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RESEARCH PAPER

Effects of a new advanced glycation inhibitor, LR-90, on mitigating arterial stiffening and improving arterial elasticity and compliance in a diabetic rat model: aortic impedance analysis

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BACKGROUND AND PURPOSE

We determined the effects of treatment with LR-90, an inhibitor of advanced glycation end products, on the mechanical properties of the arterial system in streptozotocin (STZ)-induced diabetic Sprague Dawley rats, using aortic impedance analysis, and further investigated the effects of LR-90 on the progression of aortic pathology.

EXPERIMENTAL APPROACH

STZ-induced diabetic rats were treated with or without LR-90 (50 mg L^{-1} in drinking water) for 8 weeks and compared with control groups. Arterial BP measurements, various metabolic parameters, aortic histopathology, collagen cross-linking, AGE accumulation, and RAGE protein expression in aortic tissue were determined. Pulsatile parameters were evaluated using a standard Fourier series expansion technique and impulse response function of the filtered aortic input impedance spectra.

KEY RESULTS

LR-90 reduced glycated haemoglobin and triglycerides levels, although it had no effect on the glycaemic status. LR-90 did not affect arterial BP, but prevented the diabetes-induced increase in peripheral resistance and variations in aortic distensibility, as it reduced aortic characteristic impedance by 21%. LR-90 also prevented the elevation in wave reflection factor, as indicated by a 22.5% reduction and an associated increase of 23.5% in wave transit time, suggesting it prevents the augmentation of the systolic load of the left ventricle. Moreover, LR-90 inhibited collagen cross-linking and the accumulation of AGE and RAGE in the vasculature of diabetic rats.

CONCLUSIONS AND IMPLICATIONS

Treatment with LR-90 may impart significant protection against diabetes-induced aortic stiffening and cardiac hypertrophy and provides an additional therapeutic option for treatment of AGE associated diabetic complications.

Abbreviations

AGEs, advanced glycation end products; RAGE, receptor for advanced glycation end product; HgbA1c, glycated haemoglobin; C_m, systemic arterial compliance; CO, cardiac output; EL, elastic lamellae; FC, fibrillar collagen; LR-90, methylene bis(4, 4′- (2 chlorophenyl ureido phenoxyisobutyric acid); PAS, periodic acid-Schiff; PSR, picrosirius red; PWV, pulse wave velocity; P_b, magnitude of the backward pressure; P_d, diastolic aortic pressure; P_f, magnitude of the forward pressure; P_m, mean aortic pressure; P_s, systolic aortic pressure; R_f, wave reflection factor; R_p, total peripheral resistance; SMC, smooth muscle cells; SV, stroke volume; STZ, streptozotocin; τ, wave transit time; Z_c, aortic characteristic impedance; Z_i, aortic input impedance spectra

Introduction

It has been known over the last two decades that advanced glycation end products (AGEs) and AGE-induced protein covalent cross-linking is involved in diabetes-induced tissue and vascular injury associated with type I (insulin-dependent) and type II (non-insulin-dependent) diabetes (Singh *et al.*, 2001; Basta *et al.*, 2004). AGEs are heterogeneous moieties that alter fundamental protein structure by inducing non-enzymatic glycation on lysine and arginine residues in addition to inducing alterations in biological lipid structures and in extracellular matrix architecture. Once glycated, AGE epitopes on proteins and lipids interact with their signal transduction receptor, receptor for AGE (RAGE), a multiligand member of the immunoglobulin superfamily, to mediate intracellular signalling and modulate various cellular properties (Brett *et al.*, 1993; Yan *et al.*, 2009). It is now well known that different classes of AGEs, such as carboxymethyllysine (CML), accumulate in diabetes and result in activation of RAGE, which in turn mediates generation of reactive oxygen/carbonyl species (ROS/RCS) contributing to further AGE production, generation of more ROS and activation of the inflammatory response (Soulis *et al.*, 1997; Bierhaus *et al.*, 2005; Yan *et al.*, 2010). Hyperglycaemia and AGE production have been implicated in the accelerated vascular damage associated with diabetes, which eventually manifests as microvascular complications and macrovascular disease.

AGEs mediate glycation cross-linking of the structural framework of collagen in diabetes resulting in diabetic complications in the collagen rich tissues, which includes stiffening of the elastic arteries. AGE accumulation can lead to vascular thickening with associated loss of elasticity, hypertension and endothelial dysfunction. Furthermore, reactive free radicals generated following glycation of proteins and lipids can react with collagen to form collagen-cross-links exacerbating the cardiovascular risk via activation of smooth muscle proliferation and increased vascular permeability (Rojas and Morales, 2004; Ahmed, 2005). Accumulation of AGEs is also believed to contribute to Alzheimer's disease, atherosclerosis and in the normal aging process in addition to the diabetic complications (Brownlee *et al.*, 1988; Li *et al.*, 1996). For these reasons, a variety of different compounds that inhibit AGEs have been under investigation and several natural and synthetic AGE inhibitors have been developed. These AGE inhibitors have two possible mechanisms of action: (i) attenuation of accumulated AGE to prevent architectural damage that has pathophysiological implications and (ii) mediation of chemical modification of accumulated AGEs and conversion to non-active intermediates. In addition to agents that prevent AGE formation or that disrupt AGE cross-links, several antioxidants such as vitamin E, lipoic acid, vitamin B1 derivatives, pyridoxamine and other synthetic derivatives have been reported to be effective in inhibiting AGE formation and cross-linking *in vitro* as well as in animal studies (Rahbar and Figarola, 2003; Reddy and Beyaz, 2006; Rahbar, 2007; Yamagishi *et al.*, 2008).

Our group has developed and investigated several synthetic aromatic compounds with AGE inhibitory effects over the last decade, including the lead compound LR-90 {methylene bis[4, 4′- (2 chlorophenyl ureido phenoxyisobutyric acid)]}. LR-90 inhibits AGE formation by scavenging dicarbonyl intermediates and by chelating transition metals that catalyse the production of AGE and has therapeutic potential as an inhibitor of circulating AGE accumulation and AGE deposition in tissues (Rahbar and Figarola, 2003; Rahbar, 2007). In animal studies, LR-90 demonstrated protective effects against diabetic nephropathy (Figarola *et al.*, 2003; 2008), retinopathy (Bhatwadekar *et al.*, 2008) and atherosclerosis (Watson *et al.*, 2010).

The present study determined the role of LR-90 in the modulation of diabetes-induced changes in the pulsatile nature of blood flow in arteries using aortic impedance analysis. Diabetic subjects have increased arterial stiffness, as measured by diastolic dysfunction, increased pulse wave velocity and decreased arterial compliance. The aortic characteristic impedance (Z_c) is the frequency domain representation of the aortic pressure and flow relationship that can be derived from the input impedance spectrum. Other pulsatile parameters such as wave transit time and wave reflection factor can be derived from this spectrum to explain the timing of the pulse wave reflection from the peripheral circulation. In this study, STZ-induced diabetic Sprague Dawley rats were treated with LR-90 for 8 weeks. We examined the efficacy of LR-90 in protecting against aortic pathology and assessed therapeutic potential to inhibit tissue AGE accumulation and the subsequent expression of RAGE in the aortic lumen of diabetic rats through immunohistochemical methods. Our results indicated that LR-90 (50 $mg \cdot L^{-1}$ in drinking water) mitigates arterial stiffness by preventing the decline in aortic distensibility and the early return of pulse wave reflection from the peripheral circulation induced by the diabetic condition.

Methods

Animals

The experiments were performed in male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) maintained at

the Animal Resources Center in the Division of Comparative Medicine, Beckman Research Institute (BRI) of City of Hope (COH). The rats were housed and maintained at temperature and humidity levels as specified in the Guide for the Care and Use of Laboratory Animals, Public Health Service Policy on Humane Care and Use of Laboratory Animals. The study was approved by the COH/BRI Institutional Animal Care and Use Committee and all experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Public Health Service Policy on Humane Care and Use of Laboratory Animals, and Animal Welfare Act. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Eight-week old rats, following 1 week of acclimatization, were randomly divided into four groups (n = 7) as follows: (i) non-diabetic control; (ii) STZ-induced diabetic control; (iii) LR-90-treated non-diabetic control; and (iv) LR-90-treated STZ-diabetic rats. Diabetic rats were injected with a single i.p. injection of STZ (65 mg·kg^{-1} in citrate buffer, pH 4.5), following an overnight fast. Non-diabetic animals were administered an i.p. injection of citrate buffer. Blood glucose concentrations were measured using the AlphaTrak glucose meter (Abbott Laboratories, Abbott Park, IL, USA) after 7 days following injection. A blood glucose level of >20 mmol·L^{-1} after 1 week of STZ injection was used to confirm onset of diabetes. LR-90-treated non-diabetic control and LR-90-treated STZ-induced diabetic rats were treated daily for up to 8 weeks with LR-90 via drinking water at 50 mg·L^{-1} concentration. All animals were allowed free access to regular rodent chow and housed in Opti-Rat cages with 1–2 rats per cage in a 12:12 h light : dark cycle animal room. Body weights and water consumption were checked every week. Systolic, diastolic and mean BPs were determined at weekly intervals using a standard tail cuff non-invasive BP measurement system (CODA2 Monitor Non-Invasive Blood Pressure System, Kent Scientific, Torrington, CT, USA) as described by Feng *et al.* (2008). Blood glucose, glycated haemoglobin (HbA1c) and plasma triglyceride concentrations were monitored by tail vein blood collections every 2 weeks for the 8 week duration of the experiment (five time points – 0, 14, 28, 42, 56 days). Glycated haemoglobin was measured by HPLC and plasma triglyceride concentration was measured using the Vitros 250 chemistry system (Division of Pathology, COH).

Surgical measurements and procedure

Pulsatile aortic flow volume was measured using an electromagnetic flowmeter (T402 2-channel modular flow meter, Transonic Systems Inc, Ithaca, NY, USA) and aortic pressure was measured using a pressure transducer as previously described (Lin *et al.*, 2004). Rats were anaesthetized with isoflurane (2–4%) and upon verifying appropriate depth of anaesthesia, the animals were intubated and then transferred to a rodent ventilator. The depth of anaesthesia in the rats was assessed by monitoring the rate and depth of respiration, as well as by assessing the heart rate using ECG electrodes. We also provided a circulating warm water blanket to maintain the body temperature during the surgical intervention. The pedal (digital withdrawal) reflex and the tail pinch reflex were used to measure the CNS depression in the anesthetized rats. The surgical site was prepped by clipping hair and disinfecting the skin with betadine scrub, followed by 70% ethanol application. A high-fidelity pressure catheter (1.4F Millar catheter) was used to measure the aortic pressure via placement in the isolated right carotid artery. Briefly, a blunt dissection was made along the superficial cervical platysma muscle and the carotid artery was isolated from the sheath after reflecting the salivary glands and overlying musculature. The pressure sensor was pre-warmed before being inserted into the artery. The tip of the transducer was positioned 1–2 mm distal to the electromagnetic flow probe, which was positioned around the ascending aorta. A midline skin incision was made 1 cm rostral to the manubrium and extending to the xiphoid. The incision was extended into the thoracic cavity, avoiding damage to the internal mamillary artery and then the position of aorta was located. The electromagnetic flow probe was positioned around the ascending aorta to measure aortic flow. The ECG of lead II was recorded using the PowerLab data acquisition system (ADInstruments, Inc., Colorado Springs, CO, USA). Pressure and flow signals of 5–10 beats were averaged in the time domain, using ECG peak R wave as the fiducial point. Time differences between pressure and flow signals, due to spatial distance between the flow probe and the pressure transducer, were corrected by a time-domain approach in which the foot of the pressure waveform was realigned with that of the flow signals (Mitchell *et al.*, 1994).

Aortic input impedance spectra

Aortic input impedance (Z_i) was obtained from the ratio of ascending aortic pressure harmonics to the corresponding flow harmonics using a standard Fourier series expansion technique (Chang *et al.*, 2003). Total peripheral resistance of the systemic circulation was calculated by determining the ratio of mean aortic pressure and mean aortic flow. Aortic characteristic impedance (Z_c) was determined by averaging high-frequency moduli of the aortic input impedance data points (Chang *et al.*, 2003). Systemic arterial compliance C, at any pressure P, was calculated by expanding the two-element into the three-element Windkessel model that accounts for a non-linear exponential pressure–volume relationship:

$$C(P_m) = \frac{SVb}{K + Z_c SV/A_d} \times \frac{e^{bP_m}}{e^{bP_i} - e^{bP_d}}$$

where SV is the stroke volume; K is the ratio of total area under the aortic pressure curve to the diastolic area (A_d); b is the coefficient in the pressure–volume relation (–0.0131 +/– 0.009 in aortic arch); P_i is the pressure at the time of incisura; and P_d is the end-diastolic pressure (Chang *et al.*, 2003). Wave transit time was calculated by the impulse response of the filtered Z_i. This was accomplished by the inverse transformation of Z_i after multiplication of the first 12 harmonics by a Dolph–Chebychev weighting function with the order 24 (Laxminarayan *et al.*, 1978). Time-domain reflection factor (R_f) was derived as the amplitude ratio of backward-to-forward peak pressure wave by the method proposed by Westerhof *et al.* (1972). Therefore, both wave transit time and wave reflection factor may characterize the wave reflection phenomenon in the vasculature.

Tissue collection and aorta histology

The aorta was collected immediately following the surgical procedure and rinsed in PBS buffer. Sections of aorta were stored in 10% neutral buffered formalin for microscopic examinations and further immunohistochemistry. Aorta sections were processed for light microscopy examination, and the severity of cross-linking and damage was assessed by periodic acid-methenamine silver (PAMS), picrosirius red (PSR) and trichrome staining as described previously (Rahbar, 2007; Figarola *et al.*, 2008).

AGE and RAGE immunohistochemistry

Formalin-fixed paraffin-embedded aorta sections were mounted on slides and stained with 6D12 monoclonal anti CML-AGE, polyclonal rabbit anti-RAGE antibodies as described previously (Rahbar, 2007).

Electron microscopic examination

Formalin-fixed samples of aorta from each group were processed and sections were observed and photographed with a Philips CM-10 transmission electron microscope (Philips Electronics, Mahwah, NJ, USA) as per the procedure described by Figarola *et al.* (2003). Images were used to determine the width of elastic bundles and fibrillar collagen cross-linking.

Statistical analyses

Statistical analyses were performed using GraphPad Prsim 6 (GraphPad Software Inc., San Diego, CA, USA). Results are presented as means ± SEM. Two-way ANOVA was used to determine the effects of diabetes and LR-90 treatment on the physical properties of the rat arterial system. *Post hoc* comparisons among means were analysed by Tukey's test. A $P < 0.05$ was considered to be significant.

Results

LR-90 attenuates metabolic effects of diabetes

STZ-induced diabetic rats had significantly elevated blood glucose, higher triglycerides and HbA1c levels, and lower body weight compared with age-matched normal controls (Figure 1A–D, $P < 0.05$). Treatment with LR-90 had no effect on blood glucose levels and body weight in both control and diabetic rats, but resulted in marked reductions in plasma triglyceride and glycated haemoglobin levels by 26 and 39%, respectively, in diabetic rats (Figure 1B and C, $P < 0.05$).

LR-90 had minimal effects on arterial BP

Table 1 shows the arterial pressure profile of the various treatment groups. Neither diabetes nor LR-90 treatment produced a statistically significant difference in systolic, diastolic and mean arterial pressures. However, STZ-diabetic animals elicited an increase in the magnitude of backward pressure (P_b), but exerted no effect on the magnitude of forward pressure (P_f), causing an increase in the wave reflection factor, R_f (from 0.54 ± 0.05 to 0.71 ± 0.05, $P < 0.05$; detailed in subsequent figure). Treatment with LR-90 resulted in a significant decline in the P_b, which was then reflected in the R_f (from 0.71 ± 0.05 to 0.55 ± 0.08, $P < 0.05$; detailed in subsequent figure).

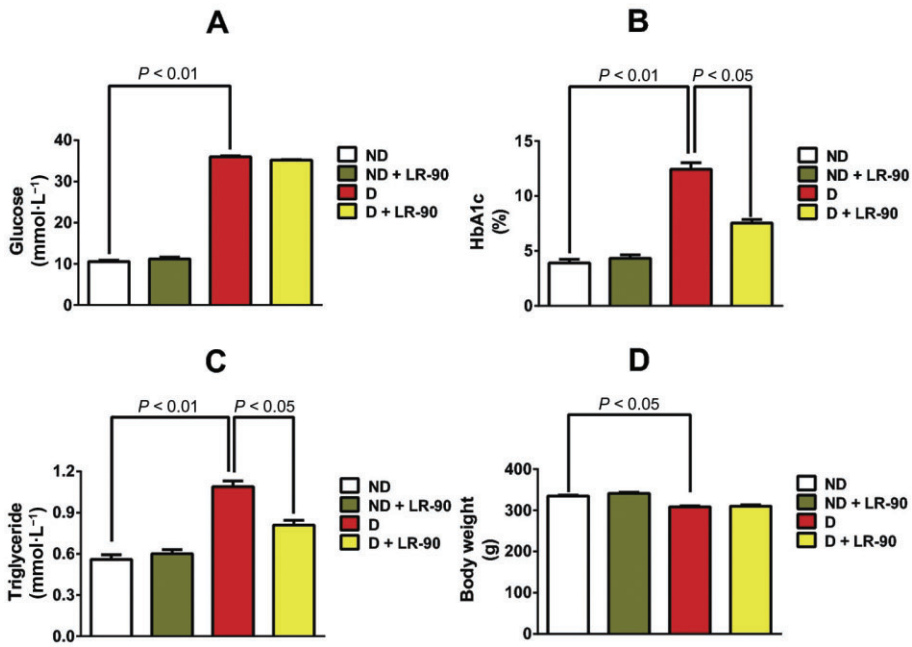

Figure 1

(A–D) Effects of LR-90 on glycated haemoglobin (HbA1c), triglyceride, glucose levels and body weight in STZ-diabetic rats. HbA1c, triglyceride and glucose levels were increased in diabetic animals, which were significantly reduced by LR-90 treatment, whereas, body weight was lowered in diabetic animals compared with the control. Non-diabetic control (ND); LR-90 treated non-diabetic (ND + LR-90); diabetic (D); diabetic rats treated with LR-90 (D + LR-90).

Table 1

Effects of diabetes and LR-90 treatment on aortic pressure profile in Sprague Dawley rats[a]

Variable	ND	ND + LR-90	D	D + LR-90
P_s (mmHg)	139.7 ± 2.6	141.5 ± 3.2	149.5 ± 2.9	143.9 ± 4.0
P_d (mmHg)	92.5 ± 3.7	93.8 ± 3.4	97.0 ± 3.1	94.9 ± 2.9
P_m (mmHg)	109.8 ± 3.9	111.3 ± 2.8	117.7 ± 3.8	110.3 ± 3.6
P_f (mmHg)	9.5 ± 0.7	10.1 ± 0.5	10.7 ± 0.3	10.2 ± 0.6
P_b (mmHg)	4.3 ± 0.5	4.6 ± 0.5	6.5 ± 0.2*	4.9 ± 0.5**

*$P < 0.05$ versus ND.
**$P < 0.05$ versus D.
[a]All values are expressed as means ± SEM ($n = 7$).
D, STZ-diabetic rats; D + LR-90, STZ-diabetic rats + LR-90; ND, normal control rats; ND + LR-90, normal control rats + LR-90; P_b, magnitude of backward pressure; P_d, diastolic aortic pressure; P_f, magnitude of forward pressure; P_m, mean aortic pressure; P_s, systolic aortic pressure.

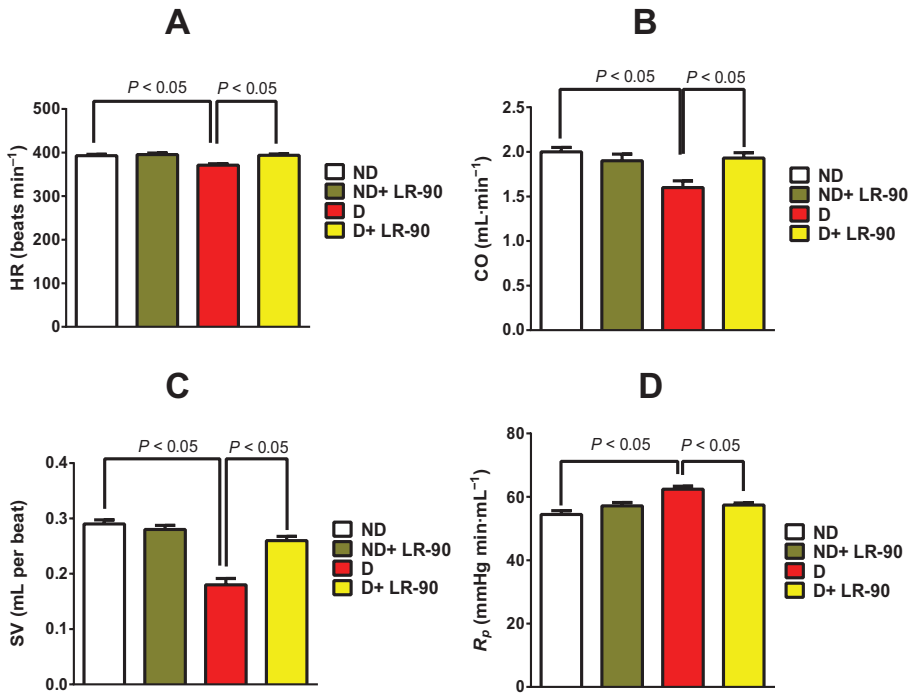

Figure 2

(A–D) Effects of LR-90 on basal heart rate (HR), cardiac output (CO), stroke volume (SV) and total peripheral resistance (Rp) in STZ-diabetic rats. Diabetes resulted in a decrease in HR, CO and SV, whereas, R_p was significantly elevated. LR-90 treatment resulted in reversal of these effects, thereby restoring the haemodynamic parameters. Non-diabetic control (ND); LR-90 treated non-diabetic (ND + LR-90); diabetic (D); diabetic rats treated with LR-90 (D + LR-90).

LR-90 improves haemodynamic parameters in diabetic rats

In comparison with age-matched control rats, STZ-diabetic rats showed significantly altered haemodynamics characterized by decreased basal heart rate (HR), cardiac output (CO), stroke volume (SV) (Figure 2A–C respectively). Moreover, these diabetic animals exhibited an increase in total peripheral resistance (Rp) (Figure 2D). After 8 weeks of treatment, LR-90-treated diabetic rats showed significant improvements in haemodynamic parameters as both CO and SV were almost normalized. More importantly, LR-90 decreased R_p in diabetic rats (Figure 2D). Interestingly, no significant changes in these haemodynamic parameters were observed in non-diabetic animals treated with LR-90.

LR-90 ameliorates the effect of diabetes on mechanical characteristics of blood flow

The average aortic pressure and flow waveforms from rats treated with LR-90 compared with that of untreated STZ-diabetic rats are illustrated in Figure 3A and B respectively. After treatment with LR-90, diabetic rats showed a decline in the rate of change of pressure over time and the aortic flow

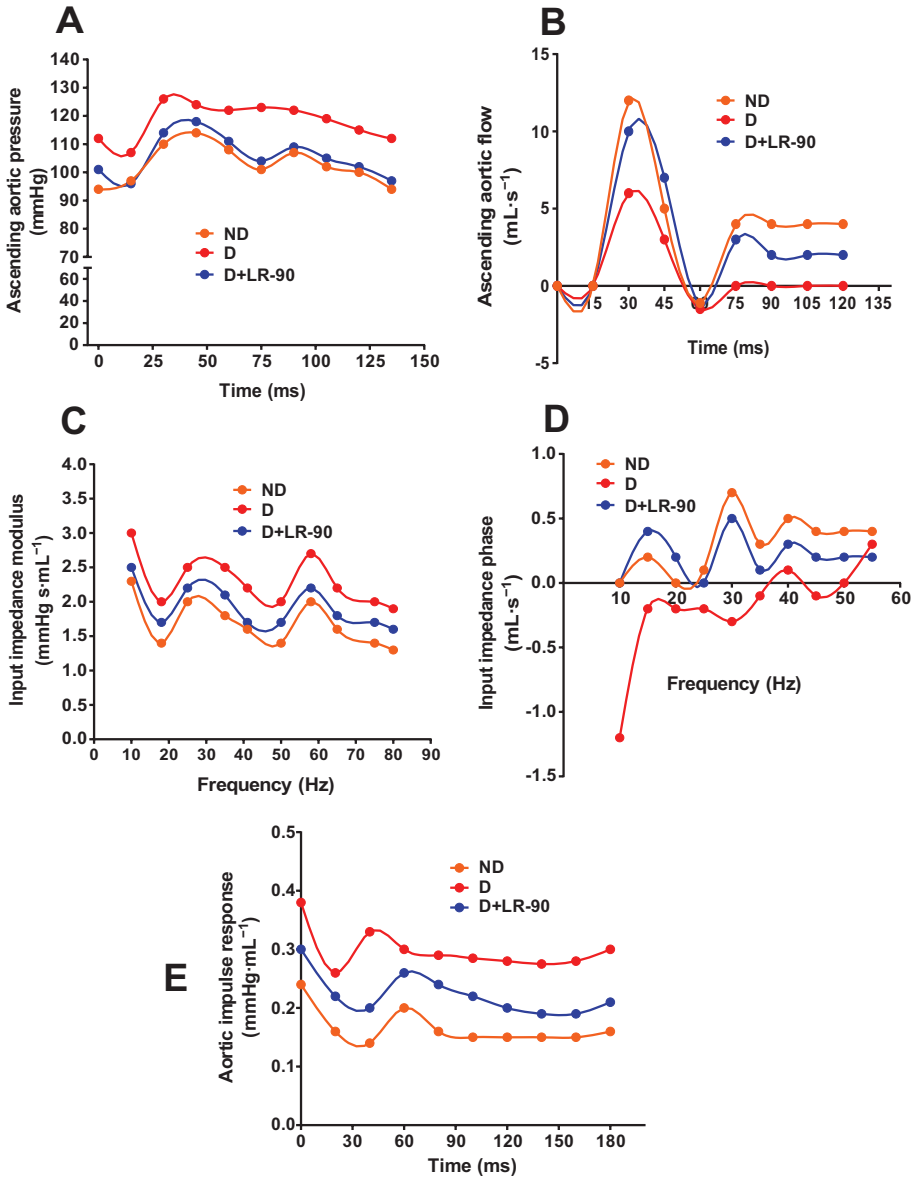

Figure 3

Top (A–B): average pressure and flow waveforms from non-diabetic control (ND), LR-90 treated non-diabetic (ND + LR-90); diabetic (D); diabetic rats treated with LR-90 (D + LR-90). Bottom (C–D): aortic input impedance spectra derived from the ascending aortic pressure and flow signals shown in the top panel. Impulse response function curve derived from the filtered aortic input impedance spectra (E). The initial peak and the discrete reflection peak from the body circulation have been demonstrated. The wave transit time in the lower body circulation can be derived as one half of the time difference between the appearance of reflected peak and the initial peak. The wave transit time was increased in the LR-90-treated group compared with the diabetic control.

rate was markedly improved compared with the untreated STZ-diabetic rats. The aortic input impedance spectra of STZ-diabetic rats were also higher compared with LR-90-treated diabetic rats (Figure 3C and D). The modulus of the impedance spectra was displaced upward and slightly shifted to the right following induction of diabetes, whereas the effects were considerably reversed following treatment with LR-90. Additionally, diabetes caused a marked increase in aortic impulse response, which was attenuated with LR-90 treatment (Figure 3E). The wave transit time was shortened in the diabetic rats with a subsequent increase in the measurement following treatment with LR-90.

Figure 4 shows the effect of diabetes and LR-90 administration on pulsatile characteristics of blood flow in arteries in terms of aortic characteristic impedance (Z_c), aortic compliance (C_m), wave transit time (τ) and wave reflection factor (R_f). We observed significant interaction between the effects of diabetes and LR-90 on the mechanical characteristics of Windkessel vessels such as Z_c, C_m, τ and R_f. Diabetes resulted in an increase in Z_c (Figure 4A) and a decrease in C_m

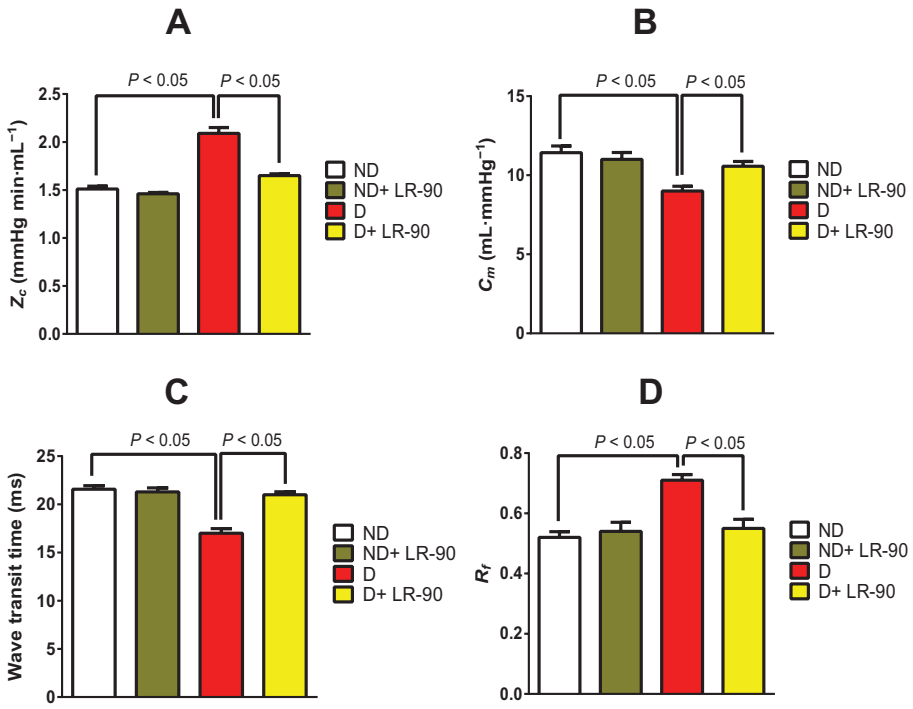

Figure 4

(A–D) Effects of LR-90 on aortic characteristic impedance (Z_c), aortic compliance (C_m), wave transit time (τ) and wave reflection factor (R_f) (A–D) in STZ-diabetic rats. An increase in both Z_c and R_f, and a decrease in C_m were observed in diabetic animals relative to control non-diabetic animals. These structural alterations were mitigated by LR-90 treatment.

(Figure 4B) compared with age-matched normal controls. These mechanical alterations were improved significantly following administration of LR-90, as manifested by a 21% decrease in Z_c and a 17% increase in C_m. Meanwhile, wave transit time was significantly lowered in STZ-diabetic rats compared with age-matched controls, indicating the early return of reflected waves from the peripheral circulation due to mechanical alterations (Figure 4C). This effect was prevented by LR-90 as evidenced by the increase in wave transit time (τ). Furthermore, wave reflection factor was significantly increased in diabetic rats compared with age-matched controls (Figure 4D). Again, this effect was reversed by treatment with LR-90, as shown by a decrease in R_f. Oscillatory components were not affected by administration of LR-90 to age-matched controls.

LR-90 prevents aortic collagen accumulation and cross-linking

Compared with age-matched normal control animals, diabetic rats had significant increase in PAMS staining (Figure 5A) and collagen accumulation (Figure 5B) in the aortic lumen, indicative of aortic fibrosis. To examine further the collagen changes in the diabetic aorta, we distinguished the different types of collagen in the aortic media wall using PSR staining under polarized light microscopy. Collagen type 1 birefringence was increased significantly (~3-fold, $P < 0.01$) in the diabetic aorta compared with non-diabetic samples (Figure 5C). Treatment with LR-90 significantly decreased both PAMS staining and collagen deposition within the aortic walls to almost similar levels as non-diabetic aorta. LR-90 also significantly reduced collagen type 1 associated birefringence by almost 50% (Figure 5C, $P < 0.05$). Electron microscopic examinations of aortic sections are shown in Figure 5D. In the non-diabetic aorta, smooth muscle cells (SMCs) were found to occupy spaces between the elastic lamellae (EL) and the fibrillar collagen (FC) bundles and were few in number. In the diabetic aorta, the EL was significantly thicker and the SMCs were withdrawn from the EL, leaving an increased amount of intercellular ground substance. In addition, the FC bundles were significantly increased and were arranged loosely and irregularly. Deposits of elastic materials were visible in the space between the two parallel elastic lamellae. Treatment with LR-90 prevented elastic lamellae thickening to an almost similar degree as that of non-diabetic control aorta. Moreover, fibrillar collagen bundles were reduced and have nearly normal arrangements compared with the untreated diabetic aorta.

LR-90 inhibits aortic AGE and RAGE protein expression

Immunohistochemical staining for AGE and its receptor, RAGE, demonstrated a significant increase in both AGE and RAGE expression in the media aortic wall of diabetic rats compared with age-matched normal control rats. AGE and RAGE appear to be co-localized in regions of the tunica media (Figure 6). After treatment with LR-90 for 8 weeks, both AGE and RAGE protein expressions were reduced by more than 60% ($P < 0.01$).

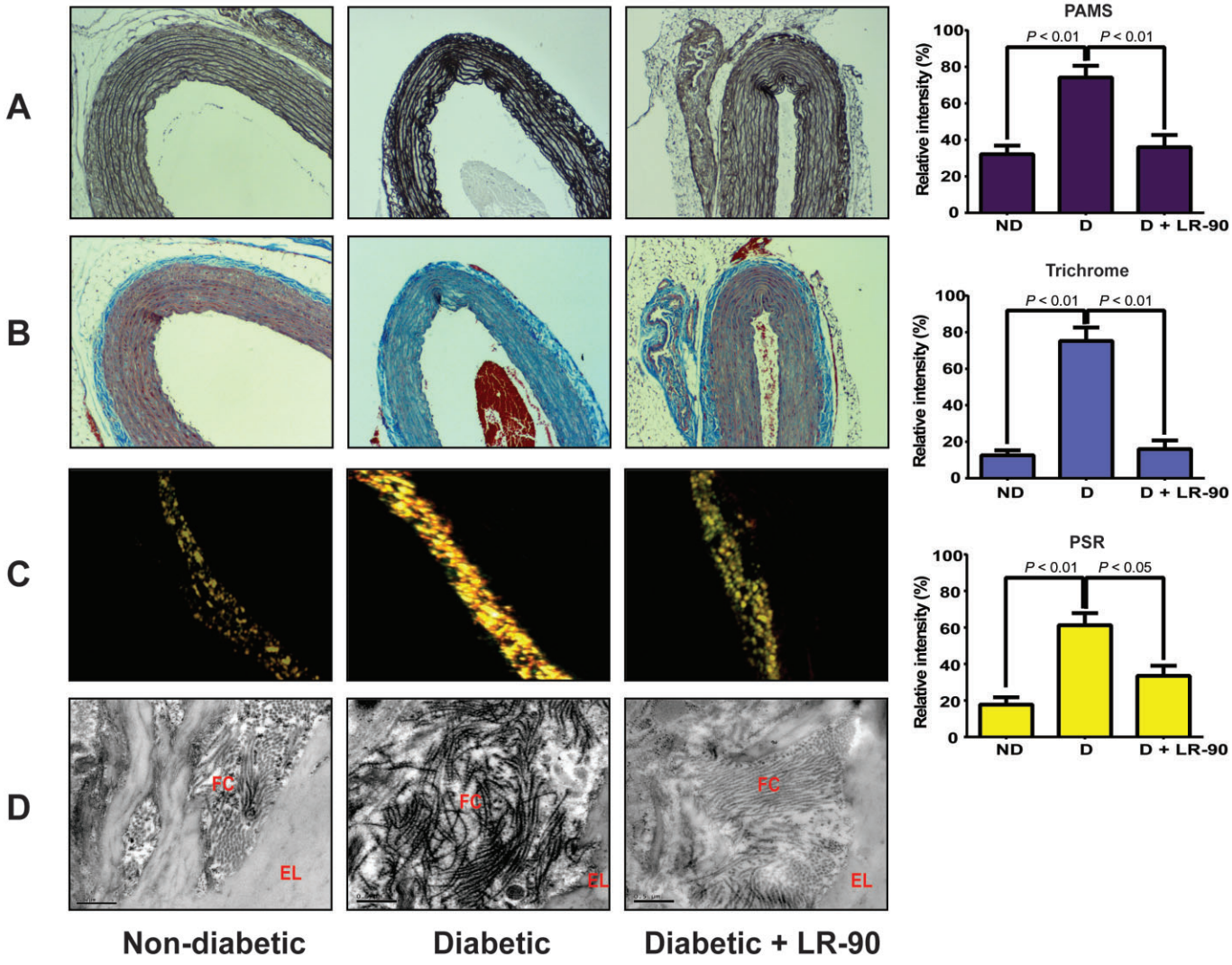

Figure 5

(A–D) Representative photomicroscopic images of sections of aorta stained with (A) PAS, (B) Masson's trichrome and (C) PSR. Relative intensity of positively stained areas was quantified using an image analysis programme. Original magnification 200×. (D) Representative electron micrograph sections of sections of aorta. Original magnification 5500×. Non-diabetic control (ND); diabetic (D); diabetic rats treated with LR-90 (D + LR-90).

Discussion

AGEs, the products of non-enzymatic glycation and oxidation of proteins and lipids, accumulate in diverse biological settings, such as diabetes, inflammation, renal failure and aging. Accumulation of AGEs and their subsequent chemical modification, cross-linking of proteins, and engagement of the RAGE have been implicated in the accelerated vascular damage associated with diabetes. AGEs are believed to directly contribute to the development and progression of diabetic vascular changes by formation of covalent cross-links between proteins, which accelerate vascular dysfunction and damage (Kass *et al.*, 2001). Binding of AGEs to RAGE activates intracellular signalling processes, thus mediating pro-inflammatory effects and AGE accumulation (Yan *et al.*, 2010). These changes result in vascular thickening with associated loss of elasticity, hypertension and endothelial dysfunction – all of which contribute to arterial stiffness in diabetic subjects and the manifestation of microvascular complications and macrovascular disease. It is known that the glycosylated proteins continue to cross-link, polymerize and form complex protein–protein cross-links with the rate of formation directly correlated to the glucose concentration and the time of exposure. Since AGE-protein adducts are stable and virtually irreversible, the ability of AGE-modified proteins to form protein–protein cross-links on collagen *in vivo* is a key determinant in the pathogenesis of the reduced vascular and myocardial compliance observed with aging and diabetes (Aronson, 2003). In diabetes, various AGEs interact with collagen forming intermolecular cross-links and modifying collagen–cell interactions that subsequently result in loss of elasticity and increased stiffness, as well as inducing

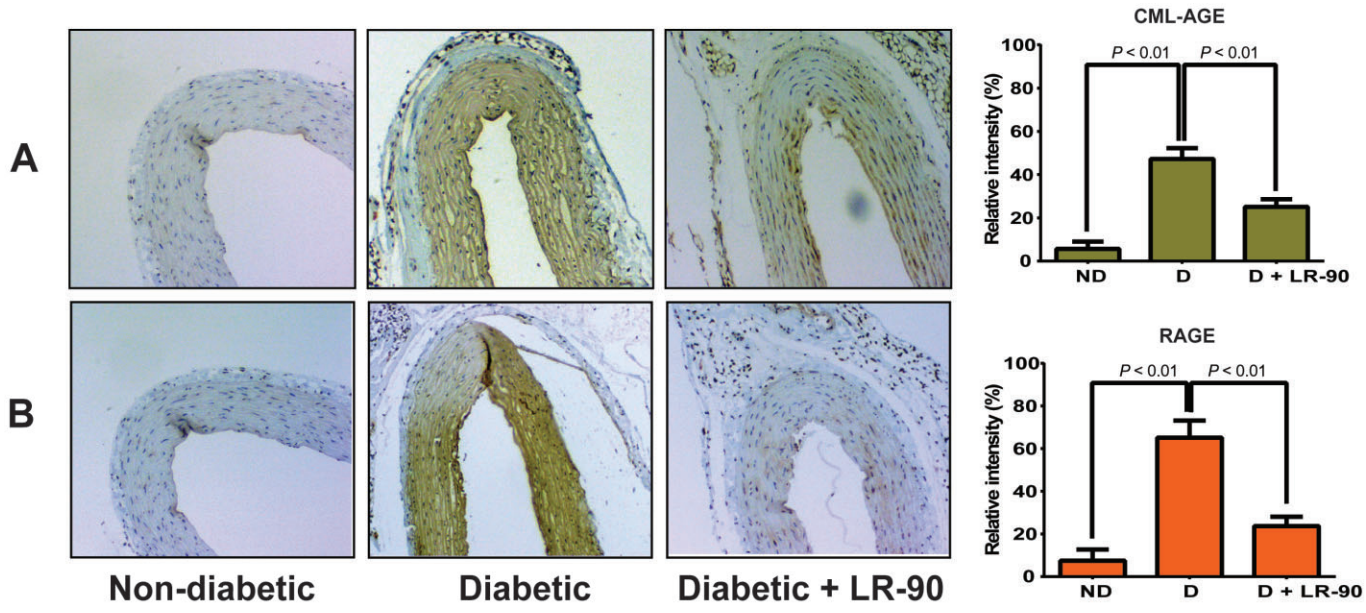

Figure 6

(A–B) Representative photomicroscopic images of sections of aorta showing increased staining for both AGE and RAGE in diabetic aorta, which were attenuated by treatment with LR-90. Original magnification ×200. Non-diabetic control (ND); diabetic (D); diabetic rats treated with LR-90 (D + LR-90).

inflammation and vascular damage (Avery and Bailey, 2006). Previous studies with aminoguanidine, an AGE inhibitor, demonstrated significant attenuation of myocardial collagen cross-linking by fluorescence studies following long-term treatment (Brownlee *et al.*, 1986; Norton *et al.*, 1996). Our present studies with LR-90, a potent AGE inhibitor, are in agreement with previous studies exhibiting marked inhibition of chemical modification of collagen and subsequent cross-linking. Our current study also demonstrated a significant reduction in the accumulation of AGE and the subsequent co-localization with its receptor, RAGE, in the vasculature of diabetic rats.

Previous studies by our group have indicated that the strong metal chelation (especially Cu^{2+}) property of LR-90, results in inhibition of auto oxidation pathways, and coupled with an ability to interact with RCS, collectively leads to effective interference with AGE formation and subsequent free radical generation (Figarola *et al.*, 2003). Our current results indicate that STZ-induced diabetes has the potential to affect elastic arteries (characteristic impedance), muscular arteries (wave reflection factor) and arterioles (peripheral resistance). Furthermore, LR-90 belongs to a group of aromatic compounds that act as allosteric effectors, synergistic with 2,3-bisphoshoglycerate in increasing the oxygen affinity of haemoglobin molecules, which have been shown to lower serum cholesterol and low-density lipoproteins in rats. In this study, LR-90 did not have an effect on blood glucose concentrations or on body weight of diabetic rats. This finding is consistent with previous studies conducted with LR-90 in STZ-diabetic rats (Figarola *et al.*, 2003). Our data also indicated that LR-90 significantly lowered the concentrations of glycated haemoglobin and triglycerides in the blood. The fact that LR-90 resulted in significant reduction in glycated haemoglobin levels without affecting the blood glucose profile indeed suggest that LR-90 prevents formation of glycation end products *in vivo*. This observation is in agreement with previous *in vitro* and *in vivo* studies that demonstrated LR-90 as a potent inhibitor of AGE formation and AGE-protein cross-linking in addition to decreasing lipid levels (Figarola *et al.*, 2008). These findings suggest that LR-90 attenuates diabetes induced vascular injury by modulating several metabolic factors involved in the pathogenesis of these complications such as dyslipidaemia and increased glycosylation of haemoglobin and other serum proteins. Furthermore, our current observations are in agreement with more recent studies using apolipoprotein E knockout mice that demonstrated the attenuation of diabetes-associated atherosclerosis following treatment with LR-90, which was attributed to the inhibition of AGE accumulation in the aorta (Watson *et al.*, 2010).

In the present study, the non-invasive BP measurements were not significantly altered between groups, which are in agreement with previous studies that have reported no change between groups following indirect BP measurements. However, previous studies observed significant alterations in the pressure profile following direct measurements (Katovich *et al.*, 1995). Similarly, we observed significant alterations in the direct aortic pressure profile of diabetic rats that were then reversed with LR-90 treatment. On the contrary, a decline in cardiac output stroke volume occurred, in the absence of any significant changes in mean aortic pressure, caused an increase of 14.7% in total peripheral resistance in STZ-induced diabetic rats. AGEs that accumulate in STZ-induced diabetes are known to induce free radical production as well as deplete NO reserve, leading to a state of oxidative stress. Furthermore, AGE formation as a result of oxidative or

non-oxidative glycation of proteins is shown to have the potential to quench NO and thus diminish the vasodilatory capacity of peripheral muscular arteries (Lang *et al.*, 2000). This process of vascular physical change can eventually lead to further cellular injury in rats with prolonged hyperglycaemia (Hayden and Reaven, 2000). Significantly, diabetes-induced physical changes in resistance vessels were mitigated by administration of LR-90 to rats for 8 weeks as reflected in the reduction of 13.9% in R_p. Prevention of diabetes-induced vasodilatory dysfunction may result from the inhibition of AGE formation and an associated subsequent effect on free radical formation and NO availability in resistance vessels.

It is of importance to note that our surgical procedure involving an open-chest rat with anaesthesia induces a fall in BP and fluctuations in pulsatile dynamics compared with a closed-chest surgical setting. However, since aortic input impedance cannot be measured in conscious animals, the effects of inhalant anesthesia on the control and treatment groups are difficult to completely evaluate. Nevertheless, the effects appear to be small compared with previous studies involving i.v. general anaesthesia and other biological or experimental variability between animals (Chang *et al.*, 2006).

Aortic characteristic impedance is frequently used as an indicator of aortic stiffness. Higher aortic charactestic impedance is associated with stiffer aortic wall (Chang *et al.*, 2006). When compared with normal control rats, aortic characteristic impedance, Z_c, increased and arterial compliance, as well as wave transit time, decreased in diabetic rats. Since Z_c is directly related to pulse wave velocity (PWV), which is inversely related to aortic distensibility, our results suggested that the decline in aortic distensibility had occurred in diabetic rats. Previous studies in diabetic patients demonstrated that there is increased carotid-femoral PWV, increased pulse pressure and shorter time to the foot of the reflected wave, all of which are strong indicators of arterial stiffness (Lacy *et al.*, 2004). The increased stiffness observed in the aorta has been implicated as an important determinant of left ventricular function and coronary blood flow (Lacy *et al.*, 2004). Studies have shown that accumulation of AGEs can increase stiffness of these elastic arteries by inducing changes in the biomechanical properties of collagen (Jakus and Rietbrock, 2004). Thus, the glycosylation of matrix proteins and the subsequent accumulation of AGEs in diabetes might be implicated in increased aortic stiffness in diabetic rats. In the current study, the diabetes-induced variation in aortic distensibility was prevented by administration of LR-90 as manifested by a reduction of 21% in Z_c.

Studies using the three-element Windkessel model suggest that an increase in low frequency harmonic of impedance may indicate a decrease in systemic arterial compliance (Westerhof *et al.*, 1973). In the present study, we used the fundamental impedance modulus as an index of the low frequency portion of the impedance spectrum and observed that the moduli of the lower harmonics increase in STZ-induced diabetic rats (Figure 1). This increase in turn supported the observation that STZ-induced diabetes lowered systemic arterial compliance in diabetic rats. The diabetes-induced increase in Z_1 and the associated lowering of systemic arterial compliance was prevented by the administration of LR-90 as evidenced by the reduction in Z_1 and by a 17% increase in C_m.

The shorter wave transit time observed in STZ-induced diabetic rats suggested that the prolonged hyperglycaemia may augment the systolic load of the left ventricle. The loading condition for the left ventricle can be impaired by changes in timing and/or magnitude of pulse wave reflection, when coupled to the arterial system. Pulse wave reflections from the peripheral circulation influence the measured aortic pressure and flow signals, which can be separated into their respective forward and backward components. The forward and backward pressures are in phase with each other, whereas the backward flow wave is inverted with respect to forward flow (Westerhof *et al.*, 1972). The early return of wave reflection, as evidenced by shorter wave transit time, would suggest that the reflection is apparent during systole rather than diastole, which in turn indicate augmented systolic load of the left ventricle coupled to the arterial system (O'Rourke *et al.*, 1987). Early return of pulse wave reflection was prevented following treatment with LR-90, as evidenced by the increase of 23.5% in wave transit time in treated diabetic rats. These results indicate that LR-90 slows down the progression of the heavy intensity pulse waves between the larger arteries and the microvasculature (Chang *et al.*, 2000).

It is interesting to note that STZ-induced diabetes contributed to significant increase in magnitude of backward pressure (P_b), whereas magnitude of forward pressure (P_f) remained unchanged. The increase in R_f was a result of this elevated P_b associated with the unchanged P_f, indicating a heavy reflection intensity in diabetic rats. Heavy reflection intensity in the arterial system was lowered following treatment with LR-90, as evidenced by the reduction of 22.5% in R_f. Thus, increased wave transit time and decreased R_f suggest that LR-90 may be effective in preventing the diabetes-induced modification of pulse wave reflection that would augment systolic load of the left ventricle.

In this study, we do not report any hypertensive effects of LR-90 when administered for 8 weeks to STZ-diabetic rats. Diabetes creates a detriment to the physical properties of resistance and Windkessel vessels in the Sprague Dawley rat model that is reversed by treatment with LR-90. Decreased aortic characteristic impedance and increased wave transit time along with unchanged aortic pressure profile indicate that LR-90 may prevent the diabetes-associated fall in aortic distensibility. Furthermore, increased wave transit time coupled with wave reflection factor suggests that LR-90 can retard diabetes-induced augmentation in systolic loading of the left ventricle coupled to the arterial system. Moreover, LR-90 markedly inhibited chemical modification of collagen and accumulation of AGE and subsequent co-localization with its receptor, RAGE, in the aorta of diabetic rats without any effect on hyperglycaemia. These results suggest that the AGE inhibitor, LR-90, can confer protective effects on elastic arteries and can delay or inhibit the progression of diabetic microvascular complications and macrovascular disease. In conclusion, our results suggest that treatment with LR-90 may impart significant protection against diabetes-induced aortic stiffening and cardiac hypertrophy. This study demonstrates that LR-90 has significant therapeutic potential as an inhibitor of circulating AGE accumulation and AGE tissue deposition and provides investigators with an additional therapeutic option for the treatment of AGE-associated diabetic complications.

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Conflict of interest

The authors declare that they have no competing interests as defined by the *British Journal of Pharmacology*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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