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Original article

Arterial stiffness and the autonomic nervous system during the development of Zucker diabetic fatty rats

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Abstract

Aim. – This study aimed to investigate the role played by sympathovagal balance in arterial stiffness, a common feature of insulin resistance and type 2 diabetes.

Methods. – We investigated the relationship between autonomic nervous system activity and arterial stiffness in Zucker diabetic fatty rats (ZDF: Gmi-fa/fa) and their age-matched controls (lean: ?/fa). Using simultaneous catheterization of the proximal and distal aorta, we measured intra-arterial blood pressure (BP), heart rate (HR), their variability (spectral analysis) and aortic pulse wave velocity (PWV) in a series of at least six conscious rats aged 6, 12, 18 and 24 weeks.

Results. – BP and PWV increased with age (P < 0.001) in both strains with no differences between strains, despite the insulin resistance already present at 6 weeks in ZDF rats. HR was significantly lower (P < 0.001) in ZDF than in lean rats. In ZDF compared with lean rats, the low-frequency (LF) component of the systolic BP variations and the LF/high-frequency (HF) component of the pulse interval (PI) variation ratio were reduced (P < 0.01 and P < 0.05, respectively), while the HF component of the PI (HF-PI) variation was raised (P < 0.05). PWV was negatively correlated with HF-PI (r = −0.37, P < 0.01), but not with biochemical parameters. HF-PI was an independent variable explaining the variation in PWV.

Conclusion. – During the development of disease of ZDF rats, sympathovagal balance might account for the lack of increase in PWV.

Keywords: Pulse wave velocity; Conscious rats; Spectral analysis; Intra-arterial blood pressure measurement

Résumé

Rigidité artérielle et système nerveux autonome pendant le développement du rat Zucker Diabetic Fatty.

Objectif. – Explorer le rôle de la balance vagosympathique dans la rigidité artérielle, souvent associée à l’insulinorésistance et au diabète de type 2.

Méthodes. – Nous avons cherché les relations entre le système nerveux autonome et la rigidité artérielle chez des rats Zucker Diabetic Fatty (ZDF : Gmi-fa/fa) et leurs témoins (Lean ?/fa) apparus selon l’âge. Nous avons mesuré la pression artérielle (PA), la fréquence cardiaque (FC), leurs variabilités par analyse spectrale et la vitesse de l’onde de pouls fémoro-aortique (VOP) grâce à un cathétérisme simultané de l’aorte proximale et distale chez au moins six rats non anesthésiés âgés de six, 12, 18 et 24 semaines.

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Résultats. – La PA et la VOP ont augmenté avec l’âge ($P < 0,001$) sans différence entre rats ZDF et Lean malgré une insulinorésistance déjà présente à six semaines chez les rats ZDF. La FC était plus basse ($P < 0,001$) chez les rats ZDF que chez les rats Lean. La composante de basse fréquence de la variabilité de la PA systolique et le rapport composante de basse fréquence/composante de haute fréquence de la variabilité de la FC était réduit chez les rats ZDF comparés aux rats Lean ($P < 0,01$ et $P < 0,05$, respectivement), alors que la composante de haute fréquence des variations de FC (HF-FC) était augmentée ($P < 0,05$). La VOP était en corrélation négative avec HF-FC ($r = −0,37$, $P < 0,01$), mais non avec les paramètres biochimiques. HF-FC était une variable indépendante expliquant la variance de la VOP.

Conclusion. – Lors du développement des rats ZDF, la balance vagosympathique pourrait participer à l’absence d’augmentation de la vitesse de l’onde de pouls.

Mots clés : Vitesse de l’onde de pouls ; Rats conscients ; Analyse spectrale ; Mesure intra-arterielle de la pression artérielle

1. Introduction

Diabetes, obesity, hypertension and dyslipidaemia all contribute to vascular remodeling and, as a consequence, arterial stiffening. Arterial rigidity, determined by an increased pulse pressure or pulse wave velocity (PWV), is a strong and independent predictor of coronary risk in subjects with diabetes, hypertension and chronic renal failure [1,2].

The autonomic nervous system plays a key role in blood pressure (BP) regulation and is also involved in obesity-induced hypertension [3]. Its influence on arterial stiffness was also suggested in the late 1990s [4,5]. In humans, arterial stiffness has been associated with high sympathetic activity in hypertensive patients with [6] and without [7] type 2 diabetes, and in healthy individuals as well [8]. In addition, depressed vagal activity has also been associated with increased arterial stiffness [9]. Previously, we have found an association between sympathetic activity and arterial distensibility in spontaneously hypertensive rats (SHR) [10] and, more recently, a negative correlation between vagal activity and PWV in Wistar–Kyoto (WKY) rats [11]. Furthermore, data obtained in a model of rats with massive obesity have suggested that enhanced vagal activity may protect against hypertension [12]. However, such findings were observed in animal models with hypertension as a unique feature [10], or with major metabolic disorders linked to obesity, but not diabetes [12].

The Zucker diabetic fatty (ZDF) rat is a model of type 2 diabetes and obesity that exhibits a number of predisposing metabolic factors for an increased BP and arterial stiffness such as obesity and dyslipidaemia [13–16]. Nevertheless, conflicting results have been reported regarding BP levels in ZDF rats. These discrepancies might be ascribed to the methods used to record BP, including tail-cuff plethysmography [13–16] and intra-arterial determinations made under anaesthesia [17,18]. As for arterial rigidity, to our knowledge, only one report has described an increased index of arterial stiffness under anaesthesia in ZDF rats aged 10 weeks [18]. Thus, exploring intra-arterial BP and PWV in conscious unrestrained ZDF rats during disease development remains a critical issue.

For this reason, power spectral analysis of heart rate variability (HRV) has been introduced to detect autonomic dysfunction in diabetic patients and can therefore serve as a clinical test of autonomic function [19,20]. Yet, to our knowledge, the autonomic nervous system has remained unexplored in ZDF rats, the report of Towa et al. [19] having been performed in Zucker fatty rats, a model of insulin resistance without diabetes. Indeed, in ZDF rats, changes in the sympathovagal balance related to the different disease stages (obesity and insulin resistance, then diabetes and, finally, weight loss) are expected.

The aims of the present study were:

- to investigate changes in intra-arterial BP and aortic PWV in conscious, freely moving ZDF rats at different times in the development of diabetes from ages 6 to 24 weeks;
- to explore autonomic nervous function with the use of power spectral analysis of BP and heart rate (HR);
- to identify the possible factors that might have an influence on BP and PWV, in particular, the metabolic changes and indices of the autonomic nervous system derived from BP and HR variability.

2. Methods

2.1. Animals

Male ZDF rats (Gmi-fa/fa; $n = 30$) and their age-matched male controls (lean ?/fa; $n = 28$) were obtained from Charles River Laboratories France (L’Arbresle, France) at 5 or 6 weeks of age and acclimatized for at least 1 week before the experiments. The animals were maintained at a temperature of 22–24 °C, with a light on from 06:00 to 18:00 every day, and given access to chow (A04; UAR, France) and tap water ad libitum. The measurements were taken when the animals were 6, 12, 18 and 24 weeks of age. The experimental protocol was approved by the animal ethics committee of the Institut National de la Santé et de la Recherche Médicale (National Institute of Health and Medical Research) in Paris, France. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health.

2.2. Blood pressure and pulse wave velocity

The technique for measuring PWV in rats has been described in detail elsewhere [11]. Briefly, the rats were anaesthetized with sodium pentobarbital (60 mg/kg intraperitoneally). Two polyethylene catheters – a PE-10 (2 cm in length, inner diameter [ID] 0.28 mm, outer diameter [OD] 0.61 mm; Clay Adams, Parsippany, NJ) connected to a PE-50 (15 cm in length, ID
according to the following formula: 2.11

catheters (an index of aortic length) divided by transit time
to determine the mean.

For calculation of PWV, the foot-to-foot method was
used to determine the time delay between the proximal
and distal aorta [11]. BP signals were analyzed beat-to-beat by Chart 5.2 software on 20-s recordings corresponding to 100–150 cardiac cycles. This software can identify the times of the lowest values (foot) of the proximal and distal BP, and of the lowest (diastolic) and highest (systolic) BP (SBP), and calculate the mean BP and pulse pressure. HR is also automatically recorded. The difference between the time of the lowest values of the proximal and distal aortic BP yields the transit time (ms). PWV was calculated as the aortic distance between the tips of the two catheters, stretched at its tip, was 54 Hz. This ensured the correct transmission of any event oscillations within this frequency limit [21].

2.3. Spectral analysis

For each rat, a stationary segment of 102.4 s (1024 values)
was extracted from the lattermost part of the recorded data. Short-term variability of the proximal SBP and pulse interval (PI) was computed using a fast Fourier transform. To perform this spectral analysis, a resampling rate of 10 Hz was chosen without interpolation – that is, SBP and PI values were replicated every 0.1 s until a new BP cycle occurred within a 0.1-s window. Each spectral component band was a harmonic of 10/1024 Hz (9.8 mHz). The first spectral component corresponded to the mean value of the variable. The power of the SBP and PI spectrum was given as units of mmHg² and ms², respectively. The low-frequency (LF) component was obtained by integrating values of the consecutive bands from 0.2 to 0.6 Hz, and the high-frequency (HF) component from bands of 1.0 to 2.5 Hz [22].

2.4. Biochemical measurements

Blood samples were collected from the proximal aorta through the catheter just after BP recording and before the rats were euthanized. Plasma glucose was analyzed using the Infinity glucose test (ThermoTrace, Melbourne, Australia). Plasma insulin concentration was assayed by ELISA (ELIT kit, Laboratoires Eurobio, Les Ulis, France); serum triglycerides and total cholesterol were determined using the IL Test (Instrumentation Laboratory, Milan, Italy); and serum free fatty acids were measured by spectrometry using the Wako NEFA C test (Wako Chemicals, Neuss, Germany).

2.5. Statistical analyses

Our results are presented as means ± SE. Two-way analysis of variance followed by Fisher’s protected least significant difference (PLSD) test for multiple comparisons were used to assess the significance of the results. To evaluate the major determinants of PWV, a robust stepwise regression analysis was carried out. The variables entered into the model were the central mean BP, HF-P1, age and body weight. The statistical analyses were performed with StatView 5.0 (SAS Institute Inc., Cary, North Carolina, USA) and NCSS 6.0 (Kaysville, Utah, USA) software. A P-value < 0.05 was considered to be statistically significant.

3. Results

Table 1 presents the changes in the rats’ body weight and biochemical parameters, haemodynamic parameters and arterial stiffness indices (PWV and β index), and the results of spectral analysis as a function of age and rat strain. Body weight increased with age (P < 0.0001) in both strains, but more in ZDF rats up to 12 weeks of age and later in lean rats (P < 0.0001 for interaction). In ZDF rats, diabetes appeared after 6 weeks of age, with plasma glucose levels that were significantly higher than in the lean control rats. In addition, plasma insulin levels were significantly higher in ZDF vs lean control rats from 6 to 18 weeks of age, but were similar in both strains at age 24 weeks. However, ZDF rats exhibited higher serum levels of total cholesterol, triglycerides and free fatty acids compared with lean control rats at all ages.

HR significantly decreased with age (P < 0.001) in both rat strains. At 6 weeks of age, HR was similar in ZDF rats as in lean rats, but was lower in the ZDF rats from age 12 to 24 weeks. Proximal aortic diastolic, systolic and mean BPs and pulse pressure increased between weeks 6 to 12 in both strains, but did not differ between ZDF and lean rats, whatever their age. The results were similar for BP measured in the distal aorta (data not shown). In both rat strains, aortic PWV increased between weeks 6 and 12 (P < 0.05 for both strains), with no significant
changes between weeks 12 and 24. However, PWV was similar in ZDF and lean rats at each age. Similar results were observed for the proximal β index.

The LF SBP significantly increased from age 6 to 12 weeks in both strains (P < 0.001), and was significantly lower in ZDF rats than in lean rats whatever the age studied (P < 0.001 for strain). In contrast, HF-PI did not change with age in either strain (no strain effect or interaction), but was significantly higher in ZDF rats than in lean control rats (P < 0.05). The LF-PI/HF-PI ratio increased significantly with age (P < 0.001), but was lower in ZDF rats than in lean rats (P < 0.05).

In the overall population of both ZDF and lean rats, PWV correlated positively with mean BP (r = 0.51, P < 0.0001), age (r = 0.37, P < 0.01) and body weight (r = 0.33, P < 0.05), but negatively with HF-PI (r = −0.37, P < 0.01). When considered separately, a trend towards a negative correlation was observed between PWV and HF-PI in both ZDF (r = −0.36, P = 0.06) and lean (r = −0.37, P = 0.07) rats. In the whole of the test population, the proximal β index also correlated with the HF-PI (r = −0.31, P < 0.05). In contrast, no correlation was observed between PWV and biochemical parameters.

Robust stepwise regression analyses showed that mean BP and HF-PI, but not age, were independent variables, explaining 44% and 10% of the variance in PWV, respectively (Table 2). The entire model explained 61% of the PWV variance. Similar results were observed when body weight instead of age was introduced into the model (Table 2).

4. Discussion

The present study was the first to measure BP and, more important, PWV in conscious, moving ZDF rats, and to investigate the role of the autonomic nervous system in this model of type 2 diabetes. The results showed that BP and PWV both increased with age in both strains with no difference between strains despite the insulin resistance that was already present at 6 weeks of age in ZDF rats. In addition, in the ZDF compared with lean rats, the LF-SBP variability and LF-PI/HF-PI

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (weeks)</th>
<th>P-value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>ZDF</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Lean</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>140 ± 6</td>
<td>326 ± 4</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>7.79 ± 0.19</td>
<td>21.60 ± 1.54</td>
</tr>
<tr>
<td>Plasma insulin (pmol/L)</td>
<td>337 ± 38</td>
<td>672 ± 102</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>2.60 ± 0.10</td>
<td>3.24 ± 0.17</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>1.08 ± 0.08</td>
<td>2.58 ± 0.56</td>
</tr>
<tr>
<td>HF-PI</td>
<td>24 ± 5</td>
<td>122 ± 19</td>
</tr>
<tr>
<td>HF-SBP (mmHg²)</td>
<td>396 ± 12</td>
<td>295 ± 13</td>
</tr>
<tr>
<td>HF-PI (mm²)</td>
<td>75 ± 2</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>121 ± 3</td>
<td>131 ± 5</td>
</tr>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>80 ± 4</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Pulse wave velocity (cm/s)</td>
<td>554 ± 43</td>
<td>653 ± 15</td>
</tr>
<tr>
<td>OH-PI (10^-3)cm²/s².mmHg</td>
<td>8.79 ± 0.68</td>
<td>8.74 ± 0.54</td>
</tr>
<tr>
<td>OH-PI/FH-PI</td>
<td>8.51 ± 1.21</td>
<td>9.08 ± 0.37</td>
</tr>
<tr>
<td>OH-PI/FH-PI ratio</td>
<td>1.08 ± 0.16</td>
<td>1.82 ± 0.18</td>
</tr>
<tr>
<td>OH-PI (mm²)</td>
<td>1.31 ± 0.21</td>
<td>2.63 ± 0.49</td>
</tr>
<tr>
<td>OH-PI/FH-PI</td>
<td>0.89 ± 0.22</td>
<td>3.01 ± 1.10</td>
</tr>
<tr>
<td>OH-PI/FH-PI ratio</td>
<td>1.27 ± 0.32</td>
<td>1.30 ± 0.49</td>
</tr>
<tr>
<td>OH-PI/FH-PI</td>
<td>0.17 ± 0.01</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>OH-PI/FH-PI ratio</td>
<td>0.16 ± 0.04</td>
<td>0.33 ± 0.09</td>
</tr>
</tbody>
</table>
The present study is also the first in which the aortic PWV and proximal $\beta$ index – PWV normalized by diastolic BP in the proximal aorta – were determined in conscious ZDF rats and lean controls. Although an age-dependent increase in PWV was observed between ages 6 and 12 weeks, the changes in PWV were similar in both rat strains. Such results were unexpected, given that obesity, dyslipidaemia, insulin resistance and diabetes have been consistently associated with arterial stiffness, at least in humans [2,25]. Interestingly, two previous studies looking at arterial stiffness in ZDF [18] and Zucker rats [26] obtained results that were different from our findings. Marsh et al. demonstrated that the augmentation index (of arterial stiffness) was increased in 10-week-old ZDF rats compared with Sprague–Dawley rats [18]. In that study, animals were examined under 2% isoflurane anaesthesia for a total time of up to 1 h, and this may have influenced the haemodynamic parameters [18]. Sista et al. found that aortic distensibility was lower in 13- to 16-week-old insulin-resistant $fa/fa$ rats than in Zucker lean control rats. However, these rats had mild hyperglycaemia without diabetes, and aortic stiffness was determined ex vivo [26]. It is well established that aortic stiffness increases with BP or age, or both [27]. In the present study, we observed a similar significant increase in PWV in both strains at 12 and 24 weeks, but not at 18 weeks, despite the similar increase in BP at 12, 18 and 24 weeks compared with 6 weeks. Although unexpected, this does not appear to be unique to ZDF rats. Indeed, a similar evolution of PWV and BP has been reported in SHR in which PWV decreased with maturation, then rose to very high levels at 15 months [28]. Again, as with BP, our data do not exclude the possibility of a significant difference in PWV between ZDF and lean rats after a longer follow-up period.

4.2. Role of sympathovagal balance

In the present study, the autonomic nervous system was studied using spectral analysis of BP and HR, which has proved to be a useful tool in both humans and animals for the assessment of the vagal and sympathetic contributions to BP and HR oscillation variations. There is also evidence to support the idea that the fluctuations in HR are synchronized to respiration and that respiratory sinus arrhythmias represent parasympathetic influences on the sinus node [22]. In rats, these HF fluctuations in HR are virtually abolished by vagal blockade using atropine, while HR fluctuations in the LF range are diminished by vagal blockade or cardiac sympathetic blockade with atenolol. The LF oscillations in SBP are reduced by $\alpha$-sympathetic blockade and enhanced during stress [22]. The LF component of SBP might be, at least in part, under the control of sympathetic activity [20].

Several hypotheses may be proposed to explain the lack of difference in BP and PWV between ZDF rats and their lean controls, including the role of sympathovagal modulation. HR was markedly decreased between week 6 and week 12 in ZDF rats,
and was significantly lower in ZDF than in lean control rats at week 12 and week 24. Such a decrease in HR has already been observed in other rat models of diabetes and obesity [12,23]. In addition, HF-PI was higher in ZDF than in lean control rats, while LF-SBP and the LF-PI/HF-PI ratio were lower in ZDF rats. Furthermore, a significant albeit slight negative association was observed between PWV and HF-PI. Likewise, an inverse relationship between arterial stiffness and HR has been reported in both humans [29] and rats [11]. Taken altogether, these results suggest that an imbalance in the autonomous nervous system – such as high parasympathetic combined with low sympathetic activity – may account for the lack of difference in BP and PWV between ZDF rats and their lean controls. However, such an imbalance has not been demonstrated in 12-week-old Zucker fatty rats – which exhibit the features of the metabolic syndrome without frank diabetes [19] – thus suggesting a role for hyperglycaemia per se.

An increase in vagal activity leads to a reduction in HR. It may also act through the smooth muscle cells of the aorta, which are believed to contribute to about one-third of the elasticity of the aortic wall [30,31]. Furthermore, a protective effect of vagal activity against hypertension has been suggested in an animal model of massive obesity. Indeed, in rats with ventromedial hypothalamic lesions, their BP was no higher than in control rats, despite their being hugely overweight with insulin resistance, normal or increased blood catecholamine levels and normal reactivity to beta-adrenoceptors [12]. In contrast, Schreihofer et al. showed that, in 14-week-old Zucker fatty rats, a high BP was associated not only with increased sympathetic vasomotor tone, but also with autonomic deficits in baroreflex control. These authors also suggested that the depressed baroreflex function may have predisposed or contributed to the later development of hypertension [3]. As regards the role of vagal activity on arterial stiffness, a negative correlation between PWV and HF-PI in WKY rats has recently been found [11]. In type 2 diabetic patients, changes in the autonomic nervous system with diabetes duration are characterized first by a reduction of cardiac vagal activity, then a subsequent sympathetic predominance and, later, by a progressive depression of sympathetic activity [32]. Interestingly, LF-SBP was found to correlate with pulse pressure in type 2 diabetic patients with hypertension [33], and HF-PI was reported to correlate negatively with carotid artery wall stiffness in type 1 diabetic patients [9].

In fact, the deleterious effect of sympathetic tone on arterial stiffness has been suggested by many authors. Recent studies of human vascular smooth muscle cells have shown that adrenergic stimulation directly modulates elasticity indices through changes in transforming growth factor-β1 expression, fibronectin, and extracellular matrix protein synthesis of elastin and collagen in the arterial wall [34]. Sympathetic activity has recently been reported to correlate with arterial stiffness in patients with type 2 diabetes [6] and in young Japanese men, after adjusting for age, BP and plasma noradrenaline levels [8]. However, the present data are correlative and, thus, are not proof of a causal relationship. An interventional study that modifies the autonomic nervous system would be necessary to establish a cause-and-effect relationship between the autonomic nervous system and arterial stiffness. Moreover, it is possible that alterations in the autonomic nervous system may be a consequence of arterial stiffening. It has been suggested that stiffness of the aorta and carotid arteries may alter baroreceptor afferent transduction. This would, in turn, attenuate efferent vagal tone and increase sympathetic vascular outflow, manifesting as reduced baroreflex sensitivity and HR variability, and increased SBP variability [3,35]. Other factors may also be responsible for the lack of increased arterial stiffness in ZDF rats compared with their lean counterparts. In ZDF rats, the mutation in leptin receptors [36] leads to leptin resistance. As leptin has recently been reported to be associated with increased arterial stiffness [37,38], leptin resistance might counteract the effects of insulin resistance, diabetes and lipid disorders in this strain of rat. Also, the aortic PWV does not reflect overall vascular compliance. Indeed, the PWV applies only to the arterial segment in which the measurement is taken [2]. In particular, the aortic PWV does not reflect the rigidity of the smaller arterioles and branch vessels which is likely to be influenced by the endothelial dysfunction observed in diabetes and in ZDF rats [15].

In the present study, although the ages of the rats were selected to study the potential role of insulin resistance without diabetes (week 6), insulin resistance and diabetes (week 12), and relative insulin deficiency and diabetes (weeks 18 and 24), no correlations were observed between PWV and biochemical parameters such as glucose and insulin values, and lipid parameters. Indeed, an increased PWV might be observed in older rats (>24 weeks of age), as a longer time interval might be necessary to allow for advanced glycation end-products to be deposited in the arterial walls, thereby increasing arterial stiffness [39]. This means that arterial stiffness might be the consequence of biochemical changes, as the latter do precede the former. However, the effects of biochemical changes during disease development in ZDF rats may also have been blunted by alterations of the autonomic nervous system (see above), at least during rat development.

5. Conclusion

ZDF rats do not develop high BP and arterial stiffness compared with lean rats, and do not appear to be an adequate model for studying atherosclerosis in type 2 diabetes. This suggests that exploring new models of type 2 diabetes, such as fat-fed streptozotocin-induced diabetes [40], may be helpful. However, the present data also suggest that, during development, sympathovagal modulation could play a major role in the lack of an increase in arterial stiffness in ZDF rats, despite their insulin resistance, obesity, diabetes and dyslipidaemia. Indeed, alterations in sympathovagal balance could lead to a reduction in HR that could, in turn, decrease arterial stiffness. It could also act directly on arterial smooth muscle cell function or structure. Finally, new treatments that enhance vagal activity could have beneficial effects on arterial elasticity, and could prove useful in the prevention of cardiovascular morbidity and mortality in patients with the metabolic syndrome.
6. Conflicts of interest

None.

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