not significant. Npx50 for HFD60 and ApoE/LDLr-/- mice was significant different from Npx50 for C57BL/6J mice. Additionaly change of FITC-albumin concentration before thrombin administration is higher in ApoE/LDLr-/- mice ($\Delta C_{[0\text{-}45min]}$= 0,020, $\Delta C_{[45\text{-}90min]}$= 0,147) than in control group ($\Delta C_{[0\text{-}45min]}$=0,006, $\Delta C_{[45\text{-}90min]}$=0,005).

	C57BL/6J	**C57/HFD60**	**ApoE/LDLr-/-**
T_1 change	-5,61±4,10%	-23.43±9.63%	-24.39±4.84%
Npx50	8±5	20±6	19±6

Conslusion: Feasibility to noninvasive assessment of endothelial function and vascular permeability was demonstrated using MRI-based method. Impaired NO-dependent vasodilation and increased permeability of the endothelium in BCA and LCA in vessels from diseased mice were showed. Further comparative *in vivo*, *ex vivo*, and *in vitro* studies are needed to apply this method to monitor improvement of endothelial function in vivo in mice treated with endothelium-targeted therapy.

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Role of ubiquitylation in the regulation of the renal Na^+-Cl^- cotransporter

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The Na^+-Cl^- cotransporter (NCC) is expressed in the apical membrane of the distal convoluted tubule cells in the kidney where it reabsorbs sodium and chloride playing a crucial role the in the regulation of blood pressure.

It has been demonstrated that NCC is an aldosterone-induced protein. Our group showed that one of the players in the pathways by which aldosterone regulates NCC is the ubiquitin-protein ligase NEDD4-2. NEDD4-2 interacts with NCC, ubiquitylates the cotransporter at the cell surface and induces a reduction in NCC membrane expression and function. The Serum/glucocorticoid-regulated kinase 1 (SGK1) phosphorylates NEDD4-2, thus preventing ubiquitylation and inhibition of NCC. Recently we showed that NCC abundance was increased in inducible nephron-specific Nedd4-2 KO mice, concomitantly with a proportional increase in NCC phosphorylation that directly mirrors its activation. We also observed that the KO mice had salt-sensitive blood pressure increase and hypercalciuria that could both be corrected by thiazide confirming the role of NEDD4-2 in NCC regulation *in vivo*.

In order to better investigate the role of ubiquitylation in NCC regulation we performed an Ubiscan assay on mouse kidney lysates and we found that NCC is ubiquitylated in 11 different lysines. To elucidate which lysines could be implicated in NCC regulation we mutated the 11 lysines in arginines, to avoid the ubiquitylation in each site, and we expressed each single mutant in HEK cells. Interestingly, biotinylation assays clearly demonstrated that some of the mutants are more expressed in the membrane compared to wild type NCC. Moreover, preliminary results showed that, in contrast with the wild type NCC, some of the mutants are completely expressed in the membrane already in normal chloride conditions and the incubation with hypotonic low chloride solution does not further increase their cell surface abundance.

These results clearly suggest a role of some ubiquitylation sites in the trafficking of NCC and/or in its stability in the membrane.

Systemic increase in serum- and glucocorticoid-inducible kinase 1 (SGK1) activity potentiates mineralocorticoid/NaCl-induced renal but not cardiac fibrosis

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The mineralocorticoids aldosterone and deoxycorticosterone acetate (DOCA) stimulate renal tubular salt reabsorption, increase salt appetite, induce extracellular volume expansion, and elevate blood pressure. Mineralocorticoid excess induces deleterious effects on cardio-renal function, including the development of fibrosis. The effects of mineralocorticoids on renal tubular Na^+ reabsorption and salt appetite involve the serum- and glucocorticoid-inducible kinase 1 (SGK1). The present experiments explored the involvement of a systemic excess of SGK1 activity in the development of mineralocorticoid/NaCl-induced cardiac and renal fibrosis. To this end, we produced mice carrying one additional genomic copy of the mouse *sgk1* gene as bacterial artificial chromosome (BAC) transgene (Tg.SGK1) modified with a mutation rendering the kinase constitutively active. The transgene followed the same expression pattern as the endogenous counterpart. Tg.SGK1 mice and wild-type littermates were uninephrectomized, treated with DOCA (75 mg/Kg) and given 1% NaCl in the drinking water for 6 weeks. The treatment led to a significant increase in blood pressure in both genotypes, slightly more pronounced in Tg.SGK1 mice. Histology after 6 weeks treatment revealed marked glomerular, perivascular and tubulointerstitial fibrosis in the kidney cortex, which was significantly greater in Tg.SGK1 mice. In contrast, treated animals developed cardiac fibrosis without significant differences between genotypes. In the absence of treatment, Tg.SGK1 mice displayed enlarged glomeruli and increased glomerular collagen content. Our results demonstrate that increased SGK1 expression is sufficient to induce glomerular damage without added stimuli and enhances tissue specific mineralocorticoid/ NaCl induced fibrosis.

Role of the Ser/Thr PIM-3 kinase in the aldosterone-regulated renal salt handling

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The kidneys play a central role in blood pressure regulation as defaults in maintaining salt balance can result in the development of hypertension, the most common disease in the human population. The renin-angiotensin-aldosterone axe (RAAS) plays a crucial role in salt handling. Aldosterone is the key hormone in the control of sodium balance, blood volume and blood pressure, acting in the aldosterone-sensitive distal nephron (ASDN) and stimulating a complex transcriptional, translational and cellular program.

We have carried out a gene expression profiling in a mouse cortical collecting duct cell model (mpkCCD), stimulated or not by aldosterone and identified the PIM-3 Ser/Thr kinase as a novel aldosterone-induced protein. PIM kinases (PIM-1, -2 and -3), and in particular PIM-3, are overexpressed in different tumors. PIM-3, the only PIM kinase family member expressed in the kidney, has similar substrate specificities to other Ser/Thr kinases (e.g. SGK1, PKB) but its role in the kidney is largely unknown.

Here we studied a possible new role of PIM-3 in the regulation of renal salt handling, both *in vitro* ($mCCD_{cl1}$ cells) and *in vivo* (PIM-3 KO mice). In mCCD cells, we confirmed that PIM-3 expression is stimulated by aldosterone and observed that shRNA-based suppression of PIM-3 reduces basal and aldosterone-induced sodium currents as well as the activation of the aldosterone-mineralocorticoid receptor pathway. In PIM-3 KO mice, we found increased circulating renin activity and elevated plasma aldosterone levels compared to

control littermates. Moreover, preliminary measurements of metabolic parameters indicate that sodium handling is impaired in PIM-3 KO mice.

In conclusion, our data suggest a possible novel function of the PIM-3 kinase and its potentially important role in the control of sodium homeostasis and blood pressure.

Towards panel of plasma biomarkers of endothelial dysfunction by LC/MS/MS technique

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The introduction of biomarkers to the clinic as a diagnostic tool requires an accurate, robust and high throughput methods. Endogenous peptides play an important role in the homeostasis in the human body. Some of them are very potent and are circulating in body fluids at low concentrations. Among common the angiotensins and endothelins isoforms are implicated in normal physiological and pathophysiological processes. A multiplexed MRM based assay for endothelin-1, endothelin-2, endothelin-3 and big endothelin-39 and angiotensin (Ang) I, Ang II, Ang 1-7, Ang III and Ang IV and Ang 1-5 in biological samples has been developed for preclinical research. A micro flow system using ultra-high performance LC (Dionex, USA) and QTRAP 5500 (ABSciex, USA) triple quadrupole mass spectrometer provided robustness of the assay. The analysis is isoform-specific and employs solid phase extraction C18 cartridges (Strata X, Phenomenex, USA) and separated by a reverse-phase C8 column (ACE C8-300, Scotland) using acetonitrile in water with 0.1% formic acid as a mobile phase. Endothelin and angiotensin peptides are ionized by electrospray in the positive ion mode $(M+3H)^{3+}$ and $(M+2H)^{2+}$ and fragmented ions are chosen for detection. Linear responses ($r > 0.98$) are obtained for peptide targets with picomole level limits of quantification. Targeted simultaneous

Development of cleavage specific monoclonal antibodies against the γ subunit of human ENaC

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In the kidney, aldosterone regulates sodium excretion through activation of the epithelial sodium channel (ENaC) expressed in the distal nephron and collecting ducts. ENaC is comprised of the three subunits alpha, beta and gamma, and the activity of the channel is affected by proteolytic cleavage of the alpha and gamma subunits at specific sites. In the γENaC subunit, dual cleavage in the extracellular domain by the intracellular pro-protein convertase furin and extracellular proteases, e.g. kallikrein and prostasin, has been shown to activate ENaC through release of an inhibitory tract peptide. To test the sequence-specific cleavage of ENaC by furin and intracellular proteases in human kidneys, we used immunogenic peptides surrounding the proposed cleavage sites in γENaC and generated a panel of mouse monoclonal antibodies. Clones were selected based on their positive and negative reactivity towards peptide mimicking uncleaved and cleaved γENaC. We isolated single clones, which recognized γENaC cleaved at the furin site, γENaC uncleaved at

the prostasin/kallikrein site, and γENaC cleaved at the kallikrein/prostasin cleavage site. Immunohistochemical labelling of human kidney section showed immunoreactive labelling of the distal nephron and collecting ducts, but with different sub-cellular localization. Western blot of human kidney cortex homogenates showed immunoreaction with bands corresponding to both intact γENaC and cleavage at the proposed cleavage sites for furin and prostasin/kallikreins. In conclusion, we have developed a panel of monoclonal antibodies, which allows the detection and quantification of proteolytically activated human γENaC.

Lipocalin-2 is a target of aldosterone action in adipose tissue: clinical and experimental studies

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Objective: In the last decade, animal and cross-sectional human studies have evidenced a strong correlation between circulating levels of aldosterone (aldo), lipocalin-2 (NGAL) or the complex NGAL-matrix metalloproteinase-9 (MMP9), and the prevalence of obesity, hyperglycemia and insulin resistance. Previously, our laboratory has demonstrated that NGAL is a primary target of aldosterone action in cardiovascular cell types, i.e., endothelial and vascular smooth muscle cells, and cardiomyocytes. Our hypothesis is that NGAL may also be a primary target of aldosterone in adipocytes and that the mechanism behind their clinical association would better explain their role in pathophysiology of adipose tissue in metabolic diseases.

Design: In order to support our hypothesis, we used multiple approaches: 1) analysis of aldo/NGAL/NGAL-MMP9 serum concentrations in a cross sectional study in 134 patients with or without abdominal obesity; 2) study of the effect of treatment with mineralocorticoid receptor antagonist (MRA) on NGAL serum concentration in obese db/db mice; 3) exploration of the mechanism linking aldosterone action and expression/secretion of NGAL in adipose tissue in adipo-MR mice, a transgenic mouse model designed to conditionally overexpress aldosterone specific receptor, i.e., mineralocorticoid receptor (MR), only in adipocytes.

Results: In patients, aldosterone and NGAL-MMP9 were positively correlated each other (r_s=0.30, p<0.0004) and independently correlated with BMI (respectively, r_s=0.35, p<0.0001 and r_s=0.47, p<0.0001) and HOMA index (respectively, r_s=0.33, p=0.0001; r_s=0.24, p=0.007). Db/db mice, treated 8 weeks with MRA, presented reduced NGAL circulating levels (3 fold reduction, p<0.01) and decreased NGAL mRNA levels in visceral adipose tissue (3 fold reduction, p<0.001). In transgenic adipo-MR mice, MR was overexpressed in perirenal visceral, subcutaneous and brown adipose tissues (3-7 fold increase, p<0.05), and in primary cultures of adipocytes derived from adipo-MR mice (20-30 fold increase, p <0.05). This was always coupled with a significantly rise in NGAL mRNA levels (2-8 fold increase, p<0.05).

Conclusion: Our results in clinical studies confirm a strong correlation between high levels of aldosterone, NGAL, overall fat mass and prevalence of metabolic diseases. We demonstrated a relationship between MR

activation in adipose tissue and NGAL expression. However, further analyses are required to better understand at molecular level the mechanism leading aldosterone to enhance NGAL expression and secretion in adipose tissue, and the relative contribution of this process to adipocytes physiology, i.e., insulin sensitivity and adipokines expression.

MECHANISMS OF RENAL CONTROL OF POTASSIUM HOMEOSTASIS IN COMPLETE ALDOSTERONE DEFICIENCY

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ABSTRACT

Aldosterone stimulates potassium (K^+) excretion in kidney and colon. To define the role of aldosterone in K^+ homeostasis, we studied aldosterone-synthase knock-out ($AS^{-/-}$) mice. $AS^{-/-}$ mice were normokalemic and tolerated a physiological dietary K^+ load (2% K^+ for 2 days) without any signs of illness. Urinary K^+ excretion rose and plasma K^+ levels remained in the normal range. However, with supraphysiological high K^+ intake (5% K^+), $AS^{-/-}$ mice decompensated and became hyperkalemic. The K^+ diet induced up-regulation of the renal secretory K^+ channel ROMK occurred in all K^+, diets, but sufficient up-regulation of the epithelial sodium channel ENaC, providing the electrochemical driving force for K^+ excretion, was detected in $AS^{-/-}$ mice only with 2% but not with 5% K^+ diet. The aldosterone-independent residual renal ENaC activity required angiotensin II (ANG II) as inhibition of the ANGII receptor AT1R diminished any detectable apical ENaC localizatrion and caused severe hyperkalemia in $AS^{-/-}$ but not in wildtype ($AS^{+/+}$) mice. Maintenance of K^+ homeostasis in $AS^{-/-}$ mice was likely also supported by a downregulation of the thiazide-sensitive NaCl cotransporter increasing the intratubular availability of Na^+ that can be reabsorbed in exchange for K^+ secreted. In contrast to the kidney, the distal colon of $AS^{-/-}$ mice did not respond to dietary K^+ loading as evident from distal colon Na^+ and K^+ channel activities assessed in Ussing-type chambers. Thus, renal adaptation to physiological potassium load can be achieved by aldosterone-independent activation of ROMK and a residual activity of ENaC to which ANG II may contribute. Furthermore, an enhanced urinary flow and a reduced thiazide-sensitive NaCl cotransporter activity may improve renal K^+ excretion in complete aldosterone deficiency.

Effects of MR activation on contractility and Ca^{2+} cycling in rat cardiomyocytes of endo- and epicardial origin

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Activation of the cardiac mineralocorticoid receptor (MR) has been shown to increase the L-type Ca^{2+} current (I_{CaL}), a key player in Ca^{2+} homeostasis and hence, regulation of cardiac contractility. Within the left ventricular free wall a marked gradient of action potential (AP) waveforms is present, with short AP duration (APD) in epicardial and long APD in endocardial myocytes. As a consequence, the Ca^{2+} influx induced by the AP is markedly larger in endocardial than in epicardial myocytes. We demonstrated that MR activation increases I_{CaL} in a similar way in endocardial and epicardial myocytes. Since the epicardial AP was markedly more prolonged by MR activation than the endocardial AP, AP-induced Ca^{2+} influx was increased 4-fold ($p<0.001$) in epicardial myocytes, while in endocardial myocytes only a 1.8-fold increase (n.s.) was noted. Here we investigate region specific effects of these changes in the transmembrane Ca^{2+} flux on the intracellular Ca^{2+} transient and unloaded fractional shortening (FS) in endocardial and epicardial myocytes isolated from the left ventricular free wall of the rat heart. Sarcomere length was assessed simultaneously with Ca^{2+} transients, which were measured ratiometrically using fura2-AM and are given as fura-ratio. After incubation for 24h under control conditions, I_{CaL} was similar in endocardial (at 0mV: -7.8±0.5pApF^{-1}, n=27) and epicardial (-8.2±0.3pApF^{-1}, n=33, n.s.) myocytes. Despite the longer APD in endocardial myocytes, the amplitude of the Ca^{2+} transient (endo: 0.20±0.01, n=52, epi: 0.23±0.02, n=44, n.s.), its decay time constant (endo: 0.72±0.04s, n=52, epi: 0.63±0.03s, n=44, n.s.), and FS (endo: 5.0±0.5%, n=52, epi: 5.1±0.5%, n=44, n.s.) were similar in endocardial and epicardial myocytes after 24h incubation under control conditions, as were the baseline of Ca^{2+} levels and sarcomere length. After 24h of MR stimulation, I_{CaL}, Ca^{2+} transients and FS were affected in a similar way in epi- and endocardial myocytes: in epicardial myocytes, I_{CaL} was increased by 49% to 12.2±0.7pApF^{-1} (n=36, $p<0.001$) while in endocardial myocytes the increase was 45 % (11.4±0.5pApF^{-1}, n=37, $p<0.001$). In epicardial myocytes the Ca^{2+} transient amplitude increased by 43% to 0.33±0.02 (n=52, $p<0.001$) and the increase was even higher in endocardial myocytes (+70%, 0.34±0.02, n=48, $p<0.001$). The decay of the Ca^{2+} transient was significantly accelerated in endocardial myocytes (0.53±0.04s, n=48, $p<0.001$), which was visible as trend also in epicardial myocytes (0.52±0.02s, n=52, n.s.). Baseline Ca^{2+} levels were not affected by MR stimulation. FS was increased by 48% (7.4±0.5%, n=52, $p<0.05$) in epicardial and by 74% (8.7±0.7%, n=48, $p<0.001$) in endocardial myocytes. To examine whether the increase in the Ca^{2+} transient was due to an increased filling of the SR, its Ca^{2+} content was assessed as the increase of the fluorescence ratio induced by a 10mM caffeine pulse after steady-state pacing. SR filling was similar in endo- and epicardial myocytes under control conditions after 24h (endo: 0.81±0.05, n=33, epi: 0.67±0.04, n=33, n.s.). MR activation only slightly influenced SR filling (endo: 0.85±0.03, n=36, n.s., epi: 0.77±0.04, n=33, n.s.). Taken together, these data indicate that the increase I_{CaL} density predicts the height of the Ca^{2+} transient and contractility in rat left ventricular endocardial and epicardial cardiomyocytes under control conditions and upon MR activation independently of differences in APD and consequently AP-induced Ca^{2+}influx.

Regulation of the mineralocorticoid receptor expression and aldosterone production by AMP-activated protein kinase

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Background

Adipose tissue dysfunction is implicated in the development of obesity-related disorders such as diabetes and hypertension. The local renin-angiotensin-aldosterone system (RAAS) appears to play a role in the development of inflammation and insulin resistance in adipose tissue and as such may be an important target for future therapies. The energy sensor AMP-activated protein kinase (AMPK) improves insulin sensitivity when activated by low energy states, and has also been shown to have anti-inflammatory actions and to prevent deleterious effects of aldosterone in the kidney.

Aim

To determine the effects of AMPK activation on mineralocorticoid receptor expression and aldosterone production in adipocytes.

Methods

Differentiated 3T3-L1 (murine) and SW872 (human) adipocytes were stimulated with an AMPK activator (either AICAR or A769662) for 4, 8 and 24 hours. Adipose tissue depots were isolated from AMPK alpha1$^{-/-}$ knockout and wild type littermate control mice. Gene expression was analyzed by real time PCR. Culture medium was collected following cell stimulations and aldosterone levels measured by ELISA.

Results

Mineralocorticoid receptor (MR) mRNA levels were increased twofold in murine and human adipocytes compared to pre-adipocytes. This was down-regulated following stimulation with A769662 (50 ± 7%) and AICAR (40 ± 15%), $p<0.05$. The glucocorticoid receptor was not affected by the differentiation process or exposure to AMPK activators. Expression of MR was unchanged between different adipose tissue depots (subcutaneous, mesenteric and epididymal) from AMPK alpha1$^{-/-}$ knockout mice and littermate controls. AICAR treatment stimulated aldosterone secretion from 3T3-L1 adipocytes (42 ± 12% increase, $p<0.05$). This was associated with a trend towards increased expression of the steroid precursor StAR ($p=0.057$).

Conclusion

Obesity is an increasingly important health concern worldwide associated with aldosterone excess. Our preliminary data showed that expression of MR is increased in differentiated adipocytes and decreased by AMPK activators. Interestingly, the AMPK activator AICAR increased aldosterone secretion in adipocytes. This may be a physiological mechanism to decrease blood flow to adipose tissue in low energy states such as starvation when storage of fats and glucose is not essential, further studies are required to determine whether this is an AMPK-dependent process.