Glucose-independent improvement of vascular dysfunction in experimental sepsis by dipeptidyl peptidase-4 inhibition

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Aims
Dipeptidyl peptidase-4 (DPP-4) inhibitors are a novel class of drugs for the treatment of hyperglycaemia. Preliminary evidence suggests that their antioxidant and anti-inflammatory effects may have beneficial effects on the cardiovascular complications of diabetes. In the present study, we investigate in an experimental sepsis model whether linagliptin exerts pleiotropic vascular effects independent of its glucose-lowering properties.

Methods and results
Linagliptin (83 mg/kg chow for 7 days) was administered in a rat model of lipopolysaccharide (LPS) (10 mg/kg, single i.p. dose/24 h)-induced sepsis. Vascular relaxation, reactive oxygen species (ROS) formation, expression of NADPH oxidase subunits and proinflammatory markers, and white blood cell infiltration in the vasculature were determined. Oxidative burst and adhesion of isolated human neutrophils to endothelial cells were measured in the presence of different DPP-4 inhibitors, and their direct vasodilatory effects (isometric tension in isolated aortic rings) were compared. In vivo linagliptin treatment ameliorated LPS-induced endothelial dysfunction and was associated with reduced formation of vascular, cardiac, and blood ROS, aortic expression of inflammatory genes and NADPH oxidase subunits in addition to reduced aortic infiltration with inflammatory cells. Linagliptin was the most potent inhibitor of oxidative burst in isolated activated human neutrophils and it suppressed their adhesion to activated endothelial cells. Of the inhibitors tested, linagliptin and alogliptin had the most pronounced direct vasodilatory potency.

Conclusion
Linagliptin demonstrated pleiotropic vasodilatory, antioxidant, and anti-inflammatory properties independent of its glucose-lowering properties. These pleiotropic properties are generally not shared by other DPP-4 inhibitors and might translate into cardiovascular benefits in diabetic patients.

Keywords
DPP-4 inhibitors • Lipopolysaccharide • Sepsis • Endothelial dysfunction • Oxidative stress

1. Introduction
Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance and beta-cell dysfunction, leading to hyperglycaemia and secondary micro- and macrovascular complications. The incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide, are secreted by the enterocendocrine cells of the gut in response to the ingestion of carbohydrate-containing nutrients. These hormones are key modulators of pancreatic islet hormone secretion and, thus, of glucose homeostasis. The glucoregulatory effects

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of incretins are the target for new therapies that have recently been developed for the treatment of T2DM. Drugs that inhibit dipeptidyl peptidase-4 (DPP-4), a ubiquitous enzyme that rapidly inactivates both GLP-1 and insulinotropic polypeptide, increase the active levels of these hormones and thereby improve islet function and glycemic control in T2DM. In addition, there is some evidence that these compounds possess antioxidant and anti-inflammatory effects that may beneficially influence cardiovascular disease, the leading cause of morbidity and mortality in T2DM patients. In recent reports, sitagliptin displayed anti-inflammatory effects in an animal model of T2DM, and alogliptin showed acute direct vasodilatory effects in isolated vessels. More importantly, the chronic treatment with alogliptin and sitagliptin reduced atherosclerosis and inflammation via effects on monocyte recruitment and chemotaxis in two models of experimental atherosclerosis (LDLR−/− and ApoE−/− mice).

T2DM has long been associated with endothelial dysfunction and vascular oxidative stress. Uncoupled endothelial nitric oxide synthase (eNOS), the mitochondrial respiratory chain, NADPH oxidases, and xanthine oxidase have been identified as significant sources of vascular reactive oxygen species (ROS) formation that can trigger endothelial dysfunction. Diabetic cardiovascular complications are at least partially mediated by inflammation and oxidative stress-mediated mechanisms. The objective of the present study was to investigate effects of the DPP-4 inhibitor, linagliptin, on vascular function, oxidative stress, and inflammation that are independent of its effects on glucose homeostasis. To this end, a model of maximal oxidative stress based on lipopolysaccharide (LPS)-induced sepsis was selected for study. We also compared the antioxidant effects as well as vasodilatory and anti-inflammatory properties of linagliptin with those of alogliptin, vildagliptin, saxagliptin, and sitagliptin in vitro and in vivo. Differences in the molecular structure of these different DPP-4 inhibitors might confer distinct pleiotropic effects independent of their effect on glucose (see Supplementary material online, Figure S1).

2. Methods

2.1 Materials

For isometric tension studies, glyceryl trinitrate (GTN) was used from Sigma-Aldrich (Munich, Germany). L-012 (8-amino-5-chloro-7-phenylpyrazido[3,4-d]pyridazine-1-(2H,3H)dione sodium salt) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Fluka or Merck.

2.2 Effects of linagliptin in vivo treatment on LPS-induced endothelial dysfunction and systemic oxidative stress

All the animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health, and permissions for the study were granted by the University Hospital Mainz Ethics Committee and the Legislator (Landesuntersuchungsamt Koblenz, Germany). The anti-inflammatory potential of linagliptin was tested in an experimental model of LPS (10 mg/kg i.p. for 24 h)-induced septic shock in male Wistar rats (400 g; 4 months; Charles River Laboratories, Sulzfeld, Germany). The rats were sacrificed by exsanguination under isoflurane anaesthesia (5% inhalant in room air) 7 days after oral linagliptin therapy and 24 h after LPS injection. The effects of sepsis and oral linagliptin co-therapy (starting 6 days before LPS injection; 83 mg/kg chow, which corresponds to plasma levels of ~100 nM and a 5 mg/kg oral dose) were assessed by isometric tension (aortic ring preparation) recordings and vascular as well as leucocyte-derived reactive oxygen and nitrogen species (RONS) formation detected using fluorescence- and chemiluminescence-based methods. Vasodilator formation from NO synthase (most probably the inducible isoform) was determined in serum by the nitrosation product of 2,3-diaminomorpholine, naphthyltriazole, by HPLC as reported elsewhere and by the photoreaction of S-nitrosothiols with the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and subsequent immunostaining with anti-DMPO. Expression of mRNA was assessed by quantitative real-time RT–PCR using TaqMan® Gene Expression assays. Protein expression was determined by western blotting. Vascular oxidative stress was also assessed by dot blot analysis of vascular tissues with the detection of nitrated and malondialdehyde (MDA)-positive proteins. The activity of DPP-4 was assessed using a specific peptide substrate with a terminal coumarin derivative (H-Ala-Pro-7-amido-4-trifluoromethycoumarin; Bachem, Bubendorf, Switzerland; final assay concentration, 100 μM) allowing quantification in a fluorescence microplate reader upon cleavage by DPP-4. A commercially available enzyme-linked immunosorbent assay kit was used to measure rat serum GLP-1-(7–36) amide levels (Linco Research, Inc., St Charles, MO, USA). Co-immunoprecipitation of adenosine deaminase (ADA) and DPP-4 was performed as described. For detailed protocols, see Supplementary material online.

2.3 Determination of myelomonocytic and dendritic cell infiltration by FACS analysis of rat aortic cells

As described by the manufacturer’s instructions, we isolated total aorta white blood cells (WBCs) by performing a complete protease/collagenase digestion step for 45 min up to 1 h at 37°C by using Liberase TM (26 U/mL; Roche, Basel, Switzerland). After the subsequent filtering of the cells by using a 70 μm cell strainer (BD Biosciences, Heidelberg, Germany), the cells were washed in Hank’s balanced complete medium, 0.06% BSA, 0.3 mM EDTA. After a centrifugation step, wells were washed in PBS including 2% FCS before the cell number had been determined. For FACS analysis of the aortic single-cell suspension, we used 5 × 10⁵ cells per aorta for staining with an anti-rat CD11b/c PerCP antibody and an anti-rat CD3 FITC antibody (all labelled antibodies were purchased from e-bioscience, San Diego, CA, USA). The stained cells were measured by a Cantoll flow cytometer by using the FACSDiva software (BD Biosciences) and analysed by excluding monocytes, macrophages, granulocytes, and myeloid dendritic cells. The protocol was modified from a previous report.

2.4 Assessment of direct antioxidant properties of DPP-4 inhibitors

For detailed protocols, see Supplementary material online.

2.5 Measurement of antioxidant properties of DPP-4 inhibitors in isolated WBCs

Handling of all human material was in accordance with the Declaration of Helsinki and was approved by the local institutional Ethics Committee. In-direct antioxidant effects of DPP-4 inhibitors were measured in isolated human leucocytes in PBS (1 mM Ca²⁺/Mg²⁺) by interfering with the oxidative burst (NADPH oxidase activation) induced by phorbol ester dibutyrate (PDBu, 1 μM), the endotoxin LPS or zymosan A (each 0.5, 5 and 50 μg/mL), and the chemotactic peptide formyl-methylionyl-leucyl-
phenylalanin (50 μM; fMLP). Polymorphonuclear leucocytes (neutrophils, PMNs) and lymphocytes/monocytes (buffy coat fraction, WBCs) were isolated by sedimentation of red blood cells with dextran and centrifugation on Ficoll as described previously. Total blood cell count and the purity of the fractions were evaluated using an automated approach using a haematology analyser KX-21N (Sysmex Europe GmbH, Norderstedt, Germany). The typical constitution of the blood cell fractions obtained by this method was recently published. The activation of WBCs was quantified by ROS formation during oxidative burst in response to PDBu, LPS, zymosan A, or fMLP using L-012 (100 WBCs) was quantified by ROS formation during oxidative burst in a Centro plate reader (Berthold Technology, Bad Wildbad, Germany). All other incubation conditions are provided in the figure legends.

2.6 Determination of the effects of linagliptin on PMN adhesion to endothelial cells

The antioxidant/anti-inflammatory effects of linagliptin were also tested by quantification of PMN adhesion to cultured endothelial cells (EA.hy 926 cells, a kind gift of C.J. Edgell, University of North Carolina at Chapel Hill, NC, USA) by different methods. The culture conditions were previously published. After the incubation of the endothelial cells in six-well plates with PMNs (1 × 10⁵, 2.5 × 10⁵, and 5 × 10⁵ PMN/mL) and LPS (0.5, 5, and 50 μg/mL) for 30 min, the endothelial cells were washed twice with warm PBS. Adherent PMNs were quantified by PDBu (1 μM)-triggered ROS formation [measured by amplex red (100 μM) plus horseradish peroxidase (0.1 μM)-enhanced chemiluminescence (ECL) on a Centro plate reader (Berthold Technology, Bad Wildbad, Germany). All other incubation conditions are provided in the figure legends.

2.7 Direct vasodilatory effects of DPP-4 inhibitors in isolated vessels

DPP-4 inhibitor-induced relaxation was determined by the isometric tension technique in isolated aortic ring segments. For detailed protocols, see Supplementary material online.

2.8 Statistical analysis

Results are expressed as mean ± SEM. Two-way ANOVA (with Bonferroni’s correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy (Prism 5 for Windows, GraphPad Software, Inc.). One-way ANOVA (with Holm–Sidak’s correction for comparison of multiple means) was used for comparisons of ROS detection, FACS analysis, adhesion assays, as well as protein and mRNA expression studies (SigmaStat 3.5 for Windows, Systat Software, Inc.). P-values <0.05 were considered to indicate statistical significance. All tests were two-sided.

3. Results

3.1 Effects of oral linagliptin treatment on vascular dysfunction and oxidative stress in septic rats

Body weight was not different between groups, and blood glucose significantly changed neither between the control and the LPS group nor between the LPS and the LPS plus linagliptin therapy group (see Supplementary material online, Figure S2). The only significant difference in blood glucose was observed between the control and the LPS plus linagliptin therapy group, keeping in mind that all values were still in the normoglycaemic range. This, however, shows that improvement of septic complications by linagliptin was not based on changes in glycaemia.

Vascular function [acetylcholine (Ach), GTN, and diethylamine NONOate-dependent relaxation] was largely impaired by single LPS administration and normalized by linagliptin therapy (Figure 1A–C). Mitochondrial and whole-blood (PDBu and zymosan A-stimulated) RONS production was substantially increased by LPS and reduced

Figure 1 Effects of oral linagliptin therapy on vascular function in LPS-induced sepsis. Vascular function was determined by isometric tension recording and relaxation in aortic ring segments in response to endothelium-dependent (ACh, A), endothelium-independent with requirement of bioactivation (GTN, B), and endothelium-independent without requirement of bioactivation (diethylamine NONOate, C) vasodilators in aortic ring segments from control, LPS, and LPS/linagliptin-treated rats. The data are mean ± SEM of 16–28 aortic ring segments from five to seven animals/group. *P < 0.05 vs. control and #P < 0.05 vs. LPS. Statistic analysis was performed by two-way ANOVA; for the sake of clarity, only one significance symbol is shown although at least four data points showed significant differences.
by linagliptin treatment (Figure 2A–C). Serum xanthine oxidase activity was increased in septic rats and normalized by linagliptin therapy (Figure 2D). Vascular oxidative stress [measured by dihydroethidine (DHE)-dependent fluorescent microtopography, protein nitration, and MDA staining] was increased in septic aorta and normalized by linagliptin (Figure 3D and Supplementary material online, Figure S3A and B), and was associated with an attenuation of the LPS-induced increase in aortic expression of eNOS, Nox1, and Nox2 isoforms of the NADPH oxidase (Figure 3C). Exposure to linagliptin was also associated with a reduction in the aortic expression of the LPS-induced inflammation markers VCAM-1, Cox-2, and NOS-2 in the aorta (Figure 3B), suggesting that antioxidant and anti-inflammatory properties of linagliptin preserve endothelial function, e.g. via decreased peroxynitrite formation but also via suppressed desensitization of the NO/cGMP signalling pathway by chronic nitric oxide challenges from NOS-2. This assumption is supported by the serum nitrite and S-nitrosothiol levels. These products of NO synthase activity (most probably the inducible isoform) were increased in septic rats and partially normalized by linagliptin therapy (Supplementary material online, Figure S4). This provides rather firm confirmation of the ‘desensitization hypothesis’, although our NOS-2 mRNA data cannot be directly translated to the protein level since NOS-2 is also regulated at the translational level.

The activity of serum DPP-4 and serum levels of GLP-1 were determined to confirm the successful oral delivery of linagliptin. There was a significant increase in serum DPP-4 activity in the septic model (LPS-challenged) rats, which was decreased below control levels by linagliptin therapy (Figure 3A). However, levels were markedly reduced below those seen in controls in the linagliptin-treated animals. Consequently, exposure to linagliptin was associated with significant increases in serum GLP-1 levels (Figure 3A). Increased DPP-4 activity was also envisaged by co-immunoprecipitation of ADA with DPP-4 (see Supplementary material online, Figure S5).

The infiltration of CD11b/c1+ cells, including the most of antigen-presenting cells (monocytes, macrophages, granulocytes, and dendritic cells), into the aortic tissue was significantly increased by LPS treatment but was not as marked and did not achieve statistical significance (vs. control) when combined with linagliptin therapy (Figure 4A). It should be noted that the difference between the LPS and LPS/linagliptin group was not significant. An additional readout for the infiltration of these immune cells into the vascular tissue was based on the quantification of myeloperoxidase (MPO) expression, an enzyme predominantly expressed in granulocytes (e.g. PMNs). The expression of MPO was increased by LPS and normalized by linagliptin (Figure 4B).

3.2 Direct antioxidant properties of DPP-4 inhibitors (not shown)

We investigated direct and indirect antioxidant as well as the anti-inflammatory effects of this DPP-4 inhibitor compared with other inhibitors. All of the DPP-4 inhibitors tested showed little more...
than marginal direct antioxidant capacity. In vitro, linagliptin alone was associated with consistent though minor suppression of peroxynitrite. However, superoxide formation from purified xanthine oxidase was not significantly modified by any of the drugs tested. With the exception of saxagliptin, all the DPP-4 inhibitors showed a significant degree of interference with 1-electron-oxidations by the hydrogen peroxide/peroxidase system, with linagliptin being the most potent compound tested.

### 3.3 Indirect antioxidant properties of linagliptin in isolated human leucocytes

Linagliptin was the most potent inhibitor of oxidative burst in isolated human leucocytes in response to NADPH oxidase activation by LPS and zymosan A. The incubation of neutrophils with LPS (0.5, 5, and 50 μg/mL) increased the RONS signal in a dose-dependent fashion and the response was suppressed in a concentration-dependent fashion by linagliptin (Figure 5A). Linagliptin was the most potent drug in suppressing LPS-triggered oxidative burst in isolated PMNs, whereas sitagliptin, alogliptin, saxagliptin, and vildagliptin appeared to have little or no effect (Figure 5B). Furthermore, linagliptin demonstrated the most pronounced inhibitory effect on zymosan A-induced RONS formation. Similar results were obtained when using isolated monocytes/lymphocytes extracted from theuffy coat sample fraction instead of isolated PMNs (Supplementary material online, Figure S6A). When the number of immune cells in the assay was decreased, linagliptin showed half-maximal inhibition of the oxidative burst in stimulated cells already at 2 μM (data not shown).

The adhesion of PMNs to cultured endothelial cells was tested in the presence of LPS by quantification of the phagocytic NADPH oxidase subunit p67phox. This protein is expressed magnitudes higher in PMNs compared with EA.hy 926 cells. The number of
adherent PMNs was augmented with increasing levels of leukocytes/LPS and decreased in a linagliptin concentration-dependent fashion (Figure 5C and Supplementary material online, Figure S6B). Qualitatively similar results on the inhibitory effects of linagliptin on the adhesion of PMNs to EA.hy 926 cells were obtained by visualization of adherent PMNs by Turk’s or carboxyfluorescein staining or quantification of the oxidative burst of adherent PMNs upon PDBu stimulation (Figure 5D).

3.4 Direct vasodilatory effects of DPP-4 inhibitor

Isometric tension recording demonstrated that several DPP-4 inhibitors have direct vasodilatory effects on aortic tissue in the concentration range of 10–100 μM (Supplementary material online, Figure S7A and B). In this respect, linagliptin was the most potent compound directly followed by alogliptin and vildagliptin, whereas sitagliptin and saxagliptin expressed vasodilatory properties that were similar to that of the dimethyl sulfoxide vehicle control. Classical inhibitors of the NO/cGMP pathway also impaired linagliptin-induced relaxations (Supplementary material online, Figure S7C and D). The subsequent ACh- and GTN-dependent relaxations (in response to acute incubations with gliptins) were enhanced by the in vitro pretreatment with DPP-4 inhibitors (not shown).

4. Discussion

We demonstrated that the DPP-4 inhibitor, linagliptin, exhibits powerful antioxidant and anti-inflammatory effects that are independent of its glucose-lowering properties. Although it had only minor direct antioxidant properties, linagliptin had an appreciable effect on endotoxin-dependent activation of isolated leucocytes. In vivo, linagliptin (at pharmacologically relevant doses) normalized vascular dysfunction and reduced oxidative stress as well as inflammation associated with LPS-induced septic shock. Whether or not these powerful antioxidant and anti-inflammatory properties translate into clinical benefit, perhaps by way of decreased vascular complications, in diabetic patients needs to be elucidated.

Considering the marked structural differences between DPP-4 inhibitors (Supplementary material online, Figure S1), it is not surprising that these drugs may demonstrate distinct pleiotropic effects beyond their pharmacological actions on DPP-4. For example, linagliptin contains a methylxanthine structure, which might suggest the direct inhibitory effects on the xanthine oxidase activity (not
shown). This action may be clinically relevant; methylxanthine derivatives (e.g. caffeine) have direct cardiovascular effects (e.g. by antagonizing the adenosine-1-receptor action, conferring diuretic effects). Whether adenosine-1-receptor inhibition contributes to the observed direct vasodilatory effects of linagliptin remains to be established. However, a direct vasodilatory property has been reported for alogliptin, which was almost as potent as that of linagliptin in the present studies. These authors reported that the alogliptin-dependent relaxation was not affected by a GLP-1 receptor antagonist, but partially inhibited by endothelial denudation, a soluble guanylate cyclase inhibitor or an NOS inhibitor and was completely abolished by a combination of the NOS inhibitor and the potassium channel blockers charybdotoxin plus apamin. Cell culture experiments revealed that alogliptin-induced activation of eNOS is mediated by Akt-mediated phosphorylation at serin1177 and activation of Src kinase resulting in increased nitric oxide formation. The data of the present study show that in large conductance vessels, linagliptin-induced vasodilation is, like ACh- and alogliptin-induced vasodilation, mainly due to the NO/cGMP signalling pathway. The inhibition of adenosine receptors by theophylline had no significant effects on linagliptin-induced vasodilation. 

Figure 5 Effects of DPP-4 inhibitors on activation of WBCs. (A) Quantification of oxidative burst in isolated human PMNs \( (5 \times 10^5 \text{ cells/mL}) \) with increasing LPS and linagliptin concentrations by ECL using the luminol analogue L-012 \( (100 \mu M) \). (B) Quantification of oxidative burst in isolated human PMNs \( (1 \times 10^5 \text{ cells/mL}) \) upon stimulation with LPS or zymosan A \( (50 \mu g/mL) \) and different DPP-4 inhibitors concentrations by ECL using the luminol \( (200 \mu M) \) and horseradish peroxidase \( (0.1 \mu M) \) assay. *\( p < 0.05 \) vs. PMN group; #\( p < 0.05 \) vs. respective LPS/ZymA stimulation. (C) EA.hy 926 cells were incubated with PMNs \( (5 \times 10^5 \text{ and } 20 \times 10^5 \text{ cells/mL}) \) and LPS \( (20 \text{ and } 50 \mu g/mL) \) termed as middle and high conditions. After 30 min, cells were washed and adherent PMNs were quantified upon lysis by the content of p67phox protein, which is not expressed by EA.hy 926 cells. *\( p < 0.05 \) vs. respective control group (blue); #\( p < 0.05 \) vs. respective higher PMN number (red); %\( p < 0.05 \) vs. respective 10 \( \mu M \) linagliptin treatment group. (D) EA.hy 926 cells were incubated with PMNs \( (20 \times 10^5 \text{ cells/mL}) \) and LPS \( (50 \mu g/mL) \). After 30 min, cells were washed and adherent PMNs were quantified by phorbol ester-induced oxidative burst measured by amplex red \( (100 \mu M) \) plus peroxidase \( (0.1 \mu M) \) fluorescence or Turk’s (undiluted) and CF-DA (20 \( \mu M \)) staining. Linagliptin \( (LG) \) concentration was 100 \( \mu M \) for amplex red and 20 \( \mu M \) for staining assays. Magnification was 20-fold; the scale bar represents 200 \( \mu m \). *\( p < 0.05 \) vs. control group (blue); %\( p < 0.05 \) vs. PMNs/LPS group. The data are mean ± SEM of at least six \( (A \text{ and } B) \), four \( (C) \), or 16 ECL and three staining \( (D) \) independent experiments/group.
effect on ACh- and linagliptin-dependent relaxation. In contrast to that mentioned above, we observed no effect of the potassium channel blocker charybdotoxin alone on ACh- and linagliptin-conferred relaxation.

For short-term in vitro challenges with different gliptins, we observed an improvement of subsequent ACh-mediated vasodilation and even improvement of nitroglycerin-dependent relaxation (not shown). This observation, at a first sight, seems to be at variance with the decrease (normalization) in the eNOS expression by long-term linagliptin in vivo therapy in LPS-treated rats (Figure 3C). Previous studies have demonstrated that eNOS in septic rats is upregulated and uncoupled as also supported by increased endothelial ROS formation in the micrographs (Figure 3D). Therefore, recoupling of eNOS by linagliptin and the normalization of eNOS expression would explain the improved endothelial function. Moreover, as outlined above, linagliptin itself has potent direct vasodilatory effects via the NO/cGMP signalling pathway (as reported by eNOS serin1177 phosphorylation for alogliptin), which may lead to a counter-regulatory decrease in eNOS expression. Another contributor of importance is the inducible NOS-2 yielding nitric oxide formation rates in the pathophysiological range that in the long run will cause a desensitization of the NO/cGMP signalling pathway. Previous reports demonstrated that the inhibition of NOS-2 in cytokine- and endotoxin-stimulated vessels results in complete normalization of endothelium-dependent vasodilation. Therefore, the normalization of NOS-2 mRNA by linagliptin (70% decrease compared with LPS treatment group) could significantly contribute to the improvement of vascular function in septic aorta by linagliptin therapy. This assumption is supported by the serum nitrite and S-nitrosothiol levels (see Supplementary material online, Figure S4) and provides rather firm confirmation of the ‘desensitization hypothesis’, although we do not know to what extent the mRNA data directly translate to the protein level.

It has previously been shown in a T2DM rat model that sitagliptin can express antioxidant and anti-inflammatory effects that are accompanied by a normalization of the serum C-reactive protein and interleukin-1β levels. In addition, the expression of the lipid peroxidation marker MDA in heart and pancreas tissue from diabetic rats was markedly decreased by sitagliptin therapy. In another study in obese and diabetic mice, sitagliptin normalized the expression of cytokines and lipoxygenase in adipocytes as well as the infiltration of macrophages in adipose tissue. The fact that these studies were performed in models of T2DM and obesity leaves the possibility that the antioxidant and anti-inflammatory effects of sitagliptin may have been mediated by the control of blood glucose levels rather than other pleiotropic effects.

More recently, treatment with alogliptin and sitagliptin was demonstrated to reduce atherosclerosis and inflammation in two experimental models of atherosclerosis (LDLR−/− and ApoE−/− mice) via effects on monocyte recruitment and chemotaxis. In vitro and in vivo assays of DPP-4 inhibition revealed appreciable effects on monocyte activation and migration. The authors could show that these effects are probably due to the inhibition of DPP-4 in bone marrow-derived CD11b+ cells and reduced recruitment of adipose tissue macrophages. In the present study, the increased DPP-4 levels and activity in septic rats were also translated to increased association with and activation of ADA, an enzyme that catalyses the break-down of adenosine to inosine, which might be further metabolized to

**Figure 6** Proposed mechanisms of LPS-induced vascular dysfunction and improvement by linagliptin therapy.
hypoxyanthine, providing the substrate for xanthine oxidase. This could contribute to the increase observed here in xanthine oxidase activity in septic rats.

A non-diabetes animal model was selected for use in the present study in order to discriminate potential pleiotropic effects from those solely attributable to improved blood glucose control. Experimental sepsis by LPS injection is characterized by an increase in the number of leucocytes adhering to the endothelium, leucocyte, and endothelial cell activation. Since linagliptin exerts potent inhibitory effects on the activation of leucocytes in whole blood, it is plausible to speculate that anti-inflammatory properties account for the observed improvement of (in vivo) vascular function by linagliptin therapy. In fact, we observed that classical inflammation markers as well as several important sources of oxidative stress in the vasculature were dramatically decreased by linagliptin. The oxidative burst inhibitory effects of linagliptin were even comparable with those demonstrated for the third-generation β-blocker nebivolol. This scenario is further substantiated by our finding that linagliptin has the potential to interfere directly with the infiltration of inflammatory cells into the vasculature (see what follows).

Recent studies suggest that WBCs play an important role in mediating angiotensin II-induced vascular dysfunction and hypertension. It is possible that the suppression of leucocyte activation may represent an important mechanistic aspect of how linagliptin (and to a lesser extent also other DPP-4 inhibitors) preserves endothelial function during inflammatory vascular disease. Activated leucocytes (most probably myelomonocytic cells) adhere to and infiltrate the vascular wall, leading to increased production of ROS primarily by the phagocytic NADPH oxidase within the vasculature and scavenging of nitric oxide; this diffuses freely from the endothelium to the smooth muscle cell layer. The reaction of endogenous nitric oxide with Nox2-derived superoxide has the potential to decrease the bioavailability of this important vasodilator and promote formation of the highly toxic intermediate peroxynitrite (ONOO−), as previously described for arterial hypertension and type 1 diabetes. Therefore, the interference of linagliptin with activation and recruitment of leucocytes to the vessel wall may serve as a novel protective effect. We characterized this action for isolated neutrophils and monocytes/lymphocytes by direct measurement of the LPS or zymosan A-induced oxidative burst in the presence of different DPP-4 inhibitors at different concentrations. We found that linagliptin was most effective at inhibiting the oxidative burst, but also decreased the PMN adhesion to endothelial cells. At this point, we can only speculate on the underlying mechanism of this action. It is not clear whether leucocytes express DPP-4 at an appreciable level and whether the observed effect is mediated by increased levels of GLP-1, but recent reports indicate that DPP-4 expression is increased in circulating blood monocytes from obese human subjects and authentic GLP-1 is able to reduce the monocyte chemotactic protein-1 or RANTES-triggered migration of isolated human monocytes and their release of matrix metallopeptidase 9. If this hypothesis is correct, similar anti-inflammatory effects should be obtained by the use of incretin analogues such as exenatide.

4.1 Conclusions
The results of the present study support the concept of pleiotropic antioxidant and anti-inflammatory properties of linagliptin (for summary, see Figure 6), which are independent of its glucose-lowering properties and not (or are to a minor extent) shared by other DPP-4 inhibitors. In particular, linagliptin reduced leucocyte adhesion to endothelial cells in the presence of LPS and improved sepsis-induced oxidative stress, which resulted in an improvement of endothelial function. These beneficial effects on vascular function could be in part related to the direct vasodilatory effects of linagliptin, although this property could also lead to synergistic hypotensive effects in the setting of sepsis. However, adverse effects by linagliptin-induced vasodilation are rather unlikely since the preliminary results on mortality of septic rats with and without linagliptin therapy are striking (Supplementary material online, Figure S8) and require further investigations using different LPS doses and detailed elucidation of the time points of death. Further studies have to demonstrate whether these pleiotropic antioxidant and anti-inflammatory properties of linagliptin may translate into improved clinical outcomes in diabetic patients.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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