Prevention and Reversal of Premature Endothelial Cell Senescence and Vasculopathy in Obesity-Induced Diabetes by Ebselen

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Abstract—Although the accelerated atherosclerosis and premature aging of the cardiovascular system in patients with metabolic syndrome have been appreciated, the mechanisms of their development and potential therapeutic interventions remain unresolved. Our previous studies implicated advanced glycosylation end products in development of premature senescence preventable with a peroxynitrite scavenger, ebselen. Therefore, the effect of ebselen on endothelial senescence and vasculopathy in a model of metabolic syndrome—Zucker diabetic rats (ZDF)—was investigated. Ebselen decreased the abundance of 3-nitotyrosine-modified proteins in ZDF rats. A 6-fold increase in the number of senescent endothelial cells in 22-week-old ZDF was prevented by ebselen. Development of vasculopathy, as collectively judged by the acetylcholine-induced vasorelaxation, NO production, angiogenic competence, and number of circulating microparticles, was almost completely prevented when ebselen was administered from 8 to 22 weeks and partially reversed when the treatment interval was 13 to 22 weeks. In conclusion, premature senescence of endothelial cells is progressively rampant in ZDF rats and is associated with the signs of severe vasculopathy. In addition, prevention of premature senescence of vascular endothelium through controlled decrease in nitrotyrosine formation was chronologically associated with the amelioration of vasculopathy, lending support to the idea of the pathogenetic role of premature senescence of endothelial cells in diabetic macrovasculopathy. (Circ Res. 2004;94:377-384.)

Key Words: metabolic syndrome • macrovasculopathy • nitric oxide • vasorelaxation • peroxynitrite

Atherosclerosis is responsible for the death of 80% of patients with diabetes, compared with only 30% in the general population of North America.1 Although the accelerated atherosclerosis and premature aging of the cardiovascular system in patients with diabetes mellitus and metabolic syndrome X have long been appreciated, the mechanisms of their development and potential therapeutic interventions remain not fully understood. Several main concepts attempt to explain the phenomenon of premature aging of the cardiovascular system by ascribing it to the oxidative stress and mitochondrial dysfunction, advanced glycosylation end products (AGE) activating a cognate receptor, RAGE, and activation of protein kinase C-β.2–5 These metabolic abnormalities lead to the increased incidence of apoptosis in endothelial cells, a possible contributor to the accelerated atherosclerosis.6 And yet a mechanistic view on the progression from oxidative stress to overt vasculopathy is missing. This study seeks to provide experimental evidence in support of an idea that premature endothelial cell senescence may represent a critical intermediate step between oxidative stress and vasculopathy.

Our previous studies of endothelial cells subjected to a microenvironment emulating diabetic milieu in that it contains AGE-modified long-lived extracellular matrix protein glycated collagen I (GC) revealed deceleration of proliferation, larger cell size, higher proportion of cells staining positive for senescence-associated β-galactosidase (SA-β-gal), and enhanced expression of p14 and p53, collectively suggestive of developing cell senescence. Despite the fact that primary cultures of human umbilical vein endothelial cells (HUVECs) used in these experiments were on the 3rd to 5th passage, the population of senescent cells doubled within 3 to 5 days of culture in GC lattices.7,8 Intriguingly, such markers of senescence as attrition of telomers and reduced telomerase activity were not prominent, in contrast to the replications of senescent HUVECs. For this reason, we surmised that the premature senescence-like phenotype acquired by HUVECs exposed to GC has mechanistically distinct
causes. Based on the observations that the production of NO was reduced whereas the expression of nitrotyrosine-modified proteins was enhanced in the prematurely senescent cells, we treated HUVECs cultured in GC with a bona fide peroxynitrite scavenger/antioxidant ebselen. Such a treatment was associated with the prevention of premature senescence, in accord with the previous observation that increased levels of peroxynitrite in endothelial cells are causally connected with the aging process. These findings prompted us to investigate the effect of ebselen on premature endothelial cell senescence in a model of metabolic syndrome/type 2 diabetes—Zucker diabetic rats—and examine the development of vasculopathy in these animals.

### Materials and Methods

#### Experimental Animals

Studies were carried out in Zucker diabetic fat (ZDF) and nondiabetic lean control (ZL) rats (Charles River Laboratories, Wilmington, Mass) aged 8 and 22 weeks. The animals were housed in animal quarters kept at 20°C to 22°C with a 12-hour light/dark cycle and were allowed free access to rat chow and water throughout the study. ZDF rats were randomly divided into the following groups. The first group was treated daily with Ebselen administered by gavage in two daily doses, 5 mg/kg body weight each (Daichi Corporation) dissolved in 5% CM-cellulose (Sigma) starting at the age of 8 to 10 weeks. Additional subgroups of ZDF animals received the above therapy starting at the age of 13 or 16 weeks and continued until euthanasia at 22 weeks. Control (vehicle) groups of ZDF and ZL rats received 5% CM-cellulose by gavage. Before euthanasia, rats were anesthetized by intraperitoneal injection of ketamine/xylazine (60/7.7 mg/kg, respectively). A midlaparotomy was performed, the abdominal aorta was cannulated with a P-50 catheter, and a mean blood pressure was measured using a pressure monitor BP-1 (WPI). Subsequently, blood was collected and the left kidney and thoracic aorta were aseptically removed and used for angiogenesis assays, Western blot analyses, and vasoreactivity studies, respectively. Glucose concentration in the blood was measured using the modified Trinder color reaction according to the manufacturer’s protocol (Raichem). The animal study protocol was approved by the institutional Animal Care and Use Committee.

#### Assays of Endothelial Cell Senescence

Staining of en face aortic preparations for SA-β-gal was performed as previously detailed. Other immunohistochemical procedures were performed on aortic cross sections. Samples of aorta were fixed in PLP solution (2% paraformaldehyde, 10 mmol/L Na-m-periodate were performed on aortic cross sections. Samples of aorta were fixed with hematoxylin. For p53, p21, and p16 analysis, the number of the total nuclei in the same preparation was counted after counterstaining with hematoxylin.

### Protein-Incorporated 3-Nitrotyrosine Assay of Plasma

All chemicals, unless otherwise stated, were purchased from Sigma Chemical Co. Water used for HPLC mobile phase and sample preparation was from a MilliQ water purification system and >18 MΩ resistance. Plasma samples (40 μL) were treated with proteinase K (1 μL, 1 U/10 mg protein) for 8 hours at 55°C. After cooling to room temperature, 120 μL of precipitation buffer (0.1 mol/L phosphoric acid, 0.23 mol/L trichloroacetic acid) was added. Samples were then incubated for an additional 5 minutes at room temperature, centrifuged for 15 minutes at 12,000g, and placed in HPLC vials for analysis of free 3-nitrotyrosine (3-NT). Quantification of 3-NT was accomplished by HPLC using a 100-mm C-18 column (Microsorb-MV, Varian), multichannel electrochemical detection (CoulArray; ESA Inc), and isocratic mobile phase consisting of 90 mmol/L sodium acetate, 35 mmol/L citric acid, 130 μmol/L EDTA, and 460 μmol/L sodium octane sulfonate (pH 4.35) running at 0.75 mL/min and 30°C. The optimum potential for detection of 3-NT was +800 mV. To maximize sensitivity for 3-NT and to ensure completeness of detection, two flanking electrodes were operated at +700 and +900 mV, respectively. Quantification of 3-NT was performed by comparing the peak area with that of an external standard. Identity of the 3-NT peak was confirmed by establishing that addition of 10 mmol/L sodium hydrosulfite, which causes chemical reduction of 3-NT to 3-aminotyrosine, silenced the electrochemical signal at the predicted retention time of 3-NT.

### Acetylcholine-Induced Vasorelaxation

Aortas were cleared of perivascular tissue and cut transversely into rings 1.5 to 2.0 mm in diameter. Vascular rings, handled carefully to avoid damage to the inner surface, were mounted on wires in the chambers of a multiwall vessel myograph (J.P. Trading) and bathed in Krebs’ buffer. The medium was gassed with 95% O2 and 5% CO2 and maintained at 37°C (pH 7.4). After equilibration (30 minutes), the rings were set to an internal circumference equivalent to 90% of full relaxation under a transmural pressure of 100 mm Hg and allowed to stabilize for 20 to 30 minutes. The rings were then depolarized with potassium chloride (KCl; 60 mmol/L) to evaluate maximal contraction. After washing with a Krebs’ buffer, the vascular preparations were contracted with phenylephrine (10−6 mol/L), and when the contractile response was stabilized (steady-state phase, 12 to 15 minutes), vasorelaxing responses to cumulative increments in the concentration of acetylcholine or NONOate were examined.

### Angiogenesis Assays

The explants of thoracic aorta (rings of ~0.5 mm) were plated in ice-cold sterile EB2-2 medium supplemented with EGM-2 kit (Clonetics) containing 2% FBS, hydrocortisone, endothelial growth factor, vascular endothelial growth factor, insulin-like growth factor-1, bovine fibroblast growth factor, and heparin. All tissue samples were rinsed three times with a fresh EB2-2 medium. Finally, the explants were embedded in growth factor–depleted Matrigel (Becton Dickinson) in tissue culture chambers (Nalgene Nunc, as previously described. Quantitative analysis of angiogenesis in explant cultures was performed under an inverted fluorescence microscope (Nikon) equipped with a CCD camera (Diagnostic Instruments). Vascular sprouts were counted daily along the perimeter of each sample under ×10 magnification. Comparative analysis of vascular sprouts between the experimental groups was performed using ANOVA for multiple comparisons followed by Tukey posttest. P<0.05 was considered statistically significant.

In vivo angiogenic competence was tested using a femoral artery ligation model in ZDF and ZL rats. Animals were studied 5 weeks postoperatively, when laser Doppler flowmetry/imaging of the cutaneous circulation and immunohistochemical analysis of capillary density in the affected and contralateral quadriceps muscle were performed using endothelial-specific staining with RECA antibody.
Measurement of NO Production by Aortic Rings and Primary Cultures of Endothelial Cells Obtained From ZL and ZDF Rats

The full length of the thoracic aorta was aseptically removed and immediately placed into EBM-2 medium. After removal of the periaortic fibroadipose tissue with fine microsurgical forceps under a dissecting microscope, the aortas were placed in fresh EBM-2 medium and cross-sectionalized with a 1.2- to 1.5-mm interval, and the resulting aortic rings were tested for NO production using a porphyrin-electroplated, Nafion-coated, carbon-fiber electrode (30 μm daily), manufactured according to Bio-Logic Instruments’ instructions. Calibration of the electrode was performed before each experiment using dilutions of freshly prepared NO-saturated Krebs-Ringer solution.

Aortic rings were placed into 100 μL of Krebs buffer, and after obtaining a stable baseline, 1 to 5 μmol/L calcium ionophore A23187 was added to the buffer and the electrochemical response was continuously recorded. N-nitroarginine methyl ester (L-NAME) (2 mmol/L) was used to verify the NO dependence of the recorded electrode current at the end of the experiments. On completion of the experiments, electrode calibration was confirmed by evaluating responses to reference NO solutions.

Isolation and Detection of Circulating Endothelial Microparticles

For platelet-free plasma preparation, microparticles were isolated from whole plasma by two sequential centrifugations for 10 minutes at 15 000g, as previously described.13 Supernatant containing microparticles was immediately placed into EBM-2 medium. After removal of the periaortic fibroadipose tissue with fine microsurgical forceps under a dissecting microscope, the aortas were placed in fresh EBM-2 medium and cross-sectionalized with a 1.2- to 1.5-mm interval, and the resulting aortic rings were tested for NO production using a porphyrin-electroplated, Nafion-coated, carbon-fiber electrode (30 μm daily), manufactured according to Bio-Logic Instruments’ instructions. Calibration of the electrode was performed before each experiment using dilutions of freshly prepared NO-saturated Krebs-Ringer solution.

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Results

Validation of the Therapeutic Dose of Ebselen

A mild hyperglycemia was registered at age 8 weeks with glucose levels of 197.9±11.7 g/dL in ZDF versus 144.8±16.2 g/dL in ZL. By the age of 22 weeks, glucose levels in ZDF rats averaged 400.2±33.6 g/dL (versus 180.9±17.8 in ZL). Ebselen-treated rats showed the same degree of hyperglycemia as the vehicle-treated age-matched counterparts. Both ZDF and ZL rats had unaltered levels of mean blood pressure throughout the observation period of 8 to 22 weeks of age (107.6±4.8 versus 103.4±3.2 mm Hg, respectively), and ebselen treatment did not affect blood pressure (data not shown). Thus, ebselen treatment changed neither the severity of diabetes nor the systemic blood pressure of ZDF rats.

Measurement of the abundance of 3-NT–modified proteins in the plasma of experimental animals showed comparably low levels in ZDF and ZL at age 8 weeks (3.17±4.54 and 2.18±2.1 pmol/mg protein, respectively) but a 6-fold increase in the plasma of ebselen-treated ZDF rats. At age 22 weeks (19.43±6.04 pmol/mg protein), NO production was significantly increased at 22 weeks of age in ZDF. Ebselen treatment significantly reduced the abundance of 3-NT–modified proteins in the vascular wall (Figures 1B and 1C). Detection of lipid peroxidation products in the vascular wall (HNE staining) showed that they increased by 22 weeks of age in ZDF but were reduced after ebselen treatment (Figures 1B and 1C). Collectively, these data indicate the prevalence of oxidative (lipid peroxidation) and nitrosative (3-NT–modified proteins) stress in the aorta of ZDF rats and that the implemented therapy was adequate and resulted in amelioration of both.

To investigate the in vivo effect of ebselen therapy on the course of vasculopathy in ZDF rats, separate experiments were performed to quantify the number of senescent endothelial cells and correlate it with a constellation of parameters characteristic of endothelial dysfunction, including measure of NO production by aortic endothelium, vascular reactivity to acetylcholine, abundance of endothelial microparticles in the circulation, and angiogenic competence of various vascular beds. These parameters are integral parts of the pathologic vascular triad of thrombogenesis (exemplified by the endothelial microparticles), endothelial dysfunction (exemplified by endothelium-dependent relaxation and NO production), and angiogenesis.

Senescent Endothelial Cells

Direct quantification of the number of senescent endothelial cells per unit surface area of the en face stained aortic preparations revealed several important findings (Figure 2). At the age of 8 weeks, no differences were detected in the frequency of SA-β-gal–positive endothelial cells (<5 cells/field), both in the areas surrounding the orifices of intercostal arteries and in those away from branching points. With the increasing duration of diabetes, the number of senescent endothelial cells increased 6-fold (to 19 cells/field) at age 22 weeks (P<0.01). This increase in the frequency of SA-β-gal–positive endothelial cells was detectable in aortas of 22-week-old ZDF rats, both in the areas away from branching points (Figure 2A) and in those surrounding the orifices of intercostal arteries (Figure 2B). Treatment with ebselen resulted in a significantly lower number of senescent cells at both sites.

Immunohistochemical analysis of cross sections obtained from aorta of experimental animals showed minimal staining of the endothelium at 8 weeks of age but a dramatic induction of p53, p21, and p16 in endothelial cells from ZDF rats at 22 weeks of age (Figures 3A and 3B). Chronic administration of ebselen abrogated the induction of these cell-cycle proteins. Taken together, these data demonstrate that ebselen therapy
initiated at age ≈8 weeks is capable of preventing premature endothelial cell senescence by the age of 22 weeks.

**Vascular Reactivity to Acetylcholine**

Vascular reactivity was examined in aortic rings (these vessels respond to acetylcholine with vasorelaxation, which is almost entirely endothelium-derived relaxing factor [NO]–dependent). Acetylcholine-induced vasorelaxation was equipotent in 7- to 8-week-old ZDF and ZL rats, as shown in online Figure 1 (available in the online data supplement at http://www.circresaha.org) (of note, severely impaired responses to acetylcholine are seen already in 9- to 10-week-old ZDF rats) but became severely impaired in 22-week-old ZDF rats. Acetylcholine-induced relaxation was almost completely restored in ZDF rats receiving ebselen treatment. To elucidate the contribution of endothelium-derived relaxing factor (NO)–dependent relaxation, in the next series of experiments, vessels were pretreated with 1 mmol/L L-NAME and stimulated by acetylcholine at increasing concentrations (data not shown). Blockade of NO production resulted in a dramatic blunting of relaxation in all experimental groups. The observed differences in relaxation responses to acetylcholine could not be ascribed to the different sensitivity or responsiveness of the vascular smooth muscle to NO, because vessels from all experimental groups showed an equally robust relaxation elicited by an NO donor, NONOate (Figure 4B).

**NO Production by Aortic Endothelium**

NO production by aortic rings obtained from 22-week-old ZDF rats, which showed defective vasorelaxation, was measured using an NO-selective microelectrode technique, as detailed in Materials and Methods. As summarized in Figure 4B, responses to the calcium ionophore were significantly blunted in ZDF rats. This observation is in accord with the previous in vitro and in vivo data. In rats receiving ebselen treatment, A23187-elicited NO production by aortic endothelium was partially restored.

**Partial Reversal of Endothelial Dysfunction in ZDF Rats Receiving Delayed Ebselen Treatment**

The reversibility of the above vascular dysfunction was tested in ebselen-treated ZDF rats receiving the therapy between weeks 13 to 22 or 16 to 22 (as opposed to the 8- to 22-week
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Figure 2. Senescent endothelial cells in aortas (A) and branching points of intercostal arteries (B) in experimental senescence. Representative images of en face aortas. By adjusting the focal distance, it is feasible to image the endothelial layer alone without interference from the medial or adventitial layers. Insets, Quantification of senescent cells. *P<0.05 vs 8-week-old ZDF; #P<0.05 vs 22-week-old ZDF.

Endothelial Microparticles
After staining with ulex europeus, as detailed in the Materials and Methods, the number of circulating microparticles was quantified using FACS analysis. The data demonstrated a significant increase in the number of circulating endothelial microparticles in ZDF rats at 22 weeks compared with ZL rats of the same age (Figure 5). Ebselen treatment significantly attenuated the increase in the number of microparticles in the circulation. Because the number of endothelial microparticles represents a surrogate marker of endothelial dysfunction, these findings lend additional support to the notion of the dysfunctional endothelium in ZDF rats and improved function after ebselen treatment.

In Vivo and Ex Vivo Angiogenesis
In the first series of experiments, the ability to form collateral blood vessels was studied in 22-week-old rats subjected at the age of 16 weeks to left femoral artery ligation. The success of the surgery and the completeness of the recovery of the circulation were assessed using laser Doppler flowmetry/imaging and histochemically by counting the density of capillaries in the affected striated muscle. There was a striking difference in the baseline perfusion of the limbs in ZDF versus ZL rats with ZDF rats, both ebselen- and vehicle-treated, displaying a 60% lower basal flow (data not shown). After ligation of the femoral artery, the drop in limb perfusion was comparable in all experimental groups and there were no significant differences in the gross recovery of circulation in the left limb. All animals, either ebselen-treated or vehicle-treated, showed partial improvement in the circulation. To examine whether this was attributable to the development of collateral circulation, sections of affected striated muscle were stained with RECA antibody recognizing rat endothelial cells, and the density of blood vessels was quantified (Figure 6). The data demonstrated that ZDF rats had a significantly lower number of capillary profiles in the affected muscle compared with the nonaffected side, the difference of which was attenuated in ebselen-treated ZDF rats. In ZL rats, the density of capillaries was within normal range on both sides.

In a series of experiments on ex vivo angiogenic competence, aortic rings were embedded in Matrigel and the number of sprouting capillaries was counted, as detailed in Materials and Methods. In both groups of animals, aortic rings showed robust and comparable degree of capillary sprouting (some differences were observed only after 8 to 10 days in culture, when ZL aortas showed an almost doubled number of capillaries) at age 8 weeks, before the development of severe diabetes. At age 22 weeks, ZDF aortas showed a significantly smaller number of sprouting capillaries and faster pruning of the existing capillaries at all times in ex vivo culture. Ebselen treatment resulted in a significant improvement of capillary sprouting and slower capillary pruning (online Figure 2).

Discussion
The data presented herein indicate that premature endothelial cell senescence is progressively rampant in the aorta, especially at the areas surrounding branching points of intercostal arteries in young ZDF rats, and is associated with the clear-cut signs of vasculopathy. These include impaired vasorelaxation and NO production and defective angiogenic competence. As suggested in our previous in vitro studies, these changes were causally associated with the accumulation of nitrotyrosine-modified proteins—evidence for oxidative and nitrosative stress. These findings prompted us to investigate whether premature endothelial cell senescence is a pathogenetic factor for the development of diabetic vasculopathy in this animal model of metabolic syndrome/type 2 diabetes. A series of studies on the prevention of premature endothelial cell senescence (through controlled decrease in oxidative and nitrosative stress, as judged by the attenuation in the accumulation of 3-NT–modified proteins and products of lipid peroxidation) have disclosed their chronological association with the amelioration of vasculopathy, thus lending circumstantial support to the idea of the pathogenetic role...
of premature endothelial cell senescence in the development of diabetic vasculopathy.

Our previous findings together with several other recent investigations implicate cellular oxidative stress in the development of premature senescence. One of the major distinguishing features of premature senescence pertains to the mechanisms of senescence; it is not driven by the loss of telomerase activity and the subsequent attrition of telomeres, because it occurs in replicative senescence. One of the important corollaries of this apparent telomere-independent...
type of senescence might be its preventability and reversibility if and when oxidative or nitrosative stress is diminished. Indeed, in vitro studies showed that premature senescence could be prevented and reversed when oxidative stress is controlled, thus providing a rationale for the present in vivo investigation.

Ebselen, a peroxynitrite scavenger and antioxidant presently undergoing phase II clinical trials in ischemic stroke, had no effect on the severity of diabetes mellitus per se but significantly attenuated the accumulation of 3-NT–modified proteins in the plasma and aortic wall when administered to ZDF rats starting at 8 weeks of age (the time when there are no detectable signs of vasculopathy and the number of senescent endothelial cells and ex vivo angiogenic competence of aortic endothelium are unimpaired). Animals were reexamined at age 22 weeks for signs of endothelial cell senescence and developing vasculopathy; both were manifest in the absence of therapy with ebselen. In a separate group of animals, ebselen therapy initiated at 8 weeks of age resulted in a complete prevention of diabetes-induced premature endothelial cell senescence by 22 weeks of age. Similarly, ebselen treatment improved ionophore-stimulated NO production by aortic rings, as well as the acetylcholine-induced NO-dependent vasorelaxation by aortic rings. This therapy was also associated with (1) the improved angiogenic competence of aortic and renal endothelial cells, which was profoundly perturbed in age-matched ZDF rats; (2) improved in vivo angiogenesis after ligation of the femoral artery; and (3) the reduction of the number of circulating endothelial microparticles, possible markers of endothelial dysfunction. Hence, five independent parameters determined the presence of endothelial dysfunction in 22-week-old ZDF rats. All these parameters showed near normalization after prolonged ebselen administration from 8 to 22 weeks, in parallel with the prevention of premature endothelial senescence. Moreover, ebselen therapy was capable of reversing the existing vasculopathy, which started at 13 weeks of age (but not at 16 weeks).

Macrovascular complications of metabolic syndrome and diabetes are among the leading causes of morbidity and mortality. Tight glucose control alone is insufficient to prevent macrovasculopathy, but there are indications that the soluble extracellular portion of AGE receptor or AGE breakers is capable of preventing these complications. Notably, both agents are acting at the upstream prereceptor mechanisms of endothelial dysfunction. Ebselen therapy, on the other hand, targets downstream, postreceptor cellular consequences of endothelial oxidative stress. One of the possible attractive features of selenorganic compounds is their combined peroxynitrite scavenging and antioxidant effect, thus potentially not only preventing additional oxidant stress but also accelerating the clearance of preformed 3-NT–modified proteins. Indeed, the fact that ebselen not only prevents but also reverses the preexisting macrovasculopathy makes it a promising therapeutic agent. It might be interesting to test its efficacy in combination with the above-mentioned upstream-acting agents in future studies.

In conclusion, premature endothelial cell senescence and vasculopathy develop in ZDF rats. In view of the previously established pathogenetic importance of oxidative stress and nitrotyrosine modification of proteins demonstrated herein, ebselen therapy was instituted, which seemed to curtail the circulating and tissue-expressed levels of 3-NT. This treatment resulted in amelioration of premature endothelial cell senescence and vasculopathy. Furthermore, the data obtained
in ZDF rats treated with ebselen are internally consistent with the hypothesis that the diabetes-induced premature endothelial cell senescence is pathogenetically linked to the development of vasculopathy and that preventing the former leads to the amelioration of the latter.

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References
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SUPPLEMENTAL FIGURES

Figure 1. Acetylcholine-induced vasorelaxation of aortic rings and NO production by aortic endothelium of 8 week old ZDF and ZL rats.

A - Cumulative dose-response curves of acetylcholine-induced vasorelaxation in phenylephrine-preconstricted aortic rings. B- the same as in A after pretreatment with L-NAME. Note that in these aortic preparations acetylcholine-induced relaxation is mediated by NO. C - Cumulative dose-response curves of NONOate-induced vasorelaxation in denuded phenylephrine-preconstricted aortic rings. D – the same as in C, after pretreatment with L-NAME

Figure 2. Ex vivo aortic ring capillary sprouting and regression in 8 week-old (A) and 22 week-old (B) rats

Endothelial sprouting was studied as detailed in Methods. Endothelial origin of sprouts was confirmed by positive staining for vonWillebrand factor and staining with Ulex europeus lectin (not shown). Capillary counting was impossible to perform when the number of sprouts exceeded 200. Two-way ANOVA analysis showed that there was a difference between ZL and ZDF curves significant at p<0.0001 (n=8), between ZL and ZDF+Ebselen at p<0.001 and between treated and untreated ZDF at the level of p<0.0001 (n=8).

* indicates p<0.01 vs ZL and ZDF+Ebselen; # p<0.05 vs ZDF+Ebselen for each time-point.
Online Fig. 1

A. Acetylcholine (M)
- ZL 8w
- ZDF 8w

B. Acetylcholine (M)

C. NONOate (M)

D. NONOate (M)
**Online Fig. 2**

**A**

![Graph A](image)

**B**

![Graph B](image)