Fatty Acids Impair Endothelium-Dependent Vasorelaxation: A Link Between Obesity and Arterial Stiffness in Very Old Zucker Rats

Natacha Sloboda,1,2 Bruno Fève,3,4,5 Simon N. Thornton,1,2 Rosine Nzietchueng,1,2 Véronique Regnault,1,2 Ginny Simon,1,2 Carlos Labat,1,2 Huguette Louis,1,2 Jean-Pierre Max,1,2 Adeline Muscat,3,4 Mary Osborne-Pellegrin,6,7 Patrick Lacolley,1,2 and Athanase Benetos1,2,8

1Institut National de la Santé et de la Recherche Médicale, U961, Vandoeuvre-les-Nancy, France.  
2Université Henri Poincaré, Vandoeuvre-les-Nancy, France.  
3Institut National de la Santé et de la Recherche Médicale, UMR S938, Paris, France.  
4Faculté de Médecine de St Antoine, Université Paris 6, France.  
5Assistance-Publique des Hôpitaux de Paris, Service d’Endocrinologie, Hôpital Saint-Antoine, France.  
6Institut National de la Santé et de la Recherche Médicale, U698, Paris, France.  
7Université Paris Diderot, Sorbonne Paris Cité, France.  
8Département de Gériatrie, CHU Nancy, Hôpital Brabois, Vandoeuvre-les-Nancy, France.  

Address correspondence to Athanase Benetos, MD, PhD, Department of Geriatrics, CHU Nancy, Hôpital Brabois, 54 510 Vandoeuvre-lès-Nancy, France.  
Email: a.benetos@chu-nancy.fr

To analyze age-related interactions between obesity, its associated metabolic disorders, and macrocirculation, we studied large artery stiffness and fatty acid responsiveness in lean and obese Zucker rats, aged 25 (adult) and 80 weeks (very old). Systolic arterial pressure was higher in old obese than in old lean rats (178 ± 10 vs 134 ± 8 mmHg, respectively). Carotid elastic modulus–wall stress curves showed increased age-dependent arterial stiffening, which was greater in obese animals. Old obese exhibited endothelial dysfunction with increased systemic oxidative stress. Adult obese had elevated plasma free fatty acid levels (1,866 ± 177 vs 310 ± 34 μg/μL in lean animals). In old obese, linoelate and palmitate increased contractility to phentylephrine and reduced relaxation to acetylcholine. Thus, obesity at 25 weeks appears to trigger accelerated arterial aging observed at 80 weeks. The early increase in free fatty acids may be a key effector in the severe arterial stiffness of the aged obese Zucker model.

Key Words: Aging—Arterial stiffness—Free fatty acids—Obesity—Vascular reactivity.

Received August 4, 2011; Accepted November 28, 2011

Decision Editor: Rafael de Cabo, PhD

The metabolic syndrome (MetS) refers to the co-occurrence of several known cardiovascular risk factors, including insulin resistance, abdominal adiposity, dyslipidemia, and hypertension (1). Likewise, obesity has been described to be responsible for the very high prevalence of MetS, which is about 39% of the adult population in the United States (2,3). Among obesity-related features, cardiovascular disease is among the leading causes of death in elderly patients (4,5). The interplay between obesity and vascular dysfunction is multifactorial and implicates hyperinsulinemia and resistance to insulin-mediated glucose disposal (6). Interestingly, the occurrence of obesity has been shown to be higher in people over 60 years old, compared with the mean occurrence in the U.S. population (33.1% vs 29.7%, respectively) (7). Taken together, these observations suggest a synergistic effect of aging and obesity in cardiovascular disease.

It has been reported that insulin resistance could play a central role in the vascular dysfunction observed during obesity or aging. Two major metabolic disturbances subsequent to insulin resistance are an increase in plasma free fatty acids (FFAs) through the loss of the antilipolytic effect of insulin (8) and an altered pattern of pro- or anti-inflammatory cytokines that can directly affect arterial wall structure and function (9). In this context, a major challenge is to understand the mechanisms that underlie the emergence of arterial dysfunction and stiffness during the natural course of aging, obesity, or the association of the two. Available rodent models that mimic human MetS represent major tools for understanding the pathophysiology of cardiovascular complications, providing the opportunity to test the interactions between visceral adiposity and aging.

The obese Zucker rat, which displays many aspects of metabolic dysfunction, such as insulin resistance, hypertension, and increased plasma lipid levels (10), has a missense point mutation (fa/fa) in the gene encoding the leptin receptor; this mutation causes hyperphagia and results in marked obesity (11). It represents an ideal model for investigating
the mechanisms that contribute to large artery dysfunction, which occur with advanced age in obese individuals. Thus, our aim was to evaluate in the obese Zucker rat the relationship between obesity, FFA disturbances, arterial stiffness, and specific biomarkers of endothelial function, oxidative stress, and inflammation. We studied “very old” (80-week-old) obese Zucker rats (OZR) and their lean (lean Zucker rats [LZR]) littermates (which have the same genetic background as OZR), which we compared with younger “adult” 25-week-old OZR and LZR. Indeed, 80 weeks of age correspond to 5 weeks before the mean life span of obese rats from our local breeding colony. We have used the terms “very old” and “great aging” to describe these 80-week-old rats in this paper. We demonstrate that aging, obesity, and interactions of the two all influence vascular function and structure, a phenotype that could depend on age- or obesity-related arterial responsiveness to FFAs.

METHODS

Animals

Male OZR (fa/fa; n = 44) and their age-matched male LZR controls (+/FA; n = 46) were obtained from our local breeding colony (Animal Facility, Faculty of Medicine, Nancy, France). The animals were maintained at a temperature of 22°C–24°C, with a 12-hour light–dark cycle (light beginning at 8 am) and given free access to water and chow (A04 standard; Scientific Animal Food and Engineering). The study was performed at the 25th and 80th week of age. Measurements of arterial stiffness were performed in 37 LZR and 36 OZR. Blood biochemical measurements were carried out in some of these animals (19 LZR and 18 OZR). The thoracic aortas of some of these animals and of others of the same ages were used for determination of the extracellular matrix (27 LZR and 23 OZR) and for Western blots (18 LZR and 18 OZR). Vascular reactivity experiments were performed on carotid arteries of nine LZR and eight OZR. All procedures were in accordance with and approved by the Animal Ethics Committee of the Institut National de la Santé et de la Recherche Médicale and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Measurement of Conscious Systolic Blood Pressure and Heart Rate

Systolic blood pressure and heart rate were measured using a tail-cuff blood pressure analyzer (Blood Pressure Analysis System, model SC-1000; Hatteras Instruments, Cary, NC) on conscious animals. See Supplementary Data for further details.

Mechanical Parameters

We recorded simultaneously arterial diameter (right carotid artery) and blood pressure (left carotid artery) in isoflurane-anesthetized rats (3% of isoflurane in 1.5 L/min dioxygen). Internal arterial diameter was measured using an ultrasonic echo-tracking device (NIUS-01; Asulab SA, Marin, Switzerland). We determined incremental elastic modulus (Einc) and circumferential wall stress (σ), as described previously (12). Circumferential wall stress and Einc characterize the intrinsic mechanical properties of the arterial wall material. To compare Einc-wall stress curves, we calculated the mean wall stress within the 800- to 1,600-kPa range of Einc (MWS_{800-1600}).

Determination of Composition of the Aortic Media

The composition of the vascular wall, with respect to its main structural components, was determined in the media of the thoracic aorta, assuming that any structural modifications at this level reflect similar changes in the contiguous carotid artery. Medial elastin, collagen, and cell protein contents were measured by biochemical techniques, using the method described previously (13). See Supplementary Data for further details.

Blood Sampling and Biochemical Assays

Overnight fasted animals were anesthetized with isoflurane (3% in 1.5 L/min dioxygen), and 2 mL of blood were drawn from the jugular vein into heparin-filled tubes. The different measurements of blood glucose and lipids were made by automatic analyses. Insulin resistance was evaluated with homeostasis model of assessment of insulin resistance [HOMA-IR (14)]. Plasma nonesterified fatty acids were measured by an enzymatic method (NEFA-HR kit; Wako Chemicals GmbH, Neuss, Germany). Plasma adipokines, monocyte chemoattractant protein-1 (MCP-1), von Willebrand factor (vWF), and insulin were measured by ELISA kits (Rand Adiponecint Elisa Kit, Circulex, CyClex Co. Ltd, Nagano, Japan; RayBio Elisa kit Rat Leptin, RayBiotech, Norcross, GA; MCP-1 Elisa Kit, Thermo Fisher Scientific, Illkirch, France; Asserachrom vWF kit, Diagnostica Stago, Asnières, France; Ultra sensitive Rat Insulin Elisa Kit, Crystal Chem, Downers Grove, IL). The determination of soluble CD146 was developed in the laboratory in 96-well plate using the mouse monoclonal antibody anti-CD146, clone P1H12 (Millipore, Molsheim, France) as capture antibody, and the biotin-conjugated mouse monoclonal anti-CD146, clone 2Q401 (US Biological, Swampscott, MA) for revelation.

Measurement of thiobarbituric acid–reactive substances (TBARS) was used for assaying lipid peroxidation in plasma (TBARS assay kit; Cayman Chemical, Ann Arbor, MI). Samples contained malondialdehyde, a naturally occurring product of lipid peroxidation. In the presence of thiobarbituric acid, a malondialdehyde–thiobarbituric acid adduct is formed under high temperature (90°C–100°C) and acidic conditions. This adduct was measured colorimetrically at 539 nm.
Measurement of superoxide dismutase (SOD) was used for assaying antioxidant activity in plasma (SOD assay kit; Cayman Chemical). This assay utilizes a tetrathiazolium salt for detection of superoxide radicals generated by xanthine oxidase. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. This assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD) activity in plasma.

Histomorphometry

At the end of the in vivo measurement of arterial mechanical parameters, the right carotid artery was rapidly excised, cleaned of blood in ice-cold saline, and mounted in a device to perfuse the arterial segment with buffered formalin at a controlled pressure. To obtain the most physiological dimensions and structure, the pressure was adjusted to the mean arterial pressure of the animal. After 2 hours of perfusion, the carotid artery was removed and stored in formalin until being embedded in paraffin.

Five-micrometer sections of carotid artery were stained using Weigert’s resorcin–fuchsin method for elastic fibers and hematoxylin after periodic acid oxidation for nuclei. Evaluation of carotid wall thickness, perimeter, medial cross-sectional area and nuclear density were then determined by computer-directed analysis (Quancoul Software, Talence, France).

Immunoblot Analyses

Tissue lysates were prepared by homogenizing frozen aortas in a lysis buffer (Roche, Meylan, France). Proteins were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Hybond C Extra Membrane (Amersham Bioscience, Glatbrugg, Switzerland). After 1 hour in blocking buffer, membranes were incubated overnight at 4°C with primary antibodies and then incubated for 1 hour at room temperature with secondary antibodies (GE Healthcare; Aulnay-sous-Bois, France). Protein bands were detected by a chemiluminescence system (Bio-Rad; Marnes-la-Coquette, France). The signal intensity was quantified by densitometry with a computerized image processing system (LAS-4000; Fuji-Sciences, Courbevoie, France). See Supplementary Data for further details.

Vascular Reactivity

Carotid artery rings (3 mm long) were obtained from OZR and LZR at 25 and 80 weeks of age after isoflurane anesthesia and exsanguination. Carotid artery rings were mounted on a wire myograph (Danish MyoTechnology, Aarhus, Denmark) filled with a physiological saline solution as described previously (15). Endothelium-dependent and endothelium-independent vasodilation were studied in carotid rings with or without functional endothelium. Tissues were preincubated with 0.5 mM palmitic acid, 0.5 mM linoleic acid diluted in bovine serum albumin 1%, or bovine serum albumin 1% alone (vehicle) for 30 minutes before phenylephrine (Phe) precontraction and then the concentration–response curves to vasorelaxant agents were obtained. See Supplementary Data for further details.

Statistical Analyses

Results are presented as mean ± standard error of the mean. Data were analyzed by use of a two-way analysis of variance, followed by the Fisher’s test for multiple comparisons to evaluate the influence of age and strain and their interaction on the different variables. Differences between dose–response curves were investigated by the sensitivity (pD2) and maximum effect (Emax). The effects of FFA treatment were analyzed using the nonparametric Mann–Whitney test. Statistical analysis was performed with NCSS 6.0 package software (Hintze JL, Kaysville, UT); p < .05 was considered statistically significant.

RESULTS

Weight, Hemodynamic, and Metabolic Parameters

Body, kidney, and heart weights of 80-week-old rats were greater than in 25-week-old rats (Supplementary Table 1). Compared with 80-week-old LZR, 80-week-old OZR exhibited greater body, kidney, and heart weights.

Table 1 shows all hemodynamic and metabolic parameters as a function of aging or obesity. In 80- versus 25-week-old LZR, aging is associated with a decrease in conscious systolic arterial pressure and heart rate, but an increase in plasma levels of glucose, insulin, triglycerides, cholesterol, and leptin. In OZR, great aging induced an increase in plasma levels of glucose, triglycerides, leptin, and creatinine, whereas it reduced the levels of insulin, HOMA-IR, HDL cholesterol, and the hematocrit.

Obesity was also associated with both early and late disturbances. Compared with 25-week-old LZR, 25-week-old OZR had increased values of conscious systolic arterial pressure, insulin, HOMA-IR, total and HDL cholesterol, triglycerides, FFAs, and leptin. Compared with 25-week-old OZR, 80-week-old OZR had decreased plasma FFA levels. Compared with 80-week-old LZR, 80-week-old OZR had higher values for conscious systolic arterial pressure, total and HDL cholesterol, triglycerides, FFAs, leptin, and creatinine. In contrast, they had a lower hematocrit value (Table 1).

These findings show that both aging and MetS each had their own impact on hemodynamic and metabolic parameters, but that the aging/obesity interaction also interfered with the magnitude of the phenotype. Moreover, at 80 weeks, compared with 25-week-old OZR, the decrease in HOMA-IR and FFA values was the consequence of insulin deficiency.

In Vivo Functional Vascular Characteristics

Echotracking was used to measure the mechanical parameters of the carotid artery. As seen in Table 2, 80-week-old
animals had lower wall stress and mean arterial pressure compared with 25-week-old rats. Arterial diameter in 80-week-old OZR was smaller compared with 80-week-old LZR. An age effect (p < .001) was observed in Einc at mean arterial pressure (lower values in the 80-week-old animals) (Table 2), and this difference was significant only in OZR. This result was due to lower mean arterial pressure in the old rats and did not reflect higher elastic properties in 80-week-old animals. The arterial wall stress/elastic modulus curves at 25 and 80 weeks of age in LZR and OZR are shown in Figure 1A. The comparison between LZR and OZR was made by calculating mean wall stress (MWS800-1,600 kPa, Figure 1B). It can be seen that, MWS800-1,600 decreased between 25 and 80 week of age in the two groups, this reduction being more pronounced in OZR. Thus, the carotid artery at 80 weeks of age was significantly stiffer in OZR than in LZR. These results demonstrate that in vivo, obesity and MetS increased arterial stiffness in 80-week-old rats.

Arterial Structure

Eighty-week-old LZR had increased medial cross-sectional area (Table 2) and thickness (Supplementary Figure 1; Supplementary Table 2) of the carotid artery wall compared with 25-week-old LZR. Eighty-week-old OZR had also increased medial cross-sectional area (Table 2) and thickness and an increased perimeter compared with 25-week-old OZR (Supplementary Figure 1; Supplementary Table 2). Eighty-week-old OZR had a decreased perimeter compared with 80-week-old LZR. Cell density (evaluated through nuclear density) was the same in all groups (Supplementary Table 2).

Eighty-week-old rats had significant differences in aortic composition compared with 25-week-old rats (increased dry weight, cell proteins and collagen, a decreased relative elastin content, and a reduced elastin/collagen ratio) (Figure 2A–E). Eighty-week-old OZR exhibited a significant increase in total fibronectin compared with 25-week-old OZR (Figure 2F). In addition, compared with 80-week-old LZR, 80-week-old OZR displayed an increase in aortic dry weight and cell protein content while having a decreased relative elastin content and elastin/collagen ratio (Figure 2A–C, E). In addition, 80-week-old OZR displayed higher total fibronectin content than 80-week-old LZR (Figure 2F).

These data not only demonstrate that carotid geometry and matrix components of the large arteries (carotid arteries and Supplementary Table 2) of the carotid artery wall compared with 25-week-old LZR. Eighty-week-old OZR had also increased medial cross-sectional area (Table 2) and thickness and an increased perimeter compared with 25-week-old OZR (Supplementary Figure 1; Supplementary Table 2). Eighty-week-old OZR had a decreased perimeter compared with 80-week-old LZR. Cell density (evaluated through nuclear density) was the same in all groups (Supplementary Table 2).

Eighty-week-old rats had significant differences in aortic composition compared with 25-week-old rats (increased dry weight, cell proteins and collagen, a decreased relative elastin content, and a reduced elastin/collagen ratio) (Figure 2A–E). Eighty-week-old OZR exhibited a significant increase in total fibronectin compared with 25-week-old OZR (Figure 2F). In addition, compared with 80-week-old LZR, 80-week-old OZR displayed an increase in aortic dry weight and cell protein content while having a decreased relative elastin content and elastin/collagen ratio (Figure 2A–C, E). In addition, 80-week-old OZR displayed higher total fibronectin content than 80-week-old LZR (Figure 2F).

Table 1. Hemodynamic and Metabolic Parameters of 25- and 80-Week-Old LZR and OZR.

<table>
<thead>
<tr>
<th></th>
<th>LZR</th>
<th>OZR</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 wk</td>
<td>80 wk</td>
<td>25 wk</td>
</tr>
<tr>
<td>Conscious SAP (mmHg)</td>
<td>158 ± 2</td>
<td>134 ± 8*</td>
<td>165 ± 2*</td>
</tr>
<tr>
<td>Conscious heart rate (bpm)</td>
<td>431 ± 21</td>
<td>365 ± 14*</td>
<td>405 ± 16</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>8.0 ± 0.3</td>
<td>11.5 ± 0.8*</td>
<td>9.4 ± 0.7</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.66 ± 0.06</td>
<td>2.02 ± 0.48*</td>
<td>10.5 ± 1.43*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>32 ± 4</td>
<td>154 ± 88</td>
<td>592 ± 114</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>1.2 ± 0.1</td>
<td>2.5 ± 0.1*</td>
<td>5.6 ± 0.3*</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>0.73 ± 0.03</td>
<td>0.65 ± 0.03</td>
<td>2.47 ± 0.1*</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.15 ± 0.01</td>
<td>0.57 ± 0.08*</td>
<td>1.55 ± 0.35*</td>
</tr>
<tr>
<td>FFA (μg/mL)</td>
<td>310 ± 34</td>
<td>151 ± 31</td>
<td>1866 ± 177*</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>0.4 ± 0.1</td>
<td>2.9 ± 0.7*</td>
<td>33.1 ± 3.0*</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>44 ± 8</td>
<td>40 ± 2</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>42 ± 2</td>
<td>53 ± 2</td>
<td>41 ± 1</td>
</tr>
</tbody>
</table>

Notes: All values are mean ± SEM. ANOVA, analysis of variance; bpm = beats per minute; FFA = free fatty acid; HOMA-IR = Homeostasis model of assessment–insulin resistance; LZR = lean Zucker rat; NS = nonsignificant; OZR = obese Zucker rat; SAP = systolic arterial pressure.

* p < .05, 80- vs 25-week-old in the same strain.

† p < .05, obese vs lean at the same age (n = 6–11 in each group).

Table 2. Hemodynamic and Arterial Mechanical Parameters of 25- and 80-Week-Old LZR and OZR.

<table>
<thead>
<tr>
<th></th>
<th>LZR</th>
<th>OZR</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 wk</td>
<td>80 wk</td>
<td>25 wk</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>111 ± 7</td>
<td>87 ± 6*</td>
<td>130 ± 6</td>
</tr>
<tr>
<td>Diameter at MAP (mm)</td>
<td>1.21 ± 0.04</td>
<td>1.24 ± 0.04</td>
<td>1.16 ± 0.04</td>
</tr>
<tr>
<td>MCSA (mm²)</td>
<td>0.112 ± 0.011</td>
<td>0.170 ± 0.010*</td>
<td>0.099 ± 0.010</td>
</tr>
<tr>
<td>Wall stress at MAP (kPa)</td>
<td>339 ± 37</td>
<td>175 ± 32*</td>
<td>379 ± 51</td>
</tr>
<tr>
<td>Einc at MAP (kPa)</td>
<td>973 ± 144</td>
<td>702 ± 125</td>
<td>1279 ± 122</td>
</tr>
</tbody>
</table>

Notes: All values are mean ± SEM. ANOVA, analysis of variance; Einc = elastic modulus; LZR = lean Zucker rat; MAP = mean arterial pressure; MCSA = media cross sectional area; NS = nonsignificant; OZR = obese Zucker rat.

* p < .05, 80- vs 25-week-old in the same strain.

† p < .05, obese vs lean at the same age (n = 14–21 in each group).
Figure 1. Mechanical properties in carotid arteries in 25- and 80-week-old lean Zucker rat (LZR) and obese Zucker rat (OZR). Incremental elastic modulus (Einc)-wall stress (WS) curve (A) from 25- to 80-week-old lean and obese rats. (B) Carotid artery mean wall stress within the 800- to 1,600-kPa range of Einc (MWS$_{800-1,600}$) in the same rats (analysis of variance, age: $p < 10^{-6}$, strain: $p < .05$, interaction: $p = .3$). Values are mean ± standard error of the mean. * indicates $p < .05$, 80- vs 25-week-old in the same strain; † indicates $p < .05$, obese vs lean at the same age ($n = 14–21$ in each group).

artery at 80 weeks of age was significantly stiffer in OZR compared with 80-week-old animals. The arterial wall stress/elastic modulus (Table 2), and this difference was significant only in OZR.

Eighty-week-old LZR had increased medial cross-sectional area (Table 2) and thickness compared with 25-week-old LZR. Eighty-week-old OZR had also increased medial cross-sectional area (Table 2) and thickness compared with 25-week-old OZR (Supplementary Figure 1; Supplementary Table 2).

Alterations of Key Signalling Pathways in the Arterial Wall

Because profound alterations of arterial structure and function were found in the specific context of great aging and obesity, we examined in aortas the expression and activation of the ERK1/2, p38 MAPK, and Akt signaling pathways, which exert key roles in the phenotypic changes of aorta) were modified by aging, as expected, but also that these age-related changes were accentuated in 80-week-old OZR.

In Vitro Vascular Reactivity

Vascular function of the carotid arteries was explored in vitro with wire myography. The response to Phe was both strain and age dependent (Figure 3A and Supplementary Table 3) because the sensitivity (pD2) and the maximal effect ($E_{\text{max}}$) were differentially disturbed: pD2 was reduced in 80-week-old LZR compared with 25-week-old LZR while $E_{\text{max}}$ was decreased in 80-week-old OZR compared with age-matched LZR.

In 80-week-old animals, endothelium-dependent relaxation was impaired in OZR compared with LZR (Figure 3B). Acetylcholine (Ach) $E_{\text{max}}$ was significantly decreased in 80-week-old OZR compared with 80-week-old LZR (Figure 3B). When we assessed the endothelium-independent relaxation on rings without endothelium stimulated with sodium nitroprusside, an NO donor, no significant differences were found between OZR and LZR (Figure 3C).

Taken together, these data indicate that in vitro, old OZR exhibit anomalies in vasoconstriction and endothelium-dependent vasodilation.

To reinforce the demonstration of endothelial dysfunction, we analyzed several reliable markers of endothelial function. In the LZR, aging did not alter the plasma level of vWF (Figure 4A). In OZR, vWF was higher at 25 weeks than in LZR and its level increased with age. Values of soluble CD146 were significantly higher at 80 weeks of age in LZR (Figure 4B), but there was only a trend toward an increase in soluble CD146 in 80-week-old OZR.

Notes:

† < .05, 80- vs 25-week-old in the same strain.

* < .05, obese vs lean at the same age ($n = 10–14$ in each group).

Figure 2. Composition of aortic media in 25- and 80-week-old lean Zucker rat (LZR) and obese Zucker rat (OZR). (A) Dry weight of thoracic aorta media (mg/cm). (B) Cell protein content, expressed as % of dry weight. (C) Elastin content (% of dry weight). (D) Collagen content (% of dry weight). (E) Elastin/collagen ratio. (F) Protein level of total fibronectin was normalized to that of tubulin, used as loading control. Values are mean ± standard error of the mean ($n = 10–14$ in each group). * indicates $p < .05$, 80- vs 25-week-old in the same strain; † indicates $p < .05$, obese vs lean at the same age.
vascular cells during senescence (16). As seen in Figure 5A, 80-week-old LZR displayed an increased phosphorylated-Akt compared with 25-week-old LZR. Eighty-week-old OZR had decreased phosphorylated-p38 MAPK and phosphorylated-Akt compared with 25-week-old OZR (Figure 5A and B). Differences in protein phosphorylation were not significant in 80-week-old OZR compared with 80-week-old LZR. However, we show that phosphorylated-p38 MAPK, phosphorylated-Akt, and phosphorylated-ERK1/2 were increased significantly in 25-week-old OZR compared with age-matched LZR. Together, these results indicate that p38 MAPK, Akt, and ERK1/2 are activated mainly in 25-week-old OZR before the emergence of increased arterial stiffening.

Reactive Oxygen Species Production and Inflammation Correlated with Vascular Stiffening in 80-Week-Old OZR

Insulin resistance, oxidative stress, and inflammation provide the basis for the macrocirculatory abnormalities detected in both obesity and aging (17). Thus, we evaluated markers of oxidative stress to assess whether they could contribute to the alterations in arterial structure and function in OZR at 80 weeks. TBARS, a marker of lipid peroxidation, were increased significantly with aging in the LZR (Figure 6A). As compared with 25-week-old LZR, age-matched OZR had increased TBARS with no further increase at the age of 80 weeks. Interestingly, the activity of SOD was not significantly modified by age or obesity in our model (data not shown).

We then evaluated specific markers of arterial inflammation. Plasma levels of MCP-1 were increased with aging in both strains (Figure 6B). In addition, MCP-1 was significantly increased in the OZR compared with LZR at both ages. There was no modification in NFκB phosphorylation in the aorta from LZR, whereas it was significantly increased in 80-week-old OZR compared with 25-week-old OZR (Figure 6C). This was consistent with the activation pattern of NFκB inhibitor, IkBζ (Figure 6D). Meanwhile, plasma adiponectin, an anti-inflammatory factor, was increased in 25-week-old OZR compared with 25-week-old LZR, whereas it was significantly reduced in 80-week-old OZR.

Figure 3. Vascular reactivity in carotid arteries in 25- and 80-week-old lean and obese rats. Concentration–response curves of carotid arteries from 25- to 80-week-old lean and obese rats to phenylephrine (Phe) (A), acetylcholine (Ach) (B), and sodium nitroprusside (SNP) (C). Values are mean ± standard error of the mean (at least four rings per animal, were used in three to five animals in each group). * Indicates p < .05, 80- vs 25-week-old in the same strain; † indicates p < .05, obese vs lean at the same age. LZR = lean Zucker rat; OZR = obese Zucker rat.

Figure 4. Endothelial dysfunction markers in 25- and 80-week-old lean and obese rats. Plasma levels of von Willebrand Factor (vWF), expressed as μg/mL (A) and soluble CD146 (sCD146), expressed as the optical density at 450 nm (B). Values are mean ± standard error of the mean (n = 10–14 in each group). * Indicates p < .05, 80-week-old vs 25-week-old in the same strain; † indicates p < .05, obese vs lean at the same age. LZR = lean Zucker rat; OZR = obese Zucker rat.
compared with 25-week-old OZR. (Figure 6E). Together, these data support a role for reactive oxygen species-induced peroxidation and inflammation in vascular dysfunction of 80-week-old OZR.

**Effects of FFAs in Vascular Impairment in 80-Week-Old OZR**

Three lines of evidence prompted us to examine the potential involvement of FFAs in the alterations of vascular reactivity in 25- and 80-week-old OZR: (i) plasma levels of FFAs were strongly increased in OZR as a consequence of insulin resistance, (ii) FFAs are known to directly influence vascular tone (18,19), and (iii) FFAs produce reactive oxygen species (20) and can activate ERK1/2, Akt, and p38 MAPK pathways (21,22) that are implicated in the contractile properties of large arteries. Thus, we tested the hypothesis that FFAs could affect vascular reactivity as a function of both age and obesity. For this purpose, carotid rings were
incubated with linoleic acid and then tested for the vasorelaxant effect of acetylcholine.

Preincubation with linoleic acid did not affect the basal tone of carotid artery rings (data not shown). Although linoleic acid was ineffective in 25- and 80-week-old LZR, it increased the contractility to Phe in 25- and 80-week-old OZR (Figure 7A). Exposure to linoleic acid did not alter the relaxation to Ach in 25- and 80-week-old LZR nor in 25-week-old OZR (Figure 7B). By contrast, linoleic acid strongly inhibited relaxation to Ach compared with vehicle in 80-week-old OZR. In the absence of endothelium, linoleic acid had no effect on contraction (Figure 7C) or relaxation (Figure 7D). Similar results were obtained in response to Phe or Ach for the two strains when palmitic acid was used instead of linoleic acid (data not shown). Thus, FFAs decreased the endothelium-dependent relaxation in 80-week-old OZR, without affecting the muscular response to sodium nitroprusside.

**DISCUSSION**

To our knowledge, this is the first study to assess both in vivo and in vitro the arterial phenotype of very old Zucker rats. Another study has also reported the relationship between insulin resistance and blood pressure in this rat strain but in much younger animals (23). We have shown that the metabolic disorders present in the obese Zucker rat accentuate arterial aging as demonstrated by the increase in arterial stiffness and the alterations in aortic structure. In vitro, this model presents alterations in vessel relaxation and contraction. Finally, we showed that FFAs inhibited endothelium-dependent relaxation and potentiated contraction in 80-week-old OZR, indicating a major dysfunction of endothelial cells, whereas the smooth muscle cell response to an NO donor remained intact. Our data clearly showed a deleterious action of obesity and associated metabolic disorders on the age-related alterations of large artery function and structure.

**Figure 7.** Effects of free fatty acids on reactivity in carotid arteries from 25- to 80-week old lean Zucker rat (LZR) and obese Zucker rat (OZR). Contraction to \(10^{-5}\)M Phe (A) and relaxation to \(10^{-4}\)M Ach (B) of intact carotid artery rings (E+) from 25- to 80-week-old lean and obese rats. Contraction to \(10^{-5}\)M Phe (C) and relaxation to \(10^{-4}\)M sodium nitroprusside (D) of carotid artery rings without endothelium (E-) from 25- to 80-week-old lean and obese rats. Contraction is expressed as a percent of the response obtained before treatment (corresponding to 100%) with bovine serum albumin (vehicle) or linoleic acid. Values are mean ± standard error of the mean (at least three rings per animal, using three to five animals in each group). "*" Indicates \(p < .05\), linoleic acid vs vehicle.
The metabolic status of the Zucker model has been previously studied. At 25 weeks of age, OZR displayed insulin resistance and dyslipidemia, which is consistent with other reports (24–26), and in younger rats (23). Plasma glucose levels are in agreement with other reported results (25,27). Coimbra and coworkers (28) studied renal injury in Zucker rats up to 60 weeks of age, but blood glucose values were not measured at this late age. However, at the age of 40 weeks, they showed that blood glucose was lower than in our very old rats (4.49 ± 1.33 in LZR vs 8.82 ± 2.38 mmol/L in OZR). Our study showed that very old OZR display several manifestations of accelerated aging such as renal failure with anemia, high systolic blood pressure, and excessive blood pressure variability as observed during anesthesia. Renal failure was found only in old OZR, which is concordant with another study, showing that this disease was the main cause of death in OZR (29). Renal failure represents an important determinant of vascular stiffness linked to the process of accelerated arterial aging. The vascular alterations observed in this study are an additional expression of excessive arterial aging in very old OZR. Interestingly, most of these cardiovascular and renal manifestations are similar to those observed in elderly patients (over 60 years old) with metabolic disorders (30).

The effects of aging on arterial function and structure have been shown in a nonobese rat model (31). Previous studies have demonstrated that large artery stiffness increases with age in rats, in parallel with the decrease in elastin/collagen ratio (32–36). The arterial wall is composed of various elements, each of which contributes to its mechanical behavior. It is has been shown that the increase in stiffness with aging is accelerated in overweight people (37) and in patients with MetS (38). It is well known that there is not only an increase in blood pressure with age but also an increase in arterial stiffness, both in humans and in animals. To measure this stiffness, we use invasive techniques in vivo in rats. Einc evaluates the elastic properties of the wall material (39,40). A higher level of Einc represents increased arterial stiffness. The arterial wall is not homogeneous and is composed of various elements, including smooth muscle cells, collagen, elastin, and other components of the extracellular matrix. All these elements contribute to the mechanical behavior of the wall material via their individual elastic moduli and the way in which they are arranged. (39,40) The Einc stress curve of the carotid artery is established using a methodology validated in rats, as previously published (41). In our model, a strain-related stiffness in OZR has been reported already, but in much younger animals (42). Here, we show that the carotid artery at 80 weeks was significantly stiffer in OZR than in LZR, indicating that arterial structure is also affected by the combination of obesity and aging. We found that, at 80 weeks of age, obesity and the associated metabolic disorders are associated with a significantly lower elastin/collagen ratio compared with LZR.

Changes in extracellular matrix production by smooth muscle cells, including that of collagen and elastin, are influenced by adhesion molecules that attach cells to the matrix (41). Accordingly, we observed in our study that fibronectin was significantly increased in 80-week-old OZR compared with age-matched LZR. Another study demonstrated that fibronectin was increased in an aldosterone- and salt-induced hypertensive rat model (43). Thus, our data argue for the promoting role of fibronectin in arterial stiffness, as this protein was described to increase cell–extracellular matrix attachments (44).

The vascular tone in isolated carotid artery rings from 25- to 80-week-old LZR and OZR is altered in response to Phe as a function of age or adiposity (either on E\textsubscript{max} or pD2; Figure 3). At 80 weeks, E\textsubscript{max} values in response to vasoconstrictive or vasodilating effectors in OZR (25,26,45–48). There is no clear consensus on the existence of a decreased relaxation in OZR. Most of the studies have been performed in younger animals and in different vascular beds. Vessieres and colleagues have shown a greater reduction of Ach relaxation in mesenteric arteries in OZR at 52 weeks than at 20 weeks of age (49,50). A major result of the present study is the demonstration of the age-dependent variations in vascular reactivity at the same site as the arterial stiffness measurement. Overall, although the contracting or relaxing properties of intact large arteries (carotid rings) are disturbed in very old and/or obese rats in response to Phe or Ach, the vasodilating effect of sodium nitroprusside is retained in the absence of endothelium. The predominance of endothelial dysfunction is also supported by the strain and/or age-related increase in the endothelium-derived factors vWF and soluble CD146. This suggests strongly that while the endothelial function is damaged, the response to NO in vascular smooth muscle cells remains unmodified, even in very old animals.

A well-known cause of reduced bioavailability of NO is the increase in reactive oxygen species production, followed by an inflammatory response (51). Indeed, in our study, greater oxidative damage was observed from 25 weeks of age in obese rats followed by a marked activation of the NFκB pathway at 80 weeks of age. A major result of our study was the increase in plasma adiponectin observed in 25-week-old OZR despite the presence of insulin resistance. It has been recently demonstrated that adiponectin reduces inflammation and oxidative stress in aorta of adiponectin-knockout mice (52). Moreover, these authors showed that adiponectin knockout mice had impaired endothelial-dependent vasodilation, suggesting that adiponectin plays a central role in the prevention of endothelial dysfunction. Previous studies have reported either higher (53) or lower (25,54) adiponectin levels in Zucker rats. The high plasma...
levels of adiponectin in 25-week-old OZR observed in our study could reflect a protective mechanism to delay the onset of endothelial dysfunction, a major contributor to arterial stiffness. The subsequent fall in adiponectin in 80-week-old OZR may correspond to a defect in the protective mechanism, resulting in a proinflammatory state, which leads to endothelial dysfunction and the related arterial stiffness.

It was recently demonstrated that acute elevation of plasma FFA activated the proinflammatory NFκB pathway (55), mediated by PKC and IKK activation (56). Some evidence also suggests that FFA-mediated activation of IKK and NFκB may be, at least in part, mediated by the Toll-like receptor 4 (57). In addition, because activation of the NFκB pathway can result in an increase in circulating MCP-1 (58), it supports the notion that FFAs represent a primary link between obesity and the development of inflammatory changes. In our study, FFAs were increased in 25-week-old OZR and can lead to oxidative damage and systemic inflammation. However, arterial structure and function were only slightly altered at this age. The early increase in FFAs, causing a modification of signaling pathways in the aorta, may be a cause of the vascular alterations observed at 80 weeks. Moreover, plasma FFAs remained higher in 80-week-old OZR compared with age-matched LZR. Interestingly, it has been suggested that FFAs could represent an important effector of the endothelial dysfunction and arterial stiffness detected in individuals with insulin deficiency or resistance (59,60). Whether age-related changes in plasma FFAs could contribute to associated variations in arterial stiffness in humans remains an open issue.

The most important fractions of FFA in rat plasma are palmitic and linoleic acids (61). These FFAs were tested based on their average plasma concentration in Zucker rats. In our study, pretreatment by both palmitic and palmitic acid exerts specific effects on vascular tone as previously shown on the adrenergic response (62–64). Therefore, the FFA-induced endothelial dysfunction observed in our model may explain the increase in systolic blood pressure in 80-week-old OZR. However, considering that FFAs increased the response to Phe in obese rats (Figure 7A), they are unlikely to be involved in the reduced sensitivity to Phe observed in vitro in OZR (Figure 3A). However, FFAs are probably involved in the decreased Ach-induced relaxation in 80-week-old OZR because they reduce this response in isolated rings. In addition to previous studies (18,19), our experiments provide further insight into the impact of FFAs on the endothelium, showing that linoleic or palmitic acids specifically target endothelium-dependent vasorelaxation in the very old. This is the first demonstration of endothelium-dependent effects of FFAs in an animal model of advanced age with increased arterial stiffness.

FFAs have also been demonstrated to mediate the phosphorylation of Akt, p38 MAPK, and ERK1/2 in vascular smooth muscle cells (21), thus inducing proliferation, apoptosis, and decreased NO production, respectively. More specifically, palmitic acid enhances p38 MAPK activation in several cell types (22,65), resulting in apoptosis or insulin resistance. In our study, we have demonstrated that phosphorylated-p38 MAPK, -Akt and -ERK1/2 were significantly increased in 25-week-old OZR compared with their control LZR, whereas this effect was lessened in 80-week-old OZR. This pattern paralleled the concentrations of FFAs measured in our animals. Indeed, 25-week-old OZR displayed the highest level of FFAs, whereas they were lower in 25-week-old LZR and 80-week-old OZR. Together, these observations suggest that the effects of FFAs on vascular relaxation may depend on the activation of p38 MAPK, Akt, and ERK1/2.

In conclusion, we have shown that obesity and its associated metabolic disorders synergize with great aging to alter vascular structure and function in vivo and in vitro, both targeting endothelial properties. The finding of an early increase in plasma FFAs before any activation of the NFκB pathway and FFA-induced impairment of carotid relaxation consolidates the hypothesis that FFAs are initiators in the pathogenesis of arterial stiffness development in this model. Our data underscore the interest of an early reduction in FFA levels, in order to prevent vascular alterations that appear later in life and are potentiated in the presence of obesity.

FUNDING
This work was supported by grants from Institut National de la Santé et de la Recherche Médicale, Henri Poincare Université of Vandoeuvre-lès-Nancy, France, « la Région Lorraine », and La Fondation pour la Recherche Médicale (FRM DCV-20070409250).

SUPPLEMENTARY MATERIAL
Supplementary material can be found at: http://biomedgerontology. oxfordjournals.org/.

CONFLICT OF INTEREST
The authors have no conflicts of interest.

ACKNOWLEDGMENT
We thank especially Anne-Laure Leblanc for technical assistance.

REFERENCES
6. Perreault L, Bergman BC, Playdon MC, Dalla Man C, Cobelli C, Eckel RH. Impaired fasting glucose with or without impaired glucose


