CLINICAL STUDY

Acute hyperinsulinemia decreases plasma osteoprotegerin with diminished effect in type 2 diabetes and obesity

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Abstract

Objective: Osteoprotegerin (OPG) is a soluble tumour necrosis factor-receptor-like molecule present in connective tissues, especially bone and vasculature. It is known to accumulate in the arterial wall in diabetes. As its synthesis in vascular cells is decreased by insulin, we wanted to elucidate the acute effects of insulin on plasma OPG concentrations in individuals with type 2 diabetes and obese individuals compared with lean controls.

Design: The study population consisted of ten type 2 diabetic, ten obese subjects, and ten lean subjects with no family history of diabetes.

Methods: All subjects underwent a 4-h euglycemic–hyperinsulinemic clamp. Plasma OPG, insulin, lactate, HbA1c, cholesterol, triglycerides, free fatty acids (FFA), and glucose disposal rate were measured before and at the end of the clamp.

Results: Baseline OPG concentrations did not differ significantly between groups. Insulin infusion decreased plasma OPG concentrations in all groups ($P < 0.01$); however, the fall in OPG was 50% less in obese and type 2 diabetic individuals ($P = 0.007$). Baseline OPG correlated with fasting insulin, baseline lactate, and low density lipoprotein cholesterol in the diabetic group, and with baseline FFA in the lean group. The relative change of OPG in response to insulin correlated inversely with HbA1c and baseline FFA in the lean group.

Conclusions: Acute hyperinsulinemia decreases plasma OPG, but with diminished effect in individuals with type 2 diabetes and obesity. Increased levels of OPG in arteries and plasma in diabetes together with the capability of plasma OPG as a cardiovascular risk predictor may be related to the described effects of insulin.

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Introduction

Osteoprotegerin (OPG) was first identified in 1997 as a secreted, bone-related glycoprotein of the tumour necrosis factor (TNF)-receptor superfamily that prevents bone resorption and bone loss (1). This effect is due to binding and neutralization of the receptor activator of nuclear factor- κ B ligand, a strong inducer of osteoclast differentiation. However, OPG is also present in connective tissues, especially in vasculature. In the arterial wall, it is present in concentrations comparable with that of bone (2), and it is believed to function as a vascular calcification inhibitor. This idea is based on the observation that a proportion of OPG knockout mice develop vascular calcifications (3), although this effect could be secondary to severe osteoporosis in these animals (4). OPG has also been considered a survival factor for endothelial cells (5), but the exact role of OPG in the arterial wall is still undetermined.

The cellular source of OPG in bone is considered to be osteoblasts, whereas in the vasculature it is ascribed to vascular smooth muscle cells (VSMC), since endothelial cells produce only smaller amounts (6). Interestingly, we have observed that the OPG concentration is increased in the deeper layers of arterial tissue from diabetes patients, independent of the presence of atherosclerosis (2). The significance of this observation is unknown, but accumulation of OPG may be a part of the generalized matrix changes seen in the arterial wall in diabetes (7, 8). The arterial accumulation of OPG could relate to the fact that production of the molecule from VSMC is highly influenced by important factors in the diabetic milieu, i.e. pro-inflammatory, hormonal, and metabolic factors. Thus, TNF- α (2), interleukin-1 (IL-1) (9), IL-4 (10), and peroxisome proliferator-activated receptor (PPAR) agonists (11) upregulate the synthesis, and interestingly insulin decreases the production (2). This direct effect of insulin on vascular cells is in agreement with other insulin effects concerning both NO synthesis, vasomotoric response

(12), expression of adhesion molecules (13), matrix synthesis (14, 15), and calcifications (16, 17). The understanding of insulin effects in vascular cells is important, since it is not known whether hyperinsulinism or lack of insulin effects in some pathways are prevailing in type 2 diabetes (18, 19).

In addition to its presence in connective tissues, OPG also circulates in blood, although the concentrations here are considerably lower than in tissue (2). A large observational study found higher plasma OPG concentrations in diabetic individuals compared with non-diabetic, although the absolute concentration difference was limited (20). Furthermore, a correlation between insulin resistance and plasma OPG has been reported in obese subjects (21) and elderly men (22), whereas a study including only women showed no relationship (23). Three studies of associations between body mass index (BMI) and OPG did not show any relationships, but information about insulin or insulin sensitivity was not reported (23–25). In a few studies, the focus was on the relationship between markers of insulin resistance and OPG, and these found a correlation with C-reactive protein and TNF- α levels in men with type 2 diabetes (26), while serum OPG was found associated with carotid intima media thickness in women with previous gestational diabetes (27).

As insulin resistance is associated with increased coronary heart disease and mortality (28–30), it is interesting that recent studies have suggested plasma OPG as a predictor of cardiovascular disease (31–33). Many authors assume that this connection is due to an association between OPG and vascular calcifications, but the determinators of the circulating levels of plasma OPG have not been established. Of note, there are controversial results from different studies concerning

the relationship between OPG and arterial disease (34, 35), and further investigation of this matter is certainly needed.

In this study, we hypothesized that OPG's involvement in arterial disease in diabetes and its capability as a predictor of cardiovascular diseases could relate to putative effects of insulin on OPG amounts, which may be defect in situations with insulin resistance as obesity and type 2 diabetes. Consequently, we investigated the effect of acute hyperinsulinemia on plasma OPG concentrations in type 2 diabetic subjects and obese individuals.

Materials and methods

Study population

The study population consisted of three groups: ten obese type 2 diabetic patients carefully matched according to BMI, age, and gender to ten healthy obese subjects, and according to age and gender to ten healthy lean subjects (Table 1). Type 2 diabetic patients were either treated by diet alone or in combination with sulfonylurea and/or metformin, which were withdrawn 1 week prior to the study together with antihypertensive and lipid-lowering drugs. The patients were all GAD65 antibody negative and without signs of diabetic retinopathy, nephropathy, neuropathy, or macrovascular complications. All the control subjects had normal glucose tolerance and no family history of diabetes. All subjects had normal results on screening blood tests of hepatic and renal functions. Informed consent was obtained from all subjects before participation. The study was approved by the Local Ethics Committee and was performed in accordance with the Helsinki Declaration.

Table 1 Baseline fasting parameters for all groups.

	Lean (n=10)	Obese (n=11)	Diabetes (n=10)
Male/female	4/6	6/4	6/4
Age (years)	53.9 $\pm$ 4.6	55.4 $\pm$ 3.9	53.9 $\pm$ 5.2
BMI (kg/m ²)	23.2 $\pm$ 1.8	31.0 $\pm$ 2.8*	29.8 $\pm$ 4.0*
Percent body fat (%)	26.6 $\pm$ 7.0	32.9 $\pm$ 6.9	35.0 $\pm$ 6.5 [§]
Fasting plasma glucose (mmol/l)	5.6 $\pm$ 0.4	5.8 $\pm$ 0.4	8.9 $\pm$ 2.1* [‡]
Fasting serum insulin (pmol/l)	31.6 $\pm$ 14.5	39.6 $\pm$ 18.4	85.2 $\pm$ 51.6 ^{†,¶}
Fasting serum C-peptide (pmol/l)	520 $\pm$ 104	633 $\pm$ 143	1181 $\pm$ 444* ^{‡,§}
HbA1c (%)	5.4 $\pm$ 0.3	5.3 $\pm$ 0.3	6.8 $\pm$ 1.1* ^{‡,§}
Total cholesterol (mmol/l)	5.6 $\pm$ 0.9	5.1 $\pm$ 0.9	4.9 $\pm$ 0.7
LDL cholesterol (mmol/l)	3.6 $\pm$ 0.7	3.2 $\pm$ 0.9	3.2 $\pm$ 0.6
HDL cholesterol (mmol/l)	1.7 $\pm$ 0.3	1.6 $\pm$ 0.3	1.2 $\pm$ 0.2 ^{†,}
Plasma triglycerides (mmol/l)	0.8 $\pm$ 0.2	1.0 $\pm$ 0.4	1.6 $\pm$ 0.5*
GDR basal (mg/mg per min ²)	74 $\pm$ 8	73 $\pm$ 6	74 $\pm$ 10
GDR clamp (mg/mg per min ²)	369 $\pm$ 74	313 $\pm$ 81	160 $\pm$ 16* [§]
Plasma lactate basal (mmol/l)	0.70 $\pm$ 0.19	0.74 $\pm$ 0.18	1.14 $\pm$ 0.34 ^{†,}
Plasma lactate clamp (mmol/l)	1.30 $\pm$ 0.24	1.19 $\pm$ 0.29	1.01 $\pm$ 0.22 [‡]
Serum FFA basal (mmol/l)	0.453 $\pm$ 0.145	0.409 $\pm$ 0.108	0.541 $\pm$ 0.186
Serum FFA clamp (mmol/l)	0.012 $\pm$ 0.006	0.023 $\pm$ 0.015	0.081 $\pm$ 0.050*

Data represent means $\pm$ s.d. * P <0.001, [†] P <0.01 and [‡] P <0.05 versus lean subjects, [§] P <0.001, ^{||} P <0.01 and [¶] P <0.05 versus obese control subjects. GDR, glucose disposal rates.

Study design

All study subjects were admitted to the Diabetes Research Centre at Odense University Hospital, Denmark. They were instructed to refrain from strenuous physical activity for a period of 48 h before the experiment. After an overnight fast, the subjects underwent a euglycemic–hyperinsulinemic clamp. After a 2-h basal tracer equilibration period, insulin was infused at a rate of 40 mU/m² per min for 4 h. A primed-constant 3-³H-glucose infusion was used throughout the 6-h study, and 3-³H-glucose was added to the glucose infusates to maintain plasma-specific activity constant at baseline levels during the 4-h clamp period as previously described in details (36). Using this protocol, physiological hyperinsulinemia at a serum insulin concentration of ~400 pmol/l was obtained in all groups during the insulin-stimulated period. In type 2 diabetic subjects, plasma glucose was allowed to decline to ~5.5 mmol/l during the initial part of the 4-h insulin infusion period before glucose infusion was initiated. Total glucose disposal rates (GDR) was calculated using Steele's non-steady-state equations adapted for labeled glucose infusates (37). Distribution volume of glucose was taken as 200 ml/kg body weight and pool fraction as 0.65. Percent body fat was determined by the bioimpedance method.

Biochemical analyses

Plasma glucose was measured at bedside on a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA, USA) by the glucose oxidase method. Total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein cholesterol, and triglycerides were all analyzed on a Modular Analytics P (Roche Diagnostics) with methods applied as recommended by the supplier. Serum insulin and C-peptide were analyzed by a two-site time-resolved immunofluorometric assay using DELFIA equipment (Perkin Elmer/Wallac, Turku, Finland). Serum-free fatty acids (FFA) were measured by an enzymatic colorimetric method (Wako Chemicals, Neuss, Germany). HbA1c was measured by cation exchange chromatography using Tosoh G7 (Medinor, Broendby, Denmark) with dedicated reagents. Lactate was analyzed on an ABL 800 Flex (Radiometer, Copenhagen, Denmark) using electrochemical sensors based on potentiometric and amperometric methods.

Osteoprotegerin measurement

The OPG concentration was measured in EDTA-plasma using a modified immunoassay based on a commercially available sandwich ELISA (R&D Systems, Minneapolis, MN, USA). The assay used specific antibodies against human OPG, and for detection Eu-labeled streptavidin was used. Bound europium was measured by time-resolved fluorometric detection using an AutoDelfia

Instrument from Perkin Elmer/Wallac. The analytical range was 62.5–20 000 ng/l with an intra-assay imprecision of 4% and an inter-assay imprecision of 7%.

Statistical analyses

Student's *t*-test was used to evaluate unpaired and paired data respectively. For statistical comparison of the changes in plasma OPG concentrations, OPG relative change was calculated by simply dividing the change in OPG concentrations ($C_{\text{end of clamp}} \div C_{\text{baseline}}$) with the baseline value. Associations between continuous variables were assessed by Pearson's correlation coefficient with normal distribution as an assumption or Spearman's test when data were not normally distributed. ANOVA was performed to disclose differences between groups using Tukey *post hoc* analyses. Two-tailed tests were used with a 5% significance level. Analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Baseline variables for all groups are shown in Table 1. Baseline OPG did not differ significantly between the three groups, as mean OPG concentrations were (mean $\pm$ s.d.) 1038 $\pm$ 302 ng/l in the lean control group, 993 $\pm$ 184 ng/l in the obese group, and 1074 $\pm$ 372 ng/l in the diabetic group (Fig. 1).

After insulin infusion, plasma OPG concentrations decreased in all groups (Fig. 2). In the type 2 diabetic group, the decrease was 141 $\pm$ 123 ng/l and in the obese group 110 $\pm$ 106 ng/l, whereas in the control group with normal body weight, it was 235 $\pm$ 97 ng/l ($P=0.006$, $P=0.009$, and $P<0.001$ respectively when compared with the baseline value; Fig. 3). ANOVA analyses showed differences in the decrease between the groups ($P=0.007$). *Post hoc* analyses revealed a larger

Figure 1 Baseline plasma OPG concentrations. (A) Diabetes; (B) obese; (C) control, lean. Horizontal bars indicate mean value, vertical bars indicate s.d.

Figure 2 Alteration in plasma OPG concentrations. *P* values are Student's *t*-test, showing a significant decrease for all groups.

decrease in plasma OPG in the lean control group than in both the diabetes and the obese group ($P=0.053$ and $P=0.029$ respectively).

Of significance, the relative change in OPG correlated significantly with HbA1c ($r=0.771$; $P=0.009$) and FFA baseline ($r=0.691$; $P=0.027$) in the lean group. No significant relationship between OPG fold change and BMI, GDR clamp, FFA clamp, lipids or glucose, and lactate levels was observed. OPG baseline values correlated inversely with FFA baseline in the lean group ($r=-0.702$; $P=0.024$), while there were no correlations in the obese group. In the diabetic group, OPG baseline concentrations correlated with LDL

Figure 3 Relative changes in plasma OPG concentrations. (A) Diabetes; (B) obese; (C) control, lean. Horizontal bars indicate mean value, vertical bars indicate s.d.

cholesterol ($r=-0.719$; $P=0.019$), fasting insulin ($r=0.770$; $P=0.009$), and lactate baseline ($r=0.800$; $P=0.005$). When correlation analysis was performed for the entire group, the only significant correlation was between lactate baseline and OPG baseline concentrations ($r=0.419$; $P=0.021$).

Discussion

In this study, we investigated the effect of insulin on plasma OPG concentrations in individuals with type 2 diabetes and obese individuals as well as in individuals with normal body weight. We found a decrease in plasma OPG concentrations after 4 h of hyperinsulinemia in all three groups. The insulin effect on plasma OPG was diminished both in individuals with type 2 diabetes and obesity compared with the lean control group. This finding is compatible with the idea that OPG may be involved in insulin effects in connective tissues, especially vasculature, and that this pathway is dysfunctional in type 2 diabetes and obesity.

The determinators of the circulating OPG level are not known, but the decrease in plasma OPG after the hyperinsulinemic clamp may be due to either alteration in the production, the mobilization, or the degradation of OPG. In a previous study of acute effects of hyperglycemia, we found no effects on plasma OPG in a set-up very similar to the present, also using the same OPG assay (38). Thus, no variations seem to occur in plasma OPG during the time of the day, where these experiments are performed. Our findings are consistent with earlier observations of the direct influence of physiological concentrations of insulin on OPG synthesis from VSMC. In these previous *in vitro* studies, we showed that OPG secretion from VSMC was diminished after insulin addition, both at RNA and protein levels (2). The *in vitro* effects on OPG synthesis were the strongest after more than 4 h of insulin treatment, suggesting that larger differences may have

been observed if longer observation periods would have been obtainable. Interestingly, also effects of insulin-sensitizing drugs (PPAR agonists) have been shown to influence OPG production *in vitro* (11). A large proportion of OPG in plasma may come from bone, but information about putative insulin effects on OPG synthesis in bone cells is lacking. Inflammatory factors such as TNF- α , IL-1, and IL-4 have been shown to alter the expression of OPG in both bone and vascular cells (2, 9, 10), but whether the observed effects of insulin on plasma OPG work through inflammatory pathways need further investigation.

Not only production from tissues, but also release from vascular surfaces may determine the plasma OPG concentration. This was demonstrated in two independent studies, where injection of heparin caused acute rise in plasma OPG (6, 39). This observation can be explained by the fact that OPG, secreted from smooth muscle cells and other stromal cells like osteoblasts, is transported to the luminal side of endothelial cells, where it binds proteoglycans. From this position, OPG can be competitively washed into the circulation by heparin (6). Since insulin may alter proteoglycan composition (14, 15, 40) it is possible that the observed effects of insulin could be explained by altered binding to surface proteoglycans. In addition to alterations in synthesis and releasability from vascular surfaces, it is also theoretically possible that the degradation of plasma OPG may partly explain the observed effects of insulin. However, nothing is yet known about OPG degradation or excretion *in vivo*. A more concise time study may help to reveal whether the mechanisms involve increased synthesis, mobilization, or elimination.

We found a clear discordance in the insulin-induced decrease of OPG in the lean controls and individuals with type 2 diabetes as well as obesity. This is compatible with the fact that many of the biological effects of insulin are severely impaired in patients with type 2 diabetes and to a lesser extent also in obesity. However, the observed ability of insulin to suppress plasma OPG does not seem to be tightly associated with insulin action on peripheral glucose metabolism, as there was no correlation between insulin-mediated decrease in OPG and insulin-stimulated GDR in any group. Moreover, there was no difference in the OPG fold change between obese non-diabetic subjects and patients with type 2 diabetes despite a significantly lower insulin-stimulated GDR in the diabetic group. Thus, the observed effects on plasma OPG could relate to direct effects of insulin on vascular cells, which previously has been observed in a series of both *in vivo* (12) and *in vitro* studies (2, 13–15, 41). As the effects of insulin on the vasculature are different from metabolic effects in muscle, fat, and liver, and also have a different timing, the lack of observed correlations are not surprising.

We observed a significant positive correlation between the relative change in OPG and FFA values at baseline in the lean control group ($P=0.027$).

In the obese group, a positive correlation was also indicated, although not statistically significant ($P=0.095$), while in the diabetic group no associations were observed. Thus, it could be speculated that FFA concentrations may play a role in insulin regulation of the plasma OPG concentration in insulin-sensitive subjects, whereas high FFA concentrations do not play a role in insulin-insensitive subjects. Interestingly, a recent study has reported synergistic effects of FFA and insulin on the production of another extracellular molecule in the arterial wall, namely matrix metalloproteinase-2 in insulin-sensitive animals (42). Whether similar mechanisms are involved concerning arterial OPG production is unknown and demands further investigations.

Our observation that plasma OPG at baseline did not differ between obese and lean persons is compatible with previous findings (24). Furthermore, our findings of similar OPG concentrations at baseline in all groups are in agreement with one earlier study showing no significant differences between plasma OPG in diabetic and non-diabetic individuals (43), but two other studies have described a difference (20, 44). As our own study have a limited number of participants, and the studies mentioned included 264, 490, and 86 subjects respectively, this matter needs elucidation through a larger study.

Also, it should be taken into consideration that high plasma OPG has evolved as a strong, independent predictor of cardiovascular diseases (32, 45). This observation has been ascribed to the idea of plasma OPG as a marker of vascular calcifications (46), a characteristic often observed in patients with glucose intolerance (47) and recently shown to involve actions of insulin (17, 41). Plasma OPG levels in patients with obesity and type 2 diabetes may therefore rely on the prevalence of cardiovascular disease in the study population. Nevertheless, our present observations fit very well with the described associations between plasma OPG, cardiovascular risk, vascular calcifications, and actions of insulin, especially when taking into consideration that OPG accumulates in the arterial wall in diabetes (2).

In conclusion, we show for the first time that insulin decreases plasma OPG acutely and that this effect is diminished in obese individuals with and without diabetes. These observations indicate that insulin effects may involve OPG. This knowledge is particularly relevant when trying to unravel the relationship between plasma OPG, vascular calcifications, diabetic arterial disease, actions of insulin, and cardiovascular risk.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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