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PREMATURE VASCULAR SENESCENCE IN METABOLIC SYNDROME: COULD IT BE PREVENTED AND REVERSED BY A SELENORGANIC ANTIOXIDANT AND PEROXYNITRITE SCAVENGER EBSELEN?

Jun Chen, Hyeong-Cheon Park, Susann Patschan, Sergey V Brodsky, Olga Gealikman, Mei-Chuan Kuo, Houwei Li, Francesco Addabbo, Fung Zhang, Alberto Nasjletti, Steven S. Gross, and Michael S Goligorsky

Departments of Medicine and Pharmacology, Renal Research Institute, New York Medical College, Valhalla, NY and Department of Pharmacology, Weill Medical College at Cornell University, New York, NY

Accelerated atherosclerosis, nephropathy, neuropathy, retinopathy and impaired wound healing are all hallmarks of metabolic syndrome, insulin resistance and type 2 diabetes (rev in 1, 2). Atherosclerosis is responsible for the death of 80% of patients with diabetes, compared with only 30% in general population of North America (3). Several concepts have been advanced to explain premature aging of cardiovascular system by ascribing it to the oxidative stress and mitochondrial dysfunction (4), advanced glycosylation end products and activation of a receptor for AGEs (5, 6), activation of protein kinase C- β (7). These metabolic derangements, together with the oxidized LDL, TNF- α , and peroxynitrite eventually lead to the increased incidence of premature senescence and apoptosis in endothelial cells, hypothetically contributing to the accelerated atherosclerosis (8, 9). Indeed, increased peroxynitrite formation in endothelial cells has been incriminated in triggering aging process (10).

Tight glycemic control has been convincingly shown to improve microvascular complications of diabetes, however, “UGPD and UKPDS trials have found only limited, if any, relationship between glycemic control and diabetic macrovascular manifestations” (11). Emerging novel therapeutic approaches based on the administration of the soluble RAGE, a decoy for AGE binding, or AGE “breakers” may have some potential in preventing and even arresting the progression of atherosclerosis by diminishing AGE binding to the endothelium and the consequent oxidative stress (12–14). Presently, cardiovascular risk reduction is based on the use of statins, blood pressure, PPARs and glycemic control, each targeting one specific risk factor (LDL, BP and glucose, respectively) (13–15). Our laboratory has demonstrated that a selenoorganic peroxynitrite scavenger and antioxidant compound, ebselen, may be effective in not only preventing, but also reversing macro- and microvasculopathy in a model of obesity and diabetes – Zucker diabetic fat rat (see below). One of the possible attractive features of selenoorganic compounds is their combined peroxynitrite scavenging and antioxidant effect (16), thus potentially not only preventing further oxidant stress, but also accelerating the

Correspondence/reprint requests: Michael S Goligorsky, New York Medical College, Basic Sciences Building, Renal Research Institute, Valhalla, NY 10595.

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clearance of preformed 3-nitrotyrosine (3-NT)-modified proteins. The target for this therapy is *downstream* of soluble RAGE and AGE breakers – on the cellular sequelae of AGE binding – oxidative and nitrosative stress, peroxynitrite formation, cell senescence and apoptosis. This brief overview focuses on the potential in vitro and in vivo therapeutic benefits of ebselen.

Stress-induced premature senescence of cultured endothelial cells: phenotypic characterization

Cellular aging has been extensively studied with reference to replicative aging, the process that is characterized by the appearance of senescence-associated β -galactosidase staining, expression of several regulators of the cell cycle, attrition of telomeres, suppression of telomerase activity, and irreversibility of the process (17,18). Hallmarks of the replication-independent cell senescence, however, remain scarcely examined (19), although premature senescence of cardiovascular system is becoming the subject of intense investigations (rev in: 20). We have previously demonstrated that glycated collagen I (GC) induces premature endothelial cell senescence, as judged by the appearance of senescence-associated β -galactosidase staining, increased cell size, rate of apoptosis, and expression of p53 and p16^{INK4a} in a dose- and time-dependent manner (21, 22). However, telomerase activity and the length of telomeres did not show significant changes, suggesting that the pathogenesis of premature senescence is distinct from that of replicative senescence.

Endothelial cell senescence was associated with decreased calcium-dependent synthesis of nitric oxide (NO), (despite an increase in the expression of eNOS) and with increased abundance of nitrotyrosine-modified proteins. In contrast to the replicative senescence, premature senescence of HUVEC was reversible: scavenging peroxynitrite with ebselen, supplementing cells with an intermediate in NO synthesis, N^ωhydroxy-L-arginine (NOHA), or with a cell-permeable SOD mimetic Mn-TBAP, prevented and reversed premature senescence.

In vivo studies of stress-induced premature senescence of vascular endothelium: Zucker diabetic fat rat model

Analysis of senescence-associated β -galactosidase, p53 and p16^{INK4a} staining of aortas obtained from Zucker diabetic rats (ZDF), compared to age-matched lean controls, confirmed the occurrence of premature senescence of endothelial cells in vivo, in an animal model of insulin-independent diabetes mellitus/obesity (Fig. 1). We have also studied a group of fructose-treated rats, which are non-obese, non-hyperglycemic, but develop insulin resistance. Our data demonstrate that fructose-treated rats develop macrovasculopathy and premature senescence of endothelial cells, similar to those developed by ZDF rats (Fig. 1,C).

Ebselen, a *bona fide* glutathione peroxidase mimetic and peroxynitrite scavenger was previously used by our laboratory in vivo to ameliorate renal injury in acute renal ischemia in the rat (23) and in vitro to prevent endothelial cell senescence (24). Based on these studies we were able to work out the therapeutic regimen in rats and in cultured cells. We have also developed a test to validate Ebselen therapeutic efficacy – measurement of 3-nitrotyrosine-modified proteins in the circulation and in tissues

MACROVASCULOPATHY

Ebselen-treated ZDF rats showed the same degree of hyperglycemia and levels of blood pressure as the vehicle-treated age-matched counterparts, Zucker lean (ZL) rats throughout the observation period of 8–22 weeks of age (25).

Measurement of the abundance of 3-nitrotyrosine (NT)-modified proteins in the plasma of experimental animals showed comparably low levels in ZDF and ZL at age of 8 weeks, but significant accumulation of 3-NT modified proteins in ZDF rats at age of 22 weeks and unchanged levels in ZL rats of the same age (Fig. 2, A). Treatment with ebselen resulted in a significant attenuation of plasma 3-NT-modified proteins. The similar dynamics was observed in the aortic wall: 3-NT-modified proteins were slightly elevated in 8 weeks-old ZDF rats, compared to ZL animals of the same age, but was dramatically increased at 22 weeks of age. Ebselen treatment significantly reduced the abundance of 3-NT-modified proteins in the vascular wall (Fig.2, B). Detection of lipid peroxidation products in the vascular wall showed that they increased by 22 weeks of age in ZDF rats, but were reduced after ebselen treatment. Collectively, these data indicate that the implemented therapy has reached the desired goal of reducing peroxynitrite burden and 3-NT modification of proteins.

Direct quantification of the number of senescent endothelial cells per unit surface area of the *en face* stained aortic preparations revealed no differences at the age of 8 weeks. The number of senescent endothelial cells increased 6-fold at age of 22 weeks, both in the areas surrounding the orifices of intercostal arteries and in those away from branching points. Treatment with ebselen resulted in a significantly lesser number of senescent cells at both sites. These data demonstrate that ebselen is capable of preventing premature endothelial cell senescence by the age of 22 weeks (Fig.2, A, B and 3).

NO production by ex vivo aortic rings was measured using an NO-selective microelectrode technique after stimulation with A23187. NO responses to the calcium ionophore were significantly blunted in ZDF rats (Fig.4). In rats receiving ebselen treatment A23187-elicited NO production by aortic endothelium was partially improved.

Vascular reactivity was examined in renal intralobar arteries and in aortic rings. Acetylcholine-induced vasorelaxation was severely impaired in ZDF rats, but completely restored in ZDF rats receiving ebselen treatment (Fig.5). 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 (not shown).

Angiogenic competence was studied as the ability to form collateral blood vessels in rats subjected to a femoral artery ligation (Fig.6). The data demonstrated that ZDF rats had a significantly lower number of capillary profiles in the affected muscle compared to the non-affected side, whereas in ZL rats the density of capillaries was within normal range on both sides. Ebselen treatment partially restored capillary density of the affected limb in ZDF rats. In a series of experiments on ex vivo angiogenic competence (26), aortic rings and sections of kidney were embedded in Matrigel and the number of sprouting capillaries was counted. In both groups of animals, aortic rings showed robust and comparable degree of capillary sprouting at age of 8 weeks. At age of 22 weeks, ZDF aortae showed significantly lesser 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. Renal cortical capillaries sprouted much better in ZDF rats, than in ZL, but only at the age of 8 weeks. At 22 weeks, ZDF rats showed a dramatic loss of angiogenic competence, which was partially restored by ebselen treatment.

The number of circulating microparticles was quantified using FACS analysis after staining with *Ulex Europeus* (25). The data demonstrated a significant increase in the number of circulating endothelial microparticles in ZDF rats at 22 weeks, compared to ZL rats of the same age (not shown). Ebselen treatment significantly attenuated the increase in the number of microparticles in the circulation. Since the number of endothelial microparticles represents a

surrogate marker of endothelial dysfunction, these findings lend further support to the notion of the dysfunctional endothelium in ZDF rats and improved function after ebselen treatment.

The reversibility of the vascular dysfunction was tested in ebselen-treated ZDF rats receiving the therapy between weeks 13–22 or 16–22 (as opposed to the 8–22 week treatment used for preventive strategy), in other words, treatment was initiated when vasculopathy was already present. Ebselen administered from 16th to 22nd week was ineffective in reversing the existing impairment of acetylcholine-induced vasorelaxation. Administration of ebselen from the 13th to 22nd week, however, was accompanied by a dramatic improvement of vasculopathy. Specifically, acetylcholine-induced vasorelaxation and NO production (Fig. 3 and Fig. 4) showed significant recovery in ZDF rats.

MICROVASCULOPATHY

ZDF rats develop progressive nephropathy with focal segmental glomerulosclerosis (FGS) and proteinuria. At 8w ZDF showed no proteinuria or nephropathy. At 22 weeks ZDF developed FGS and increased urinary protein excretion (UPE), with amelioration of both in ZDF rats receiving ebselen. ZDF rats at 22w also had significant TIS and inflammation compared to age-matched ZL, and these manifestations of nephropathy were ameliorated with ebselen (27).

Progression of nephropathy in metabolic syndrome is associated with microvasculopathy (MV) and vascular dropout. Acetylcholine-induced relaxation of microdissected interlobar arteries from ZL and ZDF rats was similar at 8 weeks, but significantly attenuated in ZDF rats aged 22 weeks. Capillary density (CD) was increased, both in the cortex ($p<0.05$) and in the inner medulla by the age of 8 weeks, but significantly decreased by the age of 22 weeks in ZDF rats (26, 27). Similarly, the angiogenic competence of cortical and medullary renal explants was increased in 8-week old ZDF ($p<0.01$), but decreased with age at 22 weeks ($p<0.001$). Treatment with ebselen partially prevented the decrease in CD and AC of renal explants and restored acetylcholine-induced vasorelaxation in 22-week old ZDF rats. In addition, treatment with ebselen resulted in a significant amelioration of the accumulated 3-NT-modified proteins and lipid peroxidation products.

In conclusion, 1) progression of nephropathy in ZDF is associated with the decreased angiogenic competence both ex vivo and in vivo; 2) this is accompanied by nitrosative and oxidative stress; and 3) scavenging peroxynitrite with ebselen prevented progression of microvasculopathy and partially restored angiogenic competence.

Senescence of endothelial progenitor cells (EPC)

EPC have been shown to participate in regenerative processes. Transplantation of EPCs augments neovascularization of ischemic/infarcted myocardium, ischemic limbs, or brain (28, 29). EPC may play a critical role in the maintenance of integrity of vascular endothelium and in its repair after injury or inflammation (30). EPC are subjected to various stressors, the same as occur in other cells that could impair their competence (31). Hyperglycemia has been reported to reduce survival and impair function of circulating EPCs (32). Circulating EPCs in asymptomatic smokers exhibit impaired functions (33). There is emerging evidence that senescence may serve as an important mechanism mediating EPC dysfunction. Decreased numbers and increased proportion of senescent EPC has been reported in patients with preeclampsia (34) or hypertension (35). Angiotensin II can induce EPC senescence through the induction of oxidative stress and influence telomerase activity (36). Oxidized low-density lipoprotein induces EPC senescence and dysfunction (37). EPC from type II diabetics exhibit impaired proliferation, adhesion, and engraftment in vascular structures (38). In addition, EPC dysfunction has been documented in type I and II diabetes, coronary artery disease,

atherosclerosis, vasculitis with kidney involvement, and end-stage renal disease (39–45). Whether ebselen can prevent or reverse premature senescence of EPC remains to be established.

In conclusion, the fact that ebselen not only prevents, but also partially reverses the preexisting macrovasculopathy and microvasculopathy makes it a promising therapeutic candidate. It might be interesting to test its efficacy in combination with other the upstreamacting agents, like blockade of RAGE or AGE-breakers, in the future studies.

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Figure 1. Senescent endothelial cells in aortae and branching points of intercostals arteries in experimental animals

Representative images of *en face* aortae of ZDF and ZL rats show senescent endothelial cells (SA β -gal positive) in aortae (A) and branching points of intercostals arteries (B). Senescent cells in the aorta and intercostals arteries of rats fed high fructose diet, as a model of insulin resistance and mild hyperglycemia (C). Insets: quantification of senescent cells. * depicts $p < 0.05$ vs 8 week-old ZDF; # $p < 0.05$ vs 22 week-old ZDF (reprinted with permission from ref. 25).

Figure 2. Increased 3-nitrotyrosine formation and peroxidation products in experimental animals
 Concentration of circulating 3-nitrotyrosine-modified (3NY) proteins in experimental animals (upper panel). Abundance of lipid peroxidation products (HNE staining) and 3-nitrotyrosine-modified proteins in the aortic wall of experimental animals (lower panel). * depicts $p < 0.05$ vs 8 week-old ZDF; # $p < 0.05$ vs 22 week-old ZDF. Abbreviations: Ebs – ebselen, 8 and 22 – animals' age of 8 and 22 weeks (reprinted with permission from ref. ²⁴).

Figure 3. Senescent endothelial cells in the sections of thoracic aorta of ZDF and ZL rats – expression of p53, p21, and p16 markers

Fresh-frozen sections of aorta obtained from 8 and 22 week-old ZDF rats treated and untreated with Ebselen (Ebs) were immunohistochemically processed to show that the expression of all three cell cycle proteins – p53, p16 and p21, is increased in ZDF vessels at 22 weeks. Treatment with ebselen resulted in the dramatic correction of premature vascular senescence. Lower panel: diagrammatic summary of the number of endothelial cells stained for the above markers. * depicts p<0.05 compared to the age-matched ZL aorta (reprinted with permission from ref. 24).

Figure 4. NO production by endothelial cells of aortic rings obtained from different experimental groups

The data represent peak responses to administration of 2 ug/ml A23187 (reprinted with permission from ref. ²⁴).

Figure 5. Acetylcholine- induced vasorelaxation of aortic rings

Cumulative dose-response curves of acetylcholine-induced vasorelaxation in phenylephrine-precontracted aortic rings. All preparations responded to NO (reprinted with permission from ref. ²⁵)

Figure 6. Recovery of microcirculation of rectus femoris muscle after ligation of the femoral artery Immunohistochemical staining of microvascular endothelium in 22 week-old ZL ischemic muscle (A), ZDF non-ischemic muscle (B), ZDF ischemic muscle (C) and ZDF+ebselen ischemic muscle (D). Inset: A bar diagram summarizing capillary density in ischemic and contralateral muscles in different experimental groups 5 weeks after ligation of the femoral artery. * depict $p < 0.05$ compared to age matched ZL (reprinted with permission from ref. 26).