Mineralocorticoid receptors modulate vascular endothelial function in human obesity

Moon-Hyon HWANG*, Jeung-Ki YOO*, Meredith LUTTRELL†, Han-Kyul KIM*, Thomas H. MEADE†, Mark ENGLISH‡, Mark S. SEGAL§ and Demetra D. CHRISTOU*

*Department of Applied Physiology and Kinesiology, University of Florida, Gainesville, FL, U.S.A.
†Department of Health and Kinesiology, Texas A&M University, College Station, TX, U.S.A.
‡Department of Cardiology, Scott & White Healthcare, Texas A&M University, College Station, TX, U.S.A.
§Department of Family & Community Medicine, Scott & White Healthcare, Texas A&M University Health Science Center, Bryan, TX, U.S.A.
||Department of Medicine, University of Florida, Gainesville, FL, U.S.A.

Abstract
Obesity increases linearly with age and is associated with impaired vascular endothelial function and increased risk of cardiovascular disease. MRs (mineralocorticoid receptors) contribute to impaired vascular endothelial function in cardiovascular disease; however, their role in uncomplicated human obesity is unknown. Because plasma aldosterone levels are elevated in obesity and adipocytes may be a source of aldosterone, we hypothesized that MRs modulate vascular endothelial function in older adults in an adiposity-dependent manner. To test this hypothesis, we administered MR blockade (eplerenone; 100 mg/day) for 1 month in a balanced randomized double-blind placebo-controlled cross-over study to 22 older adults (ten men, 55–79 years) varying widely in adiposity [BMI (body mass index): 20–45 kg/m²], but who were free from overt cardiovascular disease. We evaluated vascular endothelial function [brachial artery FMD (flow-mediated dilation)] via ultrasonography) and oxidative stress (plasma F₂-isoprostanes and vascular endothelial cell protein expression of nitrotyrosine and NADPH oxidase p47(phox) during placebo and MR blockade. In the whole group, oxidative stress (P > 0.05) and FMD did not change with MR blockade (6.39 ± 0.67 compared with 6.23 ± 0.73%; P = 0.7). However, individual improvements in FMD in response to eplerenone were associated with higher total body fat (BMI: r = 0.45, P = 0.02; and dual-energy X-ray absorptiometry-derived percentage body fat: r = 0.50, P = 0.009) and abdominal fat (total: r = 0.61, P = 0.005; visceral: r = 0.67, P = 0.002; and subcutaneous: r = 0.48, P = 0.03). In addition, greater improvements in FMD with eplerenone were related to higher baseline fasting glucose (r = 0.53, P = 0.01). MRs influence vascular endothelial function in an adiposity-dependent manner in healthy older adults.

Key words: abdominal visceral and subcutaneous fat, brachial artery, flow-mediated dilation, mineralocorticoid receptor, obesity

INTRODUCTION

More than one-third of adults worldwide are overweight or obese [1], and the prevalence of obesity increases linearly with age [2]. Obesity is associated with increased risk of cardiovascular disease [3], but the underlying mechanisms are not completely understood. Substantial evidence supports an independent role of aldosterone in the development and progression of cardiovascular disease [4–6]. According to the classic view of physiology, aldosterone is secreted by the adrenal gland and is involved in BP (blood pressure) regulation by acting on the kidney via activation of epithelial MRs (mineralocorticoid receptors) [7]. In the past decade, the non-epithelial presence of MRs has been demonstrated in cardiac and vascular cells and increasing evidence supports the direct role of MRs in modulating vascular function and contributing to cardiovascular disease [8].

Recently, findings from studies in vitro and studies performed in rodents demonstrate that adipose tissue is a secondary source of aldosterone [9] and that adipocyte-derived aldosterone contributes to vascular dysfunction in obesity [10]. In humans, several studies have shown that plasma aldosterone levels are positively related to measures of total and abdominal adiposity including BMI (body mass index) [11], waist circumference [12], abdominal visceral [13] and subcutaneous adipose tissue [14]. In addition, plasma aldosterone concentrations are elevated in obese compared with lean human subjects [15,16]. With weight loss,
aldosterone levels are significantly decreased [14,17–19], highlighting the important role of adipose tissue in the obesity-related increases in aldosterone concentration.

Obesity is also associated with impaired endothelial function [20,21], an independent predictor of future cardiovascular events, disease progression and long-term outcome [22,23]. A key component of endothelial dysfunction is decreased nitric oxide bioavailability resulting from either decreased synthesis or increased degradation because of oxidative stress [24]. Activation of vascular NADPH oxidase, eNOS (endothelial NOS) uncoupling and other factors lead to increased production of ROS (reactive oxygen species), which inactivate nitric oxide, thus leading to impaired vascular smooth muscle relaxation and vasodilation [25].

There is strong evidence supporting that aldosterone activation of MRs contributes to oxidative stress and decreased nitric oxide activity. Data from experimental models of cardiovascular disease demonstrated that MR activation increases NADPH oxidase expression and activity leading to increased superoxide production, vascular oxidative stress, decreased nitric oxide bioavailability and impaired vascular endothelial function, whereas MR blockade reverses these effects [26–29]. Human studies in patients with congestive heart failure found that 1 month of MR blockade improves endothelial function and this improvement is associated with increased NO bioactivity [30,31].

Taken together these data support a potential role for MRs in obesity-related impairments in endothelial function, but this has not been studied in human obesity. Thus, in the present investigation, we hypothesized that MRs modulate vascular endothelial function in an adiposity-dependent manner in healthy older adults. To test this hypothesis we administered the selective MR antagonist eplerenone (100 mg daily for 1 month) in a balanced randomized double-blind placebo-controlled cross-over study in healthy older adults varying widely in total and abdominal adiposity. We measured vascular endothelial function and oxidative stress markers during placebo and MR blockade.

**MATERIALS AND METHODS**

**Subjects**

A total of 22 healthy adults (55–79 years), ten men and 12 women, of a wide range of adiposity (BMI, 20.0–44.6 kg/m²; body fat, 25.6–54.1%) were studied. All subjects were sedentary, non-smokers and were free of overt cardiovascular disease and other clinical disorders (e.g. diabetes, liver and renal disease) as assessed by medical history, physical examination, resting ECG, urinalysis, blood chemistries and haematological evaluation. None of the subjects were taking antihypertensive or vasoactive drugs and subjects who were taking antioxidant supplements completed a 4-week washout prior to study enrolment. All subjects demonstrated normal ECG and BP responses to a graded exercise test on a treadmill. The graded exercise protocol is described below under the aerobic fitness section. Women were all postmenopausal, established by absence of menses for at least 2 years and follicle stimulating hormone >40 international units/l. Postmenopausal women were not on hormone replacement therapy for at least 1 year prior to data collection.

The study was carried out in accordance with the Declaration of Helsinki (2008) and was approved by the Institutional Review Boards of the University of Florida, Texas A&M University, and Scott & White Health System. The purpose, nature and risk of all procedures used were explained to the subjects and their written informed consent was obtained prior to participation.

**Study design**

Subjects were assigned to receive an MR antagonist (eplerenone; 100 mg/day) or placebo for 1 month in a balanced randomized double-blind placebo-controlled cross-over study with 1-month washout between treatments. (Figure 1). Eplerenone was chosen because it has a higher selectivity for MRs and fewer side effects than the other MR antagonist that is currently available (i.e. spironolactone).

To reduce the risk of hyperkalaemia, subjects were not enrolled in the study if their baseline serum potassium was greater than 5.5 mmol/l, serum creatinine was >1.6 mg/dl or creatinine clearance was <30 ml/min. Following study enrolment, serum potassium and BP were assessed at baseline, day 3, day 7 and weekly thereafter for each treatment. In response to 1-month treatment with eplerenone, serum potassium levels did not rise and systolic BP did not decrease excessively requiring subject withdrawal.

**General experimental procedures**

All measurements were performed in the morning, at the same time each day, in a semi-darkened temperature-controlled room after a 12-h overnight fast (including abstinence from caffeine and alcohol) and a minimum of 20 min of supine rest. Subjects took their morning dose of eplerenone or placebo exactly 1 h prior to data collection.
Vascular endothelial function (FMD)

Brachial artery FMD (flow-mediated dilation) was assessed non-invasively following established guidelines ([32], but see [33],[34]) by using an ultrasound/Doppler system equipped with a 7.5 MHz vascular transducer (Aplio XV; Toshiba).

Briefly, the subject rested in the supine position with the right arm abducted and fixed in position at heart level by using a Versaform pillow (Sammons Preston Rolyan). A pressure cuff connected to a rapid inflator/deflator system (E20 and AG 101; D. E. Hokanson) was placed around the widest part of the subject’s forearm. A dupplex ultrasound image of the brachial artery (i.e. 2D image and spectral Doppler waveforms) was obtained ∼ 7 cm proximal to the antecubital fossa. The Doppler angle of insonation for assessing blood velocity was set at ∼ 60°. Following image optimization the vascular transducer was clamped (Flexbar) in place to prevent movement during data collection. To ensure the same segment of the brachial artery was imaged in the subsequent ultrasound visit, the distance of the transducer relative to the antecubital crease was recorded, a digital photograph of the arm position was stored, and the ultrasound image was printed.

Reactive hyperaemia was induced by inflating the forearm cuff to 250 mmHg for 5 min followed by rapid deflation. ECG R-gated duplex ultrasound images of the brachial artery were digitally recorded (Vascular Imager; Medical Imaging Applications) for 1 min to establish pre-occlusion baseline and for 2 min after cuff deflation to assess peak dilatory response (the maximum brachial artery diameter). End-diastolic diameters were analysed by using a commercially available edge-detection wall-tracking software package (Brachial Analyzer; Medical Imaging Applications). Individual diameters were averaged (bin: three R-gated diameters) before identifying the peak diameter. FMD was expressed as absolute change in mm (maximum diameter–baseline diameter) and as percentage change {[(maximum–baseline diameter)/baseline diameter]×100}. To quantify the hyperaemic response, the first 15 post-occlusion spectral Doppler envelopes and at least 15 baseline spectral Doppler envelopes were recorded on super VHS tape and were analysed with the Toshiba ultrasound system software to obtain blood velocity. Blood flow (ml/min) was calculated as:

\[
\text{Blood flow} = \text{mean blood velocity} \times \left( \frac{\text{diameter}}{2} \right)^2 \times \pi \times 0.6
\]

(1)

Shear stress (dyne/cm²) was calculated as:

\[
\text{Shear stress} = \frac{8 \times \mu \times \text{mean blood velocity}}{\text{baseline diameter}}
\]

(2)

where \( \mu \) was the blood viscosity, which was assumed to be 0.035 dyne/cm² [35]. Ultrasound images and spectral Doppler were analysed by researchers blinded to the treatment (i.e. eplerenone or placebo) and subject identity.

Vascular endothelial cell collection and protein expression

Endothelial cells were collected from an antecubital vein as previously described [36–39]. Briefly, two sterile J-shaped guidewires (Daig) were sequentially advanced ∼ 10 cm through an 18-gauge intravenous catheter and retracted. Cells were recovered by washing the wires with a dissociation buffer and centrifugation. Cells were fixed with 4 % (v/v) paraformaldehyde (USB), washed thoroughly with PBS, plated on poly-L-lysine coated slides (Sigma Chemical), and stored at ∼ 80 °C until the immunofluorescence staining was performed.

For immunofluorescence staining, fixed vascular endothelial cells were rehydrated with PBS containing 50 mmol/1 glycine and non-specific sites were blocked with 5 % (v/v) donkey serum (Jackson Immunoresearch). Slides were incubated with one of the following primary antibodies followed with the corresponding secondary antibody with Alexa Fluor® 488 (Invitrogen): nitrotyrosine that is a marker of oxidative stress (Abcam) and NADPH oxidase p47phox (Millipore) that is one of the major sources of vascular superoxide. Slides were also incubated with a primary antibody for von Willebrand factor (DAKO) and the corresponding secondary antibody with Alexa Fluor® 555 (Invitrogen) to allow identification of endothelial cells. Finally, slides were mounted with Vectashield containing the nuclear stain DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories). Because of the large number of slides, staining was performed in several batches, but each subject’s slides from the eplerenone and placebo visits were included in the same batch to avoid the influence of day-to-day variability in staining. To minimize the potential confounding effect of inter-batch variability in staining, two slides of HUVECs (human umbilical vein endothelial cells) were stained in each batch and intensity for each protein of interest was expressed relative to the average HUVEC intensity in that batch.

For analysis, cells were examined with a fluorescence microscope (Eclipse 80i; Nikon Instruments) at ×100 magnification using the same exposure time. Images of endothelial cells with intact nuclei were digitally captured by a coolSNAP ES2 camera (Photometrics). Endothelial cells were identified by the presence of von Willebrand factor staining and nuclear integrity was confirmed by DAPI staining. Vascular endothelial cell protein expression was measured with NIS Elements software (version 3.2; Nikon Instruments) by quantifying Alexa Fluor® 488 intensity while correcting for background fluorescence. Vascular endothelial cell protein expression is reported as intensity per HUVEC intensity.

Blood measures

Standard blood chemistries and haematological evaluation were performed at baseline by a clinical laboratory using conventional assays. Insulin resistance was estimated using the HOMA-IR (homeostasis model of insulin resistance) [HOMA-IR = (fasting insulin μunit/ml × fasting glucose mg/dl)/405]. Plasma 8-isoprostanes were measured by the Vanderbilt University Eicosanoid Core Laboratory using gas chromatography–mass spectrometry, as previously described [40].

Height, weight and adiposity measures

Height was measured to the nearest mm using a stadiometer. Body weight was measured to the nearest 0.1 kg with an electronic scale (Tanita) while subjects were barefoot and dressed in light clothing. BMI was calculated as weight divided by height squared (kg/m²). Total percentage body fat was assessed with
dual-energy X-ray absorptiometry (DPX-IQ; GE/Lunar) as described previously [41]. Abdominal total, visceral and subcutaneous fat were measured at the level of L4–L5 using a single slice computed tomography scan and assessed by a commercially available analysis software (Slice-O-Matic v4.3, Tomovision) [42].

Resting BP
Resting BPs were recorded over the brachial artery with a semi-automated device (Dinamap, GE).

Aerobic fitness
Aerobic fitness was determined using \( \dot{V}O_{2\text{max}} \) (maximal oxygen consumption) as described previously [41]. Briefly, online computer-assisted open-circuit spirometry was used during incremental treadmill exercise. After subjects walked for 6–10 min at a comfortable speed that corresponded to 70–80% of their age-predicted maximal heart rate to warm-up, the treadmill grade was increased 2.5% every 2 min until volitional exhaustion.

Data analysis
Statistical analyses were performed using SPSS version 21. Statistical significance for all analyses was set at \( P < 0.05 \). Paired Student's t tests were used to compare FMD, blood and vascular endothelial cell markers of oxidative stress during MR blockade and placebo treatments. Bivariate relationships were determined using Pearson product moment correlation coefficients.

RESULTS
Mean values and ranges for baseline subject characteristics are presented in Table 1. Subjects varied widely in total and abdominal adiposity. At baseline, total and abdominal adiposity were negatively associated with FMD (\( r = -0.37 \) to \(-0.49, P < 0.05 \)) and positively associated with \( F_2 \)-isoprostanes (\( r = 0.43–0.68, P < 0.05 \)).

Vascular responses to MR blockade
In the whole group, mean brachial artery FMD was not different with MR blockade compared with placebo (\( P = 0.7 \); Table 2). However, individual responses to MR blockade varied from decreased to increased FMD. Subjects whose FMD improved with MR blockade had \( \sim 40\% \) higher abdominal visceral fat compared with those whose FMD either decreased or did not change with MR blockade (\( P = 0.03 \)). In agreement with these results, greater improvements in FMD in response to MR blockade were related to greater baseline BMI, total percentage body fat and total abdominal, visceral and subcutaneous fat (\( r = 0.45–0.67, P \leq 0.03 \); Figures 2 and 3). In addition, greater improvements in FMD were associated with higher baseline fasting glucose (\( r = 0.53, P = 0.01 \); Figure 4).

Baseline brachial artery diameter was not different between MR blockade and placebo treatment, whereas, baseline shear stress increased in response to MR blockade (\( P = 0.9 \) and 0.02, respectively; Table 2). However, hyperaemic shear stress and the change in shear stress from baseline did not differ between MR blockade and placebo, indicating that the post-occlusion stimulus to induce vasodilation was similar (\( P = 0.8 \) and 0.1 respectively; Table 2).

MR blockade resulted in significant reductions in systolic BP (\( P < 0.0001 \)) and smaller reductions in diastolic BP that did not reach statistical significance (\( P = 0.07 \); Table 2). However, the change in systolic BP was not related to the change in FMD in response to MR blockade (\( P > 0.05 \)). In addition, accounting for the change in systolic BP in multiple linear regression analysis did not contribute significantly to the model (\( P > 0.05 \)) and did not influence the relationship of adiposity with the change in FMD in response to MR blockade.
Plasma oxidative stress and vascular endothelial cell protein expression
MR blockade did not influence plasma \( F_2 \)-isoprostanes (6.5 ± 1.0 pg/ml in placebo compared with 5.9 ± 0.6 pg/ml with MR blockade; \( P = 0.3 \)). Similarly, vascular endothelial cell protein expression of nitrotyrosine (marker of oxidative stress) and NADPH oxidase (vascular source of superoxide) did not significantly change in response to MR blockade (0.79 ± 0.04 compared with 0.73 ± 0.22 intensity/HUVEC intensity, \( P = 0.2 \); 0.66 ± 0.04 compared with 0.57 ± 0.04 intensity/HUVEC intensity, \( P = 0.1 \) respectively). There were no correlations between (i) baseline plasma/endothelial cell oxidative stress measures, and baseline adiposity or change in FMD with MR blockade; and (ii) change in plasma/endothelial cell oxidative stress measures and change in FMD with MR blockade.

DISCUSSION
We have investigated whether MRs modulate vascular endothelial function in an adiposity-dependent manner in healthy older adults with widely varying total and abdominal adiposity. Our study demonstrates for the first time that greater improvement in vascular endothelial function with MR blockade is seen in those who have greater total and abdominal adiposity. Another important finding of our study is that greater enhancements in endothelial function in response to MR blockade are associated with higher baseline fasting glucose.

Findings from two recent studies based on animal and in vitro models have shown compelling evidence of aldosterone production in adipocytes and contribution of adipocyte-derived aldosterone to vascular dysfunction in obesity [9,10]. In humans, several studies have shown elevated plasma aldosterone levels with obesity and some have found that greater BP reduction with MR blockade was associated with higher BMI [43] and higher waist circumference [44]. Our data extend these findings by demonstrating greater increases in FMD with MR blockade are associated with higher BMI, total percentage body fat, total abdominal, visceral and subcutaneous fat.

Aldosterone might be the potential link between adiposity, insulin resistance and increased risk of cardiovascular disease. A recent review article highlighted data supporting a role for elevated plasma aldosterone levels and MR signalling in the pathophysiology of insulin resistance and vascular dysfunction [45]. Our data demonstrate that greater improvements in endothelial function with MR blockade are associated with higher baseline fasting blood glucose. These findings suggest that MRs play a larger role in vascular dysfunction in subjects with lower insulin sensitivity.
In our study, systolic BP significantly decreased in response to MR blockade, thus, one might speculate that this could have contributed to the improvements in endothelial function. However, the change in systolic BP was not related with the change in FMD in response to MR blockade. In addition, accounting for the change in systolic BP in multiple linear regression analysis did not significantly contribute to the model and did not influence the relationship of adiposity with the change in FMD in response to MR blockade. Taken together these findings argue against the assumption that reductions in BP might have played a significant role in the beneficial effects of eplerenone on vascular endothelial function.

Our study has several strengths including: (i) novelty of findings; (ii) use of balanced randomized double-blind placebo-controlled cross-over design; (iii) exclusion of subjects with overt cardiovascular or other clinical disease and medication use, which could confound the independent relationship of MRs with obesity; (iv) quantification of total percentage body fat using dual-energy X-ray absorptiometry and total abdominal, visceral and subcutaneous fat using computed tomography; and (v) rigorous procedures to ensure adherence to intervention.

Our study also has some potential limitations. We did not measure baseline plasma aldosterone to determine if it was elevated in our obese subjects. However, several studies have already established a relationship between aldosterone levels and obesity. MRs have equal affinity for aldosterone and cortisol; however, the presence of the enzyme \(11\beta\)-HSD \((11\beta\)-hydroxysteroid dehydrogenase\) in tissues (including the vascular wall) converts cortisol into corticone making aldosterone the primary MR agonist [46]. Our current data cannot address whether cortisol might have a role in the observed effects of MR blockade. Taken together these findings argue against the assumption that reductions in BP might have played a significant role in the beneficial effects of eplerenone on vascular endothelial function.

Conclusions

The present findings demonstrate, for the first time, that MRs modulate vascular endothelial function in an adiposity-dependent manner in healthy older adults. MR-blockade-related improvements in FMD are positively related to both total and abdominal adiposity. We also demonstrate that changes in vascular endothelial function with MR blockade are related to baseline fasting blood glucose. Our study suggests that MRs contribute to the pathophysiology of impaired vascular endothelial function in human obesity.

CLINICAL PERSPECTIVES

- Aldosterone contributes to vascular dysfunction in cardiovascular disease. Plasma aldosterone is elevated with total and abdominal adiposity in humans, but its influence on vascular function is unknown. We sought to examine the role of MRs in vascular endothelial function in human obesity in a balanced randomized, double-blind placebo-controlled cross-over study using 1-month MR blockade with eplerenone.
- We found that eplerenone-related improvements in FMD were positively associated with total and abdominal adiposity and baseline fasting glucose in healthy older adults. Aldosterone appears to be an important contributor to vascular endothelial dysfunction in healthy older adults with increased adiposity and fasting blood glucose.
- These findings have important clinical implications. Therapeutic use of MR blockade to treat hypertension in patients with increased adiposity might confer direct favourable effects on obesity-related vascular alterations and might reduce the risk of developing cardiovascular complications.

AUTHOR CONTRIBUTION

Moon-Hyon Hwang and Demetra Christou conceived and designed the study; Moon-Hyon Hwang, JeungKi Yoo, Meredith Luttrell, Thomas Meade, Mark English and Demetra Christou collected the data; Moon-Hyon Hwang, JeungKi Yoo, Meredith Luttrell and HanKyul Kim analysed the data; Thomas Meade and Mark English provided on-site medical supervision for the experiments; Moon-Hyon Hwang and Demetra Christou performed the statistical analysis, prepared the Figures and drafted the paper; Moon-Hyon Hwang, Mark Segal and Demetra Christou interpreted results, and edited and revised the paper; JeungKi Yoo Y, Han-Kyul Kim, Meredith Luttrell, Mark Segal, Thomas Meade and Mark English provided feedback on the paper. All authors approved the final version of the paper.
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