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Mineralocorticoid Receptor Antagonism Reverses Diabetes-related Coronary Vasodilator Dysfunction: A Unique Vascular Transcriptomic Signature

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Abstract

Coronary microvascular dysfunction predicts and may be a proximate cause of cardiac dysfunction and mortality in diabetes; however, few effective treatments exist for these conditions. We recently demonstrated that mineralocorticoid receptor (MR) antagonism reversed cardiovascular dysfunction in early-stage obesity/insulin resistance. The mechanisms underlying this benefit of MR antagonism and its relevance in the setting of long-term obesity complications like diabetes; however, remain unclear. Thus, the present study evaluated the impact of MR antagonism on diabetes-related coronary dysfunction and defines the MR-dependent vascular transcriptome in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat recapitulating later stages of human diabetes. OLETF rats were treated with spironolactone (Sp) and compared to untreated OLETF and lean Long-Evans Tokushima Otsuka rats. Sp treatment attenuated diabetes-associated adipose and cardiac inflammation/fibrosis and improved coronary endothelium-dependent vasodilation but did not alter enhanced coronary vasoconstriction, blood pressure, or metabolic parameters in OLETF rats. Further mechanistic studies using RNA deep sequencing of OLETF rat aortas revealed 157 differentially expressed genes following Sp including upregulation of genes involved in the

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molecular regulation of nitric oxide bioavailability (*Hsp90ab1*, *Ahsa1*, *Ahsa2*) as well as novel changes in α_{1D} adrenergic receptors (*Adra1d*), cyclooxygenase-2 (*Ptgs2*), and modulatory factors of these pathways (*Ackr3*, *Acsf4*). Further, Ingenuity Pathway Analysis predicted inhibition of upstream inflammatory regulators by Sp and inhibition of ‘migration of endothelial cells’, ‘differentiation of smooth muscle’, and ‘angiogenesis’ biological functions by Sp in diabetes. Thus, this study is the first to define the MR-dependent vascular transcriptome underlying treatment of diabetes-related coronary microvascular dysfunction by Sp.

Keywords

fibrosis; inflammation; cardiac; LETO

1. Introduction

Coronary microvascular dysfunction has recently been proposed as a precipitating cause of cardiac diastolic dysfunction and heart failure with preserved ejection fraction in the setting of co-morbid conditions such as obesity and diabetes [1]. Indeed, in the absence of coronary atherosclerosis, impaired coronary flow reserve (CFR; *i.e.*, microvascular dysfunction) is associated with cardiac damage (*i.e.*, positive troponin) [2] and is a powerful independent predictor of cardiac mortality in diabetic patients [3]. Few effective treatments exist, however, for coronary microvascular dysfunction in diabetes or other disease states [4]. In light of these known associations, delineation of such treatments and underlying mechanisms holds promise to improve coronary blood flow regulation in diabetes and reduce diabetes-associated cardiac diastolic dysfunction, a condition also for which no treatment currently exists.

We recently demonstrated that mineralocorticoid receptor (MR) blockade reversed obesity-associated cardiovascular dysfunction in the obese, insulin resistant Zucker rat model [5]. The MR is a ligand-activated transcription factor that is functionally expressed in both vascular smooth muscle and endothelial cells [6]. Accumulating evidence implicates vascular MR signaling underlying gene expression of vascular ion channels [7–9], receptors for vasoactive ligands [8–11], adhesion/inflammatory molecules [12–16], and fibrosis/oxidant stress signaling [14, 15, 17]; pathways critical for microvascular (dys)function and coronary blood flow control [18]. Importantly, it was recently demonstrated that MR antagonism improved CFR in patients with diabetes, independent of blood pressure [19, 20]. The nature of MR-dependent gene transcription and MR-regulated gene networks in the setting of obesity and diabetes, however, has not been assessed and holds promise for the identification of novel mechanisms of diabetes-related coronary dysfunction. Accordingly, we evaluated the hypothesis that treatment of coronary microvascular dysfunction in obesity and type 2 diabetes by MR antagonism is associated with pronounced alterations in the vascular transcriptome.

This hypothesis was evaluated utilizing the Otsuka Long-Evans Tokushima Fatty (OLETF) rat model of obesity and diabetes and their lean Long-Evans Tokushima Otsuka (LETO) counterparts [21]. The hyperphagic OLETF rat recapitulates adult-onset human obesity and

type 2 diabetes by exhibiting late onset hyperglycemia (>13 wks of age), mild obesity, and hyperlipidemia transitioning to overt diabetes by 40 wk of age [22, 23]. In this way, the OLETF rat model is a more translationally relevant model of the slow onset later stages of human diabetes as opposed to the Zucker rat, used in our prior study [5], that models earlier stages of obesity (*i.e.*, insulin resistance) from very early in life. In addition, the OLETF rat exhibits impaired CFR and structural remodeling of the coronary microcirculation [24]; however, the time course of obesity/diabetes-associated changes in coronary endothelial function and vasoconstrictor responses has not previously been evaluated in this model. Thus, we evaluated the time course of obesity/diabetes-associated changes in coronary microvascular function and whether MR antagonism could reverse *established* coronary vasomotor dysfunction. Furthermore, assessment of the vascular transcriptome in diabetes with and without MR antagonism was accomplished by RNA deep sequencing (RNA-Seq) to globally investigate potential mechanisms underlying the vascular benefits of MR antagonism in diabetes. The impact of MR antagonism on cardiac and adipose fibrosis/inflammation was also assessed.

2. Methods

2.1. Animals.

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Missouri. Animals were housed in a temperature-controlled room with a 12:12-h light:dark cycle and provided ad libitum water and standard rodent chow (Formulab 5008, Purina Mills, St. Louis, MO). Male LETO and OLETF rats (4 wk of age) were obtained from Japan SLC, Inc. The progression of coronary dysfunction was assessed in batches of both strains studied at 5–8 (LETO: n=4, OLETF: n=5), 20 (LETO: n=5, OLETF, n=4), and 32 (LETO: n=11, OLETF: n=12) weeks of age. Involvement of MR signaling was assessed in 32 week old OLETF rats randomly assigned to placebo (n=4) or spironolactone (Sp; n=5) treatment paradigms. Under isoflurane anesthesia (2–4% in 100% O₂), rats were implanted with time-release, matrix-driven delivery pellets (Innovative Research of America, Sarasota, FL) containing either placebo or Sp (sc; 20 mg·kg⁻¹·d⁻¹) for 21 days beginning at 29 weeks of age, as we previously published [5, 25]. This dose was chosen to elicit complete systemic MR inhibition *in vivo*, based on a previous report [26]. Animals were fasted 5 hours prior to euthanization, anesthetized with pentobarbital sodium (100 mg/kg), blood was collected from the abdominal aorta, processed to serum, and frozen at –80°C. Animals were euthanized by exsanguination.

2.2. Blood pressure.

Blood pressure (BP) was determined in placebo and Sp-treated OLETF rats by tail-cuff (Coda 8, Kent Scientific, Torrington, CA) during the final week of treatment. Rats were acclimated to restraints and tail cuffs for 3 days. Blood pressure was determined on the fourth day with 10–25 cuff inflations averaged for each animal, as previously reported [5].

2.3. Plasma chemistries.

Blood glucose (AlphaTrak, Abbott) was determined immediately prior to euthanasia. Serum total cholesterol, triglycerides, and potassium were measured using an Olympus AU680

automated chemistry analyzer (Beckman-Coulter, Brea, CA) and plasma insulin was determined using a rat-specific ELISA by Comparative Clinical Pathology Services (Columbia, MO). Plasma aldosterone were determined in duplicate by radioimmunoassay at the Vanderbilt Hormone Assay & Analytical Services Core (Nashville, TN).

2.4. Coronary arteriolar function.

Coronary function was assessed as previously described [5, 27]. Briefly, the heart was removed and immediately placed in ice-cold physiological salt solution (PSS) containing (in mM): 145 NaCl, 4.7 KCl, 1.2 NaH₂PO₄, 1.17 MgSO₄, 2 CaCl₂, 5 glucose, 2 pyruvate, 0.02 EDTA, 3 MOPS, and 1% bovine serum albumin, pH 7.4. Coronary arterioles (<200 μ m internal diameter) from the septum or left ventricular (LV) free wall were dissected, secured to glass micropipettes, pressurized to 60cmH₂O at 37°C, and visualized with an inverted microscope for determination of internal diameter with a video micrometer recorded using Chart software with a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO). Vessels free from leaks were equilibrated for 1 hr and viability was assessed by presence of vasoconstriction to 80 mM KCl. Prior to assessment of vasodilator responses, arterioles were precontracted to 25–45% tone with the thromboxane A₂ analog U46619 (0.1–1 μ M). Preconstrictor doses of U46619 were not different among groups (LETO: 0.5 $\pm$ 0.1 μ M; OLETf: 0.5 $\pm$ 0.1 μ M; OLETf Sp: 0.4 $\pm$ 0.2 μ M). Vasodilator responses to acetylcholine (ACh; 1 nM – 10 mM), insulin (Novolin R, Novo Nordisk; 0.1 – 100 ng/ml), or the nitric oxide (NO) donor sodium nitroprusside (SNP; 1 nM – 10 mM) were assessed by cumulative addition of agonists to the vessel bath. In addition, vasoconstrictor responses to U46619 (10 nM – 1 μ M), endothelin-1 (ET-1; 0.1 – 10 nM), or the L-type Ca²⁺ channel activator BayK-8644 (0.1 – 100 nM) were assessed. Maximum passive diameter was determined at the end of experiments by replacing bath PSS with Ca²⁺-free PSS. Coronary vasodilator responses are presented as percent maximal dilation, calculated as $[(D_d - D_b)/(D_{max} - D_b)] \times 100$, and constrictor responses are presented as percent vasoconstriction, calculated as $[(D_b - D_d)/D_b] \times 100$ where D_d is diameter after a drug intervention, D_b is baseline diameter, and D_{max} is maximal passive diameter. No differences in coronary arteriolar function were found between untreated and placebo-treated OLETf rats so these data were combined.

2.5. Real time PCR.

Cardiac and retroperitoneal adipose tissue samples were frozen immediately at sacrifice and subsequently homogenized using a tissue homogenizer (TissueLyser LT, Qiagen, Valencia, CA) followed by total RNA isolation (RNeasy Fibrous or Lipid Tissue Kit, Qiagen) and assessment of RNA purity and concentration using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). First-strand cDNA was synthesized from total RNA using the Improm-II Reverse Transcription System (Promega, Madison, WI). In placebo- and Sp-treated OLETf rats the cardiac septum was separated from the LV free wall and placed in RNA_{later} solution after which the septal coronary artery was dissected and frozen until processed for RNA isolation (NucleospinXS, Clontech, Mountain View, CA) and assessment of RNA purity and concentration. As expected, these single vessels had limited RNA yield so total RNA was amplified (ExpressArt mRNA amplification kit; AMS Biotechnology, United Kingdom) to provide sufficient RNA for PCR analysis. First-strand cDNA was

synthesized from total RNA as above. Quantitative real-time PCR was performed using the CFX Connect™ Real-Time PCR Detection System (BioRad, Hercules, CA), as previously described [5]. Primer sequences (Supplemental Table 1) were purchased from IDT (Coralville, IA) and duplicate PCR reactions were performed using iTaq UniverSYBR Green SMX (BioRad, Hercules, CA). GAPDH primers were used to amplify the endogenous control product and mRNA expression values were calculated as 2^{-CT} whereby $CT = \text{GAPDH } CT - \text{gene of interest } CT$ and are presented normalized to the appropriate control groups, which were set at 1.

2.6. RNA Sequencing (RNA-Seq).

Total RNA was extracted from thoracic aorta of placebo and Spiro treated OLETF rats and high RNA integrity confirmed for each sample using the Qubit assay (ThermoFisher). RNA-Seq was performed on aortic total RNA due to the limitation of obtaining adequate RNA yield from rat coronary artery for RNA-Seq. Genes that were differentially expressed following Sp treatment were examined in coronary samples by RT-PCR, as described above. Samples underwent ribosomal RNA reduction (Ribo-Zero Gold Kit, Illumina) and cDNA library preparation via manufacturer's protocols including purification of poly-A containing mRNA, RNA fragmentation, generation of double-stranded cDNA, and ligation of index containing sample identifier adapters. Final constructs were evaluated using the Qubit assay and diluted according to Illumina's standard sequencing protocol for sequencing on the HiSeq 4000. For sequencing, samples were loaded onto one flow cell where clusters of each oligo were replicated and fluorescently-labeled bases were attached to the complementary bases of each sequence in the sequencer. The Illumina Genome Analyzer recorded paired end 100 base pair reads with at least 79 million reads per sample (range: 79–116 million reads per sample; ~80% matched to the USCS rn5 rat reference genome). Reads were trimmed to ensure adaptor sequence removal and tiled to the reference rat genome (UCSC rn5) using Bowtie2 2.2.6 (Johns Hopkins University) and annotated with BEDTools 2.17.0. Following statistical determination of differentially expressed transcripts (described below), Ingenuity Pathway Analysis (IPA; Qiagen) was utilized for examination of the top up- and down-regulated genes and the corresponding top networks and pathways.

2.7. Statistical Analysis.

Coronary function as well as cardiac and adipose gene expression data were evaluated with one- or two-way ANOVA followed by Fishers LSD *post hoc* analysis, as appropriate. For RNA-Seq data, nonspecific filtering of genes prior to statistical testing was carried out to increase detection power, based on the requirement that a gene have an expression level greater than 220 raw counts per million (CPM) output for at least 5 libraries (5 was chosen because it was the size of each treatment group). This CPM cutoff was established empirically based on the point at which the ERCC Spike-Ins at different concentrations were no longer distinguishable in the samples. In this study, ERCC Spike-Ins were no longer distinguishable at 75 CPM and we chose to utilize a more conservative cutoff of 220 CPM. Adjustment to the P values was made by EdgeR 3.4 software to account for multiple testing with a 10% false discovery rate (FDR) and based on the method of Benjamini and Hochberg [28].

3. Results

3.1. Differential progression of coronary arteriolar vasodilator and vasoconstrictor dysfunction with age in OLETF rats.

Similar to previous reports from our group [22, 29], OLETF rats exhibited increased body weight and adipose expansion at all ages studied compared to LETO rats (Table 1 and Supplemental Table 2). Average passive diameters and percent precontraction of isolated coronary arterioles studied were similar within age groups (Supplemental Table 3). Examination of isolated coronary arterioles revealed impaired endothelium-dependent vasodilation to ACh in OLETF rats at all ages studied (5–8, 20, and 32 weeks) (Figure 1). Vasodilation to SNP was similar in 32 week old OLETF and LETO rats (data not shown). Vasoconstriction to ET-1, however, was progressively increased with age in OLETF rats. Specifically, compared to LETO rats, constriction to ET-1 was unchanged in 5–8 week old OLETF rats, increased at the highest doses in 20 week old OLETF rats, and increased at all doses examined in 32 week old OLETF rats (Figure 1).

3.2. MR inhibition attenuates cardiac and adipose pro-inflammatory and cardiac fibrosis-related gene expression in OLETF rats independent of blood pressure.

Phenotypically, in addition to increased body weight and adipose expansion, 32 week old OLETF rats exhibited elevated fasting plasma glucose, cholesterol, and triglycerides but similar systolic blood pressure compared to age-matched LETO rats (Table 1). Treatment of OLETF rats for 3 weeks with Sp resulted in no change in body or tissue (heart and adipose) weights, fasting plasma measures (glucose, insulin, cholesterol, and triglycerides), serum potassium, or systolic blood pressure compared to placebo-treated OLETF rats (Table 1). Plasma aldosterone was reduced in OLETF rats compared to LETO rats, as previously described [30], and was increased by Sp treatment (Table 1). OLETF rats exhibited increased cardiac expression of pro-inflammatory (TNF- α , VCAM-1, ICAM-1) and fibrosis-related (increased collagen III and trend for increased collagen I) genes compared to LETO rats (Figure 2A and 2B). Similarly, in retroperitoneal adipose tissue, OLETF rats exhibited increased expression of pro-inflammatory (TNF- α , VCAM-1, ICAM-1) and immune cell marker (F4/80, CD11c, CD4) genes compared to LETO rats, indicative of adipose immune cell infiltration (Figure 2C and 2D). Treatment of OLETF rats with Sp attenuated cardiac pro-inflammatory (VCAM-1, ICAM-1) and fibrosis-related (collagen III) gene expression in concert with reduced adipose pro-inflammatory (TNF- α , VCAM-1, ICAM-1) and lymphocyte (CD4) gene expression (Figure 2).

3.3. MR inhibition normalized coronary endothelium-dependent vasodilation but not coronary constriction in OLETF rats.

As demonstrated in Figure 1, compared to LETO rats, 32 week old OLETF rats exhibit impaired endothelium-dependent vasodilation to acetylcholine, no change in endothelium-independent vasodilation to SNP (data not shown), and enhanced ET-1-induced vasoconstriction. In addition, at this age, OLETF rats exhibit insulin-induced coronary vasoconstriction as opposed to insulin-induced vasodilation in LETO rats (Figure 3). Lastly, 32 week old OLETF rats exhibit increased coronary vasoconstriction to the thromboxane A₂ mimetic U46619 but not the L-type Ca²⁺ channel opener BayK-8644 (Figure 3). Treatment

of OLETF rats with Sp for 3 weeks largely normalized impairments in acetylcholine- and insulin-induced vasodilation but did not reduce the enhanced vasoconstriction induced by ET-1 or U46619 despite modestly attenuating vasoconstriction to BayK-8644 (Figure 3).

3.4. MR inhibition induces pronounced differential vascular gene expression in OLETF rats.

RNA-Seq analysis identified 157 differentially expressed transcripts (70 up- and 87 down-regulated) in whole aortas from Sp-treated compared to placebo-treated OLETF rats (Supplemental Table 4). The top up- and down-regulated mRNAs are presented in Table 2. Examination of coronary expression of these differentially expressed aortic genes in the coronary artery are presented in Figure 4 and these data reveal strong agreement between Sp-induced changes in aortic and coronary artery gene expression for many genes. Using all differentially expressed genes, IPA identified ‘NRF2-mediated oxidative stress response’, ‘glucocorticoid receptor signaling’, and ‘IL-2 signaling’ as the top regulated canonical pathways (Table 2). The top regulated gene networks identified by IPA in Sp versus placebo-treated rats included ‘drug metabolism, endocrine system development and function, lipid metabolism’ (network 1, IPA score 55, Figure 5) and ‘gene expression, cardiovascular system development and function, cellular movement’ (network 2, IPA score 37, Figure 6). Examination of potential upstream regulators mediating the measured changes in gene expression by Sp in IPA predicted inhibition of PDGF-BB, FOXO1, TNF, VEGF-A, NFκB, and IFN-γ signaling as well as activation of DHT, HDAC, NKX2–3, and NFE2L2 (*i.e.*, NRF2) signaling (Supplemental Table 5). Lastly, based on all differentially expressed genes, assessment of the potential impact of Sp on biological functions by IPA revealed predicted inhibition of angiogenesis (IPA z-score –2.508), migration of endothelial cells (IPA z-score –2.172), and differentiation of smooth muscle (IPA z-score –2.00) by MR antagonism in OLETF rats (Figure 7).

4. Discussion

Emerging evidence suggests a principle role for inappropriate MR signaling underlying the development of coronary microvascular dysfunction in the setting of obesity and type 2 diabetes [5, 19, 20]. Accordingly, the present results demonstrate that systemic MR antagonism with Sp attenuates (*i.e.*, treats) established coronary microvascular dysfunction and reduces pro-inflammatory/fibrotic gene expression in visceral adipose and cardiac tissues independent of blood pressure in a model recapitulating later stages of human type 2 diabetes. To begin to identify potential mechanisms underlying the vascular benefit of MR antagonism in diabetes, the vascular transcriptome was sequenced revealing a number of differentially regulated mRNA transcripts and networks. To our knowledge, this is the first investigation of the vascular transcriptomic signature underlying the therapeutic impact of MR antagonism in diabetes, thus providing a unique snapshot of potential MR-dependent mechanisms of diabetes-related vascular dysfunction.

The natural history of obesity and diabetes in the OLETF rat closely resembles that in humans *vis-à-vis* the late (*i.e.*, adult) onset of hyperglycemia and overt diabetes as well as the development of coronary microvascular dysfunction [22–24]. Indeed, impaired CFR has

been reported in this model as early as 15 weeks of age concomitant with coronary perivascular fibrosis [24]. Our findings extend these data by demonstrating impaired endothelium-dependent vasodilation at 5–8 weeks of age preceding the reported change in CFR and the progressive development of enhanced coronary vasoconstrictor responses beginning at 20 weeks of age. Although the temporal relationship between impaired endothelium-dependent vasodilation and reduced CFR is unclear in human patients, both are independent predictors of future cardiovascular morbidity and mortality in the absence of overt coronary atherosclerosis [3, 31, 32]. It has previously been established that coronary arteriolar dilation to ACh in rats is NO-dependent [33] and that NO inhibition and removal of the endothelium enhance ET-1-mediated coronary constriction [34, 35]. Thus, the impairment of ACh-induced vasodilation and the progressive increase in ET-1-mediated constriction in coronary arterioles of the OLETF rat may arise from reduced NO bioavailability as we previously reported in skeletal muscle feed arteries in this model [36]. Increased ET-1-induced constriction in this model may also involve sensitization of coronary ET-1 receptors as was previously reported in obese dogs [37] consistent with our previous report of reduced ET-1 type A receptors in the OLETF rat [36]. Nonetheless, these findings indicate that endothelial vasodilator dysfunction may be the earliest coronary defect in obesity leading to additional impairments of coronary function and impaired myocardial blood flow regulation.

It has recently been proposed that the coronary dysfunction associated with obesity and diabetes, as well as subsequent impairment of cardiac diastolic dysfunction, arises principally from a systemic pro-inflammatory state [1]. Our data are consistent with this paradigm demonstrating increased pro-inflammatory and pro-fibrotic gene expression in both visceral adipose and cardiac tissues of the OLETF rat. Importantly, and in line with this proposition, MR antagonism with Sp reduced both adipose and cardiac pro-inflammatory/pro-fibrotic gene expression in concert with restored coronary endothelial function with no change in blood pressure. These data are consistent with our recent report that MR antagonism reversed coronary dysfunction as well as adipose and cardiac inflammation in the obese Zucker rat model of insulin resistance [5]. Together with available evidence in patients with type 2 diabetes [19, 20], these studies demonstrate a clear impact of MR antagonism to treat obesity/diabetes-associated coronary microvascular endothelial dysfunction. Surprisingly, however, MR antagonism did not attenuate diabetes-associated increases of coronary vasoconstrictor responses despite a modest reduction of vasoconstriction to L-type Ca^{2+} channel activation. The latter finding is consistent with the recent report that vascular L-type Ca^{2+} channels are upregulated by smooth muscle MR activation in aging [8, 9]. Together, these data suggest that mechanisms other than MR activation may be principle contributors to enhanced coronary constriction despite the improvement of endothelial function following MR antagonism in the obese, diabetic OLETF rat. Importantly, it should be noted that MR antagonism in both the obese Zucker [5] and OLETF rat models is beneficial despite reduced plasma aldosterone levels indicating a limitation of plasma aldosterone as an index of pathologic MR activation.

In order to delineate potential mechanisms underlying the benefit of MR antagonism on vascular function in the setting of obesity and diabetes, we employed RNA Seq coupled with IPA analysis of differentially expressed genes. In total, RNA Seq revealed 157 differentially

expressed genes in aortas from OLETF rats following MR antagonism. MR-dependent regulation of vascular cell gene expression has previously been evaluated via short-term treatment of isolated vessels or cultured vascular cells with the MR agonist aldosterone [13, 14, 16, 38, 39]. Conversely, our study examined the impact of chronic MR antagonism with Sp *in vivo* on the vascular transcriptome in the setting of established obesity and diabetes. In this way, to our knowledge, this is the first study of its kind focused on delineating potential transcriptomic mechanisms underlying the vascular benefit of MR antagonism in obesity/diabetes. It should be noted that only four genes (*Egr-1*, *Nedd9*, *Tsc22d3*, *Zfp281*) identified in this study overlap with genes differentially expressed by acute MR activation in healthy cells/vessels *ex vivo* [14, 16, 38]. Together, these results support a growing recognition of significant context dependency of MR-dependent gene regulation [40] warranting further examination in disease states associated with inappropriate renin-angiotensin-aldosterone system activation.

Vascular transcriptomic analysis in Sp- versus placebo-treated OLETF rats revealed several differentially regulated transcripts and pathways which likely contribute to the Sp-mediated improvement of coronary microvascular vasodilation, a primary functional outcome of this study. First, Sp treatment resulted in upregulation of aortic *Hsp90ab1* mRNA as well as upregulation of aortic *Ahsa1* and *Ahsa2*, highlighted in the top regulated gene network determined by IPA. Hsp90 is a molecular chaperone and key mediator of agonist-mediated endothelial NO synthase (eNOS) activation and NO production [41]. Further, *Ahsa1* and *Ahsa2* (aka AHA1) are Hsp90 co-chaperones directly involved in agonist-induced Hsp90 phosphorylation, eNOS activation, and NO production [42]. These findings implicate improved dynamic regulation of eNOS-mediated NO production as a potential mechanism underlying enhanced coronary endothelium-dependent vasodilation following MR blockade in diabetes. Second, consistent with reduced cardiac and adipose pro-inflammatory/pro-fibrotic gene expression by PCR, RNA Seq revealed transcriptomic changes indicative of reduced vascular inflammation and oxidative stress following MR blockade. Specifically, several of the top genes downregulated by MR blockade are subunits necessary for pro-inflammatory AP-1 signaling (*Jun*, *Fosb*, *Fos*) and upstream regulator analysis by IPA was enriched for predicted inhibition of endogenous pro-inflammatory/pro-fibrotic mediators by Sp (PDGF-BB, TNF, NFκB, etc). In addition, the top canonical pathway in IPA impacted by MR blockade was ‘NRF2-mediated oxidative stress response’. NRF2 signaling is a critical positive regulator of antioxidant defenses, the activation of which attenuates vascular oxidative stress [43, 44]. Genes of this canonical pathway (*Dnaja1*, *Dnajb5*, *Ephx1*, *Fos*, *Gab1*, *Hacd3*, *Jun*, *Junb*, *Jund*, *Pik3c3*, *Rras2*, *Stip1*) are altered by Sp in a manner suggesting activation of this pathway consistent with NRF2 (NFE2L2) being identified by IPA as a predicted upstream regulator activated by Sp. These data suggest a broad impact of MR antagonism on vascular redox state in diabetes involving not only reduction of well described pro-oxidant actions of MR activation [6] but also a less well appreciated enhancement of antioxidant defenses in the vasculature warranting further exploration. Taken together, these data support the paradigm that vascular inflammation/endothelial activation underlies coronary (and ultimately cardiac) dysfunction in obesity/diabetes and that the benefit of MR antagonism involves reduced vascular inflammation and improved regulation of NO bioavailability.

Examination of potential biological functions impacted by MR antagonism via IPA lends additional support to reduced vascular inflammation and endothelial activation with Sp in diabetes. Specifically, IPA predicted that MR antagonism will inhibit migration of endothelial cells, smooth muscle cell differentiation, and angiogenesis in line with identification of VEGF-A as a Sp-inhibited upstream regulator. Endothelial activation and oxidative stress are key steps in the initiation of endothelial migration, particularly in the context of angiogenesis. Interestingly, available evidence for the role of MR signaling in angiogenesis is inconsistent. For instance, in models of hindlimb ischemia via femoral artery occlusion, MR antagonism improved angiogenesis [45] while infusion of the MR agonist aldosterone has been reported to enhance [46] or attenuate [47] angiogenesis via MR-dependent mechanisms. Thus, further studies regarding the role of vascular MR signaling in peripheral angiogenesis are warranted, particularly in the setting of obesity and diabetes where this has not been examined.

Beyond the changes in gene expression consistent with our functional data, RNA-Seq revealed other novel Sp-induced changes in the diabetic vascular transcriptome. Notably, MR antagonism resulted in downregulation of the α_{1D} adrenergic receptor (*Adra1d*) and atypical chemokine receptor 3 (*Ackr3*) gene expression in OLETF rats. Recent evidence indicates that these receptors, along with chemokine (C-X-C motif) receptor (CXCR) 4, engage in a dynamic process of hetero-oligomeric complex formation in vascular smooth muscle [48, 49]. The makeup of these complexes modulates α_{1D} -dependent responses (*i.e.*, vasoconstriction) and this previously unreported downregulation of *Adra1d* itself as well as the modulatory *Ackr3* by MR antagonism is worthy of further examination as sensitization of coronary α_1 adrenergic receptors and increased α_1 -dependent coronary vasoconstriction have been reported in prediabetic dogs [50]. Our transcriptomic analysis also revealed pronounced downregulation of prostaglandin-endoperoxide synthase 2 (*Ptgs2*; *i.e.*, cyclooxygenase 2) as well as upregulation of acyl-CoA synthetase long-chain family member 4 (*Acsl4*). These directional changes would be expected to reduce vascular production of the vasoconstrictor prostaglandin E₂ (PGE₂) by directly reducing *Ptgs2* that produces PGE₂ [51] as well as reducing membrane phospholipid substrate for *Ptgs2* via increased *Acsl4* [52]. These MR-associated alterations may provide novel mechanistic insight into the previously reported impairment of endothelium-dependent vasodilation in a cyclooxygenase-dependent manner in aldosterone-infused mice [17].

In summary, results of the present study identify a variety of novel vascular transcripts and gene networks associated with the benefit of MR antagonism to reverse coronary microvascular vasodilator dysfunction in diabetes. Importantly, this benefit occurred independent of changes in blood pressure or serum potassium. These results are clinically relevant given the growing appreciation of the role of coronary microvascular dysfunction in the etiology of cardiac disease [53] as well as the powerful association of coronary microvascular dysfunction and cardiac mortality in diabetic patients [3]. The efforts of the present study therefore provide novel insight into and the first report of potential vascular-specific targets linked to MR activation in diabetes worthy of further examination. Ultimately, enhanced understanding of vascular MR signaling in diabetes holds promise to address the critical need for improved coronary flow regulation in diabetes in order to

prevent cardiac diastolic dysfunction, the primary cardiac defect in heart failure with preserved ejection fraction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ACh	acetylcholine
BP	blood pressure
CD4	cluster of differentiation 4
CD11c	cluster of differentiation 11c
Col I	collagen I
Col III	collagen III
CFR	coronary flow reserve
ET-1	endothelin-1
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
ICAM-1	intercellular adhesion molecular-1
LETO	Long-Evans Tokushima Otsuka
MR	mineralocorticoid receptor
OETF	Otsuka Long-Evans Tokushima Fatty
PSS	physiological salt solution
SNP	sodium nitroprusside
Sp	spironolactone
TNF-α	tumor necrosis factor- α
U46619	thromboxane A ₂ mimetic

VCAM-1 vascular cell adhesion molecule-1

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Figure 1.

Differential progression of coronary vasomotor dysfunction in OLETF rats. Vasomotor responses of coronary arterioles from Long-Evans Tokushima Otsuka (LETO; left panels) control and Otsuka Long-Evans Tokushima Fatty (OLETF; right panels) rats to **(A)** the endothelium-dependent vasodilator acetylcholine and **(B)** the vasoconstrictor endothelin-1 at 5–8, 20, and 32 weeks of age. Values are mean±SE; n=3–5; *p<0.05 versus LETO, †p<0.05 versus 5–8 wk OLETF and age-matched LETO.

Figure 2.

Cardiac and adipose pro-inflammatory and fibrotic markers are elevated in Otsuka Long-Evans Tokushima Fatty (OLETF) rats and attenuated by mineralocorticoid receptor antagonism. Cardiac gene expression of pro-inflammatory (A; tumor necrosis factor- α [TNF- α], vascular cell adhesion molecular-1 [VCAM-1], and intracellular adhesion molecule-1 [ICAM-1]) and fibrosis (B, collagen I [Col I] and collagen III [Col III]) genes in hearts from Long-Evans Tokushima Otsuka (LETO), OLETF, and OLETF rats treated with spironolactone (Sp). Adipose gene expression of pro-inflammatory (C, TNF- α , VCAM-1, ICAM-1) and leukocyte (D, F4/80 and CD11c for macrophages; CD4 for T cells) genes in retroperitoneal adipose from LETO, OLETF, and OLETF Sp rats. Values are mean $\pm$ SE; n=5–8; *p<0.05 versus LETO, †p<0.05 versus OLETF, ‡p=0.08 versus OLETF, §p=0.07 versus LETO.

Figure 3.

Impaired coronary arteriolar vasodilation, but not enhanced vasoconstriction, in OLETF rats is treated by mineralocorticoid receptor antagonism. **A**, Coronary vasodilation to acetylcholine and insulin in arterioles from 32 week old Long-Evans Tokushima Otsuka (LETO) control, untreated Otsuka Long-Evans Tokushima Fatty (OLETF) rats, and OLETF treated with spironolactone (OLETF Sp). **B**, Coronary vasoconstriction to endothelin-1, thromboxane A₂ mimetic U46619, and the L-type Ca²⁺ channel opener BayK-8644. Values are mean±SE; n=5–12; *p<0.05 versus LETO, †p<0.05 versus OLETF.

Figure 4.

Gene expression of differentially expressed aortic transcripts in coronary artery by PCR. The 10 top (A) up- and (B) down-regulated aortic transcripts determined by RNA-Seq were assessed in coronary arteries from placebo- and spironolactone (Sp)-treated Otsuka Long-Evans Tokushima Fatty (OLETF) rats by RT-PCR. Values are mean $\pm$ SE; n=4-5; *p<0.05 versus OETF Placebo, †p=0.06 versus OETF Placebo.

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Figure 6. The second most highly regulated gene network in Sp versus placebo-treated OLETF rats (IPA score 37). Up-regulated hubs indicated in red and down-regulated hubs indicated in green with brighter coloring indicating greater differences between Sp and placebo-treated rats. For convenience, log fold change differences of individual genes are provided below each gene.

Table 1.

Phenotypic characteristics of 32 week old LETO and OLETF rats

Parameter	LETO			OLETF			OLETF + Spironolactone		
Body Weight (g)	471	±	14	674	±	21 [*]	617	±	28 [*]
Heart Weight (g)	1.46	±	0.04	1.65	±	0.07 [*]	1.58	±	0.04 [*]
RP Adipose Weight (g)	7.6	±	0.8	22.7	±	2.6 [*]	20.9	±	2.7 [*]
Systolic Blood Pressure (mmHg)	141	±	6	138	±	5	146	±	9
Plasma Glucose (mg·dl ⁻¹)	153	±	4	236	±	19 [*]	223	±	14 [*]
Plasma Insulin (ng·ml ⁻¹)	3.4	±	0.4	3.5	±	1.5	3.9	±	1.3
Plasma Cholesterol (mg·dl ⁻¹)	94	±	3	153	±	8 [*]	147	±	19 [*]
Plasma Triglycerides (mg·dl ⁻¹)	41	±	3	301	±	33 [*]	267	±	65 [*]
Serum Potassium (mEq·L ⁻¹)				5.0	±	0.1	4.5	±	0.4
Plasma Aldosterone (pg·ml ⁻¹)	252	±	16	135	±	14 [*]	215	±	32 ^{**}

Values are mean ± SE, n = 5–10,

^{*}
p<0.05 versus LETO,^{**}
p<0.05 versus OLETF; RP, retroperitoneal

Table 2.

Top up- and down-regulated genes and canonical pathways between spironolactone and placebo-treated OLETf treated rats

Entrez ID	Symbol	Gene Name	FDR	Log Fold
Spironolactone-treated > Placebo-treated				
26973	<i>Chordc1</i>	Cysteine And Histidine Rich Domain Containing 1	1.00E-07	1.14
28951	<i>Trtb2</i>	Tribbles Pseudokinase 2	1.87E-07	1.07
27101	<i>Cacybp</i>	Calcyclin Binding Protein	2.40E-04	1.03
54805	<i>Cnnm2</i>	Cyclin And CBS Domain Divalent Metal Cation Transport Mediator 2	6.19E-05	0.94
2288	<i>Fkbp4</i>	FK506 Binding Protein 4	7.28E-06	0.93
10963	<i>Stip1</i>	Stress Induced Phosphoprotein 1	5.53E-05	0.88
3301	<i>Dna1l</i>	Dna1 Heat Shock Protein Family (Hsp40) Member A1	2.31E-02	0.88
51330	<i>Tuftsrl2a</i>	Tumor Necrosis Factor Receptor Superfamily, Member 12A	5.36E-04	0.83
1827	<i>Rcan1</i>	Regulator Of Calcineurin 1	1.80E-03	0.82
5033	<i>P4ha1</i>	Prolyl 4-Hydroxylase Subunit Alpha 1	1.28E-04	0.82
Spironolactone-treated < Placebo-treated				
1843	<i>Dusp1</i>	Dual Specificity Phosphatase 1	2.02E-13	-1.46
9592	<i>Ier2</i>	Immediate Early Response 2	2.90E-08	-1.55
3164	<i>Nr4a1</i>	Nuclear Receptor Subfamily 4 Group A Member 1	3.92E-09	-1.59
3725	<i>Jun</i>	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	3.62E-12	-1.70
2354	<i>Fosb</i>	FosB Proto-Oncogene, AP-1 Transcription Factor Subunit	1.08E-05	-1.74
5743	<i>Pgs2</i>	Prostaglandin-Endoperoxide Synthase 2	1.87E-06	-2.13
7832	<i>Btg2</i>	BTG Anti-Proliferation Factor 2	1.32E-26	-2.17
1958	<i>Egr1</i>	Early Growth Response 1	3.08E-35	-2.24
3491	<i>Cyr61</i>	Cysteine Rich Angiogenic Inducer 61	1.39E-19	-2.43
2353	<i>Fos</i>	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	1.09E-21	-3.02
Top regulated canonical pathways				
		NRF2-mediated oxidative stress response	p value	Ratio
		Glucocorticoid receptor signaling	1.22E-08	12/193
		IL-2 signaling	9.45E-07	12/288
		Thrombopoietin signaling	5.81E-06	6/64
		IGF-1 signaling	6.36E-06	6/65
			9.96E-06	7/106

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Entrez ID	Symbol	Gene Name	FDR	Log Fold
		ERK/MAPK signaling	1.18E-05	9/199
		Estrogen-dependent breast cancer signaling	1.70E-05	6/77
		Erythropoietin signaling	2.28E-05	6/81