Glucagon-Like Peptide 1 Prevents Reactive Oxygen Species–Induced Endothelial Cell Senescence Through the Activation of Protein Kinase A

Hisko Oeseburg, Rudolf A. de Boer, Hendrik Buikema, Pim van der Harst, Wiek H. van Gilse, Herman H.W. Silljé

Objective—Endothelial cell senescence is an important contributor to vascular aging and is increased under diabetic conditions. Here we investigated whether the antidiabetic hormone glucagon-like peptide 1 (GLP-1) could prevent oxidative stress–induced cellular senescence in endothelial cells.

Methods and Results—In Zucker diabetic fatty rats, a significant 2-fold higher level of vascular senescence was observed compared with control lean rats. Dipeptidyl-peptidase 4 (DPP-4) inhibition significantly increased GLP-1 levels in these animals and reduced senescence almost to lean animal levels. In vitro studies with human umbilical vein endothelial cells showed that GLP-1 had a direct protective effect on oxidative stress (H$_2$O$_2$)–induced senescence and was able to attenuate oxidative stress–induced DNA damage and cellular senescence. The GLP-1 analogue exendin-4 provided similar results, whereas exendin fragment 9–39, a GLP-1 receptor antagonist, abolished this effect. Intracellular signaling by the phosphoinositide 3-kinase (PI3K)/Akt survival pathway did not appear to be involved. Further analysis revealed that GLP-1 activates the cAMP response element-binding (CREB) transcription factor in a cAMP/protein kinase A (PKA)–dependent manner, and inhibition of the cAMP/PKA pathway abolished the GLP-1 protective effect. Expression analysis revealed that GLP-1 can induce the oxidative defense genes HO-1 and NQO1.

Conclusion—Dipeptidyl-peptidase 4 inhibition protects against vascular senescence in a diabetic rat model. In vitro studies with human umbilical vein endothelial cells showed that reactive oxygen species–induced senescence was attenuated by GLP-1 in a receptor-dependent manner involving downstream PKA signaling and induction of antioxidant genes. (Arterioscler Thromb Vasc Biol. 2010;30:1407–1414.)

Key Words: diabetes mellitus ■ endothelium ■ reactive oxygen species ■ glucagon-like peptide 1 ■ senescence

Vascular aging is an important contributor to the progression of diabetes and cardiovascular diseases and is associated with a worsened prognosis. In diabetes and atherosclerosis there is a causal link between endothelial dysfunction and the progression of these diseases. One of the main contributors is the increase in reactive oxygen species (ROS), which increases intracellular (DNA) damage and ultimately can result in the onset of apoptosis or the induction of cellular senescence. Increased levels of cellular senescence have been observed in the vasculature of patients with coronary artery disease and in tubular compartments of patients with diabetic nephropathy. In Zucker diabetic fatty (ZDF) rats, decreased aortic vasodilatation was accompanied by an increase in senescent positive endothelial cells in the aorta. The ZDF rat develops obesity-related diabetes associated with increased levels of free fatty acids and ROS in its vasculature. Treatment of these animals with the antioxidant drug ebseilen prevented vascular senescence and reduced vasculopathy.

In the treatment of type 2 diabetes, the glucagon-like peptide 1 (GLP-1) receptor is a novel target. GLP-1 is proglucagon-derived hormone produced by the intestinal L cells in response to food intake and has a profound effect on glycemic control. GLP-1 reduces glucagon secretion from the α-cells, most pronounced are its effects on the β-cells, where GLP-1 stimulates insulin production, increases proliferation, and can reduce β-cell apoptosis. In vivo, active GLP-1 is rapidly degraded by dipeptidyl-peptidase 4 (DPP-4). Therefore, more stable GLP-1 analogues, such as exenatide, and DPP-4 inhibitors, such as vildaglitin, have been developed for the treatment of type 2 diabetes.

GLP-1 acts through the GLP-1 receptor, a G-coupled-protein receptor, which is abundantly present in the gastrointestinal tract but has also been detected at lower levels in the nervous system, the heart, vascular smooth muscle, and endothelial cells. GLP-1 is a potent vasodilator and is...
associated with the improvement of endothelial function in animal models and in type 2 diabetic patients.\textsuperscript{17, 18} Activation of the GLP-1 receptor can trigger at least 2 downstream pathways, generation of the second messenger cAMP (cAMP) followed by activation of protein kinase A (PKA), and the indirect activation of epidermal growth factor receptor followed by phosphoinositide 3-kinase (PI3K) and Akt signaling.\textsuperscript{14, 19}

We hypothesized that GLP-1 receptor activation could be involved in reducing the detrimental effects of oxidative stress and reduce cellular stress in the vasculature. Here we used an in vivo and in vitro model to investigate the effects of GLP-1 on cellular senescence.

## Methods

### Animal Studies

All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen. Male ZDF (ZDF-Leprfa/Crl) rats and lean littermate (ZDF-Leprfa/Crl) rats were obtained from Charles River (Maastricht, the Netherlands). At 10 weeks of age, ZDF animals were allocated in 2 groups (n=7 per group), and 1 group was treated with vildagliptin (Galvus, Novartis Pharma AG, Basel, Switzerland) for 15 weeks at a dose of 3 mg/kg per day (via the drinking water). Untreated ZDF rats and age-matched lean controls (n=7) rats served as controls.

### Senescence-Associated β-Galactosidase Staining

Senescence was determined by senescence-associated β-galactosidase (sa-β-galactosidase) staining.\textsuperscript{20}

### Senescence Staining of Aorta

Rat abdominal aorta was cleaned by removing fat and connective tissue and subsequently stained for senescence as described above. After staining, a part of the aorta was transversely cut and stored for cryosectioning, the rest of the aorta was cut longitudinally, and pictures were taken from the aorta with a DSLR camera equipped with a 50-mm macro lens. Photos of aortas were randomized, and the percentage of senescent cells was determined.

### Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from the Endothelial Cell Facility (University Medical Center Groningen, Groningen, the Netherlands). HUVECs were isolated from 2 umbilical cords and prepared as previously described.\textsuperscript{22} Cells were cultured on 1% precoated gelatin plates at 37°C under 5% CO\textsubscript{2}. RPMI 1640 medium was supplemented with 20% fetal calf serum, 2 mmol/L-glutamine, 5 U/mL heparin, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL endothelial cell growth factor extracted from bovine brain. Experiments were conducted with cell passage numbers between 2 and 6.

### Premature Senescence Assay

Senescence was induced by adapting our previously published endothelial senescence model.\textsuperscript{8} In short, HUVECs were seeded at 5000 cells/cm\textsuperscript{2} on 1% gelatin-coated plates. After 24 hours, medium was replaced with low-serum medium (2% fetal calf serum), and at confluence, the percentage of senescent cells was determined. Various concentrations of GLP-1, exendin-4, 8-Bromoadenosine-cAMP (8-Br-cAMP), and forskolin were administered 30 minutes before H\textsubscript{2}O\textsubscript{2} exposure, and exendin (9–39), LY294002, H89, KT 5720, and NG-nomonomethyl-l-arginine were administered 30 minutes before GLP-1 addition. Senescent staining was performed as described above. Light microscopic pictures were taken on an inverted microscope with a \( \times 10 \) objective, and the number of senescent cells and total cell number were counted per microscopic field. In each well, 4 random fields were evaluated. Data from multiple experiments were compared by calculating the relative amount of senescence, for which the 30 μmol/L \( \text{H}_2\text{O}_2 \) group was set to 1.00 in each separate experiment.

### Protein Analysis

For Western blot analysis of whole cell lysates, the following antibodies were used: anti-phosphorylated Akt (Ser473), anti-phosphorylated CREB (87G3), anti-caspase-3 (8g10) (Cell Signaling Technology, Danvers, Mass), and anti-GLP-1 receptor (H55) (Santa Cruz Biotechnology, Heidelberg, Germany). For loading control, membranes were reprobed with β-actin antibody (Sigma-Aldrich), α-tubulin (Sigma-Aldrich), or GAPDH antibody (Fitzgerald Industries International, Acton, Mass). Signals were detected by ECL and quantified by densitometry (Syngene, Cambridge, United Kingdom).

### Quantitative Real-Time Polymerase Chain Reaction

Relative expression of HO-1 and NQO1 genes was determined by quantitative polymerase chain reaction. Gene expression was determined by correcting samples for reference gene values (cytochrome A), and values were expressed relative to the control group per experiment.

### Comet Assay

DNA damage was determined by the comet single cell electrophoresis assay, as described by the manufacturer (Trevigen, Gaithersburg, Md).

### Statistics

Data represent mean values±standard errors (SE). Comparisons between groups were done by 1-way ANOVA with post hoc Dunnett correction. Comparison of the sa-β-galactosidase areas in the abdominal aortas and quantitative polymerase chain reaction expression was performed by Mann-Whitney U test. A probability value of less than 0.05 was considered significant.

Expanded methods can be found as supplemental material, available online at http://atvb.ahajournals.org.

## Results

### DPP-4 Inhibitor Vildagliptin Decreases Vascular Senescence In Vivo

Here we investigated vascular senescence in normal “lean” animals compared with ZDF rats. Senescence in abdominal aorta was determined with the well-established sa-β-galactosidase staining.\textsuperscript{20} As shown in Figure 1A, staining was most pronounced in the areas of aorta side branches, in concordance with the study of Brodsky et al.\textsuperscript{10} Determination of the amount of senescence per vessel showed a significant increase in 25-week-old ZDF animals compared with control lean animals (Figure 1B), 4.7±1.1% versus 2.3±0.5%. Histological analysis of the stained vessels confirmed that parts of the endothelial cell layer were stained positive for sa-β-galactosidase (Figure 1C). Treatment of the ZDF animals with vildagliptin resulted in a significant reduction of DPP-4 activity (Figure 1D) and an almost 6-fold increase of GLP-1 plasma levels (Figure 1E). No changes in plasma glucose and insulin levels were observed and body weights remained constant with this treatment protocol (Supplemental Table I). The vildagliptin treatment did, however, decrease cellular senescence in these animals, to levels almost comparable to those of lean rats (2.6±0.6% versus 2.3±0.5%) (Figure 1A)
and 1B). This suggests that increased GLP-1 levels by DPP-4 inhibition have a protective effect on the vasculature.

**GLP-1 Treatment Inhibits ROS-Induced Premature Senescence**

To investigate the effects of GLP-1 on endothelial senescence, we switched to the well-established HUVEC system, which is known to be sensitive to stress-induced cellular senescence. In HUVECs, cellular senescence was induced by oxidative stress via exposure to low concentrations of \( \text{H}_2\text{O}_2 \) (30 \( \mu \text{mol/L} \)), which did not induce apoptosis, as confirmed by the absence of caspase-3 cleavage under these conditions (Supplemental Figure I). This low dose, however, resulted in a significant 3.4-fold increase (from 2.00\( \pm \)0.25% to 6.82\( \pm \)0.88%) in the number of senescent cells 3 days after exposure, as determined by sa-\( \beta \)-galactosidase staining (Figure 2A). Treatment of HUVECs with GLP-1 attenuated the increase of senescent cells in a dose-responsive manner (Figure 2B), with a maximum of approximately 63% reduction of senescent positive cells compared with non-GLP-1-treated group at a concentration of 10 nmol/L GLP-1 (H\( \text{O}_2 \) group 0.99\( \pm \)0.08; 10 nmol/L GLP-1 + H\( \text{O}_2 \) group 0.37\( \pm \)0.03; control group 0.32\( \pm \)0.03).

In parallel to the sa-\( \beta \)-galactosidase staining, DNA damage was also investigated in HUVECs using the comet assay (Figure 3A). The tail DNA read-out showed that H\( \text{O}_2 \) significantly increased DNA damage by 1.6-fold relative to the control group (Figure 3B). In GLP-1 treated cells, H\( \text{O}_2 \)
exposure induced DNA damage only by 1.1-fold. Thus, GLP-1 significantly reduced DNA damage generated by H2O2 as determined by the percentage tail DNA in a comet assay. Together, these findings suggest that GLP-1 treatment can reduce ROS induced DNA damage and consequently attenuate the increase in senescent HUVECs.

The Protective Effect of GLP-1 Is Receptor Mediated

Western blot analysis of cell extracts showed GLP-1 receptor expression in HUVECs (Supplemental Figure II), which is in line with other studies showing endothelial expression.15,17 Next we used the GLP-1 analogue exendin-4 (an agonist of the GLP-1 receptor). Similar to GLP-1 treatment, exendin-4 strongly attenuated H$_2$O$_2$-induced senescence in HUVECs (Figure 4A). Cotreatment with exendin (9–39), another GLP-1 derived peptide that acts as an antagonist for the GLP-1 receptor, completely abolished the protective effect of GLP-1 on H$_2$O$_2$ induced cellular senescence (Figure 4B). These data provide evidence that the GLP-1 protective effect is GLP-1 receptor mediated.

The PI3K/Akt Survival Pathway Is Not Required for the GLP-1 Protective Effect

In pancreatic β-cells and neuronal cells, GLP-1 prevented apoptosis through the activation of PI3K and Akt. Although the amount of oxidative stress used in this study did not induce apoptosis in our HUVEC model, the PI3K/Akt signaling pathway is a general survival pathway, and we therefore tested whether this pathway could be involved in the protective effect of GLP-1. The activity of Akt kinase was determined using an antibody against phosphorylated serine 473, the activation residue of Akt. Western blot analysis with this antibody did not reveal any change in Akt activity between control and GLP-1 treated cells in the presence of H2O2 (Figure 5A and 5B), indicating that GLP-1 does not activate this kinase under these conditions in HUVECs. To further rule out the involvement of this pathway, the upstream activator of Akt, PI3K, was inhibited using the small molecule LY294002 (Supplemental Figure III). As shown in Figure 5C, inhibition of PI3K by LY294002 did not result in a decrease of the protective effect of GLP-1 in HUVECs. Together these data do not support a role of the PI3K/Akt pathway in the GLP-1-mediated effect in HUVECs.

GLP-1 Protective Effect Is cAMP/PKA Dependent

The GLP-1 receptor is a G-protein-coupled receptor and activates adenylate cyclase, resulting in cAMP production and downstream PKA activation. We therefore investigated the involvement of this intracellular signaling cascade in the GLP-1 protective effect. Treatment with forskolin, an ade-
GLP-1 Prevents Endothelial Cell Senescence

GLP-1 Activates CREB and Induces Expression of HO-1 and NQO1

A downstream target of PKA is the transcription factor CREB, which is activated by PKA phosphorylation and regulates expression of numerous genes in conjunction with other transcription factors. Western blot analysis using an anti-phosphorylated CREB antibody showed that GLP-1 treatment increased relative phosphorylated CREB levels by 52% compared with the control (Figure 6C and 6D). This effect was abolished by PKA inhibition using H89 (Figure 6C and 6D). Similar results were obtained with forskolin (Figure 6C and 6D). Quantitative polymerase chain reaction analysis of heme-oxygenase 1 (HO-1), a reported antioxidative target gene of CREB,25 showed a concomitant induction after GLP-1 treatment. Also, another antioxidative gene, NQO1 (NAD[P]H dehydrogenase quinone 1), was significantly induced by GLP-1 (Figure 6E and 6F). These data show that GLP-1 can activate the transcription factor CREB and can induce expression of antioxidative genes.

Discussion

GLP-1 analogues and DPP-4 inhibitors are novel therapeutic agents for the treatment of diabetes mellitus. These drugs are primarily designed to improve glycemic control. Interestingly, evidence is accumulating that GLP-1 and its analogues can also protect cardiovascular damage via ancillary pathways.15,26 In particular, GLP-1 has been shown to induce vasodilation and improve endothelial function.17,18 In the present study, we showed that GLP-1 can protect endothelial cells against oxidative stress–induced cellular senescence.

In ZDF rats, increased levels of vascular senescence were observed, which is in agreement with a previous study.10 Treatment with the DPP-4 inhibitor vildagliptin raised GLP-1 levels and attenuated vascular cell senescence in these animals, almost to lean animal levels. Although DPP-4 inhibitors are used to improve glycemic control in diabetic patients, several studies have reported limited effects in glycemic parameters with these overtly diabetic ZDF rats on DPP-4 inhibition or treatment with GLP-1 analogues.27–29 In line with this, analysis of blood glucose and insulin levels at the end of our experiment showed only slight, but not significant, changes in the vildagliptin-treated animals compared with the nontreated ZDF animals. However, we did not perform intermediate analysis or glucose tolerance tests and therefore cannot exclude possible changes in glucose handling in these animals on treatment. Irrespective of this, our results show that DPP-4 inhibition can attenuate vascular senescence in vivo, most likely via GLP-1.

Oeseburg et al GLP-1 Prevents Endothelial Cell Senescence

...response effect on cellular senescence in combination with GLP-1, with a complete abolishment of the GLP-1 protective effect at a concentration of 1 μmol/L H89 (Figure 6B). Similar results were obtained with another PKA inhibitor KT 5720 (Supplemental Figure IVB). These results indicate that PKA activation by GLP-1 is sufficient to prevent cellular senescence in HUVECs under these conditions.

Figure 5. Effect of GLP-1 on Akt and PI3K inhibition. A, Representative blot showing phosphorylated Akt (Ser473) and β-actin levels from the following treatment groups: 1 = control, 2 = 30 μmol/L H2O2, 3 = 10 nmol/L GLP-1 + 30 μmol/L H2O2, 4 = 10 nmol/L GLP-1, PC = positive control (30 minutes of medium with 20% FCS and growth factors), and M = molecular marker lane. Cell extracts were prepared after 1 hour of H2O2 exposure. B, Relative levels of phosphorylated Akt, after GLP-1 or H2O2 treatment or a combination of the 2. Data from 3 experiments. C, Effect of the PI3K inhibitor LY294002 on the protective effect of GLP-1. Data were obtained from 4 experiments. Open bar represents the nontreated control group. *P<0.05 compared with H2O2 groups.

nylate cyclase activator, attenuated H2O2-induced cellular senescence in HUVECs (Figure 6A). Similar results were obtained with 8-Br-cAMP, a membrane-permeable cAMP analogue (Supplemental Figure IVA). The effect of forskolin was dependent on the downstream cAMP effector PKA, as specific inhibition of PKA with H89 could abolish the forskolin-mediated protection. PKA inhibition by H89 was also sufficient to block the GLP-1-mediated protective effect on HUVECs (Figure 6B). H89 inhibition showed a dose
we observed that GLP-1 treatment had a protective effect on ROS-induced senescence in HUVECs. Second, this was associated with a reduction in DNA damage, further supporting a protective effect of GLP-1. We cannot rigorously exclude that in vivo also other potential DPP-4 targets might have exerted some effects on the vasculature. We note, however, that the only other established clinically relevant DPP-4 substrate is glucose-dependent insulinotropic polypeptide, but the receptor of this hormone is strongly downregulated in hyperglycemic ZDF rats. Therefore, we strongly believe that the observed in vivo effects must be GLP-1 mediated.

It has been shown in pancreatic β-cells that GLP-1 reduced apoptosis, stimulated survival and proliferation, and increased insulin secretion. These effects of GLP-1 have primarily been ascribed to the activation of downstream cAMP/PKA and PI3K/Akt signaling pathways in these cells. Also, the cardioprotective effect of GLP-1 after ischemia/reperfusion could be abolished by inhibiting cAMP, and PI3K, indicating that the protective effects of GLP-1 generally involve multiple pathways. Our data indicate that in endothelial cells, PI3K/Akt signaling is not activated by GLP-1, and accordingly, inhibition of this pathway could not abolish the GLP-1 protective effect. The cAMP/PKA pathway, on the other hand, was clearly required for the GLP-1 protective effect in endothelial cells. Thus, it appears that in different cell types, different pathways are involved in GLP-1 prosurvival or antisenescence mechanisms. Because the GLP-1 receptor directly activates adenylate cyclase via , it is not surprising that the cAMP/PKA pathway is generally involved in this mechanism. PI3K activation by GLP-1 is indirect, however, involving IRS2 expression or epidermal growth factor receptor transactivation, and this latter mechanism is apparently not active in endothelial cells.
We also showed here that GLP-1 could induce phosphorylation of the transcription factor CREB, in a PKA-dependent way, in endothelial cells. CREB phosphorylation results in its activation, and previous studies have shown that CREB phosphorylation is associated with cellular survival in pancreatic β-cells. CREB binding site profiling studies have predicted at least 4000 putative target genes, but probably only a few percent are controlled by cAMP, and specific target genes are difficult to define. We investigated the expression of the oxidative stress defense gene HO-1, which is at least partially regulated by CREB. We could show here that HO-1 expression in endothelial cells was significantly induced by GLP-1. This extends a previous observation in which in vivo liraglutide treatment resulted in HO-1 induction and cytoprotection of cardiac tissue. Another oxidative defense gene, NQO1, a potential downstream target of the GLP-1-induced Nrf2/CREB binding protein pathway, was also induced in endothelial cells in our study. These results indicate that GLP-1 can activate oxidative stress defensive pathways in endothelial cells.

Recently, it was also shown that GLP-1 can reduce the inflammatory response of endothelial cells on exposure to advanced glycation end product. Because advanced glycation end product formation correlates with ageing and cellular senescence, this could be a second path by which GLP-1 protects endothelial cells. Interestingly, the authors showed that GLP-1 attenuated the expression of the advanced glycation end product receptor, and this was mimicked by 8-Br-cAMP, indicating involvement of PKA signaling. This suggests that GLP-1 might activate multiple cytoprotective mechanisms and could have wide-ranging cytoprotective effects in cells. Expression profiling and proteomics approaches would be required to further map these global protective changes exerted by GLP-1.

Our data on endothelial senescence provided clear evidence that the GLP-1 protective effect in this setting was conferred by the GLP-1 receptor. HUVECs express the GLP-1 receptor and both exendin-4 and GLP-1 provided protective effects, which were abolished by the antagonist exendin (9–39). To our knowledge, this is the first study that links activation of the GLP-1 receptor to a protective effect on cellular senescence. In a recent report, GLP-1 receptor-dependent antiapoptotic effects were described for exendin-4 in mouse cardiomyocytes, which is in line with our observed antisenescence effects in endothelial cells. Surprisingly, however, they did not observe this effect in endothelial cells, but these cells were treated with very high levels of H₂O₂ (700 μmol/L). Thus, GLP-1 and exendin-4 can apparently prevent endothelial senescence and cardiomyocyte apoptosis, but not apoptosis in endothelial cells. Interestingly, Ban et al. did observe antiapoptotic effects in cardiomyocytes and endothelial cells with the “inactive” cleaved form of GLP-1. GLP-1 (9–36), in a GLP-1 receptor independent manner. Whether this form has antiapoptotic or antisenescence activity on endothelial cells in vivo is, however, not known. Because DPP-4 inhibition only increases the levels of uncleaved GLP-1 (7–36), as performed in this study, we have at this stage only evidence that uncleaved GLP-1 can attenuate endothelial senescence.

In aging and in replicative senescence, nitric oxide (NO) levels tend to deteriorate, and NO is an important factor in preventing or delaying the onset of endothelial senescence. We therefore also considered the role of NO. We could not, however, abrogate the GLP-1-induced protection (Supplemental Figure V), by N⁵-monomethyl-L-arginine, an NO synthase inhibitor, indicating that NO was not involved. Interestingly, in endothelial cells antiapoptotic effects by the cleaved GLP-1 (9 to 36) form did appear to be NO mediated, indicating that this follows a different route compared with the antisenescence effects of GLP-1.

In conclusion, we have shown that GLP-1 has a protective effect on endothelial cells via GLP-1 receptor–mediated cAMP–dependent activation of PKA. The latter was both sufficient and essential for the GLP-1-induced effect. This GLP-1 effect on endothelial cells was independent of the P38K/Akt survival pathway and did not require NO synthesis.

These findings provide further evidence for the potentially extraglycemic, pleiotropic effects of GLP-1 and DPP-4 inhibitors and could serve as a starting point to further investigate the protective mechanisms and to explore GLP-1 treatment options to delay disease and aging related endothelial senescence.

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Disclosures
None.

References


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Supplemental material

Supplemental Methods

Reagents

GLP-1 (7-37) (GLP-1), 8-Bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP), N^G-Methyl-L-arginine acetate salt (L-NMMA), H-89 dihydrochloride hydrate (H89), forskolin, LY-294,002 hydrochloride (LY294002), bradykinin, exendin-4, exendin fragment 9-39 (exendin (9-39)), and KT 5720 were all obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The Accu-Check Aviva kit (Roche, Almere, The Netherlands) was used to determine blood glucose levels at sacrifice. Serum DPP-IV activity (Quantizume Assay System, BIOMOL International, Plymouth Meeting, PA, USA), active GLP-1 (Glucagon-Like Peptide-1 (Active) ELISA Kit (Millipore Corporation, Billerica, MA, USA)) and insulin levels (enzymatic assay ALPCO Diagnostics, (Rat) EIA, ALPCO Diagnostics, Salem, NH, USA) were all determined using commercially available kits, according to the manufacturer instructions.

Senescence associated β-galactosidase staining

Cells or tissue were washed in PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes. Subsequently, cells or tissue were washed and incubated for 18 hours at 37°C with sa-β-galactosidase staining solution (150 mmol/l NaCl, 2 mmol/l MgCl₂, 5 mmol/l K₃Fe(CN)₆, 5 mmol/l K₂Fe(CN₆), 40 mmol/l citric acid / sodium phosphate dibasic at pH 6.0, containing 1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-D-galactoside)).
Comet Assay

HUVEC cells were treated as described above and 24 hours after treatment cells were harvested and approximately 700 cells were placed on a CometSlide in LMAgarose (Trevigen, Gaithersburg, MD, USA). Cells were lysed for one hour at 4°C in Lysis Solution, followed by denaturation for 30 minutes in 300 mmol/l alkaline solution, 1 mmol/l EDTA pH>13. Electrophoresis was done in an alkaline electrophoresis solution (200 mmol/l NaOH 1 mmol/l EDTA pH>13). Comet slides were stained with SYBR Green and photos were taken with a 20x objective. Pictures were analyzed with CASP 1.2.2 software. Experiments were repeated three times and in total more than 220 cells were analyzed per treatment group.

Quantitative Real-Time PCR

Total RNA was isolated from cells by Nucleospin II kit (Machery-Nagel, Düren, Germany) and converted to cDNA by QuantiTect Reverse Transcription (Qiagen, Venlo, The Netherlands).

Gene expression was measured with ABsolute QPCR SYBR Green ROX Mix (Abgene, Epsom, United Kingdom) in the presence of 5 ng cDNA and 200 nmol/l forward and reverse primers. Real-Time PCR was conducted on the Biorad CFX384 (Biorad, Veenendaal, The Netherlands) Primers used: HO-1 forward CAACAAAGTGCAAGATTCT, HO-1 reverse TGAGTGTAAGGACCACATC, NQO1 forward AACTTCAATCCCATCATT, NQO1 reverse TATAAGCCAGAACCAGACT, cyclophilin A forward ACTTCACACGCCATAATG, cyclophilin A reverse ACCCGTATGCTTTAGGAT.

References

Supplemental Table I. Characteristics of lean, ZDF and ZDF + vildagliptin groups at sacrifice. Rats were 25 weeks of age.

<table>
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<th>ZDF + vildagliptin</th>
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<td>Body weight (g)</td>
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<td>Plasma glucose (mmol/l)</td>
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<td>Plasma insulin (ng/10µl)</td>
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Data presented as means ± SE *P < 0.05 versus lean.
Supplemental Figure I. Caspase-3 cleavage in HUVEC cells treated with 10 nmol/l GLP-1 followed by 1 hour exposure to 30 µmol/l H₂O₂. Caspase-3 cleavage was measured immediately after H₂O₂ (1 hour) or 24 hours after H₂O₂ exposure. C = control group, H = H₂O₂ 30 µmol/l group, H+G = GLP-1 10 nmol/l + H₂O₂ 30 µmol/l group and G = GLP-1 10 nmol/l group. Positive control (PC) sample is HUVEC cells treated for 24h with 0.5 µmol/l staurosporine. Beta-actin levels detected on the same membrane where used as loading control.
Supplemental Figure II

Membrane shows GLP-1 receptor band at 56 kDa, GAPDH (37 kDa) level detected on the same membrane is shown as loading control.
Supplemental Figure III

Supplemental Figure III. LY294002 inhibits phosphorylation of Akt in HUVEC cells. Lane 1: Non-treated control. Lane 2: 30 minutes of LY294002 1 µmol/l treatment. Lane 3: 30 minutes 20% FCS. Lane 4: 30 minutes of 1 µmol/l LY294002 followed by 30 minutes of 20% FCS treatment. Bands represent phosphorylated Akt (60 kDa) and GAPDH levels (37 kDa) on the same membrane as loading control.
Supplemental Figure IV. A. Effect of 8-Br-cAMP on H₂O₂ induced senescence. Data were obtained from 6 experiments. B. Effect of the PKA inhibitor KT 5720 on GLP-1 mediated protective effect. Data from 4 experiments. For both graphs, data are expressed as means ± SE, open bars represents non-treated control groups, *P < 0.05 compared to H₂O₂ group.
Supplemental Figure V. Effect of NO inhibition on GLP-1 mediated cellular protection. The effect of L-NMMA inhibition on the protective effect of GLP-1. Data are expressed as means ± SE. Data obtained from 5 experiments. Open bar represents non-treated control group. *P < 0.05 compared to H₂O₂ group.