Increased Aortic Stiffness and Attenuated Lysyl Oxidase Activity in Obesity

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Objective—One potential mechanism through which obesity exerts adverse effects on the vascular system is by increasing aortic stiffness, a change known to be predictive of increased cardiovascular mortality. The aim of this study was to investigate the pathophysiology that links obesity to aortic stiffening.

Approach and Results—Obese (ob/ob) mice were used to examine physical, morphological, and molecular changes in the aorta in response to obesity. ob/ob mice had increased aortic pulse wave velocity and tissue rigidity. ob/ob aorta exhibited decreases of lysyl oxidase (LOX) activity and cross-linked elastin, and increases of elastin fragmentation and elastolytic activity. The aortas of ob/ob mice were surrounded by a significant amount of proinflammatory and pro-oxidative perivascular adipose tissue. In vitro studies revealed that the conditioned medium from differentiated adipocytes or the perivascular adipose tissue of ob/ob mice attenuated LOX activity. Furthermore, inhibition of LOX in wild-type lean mice caused elastin fragmentation and induced a significant increase in pulse wave velocity. Finally, we found that obese humans had stiffer arteries and lower serum LOX levels than do normal-weight humans.

Conclusion—Our results demonstrated that obesity resulted in aortic stiffening in both humans and mice, and established a causal relationship between LOX downregulation and aortic stiffening in obesity. (Arterioscler Thromb Vasc Biol. 2013;33:839-846.)

Key Words: aortic stiffness ■ inflammation ■ lysyl oxidase ■ obesity ■ perivascular adipose tissue

Obesity increases the risk for stroke, incident cardiovascular diseases, cardiovascular mortality, and all-cause mortality in humans. One potential mechanism by which obesity increases cardiovascular risk is its effect of increasing aortic stiffness, a change known to be associated with other subclinical vascular diseases and predictive of increased cardiovascular mortality.1 Thus, excess body fat, abdominal visceral fat, and a large waist circumference have been identified as risk factors for accelerated arterial stiffening in the general population from the young to the elderly.1-3

The passive biomechanical properties that contribute to vascular stiffness are largely determined by extracellular matrix proteins, including elastin and collagen. Elastin provides vascular elasticity and wall compliance, whereas collagen contributes to wall strength and stiffness. Abnormalities in the quantity and quality of both elastin and collagen contribute to aortic stiffening.4,5 Furthermore, extracellular matrix stability is assured by the intra- and intermolecular covalent cross-linking of elastin and collagen, initiated by lysyl oxidase (LOX), a copper-dependent amine oxidase. In the vascular wall, LOX is expressed in fibroblasts, endothelial cells, and vascular smooth muscle cells.6 LOX is synthesized as a proenzyme proLOX, which is processed by bone morphogenetic protein-1 and other proteinases to release the active enzyme LOX. LOX deficiency in mice leads to arterial aneurysms, tortuosity, and rupture.7,8 Thus, the amount and activity of LOX are essential to maintain the tensile and elastic features of blood vessels.

Most blood vessels in the body are surrounded by various amounts of perivascular adipose tissue (PVAT). While PVAT has been considered a structural support for the vasculature, there is recent evidence that PVAT actively modulates vascular responsiveness by releasing various bioactive molecules.9 Proinflammatory cytokines and superoxide produced by PVAT promote vasoconstriction in the physiological condition.10-11 However, these PVAT-derived factors are also strongly implicated in the pathological processes of vascular

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dysfunction and extracellular matrix remodeling.\textsuperscript{12,13} Because PVAT is positively correlated with body mass index and waist circumference in several human studies,\textsuperscript{14,15} it is reasonable to speculate that the adverse effect of excess body weight on the vasculature is, at least in part, the result of increased PVAT.

Although the adverse effects of obesity on the vascular system are documented in humans, the pathophysiology that links obesity to aortic stiffening has not been well established. In this study, we hypothesized that obesity and its associated increase in PVAT impair elastic fiber stability, thereby contributing to aortic stiffening. Therefore, we designed experiments to examine the in vivo and in vitro elasticity of the aorta, as well as physical, morphological, and molecular changes in the aorta, in response to obesity.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Higher Aortic PWV and Stiffness in Obese Mice**

Two- and 3-month-old obese (ob/ob) mice had significantly higher aortic pulse wave velocities (PWVs) than did control mice (Figure 1A), without changes in the heart rate (HR) (data not shown) and blood pressure (BP) (Table I in the online-only Data Supplement). The pressure-diameter curves showed the attenuated response in the diameter change of the aorta from ob/ob mice (Figure 1B), resulting in lower distensibility in ob/ob mice (Figure 1C). To directly examine material stiffness, we used atomic force microscopy on the aorta (Figure 1D). Obesity shifted the Young’s modulus-distribution higher in the aorta (median Young’s modulus: 10.1 kPa [kiloPascal] in ob/ob mice versus 6.1 kPa in controls), demonstrating directly that ob/ob aortas were stiffer than control aortas.

**Structural Proteins and Maturation Enzyme in the ob/ob Aorta**

No evidence of aortic calcification in 3-month-old ob/ob mice was shown by Von Kossa stain (data not shown). Elastin and collagen are the major structural proteins related to aortic stiffness. No difference in the soluble protein levels of tropoelastin or type 1 collagen was observed between ob/ob and control mice (Figure 2A). ProLOX levels were modestly lower and LOX levels were dramatically lower in ob/ob mice than in controls (Figure 2B), which is not accompanied by the change in bone morphogenetic protein-1 (data not shown). Fibulin-4 and -5, known to conduct proper elastogenesis via interaction with LOX,\textsuperscript{16,17} were not altered in the ob/ob aorta. Immunohistochemistry and confocal microscopy showed that the expression of LOX was normal in the adventitia and PVAT of control mice, but attenuated in ob/ob mice (Figure 2C). LOX enzyme activity was substantially suppressed in the ob/ob aorta (Figure 2D). The aortic content of desmosine, a marker for mature cross-linked elastin, was significantly lower in the aortas of ob/ob mice than of control mice (Figure 2E). These results suggest that the protein content and enzyme activity of LOX and the cross-linking of elastin are significantly reduced in the ob/ob aorta. Microscopic examination revealed more breaks in the elastic network of the aortas from ob/ob mice (Figure 2F and 2G). Elastolytic activity was significantly increased in ob/ob mice (Figure 2H). Furthermore, the level of matrix metalloproteinase (MMP)-9, but not MMP-2, was slightly increased in the ob/ob aorta (Figure 2I).

**PVAT and Aorta Geometry in ob/ob Mice**

Dissection revealed the abundant PVAT surrounding the thoracic aorta (Figure 1 in the online-only Data Supplement) and abdominal aorta in ob/ob mice. After adjusting for body length, 2- and 3-month-old ob/ob mice had ≈3–4 times more PVAT than did controls. Microscopically, the PVAT in control mice exhibited a brown adipocyte-like feature with multilocular lipid deposits, whereas the PVAT in ob/ob mice exhibited a white adipocyte-like appearance with markedly enlarged lipid droplets with unilocular lipid deposits (Figure 1B in the online-only Data Supplement). Despite markedly increased PVAT surrounding the aorta, the media thickness, lumen diameter, lumen and media cross-sectional area and lumen/media ratio were not significantly different between ob/ob and control mice (Table II in the online-only Data Supplement).

**Higher Inflammatory Cytokine Expression in the PVAT and Macrophage Infiltrate in the Aortas of ob/ob Mice**

Proinflammatory cytokines and oxidative stress have been suggested to downregulate LOX and destabilize elastic fibers in the vascular wall.\textsuperscript{6,18} Whereas there were no apparent changes in the expression of chemokines (monocyte chemotactic protein-1, CC

**Figure 1.** Measures of aortic stiffness in obese (ob/ob) mice. A, Aortic pulse wave velocity (PWV) was measured in 2- and 3-month-old male ob/ob and lean control mice. Mean body weight (BW) during the measurements of PWV is shown. Numbers inside bars= n in each group. B, Pressure-diameter relationship and (C) calculated distensibility in isolated abdominal aorta from ob/ob (n=5) and control (n=8) mice. D, Histograms and corresponding Gaussian distribution curves of Young’s modulus measured using atomic force microscopy. Young’s modulus measurements were taken on 50 sites of the thoracic aorta from 3-month-old male ob/ob and control mice (2 mice each). *P<0.05 and **P<0.01 compared with control mice.
chemokine receptor type 2, and macrophage inflammatory protein-2), adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1), and cytokines (tumor necrosis factor α and interleukin 6) in the \( \text{ob/ob} \) aortas, the expression of macrophage markers F4/80 and CD68 were \( \approx 3–4 \) times higher (Figure 3A). Similarly, the upregulation of F4/80 was significant in the \( \text{ob/ob} \) PV AT (Figure 3B). Macrophages were present in the adventitia and PV AT of \( \text{ob/ob} \) mice, but not in those of control mice (Figure 3C). In contrast to that of the aorta, the expression of monocyte chemotactic protein-1, CC chemokine receptor type 2, and tumor necrosis factor \( \alpha \) was significantly higher in the \( \text{ob/ob} \) PV AT. Immunohistochemistry consistently showed significant upregulation of CC chemokine receptor type 2 in the PVAT but not in the aorta of \( \text{ob/ob} \) mice (Figure 3D). Macrophages were present in the adventitia and PV AT of \( \text{ob/ob} \) mice, but not in those of control mice (Figure 3C).

Higher Oxidative Stress in the Aortas and PVAT of \( \text{ob/ob} \) Mice
Expression of the reactive oxygen species-reducing enzymes, catalase and superoxide dismutase 1 expression was significantly lower, and \( p47 \), \( p67 \), Cyba, and Cybb expression was significantly higher in the \( \text{ob/ob} \) aorta. Catalase and superoxide dismutase 1 expression was significantly lower, and \( p47 \), \( p67 \), Cyba, and Cybb expression was significantly higher in the \( \text{ob/ob} \) PVAT (Figure 3F). Cells suffering from a superoxide anion attack were clearly abundant in the aortas and PVAT of \( \text{ob/ob} \) mice (Figure 3G). These results suggest increased oxidative stress in the aortas and PVAT of \( \text{ob/ob} \) mice.

Factors Attenuating LOX Activity
We further examined the factors down-regulating LOX in A7r5 and C3H10T1/2 cells. We found that both tumor necrosis factor \( \alpha \) and \( \text{H}_2\text{O}_2 \) attenuated LOX activity in both cell lines (Figure 4A and 4B). We then tested the hypothesis if the factors released from mature adipocyte or PVAT would downregulate LOX. The conditioned medium from differentiated 3T3-L1 adipocytes and the PVAT from \( \text{ob/ob} \) mice decreased LOX activity in both cell lines (Figure 4C and 4D). These results suggest that the factors released from adipocytes or PVAT attenuate LOX activity.

Effect of LOX Inhibition on Aortic Stiffness in Lean and Obese Mice
We next examine the causative role of LOX downregulation in aortic stiffness. We treated wild-type C57BL/6 lean mice with \( \beta \)-aminopropionitrile (BAPN), an inhibitor of LOX. Treatment of BAPN decreased LOX activity in a dose-dependent manner (Figure 5A). Administration of BAPN for 9 weeks resulted in
significant increases on PWV (Figure 5B), without changes in the HR and BP (Table III in the online-only Data Supplement). Furthermore, BAPN treatment caused elastin fragmentation (Figure 5C) and very mild attenuation in the aortic diameter change (Figure 5D) and reduction in distensibility (Figure 5E). This was not associated with significant changes in the expression of inflammatory mediators in the aorta (Figure 5F). These results suggest that LOX downregulation is sufficient to cause elastin fragmentation and aortic stiffening in wild-type lean mice. Moreover, treatment of BAPN for only 4 weeks significantly increased PWV in ob/ob mice (Figure 5G and Table IV in the online-only Data Supplement).

Arterial Stiffening and Lower LOX Levels in Obese Humans

Carotid-to-femoral PWV was significantly correlated with body mass index ($R=0.288$, $P=0.004$) in relatively healthy human subjects (Figure 6A). The correlation remained significant in the multivariate analysis adjusted for age, mean BP, and HR ($R=0.266$, $P=0.040$). We then divided our study population into 3 groups using the Asian criteria of the World Health Organization.19 No differences in age, HR, and mean BP were found between groups (Table V in the online-only Data Supplement). Carotid-to-femoral PWV values in the overweight and obese groups were significantly higher than that in the normal weight group (Figure 6B).

To examine the correlation between arterial stiffness and LOX level in humans, another cohort participated in health examination was enrolled. Although no differences in age and HR were found, the mean BP was different between groups (Table VI in the online-only Data Supplement). Two arterial stiffness indices, stiffness index and compliance index,20,21 were measured. Obese and overweight subjects showed trends toward higher stiffness index than normal weight subjects (Figure 6C). Obese subjects had significantly lower compliance index than those of normal weight and overweight subjects (Figure 6D). Serum LOX levels in the obese group were significantly lower than those in the normal weight and overweight groups (Figure 6E). While stiffness index is not associated with LOX ($R=0.013$, $P=0.908$), compliance index is independently associated with LOX even after adjustments for age, mean BP and HR ($R=0.307$, $P=0.033$). These results suggest that obese humans have stiffer aorta and lower serum LOX levels. Although obese subjects showed a dramatic increase in the inflammatory marker high-sensitivity C-reactive protein (Table VI in the online-only Data Supplement), high-sensitivity C-reactive protein is not significantly associated with LOX ($R=-0.004$, $P=0.667$).

**Discussion**

Although obesity, usually with comorbidities, has been linked to increased aortic stiffness,2 the effects of obesity alone, independent of traditional cardiovascular risk factors, on aortic
stiffness remain unknown. Recently, Rider et al.22 showed that obesity, without other cardiovascular risk factors, is associated with increased aortic stiffness, and that a significant loss of body weight ameliorates aortic stiffening. Thus, obesity alone may be associated with aortic stiffening in humans. In our study, 2 independent cohorts, consisting of subjects without major cardiovascular risk factors, unequivocally showed increases of arterial stiffness indices in obese subjects. Similarly, aortic stiffness has been shown in obese animals. For example, older (7–9 months old) and younger (2.5–3 months old) ob/ob mice, as well as db/db mice, exhibited higher aortic PWVs and lower aortic compliance.23–25 In addition, mechanical testing of the aortas from obese Zucker (fa/fa) rats showed greater stiffness in the circumferential and longitudinal directions.26 However, the mechanistic insight into the link between obesity and aortic stiffening has been lacking.

PWV measurements can be influenced by several factors, such as age, BP, and HR.27 Although BP and HR were not significantly different in ob/ob and BAPN-treated experiments, a small BP difference could still alter PWV and should be taken into consideration. To directly address material stiffness and elastic properties, namely Young’s modulus, atomic force microscopy measurements were taken. Atomic force microscopy has been used to characterize the topographical and mechanical properties of biological samples.28 This system is particularly advantageous given its ability to measure local elasticity of the low modulus materials down to kPa range. The atomic force microscopy-measured elasticity range of aortas in our study agrees with previous findings of the average of 5–8 kPa in the medial layer of the arterial section.29 Thus, the increases in PWV and Young’s modulus support the notion of greater wall stiffness in the aortas of obese mice.

Increased vascular stiffness has been ascribed to numerous factors, including decreased elastin synthesis, increased collagen content, and altered elastic fiber organization.30,31 In the current study, because we analyzed 2 main structural proteins, elastin and collagen, using Western blotting, we did not observe any quantitative changes in their soluble products. Interestingly, we found impairments in the quality of structure proteins, reflected by increased elastic fiber fragmentation, in the aortas of obese mice. Fragmentation of elastic fibers impairs the cushioning effect of the aorta and leads to an increase in aortic stiffness.32 Elastic fiber fragmentation may result from the defective maturation of elastic fiber formation in the early period of elastogenesis or from increased elastolysis in the later stage.33

LOX deficiency causes an extended fragmentation of elastic fibers, disruption of vascular smooth muscle cell contact, and breaks of both internal elastic lamina and lamellae.7,8 Thus, LOX inhibition can prevent normal formation of desmosine cross-bridges, leading to weakening of the mechanical strength of the aorta. Interestingly, we found that the downregulation of LOX was conspicuous in the adventitia of the aortas and PVAT from obese mice, which was accompanied by fewer desmosine cross-bridges in the aorta. In our study, we found serum LOX levels was reduced in obese humans and independently correlated with compliance index. It remains challenging whether serum LOX level is a surrogate of tissue LOX level. However, considering the difficulty in access of human aortic tissues, measurement of serum LOX values in humans would be an alternative. This is supported by studies demonstrating the serum LOX activity and its association with the representative fibrogenesis status within the tissue.34 Nevertheless, this study is the first demonstration of the potential contribution of LOX downregulation and impaired elastic fiber stability on aortic stiffening in obesity.
Our finding regarding LOX is slightly against the view that cross linking increases stiffness, and crosslink breakers reduce stiffness. Arterial effective stiffness and elasticity are attributed by the forces from both elastic and collagenous fibers. Thus, at lower or physiological stress, more load is carried by elastic fibers, whereas at higher stress, collagen become the major mechanical component that carry more of the overall load.35,36 Because LOX catalyzes cross-links in both elastin and collagen, the consequence of LOX inhibition can be complicated by alternations in both fibers. The discrepancy on stiffness can be found in several studies. For example, Brüel et al37 showed that BAPN treatment reduced aortic strength and stiffness, particularly at higher stress, due to reduced collagen cross-links. However, others did not show apparent differences on distensibility after BAPN treatment.38,39 In our study, we found prominent elastin fragmentation in BAPN-treated mice, and BAPN treatment increased PWV in both wild-type and ob/ob mice. Importantly, PWV was measured at a condition of physiological pulsatile hemodynamics. However, we only observed very mild attenuation in the pressure-diameter relationship in BAPN-treated mice. It is worth noting that the ex vivo circumferential direction measurement neglects the force-length behavior in the longitudinal direction because arteries are stretched longitudinally in vivo, which is more

Figure 5. Effect of lysyl oxidase (LOX) inhibition on aortic stiffness in lean and obese mice. A, LOX enzyme activity in the aortic lysate; (B) aortic pulse wave velocity (PWV); and (C) elastin fragmentation of male C57BL/6 mice received daily administration of β-aminopropionitrile (BAPN) at 300 and 600 mg/kg per day for 9 weeks. Data in A are normalized to untreated group. D, Pressure-diameter relationship and (E) calculated distensibility in isolated abdominal aorta from BAPN-treated (n=6) and control (n=5) mice. F, Expression of genes for inflammatory mediators in the aortas of BAPN-treated and control mice (n=6 each). G, Aortic PWV of 3-month-old obese (ob/ob) mice received daily administration of BAPN at 600 mg/kg per day for 4 weeks. Numbers inside bars=n in each group. *P<0.05 and **P<0.01 compared with untreated group.

Figure 6. Arterial stiffness indices and serum lysyl oxidase (LOX) levels in humans. A, The correlation between pulse wave velocity (PWV) and body mass index (BMI) in 100 healthy humans. B, PWV in 3 groups using clinical definitions of obesity. C, stiffness index; (D) compliance index; and (E) serum LOX levels in 3 groups using clinical definitions of obesity. Individuals were categorized as normal weight (BMI<23), overweight (23≤BMI<27.5), or obese (BMI≥27.5). Numbers inside bars=n in each group. Results are shown as mean±SD. *P<0.05, **P<0.01, and ***P<0.001.
appropriately applied in PWV measurement. In addition, the effect of reduced elastin cross-linking on effective stiffness is likely masked by reduced collagen cross-linking in the circumferential direction measurement.

Although the elastic fibers last the lifetime of the organism, they are subject to proteolytic degradation and chemical alterations that change their mechanical properties. Thus, qualitative alterations of elastic fibers, such as elastin fragmentation and disorganization, would be more critical in determination of mechanical behavior of the aorta in adult animals. However, in some pathological conditions, elastin expression is increased, but the newly synthesized elastin does not assemble properly. Thus, the reduction of PWV in human studies of 1-year period of weight loss\textsuperscript{22,40} may be attributed to a reduction in elastin fragmentation and an increase in mechanically functional elastic fibers. These are likely caused by the improvement in the hormonal and metabolic environment, which recreates the precise temporal, spatial, and structural interactions necessary for elastic fiber assembly. On the other hand, it is still debatable whether weight loss intervention in a short duration (3–6 months) improves arterial stiffness, because 2 human studies revealed different conclusions.\textsuperscript{41,42} It is questionable whether relatively rapid weight loss prevents fatigue fracture, crosslinking, or degradation of elastin over weeks. Thus, the reduction of arterial stiffness in a relatively short time frame may be attributed to changes in local, humoral, or neural modulation of smooth muscle tone. For example, improved nitric oxide bioavailability, reductions in angiotensin II, and reductions in sympathetic neural activity may contribute to the favorable changes in arterial stiffness observed with short-term weight loss.

Elastic fiber fragmentation may also result from an upregulation of elastase activity in association with an increased susceptibility to elastolysis of the elastic fiber formed.\textsuperscript{43} A loss of proper organization and arrangement of the elastic fiber network attributable to elastolysis impairs aortic wall elastic properties and structural integrity. Inflammation and macrophage infiltration of the aortic wall have been shown to induce activation of MMPs and increase elastolysis.\textsuperscript{44} In addition, LOX inhibition renders the presence of soluble forms of extracellular matrix proteins and increases their susceptibility to degradation by proteases.\textsuperscript{45} Our data showed that aortic lysate from obese mice had increased elastolytic activity, which is accompanied by modestly increased aortic MMP-9 and significantly increased circulating MMP-9 levels (data not shown). However, the role of MMPs on arterial stiffness is controversial. While a human study reported positive association between arterial stiffness and serum levels of MMP-9,\textsuperscript{46} another study showed negative association.\textsuperscript{47} Thus, a direct measurement of the level or activity of MMPs in the aorta may be better to determine the local tissue elastase activity.

Obesity is accompanied by low-grade inflammation. Although macrophage infiltration was found in the ob/ob aorta, reflected by increased expression and staining of macrophage markers, we found no apparent changes in the expression of cytokines or chemokines in the ob/ob aorta. Interestingly, the increased expression of cytokines and chemokines were prominent in the PVAT physically attached to the adventitia. These chemokines may recruit immune cells into PVAT through the vasa vaso rum of the adventitia. These findings suggest that proinflammatory and hypertrophied PVAT is important in mediating the vascular inflammatory response in obesity.

In summary, our results demonstrated that obesity resulted in aortic stiffening in both humans and mice. Both destabilized elastic fiber networks and increased elastolytic activity in the aortas of obese mice contributed to aortic stiffening. The downregulation of LOX in wild-type lean mice caused elastin fragmentation and induced a significant increase in PWV. Proinflammatory and pro-oxidative PVAT surrounding the aorta provide an explanation for LOX attenuation in the aortas of obese mice. Moreover, obese humans have stiffer arteries and lower serum LOX levels. Thus, our study highlights the importance of the clinical observation of aortic stiffening in obesity. More important, our study points out potential avenues for exploration of PVAT, LOX, and elastic fiber crosslinking that could illuminate the mechanism behind this clinical observation.

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Disclosures
None.

References
Our results demonstrated that obesity resulted in aortic stiffening in both humans and mice, and established a causal relationship between lysyl oxidase downregulation and aortic stiffening. This study highlights lysyl oxidase downregulation and pro-inflammatory, pro-oxidative perivascular adipose tissue as key mediators of aortic stiffening in obesity, and points out potential avenues for exploration of perivascular adipose tissue, lysyl oxidase activity, and elastic fiber crosslinking that could illuminate the mechanism behind this clinical observation. Finally, understanding these processes has several clinical implications, including the interpretation and development of therapeutic strategies for aortic stiffening in obesity. Because increased stiffness may be detected in the early stage of obesity, more aggressive treatments and monitoring procedures should be instituted for patients at a higher risk for developing accelerated vascular stiffness.
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Materials and Methods

Animals
Leptin-deficient (ob/ob) mice and control littermates, obtained from The Jackson Laboratory (Bar Harbor, ME), were fed regular chow (Purina Laboratory Rodent Diet 5001; PMI Nutrition International, Richmond, IN). β-aminopropionitrile fumarate (BAPN; 300 and 600 mg/kg/day) was administrated by intraperitoneal injection on eight-week-old male C57BL/6 mice, obtained from National Laboratory Animal Center (Tainan, Taiwan). Animals were housed in a specific-pathogen-free barrier facility and were handled in accordance with procedures approved by the Institutional Animal Care and Use Committees of National Cheng Kung University.

PWV measurement in mice
Mice were anesthetized with avertin and placed on a temperature-controlled electrocardiogram board (Indus Instruments, Houston, TX). Doppler spectrograms of aortic flow at the aortic arch and abdominal aortic site 30 mm apart were acquired with a 20-MHZ pulsed Doppler probe (Indus Instruments). Aortic PWV was calculated by dividing the distance between two measurement locations by the time difference between pulse arrivals relative to the R-wave of the electrocardiogram.

Pressure myography
Abdominal aorta containing the segment from genital artery to inferior mesenteric artery was mounted on glass microcannulas in a temperature-controlled vessel chamber (Living Systems Instrumentation, Burlington, VT). The chamber, positioned on the stage of an inverted microscope (Nikon TS100) with a video camera, was filled with HEPES-PSS equilibrated with 95% O2/5% CO2 at 37°C. The aortic segment was exposed to step increases in intraluminal pressure with HEPES-PSS. The changes in aortic diameter and intraluminal pressure were recorded with a data acquisition system. Aortic distensibility was calculated by: distensibility = (D_{n+1}-D_n) / (P_{n+1}-P_n) × D_n (D = diameter, P = pressure).

Atomic force microscopy (AFM)
An AFM (NanoWizard II; JPK Instruments AG, Berlin, Germany) and cantilevers
(T1L450B; NanoSensor, Neuchatel, Switzerland) with a nominal spring constant of 0.2-0.4 N/m were used. Tissue pieces approximately 2 mm² in area were cut from the thoracic aorta and placed under the corners of glued coverslips, leaving nearly the entire lumen of the aortic wall exposed face-up in a PBS-filled Petri dish. Stiffness was assessed by applying a 3-nN load on the aortic tissue directly through the cantilever tip. The Young's modulus of aortic tissue was calculated from the force-distance curves derived using a modified Hertz model (JPK Instruments) built into Elasticity Fit processing software.

**Immunoblot analysis**

Twenty micrograms of total proteins were subjected to electrophoresis, transferred to PVDF membranes, and probed with antibodies against tropoelastin (MAB2503, Millipore), collagen type I (234167, Merck-Calbiochem), LOX (sc-66948, Santa Cruz Biotechnology), fibulin-4 (sc-98443, Santa Cruz Biotechnology), fibulin-5 (12188-1-AP, Protein Tech), MMP-2 (ab37150, Abcam), and MMP-9 (AB19016, Millipore). Immunoreactive proteins were detected using an enhanced chemiluminescence Western blotting detection system (GE Healthcare, Pittsburgh, PA).

**LOX enzyme activity**

Aortic tissues were homogenized and incubated with the buffer containing the substrate 1,5-diaminopentane (Fluka, St. Louis, MO). The fluorescence from released hydrogen peroxide in horseradish peroxidase (HRP)-coupled reactions was measured using a fluorescence microplate reader with excitation and emission wavelengths at 538 and 590 nm. Parallel assays were prepared with 100 mM of added BAPN to completely inhibit the LOX activity, and the difference in fluorescence intensity was recorded. The LOX activity was calculated as the increase in fluorescence units over time above the BAPN controls divided by cell protein content. Human serum LOX levels were determined by an ELISA kit (USCN Life Science).

**Desmosine analysis**

Homogenates of aortas were hydrolyzed in 6N HCl at 110°C for 24 h. The samples were evaporated to dryness and dissolved in 0.4 ml of distilled water. Desmosine
content was determined using a modified immunoassay as previously described. The protein content of hydrolysates was determined using a bicinchoninic acid (BCA) protein assay (Thermo Scientific Pierce).

**Elastolytic Activity**

Proteins were extracted from aortic tissues without adding any protease inhibitors. Elastase activity was evaluated by incubating protein extracts with the DQ-elastin substrate (EnzChek elastase activity assay kit; Invitrogen) at 37°C for 4 h. The fluorescence from digested DQ-elastin was measured using a microplate fluorometer at 485/538 nm.

**Histological examination and elastic fiber stain**

Aortic tissues isolated from male mice were fixed in 10% buffered formalin and embedded in paraffin. Sections (5 μm thick) were cut and stained with hematoxylin and eosin. The elastic fiber network was evaluated on longitudinal sections of thoracic aorta stained with Van Gieson’s stain (Accustain elastic stain kit; Sigma-Aldrich, St. Louis, MO). The number of elastin breaks in the whole section of aortic tissue was evaluated by an investigator blinded to the genotype.

**Immunohistochemical and immunofluorescence staining**

Formalin-fixed paraffin-embedded tissue sections (5 μm thick) were deparaffinized and boiled in 10 mM of sodium citrate (pH 6.0) for 10 min. Sections were immersed in 3% hydrogen peroxide for 10 min, blocked in blocking buffer (Thermo Scientific Pierce, Rockford, IL) for 30 min, and incubated overnight with primary antibodies against LOX and CCR2 (1:200) (ab25788; Abcam, Cambridge, MA). Secondary antibody staining was done using a kit (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, CA) and detected with 3,3′-diaminobenzidine (DAB) substrate-chromogen solution (Dako, Glostrup, Denmark). The slides were counterstained with hematoxylin. For the immunofluorescence staining of macrophages, frozen sections (20 μm thick) were blocked with blocking buffer (Thermo Scientific Pierce) for 30 min, and incubated overnight with antibodies to F4/80 (1:50) (ab6640; Abcam) followed by anti-rat IgG conjugated with Alexa Fluor 555 (A21434; Invitrogen, Carlsbad, CA). To detect reactive oxygen species (ROS),
dihydroethidium hydrochloride (1:500) (Invitrogen) was applied to the freshly cut frozen aortic sections for 30 min at 37°C, which were then examined using a fluorescence microscope (Olympus, Tokyo, Japan).

**RNA analysis**

Aorta, including adventitia but devoid of PVAT, and a layer of PVAT lying on the outside of adventitia were dissected, respectively. Tissues were stored in RNA Later (Ambion, Austin, TX), and RNA was extracted using REzol reagent (Protech Technology, Taipei, Taiwan). mRNA was analyzed with SYBR green-based real-time quantitative RT-PCR assays (Applied Biosystems, Foster City, CA), with cyclophilin A as the reference gene in each reaction. Because of limited availability, each RNA sample extracted from the PVAT of control lean mice represents a pool of RNA samples from 3-4 mice. Sequences of the primers used for RT-PCR assays are shown in Supplementary Table VII.

**Cell culture**

A7r5 and C3H10T1/2 cells were grown in Dulbecco’s Modified Eagle’s Media (DMEM) containing 10% fetal bovine serum (FBS). Cells were treated with various stimuli for 24 or 48 hr, and the cell lysates were used for determination of LOX activity. For the preparation of the conditioned medium from 3T3-L1 preadipocytes, pre-differentiated or day-8 post-differentiated 3T3-L1 cells were incubated in DMEM with 0.2% BSA for 24 hr. The conditioned medium was collected to treat A7r5 and C3H10T1/2 cells. For the preparation of the conditioned medium from PVAT, 40 mg of PVAT was collected, cut into pieces 0.2–0.3 cm³ in size, and conditioned at 37°C in 1 ml of DMEM with 0.2% BSA for 24 h. Varies amount (10, 40, and 100 μl) of the PVAT-conditioned medium was used to treat A7r5 and C3H10T1/2 cells.

**Human participants**

The first cohort included 100 asymptomatic hospital staff members for the assessment of carotid-to-femoral PWV (PWVcf). Another cohort consisting of 100 asymptomatic subjects participated in health examination was recruited for determination of stiffness index (SI) and compliance index (CI) and collection of serum samples. All participants included in this study are Asian and were apparently healthy, free of any cardiovascular disease symptoms, and normal in sinus rhythm. All participants
provided written informed consent for this study, which was approved by the Human Research Committee of National Cheng Kung University Hospital.

**Arterial stiffness measurement in humans**

$PWV_{cf}$ was measured by applanation tonometry in the first cohort. Right carotid and femoral pulse waves were detected directly using a piezo-resistive pressure transducer (SPT 301; Millar Instruments, Houston, TX) coupled to a Sphygmocor device (AtCor, Sydney, Australia). The time delay between carotid and femoral pulse waves was calculated using the electrocardiographic R wave as the starting point and the arrival of the pulse wave as the end point. The distance on tape measures referred to the length difference between suprasternal notch-to-carotid pulse and suprasternal notch-to-femoral pulse. $PWV_{cf}$ was calculated by dividing the distance between these two pulses by the time delay. Two arterial stiffness indices, SI and CI, were determined in the second cohort. SI was measured in the right index finger by a commercially available photoplethysmograph (MicroMedical, Gillingham, UK).\(^2\) CI was measured using our newly developed dual-channel photoplethysmography system.\(^3\)

**Data analysis**

Values are reported as means ± SEM, except for Figure 6. Student’s $t$ test was used for comparisons between groups within each experiment, and significance was set at $P < 0.05$. The association was examined by the univariate analysis using Spearman’s correlation test, and by the multivariate linear regression analysis controlling for age, mean blood pressure (BP), and heart rate (HR).

**References**


3. Chen JY, Tsai WC, Wu MS, Hsu CH, Lin CC, Wu HT, Lin LJ, Chen JH. Novel
Supplemental Figure I. Gross appearance and microscopic examination of PVAT in ob/ob mice.

**A**, Gross appearance (left panels), and tissue weight expressed as a percentage of body length (BL; right panel) of the PVAT surrounding the thoracic aorta of 2- or 3-month-old male ob/ob and control mice. Dashed lines indicate the margins of the PVAT. ***P < 0.001 compared with control mice. **B**, Microscopic examination of the PVAT surrounding the thoracic aortas of 3-month-old male ob/ob and control mice. Each scale bar is 50 μm.
Supplemental Table I. Blood pressure of 3-month-old male control and *ob/ob* mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>control (n= 6)</th>
<th><em>ob/ob</em> (n=5)</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mmHg)</td>
<td>108.65 ± 2.09</td>
<td>102.48 ± 2.11</td>
<td>0.0552</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>91.81 ± 2.31</td>
<td>88.79 ± 2.41</td>
<td>0.3669</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>97.42 ± 2.15</td>
<td>93.36 ± 2.30</td>
<td>0.2066</td>
</tr>
</tbody>
</table>

*P*-value is resulted from Student’s *t* test for comparison between two groups.

Supplemental Table II. Thoracic aorta geometry of 2~3-month-old male control and *ob/ob* mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>control (n= 9)</th>
<th><em>ob/ob</em> (n=7)</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Media thickness (μm)</td>
<td>42.35 ± 2.98</td>
<td>41.69 ± 2.94</td>
<td>0.8802</td>
</tr>
<tr>
<td>Lumen diameter (mm)</td>
<td>0.61 ± 0.02</td>
<td>0.67 ± 0.03</td>
<td>0.0844</td>
</tr>
<tr>
<td>Lumen CSA (mm²)</td>
<td>0.30 ± 0.02</td>
<td>0.36 ± 0.03</td>
<td>0.0839</td>
</tr>
<tr>
<td>Media CSA (mm²)</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.7209</td>
</tr>
<tr>
<td>Lumen/media ratio</td>
<td>3.47 ± 0.19</td>
<td>3.99 ± 0.50</td>
<td>0.3071</td>
</tr>
</tbody>
</table>

*P*-value is resulted from Student’s *t* test for comparison between two groups. CSA, cross-sectional area.
Supplemental Table III. Blood pressure and heart rate of BAPN-treated wild-type lean mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>control (n= 6)</th>
<th>BAPN (n= 6)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mmHg)</td>
<td>109.10 ± 2.09</td>
<td>109.40 ± 2.01</td>
<td>0.9260</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>69.67 ± 2.31</td>
<td>68.08 ± 2.64</td>
<td>0.6587</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>82.82 ± 2.18</td>
<td>81.85 ± 2.20</td>
<td>0.7602</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>496.60 ± 18.72</td>
<td>473.20 ± 32.71</td>
<td>0.5487</td>
</tr>
</tbody>
</table>

BAPN: 600 mg/kg/day
$P$-value is resulted from Student’s $t$ test for comparison between two groups.

Supplemental Table IV. Blood pressure and heart rate of BAPN-treated $ob/ob$ mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>control (n= 8)</th>
<th>$ob/ob$ (n=5)</th>
<th>$ob/ob$+BAPN (n=6)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mmHg)</td>
<td>115.00 ± 5.09</td>
<td>100.60 ± 8.93</td>
<td>99.86 ± 5.70</td>
<td>0.2022</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>67.53 ± 3.86</td>
<td>64.01 ± 3.22</td>
<td>59.15 ± 2.66</td>
<td>0.2154</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>83.37 ± 3.96</td>
<td>76.20 ± 1.57</td>
<td>72.72 ± 3.20</td>
<td>0.0837</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>450.40 ± 12.18</td>
<td>457.30 ± 17.21</td>
<td>484.80 ± 11.36</td>
<td>0.1828</td>
</tr>
</tbody>
</table>

BAPN: 600 mg/kg/day
$P$-value is resulted from one-way ANOVA test for comparison between groups.
### Supplemental Table V. Clinical characteristics of the first group of human subjects

<table>
<thead>
<tr>
<th></th>
<th>Normal weight (N=39)</th>
<th>Overweight (N=44)</th>
<th>Obese (N=17)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>20 (51%)</td>
<td>23 (52%)</td>
<td>9 (53%)</td>
<td>0.860</td>
</tr>
<tr>
<td>Age, y</td>
<td>36±9</td>
<td>37±9</td>
<td>38±8</td>
<td>0.622</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>58±7</td>
<td>70±8</td>
<td>79±10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21±2</td>
<td>25±1</td>
<td>29±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>86±9</td>
<td>87±8</td>
<td>90±10</td>
<td>0.266</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>68±9</td>
<td>70±9</td>
<td>70±9</td>
<td>0.812</td>
</tr>
<tr>
<td>PWV&lt;sub&gt;cf&lt;/sub&gt;, m/s</td>
<td>6.2±0.9</td>
<td>6.9±1.2</td>
<td>7.1±1.5</td>
<td>0.010</td>
</tr>
</tbody>
</table>

*P*-value is resulted from one-way ANOVA test for comparison between groups.

Results are shown as mean ± SD.

### Supplemental Table VI. Clinical characteristics of the second group of human subjects

<table>
<thead>
<tr>
<th></th>
<th>Normal weight (N=19)</th>
<th>Overweight (N=49)</th>
<th>Obese (N=32)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>9 (47%)</td>
<td>22 (45%)</td>
<td>13 (47%)</td>
<td>0.782</td>
</tr>
<tr>
<td>Age, y</td>
<td>43±12</td>
<td>44±11</td>
<td>45±10</td>
<td>0.767</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>53±7</td>
<td>65±8</td>
<td>87±11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21±1</td>
<td>25±1</td>
<td>32±3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>94±7</td>
<td>100±12</td>
<td>103±9</td>
<td>0.007</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>76±13</td>
<td>72±12</td>
<td>80±8</td>
<td>0.055</td>
</tr>
<tr>
<td>hs-CRP, µg/ml</td>
<td>0.9±1.2</td>
<td>1.7±2.1</td>
<td>3.5±2.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lox, pg/ml</td>
<td>318.3±59.2</td>
<td>352.1±88.4</td>
<td>245.6±77.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stiffness index, m/s</td>
<td>7.2±1.5</td>
<td>8.0±2.1</td>
<td>8.4±2.5</td>
<td>0.169</td>
</tr>
<tr>
<td>Compliance index, unit</td>
<td>4.6±2.9</td>
<td>4.6±2.4</td>
<td>3.2±1.6</td>
<td>0.016</td>
</tr>
</tbody>
</table>

*P*-value is resulted from one-way ANOVA test for comparison between groups.

Results are shown as mean ± SD.
Supplemental Table VII. Sequences of primers used for real-time quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence Forward</th>
<th>Sequence Reverse</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophilin A</td>
<td>GGC CGA TGA CGA GCC C</td>
<td>TGT CTT TGG AAC TTT GTC TGC AA</td>
<td>64 bp</td>
</tr>
<tr>
<td>IL-6</td>
<td>AGG ATA CCA CTC CCA ACA GAC</td>
<td>TGT CAT CAT CGT TGT TCA TAC</td>
<td>138 bp</td>
</tr>
<tr>
<td>CD68</td>
<td>AGC TGC CTG ACA AGG GAC ACT</td>
<td>AGG AGG ACC AGG CCA AGT AT</td>
<td>89 bp</td>
</tr>
<tr>
<td>MIP-2</td>
<td>GCC CCC AGG ACC CCA</td>
<td>CTT TTT GAC CGC CCT TGA GA</td>
<td>62 bp</td>
</tr>
<tr>
<td>TNF-a</td>
<td>CAT CTT CTG AAA ATT CGA GTG ACA A</td>
<td>TGG GAG TAG ACA AGG TAC AAC CC</td>
<td>175 bp</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CCC ACT CAT CGT CGT CTA CT</td>
<td>TCT GGA CCC ATT CCT TCT TG</td>
<td>164 bp</td>
</tr>
<tr>
<td>F4/80</td>
<td>CTT TGG CTA TGG GCT TCC AGT C</td>
<td>GCA AGG AGG ACA GAG TTT ATC GTG</td>
<td>165 bp</td>
</tr>
<tr>
<td>SOD1</td>
<td>GCG GTG AAC CAG TTG TGT GTC A</td>
<td>CAG TCA CAT TGC CCA GGT CTC C</td>
<td>192 bp</td>
</tr>
<tr>
<td>Catalase</td>
<td>AGA GGA AAC GCC TGT GTG AGA</td>
<td>TCA GGG TGG AGG TCA GTG AA</td>
<td>100 bp</td>
</tr>
<tr>
<td>p47</td>
<td>ACC TGA AAC TGC CCA CTG AC</td>
<td>TCT CGG AAC TCT TCT CG</td>
<td>150 bp</td>
</tr>
<tr>
<td>p67</td>
<td>GCA GTG GCC TAC TTC CAG AG</td>
<td>ACC TCA CAG GCA AAC AGC TT</td>
<td>158 bp</td>
</tr>
<tr>
<td>Cyba</td>
<td>TTC CTG TCT CTT GTG CCT GC</td>
<td>TTC TCT CGG ACC TCT GCG GG</td>
<td>215 bp</td>
</tr>
<tr>
<td>Cybb</td>
<td>GGA GTT CCA AGA TGC CTG GA</td>
<td>CCA CTA ACA TCA CCA CCT CAT AGC</td>
<td>101 bp</td>
</tr>
<tr>
<td>CCR2</td>
<td>ATT CTC CAC ACC CTG TCT CG</td>
<td>GAT TCC TGG AAG GTG GTC AA</td>
<td>140 bp</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CAA TTC ACA CTG AAT GCC AGC TC</td>
<td>CAA GCA GTG CGT CTC GTC CA</td>
<td>150 bp</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>AAC GAG GCT GGA ATT AGC AGA A</td>
<td>CCC TCT TTG ACA CTC TTA GAT GGA A</td>
<td>101 bp</td>
</tr>
</tbody>
</table>