Liraglutide Reduces Oxidative Stress And Restores Heme Oxygenase-1 and Ghrelin Levels in Patients with Type 2 Diabetes: A Prospective Pilot Study

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Context: Liraglutide is a glucagon-like peptide-1 analog and glucose-lowering agent whose effects on cardiovascular risk markers have not been fully elucidated.

Objective: We evaluated the effect of liraglutide on markers of oxidative stress, heme oxygenase-1 (HO-1), and plasma ghrelin levels in patients with type-2 diabetes mellitus (T2DM).

Design and Setting: A prospective pilot study of 2 months' duration has been performed at the Unit of Diabetes and Cardiovascular Prevention at University of Palermo, Italy.

Patients and Intervention(s): Twenty subjects with T2DM (10 men and 10 women; mean age: 57 $\pm$ 13 y) were treated with liraglutide sc (0.6 mg/d for 2 wk, followed by 1.2 mg/d) in addition to metformin (1500 mg/d orally) for 2 months. Patients with liver disorders or renal failure were excluded.

Main Outcome Measure(s): Plasma ghrelin concentrations, oxidative stress markers, and heat-shock proteins, including HO-1 were assessed.

Results: The addition of liraglutide resulted in a significant decrease in glycated hemoglobin (HbA1c) (8.5 $\pm$ 0.4 vs 7.5 $\pm$ 0.4%, $P < .0001$). In addition, plasma ghrelin and glutathione concentrations increased (8.2 $\pm$ 4.1 vs 13.6 $\pm$ 7.3 pg/ml, $P = .0007$ and 0.36 $\pm$ 0.06 vs 0.44 $\pm$ 0.07 nmol/ml, $P = .0002$, respectively), whereas serum lipid hydroperoxides and HO-1 decreased (0.11 $\pm$ 0.05 vs 0.04 $\pm$ 0.07 pg/ml, $P = .0487$ and 7.7 $\pm$ 7.7 vs 3.6 $\pm$ 1.8 pg/ml, $P = .0445$, respectively). These changes were not correlated with changes in fasting glycemia or HbA1c.

Conclusions: In a 2-months prospective pilot study, the addition of liraglutide to metformin resulted in improvement in oxidative stress as well as plasma ghrelin and HO-1 concentrations in patients with T2DM. These findings seemed to be independent of the known effects of liraglutide on glucose metabolism. (***J Clin Endocrinol Metab*** **100: 603–606, 2015)**

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Abbreviations: BMI, body mass index; GLP-1, glucagon-like peptide-1; GSH, gluthatione; HbA1c, glycated hemoglobin; HO-1, heme oxygenase-1; Hsp60, heat shock protein 60; LDL, low-density lipoprotein; LOOH, lipid hydroperoxides; ROS, reactive oxygen species; T2DM, type-2 diabetes mellitus.

Glucagon-like peptide-1 (GLP-1) analogs such as liraglutide have been shown to be effective therapeutic agents for type 2 diabetes mellitus (T2DM). They may have beneficial metabolic actions that can potentially reduce cardiovascular risk (1), independent of their antihyperglycemic effect. These include weight loss and decrease in oxidative stress.

A known effect of liraglutide is promotion of weight loss, thought to be mediated by induction of satiety and retardation of gastric emptying (2). In animal models (3) liraglutide regulated plasma ghrelin concentrations independently of GLP-1 levels. Given that ghrelin is known to regulate food intake, energy expenditure, and appetite (4), the effects of liraglutide on body weight could be mediated by its effects on ghrelin. In experimental animals, liraglutide has been shown to also decrease oxidative stress independent of its glucose-lowering effects (5), an effect that may contribute to decreasing atherogenesis and cardiovascular risk.

Markers of oxidative stress such as reactive oxygen species (ROS), lipid hydroperoxides (LOOH), heme oxygenase-1 (HO-1), and gluthatione (GSH) are abnormal in T2DM subjects (6). Liraglutide's specific actions on the above-mentioned parameters are largely unknown, with the exception of sparse data from animal models and a brief research letter in patients with T2DM (7). Therefore, the goal of the present study was to investigate potential nonglycemic effects of liraglutide that may reduce cardiovascular risk, including those on oxidative stress and plasma concentrations of ghrelin, in patients with T2DM.

Materials and Methods

Patients and methods

We studied 20 subjects with T2DM whose clinical characteristics are reported in Table 1. All subjects were naïve to incretin-based therapies and were taking metformin (doses ranging from 1500–2000 mg/d) only. Liraglutide was given sc (0.6 mg/d for 2 wk, followed by 1.2 mg/d) for 2 months. The mean glycated hemoglobin (HbA1c) was 8.5 ± 0.4% and none of the subjects had known diabetic complications or clinical evidence of cardiovascular disease. The study design included a medical examination and biochemical analyses. The procedures adopted were in agreement with the Helsinki Declarations and were approved by the Ethics Council of the University of Palermo. The study was registered in clinicaltrials.gov (NCT01715428).

All patients gave written informed consent to participate in the study. They underwent a medical examination and were excluded if they had clinical evidence of liver dysfunction or renal failure. Waist circumference, height, and weight were recorded, and body mass index (BMI) was calculated as kg/m^2. Patients were followed for 2 months.

Table 1. Patients' Baseline Characteristics (n = 20)

Characteristic	
Age, y	57 ± 13
Women, n (%)	10 (50)
Diabetes duration, y	6 ± 3
Smoking habit, n (%)	2 (10)
Family history of cardiovascular diseases, n (%)	13 (65)
Systolic blood pressure, mm Hg	129 ± 18
Diastolic blood pressure, mm Hg	78 ± 8
Hypertension, n (%)	15 (75)
Dyslipidemia, n (%)	16 (80)
Obesity,[a] n (%)	4 (20)
Diabetic complications, n (%)	1 (5)
Use of anti-hypertensive therapies, n (%)	15 (75)
Use of lipid-lowering drugs, n (%)	10 (50)
Aspirin use, n (%)	9 (45)

[a] Obesity: BMI>30.

The data are presented as mean ± SD or n (%).

Biochemical analyses

Blood samples were obtained after a 14-hour fast at baseline and after 2 months of liraglutide therapy. They were centrifuged within 30 minutes of collection, and aliquots were made of both serum and plasma. All biochemical analyses were run blinded on never-frozen samples, with the exception of HO-1 and ghrelin concentrations, which were measured on aliquots immediately frozen and stored at −80° C.

Preprandial plasma ghrelin concentrations were measured by ELISA, according to the manufacturer's protocol (Bio Vendor) (8). Serum LOOH levels were evaluated following the oxidation of Fe^{2+} to Fe^{3+} in the presence of xylenol orange at 560 nm (9). The limit of detection for this assay was 0.025 nmol/L. Levels of GSH were measured in serum using a spectrophotometric assay based on the reaction of thiol groups with 2,2-dithio-bis-nitrobenzoic acid (10). The production of ROS was assessed using fluorescent probe dihydroethidium staining (Sigma) (11). Heat shock protein 60 (Hsp60) and HO-1 concentrations were assessed by ELISA using commercial kits (Enzo Life Science AG), as previously described (12). Intra- and interassay coefficients of variation were less than 10% for measurements of both Hsp60 and HO-1.

Statistical analysis

Statistical analysis was performed using Statview 5.0 (SAS Institute). Univariate analysis was performed using paired *t* test. We used the Spearman rank correlation method to evaluate whether changes in cardio-metabolic risk factors were associated with changes in other measured parameters. All variables were tested for normality performing the Kolmogorov-Smirnov normality test. Because concentrations of LOOH, ghrelin, HO-1, and Hsp60 were not normally distributed; the nonparametric Wilcoxon test was performed for these variables.

Results

None of the subjects discontinued metformin or liraglutide, and the dosages of antihypertensive medications, lipid-lowering agents, and aspirin remained unchanged dur-

ing the course of the study. As shown in Table 2, liraglutide therapy led to significant reductions in HbA1c, ghrelin, and GSH, with a concomitant decrease in LOOH and HO-1. Further (data not shown), we found inverse correlations between ghrelin and waist circumference ($r = -0.467$, $P = .0379$), as well as between LOOH and total and low-density lipoprotein (LDL) cholesterol ($r = -0.498$, $P = .0254$ and $r = -0.468$, $P = .0376$, respectively). Hsp60 inversely correlated with ROS ($r = -0.495$, $P = .0263$). No other significant correlations between the evaluated parameters were found.

Discussion

This is the first study evaluating the effect of liraglutide on parameters of oxidative stress, ghrelin, HO-1, and Hsp-60 in T2DM patients. Consistent with data from animal models (13), we found that liraglutide reduced lipid peroxidation as measured by LOOH formation. However, we did not find any significant effect on plasma ROS levels in contrast with Okada et al (7), who recently reported that liraglutide decreased diacron-reactive oxygen metabolites in Japanese subjects with T2DM. In addition, we found that liraglutide significantly increased plasma GSH content, inconsistent with previous observations showing that GLP-1 treatment may increase antioxidative activity by a reduction in the oxidized GSH/total GSH ratio (14). However, whether this effect is mediated by an increased expression or activity of enzymes related to GSH biosynthesis remains to be evaluated. Correlation analysis revealed that liraglutide's effects on oxidative stress seem to be independent of glycemic control; therefore, changes in LOOH and GSH were not associated with any change found in other variables evaluated, with the exception of an inverse correlation between LOOH and total or LDL-cholesterol.

In vitro studies suggest that the antioxidant properties of liraglutide may be linked to induction of HO-1, a microsomal enzyme able to suppress oxidative stress (12). Oeseburg et al (15) have shown that GLP-1 can induce increased expression of the oxidative stress defense gene HO-1 in endothelial cells. Patients with T2DM have abnormal elevated plasma levels of HO-1 (16) and the present study shows that liraglutide lowers its plasma concentrations. Correlation analysis revealed that changes in HO-1 were not associated with changes found in the other evaluated parameters. Hsp60, human mitochondrial chaperonin, is known to play an important role in cardiovascular homeostasis and has been associated with cardiovascular risk (17). We did not find any significant effect of liraglutide on Hsp60, despite a slight mean reduction in its plasma levels. However, changes in Hsp60 levels were inversely associated with changes in ROS.

Our findings show for the first time that liraglutide can increase plasma ghrelin concentrations in patients with T2DM. In another study, Senda et al (18) reported that liraglutide restored ghrelin levels in a 25-year old woman with the Prader-Willi syndrome. Liraglutide's effect on ghrelin may have an important indirect role on body weight regulation, because ghrelin physiologically increases during fasting and decreases after food intake [reviewed in Rizzo et al (4)]. This is supported by the inverse association between changes in ghrelin and changes in waist circumference in our subjects.

The strengths of our study include the blinded measurements of all biochemical analyses, and the excellent

Table 2. Effects of 2 Months of Liraglutide Therapy in Subjects With Type 2 Diabetes

Characteristic	Before Therapy	After Therapy	*P*
BMI, kg/m^2	28.7 ± 5.3	28.5 ± 4.2	.6755
Waist circumference, cm	105 ± 15	103 ± 12	.1562
Fasting glycemia, mmol/L	8.7 ± 3.6	7.6 ± 2.2	.0756
HbA1c, %	8.5 ± 0.4	7.5 ± 0.4	<.0001
Total Cholesterol, mmol/L	4.4 ± 1.0	3.9 ± 0.8	.0532
Triglycerides, mmol/L	1.9 ± 0.7	1.7 ± 0.5	.2358
HDL-cholesterol, mmol/L	1.1 ± 0.3	1.1 ± 0.2	.2226
LDL-cholesterol, mmol/L	2.5 ± 0.9	2.0 ± 0.7	.0752
LOOH, nmol/L	0.11 ± 0.04	0.04 ± 0.07	.0487
ROS, arbitrary units	6561 ± 996	6379 ± 1151	.5953
GSH, nmol/ml	0.36 ± 0.06	0.44 ± 0.07	.0002
Ghrelin, pg/ml	8.2 ± 4.1	13.6 ± 7.3	.0007
HO-1, ng/ml	7.7 ± 7.7	3.6 ± 1.8	.0445
Hsp60, ng/ml	7.1 ± 5.7	6.6 ± 4.8	.6149

Abbreviation: HDL, high-density lipoprotein.

The data are presented as means ± SD.

The paired *t* test was used, except for concentrations of LOOH, ghrelin, HO-1, and Hsp60, which were not normally distributed; thus, the nonparametric Wilcoxon test was performed.

adherence to treatment intervention. A potential limitation is the lack of a control group consisting of subjects on metformin therapy alone. Previous studies have shown that metformin administration has no appreciable effect on ghrelin (19) and GSH concentrations (20) in subjects with T2DM. It is therefore unlikely that metformin was responsible for the significant modulation of these parameters when administered with liraglutide, suggesting that the changes were more likely attributable to the latter. Further, the significant changes in the measured parameters upon adding liraglutide reduce the probability of these findings occurring by chance alone. The coadministration of other drugs for cardiovascular prevention (including antihypertensive agents, statins, and aspirin) could affect the measurements. However, the doses of these medications were kept essentially constant during the intervention to avoid confounding. We did not perform any metabolic studies such as oral glucose tolerance or mixed meal tests, or evaluate parameters of cardiovascular function, during the course of our pilot study.

In conclusion, we report novel metabolic effects of liraglutide in patients with T2DM. Our study is the first to examine the effects of liraglutide on ghrelin, oxidative stress and HO-1 and Hsp60 in patients with T2DM. The improvement in these parameters independent of glycemic control could contribute to reduced cardiovascular risk in this patient population.

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