Obesity-stimulated aldosterone release is not related to an S1P-dependent mechanism

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Abstract

Aldosterone has been identified as an important factor in obesity-associated hypertension. Here, we investigated whether sphingosine-1-phosphate (S1P), which has previously been linked to obesity, increases aldosterone release. S1P-induced aldosterone release was determined in NCI H295R cells in the presence of S1P receptor (S1PR) antagonists. In vivo release of S1P (100–300 µg/kg_{bw}) was investigated in pithed, lean Sprague Dawley (SD) rats, diet-obese spontaneous hypertensive rats (SHRs), as well as in lean or obese Zucker rats. Aldosterone secretion was increased in NCI H295R cells by S1P, the selective S1PR1 agonist SEW2871 and the selective S1PR2 antagonist JTE013. Treatment with the S1PR1 antagonist W146 or fingolimod and the S1PR1/3 antagonist VPC02319 decreased baseline and/or S1P-stimulated aldosterone release. Compared to saline-treated SD rats, plasma aldosterone increased by ~50 pg/mL after infusing S1P. Baseline levels of S1P and aldosterone were higher in obese than in lean SHRs. Adrenal S1PR expression did not differ between chow- or CD-fed rats that had the highest S1PR1 and lowest S1PR4 levels. S1P induced a short-lasting increase in plasma aldosterone in obese, but not in lean SHRs. However, 2-ANOVA did not demonstrate any difference between lean and obese rats. S1P-induced aldosterone release was also similar between obese and lean Zucker rats. We conclude that S1P is a local regulator of aldosterone production. S1PR1 agonism induces an increase in aldosterone secretion, while stimulating adrenal S1PR2 receptor suppresses aldosterone production. A significant role of S1P in influencing aldosterone secretion in states of obesity seems unlikely.

Key Words

- ▶ renin–angiotensin system
- ▶ obesity
- ▶ steroids
- ▶ hypertension
- ▶ sphingosine-1-phosphate

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Introduction

The Framingham Offspring Study suggested that increased abdominal fat mass accounts for 65–75% of the risk for hypertension (Garrison *et al.* 1987). Among other hormonal systems, the renin–angiotensin–aldosterone system (RAAS) has been established as a major determinant of obesity-induced hypertension (Rahmouni *et al.* 2005). The RAAS is activated in the fat

tissue of obese rats and humans (Giacchetti *et al.* 2002, Boustany *et al.* 2004, Engeli *et al.* 2005). Accordingly, ACE is upregulated in adipocytes (Bloem *et al.* 1995, Cooper *et al.* 1998, Karlsson *et al.* 1998), and obese individuals demonstrate elevated circulating angiotensin II (AngII) levels (Harte *et al.* 2005). In contrast, weight loss not only normalizes enzyme activity of the RAAS but

also attenuates blood pressure (Engeli *et al.* 2005). The potency of AT₁ receptor blockers to lower body weight particularly in obese individuals and the findings that body weight of double AT_{1A}/AT_{1B} receptor knockout mice is lower than in wild-type controls further strengthen the functional cross-talk among weight, blood pressure regulation and RAAS activity (Gembarde *et al.* 2008, Müller-Fielitz *et al.* 2015).

Aldosterone is the second key player of the RAAS. Aldosterone production in zona glomerulosa (ZG) cells is regulated by distinct extracellular stimuli (potassium, AngII, ACTH) via Ca²⁺ mobilization following cAMP-dependent (ACTH) or -independent (AngII, potassium) mechanisms. Aldosterone plasma levels positively correlate with fat mass in obesity (Lamounier-Zepter *et al.* 2006, Krug & Ehrhart-Bornstein 2008) and with obesity-induced hypertension (Kidambi *et al.* 2007). The following findings indicate that aldosterone excess is not an epiphenomenon to higher AngII levels, but rather results from adrenal hypersensitivity to AngII among overweight individuals: (1) AngII-mediated aldosterone secretion was higher in obese than in lean individuals (Bentley-Lewis *et al.* 2007, Müller-Fielitz *et al.* 2012b); (2) adipocytes secrete mineralocorticoid-releasing factors (MRFs), which themselves enhance aldosterone release independently of AngII (Ehrhart-Bornstein *et al.* 2003, 2004); (3) adipocytes sensitize adrenocortical cells to AngII via ERK1/2-dependent upregulation of steroidogenic acute regulatory (StAR) protein (Krug *et al.* 2007); (4) ZG growth and the expression of aldosterone synthase and AT_{1B} receptors were selectively heightened in obesity under concomitant AngII stimulation, which further enhanced circulating aldosterone levels and augmented blood pressure response to AngII (Müller-Fielitz *et al.* 2012b); and (5) the rise in blood pressure after chronic AngII stimulation was diminished in adrenalectomized obese Zucker rats in which plasma aldosterone levels could only be scarcely detected (Müller-Fielitz *et al.* 2012b).

However, MRFs are still being identified, including at least two that differ in heat sensitivity and molecular mass (< or >50 kDa) (Ehrhart-Bornstein *et al.* 2003, 2004). Based on observations that plasma LDL is increased in hypertensive obese patients, Ehrhart-Bornstein's group also showed that LDL dramatically increased aldosterone release in human adrenocortical NCI H295R cells. Thus, aldosterone excess in hypertensive individuals may partially result from elevated LDL levels. However, it was speculated that other MRFs may also be involved (Lamounier-Zepter *et al.* 2006). Since the identified MRF was shown to increase aldosterone release via

ERK1/2-dependent upregulation of StAR activity (Krug *et al.* 2007) and S1P induces both StAR mRNA expression and ERK1/2 activation (Brizuela *et al.* 2007, Lucki *et al.* 2012), we hypothesized that S1P acts as an MRF. The molecular mass of S1P is <50 kDa and it is heat-sensitive (>99°C), both thought to be essential features of MRF (Lamounier-Zepter *et al.* 2006). S1P is a bioactive sphingolipid and interacts with its particular G_i protein-coupled receptors, termed S1PR1-5 (Maceyka & Spiegel 2014, Proia & Hla 2015). S1PRs are highly expressed in the human adrenal gland (Wang *et al.* 2014). S1P interacts not only in cellular processes of immunity, inflammation and inflammatory disorders (Maceyka & Spiegel 2014, Proia & Hla 2015), but is also involved in cardiovascular and metabolic functions (Bellini *et al.* 2015, Borodziejcz *et al.* 2015). Interestingly, there is clear evidence that S1P levels are higher in plasma and adipose tissue of obese subjects than in lean individuals (Samad *et al.* 2006, Blachnio-Zabielska *et al.* 2012a, Kowalski *et al.* 2013), thus supporting the hypothesis that S1P may indeed be the missing link between obesity and hypertension due to adrenal hypersensitization. Thus, we hereby aimed to investigate whether S1P stimulates aldosterone release in a human adrenocortical cell line and, if so, which S1PR would be involved. Furthermore, S1P-induced aldosterone release was expected to be higher in obese than in lean rats.

Materials and methods

Animals

The study was carried out according to the National Institutes of Health guidelines for the care and use of laboratory animals. Animal care and experimental procedures were approved by the ethics committee of the local regulatory authority. Male SD rats, SHR and lean and obese Zucker rats were used in the experiments (all from Charles River, Sulzberg, Germany). The animals were kept at room temperature with a 12-h/12-h dark (14:00–02:00 h)/light (02:00–14:00 h) cycle.

Preparation of S1P stock solution

S1P was obtained from Avanti Polar Lipids (Alabaster, AL, USA). A methanolic S1P stock solution was prepared. S1P (0.5 mg/mL) was added to a methanol/water mixture (95/5), heat-mixed (50°C) and sonicated until S1P was suspended. Subsequently, 100 µL aliquots of this mixture were stored (–20°C) in glass tubes. For usage,

aliquots were warmed up to room temperature and the methanol/water were evaporated under nitrogen flow. The dry residue was dissolved in saline (0.9%), supplemented with bovine serum albumin (BSA, 4 mg/mL).

Cell culture

In vitro assays of S1P-induced aldosterone release were performed by using the human adrenocortical carcinoma NCI H295R cell line. Only cells originating from the 4th to 20th passage were used. In accordance with previous work, cells were grown in 20 mL Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12; from VWR International, Darmstadt, Germany) containing 15 mmol/L HEPES and 2.5 mmol/L L-glutamine, supplemented with 1.125 g/L NaHCO₃, insulin (66 nmol/L), hydrocortisone (10 nmol/L), 17 β -estradiol (10 nmol/L), transferrin (10 mg/mL), selenite (30 nmol/L), penicillin (100 U/mL), streptomycin (100 mg/mL) and 2% fetal bovine serum (Krug *et al.* 2007). NCI H295R cells were grown in 75-mL flasks at 37°C in a humidified CO₂/O₂ atmosphere (5%/95%). The medium was changed every 2 days, and cells were subcultured every 7 days using Accutase (PAA Laboratories, Pasching, Austria) for cell detachment. The cell suspension was centrifuged (70 g, 6 min, 21°C), and the cell pellet was then incubated with 100 μ L AccuMax (PAA Laboratories) and resuspended with medium to achieve a cell density of 75000 cells/mL. Of this cell suspension, 2 mL was used for subculture in 75-mL flasks containing 20 mL medium. For experiments, 500- μ L portions of the cell suspension (and 500 μ L additional medium) were incubated in 24-well culture plates for 96 h. After 95% cell confluence was reached, the cells were used for experiments.

Aldosterone release from the human adrenocortical carcinoma cell line NCI-H295

Cells were purged with PBS buffer (PAA Laboratories) twice and then 500 μ L medium supplemented with 0.01–10 μ M S1P, 0.1–30 μ M SEW2871 (Tocris, Wiesbaden, Germany), 1–30 μ M FTY720 (Tocris), 20 μ M forskolin (Sigma-Aldrich) or 0.01–10 μ M AngII was added. To confirm AT₁ receptor specificity for AngII-induced aldosterone release, additional experiments were performed in the presence of 10 μ M losartan. S1PR specificity in aldosterone release was determined by preincubation with the S1PR1 antagonist W146 (10 μ M, Tocris), the S1PR2 antagonist JTE013 (10 μ M, Tocris) and the S1PR1/3 antagonist VPC02319 (10 μ M, Avanti Polar Lipids) or a combination of the aforementioned receptor ligands. Control experiments

were performed without S1P antagonists. After 30-min preincubation, medium was removed and 500 μ L fresh medium containing the appropriate S1P antagonist and 10 μ M S1P or vehicle was added. After 24 h, medium was collected and stored at –20°C until aldosterone analyses. Cells were washed twice with 500 μ L PBS buffer and also stored at –20°C until protein was quantified according to Lowry.

Pithed rat preparation

Studies on aldosterone response to S1P were performed in a 'pithed rat' model, to only consider vascular reactivity to S1P for blood pressure effects. Rats were pithed at the beginning of the light cycle as previously described (Raasch *et al.* 2003, Müller-Fielitz *et al.* 2012b). Briefly, animals were anesthetized with ether and artificially respired. The medulla and thoracolumbar portions of the spinal cord were destroyed using a steel pithing rod. Catheters were placed into a carotid artery and both femoral veins. Both vagal nerves were severed. Blood pressure was measured via the carotid catheter. After preparation, pithed rats were allowed to recover for approx. 1 h until blood pressure and heart rate were constant. Thereafter, S1P was injected.

S1P effects on plasma aldosterone in pithed SD rats

S1P was intravenously injected as a bolus within 30 s (100 or 200 μ g/kg). Then, 1 μ g was injected per kg body weight. S1P stock solutions were prepared as described earlier and further diluted to final concentrations with BSA (4 mg/mL in saline) in glass vessels by vortexing (30 min, 37°C). Control animals only received vehicle. At baseline and 3, 5, 10, 15 and 20 min after injections, 200- μ L blood samples were drawn via the femoral vein catheter to determine plasma aldosterone and blood glucose levels. Volume depletion was compensated by infusing hydroxyethyl starch (6%; Fresenius, Homburg, Germany) over 30 s. For control experiments, additional SD rats received AngII (10 or 30 μ g/kg) and blood samples were acquired in the same manner for aldosterone and glucose analyses. After the experiments, rats were euthanized by decapitation.

S1P effects on plasma aldosterone of diet-induced obese, pithed rats

Aldosterone release was also investigated in a nongenetic obese rat model. Here, SHR_s ($n=12$ in each group) were

fed with a high-caloric cafeteria diet (various chocolate/cookie bars) for 42 weeks. The diet was abundantly offered, supplementary to chow, whereas controls received chow only (Miesel *et al.* 2010). At regular intervals, blood pressure and heart rate were determined by plethysmography (Raasch *et al.* 2002) and blood samples were withdrawn from a tail nick to measure blood glucose, plasma aldosterone and S1P levels. For S1P-induced aldosterone release, 200 µg/kg S1P was intravenously injected as described earlier in chow- vs CD-fed SHR. Procedures for blood sampling and determining aldosterone and glucose levels were similar to the protocol used in SD. After decapitation, adrenals were harvested for S1PR mRNA quantification.

S1P effects on plasma aldosterone in lean and obese Zucker rats

One hour after rats had been pithed, vehicle (1 µL/kg_{bw}) was injected. A 200-µL blood sample was drawn before and 3, 5, 10 and 15 min later. Then, 20, 40 and 60 min after administering vehicle, we repeatedly injected S1P at increasing doses (100, 200 and 300 µL/kg_{bw}, respectively). Again, 200 µL blood was sampled 3, 5, 10 and 15 min after each S1P injection. Each blood withdrawal was compensated by an infusion of 200 µL hydroxyethyl starch (6%; Fresenius, Homburg, Germany) over 30 s.

Biochemical analysis

Plasma concentrations of aldosterone (07-108202, MP Biomedicals, Germany), corticosterone (07-120103, MP Biomedicals, Germany) and AngII (ED29051, IBL, Germany) were determined by radioimmunoassays. Assays were performed as recommended by the manufacturer. Blood glucose was determined using glucose sensors (Ascensia ELITE, Bayer, Leverkusen, Germany).

RNA isolation and cDNA synthesis

Total RNA from one adrenal was extracted on the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems). The amount of total RNA was determined using a RiboGreen RNA quantitation assay (Invitrogen). Isolation of genomic DNA was avoided by thorough treatment with DNase I. First-strand cDNA was synthesized using oligo-(dT)15 primer and AMV reverse transcriptase (Invitrogen). cDNA was stored at -20°C until further analysis.

Quantitative real-time PCR (qPCR)

mRNA levels were quantified by quantitative real-time polymerase chain reaction using SYBR Green reagent (Invitrogen) on an ABI Prism 7000 platform (Applied Biosystems) according to established, standard protocols and using primers for S1PR1 (sense: 5'-GCATCCCAGTGGTTAAGGCT-3, antisense: 5'-TCCTGCTAACAGGTCCGAGA-3), S1PR2 (sense: 5'-CTTCATCACGCTCTCTGCCT-3, antisense: 5'-GCCACCCAGAATCAGCGATA-3), S1PR3 (sense: 5'-CAGATGAGCCTTGC AGAACG-3, antisense: 5'-AGTATCATTTCCCCAAGACTGGC-3), S1PR4 (sense: 5'-CATTGG GACAAGCAACCTGC-3, antisense: 5'-CTGTGTGGTTTCGCAGCTGTA-3) and S1PR5 (sense: 5'-gtgctccgtggacatctggcagga-3, antisense: 5'-caggttctccagcacgatga-3). All primers were obtained from Invitrogen.

Determination of S1P in plasma of DIO rats

S1P was quantified in plasma according to a modified method of Butter (Butter *et al.* 2005) after liquid/liquid plasma extraction; then, reversed-phase isocratic HPLC was performed with a precolumn derivatization with o-phthalaldehyde (OPA) and fluorescence detection (ex/em 340/455 nm). For S1P extraction, a 100-µL plasma sample (or spiked standard samples) was diluted with 100 µL methanol (containing 100 ng/mL C17-S1P (Avanti Polar Lipids) as internal standard) and 900 µL NaCl (1 mmol/L) and was mixed with 10 µL HCl (37%) and 3000 µL dichloromethane/2-propanol (1/1) in an 8-mL glass tube for 10 min. Afterward, the mixture was centrifuged (15 min, 2000 g, 19°C). The aqueous phase was discarded and 2 mL of the organic phase was dried under nitrogen evaporation. The dry residue was resolved with 400 µL of the mobile phase. An aliquot of 50 µL was mixed for 30 min with 300 µL mobile phase and 20 µL OPA reagent. The OPA reagent contained 100 µL OPA stock solution (50 mg in 5 mL ethanol), 5 µL mercaptoethanol and 5 mL 500 mM borate buffer (pH 10.4). Subsequently, 20 µL was injected onto the columns (LUNA C18(2), 3 µm, 75 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) at 40°C by using a mixture of 220 mL K₂HPO₄ (0.07 mol/L, pH 7.2) and 780 mL methanol as mobile phase (flow rate: 0.6 mL/min). Each sample was derivatized in triplicate with OPA and injected into the HPLC system. Means were included in the evaluations. Peak areas of chromatograms were considered for calculating individual plasma concentrations by using

the method of external standards of S1P. Linearity between peak area and concentrations of S1P were assured within a concentration range of 3.25–200 ng/mL. Standard curves were determined in triplicate within a concentration range of 3.25–200 ng/mL to assure precision and reproducibility during measurements. Slopes (13583 ± 66) and goodness of curve fitting ($r = 0.9994 \pm 0.0002$) did not differ between each standard curve.

Calculations and statistics

Means and upper and lower quartiles are depicted as boxplots. Whiskers indicate variability between the 10th and 90th percentiles. In line graphs and tables, data are expressed as means $\pm$ S.E.M. Differences were considered to be statistically significant at $P < 0.05$.

In cell culture experiments, each experiment yielded 3–6 values for each stimulation condition. Each experiment was independently conducted thrice ($n = 3$). Means were calculated from the values of each condition for every experiment separately and were normalized for mean basal secretion. Data are presented as means of three independent repetitions. Groups were compared using paired *t*-test (2 groups) or one-way randomized-block ANOVA with subsequent Bonferroni's test for multiple comparisons as *post hoc* test (> 2 groups). Concentration response curves were calculated by GraphPad Prism 5 for Windows by nonlinear regression analyses using individual means. Results are presented as EC_{50} or IC_{50} values including 95% confidence intervals.

For *in vivo* experiments, statistical analysis was performed by ANOVA, followed by appropriate *post hoc* tests (Bonferroni or Dunnett). Wilcoxon Signed-Rank test was used when variances differed significantly between groups. A 2-ANOVA, followed by Bonferroni's or Dunnett's *post hoc* test for multiple comparisons, was performed to examine the effects of two variables. All correlation analyses were performed by the two-tailed Pearson test.

Results

Aldosterone release from the human adrenocortical carcinoma cell line NCI H295R

AngII dose dependently enhanced aldosterone release from NCI H295R. Losartan blocked AngII-induced effects in aldosterone, confirming the AT_1 -dependent mechanism (Fig. 1A). As a second control, aldosterone release was demonstrated to be increased by forskolin (Fig. 1B and C).

Aldosterone release was also increased by S1P (Fig. 1B) and S1PR1 agonist SEW2871 (Fig. 1C) in concentrations $> 3 \mu M$. However, the magnitude of the secretion rate was lower than for AngII or forskolin. In contrast, inverse S1PR1 agonist FTY720 dose dependently attenuated aldosterone release by approx. 60% (Fig. 1D). To discover which S1PR might be involved, aldosterone release from NCI H295R was determined in the presence of various S1P antagonists under baseline conditions and costimulations with S1P. The specific S1P1 antagonist W146 inhibited aldosterone release in the presence or absence of S1P (Fig. 2A). In contrast, aldosterone release was enhanced by the S1PR2 antagonist JTE013 independently of costimulation with S1P (Fig. 2B). VPC02319 was also supposed to lower aldosterone release, although secretion only tended to be reduced in the presence of S1P (Fig. 2C). The combination of VPC02319 and JTE013 reduced aldosterone release below baseline conditions. Since aldosterone secretion in the presence of S1P exceeded secretion at baseline conditions despite the double blockade VPC02319 and JTE013, we presume that S1PR4 or S1PR5 is involved in aldosterone release (Fig. 2D).

In vivo effects of S1P on plasma aldosterone

Baseline aldosterone levels were similar for controls and SD rats that were treated with $100 \mu g/kg_{bw}$ S1P (205 ± 19 vs 192 ± 24 pg/mL, $P > 0.05$), but slightly lower in rats that were treated with $200 \mu g/kg_{bw}$ S1P (211 ± 19 vs

Figure 1

Aldosterone release in response to 24 h of incubation with AngII (A, $n = 4$), S1P (B, $n = 9$), S1PR1-agonist SEW 2871 (C, $n = 9$) and FTY720 (D, $n = 8$) in NCI H295R cells. Forskolin (FC, $20 \mu M$) was used as a positive control. Means $\pm$ S.E.M., * $P < 0.05$ vs controls tested by ANOVA and Bonferroni's (A: $P_{AngII} < 0.0001$, D: $P_{FTY720} < 0.0001$) or Wilcoxon Signed-Rank Test (B, C).

Figure 2

Effects of S1PR1 antagonist W146 (A, $P_{\text{treatment}} = 0.0014$), S1PR2 antagonist JTE013 (B, $P_{\text{treatment}} = 0.0001$), S1PR1/3 antagonist VPC02319 (C, $P_{\text{treatment}} = 0.0011$) and the combination of VPC02319 and JTE013 (D, $P_{\text{treatment}} = 0.0001$) on aldosterone release in NCI H295R cells in the presence or absence of S1P (0 vs 10 μM). P -Values given in the figures originated from 2-ANOVA by testing the S1P effects. P -Values given in legends originated from 2-ANOVA by testing the effect of the antagonists. Bonferroni's *post hoc* test for multiple comparisons was only performed if F reached $P < 0.05$ and there was no significant variance inhomogeneity. * $P < 0.05$ vs controls tested by Bonferroni's *post hoc* test.

$132 \pm 20 \text{ pg/mL}$, $P < 0.05$). In response to $200 \mu\text{g/kg}_{\text{bw}}$ S1P, plasma aldosterone increased by approx. 50 pg/mL during the observation period of 20 min, whereas aldosterone levels of saline-treated SD tended to be decreased (Fig. 3B). This was not observed at $100 \mu\text{g/kg}_{\text{bw}}$ S1P (Fig. 3A). Using 10 or $30 \mu\text{g/kg}_{\text{bw}}$ AngII as a positive control, a marked and long-lasting rise in plasma aldosterone in response to AngII was noted. Both AngII doses had almost the same effects (Fig. 3C). Serving as controls to demonstrate adrenal reactivity, we additionally determined corticosterone levels in AngII-treated SD rats. Baseline levels of corticosterone prior to AngII (10 or $30 \mu\text{g/kg}_{\text{bw}}$) were within the normal range (155 ± 31 and $168 \pm 28 \text{ ng/mL}$). In response to 10 or $30 \mu\text{g/kg}_{\text{bw}}$ AngII, corticosterone increased within 20 min to 382 ± 27 (10 $\mu\text{g/mL}$, $P < 0.05$ vs baseline) and $361 \pm 23 \text{ ng/mL}$ (30 $\mu\text{g/mL}$, $P < 0.05$ vs baseline), which is in line with previous findings (Sowers *et al.* 1981). Blood glucose in SD rats was within the normal baseline range, independently of treatment regimen (saline vs $100 \mu\text{g/kg}_{\text{bw}}$ S1P (78.7 ± 1.5 vs $76.8 \pm 2.5 \text{ mg/dL}$), saline vs $200 \mu\text{g/kg}_{\text{bw}}$ S1P (88.2 ± 3.0 vs $87.1 \pm 2.0 \text{ mg/dL}$) or 10 vs $30 \mu\text{g/kg}_{\text{bw}}$ AngII (77.1 ± 3.6 vs $79.4 \pm 3.5 \text{ mg/dL}$). While glucose was not affected by S1P, glucose increased after AngII, not showing any differences between low and high AngII treatments (Fig. 3). Conspicuously, mean arterial

Figure 3

Effects of $100 \mu\text{g/kg}_{\text{bw}}$ S1P (A), $200 \mu\text{g/kg}_{\text{bw}}$ S1P (B) or AngII (C) on aldosterone (S1P₂₀₀: $P_{\text{time} \times \text{treatment}} = 0.001$, AngII: $P_{\text{time} \times \text{treatment}} = 0.0001$), MAP or glucose in pithed SD rats. Means and upper and lower quartiles are depicted as boxplots. Whiskers indicate variability between the 10th and 90th percentiles. In line graph, data are expressed as means $\pm$ s.e.m. P -Values given in the figures originated from 2-ANOVA by testing the diet effects. P -Values given in legends originated from 2-ANOVA by testing the time effect. Bonferroni's *post hoc* test for multiple comparisons test was only performed if F reached $P < 0.05$ and there was no significant variance inhomogeneity. * $P < 0.05$ vs controls tested by Bonferroni's *post hoc* test, † $P < 0.05$ vs before injection.

pressure (MAP) decreased slightly, but significantly 20 ms after injecting S1P (approx. 5 mmHg), followed by a sharp increase of 25 mmHg (100 µg/kg_{bw} S1P) and 40 mmHg (200 µg/kg_{bw} S1P), respectively, 15 ms later, thus serving as a positive control for S1P function. These diametral effects on blood pressure may be related to different mechanisms since S1P activates vasorelaxation via an eNOS/NO-dependent pathway in endothelial cells and vasoconstriction mediated by RhoA/ROK pathways in smooth muscle cells (Igarashi & Michel 2009). In response to AngII, MAP greatly increased compared to baseline state. Indeed, 30 µg/kg_{bw} AngII was slightly more potent than 10 µg/kg_{bw} AngII.

When SHR_s were fed with CD, the energy intake markedly increased, resulting in obesity and hyperglycemia (Fig. 4). While heart rate is consistently higher in CD- than in chow-fed SHR_s, blood pressure remained almost the same (Fig. 4). At week 28, blood pressure in CD-fed animals tended to be higher than in chow-fed rats ($P=0.063$). Plasma concentrations of S1P and aldosterone at week 42 were higher in CD- than in chow-fed SHR_s (Fig. 5A and B). S1P only tended to correlate with aldosterone in a positive manner (Fig. 5C). To verify whether S1P-induced aldosterone release might be affected by obesity, we determined aldosterone plasma concentrations in response to S1P injections. Consistent with baseline levels after 42 weeks, aldosterone prior to i.v. S1P was higher in CD- than in chow-fed SHR_s (Fig. 6A). Plasma aldosterone was affected by S1P (2-ANOVA: $P_{\text{time}}=0.0017$). In obese, but not in lean SHR_s, a short-lasting increase in plasma aldosterone

was observed. However, 2-ANOVA did not demonstrate any difference for diet ($P^{\text{diet}}=0.179$, Fig. 6C). Despite higher glucose levels before S1P in the obese animals, S1P did not affect blood glucose either in lean or obese SHR_s (Fig. 6B and D). In this experimental setting, S1P induced a short-lasting increase in MAP, showing no difference between lean and obese animals (Fig. 6E). S1PR mRNA levels in adrenal glands did not differ between chow- and CD-fed rats. S1PR1 showed the highest and S1PR4 showed the lowest expression pattern. Interestingly, the S1PR3 expression was reduced in CD-fed rats (Table 1). S1PR5 receptor mRNA could not be detected.

Body weight differed between lean and obese Zucker rats (345 ± 3 vs 511 ± 5 g, $P<0.0001$, $n=11$ each group). In contrast to our findings in lean and obese SHR_s, baseline aldosterone levels before S1P injection did not differ between lean and obese Zucker rats (207 ± 26 vs 229 ± 25 pg/mL, $P=0.54$). In response to vehicle and 100 µg/kg_{bw} S1P, aldosterone time dependently decreased in both lean and obese Zucker rats. After injecting 200 and more distinctly after 300 µg/kg_{bw} S1P, aldosterone exceeded both baseline and vehicle levels, particularly in the obese rats, as 2-ANOVA showed a statistically significant dose effect (Fig. 7A and B). In Zucker rats, S1P-induced aldosterone release was not affected by obesity: the significance level was only 0.54 in 2-ANOVA testing of the corresponding AUCs of the lean vs obese Zucker rats. However, AUC values of obese Zucker rats treated with 300 ng/mL S1P were higher than those in animals receiving saline or 100 ng/mL S1P (Fig. 7C). This was not observed in lean animals.

Figure 4

Influence of CD feeding on body weight (A, $P_{\text{time}}^{\text{2-way ANOVA}} < 0.0001$), energy intake (B, $P_{\text{time}}^{\text{2-way ANOVA}} < 0.0001$), water intake (C, $P_{\text{time}}^{\text{2-way ANOVA}} < 0.0001$), blood pressure (D, $P_{\text{time}}^{\text{2-way ANOVA}} < 0.0001$), heart rate (E, $P_{\text{time}}^{\text{2-way ANOVA}} < 0.002$) and blood glucose (F, $P_{\text{time}}^{\text{2-way ANOVA}} < 0.0001$) in SHR_s. P -Values given in the figures originated from 2-ANOVA by testing the diet effects. P -Values given in legends originated from 2-ANOVA by testing the time effect. Bonferroni's *post hoc* test for multiple comparisons was only performed if F reached $P<0.05$ and there was no significant variance inhomogeneity. * $P<0.05$ vs controls tested by Bonferroni's *post hoc* test. Means $\pm$ s.e.m., $n=12$ each group.

Figure 5

Influence of CD feeding (for 42 weeks) on baseline plasma concentrations of S1P (A) and aldosterone (B) of SHR. Means and upper and lower quartiles are depicted as boxplots. Whiskers indicate variability between the 10th and 90th percentiles; * $P<0.05$ chow vs CD after Student's t -test, $n=11$ each group. Correlation analysis between S1P and aldosterone (C) was performed by the 2-tailed Pearson test.

Discussion

We clearly demonstrated that S1P dose dependently stimulates aldosterone release from adrenocortical cells by using S1P concentrations $>3\mu\text{M}$. We could also verify in the pithed rat model that S1P releases aldosterone via a direct humoral pathway *in vivo*. However, it remains uncertain as to whether S1P increases aldosterone release in obesity, thus acting as an MRF.

S1P effects on aldosterone release *in vitro*

It seems likely that S1PR1 dominates signaling: firstly, this receptor was found to be highly expressed compared to S1PR2, S1PR3 and S1PR4 in adrenals in our study and by others, too (Wang *et al.* 2014); secondly, aldosterone release was strongly stimulated by the specific and high-affinity S1PR1 agonist SEW2871 (EC_{50} 13.6 nM), not activating S1PR2-5 even at a concentration of 10 μM (Hale *et al.* 2004), and inhibited by the S1PR1 receptor antagonist W146 and FTY720 (Fig. 8). W146 specifically blocks S1PR1 (pKi 7.74) but not S1PR2, S1PR3 and S1PR5 (Sanna *et al.* 2006, Gonzalez-Cabrera *et al.* 2008). FTY720, established as fingolimod in treating multiple sclerosis, acts as a functional S1PR1 antagonist after sphingosine

kinase-mediated conversion into FTY720-P. It binds at S1PR receptors and induces its internalization and degradation (Brinkmann 2009). As S1PR3 is closely related to S1PR1 in terms of evolution (Sanchez & Hla 2004), we wanted to determine whether S1PR3 might also be involved. Indeed, the S1PR3 antagonist VPC02319 did lower aldosterone release (Fig. 8). However, VPC02319 unfavorably blocks not only S1PR3 but also S1PR1 (pKi values are 7.86 and 5.93 for S1PR1 and S1PR3 (Davis *et al.* 2005, Foss *et al.* 2007)), thus restricting any conclusion that a decrease in aldosterone secretion is related to S1PR3. To overcome this pharmacological disadvantage, the use of siRNA might represent an appropriate approach to selectively knockdown gene expression of S1PR1 and S1PR3 to clearly determine whether S1PR3 is also involved in aldosterone secretion (van Hooren *et al.* 2014, Li *et al.* 2015). We refrained from performing these experiments as (1) the knockdown efficacy (determined by qPCR or Western blots) was certainly not quantitative, thus limiting this genetic approach (van Hooren *et al.* 2014, Li *et al.* 2015), (2) the relevance of S1PR3 is suggested to be inferior compared to S1PR1 as its adrenal expression is sixfold lower (Table 1) and (3) our data support the findings of others, showing that aldosterone release stimulated by the S1PR1/R3 agonist VPC 24191 was completely abolished

Figure 6

Plasma concentrations of aldosterone and glucose before (A, B) and after S1P injection (200 $\mu\text{g}/\text{kg}_{\text{BW}}$) in chow- or CD-fed pithed SHR. Means and upper and lower quartiles are depicted as boxplots. Whiskers indicate variability between the 10th and 90th percentiles. In line graph, data are expressed as means $\pm$ S.E.M., * $P<0.05$ vs chow by Student's t -test; * $P<0.05$ vs 0 by Wilcoxon Signed-Rank test or Student's t -test.

Table 1 mRNA levels of S1P receptors in adrenal glands of chow- vs CD-fed SHR. Means $\pm$ S.E.M.

Copies/ng RNA	Chow-fed SHRs (n=10)	CD-fed SHRs (n=10)	P
S1P1 ($\times 10^3$)	255.9 $\pm$ 20.1	227.5 $\pm$ 19.1	0.318
S1P2 ($\times 10^3$)	78.6 $\pm$ 7.6	69.4 $\pm$ 7.6	0.404
S1P3 ($\times 10^3$)	42.4 $\pm$ 4.2	31.6 $\pm$ 2.7	0.046
S1P4 ($\times 10^3$)	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1	0.636

by using VPC02319 (Brizuela *et al.* 2007) and that the expression of StAR protein was also enhanced after S1PR1/S1PR3 stimulation (Lucki *et al.* 2012) (Fig. 8). StAR is a transport protein that regulates cholesterol transfer within the mitochondria as the rate-limiting step in the production of steroid hormones. Accordingly, StAR protein and mRNA were elevated in parallel to adipocyte-stimulated aldosterone release (Krug *et al.* 2007). We, however, do concede that results obtained with VPC23019 should be interpreted with caution as suggested recently (Pyne & Pyne 2011, Salomone & Waeber 2011).

S1P enhanced aldosterone release to a lesser extent than forskolin or AngII. This may indicate that aldosterone release by S1P, AngII or forskolin stimulation is related to different signaling pathways. Forskolin and AngII act via the cAMP- or the PLC/IP3/DAG-dependent pathway, which both mobilize intracellular Ca^{2+} (Gambaryan *et al.* 2006). However, S1P – upon binding to the Gi protein-coupled S1PP1/S1PP3 – was claimed to activate not only PI3K/PKB (phosphatidylinositol 3-kinase/protein kinase B), but also MEK/ERK 1/2 (mitogen-activated protein kinase kinase/extracellularly regulated kinases 1/2), which further induces phospholipase D (PLD) activation. This corresponds nicely to the fact that MRF stimulates aldosterone release not via cAMP/PKA but rather via an ERK 1/2-related signaling pathway (Krug *et al.* 2007) (Fig. 8). Moreover, S1P causes calcium increase and activation of the PKC isoforms α and δ , which are upstream of PLD (Brizuela *et al.* 2006, 2007).

Here, we show that S1PR2 exerts inhibitory properties on aldosterone release since the S1PR2 antagonist JTE013 increases aldosterone secretion (Fig. 8). JTE013 is a specific S1PR2 inhibitor (pKi 7.74) by not blocking S1PR1 and S1PR3, respectively (Ohmori *et al.* 2003, Parrill *et al.* 2004). As JTE013 was demonstrated to inhibit vasoconstriction not only to S1P, but also to the prostanoid analog U46619 or endothelin-1, it was claimed that JTE013 elicits its biological function independent of S1PR2 (Salomone & Waeber 2011). However, we demonstrated here that JTE013 enhanced both baseline and S1P-induced aldosterone releases,

Figure 7

Aldosterone levels in response to S1P in lean (A; $P_{\text{S1P dose}} = 0.420$) and obese (B; $P_{\text{S1P dose}} = 0.003$) Zucker rats. AUCs (C; $P_{\text{S1P dose}} = 0.001$) were calculated considering the delta values. In line graph, data are expressed as means $\pm$ S.E.M. Means and upper and lower quartiles are depicted as boxplots. Whiskers indicate variability between the 10th and 90th percentiles. P-Values given in the figures originated from 2-ANOVA by testing the diet effects. P-Values given in legends originated from 2-ANOVA by testing the dose effect of S1P. $^{\dagger}P < 0.05$ vs 0 by Wilcoxon Signed-Rank test; $^*P < 0.05$ vs 0 $\mu\text{g/kg}$ S1P $^{\dagger}P < 0.05$ vs 100 $\mu\text{g/kg}$ S1P by Bonferroni's or Dunnett's *post hoc* test; $n = 11$ each group.

thus strengthening the conclusion that the observed aldosterone response is dependent on S1PR2 and does not likely represent an S1P off target effect. Moreover, it was suggested that JTE013 has S1PR4 antagonistic properties (Pyne & Pyne 2011). Considering that the adrenal

Figure 8

Effects of different S1PR subtypes on aldosterone secretion: Aldosterone release is stimulated by the specific S1PR1 agonist SEW2871 and inhibited by the S1PR1 antagonist W146 and FTY720. VPC02319 lowered aldosterone release, suggesting a functional impact of S1PR3 as VPC02319 similarly blocks S1PR1 and S1PR3. The combination VPC02319 + JTE013 reduced S1P-induced aldosterone secretion, indicating that S1PR4 is also involved, considering, too, that S1PR4 is more likely to be expressed in adrenals than S1PR5. PI3K/PKB (phosphatidylinositol 3-kinase/protein kinase B); MEK/ERK 1/2 (mitogen-activated protein kinase kinase/extracellularly regulated kinases 1/2); phospholipase D (PLD) activation; steroidogenic acute regulatory protein (StAR), — stimulation; ⊥ inhibition.

expression of S1PR4 is lower than that of S1PR2 (Table 1), however, we assume that its functional importance is also minor, which then does not necessarily require specific siRNA knockdown experiments to confirm the pharmacological results. Considering adrenal S1PR1

and S1PR2 expressions (shown here and in Wang *et al.* (2014)), both receptors might balance S1P-mediated aldosterone release. S1PR2 are $G_{i/o}$ but also G_q and $G_{12/13}$ coupled, which were thought to activate Rac and Rho, respectively, upon S1P stimulation (Brinkmann 2007). Endothelial barrier function was also shown to be regulated by the opposing effects of S1PR1 and S1PR2: while activation of S1PR1- $G_{i/o}$ in endothelial cells stimulates Rac, thus increasing endothelial barrier function, stimulation of S1PR2- $G_{12/13}$ activates the Rho pathway, thereby decreasing endothelial barrier function (Brinkmann 2007). The Rac/Rho pathways – downstream at the initial activation of phospholipase C – also mediate AngII-stimulated aldosterone release (Otis & Gallo-Payet 2007).

Despite blocking S1PR1, S1PR2 and S1PR3, S1P incubation could still affect aldosterone release, thus suggesting that S1PR4 and S1PR5 play a role in regulating aldosterone. ERK1/2 signaling is activated by S1PR4 and inhibited by S1PR5 stimulation (Kluk & Hla 2002). Hence, it seems more likely that reduction of aldosterone release – despite blockage of S1PR1, S1PR2 and S1PR3 – is associated with an S1PR4-related pathway (Fig. 8). The human NCI H295R cells do express S1PR5 but not S1PR4 (Ozbay *et al.* 2006), which, however, gives rise to doubts as to whether S1PR4-ERK1/2 stimulation actually leads to aldosterone release in our experiments. In contrast to Kluk and Hla, we detected low mRNA levels of S1PR4 in adrenals of rats, while S1PR5 fell below the detection limit.

Figure 9

Correlation analyses between plasma levels of aldosterone and leptin and triglycerides (TG), respectively, from lean or obese spontaneous hypertensive rats (SHRs) or Zucker rats. Data originate from data previously published by our group (Müller-Fielitz *et al.* 2012a,b).

S1P effects on plasma aldosterone *in vivo*

In agreement with the literature (Lamounier-Zepter *et al.* 2006, Kidambi *et al.* 2007, Krug & Ehrhart-Bornstein 2008), aldosterone plasma levels were found to be increased in obesity in our study (Figs 5 and 6). This was further suggested to be related to an enhanced sensitization of the adrenals toward AngII in obesity (Bentley-Lewis *et al.* 2007, Müller-Fielitz *et al.* 2012b) but also to an extra-adrenal mechanism as AngII-induced aldosterone release from adipocytes and Cyp11 β 2 mRNA expression were higher in isolated adipocytes from db/db mice, thus provoking a greater amount of aldosterone release in vehicle, but not in candesartan-treated mice (Briones *et al.* 2012). However, as outlined in the introduction, MRF is not AngII (Ehrhart-Bornstein *et al.* 2003, 2004). Plasma aldosterone increased in response to 200 μ g/kg_{bw} S1P in lean SD rats, but not in lean SHR, while lower S1P doses also lacked effects in SD rats. The extent of aldosterone release was markedly higher when SD rats were treated with AngII (Fig. 3). On the one hand, this supports our cell model results and suggests that S1P is an *in vivo* liberator of aldosterone, but, on the other hand, implies that the relevance of S1P as a modulator for aldosterone levels may be minor. Differences in S1P-stimulated aldosterone release between SD rats and SHR might be attributed to distinct adrenal susceptibilities to various stimuli. Supporting this assumption, plasma renin activities and AngII plasma levels were demonstrated to be higher in SHR than SD rats, while aldosterone levels were lower under low sodium conditions (Williams *et al.* 1982). In addition, AngII-stimulated aldosterone release was also markedly lower in isolated glomerulosa cells from SHR than in SD rats, which indicates a decreased adrenal responsiveness to AngII (Williams *et al.* 1982).

Components of metabolic syndrome such as body weight, blood pressure and glucose levels were increased in rats in which obesity was a consequence of CD feeding or deficient leptin receptors. In our DIO rat model, plasma levels of S1P and aldosterone were simultaneously increased, while adrenal S1PR expression remained almost unchanged. This corresponds to studies in which elevated plasma levels of aldosterone (Lamounier-Zepter *et al.* 2006, Müller-Fielitz *et al.* 2012a) and S1P (Samad *et al.* 2006, Blachnio-Zabielska *et al.* 2012a,b, Kowalski *et al.* 2013, Choi & Snider 2015) were observed. In addition to S1P, ceramides and sphingosine are also increased in plasma of obesity mice (Samad *et al.* 2006). Sphingosine is phosphorylated by the sphingosine kinase 1 (Sphk1) to form the bioactive S1P. Accordingly, Sphk1 mRNA

expression and Sphk1 activity were found to be higher in white adipose tissue of obese rats than in lean controls (Tous *et al.* 2014). In particular, Sphk1 was assessed to be the crucial enzyme in regulating the balance between ceramides and S1P, both having opposite effects and involved in cell death or proliferation (Van Brocklyn & Williams 2012). Regarding a functional context, a C18 ceramide-dependent mechanism was found to be involved in aldosterone-induced vascular endothelial cells (Zhang *et al.* 2016). However, C2 ceramide did not induce aldosterone secretion in bovine glomerulosa cells (Brizuela *et al.* 2006). So far, though, we do not know whether other ceramides lower aldosterone secretion.

The aldosterone response to S1P stimulus was only minor and short-lasting in CD-fed obese SHR compared to their lean controls. Since adrenal susceptibility to S1P is thought to be low in SHR, exogenously administered S1P might reasonably be less efficient. S1PR1 expression strengths were equal in lean and obese rats. Compared to saline, the S1P-stimulated aldosterone release was higher in obese than in lean Zucker rats but only by giving maximal doses of 300 μ g/kg. However, AUC analyses only showed low obesity-related S1P efficacy. Thus, we conclude that aldosterone release from adrenals or other sources (e.g., adipocytes) is not likely enhanced by S1P in obesity.

Obesity is a pervasive risk factor for oxidative stress and for cardiovascular diseases, too (Niemann *et al.* 2017). As reactive oxygen species can increase (Jin *et al.* 2004, Ader *et al.* 2008) or decrease (Gomez-Brouchet *et al.* 2007) Sphk1 activity, it may be speculated as to whether an obesity-dependent increase in S1P might be related to oxidative stress, thus affecting aldosterone secretion. Considering that only the highest S1P levels increase aldosterone release in obese, but not in lean rats (Fig. 9), we conclude that this mechanism is only of minor importance. This assumption is strengthened by recent observations of the interaction between oxidative stress and aldosterone, showing that, in porcine adrenocortical cells, oxidative stress decreased mRNA levels of CYP11A1, CYP21 and 3 β -HSD, indicating a downregulation of aldosterone production (Wang *et al.* 2015). However, others have shown that mineralocorticoid receptor antagonism ameliorated elevated cardiac oxidative stress in obese Zucker rats (Bender *et al.* 2015) and prevented oxidative stress, but did not reduce weight gain after western diet in mice (Bostick *et al.* 2015). Furthermore, angiotensin II facilitated CYP11B2 activity and the ensuing aldosterone production via activation of the AT₁ receptor–NADPH oxidase–H₂O₂ signaling pathway (Rajamohan *et al.* 2012).

S1P seems thus rather unlikely to be MRF in obesity. Addressing this issue, others demonstrated that human adrenal ZG cells not only express leptin receptors but, moreover, that leptin increases aldosterone synthase expression and aldosterone production in mice. Obesity-mediated hyperaldosteronism requires functional leptin receptors as both plasma aldosterone and adrenal CYP11B2 expression were increased in high fat diet-fed, leptin receptor-deficient (db/db) mice compared to regular diet-fed controls (Huby *et al.* 2015). We confirm the observation of Huby *et al.* that plasma levels of leptin and aldosterone correlate in CD- and chow-fed SHR but not in lean and obese Zucker rats (Fig. 8). However, data are inconclusive with regard to findings of the Bornstein group showing that leptin had no stimulatory effect on steroidogenesis in NCI H295R, thus concluding that leptin could not serve as the sought MRF (Ehrhart-Bornstein *et al.* 2003). Low-density lipoprotein (LDL) was found to increase aldosterone release in NCI H295R and to sensitize adrenocortical cells to AngII (Lamounier-Zepter *et al.* 2006). The fact that LDL was observed to be higher in obese than in lean hypertensive individuals further supports the idea of LDL acting as MRF (Lamounier-Zepter *et al.* 2006). Accordingly, very low-density lipoprotein (VLDL) also increased aldosterone secretion, expression of StAR protein and aldosterone synthase in NCI H295R cells (Xing *et al.* 2012). VLDL was suggested to stimulate aldosterone release in obesity since plasma levels of triglycerides – an indirect parameter to assess VLDL levels – positively correlated with CYP11B2 mRNA transcript levels in normal- and sucrose-diet groups (Xing *et al.* 2012). We again confirmed these *in vitro* findings by correlating TG and aldosterone levels in CD-fed and normally fed rats (Fig. 8).

In summary, S1P stimulates aldosterone release *in vivo* and *in vitro* predominantly via an S1PR1 pathway. However, S1P-induced aldosterone release was affected only slightly, if at all, in obesity, thus not supporting our hypothesis of S1P being a major player in hyperaldosteronism leading to obesity-induced hypertension.

Declaration of interest

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

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Author contribution statement

S W, H M F and W R performed the research. W R and S W designed the research study. S W, H M F and W R analyzed the data and W R and S W wrote the paper.

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