Distinct Endothelial Cell Responses in the Heart and Kidney Microvasculature Characterize the Progression of Heart Failure With Preserved Ejection Fraction in the Obese ZSF1 Rat With Cardiorenal Metabolic Syndrome

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Background—The combination of cardiac and renal disease driven by metabolic risk factors, referred to as cardiorenal metabolic syndrome (CRMS), is increasingly recognized as a critical pathological entity. The contribution of (micro)vascular injury to CRMS is considered to be substantial. However, mechanistic studies are hampered by lack of in vivo models that mimic the natural onset of the disease. Here, we evaluated the coronary and renal microvasculature during CRMS development in obese diabetic Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) rats.

Methods and Results—Echocardiographic, urinary, and blood evaluations were conducted in 3 groups (Wistar-Kyoto, lean ZSF1, and obese ZSF1) at 20 and 25 weeks of age. Immunohistological evaluation of renal and cardiac tissues was conducted at both time points. At 20 and 25 weeks, obese ZSF1 rats showed higher body weight, significant left ventricular hypertrophy, and impaired diastolic function compared with all other groups. Indices of systolic function did not differ between groups. Obese ZSF1 rats developed hyperproliferative vascular foci in the subendocardium, which lacked microvascular organization and were predilection sites of inflammation and fibrosis. In the kidney, obese ZSF1 animals showed regression of the peritubular and glomerular microvasculature, accompanied by tubulointerstitial damage, glomerulosclerosis, and proteinuria.

Conclusions—The obese ZSF1 rat strain is a suitable in vivo model for CRMS, sharing characteristics with the human syndrome during the earliest onset of disease. In these rats, CRMS induces microvascular fibrotic responses in heart and kidneys, associated with functional impairment of both organs. (Circ Heart Fail. 2016;9:e002760. DOI: 10.1161/CIRCHEARTFAILURE.115.002760.)

Key Words: animal model ■ cardiorenal syndrome ■ endothelial cell ■ heart failure ■ mortality

Cardiorenal syndrome (CRS) represents the interdependent relation between the heart and kidneys during disease onset and progression. This important link between cardiac and renal pathophysiology is demonstrated by the high prevalence of chronic kidney disease (CKD) in patients with heart failure and vice versa, whereas dysfunction in both organs is strongly associated with increased morbidity and mortality.1

See Clinical Perspective

Recent findings have also highlighted the connection between CRS and metabolic risk factors: metabolic syndrome is a multitude of risk factors that includes high blood pressure, hyperglycemia, obesity, and dyslipidemia from which at least 3 occur together, thereby functioning as a synergistic complex that drastically increases the risk of cardiovascular disease.2 This syndrome is growing ever more...
prevalent, affecting \( \approx \text{20%} \) of adults in the aging Western population.\(^3\) A significant amount of evidence clearly shows a strong relation between metabolic syndrome and onset and progression of cardiovascular disease.\(^4,5\) In regard to renal disease, a clear association between metabolic syndrome and renal dysfunction has also been described.\(^4,6\) Thus, metabolic risk factors seem to be the driving force behind cardiovascular and CKD disease, and hence CRS. Similarly, the association between all individual components of metabolic syndrome and CRS development is now well established and is increasingly recognized as a separate disease-entity termed cardiorenal metabolic syndrome (CRMS).\(^1,6,7\)

At present, rodent CRS models typically involve renal ablation and coronary ligation, either alone or in combination to directly induce diastolic and systolic dysfunction.\(^8\) An in vivo model that uses metabolic triggers may be more relevant for studies that focus on onset and early progression of CRS. Here we propose the obese diabetic Zucker fatty/ Spontaneously hypertensive heart failure F1 hybrid (ZSF1) rat strain as a model for CRMS.\(^9\) Recently, we have reported that obese ZSF1 (ZSF1 Ob) rats develop heart failure with preserved ejection fraction (HFpEF) between weeks 10 and 20 of natural aging.\(^9\) The ZSF1 strain also autonomously develops renal disease, making it suitable for studying CKD in relation to metabolic triggers.\(^10,11\) HFpEF development in ZSF1 Ob rats was characterized by progressive left ventricle (LV) diastolic dysfunction, concentric LV remodelling, and hypertrophy.\(^12\) This was preceded by insulin resistance, glycosuria, and proteinuria.\(^9\) This phenotype is consistent with CRMS in humans, which is defined by the presence of metabolic syndrome in addition to insulin resistance, microalbuminuria, and reduced renal function.\(^13\) Therefore, these data indicate that the obese ZSF1 strain may be used as an in vivo model of CRS early progression against a well-defined background of metabolic injury, thus providing a model to study CRMS.

ZSF1 Ob rats also experienced increased vascular stiffness and lack of NO response, consistent with endothelial dysfunction.\(^14\) Persistent nitrosative/oxidative stress and inflammation have been observed in the coronary microvascular bed of patients with HFpEF.\(^15,16\) Vascular oxidative stress leading to endothelial dysfunction and inflammation has been proposed as one of the key pathways in HFpEF\(^17\) and CRS,\(^18–20\) and it is strongly linked to metabolic risk factors.\(^19,21\) However, details of the cardiac and renal microvasculature response in CRMS remain to be investigated. Consequently, we studied the microvascular changes in the heart and kidney during progression of HFpEF and renal dysfunction in ZSF1 Ob rats.

**Methods**

**Ethics**

All animal studies were carried out in accordance with the Council of Europe Convention (Directive [2010/63/EU]) for the protection of vertebrate animals used for experimental and other scientific purposes with the approval of the National and Local Animal Care Committee of Faculty of Medicine of Porto. Animal experiments were performed according to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

**Results**

**Animal Model**

Nine-week-old male Wistar Kyoto (WKY, \( n = 12 \)), ZSF1 Lean (ZSF1 Ln, \( n = 10 \)), and ZSF1 Obese rats (ZSF1 Ob, \( n = 12 \)) were obtained from Charles River (Barcelona, Spain) and housed in a light, temperature, and humidity controlled environment after a 12-hour light–dark cycle with free access to water and standard diet (Purina diet no. 5008). Phenotypic evaluation was conducted after 1 week of acclimatization, consisting of metabolic and echocardiography studies. Weight gain was monitored every third day. In weeks 18 and 24, phenotypic evaluation was repeated. Cardiac hemodynamic measurements followed by euthanasia were performed at 20th and 25th week. Heart and kidneys were excised, weighed, fixed in formaldehyde, and embedded in paraffin for further analysis.

**Echocardiography Evaluation**

Rats were anesthetized by inhalation of sevoflurane (8 and 2.5%–3% for induction and maintenance, respectively; Penlon Sigma Delta), mechanically ventilated while homeostasis was maintained by anesthetic monitoring. After applying echocardiography gel a linear 15-MHz probe (Sequoia 15L8W) was positioned on the thorax. Systolic and diastolic wall thickness and cavity dimensions were recorded in M-mode and 2-dimensional (2D) echocardiography, at the level just above the papillary muscles in the parasternal short-axis view. The long-axis diastolic dimensions of the LV and transverse aortic root diameter were recorded by 2D and M-mode echocardiography, respectively, in the parasternal long-axis view. Aortic flow velocity was recorded by pulsed-wave Doppler above the aortic valve. Mitral flow velocity tracings were obtained with pulsed-wave Doppler above the mitral leaflets, peak systolic tissue velocity, and E′ were measured with tissue Doppler at the medial mitral annulus and lateral mitral annulus, respectively, and left atrial diameters were measured at their maximum, by 2D echocardiography in the 4-chamber view. Acquisitions were conducted while suspending mechanical ventilation and recordings were averaged from 3 consecutive heartbeats (Siemens Acuson Sequoia C512). LV mass and volumes were calculated by the 2D area-length method and M-mode. For evaluation of diastolic function, peak velocity of early (E), and late (A) mitral inflow and the ratio of E to E′ were measured as an indicator of LV filling pressure. Myocardial performance index was retrieved from the mitral flow pattern. Volumes and masses were indexed for body surface area as defined by 9.1×body weight (BW)\(^19\).

**Statistics**

Groups were compared by 2-way ANOVA for repeated measurements, followed by Student–Newman–Keuls post hoc test using SigmaPlot 12.3 (Systat Software Inc, San Jose, CA). Data are presented as means±SD. \( P<0.05 \) was considered significant. Alternatively, 1-way ANOVA Kruskal–Wallis test was used, followed by Dunn multiple comparison test. Detailed description of the methods is available in the Data Supplement.

**Results**

**HFpEF Progression in ZSF1 Ob Animals**

ZSF1 Ob rats had LV hypertrophy, as shown by increased echocardiographic indices of LV mass and LV posterior wall thickness at end diastole versus WKY and ZSF1 Ln at 25 weeks, and increase in LV+interventricular septum weight/tibial length versus WKY and ZSF1 Ln at 20 and 25 weeks (Tables 1 and 2). Echocardiographic data showed preserved systolic function in all groups as assessed by ejection fraction, fractional shortening, and cardiac index at 20 or 25 weeks (Table 2). In contrast, ZSF1 Ob presented increased ratio of E to early diastolic TD mitral annulus velocity (E′), significant increase in E/E′/end-diastolic volume ratio, and increase of left atrial area (Table 2), indicative of impaired...
diastolic function. Hemodynamic evaluation showed significant increase in Tau, end-diastolic pressure, and end-diastolic pressure volume relationship $\beta_1$ in ZSF1 Ob rats at 25 weeks versus WKY and ZSF1 Ln animals at 20 and 25 weeks (Table I in the Data Supplement). Furthermore, right ventricular weight/tibial length and lung weight/tibial length were increased in ZSF1 Ob at 25 weeks versus WKY and ZSF1 Ln at 20 and 25 weeks (Table 1). Systolic, diastolic, and mean blood pressure levels were increased in the ZSF1 Ob at 25 weeks versus WKY and ZSF1 Ln at 25 weeks (Table II in the Data Supplement).

ZSF1 Ob Animals Develop Multiple Subendocardial Microstructures of High Cell Density That Are Enriched in Lectin+ ECs

Hematoxylin/eosin examination of heart cross-sections revealed no major differences in tissue morphology between WKY and ZSF1 Ln at 20 or 25 weeks. However, multiple areas with high cell density were observed at subendocardial locations of ZSF1 Ob rats at both time points, which were absent in WKY and ZSF1 Ln (Figure IAI and IBI versus IBI and IBII in the Data Supplement). High magnification assessment identified these ZSF1 Ob-specific areas as irregular star-shaped foci composed of >50 mononuclear cells, located in the interstitial space between the cardiomyocytes (Figure 1C–1G). Erythrocytes were detected in the extravascular space (Figure 1D and 1F), or in the lumen of capillary-like arrangements (Figure 1D and 1H), suggesting that these foci may contain vascular cells.

Lectin+ ECs were organized in a well-structured microcapillary network in both WKY and ZSF1 Ln at 20 and 25 weeks (Figure 2A and 2B). Similarly, a well-organized microcapillary network was observed in ZSF1 Ob at both 20 and 25 weeks in the areas outside the foci (Figure 2A and 2B). Quantification of lectin staining showed a significant increase in capillary area in ZSF1 Ob at 20 weeks of age compared with WKY and ZSF1

| Table 1. Morphometrics of WKY, ZSF1 Lean, and ZSF1 Obese Rats |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                     | WKY 20          | ZSF1 Ln 20      | ZSF1 Ob 20      | WKY 25          | ZSF1 Ln 25      | ZSF1 Ob 25      |
| Body weight, g      | 360±31          | 410±26          | 595±31*         | 368±24          | 469±23†‡        | 633±36*†        |
| TL, mm              | 39.4±0.8        | 41.9±0.9§       | 40.1±1.3        | 38.8±0.5        | 40.9±0.8§       | 39.2±1.0        |
| LV+IVS weight/TL, mg/mm | 30.1±3.9        | 28.2±1.0        | 36.4±2.5*       | 32.6±4.0        | 35.0±3.3‡       | 37.4±2.2†       |
| RV weight/TL, mg/mm | 3.4±1.1         | 2.7±0.4         | 4.1±0.6I        | 3.1±0.8         | 3.6±0.5         | 4.8±1.2*        |
| Lung weight/TL, mg/mm | 34.9±5.3        | 40.6±7.8        | 55.1±5.2*       | 45.4±6.1†       | 48.8±3.3†       | 64.7±3.0*†      |

Two-way ANOVA with Student–Newman–Keuls post hoc test. n=5 to 6 animals per group. Values are mean±SD. IVS indicates interventricular septum; LV, left ventricle; RV, right ventricle; TL, tibial length; and WKY, Wistar Kyoto.

*P<0.05 vs WKY and ZSF1 Ln 20 or 25; †P<0.05 vs week 20; ‡P<0.05 vs WKY 20 or 25; §P<0.05 vs WKY and ZSF1 Ob 20 or 25; IP<0.05 vs ZSF1 Ln.

| Table 2. Echocardiographic Data for WKY, ZSF1 Lean, and ZSF1 Obese Rats |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                     | WKY 20          | ZSF1 Ln 20      | ZSF1 Ob 20      | WKY 25          | ZSF1 Ln 25      | ZSF1 Ob 25      |
| HR, beats per minute | 299±18          | 337±5           | 309±24          | 298±55          | 301±33          | 282±24          |
| dLVPW, mm           | 1.40±0.00       | 1.45±0.07       | 1.47±0.11       | 1.11±0.05*      | 1.25±0.15*,†    | 1.46±0.11†‡     |
| LV mass, mg         | 483±93          | 628±14          | 797±35†         | 386±81          | 604±142†        | 976±201*,‡      |
| FS, %               | 35.4±3.7        | 41.2±4.4        | 37.9±1.8        | 36.2±7.4        | 34.8±6.1        | 35.4±5.3       |
| EF, %               | 70.8±4.8        | 77.3±4.7        | 73.3±2.1        | 71.0±9.0        | 69.2±7.9        | 69.7±6.6       |
| S, mm/s             | 45±3            | 59±6†           | 58±4†           | 48±7            | 54±4            | 52±4           |
| CI, μL/min per cm²  | 212±30          | 187±50          | 215±19          | 172±32          | 178±11          | 220±40         |
| MPI (Tei)           | 0.60±0.01       | 0.59±0.01       | 0.62±0.02       | 0.64±0.03       | 0.62±0.05       | 0.60±0.04      |
| E/A                 | 1.79±0.42       | 1.40±0.08       | 1.33±0.15†      | 1.68±0.24       | 1.56±0.13       | 1.30±0.19†     |
| E′                  | 12.7±0.5        | 11.9±2.3        | 16.7±0.9†       | 12.2±1.6        | 11.9±2.1        | 14.3±1.4*‡     |
| E′/E/DV (log²)      | 22.9±4.7        | 13.2±1.6        | 11.4±0.6†       | 21.4±9.9        | 12.5±5.0†       | 9.0±2.5†       |
| LAA, mm²            | 2.48±0.08       | 2.81±0.30       | 3.51±0.06‡      | 2.25±0.14       | 2.70±0.30       | 3.71±0.13‡     |
| EDVI, μL/cm²        | 1.30±0.17       | 1.87±0.17       | 2.35±0.22       | 1.37±0.41       | 1.87±0.46       | 2.45±0.49      |

Two-way ANOVA with Student–Newman–Keuls post hoc test. n=2 to 4 animals per group at 20 weeks; n=5 to 6 animals per group at 25 weeks. Values are mean±SD. CI indicates cardiac index; dLVPW, left ventricular posterior wall measured in diastole; E/A, ratio between peak E and A waves of pulsed-wave Doppler mitral flow velocity; EDVI, end-diastolic volume indexed for body surface area; E′/E′, ratio between peak E-wave velocity of pulsed-wave Doppler mitral flow and peak E′-wave velocity of tissue Doppler at the lateral mitral annulus; EF, ejection fraction; EVD, end-diastolic volume; FS, fractional shortening; HR, heart rate; LAA, left atrial area; MPI, myocardial performance or Tei index; S, peak systolic tissue Doppler velocity at the mitral annulus; and WKY, Wistar Kyoto.

*P<0.05 vs week 20; †P<0.05 vs WKY 20 or 25; ‡P<0.05 vs WKY and ZSF1 Ln 20 or 25.
and regulation of blood flow. Immunostaining for NG2 identified vascular pericytes located in the subendocardial foci, further validating their vascular identity. In the WKY and ZSF1 Ln, NG2+ pericytes were detected at their characteristic peri-EC localization, as shown by lectin double staining (representative cross-section of WKY at 25 weeks is shown in Figure 3A). In contrast, in ZSF1 Ob, the number of NG2+ pericytes was markedly increased in the regions outside the foci (Figure 3A). In the subcardiac foci, NG2+ pericytes were also observed in close distance to lectin+ ECs (Figure 3A and 3B). Although some coverage of vascular structures could be recognized, the majority of the NG2+ pericytes form atypical multilayered clusters mixed with lectin+ ECs (Figure 3B).

To further validate the vascular content of the ZSF1 Ob foci, pericytes were stained with a second vascular mural cell marker PDGFRβ in combination with a second EC marker, JG12. Immunostaining experiments using cultured human vascular cells demonstrate that NG2 and PDGFRβ are suitable markers for mural cells, in particular pericytes, being minimally expressed by endothelial cells (Figure 1A in the Data Supplement). PDGFRβ+ pericytes were detected in WKY and ZSF1 Ln at peri-EC localization (Figure 3C). In the ZSF1 Ob, PDGFRβ+ pericytes accumulated in the foci and were atypically organized with limited perivascular coverage (Figure 3C and 3D). In the capillary network directly surrounding these foci, an increase in PDGFRβ+ pericytes was observed when compared with the capillary network in WKY and ZSF1 Ln (Figure 3E and 3F). The number of vascular foci increased by 9- and 20-fold in ZSF1 Ob versus WKY and by 4- and 27-fold in ZSF1 Ob versus ZSF1 Ln at 20 and 25 weeks, respectively, when quantified using the NG2+ signal (P<0.001; Figure 3G, left graph). Similarly, quantification of foci using the PDGFRβ+ signal showed increases of 7- and 25-fold in ZSF1 Ob versus WKY and of 4- and 29-fold in ZSF1 Ob versus ZSF1 Ln at 20 and 25 weeks, respectively (P<0.001; Figure 3G, right graph). The number of foci also increased in ZSF1 Ob over time based on both NG2 and PDGFRβ methods of quantification (P<0.001; Figure 3G). Combined, these data identify the subendocardial structures as vascular cells enriched foci that specifically develop in ZSF1 Ob rats.

Increased Endothelial Cell Proliferative Activity, Fibrosis, and AGTR1 Expression in the Vascular Foci of ZSF1 Ob Animals

The large number of vascular cells in the foci point toward increased endothelial cell proliferation. We used a combination of lectin and Ki67 staining to assess the number of proliferating endothelial cells. In ZSF1 Ob foci, lectin+ ECs were enriched in nuclear Ki67 signals when compared with the surrounding tissue that displayed a regular organization of microvascular network (Figure 4A and 4C). These results were verified using a second AGTR1/Ki67 staining combination, which demonstrated a similar preferential signal of Ki67 in AGTR1+ ECs located in the foci (Figure 4B and 4D).

Fibrogenic activity in these pericyte-enriched foci was investigated by histological Periodic acid-Schiff examination, a staining method that is often used to detect extracellular deposition of (vascular) basal membrane glycoproteins. Extracellular fibril-like Periodic acid-Schiff+ structures were

ZSF1 Ob-Specific Subendocardial Foci Are Enriched in NG2+ and PDGFRβ+ Pericytes That Form Disorganized Vascular Clusters With Lectin+ and JG12+ ECs

Pericytes are vascular mural cells that are typically found in the capillary bed. They are vital for microvascular survival...
detected in between vascular cells located in the foci, pointing toward active glycoprotein deposition (WKY cross-section compared with ZSF1 Ob foci at 25 weeks; Figure 5A–5F).

Double staining for lectin+ ECs and fibronectin showed limited deposition of these early fibrosis-associated extracellular matrix components in the regular capillary network of WKY and ZSF1 Ln (Figure 5G). In contrast, fibronectin deposition was increased in the subendocardial foci of ZSF1 Ob, and in the vascular network surrounding the foci (Figure 5G and 5H). Double staining for PDGFRβ+ pericytes and collagen IV, a vascular basal membrane component predominantly produced by vascular cells, showed robust collagen IV deposition in the regular capillary network of WKY and ZSF1 Ln (Figure 5I). Similarly, collagen IV was detected in the PDGFRβ+ foci and direct surrounding capillaries of ZSF1 Ob rats (Figure 5I and 5J). Activation of the renin–angiotensin–aldosterone system (RAAS) may contribute to CRMS and HFpEF development in the ZSF1 Ob animals. Immunostaining for AGTR1, the angiotensin II receptor most associated with cardiac hypertrophy, VSMC hyperproliferation, and vascular constriction, showed increased expression of AGTR1 by cardiomyocytes and interstitial perivascular cells in ZSF1 Ob versus WKY and ZSF1 Ln controls in areas outside the vascular foci (Figure 5K and 5L). AGTR1 was highly expressed by both lectin+ ECs and perivascular cells in the ZSF1 Ob-specific vascular foci (Figure 5M).

**Vascular Foci of ZSF1 Ob Animals Are Preferred Recruitment Sites for Circulatory Immune Cells**

To assess whether inflammatory cells contribute to the fibrogenic process, double staining for lectin+ ECs and CD3+ T cells was conducted. Perivascular CD3+ T cells were absent in the microvascular bed of WKY and ZSF1 Ln (Figure 6A). In contrast, perivascular CD3+ T cells were detected in limited numbers in ZSF1 Ob in the vascular foci, whereas the remaining capillary network showed no CD3+ T-cell recruitment. Similarly, double staining for lectin+ ECs and ED1+ macrophages showed an absence of perivascular macrophages in WKY and ZSF1 Ln capillary networks, whereas in ZSF1 Ob, ED1+ macrophages accumulated in the subendocardial foci, indicating active recruitment of circulating immune cells at these sites (Figure 6B).

**Renal Function Declines in ZSF1 Ob Rats**

Compared with WKY, plasma urea was not affected in ZSF1 Ln and Ob at 20 and 25 weeks. At 20 and 25 weeks, a 9- and 38-fold increase in protein/creatinine ratio was observed, respectively, in ZSF1 Ob compared with WKY (P<0.001; Table 3). Similarly, at 20 and 25 weeks, a 6- and 37-fold increase in protein/creatinine ratio was observed in ZSF1 Ob compared with ZSF1 Ln (P<0.001; Table 3). Oxidative stress, measured by thiobarbituric acid reactive substance,
was increased in ZSF1 Ob compared with WKY and ZSF1 Ln at both time points ($P<0.001$; Table 3). No differences in protein/creatinine and thiobarbituric acid reactive substance/creatinine ratio were observed between WKY and ZSF1 Ln. Calculated per body weight (per 100 g), creatinine clearance was significantly reduced in ZSF1 Ob rats ($1.2\pm0.1$ mL/min per 100 g versus $0.7\pm0.1$ mL/min per 100 g for ZSF1 Ln and ZSF1 Ob, $P<0.05$, based on Student $t$ test).

**ZSF1 Ob Rats Show Degradation of Renal Endothelium and Develop Progressive Glomerulosclerosis**

The highest chronic tubulointerstitial damage and glomerulosclerosis scores were measured in ZSF1 Ob compared with WKY and ZSF1 Ln at 20 and 25 weeks (Figure 7A and 7B). Compared with WKY, glomerulosclerosis was increased in ZSF1 Ln (11±5 versus 23±5 and 13±5 versus 24±5, $P<0.05$). With age glomerulosclerosis increased in ZSF1 Ob (39±10 versus 53±14, $P<0.01$). Peritubular and glomerular endothelium were visualized on JG12-stained slides (Figure 8A). Compared with WKY and ZSF1 Ln, the percentage of peritubular and glomerular endothelium decreased in ZSF1 Ob (Figure 8B and 8C).

**Discussion**

The main findings of this study are (1) the obese ZSF1 rat strain is a suitable in vivo model for CRMS, sharing characteristics with the human syndrome during the earliest onset of

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** Subendocardial foci in Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) Ob rats are also enriched with NG2+ and PDGFRβ+ pericytes. A, Representative cross-sections of the left ventricles (LVs) of Wistar Kyoto (WKY) and ZSF1 Ob at 25 weeks, stained for NG2+ pericytes (green signal) and lectin+ endothelium (red signal), imaged by confocal microscopy. B, High magnification detail showing disorganized clusters of lectin+ ECs and NG2+ pericytes in the subendocardial foci of ZSF1 Ob. Arrows indicate lectin+ vascular structures with open lumens. C, Representative cross-sections of the LVs of WKY and ZSF1 Ob at 29 weeks, stained for JG12+ endothelium (green signal), and PDGFRβ+ pericytes/mural cells (red signal), imaged by confocal microscopy. Cell nuclei are stained with (Continued)
disease. (2) in obese ZSF1 rats, CRMS induces microvascular fibrotic responses in heart and kidneys, associated with functional impairment of both organs.

**ZSF1 Ob Rats as a Suitable Model for CRMS—Metabolic Syndrome as a Trigger for Chronic CRS**

Humans with metabolic derangements such as obesity, hypertension, hyperglycemia, and dyslipidemia—all recognized as important risk factors for CKD and cardiovascular disease—often present with preclinical diastolic dysfunction, diastolic heart failure, or HFpEF. In current and previous reports, we could validate that the ZSF1 Ob animals share these characteristics with patients with metabolic syndrome: ZSF1 Ob rats are obese (65% and 45% increase compared with WKY and ZSF1 Ln, respectively, Table 1), hypertensive (46% and 19% increase in mean blood pressure compared with WKY and ZSF1 Ln, Table II in the Data Supplement), and show elevated fasting plasma glucose levels (65% and 45% increase compared with WKY and ZSF1 Ln, respectively), whereas other studies have reported hypertriglyceridemia (increase of...
>25-fold) and hypercholesterolemia (increase of >5-fold) in ZSF1 Ob versus ZSF1 Ln.\textsuperscript{10,28}

Furthermore, CRMS in humans is defined by the presence of metabolic syndrome in addition to insulin resistance, microalbuminurina, and reduced renal function.\textsuperscript{13} Current and previous findings demonstrate that ZSF1 Ob develops proteinuria from 10 weeks onwards,\textsuperscript{9} whereas early signs of HFpEF were detected from 14 weeks onwards. Similarly at 10 weeks, increased oral glucose tolerance and insulin resistance, glycosuria and significant weight gain can be detected.\textsuperscript{9} These observations indicate that the presentation of metabolic risk factors accompanies the onset of renal dysfunction (signified by proteinuria) and precedes HFpEF development in ZSF1 Ob rats, which is consistent with a pathophysiological course of CRMS in ZSF1 Ob in which metabolic and renal complications induce cardiac dysfunction.\textsuperscript{18} On the basis of these combined data, we therefore propose ZSF1 Ob as a small animal model for metabolic syndrome–induced CRS (and thus CRMS), in which the combined metabolic risk factors act together to trigger early onset and progression of CKD and HF, creating the downward spiral of disease progression that is so typical for patients with chronic CRS.\textsuperscript{10}

**Microvascular Fibrotic Changes in ZSF1 Ob Rats**

CRMS was first suggested as a disease entity in 2004.\textsuperscript{29} Already in this first publication, a relation with endothelial dysfunction was proposed.\textsuperscript{29} Endothelial injury is implicated in CRS,\textsuperscript{20} whereas in HFpEF patients, vascular changes represented by endothelial dysfunction have been reported.\textsuperscript{30} A direct link between heart failure, vascular activation, metabolic comorbidities, and CKD was presented by Shestakova et al.,\textsuperscript{19} who demonstrated a correlation between endothelial dysfunction and the hallmarks of HF in CRS patients with type I diabetes mellitus. In line with these studies, our data show changes in the coronary microvascular bed of ZSF1 Ob rats: endothelial and mural cells form atypical vascular patches of cells, with remarkable increases in proliferation rate. Not only do these structures lack functional organization they also seem to act as origin sites of fibrosis, for we could demonstrate enriched deposition of fibrosis-associated extracellular matrix components such as fibronectin and collagen type IV in these vascular foci.

In CRS rat models that use subtotal nephrectomy in combination with coronary ligation to initiate ischemic heart disease, a decrease in LV microvascular density was reported to be associated with systolic dysfunction.\textsuperscript{31,32} Other clinical and experimental studies provide evidence that coronary microvascular rarefaction in diastolic and hypertrophic heart failure promotes tissue hypoxia, cell death, and fibrosis, all contributing to the progression from compensated hypertrophy to contractile dysfunction.\textsuperscript{33,34} In adults, vascular proliferation is part of the compensatory and repair response to local hypoxia and tissue damage in which endothelial cells proliferate and migrate into the ischemic injury site during macrophage influx and phagocytosis of necrotic tissue. As repair proceeds, dead

![Figure 4. Subendocardial foci in Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) Ob rats are sites of increased endothelial cell proliferation.](http://circheartfailure.ahajournals.org/)

**Figure 4.** Subendocardial foci in Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) Ob rats are sites of increased endothelial cell proliferation. Representative images of subendocardial foci of ZSF1 Ob at 25 weeks, stained for (A) lectin+ endothelium (red signal) and Ki67 cell proliferation marker (green signal), and (B) JG12+ endothelium (green signal), and Ki67 (red signal), followed by confocal imaging. Cell nuclei are stained with DAPI (blue signal). High magnification detail panels showing (C) lectin-Ki67 staining and (D) JG12-Ki67 staining.
Figure 5. Onset of fibrosis at the subendocardial foci of Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) Ob rats. Representative Periodic acid-Schiff (PAS)–stained cross-sections of the left ventricles (LVs) of Wistar Kyoto (WKY) and ZSF1 Ob at 25 weeks. A, Extra cellular matrix (ECM) glycoprotein deposition in the interstitial space between cardiomyocytes in WKY. B and D, Accumulation of ECM glycoprotein in subendocardial foci of ZSF1 Ob. E and F, High magnification detail showing PAS signal distribution in subendocardial foci of ZSF1 Ob. G, Representative cross-sections of the LVs of WKY and ZSF1 Ob at 25 weeks, stained for ECM fibronectin (green signal) and lectin+ endothelium (red signal) at low magnification (left panel in each row) and at high magnification (remaining panels). H, Representative example of overlap between fibronectin and lectin+ endothelial distribution in subendocardial foci of ZSF1 Ob at low (top) and high magnification (bottom). I, Cross-sections of the LVs of WKY and ZSF1 Ob at 25 weeks, stained for vascular basal membrane (Continued)
tissue is replaced by granulation tissue, characterized by the deposition of a provisional matrix enriched in glycoproteins and fibronectin.35,36 The vascular foci that developed in ZSF1 Ob rats resembled this process of scar formation, with excessive endothelial cell proliferation, fibronectin deposition, and local recruitment of macrophages.

Vascular cells in the foci contribute to the formation of granulation matrix, demonstrated by deposition of the typical vascular basal membrane component collagen type IV. Collagen type IV is predominantly produced by pericytes and not by fibroblasts, as shown by quantitative polymerase chain reaction using in vitro human primary vascular cell types (Figure IB in the Data Supplement).

Extensive myocardial fibrosis is one of the hallmarks of CRMS. Previously, we reported that at 20 weeks, cardiac hypertrophy was increased in ZSF1 Ob versus ZSF1 Ln and WKY, but overall levels of myocardial fibrosis remained comparable between groups.9 In contrast, glomerulosclerosis score in the kidneys (Figure 8B) was significantly increased in ZSF1 Ob versus the other 2 groups. Thus, the effect of metabolic risk factors on kidney fibrosis seems to precede the effects on fibrosis in the myocardium in the ZSF1 Ob animals. In this study, we could demonstrate that onset of fibrosis is present in the subendocardial vascular foci at 20 and 25 weeks in ZSF1 Ob rats. This was accompanied by vascular inflammation, yet another hallmark of CRMS.

On the basis of our observations to date, we hypothesize that the hyperproliferative vascular reaction in ZSF1 Ob heart is part of a destructive response that leads to replacement of functional vascular structures by fibrotic tissue with semi and nonfunctional microvascular clusters, which may ultimately deteriorate into large fibrotic areas that are completely devoid of vascular support, in line with the previously reported vascular rarefaction phenotype. Additional studies are planned to further elucidate this highly dynamic process and to validate if microvascular hyperproliferation of pre-existing vessels indeed precedes fibrogenesis and vascular rarefaction in CRMS.

Potential Impact of Current Findings on the Field of CRMS Research

Chronic CRS type 2, which is initiated by chronic cardiac dysfunction (such as congestive HF), and type 4, initiated by chronic renal dysfunction (resulting in LV hypertrophy and diastolic HF), are currently under-recognized syndromes in the cardiovascular research community in comparison with acute CRS types. This is largely because of the lack of a suitable animal model to mimic the human condition. However, the population of patients with chronic types of CRS is substantial: in adult patients with congestive HF, it has been reported that 31% of NYHA III and 39% of NYHA IV patients had renal dysfunction.37 Recent findings presented in the PREVEND study also showed that early onset of renal dysfunction, indicated by albuminuria, is a strong predictor for new onset of HFpEF, but not HFrEF.38 Others have also demonstrated that the prevalence of LV hypertrophy is significantly increased in patients with CKD from mild renal impairment onwards.39 These findings are consistent with the concept that the synergy between renal and cardiac dysfunction is a driving factor in the early disease onset of HFpEF. An improved understanding of the disease mechanism of, in
particular, chronic CRS would greatly aid in the development of new diagnostic and treatment options that are specifically targeted to this large subgroup of cardiac and renal patients.

The ZSF1 Ob strain presents a suitable model to investigate the contribution of different disease pathways to CRMS, as the disease course resembles the onset phase of chronic CRS within the first 10 to 25 weeks of the animals’ lifespan. In particular, studies that investigate the relationship between metabolic risk factors and endothelial dysfunction could shed further light on the contribution of vascular injury to HF in chronic CRMS.

**Limitations of This Study**

The ZSF1 Ob model described in this article is not suitable for studying type 1 and type 3 CRS because these forms require acute cardiac and renal failure to adequately mimic the human conditions, and may involve different disease mechanisms compared with the chronic forms (see review of disease pathogeneses of CRS subtypes). A more suitable model for acute CRS would be surgical induction methods for myocardial infarction such as coronary ligation, and subtotal nephrectomy for severe renal dysfunction. Previous studies have indicated that a dual injury model to heart and kidneys is needed to produce a more robust acceleration of renal failure and heart failure with reduced ejection fraction that are typical for acute CRS. However, the causal relationship between metabolic risk factors and chronic CRS can be better studied in the ZSF1 Ob model, as this would best imitate the natural course of the disease in patients with chronic CRS. The addition of ZSF1 Ob to our arsenal of animal models for CRS will greatly expand our means to adequately study mechanisms in the earliest phases of disease.

In addition to the proposed mechanism of vascular fibrosis-mediated CRMS, other disease mechanisms could trigger CRS, including low-cardiac output leading to impaired renal perfusion, which vice versa leads to kidney-mediated RAAS and sympathetic activation. Initial renal dysfunction could also induce chronic CRS via RAAS and sympathetic activation, in addition to Na+ and H2O overload, hypertension, accumulation of uremic toxins and calcium and phosphate abnormalities that all contribute to cardiovascular complications. Furthermore, renal impairment and subsequent dysregulation of erythropoietin contributes to anemia, which further aggravate hypoxia in both organs. In this study, we have not specifically assessed the contribution of these mechanisms to CRS development in the ZSF1 Ob model. However, cardiac output represented by the indexed cardiac output (cardiac index, in Table 2) is similar between the 3 groups at both time points assessed. In addition, ZSF1 Ob animals are indeed hypertensive, and expression of AGTR1, the main receptor for RAAS-mediated effects on the cardiovascular system, was increased in LV sections of the ZSF1 Ob group, particularly at the vascular foci. Further systematic evaluation will help identify for which specific pathological mechanisms of chronic CRS, the ZSF1 Ob strain is a suitable research model.

In conclusion, this study demonstrates that the ZSF1 Ob rat strain is a suitable in vivo model for CRMS, sharing many characteristics with the human CRS during the earliest onset of the disease, as triggered and mediated by metabolic risk factors. Further studies in the classic pathways of CRMS using this ZSF1 Ob model will enhance our understanding of the contribution of microvascular and metabolic changes during the onset of CRMS.

**Table 3. Biochemical Variables for WKY, ZSF1 Lean, and ZSF1 Obese Rats**

<table>
<thead>
<tr>
<th></th>
<th>WKY 20</th>
<th>ZSF1 Ln 20</th>
<th>ZSF1 Ob 20</th>
<th>WKY 25</th>
<th>ZSF1 Ln 25</th>
<th>ZSF1 Ob 25</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>36.1±1.9</td>
<td>28.3±8.9</td>
<td>21.1±5.5*</td>
<td>34.2±8.7</td>
<td>36.0±8.5</td>
<td>22.4±7.6†</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>4.1±0.2</td>
<td>5.3±0.7*</td>
<td>6.3±1.0*</td>
<td>7.3±1.1</td>
<td>6.7±0.5‡</td>
<td>5.9±0.7*</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein/creatinine ratio</td>
<td>119±17</td>
<td>181±9</td>
<td>1086±871†</td>
<td>126±8</td>
<td>130±5</td>
<td>4774±239‡</td>
</tr>
<tr>
<td>TBARS/creatinine ratio</td>
<td>4.1±0.2</td>
<td>3.8±0.6</td>
<td>19.3±15.1†</td>
<td>3.0±0.1</td>
<td>2.3±0.1</td>
<td>49.7±8.5†</td>
</tr>
</tbody>
</table>

Two-way ANOVA with Student–Newman–Keuls post hoc test. Plasma sample size: n=3 to 6 animals per group at 20 weeks. n=5 to 6 animals per group at 25 weeks. Urine plasma size: n=2 to 4 animals per group at 20 weeks. n=4 animals per group at 25 weeks. Values are mean±SD. TBARS indicates thiobarbituric acid reactive substance; and WKY, Wistar Kyoto.

†P<0.05 vs week 20; *P<0.05 vs WKY 20 or 25; †P<0.05 vs WKY and ZSF1 Ln 20 or 25.

Figure 7. Increase in tubulointerstitial damage and glomerulosclerosis in the kidneys of Zucker fatty/Spontaneously hypertensive heart failure F1 hybrid (ZSF1) Ob rats compared with Wistar Kyoto (WKY) and ZSF1 Ln rats. Tubulointerstitial damage (A) and glomerulosclerosis (B) scores for WKY and ZSF1 Ln and ZSF1 Ob at 20 and 25 weeks. Mean±SD. *P<0.05, **P<0.01, ***P<0.001.
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Disclosures

None.

References

13. van Dijk et al. Vascular Response in CRMS in Obese ZSF1 Rats

Cardiorenal syndrome embodies the relationship between the heart and kidney during disease and is strongly associated with increased morbidity and mortality. Cardiorenal syndrome is often triggered by metabolic risk factors, such as obesity, hypotension, hyperglycemia, and dyslipidemia. The strong connection between metabolic risk and cardiorenal syndrome led to the recognition of CRMS as a distinct disease entity. At the moment, there is a growing need to elucidate the disease mechanisms associated with CRMS as the number of patients having CRMS is rising with the increasing prevalence of metabolic disease, whereas specific therapeutic options for CRMS remain unavailable. Cardiorenal syndrome animal models typically involve renal ablation and coronary ligation, whereas an in vivo model that uses metabolic triggers may be more relevant for CRMS. Here we propose the obese ZSF1 rat strain as a suitable model to study CRMS. Renal and cardiac (diastolic) dysfunction develop gradually in ZSF1 animals, triggered and mediated by metabolic factors mimicking the onset of CRMS in humans. Renal and cardiac dysfunction is associated with microvascular endothelial injury, one of the characteristics of CRMS. Here we demonstrate that obese ZSF1 rats develop hyperproliferative vascular foci in the subendocardium, which lack microvascular organization. Obese ZSF1 animals also show regression of the peritubular and glomerular microvasculature in the kidney. Both altered microvascular responses are accompanied by a fibrotic phenotype. Clarification of the role of the microvasculature in the onset and progression of CRMS may help to develop new therapeutic strategies.
Distinct Endothelial Cell Responses in the Heart and Kidney Microvasculature Characterize the Progression of Heart Failure With Preserved Ejection Fraction in the Obese ZSF1 Rat With Cardiorenal Metabolic Syndrome


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SUPPLEMENTAL MATERIAL

Methods

Measurement of biochemical variables
Plasma samples were treated with chloroform to remove lipids which interfere with plasma analysis. Chloroform and plasma (1:1) were centrifuged at 1000 x g for 30 minutes. Supernatant was transferred to a new tube and centrifuged at 10000 x g for 45 minutes. Supernatant was transferred to a new tube and used for creatinine and urea analysis. Plasma and urine creatinine were enzymatically measured (DiaSys PAP FS; DiaSys Diagnostic Systems, Holzheim, Germany). Plasma urea was enzymatically measured (DiaSys Urea CT FS; DiaSys Diagnostic Systems, Holzheim, Germany). Total protein excretion was measured using Bradford method (BioRad Laboratories, Veenendaal, Netherlands). Thiobarbituric Acid Reactive Substances (TBARS) as indicators for the level of systemic oxidative stress were measured colorimetrically (Cayman Chemical, Ann Arbor, MI).

Haemodynamic evaluation
Animals were sedated with 100 µg.kg⁻¹ and 5 mg.kg⁻¹ intraperitoneal fentanyl and midazolam, respectively), followed by anaesthesia (8 and 2.5–3% sevoflurane for induction and maintenance, respectively; Penlon Sigma Delta), endotracheal intubation, and mechanical ventilation (TOPO, Kent scientific). Fluid replacement with warm Ringer’s lactate at 32 mL.kg⁻¹.h⁻¹ (NE-1000, New Era Pump Systems) was administered through a peripheral dorsal foot vein catheter (24G) and animals were maintained at 38°C on a heating pad. A flowmeter probe was transiently placed in the ascending aorta for cardiac output (CO) calibration (2.5PS, Transonic). A pressure-
volume catheter (SPR-847 Millar Instruments) was inserted in the LV apex and placed along the LV long axis. 3-0 silk threads were placed around the inferior vena cava to enable transient occlusions. Parallel conductance was determined by 40 µL 10% hypertonic saline injection and slope factor α was derived by simultaneous measurement of CO by a flowmeter placed around ascending aorta (TS420, Transonic). After a stabilization period of 30 minutes, recordings were obtained at suspended end-expiration. Transient 5-7 cycle occlusions of the inferior vena cava occlusions were performed to obtain load independent indexes of LV chamber stiffness by fitting an exponential function to the end-diastolic pressure-volume relationship (EDPVR) as previously described. Single beat occlusions of the ascending aorta were performed to assess diastolic response to isovolumic afterload as reported. Three separate acquisitions were obtained and averaged in each animal. Data with arrhythmia, heart rate changes higher than 2% or evidence of incomplete afterload elevations were excluded. Resting periods were allowed between each intervention. Signals were continuously acquired (MPVS 300, Millar Instruments), recorded at 1000 Hz (ML880 PowerLab 16/30, ADinstruments), and analyzed (PVAN 3.5, Millar Instruments). To account for large differences in body weight between groups, volumes were indexed for body surface area as estimated by 9.1*body weight in grams¾. Upon completion of experiments, blood (4mL) was collected for volume calibration (910–1048, Millar instruments) and storage. Finally the anesthetized animals were euthanized by exsanguination.

RV and LV + interventricular septum (IVS) were weighed after dissection, and tibia length (TL) was measured. Samples were snap-frozen and stored at -80ºC or processed for histology. Weights were normalized to TL.
Renal and cardiac immunohistology

Three to five µm sections were sliced from formaldehyde-fixed, paraffin-embedded kidneys and hearts. For the analysis of the kidneys, tubulo-interstitial (TI) damage and glomerulosclerosis (GS) were scored on Periodic-acid Schiff (PAS) stained sections in a blinded manner. TI damage was scored on a scale of 1-5 in at least 10 different non-overlapping fields per animal. Scored variables include the amount of per-tubular inflammatory infiltrate, interstitial fibrosis, tubular atrophy and tubular dilatation. GS was scored on at least 30 separate glomeruli by quadrants, on a scale of 0 to 4, with 0 meaning no quadrant affected and 4 meaning that the whole glomerulus was affected. Scored variables for GS were matrix expansion, sclerosis, adhesion of Bowman’s capsule and dilation. For both TI and GS a total damage score was calculated.

Peritubular and glomerular endothelium was stained with a primary antibody for JG12 (mouse anti-JG12, BMS1104, 1:200, Bender Medsystems GmbH, Vienne, Austria). Deparaffinized sections were subjected to heat-induced antigen retrieval by incubation with citrate/HCl buffer (pH 6.0) at 100°C for 20 minutes. As a secondary antibody brightvision-HRP (ImmunoLogic, Duiven, Netherlands) was used. Positive cells were visualized with Vector Nova Red (Vector Laboratories, Burlingame, CA) and counterstained with hematoxilin. Sections were scanned using an Aperio ScanScope XT (Aperio Technologies, Vista, CA) and analyzed using Adobe Photoshop (Adobe Systems, San Jose, CA) and ImageJ software. Ten tubular fields and 30 glomeruli were selected and the percentage of JG12+ area was calculated, to correct JG12+ area for glomerular size.

For the analysis of the heart, deparaffinized cardiac sections were subjected to heat-induced antigen retrieval with antigen retriever 2100 (Aptum Biologics Ltd, UK) with
citrate/HCl buffer (pH 6.0) for 20 minutes. After a brief wash with PBS, sections were blocked with superblock (Life Technologies, Netherlands) for 1 hour before incubation with the following primary antibodies at 4°C overnight. The following antibodies were used; rabbit anti-NG2 (1:100, Abcam ab5320), rabbit anti-PDGFRβ (1:100, Abcam ab32570), biotin labelled anti-Lectin (1:200, Sigma-Aldrich L-3759), mouse anti-JG12 (1:100, eBioscience BMS1104), rabbit anti-Ki67 (1:100, Thermo Scientific RM-9106), goat anti-Collagen IV (1:50, Millipore AB769), rabbit anti-Fibronectin (1:100, Sigma Aldrich F3648), mouse anti-CD68 (1:100, Abcam ab31630), and mouse anti-CD3 (1:100, Dako M7254). Appropriate secondary antibodies (Invitrogen, Netherlands) were used for visualization under fluorescent microscope. Nuclei were counterstained with DAPI (1 µg/ml, Sigma-Aldrich).

Images were taken by Olympus BX53 microscope. The numbers of NG2+ or PDGFRβ+ foci were counted for the whole heart section by microscope examination. For quantification of Lectin staining, pictures from the left ventricle were randomly obtained from lectin stained heart sections (four in the endocardium, four near the epicardium). Images were analysed using ImageJ (v1.49) by quantification of the percentage of the lectin+ area per image area.

**Immunofluorescence cell staining**

Human umbilical vein endothelial cells (HUVECs), pericytes and vascular smooth muscle cells (VSMCs) were seeded on sterile glass cover slips and cultured in EGM2 (Lonza), DMEM (Gibco, #41965), and SmGM-2 (Lonza) medium respectively for four days at 37°C. Cells were first washed with PBS and then fixed with cold methanol for 10 minutes. Cells were blocked with Super blot (ScyTek Laboratories #AAA125) for 1 hour at room temperature. After that, cells were incubated with rabbit anti-NG2
(1:100, Abcam ab5320) or rabbit anti-PDGFRβ (1:100, Abcam ab32570) antibodies over night at 4 °C. After washing with PBS, this was followed by incubation with Alex Flour 488-conjugated goat anti rabbit secondary antibody for 1 hour at room temperature. Nuclei were counterstained with DAPI. Images were taken with the same setting by Olympus BX53 microscope with 20x objective.

**QPCR analysis**

Total RNA of HUVECs, pericytes, VSMCs and fibroblasts was isolated using RNAeasy kit (Qiagen) and treated with DNase to remove genomic DNA contamination. cDNAs were generated using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Quantitative PCR was performed on the BioRad CFX96 RT system using SYBR Green, and mRNA expression levels were normalized to the housekeeping gene β-actin. The PCR primers used are as follows:

β-actin forward: 5’-TCCCTGGAGAAGAGCTACGA-3’,
β-actin reverse: 5’-AGCACTGTGTTGGCGTACAG-3’,
COL4a1 forward: 5’-ACGGGGGAAAACATAAGACC-3’,
COL4a1 reverse: 5’-TGGCGCACTTCTAAACTCCT-3’,
COL4a2 forward: 5’-TTATGCACCTGCTAAAGAGGAGC-3’,
COL4a2 reverse: 5’-CCCTTAACTCCGTAGAAACCAAG-3’.
Supplemental Figure 1. NG2 and PDGFRβ expression in human vascular cell types. (A) Representative immunofluorescent staining of mural cells derived from human origin (pericytes and VSMCs) and HUVECs. NG2 (red), PDGFRβ (green), and nuclei (blue). 20X magnification. (B). QPCR analysis of COL4a1 and COL4a2 expression levels in human derived vascular cells and fibroblasts. N =3. Mean ± SD. ***P<0.001 versus all.
Supplemental Table 1. Hemodynamics for WKY, ZSF1 lean and ZSF1 obese rats

<table>
<thead>
<tr>
<th></th>
<th>WKY 20</th>
<th>ZSF1 Ln 20</th>
<th>ZSF1 Ob 20</th>
<th>WKY 25</th>
<th>ZSF1 Ln 25</th>
<th>ZSF1 Ob 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (cm²)</td>
<td>428 ± 13</td>
<td>490 ± 6 †</td>
<td>651 ± 10 ‡</td>
<td>467 ± 20 *</td>
<td>545 ± 19 * †</td>
<td>671 ± 25 * ‡</td>
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<tr>
<td>HR (bpm)</td>
<td>306 ± 30</td>
<td>334 ± 6</td>
<td>300 ± 32</td>
<td>300 ± 34</td>
<td>311 ± 23</td>
<td>245 ± 13 * ‡</td>
</tr>
<tr>
<td>EF(%)</td>
<td>58 ±2</td>
<td>56 ± 4</td>
<td>61 ± 7</td>
<td>75 ± 1 *</td>
<td>66 ± 9</td>
<td>73 ± 6 *</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>10.0 ± 0.9</td>
<td>11.2 ± 1.7</td>
<td>12.3 ± 1.5 †</td>
<td>9.8 ± 1.7</td>
<td>10.1 ± 1.0</td>
<td>13.4 ± 1.6 ‡</td>
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<tr>
<td>EDP (mmHg)</td>
<td>9.5 ± 2.1</td>
<td>8.8 ± 1.7</td>
<td>16.8 ± 3.1 ‡</td>
<td>8.8 ± 2.4</td>
<td>9.6 ± 2.4</td>
<td>14.0 ± 4.0 ‡</td>
</tr>
<tr>
<td>ESPVR EesI (mmHg/µl/cm²)</td>
<td>0.11 ± 0.01</td>
<td>0.15 ± 0.06</td>
<td>0.19 ± 0.10</td>
<td>0.13 ± 0.09</td>
<td>0.19 ± 0.03 †</td>
<td>0.29 ± 0.09 †</td>
</tr>
<tr>
<td>EDPVR βI (µl/cm²)</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.003 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.000</td>
<td>0.006 ± 0.002 ‡</td>
</tr>
<tr>
<td>dP/dt_max (mmHg/s)</td>
<td>7524 ± 1220</td>
<td>8864 ± 3099</td>
<td>10942 ± 1224 ‡</td>
<td>9061 ± 1203</td>
<td>10681 ± 1193 #</td>
<td>8572 ± 1020 *</td>
</tr>
<tr>
<td>dP/dt_min (mmHg/s)</td>
<td>-8181 ± 2189</td>
<td>-10962± 1105</td>
<td>-10735 ± 1555</td>
<td>-8753 ± 2278</td>
<td>-10839 ± 1334 #</td>
<td>-8374 ± 977 *</td>
</tr>
</tbody>
</table>

BSA, body surface area; HR, heart rate; EF, ejection fraction; Tau, time constant of isovolumetric relaxation; EDP, end diastolic pressure; ESPVR EesI, slope of linear end-systolic pressure-volume relationship for indexed volumes; EDPVR βI, chamber stiffness constant for indexed volumes, derived from exponential end-diastolic pressure-volume relationship; dP/dt_max, maximum rate of pressure rise; dP/dt_min, maximum rate of pressure fall. For the purpose of volume indexation, BSA was estimated as 9.1*(body weight in g)²/³. N = 4-11 animals per group at 20 weeks. N = 6 animals per group at 25 weeks. Values are mean ± SD. *p<0.05 vs. week 20; †p<0.05 vs. WKY 20 or 25; ‡p<0.05 vs. WKY and ZSF1 Ln 20 or 25; #p<0.05 vs ZSF1 Ob 20 or 25. Two way ANOVA with Student-Newman-Keuls post hoc test.
### Supplemental Table 2. Blood pressure for WKY, ZSF1 lean and ZSF1 obese rats

<table>
<thead>
<tr>
<th></th>
<th>WKY 25</th>
<th>ZSF1 Ln 25</th>
<th>ZSF1 Ob 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>352 ± 40</td>
<td>328 ± 20</td>
<td>353 ± 28</td>
</tr>
<tr>
<td>Sys (mmHg)</td>
<td>123 ± 6</td>
<td>147 ± 5 †</td>
<td>170 ± 15 ‡</td>
</tr>
<tr>
<td>Dias (mmHg)</td>
<td>86 ± 6</td>
<td>108 ± 4 †</td>
<td>130 ± 17 ‡</td>
</tr>
<tr>
<td>Mean (mmHg)</td>
<td>98 ± 6</td>
<td>120 ± 5 †</td>
<td>143 ± 16 ‡</td>
</tr>
</tbody>
</table>

HR, heart rate; Sys, systolic blood pressure; Dias, diastolic blood pressure; Mean, mean blood pressure. N = 6 animals per group. Values are mean ± SD. †p<0.05 vs. WKY 20 or 25; ‡p<0.05 vs. WKY and ZSF1 Ln 20 or 25. One way ANOVA with Tukey post hoc test.
References
