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Vascular-Protective Effects of High-Density Lipoprotein Include the Downregulation of the Angiotensin II Type 1 Receptor

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Abstract—There is growing evidence that a cross-talk exists between the renin-angiotensin (Ang) system and lipoproteins. We investigated the role of high-density lipoprotein (HDL) on Ang II type 1 receptor (AT1R) regulation and subsequent Ang II–mediated signaling under diabetic conditions. To investigate the effect of HDL on AT1R expression in vivo, apolipoprotein A-I gene transfer was performed 5 days after streptozotocin injection. Six weeks after apolipoprotein A-I gene transfer, the 1.9-fold ($P=0.001$) increase of HDL cholesterol was associated with a 4.7-fold ($P<0.05$) reduction in diabetes mellitus–induced aortic AT1R expression. Concomitantly, NAD(P)H oxidase activity, *Nox 4*, and $p22^{phox}$ mRNA expression were reduced 2.6-fold, 2.0-fold, and 1.5-fold ($P<0.05$), respectively, whereas endothelial NO synthase dimerization was increased 3.3-fold ($P<0.005$). Apolipoprotein A-I transfer improved NO bioavailability as indicated by ameliorated acetylcholine-dependent vasodilation in the streptozotocin-*Ad.hapoA-I* group compared with streptozotocin-induced diabetes mellitus. In vitro, HDL reduced the hyperglycemia-induced upregulation of the AT1R in human aortic endothelial cells. This was associated with a 1.3-fold and 2.2-fold decreases in reactive oxygen species and NAD(P)H oxidase activity, respectively ($P<0.05$). Finally, HDL reduced the responsiveness to Ang II, as indicated by decreased oxidative stress in the hyperglycemia+HDL+Ang II group compared with the hyperglycemia+Ang II group. In conclusion, vascular-protective effects of HDL include the downregulation of the AT1R. (*Hypertension*. 2009;53:682-687.)

Key Words: high-density lipoprotein ■ angiotensin AT1 receptor ■ endothelial function ■ diabetes mellitus

Diabetes mellitus is associated with endothelial dysfunction, an early phase of atherosclerosis further characterized by a reduction in NO bioavailability, without significant morphological changes of the vessel wall.[1,2] Both diabetes mellitus–associated hyperglycemia and increased angiotensin (Ang) II levels[3] induce reactive oxygen species (ROS), which contribute to endothelial dysfunction, partly via oxidative degradation of NO. Recent studies have demonstrated that ROS are predominantly produced by vascular NAD(P)H oxidase (NOX),[4] whereas "uncoupled" endothelial NO synthase (eNOS; when eNOS produces $O_2^{\cdot-}$ rather then NO) is another important source of ROS in diseased, including diabetic, blood vessels.[5] Numerous studies have shown that activation of the Ang II type 1 receptor (AT1R) contributes to the induction of oxidative stress and apoptosis of vascular cells, thus contributing to the initiation and progression of endothelial dysfunction.[6] Ang II not only increases NOX activity but also uncouples eNOS in diabetic mice.[7] The expression levels of the AT1R define the biological efficacy of Ang II and have been shown to be regulated by several agonists, such as Ang II, glucose, insulin, ROS, low-density lipoprotein (LDL), and many others,[8,9] including diabetes mellitus.[10,11] The importance of the enhanced vascular AT1R expression and Ang II–mediated signaling in diabetes mellitus–associated endothelial dysfunction follows from the finding that AT1R antagonism in diabetes mellitus improves endothelial function.[7,12]

The vascular protective effects of high-density lipoprotein (HDL) are well documented: low plasma HDL is an independent predictor of endothelial dysfunction in healthy individuals and diabetic patients,[13,14] and elevation of plasma HDL by drug treatment with niacin or by infusion of synthetic HDL leads to a significant improvement of impaired endothelial function.[15] Recently, HDL-mediated reduction in NOX activity has been demonstrated.[16]

There is growing evidence that there exists a cross-talk between the renin-Ang system and lipoproteins: (1) AT1R expression is increased by LDL[8] and oxidized LDL[17] in
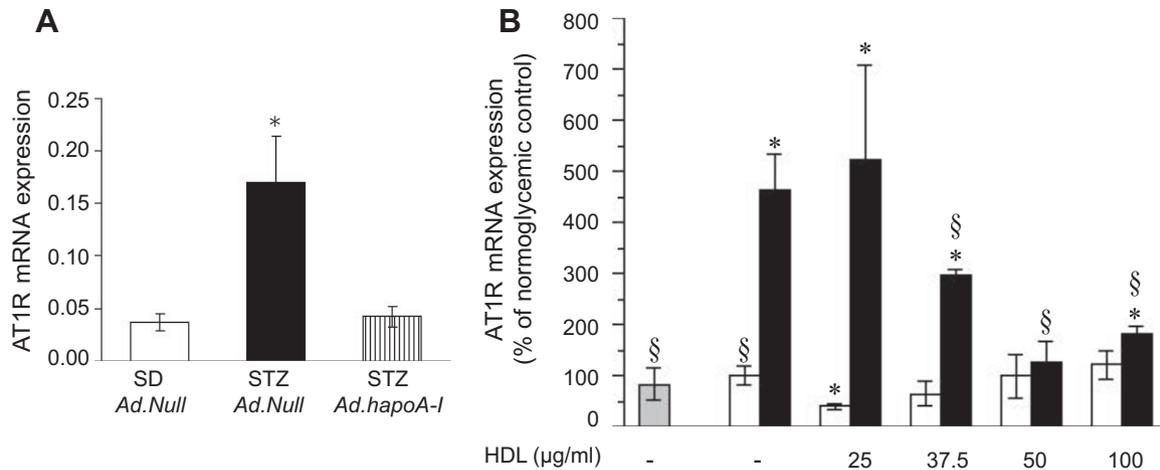
Figure 1. Reduced AT1R mRNA expression after apo A-I gene transfer in the diabetic aorta and after HDL supplementation in HAECs under HG. A, Bar graph representing aortic AT1R mRNA expression normalized toward 18S. Data are represented as the mean±SEM (n=4); *$P<0.05$ vs SD-*Ad.Null* and STZ-*Ad.hapoA-I*. B, Bar graph representing HAEC AT1R mRNA expression normalized toward ribosomal L32 and depicted as the percentage of the normoglycemic control group set as 100%. Gray bar, mannitol; open bars, normoglycemic conditions; and closed bars, HG. Data are represented as the mean±SEM (n=4 to 6); *$P<0.05$ vs C; §$P<0.05$ vs HG.

vascular smooth muscle cells and human aortic endothelial cells (HAECs), respectively; (2) Ang II facilitates the oxidation of LDL and its uptake by scavenger receptors on monocytes/macrophages,[18] whereas it inhibits macrophage expression of the ATP-binding cassette transporter A1,[19] which regulates the transport of cholesterol and phospholipids to apolipoprotein (apo) A-I, the major apo of HDL; and (3) AT1R blockade reduces LDL cholesterol and increases HDL cholesterol in diabetic patients.[20]

We hypothesized that the vascular-protective effects of HDL may include the downregulation of the AT1R and thereby reduce Ang II–mediated signaling. To further explore this hypothesis, we investigated the influence of HDL on AT1R regulation in vivo in an experimental model of diabetes mellitus–induced endothelial dysfunction and in vitro in HAECs under hyperglycemia (HG) in the presence or absence of Ang II.

Methods

For detailed methodology, please see the data supplement (available online at http://hyper.ahajournals.org). In brief, diabetes mellitus was induced by a single injection of streptozotocin (STZ; 70 mg/kg; IP) in 8-week-old male Sprague-Dawley (SD) rats. Five days after STZ injection, IV gene transfer with 3×10^{12} particles per kg of the E1E3E4-deleted adenoviral vector *Ad.hapo A-I*, which induces hepatocyte-specific expression of human apo A-I in the absence of significant hepatotoxicity,[21] or of the control vector *Ad.Null*, which contains no expression cassette,[21] was performed. Six weeks after gene transfer, rats were euthanized, and endothelium-dependent vasorelaxation was determined. Aorta was snap frozen for subsequent rat *AT1R*, *Nox 1*, *Nox 2* (*gp91phox*), *Nox 4*, *p22phox* and *extracellular (ec)-superoxide dismutase (SOD)* mRNA expression, eNOS protein level, eNOS dimerization, NAD(P)H oxidase, and SOD activity analysis. In vitro, the effects of HDL on *AT1R* mRNA and eNOS protein expression and Ang II responsiveness in HAEC were determined.

Results

Human Apo A-I Gene Transfer in STZ-Induced Diabetic Rats

Ad.hapoA-I gene transfer resulted in persistent hepatocyte-specific expression of human apo A-I for the entire duration of the experiment, 6 weeks, leading to 1.9-fold ($P=0.001$) increased levels of HDL cholesterol at the day of sacrifice compared with STZ-*Ad.Null* rats. Human apo A-I transfer did not result in significant changes of blood glucose concentrations or in alterations of LDL cholesterol levels (supplemental Figure S1A through S1D).

Human apo A-I gene transfer reduces AT1R expression in the diabetic aorta, and supplementation of HDL reduces HG-induced AT1R expression in HAECs. Aortic AT1R expression was 4.7-fold ($P<0.05$) increased in STZ-induced diabetic rats compared with SD-*Ad.Null* rats. *Ad.hapoA-I* gene transfer normalized AT1R expression in STZ-induced diabetic rats to levels found in SD-*Ad.Null* rats (Figure 1A). The *AT1R* mRNA expression was 4.6-fold ($P<0.0005$) increased in HAECs under HG. HDL supplementation reduced *AT1R* mRNA expression in a dose-dependent manner, reaching *AT1R* mRNA levels of controls at 50 μg/mL (25 μg/mL: *P* value not significant; 37.5 μg/mL: 2.2-fold [$P<0.05$]; 50 μg/mL: 3.6-fold [$P<0.01$]; and 100 μg/mL: 2.6-fold [$P<0.05$] lower versus HG, respectively; Figure 1B). Under normoglycemic conditions, HDL had no significant effect on *AT1R* mRNA expression with the exception of 2.5-fold–lower ($P<0.05$) expression levels at an HDL concentration of 25 μg/mL (Figure 1B).

Human Apo A-I Gene Transfer Reduces NAD(P)H Oxidase Activity in the Diabetic Aorta

Nox 1 and *Nox 2* mRNA expressions were not significantly induced under diabetic conditions. Human apo A-I gene transfer resulted in a 2.0-fold and 1.5-fold ($P<0.05$) reduction of diabetes mellitus–upregulated aortic *Nox 4* and *p22phox* mRNA expression, respectively, and decreased the diabetes mellitus–induced NOX activity by 2.6-fold ($P<0.05$). In STZ-induced diabetic rats, mRNA expression of the antioxidative *ec-SOD* was 1.6-fold ($P<0.05$) increased after apo A-I gene transfer, whereas the diabetes mellitus–induced SOD activity was 3.5-fold ($P<0.05$) decreased. (Figure 2A through 2D).
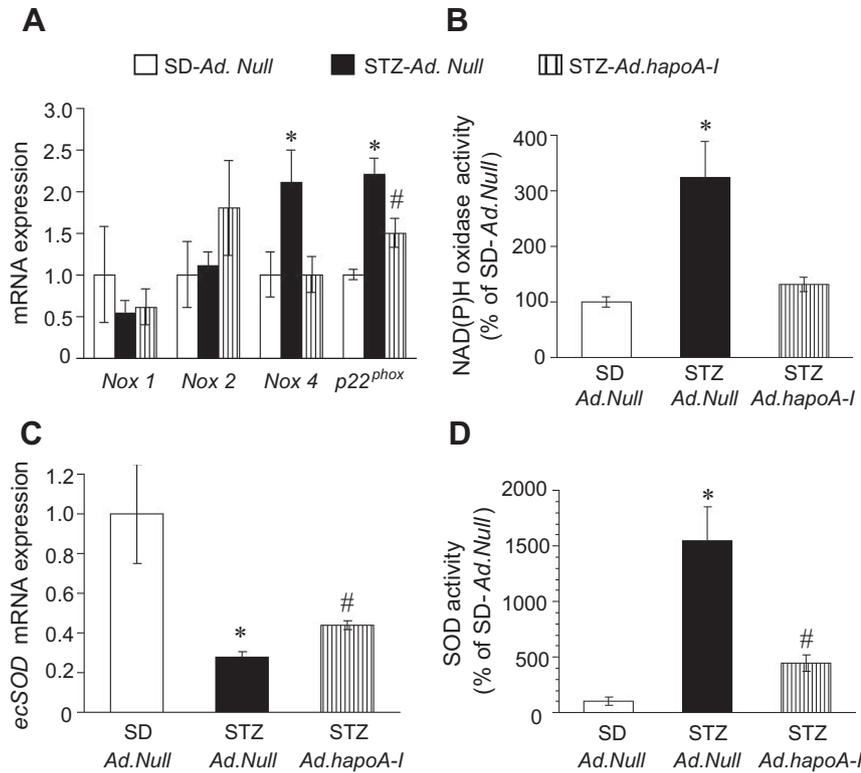
Figure 2. Effect of apo A-I gene transfer on markers of vascular oxidative stress. Bar graphs representing aortic (A) *Nox 1*, *Nox 2*, *Nox 4*, and *p22^phox* mRNA expression normalized toward 18S, with SD-*Ad.Null* set as 1, with open bar: SD-*Ad.Null*, black bar: STZ-*Ad.Null*, and dashed bar: STZ-*Ad.hapoA-I*. B, NAD(P)H oxidase activity represented as the percentage of the nondiabetic control group SD-*Ad.Null* set as 100%. C, *ec-SOD* mRNA expression normalized toward 18S with SD-*Ad.Null* set as 1. D, SOD activity (U/mL/μg protein) represented as the percentage of the nondiabetic control group SD-*Ad.Null* set as 100%. Data are represented as the mean±SEM (n=4); *$P<0.05$ vs SD-*Ad.Null* and STZ-*Ad.hApoA-I*; #$P<0.05$ vs SD-*Ad.Null*.

HDL Reduces the Responsiveness to Ang II in HAECs Under HG

HG induced 2′-7′-dichloro-dihydrofluorescein fluorescence and NOX activity by 1.3-fold and 2.2-fold, respectively ($P<0.005$), which was significantly reduced after HDL supplementation. The oxidative stress response toward Ang II was most pronounced under hyperglycemic conditions (Figure 3A and 3B).

Human Apo A-I Gene Transfer Restores eNOS Dimerization in the Diabetic Aorta

Total eNOS levels increased 2.5-fold ($P<0.05$) in STZ-induced diabetic rats relative to controls, whereas apo A-I gene transfer reduced diabetes mellitus–induced eNOS toward levels not significantly different from SD-*Ad.Null* (Figure 4A). In contrast, the ratio of eNOS dimer:monomer in

STZ-induced diabetic rats was 3.0-fold ($P<0.005$) lower compared with that in SD-*Ad.Null*, as assayed by low temperature SDS-PAGE and immunoblotting. Apo A-I gene transfer increased the eNOS dimer:monomer ratio by 3.3-fold ($P<0.005$) versus the STZ-induced diabetic group, leading to ratios not significantly different from SD-*Ad.Null* (Figure 4B). In vitro, under HG, eNOS expression was 1.7-fold ($P<0.05$) increased and normalized to normoglycemic control levels in the presence of HDL in HAECs (Figure 4C).

Vascular Reactivity Is Improved After Human Apo A-I Gene Transfer

To evaluate whether NO bioavailability was increased in vivo as a consequence of reduced NOX activity, restored SOD activity, and enhanced eNOS coupling, endothelium-dependent relaxation was evaluated in vivo. Acetylcholine-
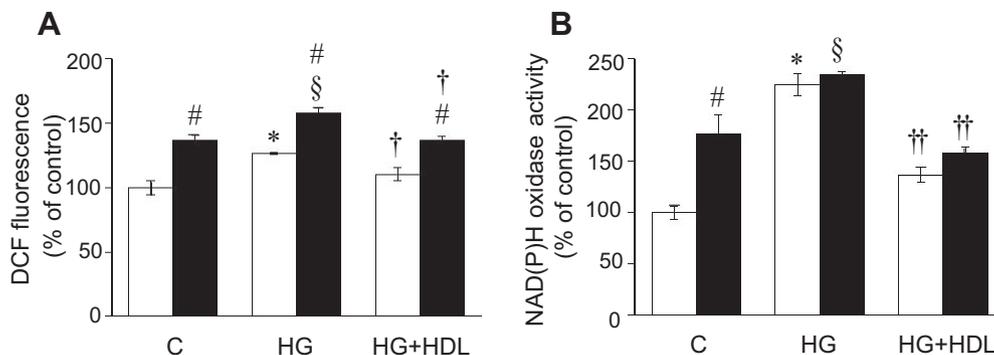
Figure 3. HDL reduces the responsiveness to Ang II in HAECs under HG. A, 2′-7′-Dichloro-dihydrofluorescein (DCF) fluorescence as a marker for ROS production and (B) NAD(P)H oxidase activity in HAECs represented as the percentage of the normoglycemic control group set as 100% with C indicating control; HG+HDL, HG with 50 μg/mL of HDL (n=4). □, basal; ■, in the presence of 1 μmol/L Ang II. *$P<0.005$ vs C and HG+HDL; §$P<0.05$ vs C+Ang II and HG+HDL+Ang II; #$P<0.05$ vs basal; †$P<0.05$ vs HG; ††$P<0.005$ vs HG.

Figure 4. Human apo A-I gene transfer preserves eNOS dimerization in the diabetic aorta, and HDL supplementation on HAECs decreases eNOS expression. A, Representative Western Blot and bar graph depicting the aortic eNOS:β-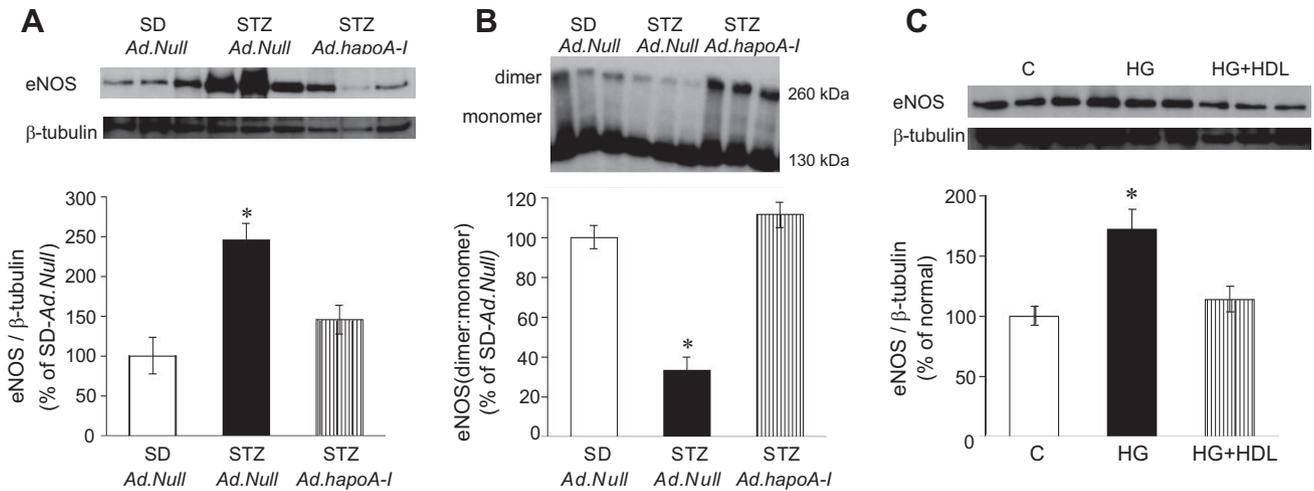tubulin ratio, represented as the percentage of the nondiabetic control group SD-*Ad.Null* set as 100%. Data are represented as the mean$\pm$SEM (n=3); *$P<0.05$ vs SD-*Ad.Null* and vs STZ-*Ad.hapoA-I*. B, Representative blot and bar graph depicting the ratio of eNOS band intensity (dimer:monomer; n=3 to 4); *$P<0.005$ vs SD-*Ad.Null* and vs STZ-*Ad.hapo A-I*. C, Western blot and bar graph depicting the eNOS:β-tubulin ratio from HAECs, represented as the percentage of the normoglycemic control group set as 100% with C indicating control; HG+HDL, HG with 50 μg/mL of HDL (n=3); *$P<0.05$ vs C and vs HG+HDL.

induced endothelium-dependent relaxation was significantly impaired in aortic rings of STZ-induced diabetic rats compared with SD-*Ad.Null* ($P<0.05$). Endothelium-dependent vasorelaxation was not significantly different in STZ-induced diabetic rats injected with *Ad.Null* (n=24) or saline (n=20). Therefore, both control diabetic groups were pooled in SD-STZ. Apo A-I gene transfer significantly improved vascular reactivity to acetylcholine in STZ-induced diabetic rats to control levels ($P<0.05$; Figure S2A). Endothelium-independent papaverine-induced relaxations were not different among all of the groups (Figure S2B).

Discussion

Our study reveals the downregulation of the AT1R as a novel vascular-protective mechanism of HDL, as demonstrated in vivo in the aorta of STZ-induced diabetic rats and in vitro in HAECs. We postulated that the reduction in aortic AT1R expression after apo A-I transfer is a significant mediator of subsequent reduced NOX activity and reduced eNOS uncoupling leading to improved endothelial function in experimental diabetes mellitus.

Under diabetic conditions, the renin-Ang system is activated and vascular AT1R expression is enhanced,[11,22] thereby contributing to diabetes mellitus–associated endothelial dysfunction.[12] Given the known vascular (endothelial)-protective effects of HDL,[13–15] we hypothesized that HDL could also regulate vascular AT1R expression under diabetic conditions and could thereby influence Ang II–mediated signaling. To investigate this hypothesis, we increased HDL in vivo via human apo A-I gene transfer, leading to hepatocyte-specific human apo A-I expression in STZ-induced diabetic rats and supplemented HDL to HAECs under HG in the presence or absence of Ang II. In vivo, we chose a gene transfer strategy, because currently available drugs, such as fibrates, nicotinacids, and statins, only moderately and not exclusively increase HDL.[23] The STZ-induced diabetes mellitus model is charac-

terized by severe HG and is, in contrast to other diabetic animal models, not associated with reduced HDL levels.[24,25] Apo A-I gene transfer did not affect glucose or LDL cholesterol levels, 2 factors known to influence *AT1R* expression and reduced aortic AT1R mRNA in STZ-induced diabetic rats to levels similar to nondiabetic controls. In addition, a dose-dependent effect of HDL in attenuating HG-induced elevated AT1R expression was observed. Under normoglycemic conditions, downregulation of physiological AT1R expression was only observed at 25 μg/mL, the lowest investigated dose, indicating a narrow concentration window in which HDL has an effect on AT1R expression under nonpathological conditions.

Because the expression levels of the AT1R define the biological efficacy of Ang II, HDL-mediated downregulation of the AT1R observed in the aorta of STZ-induced diabetic rats and in HAECs under HG reduces subsequent Ang II–mediated signaling. Consistent with the previously demonstrated role of the AT1R in mediating increased NOX activity and eNOS uncoupling in diabetes mellitus,[7] reduction of AT1R by increased HDL cholesterol is likely the predominant mediator of decreased expression of the NOX components p22[phox] and Nox 4, reduced NOX activity, and decreased eNOS uncoupling in vivo. This is further corroborated by the in vitro experiments in the current study. Here, the HDL-mediated downregulation of the AT1R in HAECs was associated with a decrease in HG-induced oxidative stress, indicated by reduced 2'-7'-dichloro-dihydrofluorescein fluorescence and reduced NOX activity. In addition, the responsiveness to Ang II was directly evaluated. In agreement with reduced AT1R expression levels, HDL decreased the HG-induced response to Ang II, as indicated by significantly lower 2'-7'-dichloro-dihydrofluorescein fluorescence and NOX activity in the HG+HDL+Ang II group compared with the HG+Ang II group. Our finding that HDL reduces AT1R expression and subsequent Ang II–mediated signaling supports the recent observation of Tölle et al,[16]
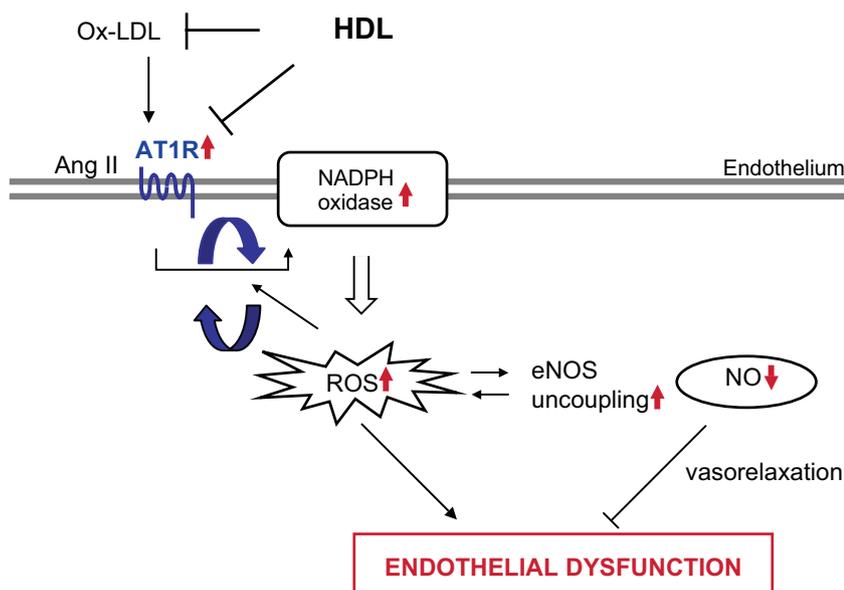
Diabetes mellitus

Figure 5. Hypothetical scheme representing how HDL via AT1R regulation improves endothelial dysfunction in diabetes mellitus. HDL reduces the upregulated AT1R under diabetes mellitus, leading to reduced Ang II–mediated signaling. This results in decreased NAD(P)H oxidase activity and eNOS uncoupling and subsequent reduced ROS formation. ROS induce AT1R expression in endothelial cells, implying that when ROS are reduced, subsequently, less AT1R expression is induced, leading to less ROS formation, consistent with an amplification mechanism. Intrinsic antioxidative features of HDL, including the reduction in oxidized (ox)-LDL, known to induce AT1R expression in endothelial cells, may further contribute to the downregulation of the AT1R. The HDL-mediated reduction in oxidative stress and the increase in NO bioavailability finally lead to an improvement in diabetes mellitus–associated endothelial dysfunction.

who demonstrated that HDL decreases NOX-dependent ROS generation via inhibition of the activation of Rac1, which is a downstream AT1R-dependent mediator of Ang II.[26]

We suggest that reduced peroxynitrite formation as a result of lower NOX activity,[27] after apo A-I transfer, decreased eNOS uncoupling and improved NO bioavailability, as evidenced by improved endothelial function. In addition, the increased eNOS dimer:monomer ratio, as a consequence of reduced NOX activity,[28] may also have contributed to enhanced NO bioavailability, because oxygen reduction is always uncoupled from NO formation in monomers. Increased eNOS expression under HG or diabetes mellitus[5,29–31] has been considered to represent a feedback response to reduced NO bioavailability.[32] Apo A-I transfer in STZ-induced diabetes mellitus and HDL supplementation to HAECs under HG decreased the diabetes mellitus– or HG-induced eNOS expression, suggesting that this feedback mechanism was reversed by increased NO bioavailability induced by increased HDL cholesterol.

The exact mechanism by which HDL affects AT1R regulation under diabetes mellitus requires further fundamental studies. Because oxidized LDL[17] and ROS[33] play a role in the induction of the AT1R in HAECs, it is tentative to postulate that HDL via intrinsic antioxidative features may contribute to the downregulation of the AT1R under diabetes mellitus, which results in less NOX activity and ROS formation, and, in turn, may reduce AT1R expression (see Figure 5). We demonstrated previously that apo A-I transfer in STZ-induced diabetic rats reduced systemic oxidative stress, as indicated by decreased plasma thiobarbituric acid reactive substances.[34] This effect is likely mediated by an increase in activity of paraoxonase or platelet activating factor-acetylhydrolase, 2 enzymes with antioxidative features, associated with HDL, and known to be increased after apo A-I gene transfer.[35] However, the recent finding that sphingosine-1-phosphate and sphingosylphosphorylcholine, 2 lipid components of HDL without antioxidative properties, mimicked the capacity of HDL to reduce ROS generation[16] suggests that other intrinsic features of HDL may contribute to the downregulation of the AT1R.

In conclusion, we define the downregulation of the AT1R as a novel additional vascular protective effect of HDL. We thereby further strengthen the existence of a cross-link between HDL and the renin-Ang system.

Perspectives

We demonstrated in vivo and in vitro in HAECs HDL-mediated downregulation of the AT1R, which took place in combination with reduced NOX activity and improved NO bioavailability and resulted in improved endothelial function. The STZ model allowed us to investigate the effect of an increase of HDL on AT1R regulation, independent of alterations in glucose and LDL cholesterol levels. On the other hand, we have to take into account that the characteristic lipid profile and severe HG of the STZ model disable a direct translation of our findings to type II diabetic animal models and diabetic patients.

Disclosures

None.

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ONLINE SUPPLEMENT

Vascular-protective effects of HDL include the down-regulation

of the AT1 receptor

Running title: HDL and AT1 receptor

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Supplemental Methods

Animals

All animal experiments were conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Ethical Committee of LAGeSo of Berlin (Germany). Eight weeks old male Sprague Dawley (SD) rats (300-330 g, Charles River Laboratories, Wilmington, MA, USA) were maintained on a 12 h light/dark cycle and fed with a standard chow *ad libitum* (n=8/group). Diabetes mellitus was induced by a single injection of streptozotocin (STZ; 70 mg/kg; i.p.) prepared in 0.1 M sodium citrate buffer, pH 4.5 (Sigma, Steinheim, Germany) as described in detail previously.[1] Only rats with blood glucose levels $\geq$ 300 mg/dl 4 days (d) after STZ injection were included in the study. Intravenous gene transfer was performed 5 d after STZ-injection in diabetic rats with 3 x 10^{12} particles/kg of the E1E3E4-deleted adenoviral vector *Ad.hapoA-I* (containing the 1.5 kb hepatocyte-specific *human α_1-antitrypsin* promoter upstream of the genomic human *apo A-I* sequence and 4 copies of the human *apo E* enhancer), that induces hepatocyte-specific expression of human apo A-I in the absence of significant hepatotoxicity[2], or of the control vector *Ad.Null* (that does not contain an expression cassette).[2] Age-matched non-diabetic SD rats injected with the same dose of *Ad.Null* were used as controls. Blood was withdrawn by retro-orbital puncture at day 12 and 20 after gene transfer for determination of human apo A-I concentrations and at the day of sacrifice for analysis of human apo A-I concentrations and HDL-C levels.

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Human apo A-I ELISA

Human apo A-I levels were determined by sandwich ELISA as described previously.[3]

Plasma lipid and lipoprotein analyses

Lipoproteins were separated from 300 µl of plasma by density gradient ultracentrifugation essentially as previously described.[4] Plasma density was adjusted to 1.23 g/ml with NaBr and the volume was made up to 500 µl with NaBr 1.23 g/ml before transfer into Ultra-Clear (Beckman Coulter GmbH, Krefeld, Germany) centrifugation tubes. Plasma was carefully over-layered with a density gradient of 500 µl NaBr 1.21 g/ml, 750 µl NaBr 1.063 g/ml, 750 µl NaBr 1.019 g/ml, 1000 µl NaBr 1.006 g/ml and 1500 µl isotonic saline buffer. All NaBr solutions contained 0.05 % EDTA, pH 7.0 to avoid oxidation of lipoproteins during centrifugation. After 22 h of centrifugation at 30 500 rpm, fractions were isolated from the meniscus downwards. All steps were carried out at 20°C. Subsequently, total cholesterol in every lipoprotein fraction was determined enzymatically. Precipath L (Roche Diagnostics, Basel, Switzerland) was used as a standard. After 1 h of incubation at 37°C, optical density was measured at 490 nm.

Vasorelaxation studies in isolated rat aortic rings

Six weeks after gene transfer, thoracic aortae from anaesthesized male SD rats were rapidly excised, cleaned of connective tissue, and cut into rings 2 to 3 mm

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in length for organ-chamber experiments as described previously.[5] Briefly, rings were mounted on platinum hooks in 10 ml jacketed organ baths containing modified Krebs-Henseleit solution (composition, in mmol/l: NaCl 144, KCl 5.9, $CaCl_2$ 1.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and D-glucose 11.1) and 1 µmol/l diclofenac. Tension was gradually adjusted to 2 g over 1 h. The solution in the bath was maintained at 37°C with a gas mixture of 5% CO_2 and 95% O_2. Following equilibration and submaximal pre-contraction with phenylephrine (0.05 µmol/l), relaxation to increasing concentrations (10 nmol/l to 10 µmol/l) of the endothelium-dependent vasodilator acetylcholine was performed to obtain cumulative concentration-response curves. Maintenance of smooth-muscle integrity was confirmed by evaluation of endothelium-independent vasodilation to papaverine (100 nmol/l to 100 µmol/l). Vasorelaxation is expressed as percentage of pre-contraction with phenylephrine.

Western Blot and low temperature SDS-PAGE

Aortic samples were homogenized in lysis buffer containing proteinase and phosphatase inhibitors. For Western blot, an equal amount of protein (10-30 µg) was loaded into a 10% SDS-polyacrylamide gel. For the investigation of eNOS homodimer formation, non-boiled proteins were resolved by 6% SDS-polyacrylamide gel at 4°C. eNOS (BD Biosciences, San Diego, CA, USA) and β tubulin (Santa Cruz Biotechnology Inc, CA, USA) were detected by each specific antibody. The blots were visualized with chemiluminescene (ECL) system

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(Amersham Bioscience, Buckinghamshire, UK). Quantitative analysis of the intensity the bands was performed with TINA Software.

Real-time RT-PCR Quantification

Rat aortic RNA was isolated by Trizol (Invitrogen, Carlsbad, CA, USA), followed by DNAse treatment and reverse transcribed with M-MLV reverse transcriptase (Invitrogen). mRNA was isolated from HAEC using a µMACS mRNA isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's protocol and subsequently reverse transcribed with Superscript III (Invitrogen). Quantitative real-time RT-PCR (ABI PRISM® 7900 HT Sequence Detection System software version 2.2.2., Perkin Elmer) of 100 ng cDNA of rat aortic samples and 40 ng cDNA of HAEC was used to quantify rat aortic *AT1R, Nox1, Nox2 (gp91phox), Nox4, p22phox, extracellular superoxide dismutase (ec-SOD)* and *18S* cDNA levels and human *AT1R* and *ribosomal L32* cDNA levels, respectively. The sequences of the primer sets used in this study were as follows: for rat AT1R FOR: 5'-GGTTCAAAGCCTGCAAGTGAA-3' and REV: 5'-GAGTGAGCTGCTTAGCCCAAA-3', for rat Nox1 FOR: 5'-CAGTGAGGAT GTCTTCCAGTACGA-3' and REV: 5'-TTCAAGAAGG AAGCAAAGGGAGT-3', for rat Nox2 FOR: 5'-TCTCAGGCCAATCACTTTGCT-3' and REV: 5'-AGTTGG GCCGTCCATACAGA-3', for rat Nox4 FOR: 5'-ACTACTACATCCAC CAGATGTTGGG-3' and REV: 5'-TGAGGTTCAGGACAGATGCAGAA-3', for rat p22^{phox} FOR: 5´-CTCTATTGTTGCAGGAGTGCTCAT-3´and REV: 5´-GGTGGAG CCCTTTTTCCTCTT-3, for rat ec-SOD FOR: 5'-GGAGATCTGGATGGAGCT

AGGA-3' and REV: 5'-CCTGCAGACTGCGTGCAT-3' and for rat 18S FOR: 5'-TT

AAGTCCCTGCCCTTTGTACAC-3' and REV: 5'-GATCCGAGGG

CCTCACTAAAC-3'; for human AT1R FOR: 5'-AGCAACAGGAGATGAGAGT

TCCA-3' and REV: 5'-TGAAAACCGGCACGAAAAC-3' and human L32 FOR: 5'-

AGGAGAGACACCGTCTGAACAAG-3' and REV: 5'-GAACCAGGATGG

TCGCTTTC 3'.

Cell culture

HAEC (Lonza Walkersville, Walkersville, MD, USA) were cultured in EBM-2

basal medium supplemented with EGM-2 Single Quots (Lonza, USA) in the

presence of 5 mM glucose (control (C)) or 5 mM glucose with 25 – 37,5 – 50 –

100 µg protein/ml of HDL (MP Biomedicals, Irvine, CA, USA), 5 mM glucose and

25 mM mannitol (M) (osmotic control), 30 mM glucose (HG) or 30 mM glucose

with 25 – 37,5 – 50 – 100 µg/ml of HDL (HG+HDL); or HAEC were cultured as

stated elsewhere. All experiments were performed with cells from passage 4-7.

NAD(P)H oxidase activity of HAEC and aorta

Confluent HAEC were serum starved and subsequently incubated in C, HG,

HG+HDL medium for 8 h, followed by 4 h incubation with 1 µM Ang II (Bachem,

King of Prussia, PA, USA). NADPH oxidase activity of HAEC was analyzed

according to Griendling *et al.*[6] In brief, cells were washed in ice-cold PBS. Then,

cells were scraped from the plate in 1 ml of ice-cold PBS and centrifuged for 10

min at 4°C at 10 000 rpm. The supernatant was discarded and the pellet

resuspended in 90 µl of lysis buffer containing protease inhibitors (20 mmol/l monobasic potassium phosphate (pH 7.0), 1 mmol/l EGTA, aprotinin, leupeptin and phenylmethylsulfonyl fluoride). The cell suspension was mixed several times during the 30 min incubation time on ice. Then, the cell suspension was centrifugated and the supernatant/homogenate was stored on ice until use.

Snap-frozen aortic rings were homogenized in 200 µl of the above lysis buffer, followed by 30 min incubation on ice. Next, the homogenate was centrifuged, the supernatant collected and stored on ice until use.

NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescent detection of superoxide in a luminometer (Berthold Detection Systems, Oak Ridge, USA). The reaction buffer contained 1 mM EGTA, 150 mM sucrose, 500 µM lucigenin and 1 mM of NADPH (180 µl). The reaction was started by addition of 20 µl of protein homogenate. Luminescence was measured as the rate of photon counts per µg protein, following subtraction of the counts obtained from a buffer blank.

Superoxide dismutase activity

Aortic rings were homogenized in 200 µl of cold 20 mM HEPES buffer pH 7.2 containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. Next, the homogenate was centrifuged at 1 500 g for 5 min at 4°C, the supernatant was collected and stored on ice until performing the assay according to the instructions of the manufacturer (Cayman Chemical Company, Ann Arbor, MI,

USA). Absorbance was read at 450 nm. Superoxide dismutase activity (U/ml) was normalized towards protein content and represented as % of SD-*Ad.Null*.

DCF Fluorescence

Intracellular ROS in HAEC were monitored by oxidation of the cell-permeable fluorescent probe 2´-7´-dichloro-dihydrofluorescein (DCF) diacetate (DCFH-DA) (Invitrogen), which reacts with ROS to form the fluorescent product DCF [7]. Confluent HAEC ($3x10^4$/well) in a 96-well plate were serum starved and subsequently incubated in C, HG, HG+HDL medium for 8 h, followed by 4 h incubation with 1 µM Ang II. Next, medium was removed and cells were incubated with 20 µM of DCFH-DA (Invitrogen) for 45 min. After 2 washes with PBS, cells were incubated for 30 min in serum-free medium. The fluorescence intensity was then read and quantified in a Spectramax Gemini fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 485 nm excitation wavelength and 530 nm emission wavelength. Fluorescence intensity was corrected for cell count and expressed as a percentage of normal control.

To quantify the amount of cells, cells were fixated with 4% formaldehyde, washed with distillated water and incubated with cell staining buffer for 30 min. After 3 washes with distillated water, cells were incubated with 1% SDS for 1 h. Then, the absorbance of each well was read at 595 nm.

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Statistical analysis

Data are presented as mean ± SEM. Paired and unpaired Student's t tests or 1-way ANOVA when comparing multiple groups, were used for statistical analysis. Differences were considered to be significant at $p < 0.05$.

Supplementary References

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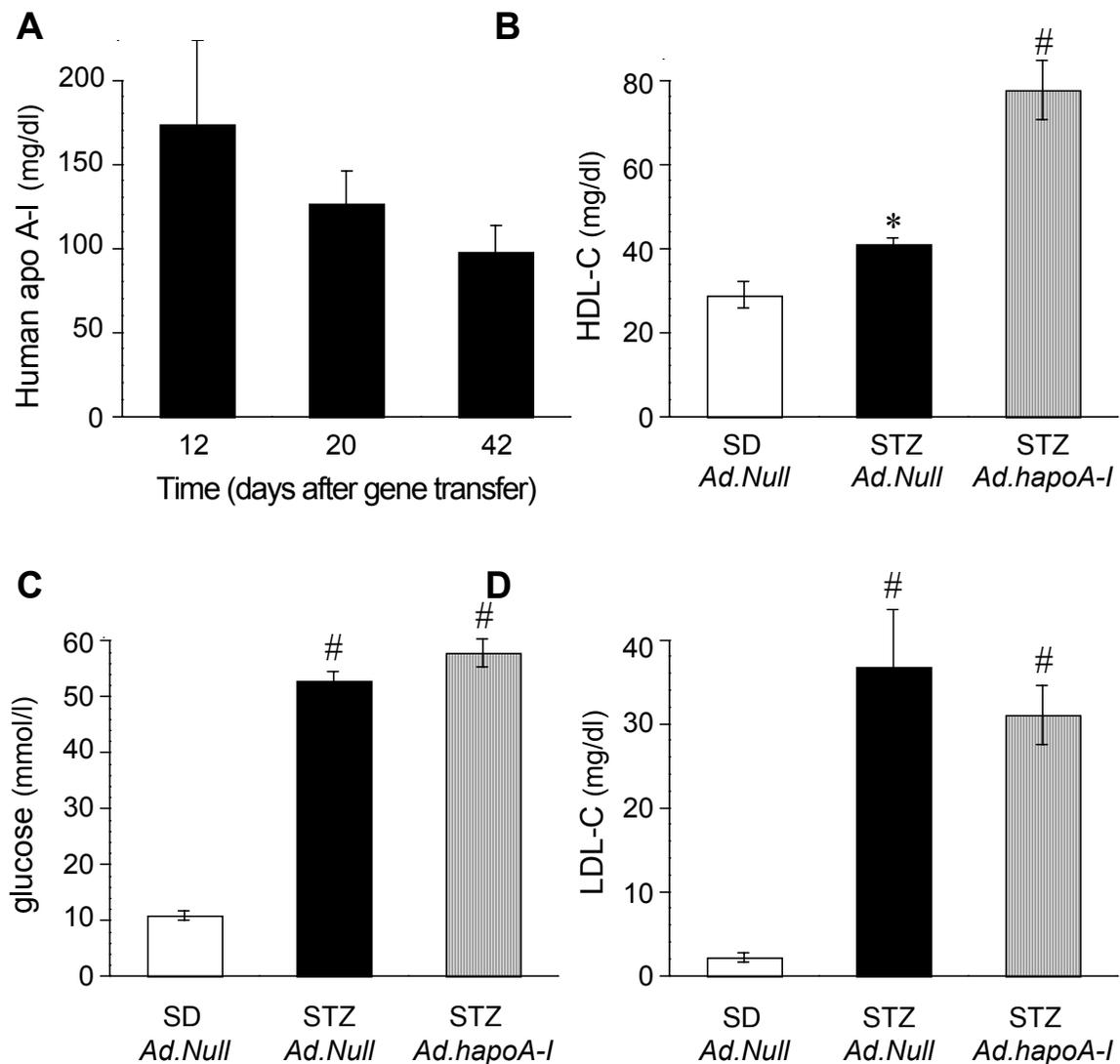
Figure S1

Figure S1. Human apo A-I gene transfer increases HDL cholesterol levels in the absence of altered glucose and LDL cholesterol levels. A) Time course of human apo A-I expression in streptozotocin-(STZ) induced diabetic rats (n=5); **B)** HDL cholesterol (C) levels; **C)** glucose levels and **D)** LDL-C levels in SD-*Ad.Null*, STZ-*Ad.Null* and STZ-*Ad.hapoA-I* 42 days after gene transfer. Data are represented

as the mean $\pm$ SEM (n=5); * p<0.01 vs. SD-*Ad.Null* and STZ-*Ad.hApoA-I*, #
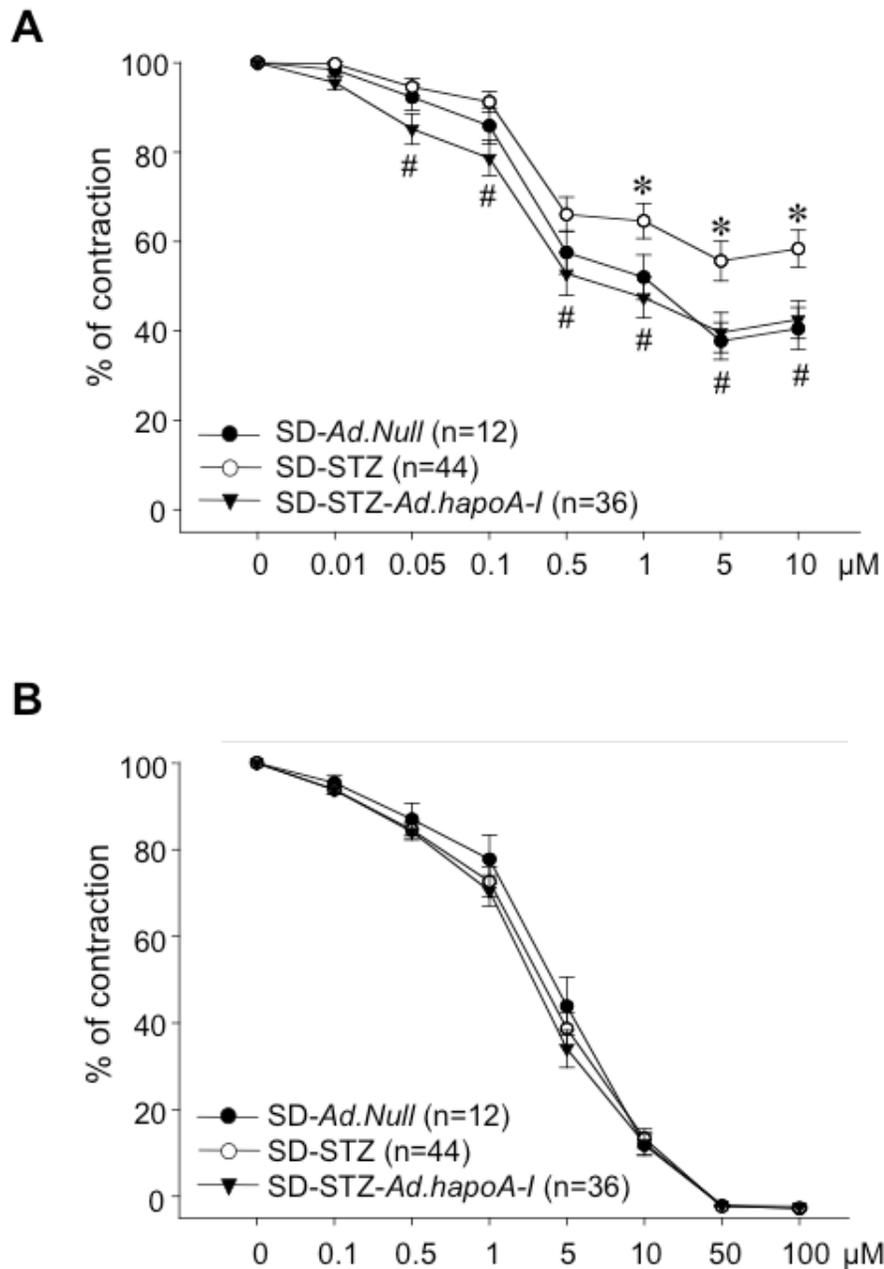
p<0.001 vs. SD-*Ad.Null.*

Figure S2

Figure S2. Human apo A-I gene transfer improves diabetes-associated endothelial dysfunction. **A)** Acetylcholine-induced endothelial-dependent relaxation and **B)** papaverine-induced endothelial-independent relaxation. * $p < 0.05$ vs. SD-*Ad.Null*, # $p < 0.05$ vs. SD-STZ.