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SHORT COMMUNICATION

Link between plasma ceramides, inflammation and insulin resistance: association with serum IL-6 concentration in patients with coronary heart disease

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Abstract
Aims/hypothesis Ceramides and IL-6 have a role in immune–inflammatory responses and cardiovascular diseases, and are suggested to be involved in insulin and glucose metabolism. We sought to assess the associations of circulating levels of IL-6, TNF-α and high-sensitivity C reactive protein (hsCRP), which are inflammatory markers related to insulin resistance (IR), with the plasma lipid metabolites ceramides and diacylglycerols (DAG) in patients with CHD.
Methods Cross-sectional analyses were carried out on data from 33 patients with CHD. Serum levels of the inflammatory markers and plasma lipid metabolites (lipidomics approach performed by ultra-performance liquid chromatography coupled to electrospray ionisation MS) were measured at the same time point as insulin resistance (IR) (HOMA-IR index).
Results Serum circulating levels of IL-6 were strongly correlated with plasma ceramide concentrations (r=0.59, p<0.001). Adjustments for serum TNF-α or hsCRP levels, smoking, BMI, age, sex or HOMA-IR did not change the results (p<0.001). After adjustments for the effect of serum inflammatory markers (TNF-α or hsCRP), HOMA-IR and BMI the correlation between plasma DAG and serum IL-6 (r=0.33) was also significant (p<0.03). In a linear regression model, circulating levels of both ceramides and TNF-α had a significant independent influence on circulating levels of IL-6, altogether accounting for 41% of its variation (p<0.001).
Conclusions/interpretation Our results strongly suggest that the link between ceramides, IR and inflammation is related to the inflammatory marker IL-6. Ceramides may contribute to the induction of inflammation involved in IR states that frequently coexist with CHD.

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Keywords Cardiovascular disease · Ceramides · Diacylglycerols · IL-6 · Inflammation · Insulin resistance · Lipidomics

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Abbreviations

CRP	C-reactive protein
CVD	Cardiovascular disease
DAG	Diacylglycerols
hsCRP	High-sensitivity C-reactive protein
IR	Insulin resistance

Introduction

The sphingolipid ceramide has emerged as an important signal transduction metabolite, with a role in immune–inflammatory responses and with a potential role as an antagonist of insulin signalling [1]. The proinflammatory cytokines IL-6 and TNF-α are associated with insulin resistance (IR) and the metabolic syndrome [2]. These inflammatory markers are known to be involved in the hepatic production of inflammatory proteins such as C-reactive protein (CRP), which has been shown to increase the risk of type 2 diabetes mellitus and cardiovascular disease (CVD) [3, 4]. Other lipid metabolites, such as diacylglycerols (DAG), are related to IR, a core feature of type 2 diabetes [5].

Therefore, we sought to assess the association of circulating levels of IL-6, CRP and TNF-α with total plasma content of ceramides and DAG in patients with CHD, in whom IR is commonly present [6]. These individuals had participated in a previous dietary intervention and lipidomics study [7, 8].

Methods

Individuals (n=33) who were part of this cross-sectional study had volunteered originally for a study investigating the effect of fatty or lean fish on cardiovascular risk markers [7]. Briefly, patients who had been admitted to Kuopio University Hospital because of myocardial infarction or unstable ischaemic attack during the previous 3–36 months participated in the study. They gave written consent for participation in the study, which was approved by the Research Ethics Committee, Hospital District of Northern Savo.

Blood samples for the serum markers and lipidomics analyses were drawn after a 12 h overnight fast. High-sensitivity ELISA kits were used for IL-6 and TNF-α measurements (Quantikine; R&D Systems, Minneapolis, MN, USA). High-sensitivity CRP (hsCRP) was determined by an image immunochemistry system (Immulite 2000 DPC; Los Angeles, CA, USA). Serum insulin and plasma glucose were analysed as previously described [7]. Data on plasma ceramides and DAG were assessed by lipidomics analyses using ultra-performance liquid chromatography coupled to electrospray ionisation MS, described in more detailed elsewhere [8]. An internal standard mixture containing ten lipid classes was used. The lipids were extracted with chloroform/methanol (2:1 [vol./vol.], 100 μl to 50 μl plasma) and measurements were done in replicate.

The mean±SD age, BMI, fasting plasma glucose concentration, serum total cholesterol and LDL- and HDL-cholesterol, and median (interquartile range) triacylglycerols, fasting serum insulin concentration, and HOMA of IR (HOMA-IR) index [9] of the patients were respectively: 61.0±5.8 years, 27.2±2.6 kg/m^2, 5.80±0.63 mmol/l, 4.05±0.82 mmo/l, 2.15±0.60 mmo/l, 1.36±0.41 mmo/l, 1.09 mmol/l (0.75–1.94), 73.6 pmol/l (43.8–99.3), and 2.77 (1.58–3.81). All the participants were using beta blockers and statins. Further details of patients' medication use and other clinical and biochemical variables are described elsewhere [7].

Statistical analyses Correlations were performed using Pearson's correlation coefficient (r) adjusted by partial correlation for serum concentrations of TNF-α or hsCRP, HOMA-IR, BMI, age, sex or smoking. Multivariate linear regression analyses were carried out for testing the independent effect of plasma ceramides and other independent variables on IL-6 circulating levels. Except for HOMA-IR, variables associated with serum IL-6 and TNF-α, and also BMI, age, sex and smoking were tested one by one together with ceramides as independent variables in the models. The final explanatory variables were selected if their effects were significant at p<0.10. Variables with a skewed distribution were $\log_{10}$ transformed before the analyses and are presented as medians (interquartile range). p<0.05 was considered to be statistically significant. Analyses were performed using SPSS software version 14.0 (SPSS, Chicago, IL, USA).

Results

In the population studied, the median (interquartile range) for IL-6, TNF-α and hsCRP serum concentrations were 1.20 pg/ml (0.99–2.15), 1.22 pg/ml (0.94–1.51) and 0.72 mg/l (0.43–2.73), respectively. The mean±SD for total ceramides and DAG were respectively 3.82±1.27 μmol/l and 4.54±1.74 μmol/l. The subspecies of serum ceramides detected were d18:1/C23:0 and d18:1/C24:1. Their respective concentrations (median, interquartile range) were 1.36 μmol/l (0.94–1.72) and 2.58 μmol/l (1.70–3.02).

Circulating levels of both plasma ceramides and serum IL-6 correlated with HOMA-IR (r=0.33, p=0.06 and r=0.37, p=0.04, respectively). As described in Table 1, serum

Table 1 Correlations (r) among serum inflammatory markers and plasma ceramide and DAG concentrations in patients with CHD (n=33)

Analyte	Serum IL-6		Serum TNF-α		Serum hsCRP	
	r	p value	r	p value	r	p value
Ceramides[a]	0.59	<0.001	0.24	0.18	0.09	0.61
DAG	0.33[b]	0.060	−0.06[a]	0.75	0.00[a]	1.00
hsCRP[a]	0.46	0.008	0.31	0.08	–	–
TNF-α[a]	0.47	0.006	–	–	0.31	0.08

[a] Adjustments for age, sex, BMI, smoking, hsCRP, TNF-α or HOMA-IR did not alter the results

[b] p<0.03 after adjustment for BMI, HOMA-IR, hsCRP or TNF-α

IL-6 concentration correlated with both TNF-α and hsCRP levels. Interestingly, serum IL-6, but not TNF-α or hsCRP, correlated with plasma ceramides. Although the correlation between serum IL-6 levels and plasma DAG did not reach conventional statistical significance, after adjustments for the effect of serum inflammatory markers (TNF-α or hsCRP), HOMA-IR or BMI the correlation was significant (Table 1). No correlation was found between TNF-α or hsCRP levels and plasma DAG (p>0.50). Because ceramides subspecies d18:1/C23:0 and d18:1/C24:1 were highly correlated (r=0.80, p<0.000001), and did not differ in respect of their association with the outcomes of interest (data not shown), we used the total plasma ceramides for our analyses.

We hypothesised that plasma ceramide concentrations could influence serum IL-6 levels. Because production of hsCRP is influenced by IL-6 and correlates with TNF-α concentration, hsCRP was not considered in the models for testing the independent effect of ceramides on IL-6. The circulating levels of ceramides and TNF-α independently influenced IL-6 concentrations, altogether accounting for 41% of its variation (ceramides: β=0.47, p=0.003; TNF-α: β=0.31, p=0.04; HOMA-IR: β=0.13, p=0.40; R^2=0.41, p=0.001). The results were not different when HOMA-IR was taken out of the model or if a step-wise regression model approach was used, considering also BMI, age, sex and smoking as independent variables (ceramides: β=0.51, p=0.002; TNF-α: β=0.33, p=0.02; R^2=0.41, p=0.001). When analysed according to tertiles of plasma ceramide concentrations, individuals who had higher ceramide levels had higher serum IL-6 concentrations (Fig. 1).

Discussion

Our results clearly show a strong association between circulating levels of IL-6 and plasma ceramide concentrations in patients with established CHD. As far as we know, no data on this association have been reported earlier. Although a causal link between circulating IL-6 and CVD or type 2 diabetes is not yet established, our findings are of relevance because serum IL-6 concentration has been suggested as a risk factor for diabetes, and one of the putative links between obesity, IR, CVD and the metabolic syndrome [2]. IL-6 is involved in the hepatic production of CRP, which is associated with increased risk of type 2 diabetes and CVD [3, 4]. Moreover, animal studies suggest that ceramides can induce IR [1], and in humans have been associated with insulin sensitivity [10].

The present results suggest that the proposed adverse effect of IL-6 on IR and type 2 diabetes per se and possibly through its role in enhancing hepatic production of CRP could be initiated by ceramides. The sphingomyelin pathway and ceramides themselves are reported to play a role in the regulation of *IL6* gene expression [11], probably through the activation of transcription factors such as the nuclear factor kappa-B (NFκB) [11, 12]. Ceramides can act as inducers of proinflammatory cytokines through the

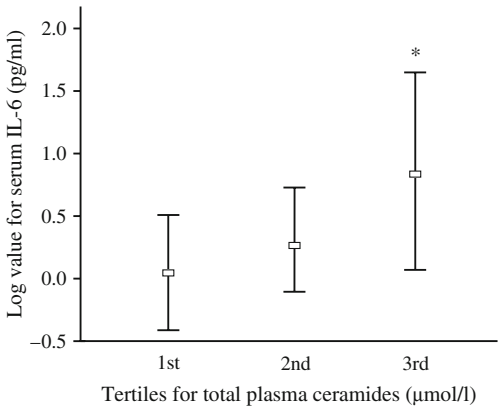

Fig. 1 Serum IL-6 concentration (mean±SD of $\log_{10}$ values) according to tertiles of plasma ceramides concentration. First tertile (n=11), 1.7–2.9 μmol/l; second tertile (n=11), 3.0–4.4 μmol/l; third tertile (n=11), 4.5–5.9 μmol/l. The respective medians (interquartile range) of IL-6 for each ceramide tertile were: first tertile, 1.09 pg/ml (0.84–1.43); second tertile, 1.19 pg/ml (1.03–1.69); and third tertile, 2.12 pg/ml (1.28–4.48). General linear model univariate analysis: p=0.02. *p =0.02 for third vs first tertile after Bonferroni post hoc test. All statistical analyses were adjusted for serum TNF-α concentration

activation of kinases (e.g. I-kappaB-kinase β [IKKβ]), which enables the activation of transcription factors such as NFκB [1]. Upregulation of ceramides and NFκB seem to work sequentially to promote the production of inflammatory molecules such as IL-6. Moreover, a role for NFκB and IKKβ in the development of IR and type 2 diabetes has been described and has been linked to inflammation [13].

Increased ceramide synthesis in response to excessive TNF-α is associated with an inhibition of insulin signalling [1]. TNF-α is known to be involved in the regulation of IL-6 production [12]. We did not observe any association between the circulating levels of TNF-α and ceramides. Conversely, recent findings showed an association between TNF-α circulating levels and plasma concentration of ceramide subspecies [10]. This observation was made in obese type 2 diabetes patients in whom IR is much more exacerbated. In our study only 15% of the participants were obese (BMI>30 kg/m^2), their serum levels of TNF-α were lower, and all were on statin therapy.

We found an association between total plasma DAG and IL-6 concentrations, which was stronger after adjustments for IR, BMI or serum inflammatory levels. DAG have been implicated in the development of IR via activation of specific protein kinase C (PKC) isoforms [5]. PKC is known to induce IL-6 synthesis [14]. However, ceramides seemed to be more strongly associated with IL-6 levels than DAG, independently of the degree of IR as assessed by HOMA-IR index.

The lack of a prospective arm might limit the generalisation of the present results. As this study is cross-sectional, we cannot assess any causal links based on the observed results. The small sample size could limit the statistical power for detecting significant results. Nonetheless, this is a homogeneous group of patients, which is important to better characterise the population under study. Because all participants were using statins, which lower plasma levels of hsCRP, correlation data involving this marker should be interpreted with caution.

In conclusion, we suggest that the link between ceramides, IR and inflammation is related to the inflammatory marker IL-6. Ceramides may contribute to the induction of inflammation involved in IR states that frequently coexist with CHD.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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