Original

Pleiotropic Effects of Linagliptin Monotherapy on Levels of Nitric Oxide, Nitric Oxide Synthase, and Superoxide Dismutase in Hemodialysis Patients with Diabetes

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Abstract : Linagliptin is an anti-diabetic drug and the only bile-excreted dipeptidyl peptidase-4 inhibitor. Malnutrition-inflammation-atherosclerosis syndrome is an important prognostic factor for hemodialysis patients, and we previously reported anti-inflammatory effects of linagliptin in hemodialysis patients with diabetes. Inflammation can accelerate oxidative stress, vasoconstriction, and platelet aggregation. However, few studies have investigated the pleiotropic effects of linagliptin treatment on inflammation in hemodialysis patients. In this study, we have extended our previous investigations of these effects in a longer and more thorough follow-up of hemodialysis patients with diabetes. We examined 20 hemodialysis patients with diabetes who were not receiving oral diabetes drugs or insulin therapy and who exhibited inadequate glycemic control （glycated albumin levels ＞ 20%）. Linagliptin （5 mg） was administered daily, and we evaluated the patients' superoxide dismutase, 8-hydroxydeoxyguanosine, nitric oxide, nitric oxide synthase, and asymmetric dimethylarginine levels in serum at baseline and after 1, 3, and 6 months of treatment. After 6 months of treatment, superoxide dismutase levels had significantly decreased from 8.8 ± 0.5 U/ml to 7.0 ± 0.5 U/ml. Nitric oxide synthase levels were significantly increased at 3 and 6 months （maximum, 94.2 ± 13.2 µg/ml ; baseline, 31.6 ± 5.5 µg/ml）. After 3 months of treatment, nitric oxide levels had significantly increased from 64.5 ± 6.6 µmol/l to 104 ± 15.4 µmol/l, and remained significantly elevated at 6 months. Asymmetric dimethylarginine and 8-hydroxydeoxyguanosine levels did not change during the 6-month treatment course, and no patients exhibited hypoglycemia or other significant adverse effects. Linagliptin treatment significantly changed various markers of inflammation relevant to the atherosclerosis in malnutrition-inflammation-atherosclerosis syndrome. Therefore, linagliptin monotherapy has pleiotropic effects on inflammation in hemodialysis patients with diabetes, and may improve their prognosis.

Key words : inflammation, anti-oxidation, hemodialysis, linagliptin, atherosclerosis

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Introduction

Hemodialysis（HD）patients have a high prevalence of protein-energy malnutrition, inflammation, and atherosclerotic cardiovascular disease. Since these three pathophysiological conditions can occur concomitantly in HD patients via the actions of pro-inflammatory cytokines, they are referred to together as malnutrition-inflammation-atherosclerosis syndrome（MIA syndrome）. MIA syndrome is an important prognostic factor for HD patients[1]. In addition, diabetes mellitus can induce end-stage renal disease and promote the inflammatory status in MIA syndrome. Although insulin injections are central to the treatment of HD patients, eyesight failure due to diabetic retinopathy and aging-associated dementia can contraindicate multiple daily insulin injections[2]. Moreover, among HD patients, many oral anti-diabetic drugs induce critical side effects, such as hypoglycemia and lactic acidosis. Therefore, new oral anti-diabetic drugs with fewer side effects are needed for HD patients. In this context, dipeptidyl peptidase-4（DPP-4）inhibitors are well tolerated, have a lower incidence of hypoglycemia, and provide a good safety profile.

Linagliptin is an anti-diabetic drug that is also the only bile-excreted DPP-4 inhibitor, thus it does not require a reduced dose in HD patients with diabetes. We previously reported the anti-inflammatory effects of linagliptin in HD patients with diabetes, wherein after initiating linagliptin treatment, the levels of prostaglandin E2, interleukin 6, and glycated albumin（GA）decreased significantly, whereas levels of glucagon-like peptide-1（GLP-1）increased significantly[3]. Interestingly, inflammation is known to accelerate oxidative stress, vasoconstriction, and platelet aggregation, all of which are related to the atherosclerosis in MIA syndrome. Consequently, linagliptin therapy also reduces the risk of cardiovascular and cerebrovascular diseases（CCV）[4], which are associated with systemic atherosclerosis and related prognostic factors. Given the anti-inflammatory effects of linagliptin, this drug may suppress oxidative stress, vasoconstriction, and platelet aggregation, although very few studies have investigated such pleiotropic effects of linagliptin.

The present study was undertaken to investigate the pleiotropic effects of linagliptin monotherapy treatment on inflammation in HD patients with diabetes, in an extension and more thorough follow-up of our previous study[3].

Methods

Patients

For the present study, we included 20 HD patients（16 men, 65.5 ± 2.8 years old）with diabetes and inadequate glycemic control（GA levels of $> 20\%$）who were also adhering to diet and exercise therapy. We selected GA to reflect glycemic control, as hemoglobin A1c（HbA1c, the more common parameter）is artificially reduced in HD patients[5-7]. Among the 20 patients that we included, 4 had a history of insulin therapy, 8 were taking other oral anti-diabetic drugs, and 8 had been treated with both insulin and other oral anti-diabetic drugs. However, their therapy was subsequently discontinued before or after the initiation of maintenance dialysis therapy, with an average washout period of > 2 years. Thus, at the start of the study, no patient was using a prescribed oral anti-diabetic drug or receiving insulin injections, and their glycemic control had

become inadequate. In addition, no patient was taking non-steroidal anti-inflammatory drugs or allopurinol. During the treatment period, the patients received a once-daily oral dose of linagliptin（5 mg）. The ethics committee of Saiyu Soka Hospital approved the study design, and all patients provided informed consent.

Efficacy

The following efficacy parameters were examined at baseline and after 1, 3, and 6 months of treatment : oxidative stress（superoxide dismutase［SOD］activity and high sensitivity 8-hydroxy-deoxyguanosine［8-OHdG］levels）and vasodilatation and platelet aggregation dysfunction factors（nitric oxide［NO］, nitric oxide synthase［NOS］, and asymmetric dimethylarginine［ADMA］ levels）.

Safety assessments

To assess safety through this study, we undertook monitoring for all adverse events including hypoglycemic events, which were assessed via blood glucose measurements. To address possible hypoglycemic events, a 24-h treatment support system was made available to all patients, allowing them to receive immediate treatment if they experienced any symptoms of hypoglycemia. Moreover, participants underwent capillary glucose monitoring before commencing their HD treatment. Participants also underwent regular hematological and blood biochemical assessments and evaluations of their vital signs and physical condition.

Blood samples

For the present study, we evaluated the same blood samples that were used in our previous study[3]. These blood samples were taken from the arterial side of the arteriovenous fistula, before the start of HD treatment and 1−2 h before eating. Caution was exercised to prevent hemolysis of the samples ; the plasma was obtained via centrifugation and was subsequently stored at −70℃ until the analysis.

Measurement of parameters

SOD activity was measured using a batched-reagent set at a single laboratory（SRL Laboratory, Hachioji, Tokyo, Japan）via an improved nitrite method, which estimates the activity based on the decreasing rate of nitrite that is produced by hydroxylamine and superoxide anions. The patients' 8-OHdG levels were measured using the high-sensitivity 8-OHdG enzyme-linked immunosorbent assay（ELISA ; Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd., Fukuroi, Shizuoka, Japan）, which evaluates 8-OHdG as a product of oxidized DNA formed by hydroxyl radicals, singlet oxygen, and direct photodynamic action. NO levels were measured using the QuantiChrom™ Nitric Oxide Assay Kit（BioAssay Systems, Hayward, CA, USA）, which measures NO production after the reduction of nitrate to nitrite via the improved Griess method. NOS activity was measured using the EnzyChrom™ Nitric Oxide Synthase Assay Kit （BioAssay Systems, Hayward, CA, USA）, which uses a 2-step process, whereby NOS produces

Table 1.　Baseline patient characteristics

Parameter	Mean ± standard error
Age	65.5 ± 2.8
Sex (male)	16
Diabetes treatment period (years)	10.4 ± 2.1
Dialysis treatment period (years)	6.0 ± 1.9
Body mass index (kg / m^2)	21.9 ± 0.8
Smoking (n)	6
Antiplatelet drugs (n)	9
Vasodilator drugs (n)	2
HMG-CoA reductase inhibitors (n)	6
Angiotensin receptor blockers (n)	3
Hemoglobin (g / dl)	10.6 ± 0.3
Albumin (g / dl)	3.5 ± 0.1
Potassium (mEq / l)	4.4 ± 0.2
Phosphorus (mg / dl)	4.9 ± 0.2
Urea nitrogen (mg / dl)	55.8 ± 3.6
Serum creatinine (mg / dl)	9.2 ± 0.5
High-sensitivity C-reactive protein (mg / dl)	0.2 ± 0.1

HMG-CoA = hydroxymethylglutaryl coenzyme A

NO that is subsequently detected and quantified. ADMA levels were measured using reverse-phase high-performance liquid chromatography with AccQ Tag amino acid analysis. This method uses 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (a derivatizing agent), which has a derivatized amino acid yield of 100%[8].

Statistical analysis

　　JMP statistical software (version 10 ; SAS Institute, Cary, NC, USA) was used for all statistical analyses, and the results are presented as mean ± standard error. Significance was tested using the paired t-test, and differences with a *P*-value of < 0.05 were considered statistically significant.

Results

Patient history

　　Table 1 details the baseline characteristics of the 20 patients included in this study. Six patients were smokers, nine were receiving antiplatelet drugs, two were receiving vasodilator drugs, six were receiving 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, and three patients were receiving angiotensin receptor blockers. Each patient had received treatment for type 2 diabetes prior to the start of maintenance dialysis therapy, with a mean treatment period of 10.4 ± 2.1 years. Dialysis treatment was conducted three times per week in 4-h sessions, with a mean treatment duration of 6.0 ± 1.9 years. During HD treatment, the mean blood flow was 201.0 ± 4.9 ml / min (range, 160−250 ml / min), the mean volume of the dialysate was 515.0 ± 10.9 ml / min (range, 500−700 ml / min), the mean dialysis time was 4.1 ± 0.1h (range, 4−4.5 h), the
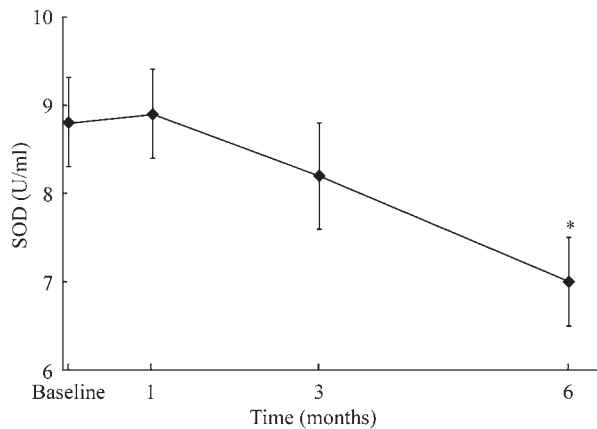
Fig. 1. Superoxide dismutase (SOD) levels before and after starting linagliptin. The patients' SOD levels decreased significantly from 8.8 ± 0.5 U / ml at baseline to 7.0 ± 0.5 U / ml after 6 months of linagliptin therapy. Values are expressed as mean ± standard error. *P < 0.05.
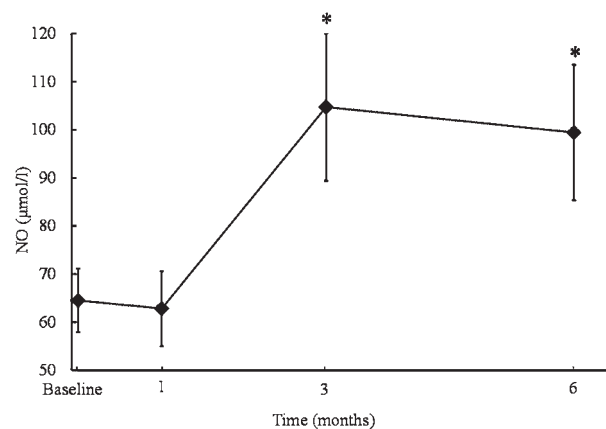

Fig. 2. Nitric oxide (NO) levels before and after starting linagliptin. The patients' NO levels increased significantly from 64.5 ± 6.6 μmol / l at baseline to 104 ± 15.4 μmol / l after 3 months of linagliptin therapy, and the NO levels remained significantly elevated at 6 months. Values are expressed as mean ± standard error. *P < 0.05.

mean membrane area was 1.8 ± 0.1 m^2 (range, 1.5–2.1 m^2), and the total glucose concentration in the dialysates was 100 mg / dl.

Effect of linagliptin on SOD, NO, and NOS levels

The patients' SOD levels decreased significantly from 8.8 ± 0.5 U / ml at baseline to 7.0 ± 0.5 U / ml at 6 months after starting linagliptin (Fig. 1). In contrast, the patients' NO levels increased significantly from 64.5 ± 6.6 μmol / l at baseline to 104 ± 15.4 μmol / l at 3 months after starting linagliptin (Fig. 2), along with their NOS levels, which reached a maximum value of 94.2 ± 13.2 μg / ml (vs. 31.6 ± 5.5 μg / ml at baseline) at 6 months (Fig. 3). There were no changes in the levels of ADMA and 8-OHdG (Table 2).

No adverse effects of linagliptin treatment

Hypoglycemia is a potential side effect of diabetes therapy, although a meta-analysis of clinical trial data revealed that only a small number of hypoglycemic events were associated with vildagliptin and sitagliptin treatment (other DPP-4 inhibitors)[9]. Notably, no patient exhibited hypoglycemia or other significant adverse effects over the 6-month course of the present study.

Discussion

Inflammation is an important prognostic factor for HD patients, and the pleiotropic effects of inflammation are relevant when treating HD patients. Herein, we confirmed and further investigated these effects, showing that linagliptin treatment significantly decreased the levels of SOD over a 6-month follow up, while increasing the levels of NO and NOS.

In our previous report on the anti-inflammatory effects of linagliptin in HD patients with
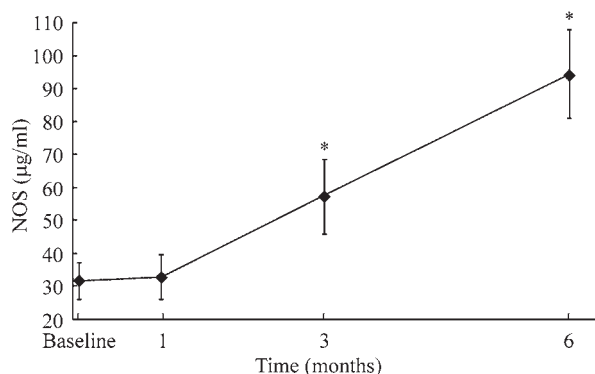
Fig. 3. Nitric oxide synthase (NOS) levels before and after starting linagliptin. The patients' NOS levels increased significantly at 3 months after starting linagliptin (57.2 ± 11.3 μg / ml vs. 31.6 ± 5.5 μg / ml), and the NOS levels increased further to 94.2 ± 13.2 μg / ml after 6 months of linagliptin therapy. Values are expressed as mean ± standard error. *$P < 0.05$.

Table 2. The response of oxidative stress markers to linagliptin therapy

Parameter	Before therapy	1 month	3 months	6 months
ADMA (nmol / ml)	0.64 ± 0.03	0.66 ± 0.03	0.69 ± 0.04	0.70 ± 0.03
8-OHdG (ng / ml)	0.34 ± 0.02	0.37 ± 0.02	0.32 ± 0.02	0.38 ± 0.03

ADMA = asymmetric dimethylarginine; 8-OHdG = high sensitivity 8-hydroxydeoxyguanosine

diabetes[3], we proposed four potential mechanisms underlying such effects: increased GLP-1, suppression of DPP-4 (CD26), an effect of the xanthine-related skeletal systems, or an anti-diabetic effect. In the present study, we confirmed the anti-inflammatory effects of linagliptin, and additionally observed a significant decrease in the levels of oxidative stress marker, SOD. In this context, the increased levels of GLP-1 could have both anti-oxidant and anti-inflammatory effects[10-12]. In addition, DPP-4 is expressed as CD26 on the membranes of various cells, including leukocytes, where it acts as an inflammatory mediator, with roles in T-cell activation, DNA synthesis, cell proliferation, cytokine production, and signaling activation. Therefore, oxidative processes might also be modulated by DPP-4 inhibition[13, 14], and a recent clinical trial involving a single daily dose of linagliptin (5 mg) administered to patients with diabetes showed that DPP-4 was inhibited by > 80% for 24 h after treatment[15]. Indeed, linagliptin exhibited stronger inhibitory activity and selectivity for DPP-4 compared to other inhibitors tested. Furthermore, among the nine types of DPP-4 inhibitors, linagliptin is the only one with a xanthine-based skeletal structure, and the pharmacological mechanisms underlying the anti-oxidant and anti-inflammatory activities associated with such a molecular structure remain unclear[16]. Thus, it is possible that this structural nature of linagliptin suppresses the degradation of cyclic adenosine monophosphate (cAMP) via phosphodiesterase inhibition (thereby increasing intracellular cAMP concentration).

Finally, hyperglycemia induces monocytes to release interleukin 6 (an inflammatory cytokine) via protein kinase C induction[17], and also stimulates pro-inflammatory cytokine production via c-Jun N-terminal kinase in monocytic THP-1 cells[18]. We have previously confirmed the antidiabetic effects of linagliptin in the patients of this study[3], finding that the GA levels, a parameter of glycemic control, were significantly decreased after starting linagliptin therapy, and there was very little difference in the effects of linagliptin therapy among the patients. Therefore, improved glycemic control may help prevent oxidative stress in HD patients with diabetes, although further studies among HD patients are needed to confirm the anti-oxidant effect of linagliptin.

In patients with type 2 diabetes, GLP-1 promotes an eNOS-mediated increase in vasodilatation, and prevents tumor necrosis factor α-induced expression of plasminogen activator inhibitor-1 and vascular cell adhesion molecule-1 in vascular endothelial cells[19, 20]. However, little is known about how linagliptin affects vasodilatation and platelet aggregation dysfunction in HD patients with diabetes. In our previous study, GLP-1 levels increased 2.5-fold after linagliptin treatment[3], and NO (which promotes vasodilatation and platelet aggregation dysfunction) increased with increasing NOS levels in the present study. In addition, ADMA (an endogenous NOS inhibitor) is dependent on GLP-1 receptor signaling[21], and the absence of any significant increase in ADMA levels in the present study might have contributed to the increased levels of NO.

Oxidative stress, vasodilatation, and suppression of platelet aggregation are other important atherosclerosis-related factors. Therefore, the decreased SOD levels and increased NO and NOS levels observed herein might also reflect an anti-atherosclerotic effect of linagliptin treatment[22, 23]. Peripheral arterial disease results from systemic atherosclerosis[24], and is an important prognostic factor for HD patients[25]. In this context, the ankle brachial pressure index (ABI) is particularly useful in functionally evaluating peripheral arterial disease, and the rate of ABI decline predicts cardiovascular mortality in HD patients[26]. Previously, we used this index in comparing a cohort of 20 HD patients after 2 years of linagliptin treatment (the linagliptin group) with 20 HD patients with diabetes who received once-daily doses of 6.25 mg alogliptin (the alogliptin group). Our unpublished results revealed a decrease in ABI from 0.97 ± 0.03 to 0.92 ± 0.07 in the linagliptin group, compared to that in the alogliptin group from 0.97 ± 0.05 to 0.83 ± 0.05. In addition, significantly fewer patients who received linagliptin (n = 6) showed a decrease in ABI, compared to the patients who received alogliptin (n = 14, $P < 0.05$). This difference suggests that not only does linagliptin have anti-atherosclerotic effects, but that such effects are stronger than those exerted by alogliptin.

This study has several important limitations that should be considered when interpreting our results. First, the present study did not include a control group and it included only a small number of participants. However, as none of the patients were receiving diabetic drugs before or after the initiation of maintenance dialysis therapy, any improvement that we observed in the parameters was likely related to the linagliptin treatment. Second, we did not observe any change in the levels of 8-OHdG (an oxidative stress marker) during the linagliptin treatment, suggesting that the anti-oxidant effect of linagliptin was not systemic. However, as SOD is the enzyme that catalyzes the conversion of superoxide into oxygen and hydrogen peroxide, the anti-

oxidant effects of linagliptin could be related to this activity. Third, there are other markers of both oxidative stress and vasodilation, and in further studies we plan to evaluate the effects of linagliptin using various other physiological markers. Moreover, we measured the enzyme activities of SOD and NOS in this study, but not the expression levels of their respective isozymes, and since each enzyme has three types of isozymes, future studies should address this issue.

In conclusion, among HD patients with diabetes, linagliptin has pleiotropic effects on inflammation that could significantly affect patient prognosis. We therefore recommend linagliptin monotherapy as a potential treatment strategy for HD patients with diabetes, given that inflammation and atherosclerosis are important prognostic factors for these patients.

Conflict of interest disclosure

None of the authors have any conflicts of interest to disclose.

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