Circulatory neutrophil chemokines in statin-treated diabetic patients

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ABSTRACT

الأهداف: تزيد نسبة الإصابة بتصلب الشرايين، والتي تعتبر السبب الرئيس في معدلات الأمراض و الوفيات في مرضى السكري. وتلعب كريات الدم البيضاء المنشَطة دوراً أساسياً في هذه العملية، حيث تعتبر بعض أنواع بروتينات السيتوكاينز (Cytokines) والتي تنشط خلايا الدم المتعادلة (Neutrophils) رابطاً مرضياً هاماً لحدوث مضاعفات أمراض الأوعية الدموية في مرضى السكري. لذا تهدف هذه الدراسة لمعرفة تأثير الأستاتين (Statin) على مستوى بروتينات السيتوكاينز الجاذبة للخلايا المتعادلة مثل بروتينات الـ GCP–2, GRO–α, ENA–78.

الطريقة: تحتوي عينة هذه الدراسة على 91 مريضاً بالسكري (منهم 46 يتم علاجهم بالأستاتين، و45 لا يتناولون أستاتين) و28 شخصاً من الأصحاء. تم قياس مستوى البروتينات الجاذبة GCP–2, GRO–α, ENA–78 في بلازما الدم لدى المجموعات الثلاث.

النتائج: كان مستوى بروتينات GCP–2 و ENA–78، وليس GRO–α، أعلى إحصائياً في مرضي السكري مقارنةً بالأشخاص الأصحاء ($p<0.05$). وعمل علاج الأستاتين على خفض مستوى كل من GCP–2 وGRO–α. ولم يتأثر مستوى ENA–78 بالأستاتين. لم يكن هناك توافق إحصائي بين مستويات بروتينات السيتوكاينز و دالة كثافة الجسم، و لا مستوى سكر الدم في المجموعات التي تمت دراستها. وقد تمت الدراسة في مستشفى الملك خالد الجامعي بالرياض، في الفترة من يناير 2006م إلى يوليو 2007م.

خاتمة: يصاحب مرض السكري ارتفاع في مستوى بروتينات الـ GCP–2 و ENA–78 ولا ينطبق ذلك على بروتينات الـ GRO–α. ويلعب الأستاتين (Statin) دوراً أساسياً في خفض مستوى البروتينات الجاذبة للخلايا المتعادلة GCP–2.

Objective: To assess the effect of statins on the circulatory levels of neutrophil chemokines, namely, granulocyte chemotactic protein-2 (GCP-2), growth regulated oncogene-alpha (GRO-α) and epithelial-cell-derived neutrophil-activating peptide-78 (ENA-78) in patients with diabetes.

Methods: We studied 91 diabetic patients (46 were statin-treated and 45 were not) and 28 healthy subjects. We measured the levels of GCP-2, GRO-α, and ENA-78 in the serum for the 3 groups using an enzyme linked immunosorbent assay. This cross-sectional study was conducted at King Khalid University Hospital (KKUH), Riyadh, Kingdom of Saudi Arabia, from January 2006 to July 2007.

Results: Circulating levels of GCP-2, ENA-78, and not GRO-α, were significantly higher in diabetic patients as compared to healthy subjects ($p<0.05$). Statins dropped the levels of both GCP-2 and GRO-α. The ENA-78 levels were not affected by statin therapy. There was no correlation between the levels of these chemokines with the body mass index and glycemia in the population studied.

Conclusion: Diabetes is associated with an elevation of GCP-2 and ENA-78, and not GRO-α. Statins have a significant role in reducing the level of GCP-2.

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Diabetes is a chronic inflammatory state associated with insulin resistance.[1-3] Diabetics have a higher morbidity and mortality due to cardiovascular disease (CVD) than non-diabetics. It is currently understood that inflammation, cytokines in particular, plays a role in CVD.[4,5] The production of proinflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin-1b (IL-1b) has been reported in both CVD and diabetes.[6] Growth factors, cytokines, and diabetic metabolic changes such as hyperglycemia, the glycosylation and oxidation of lipoproteins, compositional changes of lipoproteins, platelet aggregation, and the generation of free radicals may all contribute to the pathogenesis of diabetic macrovascular disease.[7] Cytokines are proteins produced by different cell types that regulate the function of other cell types in different tissues. They are important in the regulation and mediation of many events that occur in inflammation. Chemokines are low molecular weight, 8-10 kilodalton, chemotactic cytokines. They represent the chemotactic signals that are produced by different cell types to initiate leukocyte infiltration into inflamed or injured tissue.[8] Cytokine activity plays an important role in the pathogenesis of CVD including atherosclerosis, acute myocardial infarction, congestive heart failure, and myocarditis.[9] Interleukin-8 (IL-8), granulocyte chemotactic protein-2 (GCP-2), growth regulated oncogene-alpha (GRO-α), and epithelial-cell-derived neutrophil-activating peptide-78 (ENA-78) are α-chemokines that are capable of binding to cysteine-X amino acid-cysteine (CXC) chemokine receptor-2 (CXCR-2), which is found on the surface of neutrophils.[10] These chemokines are able to induce neutrophil migration, degranulation, and they exert many biological effects in neutrophils.[11] The role of cytokines in diabetes is also under intense investigation.[12] Hyperglycemia has been shown to induce proinflammatory cytokines and chemokine genes in monocytic cells.[13] Increased blood levels of IL-8, IL-6, IL-1b and TNF-α have been reported in diabetic patients.[6,14,15] The TNF-α has a role in impairing the insulin action in peripheral tissue.[16] Increased blood levels of IL-6 are also reported in type 1 diabetes.[17] Furthermore, it has been reported that inflammation is an initiating factor in insulin insensitivity, and that proinflammatory cytokines link inflammation to insulin resistance.[18-20] Statin, a 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA)-reductase inhibitor, is known to suppress the progression of atherosclerosis by its pleiotropic effects including the improvement of thrombus formation, antioxidant effect, and anti-inflammatory action.[21] One study showed that statin was protective against the development of diabetes mellitus.[22] We hypothesized that statin therapy will reduce the levels of the most potent polymorphonuclear neutrophils (PMNs) chemoattractants and activators, such as GCP-2, GRO-α, and ENA-78, which may reduce the level of inflammation. Although serum levels of IL-8 are known to be raised in patients with diabetes, levels of other chemokines such as GCP-2, GRO-α, and ENA-78 have not been measured. In this study, therefore, we measured the levels of these chemokines in 3 different groups: nondiabetic subjects as control group, diabetic patients that have not been treated with statins, and diabetic patients treated with statins.

Methods. Three groups were studied, nondiabetic men and women as healthy control group, diabetic patients that have not been treated with statins (DM-statin), and diabetic patients treated with statins (DM+statin). Patients were enrolled from the Endocrine Clinic, Obesity Research Center, King Khalid University Hospital (KKUH), Riyadh, Kingdom of Saudi Arabia from January 2006 to July 2007. Patients treated with statins were either on Simvastatin or Atorvastatin at 10-40 mg daily dose. Exclusion criteria include patients with acute or chronic infection, renal failure, and patients receiving steroid therapy. The protocol was approved by the College of Medicine Research Center (CMRC) review board, and all patients had agreed to participate in the study. Patient's characteristics are summarized in **Table 1**. In the statin untreated group, 9% were on diet only, 51% were on oral hypoglycemic agents, 20% were on insulin, and 20% were on oral hypoglycemic agents plus insulin. Fifteen milliliters of blood were drawn in a citrate tube from all patients. The GCP-2, GRO-α, and ENA-78 concentrations were determined by enzyme linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN). The intraassay coefficient of variation (CV) and interassay CV were 5.4% and 6.8% for GCP-2 assay, 2.7% and 5.3% for GRO-α assay, and 3.8% and 6.7% for ENA-78 assay. The minimum detectable dose for these 3 assays was: less than 10 pg/mL for GCP-2, less than 15 pg/mL for GRO-α, and from 0.4-8.0 pg/mL for ENA-78.

Data were expressed as mean±standard error of the mean (SEM) values. Comparisons between control group, diabetic group with statin, and diabetic without statin were made using Bonferroni test for making post-ANOVA pair-wise mean comparisons. Correlation was evaluated by Pearson's test. Results were considered statistically significant when $p<0.05$. Standard statistical software (SPSS, version 12, Chicago, IL) was used for the analyses.

Results. **Table 1** shows the demographic characteristics of healthy subjects, diabetic patients not treated with statins, and diabetic patients treated with statins. There

Table 1 - Demographics of healthy subjects, diabetic patients not treated with statins (DM-Statin) and diabetic patients treated with statins (DM+Statin).

Parameters	Control	DM–Statin	DM+Statin
Male (n)	12	11	22
Female (n)	18	34	34
Age	47.1±1.3	50.7±1.7	60.1±1.4*
Height	159.3±1.75	157.7±0.96	157.6±1.17
Weight	73.7±2.4	74±2	76.1±2.3
BMI (kg/m^2)	29.19±1	29.9±0.9	30.1±0.95
Fasting blood glucose	5.65±0.25	8.9±0.4*	9.4±0.5*
HbA1c	5.9±0.3	8.2±0.2*	10.1±1.7*
Duration of diabetes (years)	-	12.7±6.9	11.0±8.2
Total cholesterol	5.2±0.12	4.73±0.1	4.85±0.16
LDL- cholesterol	3.3±0.12	3.62±0.15	3.66±0.31
HDL- cholesterol	1.17±3.56	1.13±7.5	1.15±8.8
Triglycerides	1.46±0.12	1.62±0.11	2.06±0.10*

BMI - body mass index, HbA1c - Hemoglobin A1c, LDL - low density lipoprotein, HDL - high density lipoprotein, *p<0.05 compared to healthy subjects

Table 2 - Correlations between serum GCP-2, Gro-α, and ENA-78 concentrations and laboratory parameters of all studied groups.

Parameters	GCP-2		GRO-α		ENA-78	
	r	n	r	n	r	n
BMI	–0.06	78	0.15	110	0.08	98
HbA1c	0.13	75	–0.04	107	0.06	96

n - number of patients, r - Pearson's product - moment correlation coefficient, BMI - body mass index, HbA1c - hemoglobin A1c, p>0.05

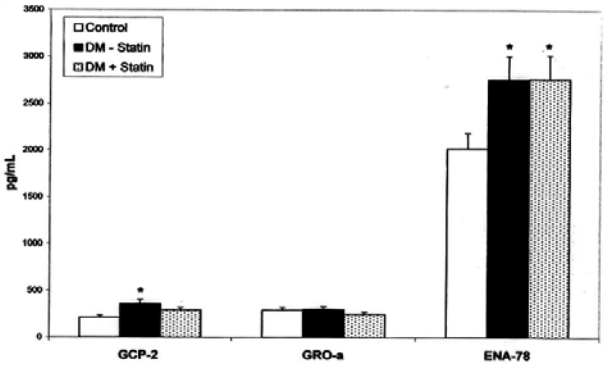

Figure 1 - Serum GCP-2 concentration: DM-statin versus healthy subjects p< 0.05, DM+Statin versus healthy subjects p> 0.05, DM-statin versus DM+statin p> 0.05. Serum GRO-α concentration: DM-statin versus healthy subjects p> 0.05, DM+Statin versus healthy subjects p> 0.05, DM-statin versus DM+Statin p> 0.05. Serum ENA-78 concentration: DM-statin versus healthy subjects p< 0.05, DM+statin versus healthy subjects p< 0.05, DM-Statin versus DM+statin p> 0.05. Results are expressed as means ± SEM, *p<0.05 compared to healthy subjects.

was no statistical significant difference between the last 2 groups in any of the measured parameters. However, diabetic patients treated with statins were significantly older and had higher serum triglycerides levels when compared to healthy subjects. **Figure 1** shows levels of neutrophil chemokines (GCP-2, GRO-α, and ENA-78) in the serum of healthy subjects, diabetic patients not treated with statins, and diabetic patients treated with statins. All values (in pg/mL) are means ±SEM. The circulatory levels of GCP-2 in diabetic patients not treated with statins were significantly higher than healthy subjects (358.7±41.9 and 212.1±24.8 pg/mL). We found that GCP-2 levels in diabetic patients treated with statins were lower than diabetic patients not treated with statins (289±30.5 and 358.7±41.9 pg/mL) and higher in comparison with healthy subjects (289±30.5 and 212.1±24.8 pg/mL). However, the difference was not statistically significant. The GRO-α was higher in diabetic patients not treated with statins than either control group or diabetic patients treated with statins (diabetic patients without statin: 298.8±32.2 pg/mL, healthy subjects: 287.5±34 pg/mL, diabetic patients with statin: 247.5±27pg/mL), however, the increase did not reach statistical significance. Statin therapy inhibited GRO-α in the diabetic group treated with statins in comparison with the other 2 groups, however, this was not statistically significant. The ENA-78 levels were significantly higher in both diabetic groups in comparison with healthy subjects (diabetic patients without statin: 2757±251.7 pg/mL, diabetic patients with statin: 2764.6±252.4 pg/mL, healthy subjects: 2017.9±167.7 pg/mL). There was no difference between the 2 diabetic groups. **Table 2** shows no correlation between the levels of these chemokines with the body mass index and glycemia in the population studied. In the statin treated group, there was a significant correlation between the levels of GRO-α with the body mass index (r=0.318, p<0.05).

Discussion. The PMNs have an important role in the pathogenesis of diabetes related chronic complications.[23] The PMNs are cells of inflammatory response and once activated, they show the ability to aggregate and adhere to the endothelium. The attraction of PMNs and other leukocytes to tissues is essential for inflammation, and the host response to infection.[8] Their stimulation results in reactive oxygen species (ROS) production, intensified arachidonic acid metabolism, and secretion of enzymes from PMN's granules. Both ROS and PMN's enzymes could contribute to the destruction of endothelial cells.[24] Several studies have demonstrated that neutrophil activation and platelet-neutrophil interactions are increased in type 2 diabetes.[25] The activation of PMNs is controlled by

chemokines.[10] Results from other studies support the role of inflammation in the development of diabetes.[26] One study has demonstrated that a component of the inflammatory response, proinflammatory cytokines, was increased in CVD, and further increased in diabetic women with CVD.[6] Hyaden and Tyagi[27] hypothesized that type 2 diabetes mellitus is a vascular disease (atherosclerosis) and inflammation, in particular cytokines, plays a key role in it. Esposito et al[14] reported that hyperglycemia acutely increased circulating IL-6 and TNF-α. Several studies have reported previously that diabetic patients have increased levels of IL-8, IL-6, and TNF-α either in the serum or vitreous fluid.[6,15,28-30] Insulin therapy could return the levels of these inflammatory cytokines to normal.[15] Statins have shown to lower serum cholesterol levels markedly and reduce cardiovascular morbidity and mortality.[31] In type 2 diabetes mellitus, high dose atorvastatin induced a strong reduction in C-reactive protein (CRP) levels, a non specific marker of inflammation, and showed no significant reduction in plasma IL-6 levels.[32,33]

In this study, we hypothesized that diabetes mellitus increases the circulatory levels of neutrophil chemokines such as GCP-2, GRO-α, and ENA-78. We also hypothesized that treatment with statins could inhibit the increased levels of neutrophil chemokines. We found that GCP-2 and ENA-78 were significantly increased in diabetic patients. It was noted in this study that these significant elevations of GCP-2 were inhibited, but it did not normalize completely in response to statin therapy. This points toward the presence of another mechanism that controls the circulating levels of GCP-2. This mechanism is probably not related to glycemia, since HbA1C was not higher in the statin compared to the non-statin treated group. However, ENA-78 concentrations were not affected by statin therapy. Our data also show the same GRO-α levels in both diabetic groups as compared to healthy subjects. The intriguing result is that the use of statins was associated with lower concentrations of GCP-2, and GRO-α, and not ENA-78. The implications of this observation are twofold. First, increased levels of neutrophil chemokines, GCP-2, and ENA-78 in diabetic patients are important indicators of the existence of inflammation in these patients. Second, statin doses usually administered in humans may not be sufficient to exert major effect on inflammatory reactions.

Visceral obesity is associated with the metabolic syndrome and a state of chronic low-grade inflammation, which may result in the development of type 2 diabetes and cardiovascular disease.[34] The concentration in blood of many adipokines, hormones, and acute-phase proteins is altered in human obesity. Blood levels of IL-10, IL-6, IL-8, TNF-α, and hepatocyte growth factor have all been reported to be elevated in obesity.[35-38] Therefore, in our study, we compared circulating GCP-2, GRO-α, and ENA-78 concentrations to laboratory parameters of disease activity. We did not find any correlation between these different chemokines with the BMI or HbA1C in the population studied. However, in the statin treated group, there was a significant correlation between the levels of GRO-α with the BMI ($r=0.318$, $p<0.05$). These results are inconsistent with previous studies that have shown a good correlation between IL-6 and other cytokines with BMI and body fat mass. This disparity, however, may be due to the different populations studied.[39-41]

The limitations to this study include the inability to determine the exact period of statin therapy. Most patients were referred from other clinics, and have already been on a statin without documentation of the starting date. Studies with a larger number of patients are needed to confirm these study results.

In conclusion, the findings of this study indicate that diabetic patients have significantly increased GCP-2 and ENA-78 in comparison with healthy controls, which indicate the potential additive effects of inflammation in this disease. In contrast, diabetic patients have the same serum GRO-α concentrations as the controls. Thus, the increased concentrations of GCP-2 and ENA-78 as a result of diabetes could work synergistically, and could be a contributor to more diabetic complications in these patients. Statin therapy has a significant effect on the levels of neutrophil chemokines. This further supports the beneficial effect of statin therapy in patients with diabetes. Further studies are needed to clarify whether levels of circulating neutrophil chemokines are associated with higher cardiovascular complications in patients with diabetes.

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