Basic Science

**GLP-1 analog liraglutide protects against oxidative stress and albuminuria in streptozotocin-induced diabetic rats via protein kinase A-mediated inhibition of renal NAD(P)H oxidases**

Hari Hendarto, Toyoshi Inoguchi, Yasutaka Maeda, Noriko Ikeda, Jing Zheng, Ryoko Takei, Hisashi Yokomizo, Eiichi Hirata, Noriyuki Sonoda, Ryoichi Takayanagi

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Accumulating evidence has implicated that GLP-1 may have a beneficial effect on cardiovascular and renal diseases but the mechanism is not fully understood. Here we show that GLP-1 analog, liraglutide, inhibits oxidative stress and albuminuria in streptozotocin (STZ)-induced type 1 diabetes mellitus rats, via a protein kinase A (PKA)-mediated inhibition of renal NAD(P)H oxidases. Diabetic rats were randomly treated with subcutaneous injections of liraglutide (0.3 mg/kg/12 h) for 4 weeks. Oxidative stress markers (urinary 8-hydroxy-2′-deoxyguanosine and renal dihydroethidium staining), expression of renal NAD(P)H oxidase components, transforming growth factor-β (TGF-β), fibronectin and urinary albumin excretion were measured. In vitro effect of liraglutide was evaluated using cultured renal mesangial cells. Administration of liraglutide did not affect plasma glucose levels or body weights in STZ diabetic rats, but normalized oxidative stress markers, expression of NAD(P)H oxidase components, TGF-β, fibronectin and urinary albumin excretion, all of which were significantly increased in diabetic rats. In addition, in cultured renal mesangial cells, incubation with liraglutide for 48 h inhibited NAD(P)H-dependent superoxide production evaluated by lucigenin chemiluminescence in a dose-dependent manner. This effect was reversed by both PKA inhibitor H89 and adenylate cyclase inhibitor SQ22536, but not by Epac2 inhibition via its small interfering RNA. Liraglutide may have a direct beneficial effect on oxidative stress and diabetic nephropathy via a PKA-mediated inhibition of renal NAD(P)H oxidase, independently of a glucose-lowering effect.

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1. Introduction

Diabetic nephropathy is a leading cause of end-stage renal failure worldwide. Establishment of therapeutic strategies targeting the causative mechanisms of diabetic nephropathy has become increasingly urgent. In recent years, evidence has suggested that oxidative stress may play an important role in the development of diabetic nephropathy [1-4]. Among the possible sources of reactive oxygen species (ROS) production, we and other investigators have shown that NAD(P)H oxidase may be a major source in diabetic renal tissues [5-9]. NAD(P)H oxidase may therefore be a therapeutic target for attenuating ROS production in diabetic kidneys, thereby preventing the development of diabetic nephropathy.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone produced by intestinal L cells in response to food intake. It is considered as an effective therapeutic agent for type 2 diabetes mellitus because it regulates plasma glucose levels by stimulating insulin secretion and inhibiting glucagon secretion in a glucose-dependent manner. It may also have other beneficial effects, such as stimulating beta cell proliferation, protecting against beta cell apoptosis, inducing satiety, and delaying gastric emptying [10,11]. Currently, the GLP-1 receptor agonist exendin-4 [12] and the GLP-1 analog liraglutide [13] are used to treat type 2 diabetes mellitus. GLP-1 acts through the GLP-1 receptor, which is abundantly produced by intestinal L cells in response to food intake.

GLP-1 receptor agonists may have a direct beneficial effect on the development of diabetic nephropathy [5,9]. Among the possible sources of reactive oxygen species (ROS) production, we and other investigators have shown that NAD(P)H oxidase may be a major source in diabetic renal tissues [5-9]. NAD(P)H oxidase may therefore be a therapeutic target for attenuating ROS production in diabetic kidneys, thereby preventing the development of diabetic nephropathy.

The major downstream pathway of GLP-1 receptor activation is generation of the second messenger cAMP followed by activation of PKA or Epac2 [20,21]. We hypothesized that GLP-1 receptor agonists may have a direct beneficial effect on the development of diabetic nephropathy through inhibition of renal NAD(P)H oxidases because NAD(P)H oxidases have been reported to be inhibited by PKA activation in phagocytes [22,23]. In the present study, we show that the GLP-1 analog liraglutide may protect against increased oxidative stress and albuminuria in rats with streptozotocin-induced type 1 diabetes mellitus through cAMP-PKA pathway-mediated inhibition of renal NAD(P)H oxidases.

2. Methods

2.1. Ethical approval

The experimental procedures were approved by The Animal Care and Use Committee, Kyushu University.

2.2. Experimental animals

Male Wistar rats were purchased from Japan SLC (Shizuoka, Japan) and given standard rat chow and water ad libitum. Diabetes was induced in 7-weeks-old rats by injecting streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mol/L citrate buffer pH 4.5 at a dose of 80 mg/kg body weight. Rats with fasting blood glucose levels >250 mg/dl were considered diabetic. Rats given injections of citrate buffer alone served as non-diabetic controls. One week after the induction of diabetes, half of the diabetic rats were randomly selected and treated with subcutaneous injections of the GLP-1 analog liraglutide for 4 weeks at the dose of 0.3 mg/kg/12 h as previously reported [24,25]. Twice daily dosing was used because the pharmacokinetic half-life of liraglutide is only approximately 4 h in rats [25]. The remaining diabetic rats were treated with injections of phosphate buffer and were the controls. After 4 weeks all rats were sacrificed by exsanguination after these treatments under deep anesthesia by intraperitoneal injection of ketamine (40 mg/kg) and xylazine (20 mg/kg) on the day of experiments. Their kidneys were immediately excised and stored at −80 °C for the experiments.

2.3. Urine analysis

A 24 h urine sample was collected using a metabolic cage at week 4. The well-mixed urine was centrifuged at 7500 × g for 5 min, purged of air with a stream of nitrogen to prevent artificial formation of oxidative stress products, and then stored at −80 °C until analysis. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels were measured using a competitive enzyme-linked immunosorbent assay (ELISA) kit (8OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan), as previously described [4]. Urinary malondialdehyde (MDA) concentrations were measured using a TBARS assay kit (Cayman Chem). Urinary albumin concentrations were measured using a Rat Albumin ELISA Kit (AKRAL-120 or 121; Shibayagi, Shibukawa, Japan) and urinary creatinine concentrations were measured using a Creatinine (urine) Assay Kit (Cayman Chem). The results of these studies are expressed as values corrected according to the urinary creatinine level.

2.4. Detection of superoxide using dihydroethidium in situ

Dihydroethidium (DHE) (Invitrogen, Carlsbad, CA, USA) staining was performed as previously described [26]. Briefly, rats were administered 1 mL of DHE (1 mg/ml) intravenously in phosphate-buffered saline (PBS) through the right jugular vein under isoflurane anesthesia. Two hours after the DHE injection, the rats were killed by transcardial perfusion with 50 mL of 4% formaldehyde in PBS. Their kidneys were frozen immediately in O.C.T. compound (Tissue-Tech II; Sakura Fine Chemical, Tokyo, Japan) and sectioned at 10 μm thickness on a cryostat. The kidney sections were subjected to nuclear staining with Hoechst 33258 (Invitrogen) in PBS for 15 min in a dark chamber and mounted after a rinse in distilled H2O. Fluorescence images were obtained using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan). The relative fluorescence intensity in glomeruli was quantified using Adobe Photoshop software (version 6.0; Adobe Systems, Mountain View, CA, USA).
2.5. Isolation of glomeruli

Rat glomeruli were isolated by a graded sieving technique. Renal perfusion was performed under deep anesthesia. Kidneys were removed, decapsulated, and placed in cold PBS. They were separated into the cortex and medulla, and cortical tissue was then minced to a fine paste with a razor blade in Hank’s balanced salt solution (HBSS; Invitrogen) with collagenase A (Roche Diagnostics K.K., Tokyo, Japan). The minced cortex was pressed against a stainless steel grid. Large, fibrous tissues were retained on the grid surface, whereas glomeruli and tubular segments passed through. The glomeruli were then isolated by filtration through a 75-μm stainless steel grid. Those retained on the sieve were collected, washed by centrifugation (4 °C, 2000×g), and suspended in HBSS. Tissue was maintained at 4 °C during the entire isolation procedure. More than 95% of purified glomeruli were observed by optical microscopy.

Table 1 – Characteristics of the study.

<table>
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<th>Characteristic</th>
<th>Controls</th>
<th>Lira</th>
<th>STZ</th>
<th>STZ+Lira</th>
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<td>550.5±14.4††</td>
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<td>108.6±3.4</td>
<td>126.8±3.8‡</td>
<td>112.7±2.1†</td>
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Lira, nondiabetic rats with liraglutide injection; STZ, streptozotocin (STZ)-induced diabetic rats; STZ+Lira, STZ-induced diabetic rats with liraglutide injection; ANOVA, analysis of variance; PLSD, protected least significant difference.

Data are means±SEM.

⁎P<0.01 and §P<0.05 vs control; †P<0.01 vs Lira; ‡P<0.01 vs STZ (ANOVA with Fisher’s PLSD test).

Fig. 1 – Effect of liraglutide on oxidative stress and albuminuria in diabetic rats. White bars represent the nondiabetic controls rats. Black bars represent the streptozotocin (STZ)-induced diabetic rats. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) excretion levels (A) and urinary malondialdehyde (MDA) excretion levels (B). Albumin excretion levels (C) were adjusted according to the urinary creatinine levels (D). Data are means±SEM. *P<0.01 (analysis of variance, or ANOVA). Only significant P values are indicated.
they were then divided in half for protein extraction and RNA purification. They were sonicated in either lysis buffer (0.25 M sucrose, 1 mmol/L EDTA) or cell lysis buffer supplied in the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), respectively, for western blot analysis and real-time polymerase chain reaction (PCR) examination.

2.6. RNA preparation and quantification of mRNA expression levels

Total RNA was extracted from the frozen tissue samples with ISOGEN (Nippon Gene, Tokyo, Japan) containing phenol and guanidine isothiocyanate. RNA concentrations were determined spectrophotometrically. The RNA was then incubated with DNase I (Invitrogen) at 20 °C for 15 min to remove DNA contamination. Total RNA (4 μg) was denatured at 62 °C for 5 min. cDNA was synthesized using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen) in a final reaction volume of 20 μL at 50 °C for 50 min. The reaction was terminated by heating at 85 °C for 5 min. RNA from isolated glomeruli was purified with the RNeasy Mini Kit (Qiagen) according to the instructions provided with the kit, followed by reverse transcription using QuantiTect Rev. Transcription Kit (Qiagen) for cDNA synthesis. Quantification of mRNA expression levels was performed on a Chromo4 Real-time PCR Detector with iQ SYBR Green Supermix reagent (Bio-Rad Laboratories, Hercules, CA, USA). PCR reactions for each target cDNA were done at the conditions shown in Supplementary Table 1. β-Actin was used as an internal control. The specificity of PCR amplification was confirmed by melting curve analysis and agarose gel electrophoresis.

2.7. Western blot analysis

For total protein extraction and western blot analysis of NOX4, gp91phox, p47phox, and β-actin, renal tissues were homogenized in lysis buffer (0.25 mol/L sucrose, 1 mmol/L EDTA), and centrifuged for 5 min at 16,000 rpm. Protein concentrations were determined using a BCATM Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Protein concentr

Fig. 2 - Effect of liraglutide on superoxide production in renal tissue of diabetic rats. Superoxide was detected by dihydroethidium (DHE) staining in situ. Control, control rats (A, E, I); Control+liraglutide, liraglutide-treated control rats (B, F, J); STZ, rats with STZ-induced diabetes (C, G, K); STZ+liraglutide, liraglutide-treated rats with STZ-induced diabetes (D, H, L). Arrows indicate glomeruli. A–D, Dihydroethidium (DHE) fluorescence; and E–H, Hoechst 33258 (nuclear).
20 μg per lane, was separated discontinuously on sodium dodecyl sulfate polyacrylamide gels (5%–15%) and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). After blockade of nonspecific binding sites, membranes were incubated overnight at 4 °C with rabbit polyclonal anti-NOX4 (1:1500) (Abcam, Cambridge, MA, USA), goat polyclonal anti-gp91phox (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-p47phox (1:500) (Abcam), and mouse monoclonal anti-β-actin (1:10,000) (Sigma-Aldrich) followed by horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G (IgG) antibody (1:5000) (GE Healthcare UK, Buckinghamshire, UK), donkey anti-goat IgG antibody (1:5000) (Santa Cruz Biotechnology), or sheep anti-mouse IgG antibody (1:5000) (GE Healthcare UK) as a secondary antibody. We used the ECL Plus system (GE Healthcare UK) for detection.

2.8. In vitro study

Normal human mesangial cells (NHMCs) were purchased from Lonza (Walkersville, MD, USA). Mesangial cells were cultured in a Mesangial Cell Growth Medium (Lonza) containing 5% fetal calf serum (FCS). The cells from the second to the fourth passages were used in the experiments. Cellular production of superoxide anion was determined by the lucigenin-enhanced chemiluminescence assay, as previously described, with minor modifications[27,28]. For the experiments, after NHMCs were incubated with or without various agents for indicated intervals, they were detached with trypsin/EDTA and resuspended in modified HEPES buffer containing (mmol/L) NaCl 140, KCl 5, MgCl2 0.8, CaCl2 1.8, Na2HPO4 1.0, HEPES 25, and 1% glucose (pH 7.2). The cell suspension was gently agitated with 0.1% Triton-X100 for cell permeabilization. After preincubation with dark-adapted lucigenin (50 μmol/L) for 10 min at 37 °C, NAD(P)H (100 μmol/L) was added to the cells immediately before recording. Light emission was recorded every 10 s for 10 min and was expressed as relative light units (RLU). An NAD(P)H oxidase inhibitor, diphenylene iodonium chloride (DPI) (10 μM), was preincubated with NHMCs for 10 min to demonstrate the experimental specificity for NAD(P)H oxidase activity. Experiments were performed in triplicate; all results are from at least three independent experiments. Superoxide production was calculated as the sum of relative light units per micrograms of protein.

Protein content was measured using a BCA Protein Assay Kit (Pierce Biotechnology). For small interfering RNA (siRNA) experiments, we used Stealth RNAi oligonucleotides targeted against RAPGEF4 (Epac2) (Invitrogen). The target sequences used for siRAPGEF4 were as follows: sense 5’-UGUUCUUAA-GUCUGACUGUAUUCG-3’ and antisense 5’-CGAAUACAGUCA-GACUUAAAGAACA-3’. As a negative control, we used Stealth RNAi Negative Universal Control LO (Invitrogen). siRNA transfection was conducted according to the modified manufacturer’s instructions. siRNA and negative control siRNAs were diluted to a final concentration of 10 nmol/L in Opti-MEM I Reduced Serum Medium (Invitrogen). Then, equal volumes of diluted siRNA and Lipofectamine RNAiMAX

![Fig. 3](image-url) - Effect of liraglutide on mRNA levels of NAD(P)H oxidase components in diabetic rats. White bars, nondiabetic control rats; black bars, rats with STZ-induced diabetes. The relative expression levels of NOX4 (A), p22phox (B), gp91phox (C), and p47phox (D) mRNA in rat glomerular homogenates were semi-quantified by a real-time polymerase chain reaction (PCR) method. β-Actin was the internal control. Results are shown as percentages of the controls. Data are means±SEM. *P<0.05, **P<0.01 (ANOVA). Only significant P values are indicated.
(Invitrogen) were mixed gently and incubated for 15 min at room temperature to allow complexes to form. NHMCs were plated at a density to achieve 50%–80% confluence on the day of transfection. The medium was replaced with one lacking antibiotics. The cells were then incubated with siRNA-lipofectamine complexes at 37 °C for 24 h before the following experiments. siRNA-mediated downregulation of RAPGEF4 expression was confirmed by real-time PCR analysis. We also measured the cAMP concentration in NHMCs using commercially available kits (Cyclic AMP EIA Kit; Cayman Chemical, Ann Arbor, MI, USA) in accordance with the manufacturer’s instructions. Briefly, a cell lysate for each condition was collected after removing the medium.

The effects of liraglutide on the expression of NAD(P)H oxidase components, NOX4 and p22phox were also evaluated in cultured NHMCs. After preincubation of the cells with the media containing 0.5% FCS and 5.5 mmol/L or 25 mmol/L glucose for 5 days, liraglutide (1 μmol/L) and/or SQ22536 (400 μmol/L) was added for 48 h. Then, the extracted mRNA levels for NOX4 and p22phox were measured by real-time PCR as described above and levels of each mRNA were normalized to the levels of β-actin. PCR reactions for each target cDNA were done at the conditions shown in Supplementary Table 2.

The effect of liraglutide on intracellular production of superoxide was also evaluated in cultured NHMCs using DHE staining as previously described [29]. NHMCs (Lonza, Walkersville, MD, USA) were plated in a glass-bottom dish (MatTek Co, Ashland, MA). When cells were confluent, they were incubated in 5.5 mmol/L or 25 mmol/L glucose, with or without various agents for indicated intervals. The cells were then loaded with DHE 20 μmol/L (Invitrogen, Carlsbad, CA, USA) and the incubation was continued for 5 min. Nuclear staining was then performed using Hoechst 33258 (Invitrogen) in PBS for 10 min in a dark chamber and rinsing in distilled H2O. Fluorescence images were obtained using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan).

Fig. 4 – Effect of liraglutide on the protein levels of NAD(P)H oxidase components in diabetic rats. Western blot analysis for NOX4 (A) and gp91phox and p47phox (B) in rat glomerular homogenates. Cont, nondiabetic control rats; Cont+Lira, liraglutide-treated control rats; STZ, rats with STZ-induced diabetes; STZ+Lira, liraglutide-treated rats with STZ-induced diabetes. Extra bands were seen around 40 kDa upon immunoblotting for p47phox, as indicated in the manufacturer’s instruction manual. Quantification of NOX4 (C), gp91phox (D), and p47phox (E) intensities relative to β-actin intensity. White bars represent the control rats; black bars represent the rats with STZ-induced diabetes. Results are shown as the fold-change relative to the controls. Data are means ±SEM. *P < 0.05, **P < 0.01 (n=8 each; ANOVA). Only significant P values are indicated.
2.9. Statistical analysis

All data are expressed as means±SEM. Statistical analysis was performed with Student’s t test or one-way analysis of variance (ANOVA) with Fisher’s protected least significant difference (PLSD) test. \( P<0.05 \) was considered statistically significant.

3. Results

3.1. Urinary oxidative stress marker and albumin excretion

Administration of liraglutide (0.3 mg/kg/12 h) was started 1 week after the onset of diabetes. Characteristics of the experimental rats are summarized in Table 1. Liraglutide significantly decreased body weight by day 28 in control rats, but not in diabetic rats. It did not affect the plasma glucose level in either group. Liraglutide also decreased food intake but not in diabetic rats. It decreased the systolic blood pressure in diabetic rats slightly but significantly.

After administration of liraglutide for 4 weeks, we evaluated 24-h urinary excretion levels of both 8-OHdG and MDA, asymptotic oxidative stress markers, and albumin. Both urinary excretion levels of 8-OHdG and MDA in diabetic rats were significantly higher than in control rats and were significantly reduced by liraglutide (Fig. 1A and 1B). Urinary albumin excretion levels in diabetic rats (21.4±4.7 mg/gCre; \( P<0.001 \) vs controls) were significantly higher than in control rats (4.7±0.7 mg/gCre) and were also significantly reduced by liraglutide (9.8±1.7 mg/gCre; \( P<0.01 \) vs STZ rats) (Fig. 1C and 1D).

3.2. Renal oxidative stress marker and NAD(P)H oxidase expression

To evaluate superoxide production in the kidneys, we performed dihydroethidium (DHE) staining. It clearly revealed a massive increase in superoxide production in the diabetic kidneys (Fig. 2C, G, K) and its attenuation by liraglutide (Fig. 2D, H, I). In addition, we measured the expression of NAD(P)H oxidase, which is considered to be a major source of superoxide production in the kidneys. The mRNA levels of NOX4, a major component of NAD(P)H oxidase in the kidneys, and the other components [p22phox, gp91phox (Nox2), p47phox] were markedly increased in diabetic kidneys, and these increases were all reduced by liraglutide (Fig. 3A–D).

Western blot analysis confirmed that the protein levels of NOX4, gp91phox, and p47phox in the glomerular homogenates of diabetic rats were higher than those of the controls (1.7-fold, \( P<0.05 \) for NOX4; 2.0-fold, \( P<0.02 \) for gp91phox; 2.1-fold, \( P<0.02 \) for p47phox), and these increases were significantly reduced by liraglutide to the control levels (Fig. 4A–E). We also evaluated the expression of transforming growth factor-\( \beta \) (TGF-\( \beta \)), a key cytokine that mediates extracellular matrix accumulation and glomerular expansion in diabetes, and a predominant matrix protein, fibronectin. The mRNA levels of TGF-\( \beta \)1 and fibronectin in glomerular homogenates were higher in diabetic rats than in the control homogenates: 18.9-fold (\( P<0.001 \) vs control) and 2.6-fold (\( P<0.05 \) vs control), respectively. These increases were significantly reduced by liraglutide: \( P<0.001 \) vs STZ rats (Fig. 5A) and \( P<0.05 \) vs STZ rats (Fig. 5B).

3.3. In vitro effect of liraglutide

We next investigated the molecular mechanisms for the inhibitory effect of liraglutide on the activity of NAD(P)H oxidase using cultured normal human mesangial cells (NHMCs), which were confirmed to have GLP-1 receptor (Supplementary Fig. 1). The activity of NAD(P)H oxidase was evaluated by the lucigenin chemiluminescence method. NADPH-dependent superoxide production from mesangial cells was completely inhibited by preincubation with NAD(P)H oxidase inhibitor diphenylene iodonium chloride (10 \( \mu \)M) for 10 min, and was significantly reduced by preincubation with liraglutide for 48 h, in a dose-dependent manner (10 nmol/L–1 \( \mu \)mol/L) (Fig. 6A). This inhibitory effect of liraglutide was reversed by simultaneous incubation with the protein kinase A (PKA) inhibitor H89 and the adenylate cyclase inhibitor.
cAMP levels. These results suggested that the effect of Epac2 inhibition, another downstream pathway of increased SQ22536 and the PKA inhibitor H89. It was not reversed by oxidase was reversed by the adenylate cyclase inhibitor the production of cAMP\[20,21\]. Mesangial cell expressed GLP-1 are mediated through the activation of adenylate cyclase and dependent superoxide production from cultured renal mesangial cell lines. The detailed molecular mechanisms should be evaluated in further studies.

ROS production from NAD(P)H oxidase is regulated by induction of the individual subunits, as well as its activation in vascular cells and other nonphagocytic cells [34]. Of particular note, ROS production derived from NOX4 that is the main component of NAD(P)H oxidase in kidneys [35] is regulated by its expression levels rather than its activation [34]. We previously showed that increased expression of NOX4 may play an important role in the increased production of ROS in diabetic renal tissues [8]. Gorin et al reported that down-regulation of NOX4 induced by antisense oligonucleotides attenuated oxidative stress, renal hypertrophy, and increased renal expression of fibronectin in rats with STZ-induced diabetes [36]. In addition, a NAD(P)H oxidase inhibitor was also reported to inhibit oxidative stress and prevent renal damage in diabetic animal models [37]. Among various sources for ROS production, NAD(P)H oxidase NOX4 may be a therapeutic target for diabetic nephropathy. In the present study, we showed that liraglutide normalized the increased expression of NOX4 as well as other components, gp91phox, p22phox, and p47phox in diabetic renal tissues. However, the detailed regulatory mechanisms for the expression of NAD(P)H oxidase components including NOX4 are generally not fully understood. Recent report has shown that transcription factor Ets-1 is a critical mediator of ROS production by Ang II by regulating the expression of NAD(P)H oxidase components such as p47phox [38]. Since Ets-1 is known as a downstream transcriptional effector of ROS in several different cell types [39], this finding implicates that rapid activation of NAD(P)H oxidase causes the expression of Ets-1 by redox sensitive mechanism, which in turn induces the expression of NAD(P)H oxidase components such as p47phox for the booster effect of ROS generation. In diabetic renal tissues, rapid activation of NAD(P)H oxidase by high glucose levels and/or increased Ang II levels might induce NOX4 overexpression for the similar booster effect. Thus, a possible mechanism is that liraglutide-induced inhibition of NAD(P)H oxidase activity (e.g., gp91phox) may inhibit such a vicious cycle and thus normalize the expression of NAD(P)H oxidases. This hypothesis was consistent with our finding that exposure of the cells with high glucose levels (25 mmol/L) for 5 days, but not for shorter period, significantly induced an increase in NOX expression and this increase was also significantly inhibited by liraglutide.

4. Discussion

A previous study showed that long-term treatment with GLP-1 analog, exendin-4, ameliorated diabetic nephropathy in db/db mice with type 2 diabetes mellitus [30]. However, the underlying mechanism was not fully understood, because exendin-4 treatment ameliorated various metabolic abnormalities in db/db mice, including body weights, adipose tissue weights, circulating free fatty acid and triglyceride concentrations, and improved insulin sensitivity [30]. Therefore, in the present study, we used rats with STZ-induced diabetes. In these type 1 diabetes mellitus models, liraglutide treatment did not significantly affect plasma glucose levels or body weights. It significantly affected systolic blood pressure in diabetic mice, but this effect was very small. The present study showed that liraglutide still normalized urinary albuminuria and expression of renal transforming growth factor-β and fibronectin in diabetic rats in parallel with normalization of oxidative stress markers and expression of renal NAD(P)H oxidase components (Nox4, gp91phox, p22phox, p47phox), independently of a glucose-lowering effect. In the present study, to confirm the direct effect of liraglutide on renal NAD(P)H oxidase, we showed that liraglutide inhibited NADPH-dependent superoxide production from cultured renal mesangial cells. It is well established that the main effects of GLP-1 are mediated through the activation of adenylyl cyclase and the production of cAMP [20,21]. Mesangial cell expressed GLP-1 receptors and the inhibitory effect of liraglutide on NAD(P)H oxidase was reversed by the adenylyl cyclase inhibitor SQ22536 and the PKA inhibitor H89. It was not reversed by Epac2 inhibition, another downstream pathway of increased cAMP levels. These results suggested that the effect of liraglutide on NAD(P)H oxidase was mediated by the cAMP–PKA pathway. This finding is consistent with previous reports showing that NAD(P)H oxidase activity is regulated by the cAMP–PKA pathway in phagocytes [22,23]. We and other investigators have previously shown that high glucose levels and increased local angiotensin II (Ang II) levels stimulate reactive oxygen species (ROS) production in vascular and renal tissues in the presence of diabetes via protein kinase C (PKC)-dependent activation of NAD(P)H oxidase [9,31,32]. PKC activation activates small G protein Rac 1 and phosphorylates p47phox, by which mechanisms NAD(P)H oxidase is considered to be activated in diabetic vascular and renal tissues [32,33]. Since PKA activation has been reported to down-regulate phosphorylation of p47phox or inhibit activation of another small G protein, Rap1A, which acts as the final activation switch of NAD(P)H oxidase by its interaction with cytochrome b558 [22,23], liraglutide may inhibit NAD(P)H oxidase activity in diabetic renal tissues via such molecular mechanisms. The detailed molecular mechanisms should be evaluated in further studies.

Very recently, Kodera et al reported that another GLP-1 analog, exendin-4, ameliorated renal injury through its anti-inflammatory action in STZ-diabetic rats [40]. They showed that exendin-4 directly attenuated release of pro-
inflammatory cytokines from macrophages and ICAM-1 production on glomerular endothelial cells. Macrophages and endothelial cells have several isoforms of NAD(P)H oxidases. The ICAM-1 expression in endothelial cells and activation of macrophages are in part regulated by oxidative stress-induced signals such as the C-Jun NH(2)-terminal kinase (JNK) pathway and NF-κB pathway [41,42]. Therefore, it is possible that such anti-inflammatory effects of exendin-4 also may be in part mediated by its inhibitory effect of NAD(P)H oxidases. At least, both effects may be closely linked by the common molecular mechanism. Further studies are needed to clarify the relationship between both effects and the detailed
Fig. 6 – Liraglutide decreased NAD(P)H oxidase activity via the cAMP–protein kinase A (PKA) pathway in normal human mesangial cells (NHMCs). All results are means ± SEM. Only significant P values are indicated. A, NADPH oxidase activity was decreased by liraglutide in a dose-dependent manner. NHMCs were preincubated with or without liraglutide (at doses of $10^{-8}$–$10^{-6}$ mol/L) for 48 h. Diphenylene iodonium (DPI) (10 μM) was preincubated with NHMCs for 10 min to demonstrate the experimental specificity for NADPH oxidase activity. Lucigenin-enhanced chemiluminescence assay followed. Chemiluminescence levels were reported in relative luminescence units (RLU) adjusted by the total protein concentration. $^# P<0.05$ vs control, $^* P<0.01$ vs DPI, $^* P<0.05$, and $^{**} P<0.01$ (ANOVA; n=3 each). B, After preincubation with $10^{-6}$ mol/L liraglutide, NHMCs were incubated with PKA inhibitor H89 (at doses of 1 and 10 nmol/L) and the adenylate cyclase inhibitor SQ22536 (at doses of 40 and 400 μmol/L) for 2 h. NADPH oxidase activity is shown as a percentage relative to the control, which was not stimulated by NADPH. $^* P<0.05$ vs control; $^* P<0.05$ and $^{**} P<0.01$, ANOVA (n=3 each). C, Epac2 siRNA or a negative universal control was induced in NHMCs with or without $10^{-6}$ mol/L liraglutide preincubation. Lucigenin-enhanced chemiluminescence assay was performed 24 h after induction of siRNA. Chemiluminescence levels are indicated as relative luminescence units adjusted according to the total protein concentration. $^* P<0.01$ vs controls; $^* P<0.01$ (ANOVA; n=3 each). D, Intracellular cAMP concentration was inversely correlated with NAD(P)H oxidase activity in NHMCs. The NHMCs were preincubated with or without liraglutide (at doses of $10^{-8}$–$10^{-6}$ mol/L) for 48 h. After preincubation with $10^{-6}$ mol/L liraglutide, the NHMCs were incubated with PKA inhibitor H89 (10 nmol/L) and the adenylate cyclase inhibitor SQ22536 (400 μmol/L) for 2 h. Liraglutide increased the intracellular cAMP concentration in a dose-dependent manner. Inhibition of adenylate cyclase completely reversed the liraglutide-induced elevation of cAMP levels. $^* P<0.05$ and $^{**} P<0.01$ vs control (ANOVA; n=6 each). Expression levels of NOX4 (E) and p22phox (F) mRNA under the normal or high glucose conditions. NHMCs were exposed to the medium containing 5 or 25 mmol/L glucose. White bar=normal glucose (5 mmol/L). Black bar=high glucose (25 mmol/L). Liraglutide (1 μmol/L) and SQ22536 (400 μmol/L) were added 48-h before cell lysis. Data are means ± SEM. $^# P<0.05$ vs control; $^* P<0.05$ and $^{**} P<0.01$ (ANOVA; n=6 each).
molecular mechanisms. On the other hand, GLP-1 has been reported to enhance sodium excretion and reduce glomerular hyperfiltration in obese men [43]. These hemodynamic effects might be involved in the beneficial effect of liraglutide on diabetic nephropathy. Further studies are also needed to clarify the role of hemodynamic effects.
In conclusion, liraglutide may have a direct beneficial effect on oxidative stress and diabetic nephropathy, at least in part, via a PKA-mediated inhibition of renal NAD(P)H oxidase, independently of a glucose-lowering effect. Its clinical efficacy should be tested in human trial.

Appendix A. Supplementary data
Supplementary materials related to this article can be found online at doi:10.1016/j.metabol.2012.03.002.

Author contributions
H. Hendarto, T. Inoguchi, R. Takayanagi designed the experiments; H. Hendarto, Y. Maeda, N. Ikeda, J. Zheng, R. Takei, H. Yokomizo, E. Hirata performed and analysed data of experiments; H. Hendarto, T. Inoguchi, Y. Maeda, N. Sonoda interpreted results of experiments; H. Hendarto, T. Inoguchi, Y. Maeda prepared figures and drafted manuscript; H. Hendarto, T. Inoguchi, Y. Maeda, N. Sonoda, R. Takayanagi edited and revised manuscript.

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Conflict of interest
The authors reported no potential conflict of interest.

REFERENCES

Fig. 7 – Effect of liraglutide on superoxide production in normal human mesangial cells (NHMCs). (A–O) Superoxide production was evaluated by dihydroethidium staining in vitro. NHMCs were exposed to the medium containing normal glucose; 5.5 mmol/L (A, B, F, G, K, L) or high glucose; 25 mmol/L (C, D, E, H, I, J, M, N, O). NHMCs were preincubated with liraglutide 1 μmol/L (B, D, E, G, I, J, L, N, O) and SQ22536 400 μmol/L (E, J, O) for 48 h. (A–E) dihydroethidium (DHE) fluorescence; and (F–J) Hoechst 33258 (nuclear). Relative intensities of DHE staining levels were compared to controls (P). Data are expressed as means±SEM. White bar=normal glucose. Black bar=high glucose. *P<0.05, only significant p values are indicated.


