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Changes in adipose tissue gene expression and plasma levels of adipokines and acute-phase proteins in patients with critical illness

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Abstract

Insulin resistance develops rapidly during critical illness. The release of adipokines from adipose tissue is thought to play a key role in the development of insulin resistance, as are elevated levels of acute-phase proteins. The aim of this study was to identify changes in adipose tissue gene expression and plasma levels of adipokines and acute-phase proteins during critical illness. From 8 patients with subarachnoidal hemorrhage, consecutive blood samples and adipose tissue biopsies were obtained at 3 time points, twice during intensive care (1-2 days [IC1] and 7-9 days after subarachnoidal hemorrhage) and once after 8 months (recovery). The patients received a continuous insulin infusion to maintain normal glucose levels reflecting insulin resistance. The DNA microarray analysis showed increased zink-alpha2 glycoprotein (ZAG) and phospholipase A2, group IIA messenger RNA levels during intensive care compared with recovery ($P < .05$). Real-time polymerase chain reaction confirmed the increased expression of ZAG and phospholipase A2, group IIA. Plasma levels of ZAG, serum amyloid A, and C-reactive protein were higher at 7 to 9 days after subarachnoidal hemorrhage compared with either IC1 or recovery ($P = .0001$); and plasma levels of retinol-binding protein 4 and adiponectin were lower at IC1 compared with recovery ($P = .05$). The described changes in adipose tissue gene expression and plasma levels of adipokines and acute-phase proteins may influence the development of insulin resistance during critical illness.

1. Introduction

In 1878, Claude Bernard [1] described hyperglycemia during hemorrhagic shock; and it is now well known that acute illness or injury may result in insulin resistance, glucose intolerance, and hyperglycemia, collectively termed *diabetes of injury* [2,3]. A recent study in patients who underwent uncomplicated elective open cholecystectomy demonstrated an approximately 50% reduction in insulin sensitivity after surgery. The insulin resistance remained for at least 5 days and normalized after approximately 3 weeks [3]. Although reductions in morbidity and mortality have been demonstrated in intensive care patients who receive close glucose monitoring and insulin infusions [4], the causes of the acute insulin resistance remain unknown.

Many of the acute metabolic changes seen in critically ill patients are similar to those seen in patients with the metabolic syndrome, a condition that takes years to develop [5]. It has been proposed that adipose tissue plays a key role in the development of the metabolic syndrome via the secretion of a variety of hormones, cytokines, growth factors, and other biologically active mediators referred to as *adipokines*. The number of identified adipokines is constantly increasing, and their potential role in physiology and pathophysiology is under investigation [6]. Furthermore, there is a well-established link between the metabolic syndrome and a proinflammatory state including higher levels of cytokines and acute-phase proteins [7]. However, no studies have explored the involvement of adipose tissue in trauma and critical illness–induced insulin resistance.

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In an attempt to identify adipokines and acute-phase proteins involved in the development of insulin resistance, we performed a study on critically ill patients with subarachnoidal hemorrhage and investigated changes in plasma levels and gene expression in adipose tissue.

2. Materials and methods

2.1. Study layout

The participants in this study (4 men and 4 women; mean age, 54.7 ± 9.3 years; mean body mass index, 27.1 ± 3.6 kg/m^2 [range, 21.4-32.1 kg/m^2]) were recruited at the neurosurgical intensive care unit (NICU) at Sahlgrenska University Hospital. Informed consent was obtained from all study subjects or next of kin. The regional ethical review board at University of Gothenburg approved the study.

The participants were severely ill at admission, with an average Acute Physiology and Chronic Health Evaluation II score of 14.9 (range, 10-23). The aneurysm related to the subarachnoidal hemorrhage was verified with intracerebral angiography. Inclusion criteria included admittance to the NICU within 2 days of hemorrhage, an expected NICU stay of 1 week, and permanent residency in the Gothenburg area, Sweden, to allow follow-up sampling.

The participants remained in the NICU for 5 to 18 days (mean, 11.5 days). The day of the subarachnoidal hemorrhage was set as day 0. Blood samples and subcutaneous abdominal adipose tissue biopsies were taken at 3 time points, twice during intensive care treatment and once after recovery. The first sampling (IC1) was performed 1 to 2 days after subarachnoidal hemorrhage. The second sampling (IC2) was performed at 7 to 9 days after subarachnoidal hemorrhage. To obtain normal values for reference, a third fasting sampling was obtained after complete neurologic and metabolic recovery. This third sampling took place after an average of 8 months.

Plasma glucose levels were maintained within normal limits (4-6 mmol/L) by a continuous insulin infusion. For this study, *insulin resistance* was defined as a state where the endogenous insulin production is not sufficient and exogenous insulin is required to maintain glucose levels within normal limits. All patients required exogenous insulin infusions for between 3 and 15 days (mean, 7.8 days). Initially, the nutritional intake goal was 10 to 15 kcal/(kg 24 h); this level was gradually increased to a maximum of 25 kcal/(kg 24 h).

2.2. RNA isolation

The subcutaneous abdominal adipose tissue biopsies were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Isolation of RNA was performed using Qiagen Lipid Tissue kit (Qiagen, Hilden, Germany). The concentration was measured spectrophotometrically with an A_{260}/A_{280} ratio of 1.8 to 2.0, and the quality was verified by agarose gel electrophoresis.

2.3. Microarray analysis

For the DNA microarray analysis, preparation of complementary RNA and hybridization to DNA microarrays were performed according to the *Affymetrix Gene Chip Expression Analysis* manual. Briefly, RNA was reverse transcribed into complementary DNA (cDNA); and biotin-labeled target complementary RNA was prepared by in vitro transcription (Enzo Diagnostics, Farmingdale, NY) followed by hybridization to DNA microarrays, Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) according to the "Minimum Information About a Microarray Experiment" guideline [8]. These arrays are composed of 54 675 probe sets representing more than 47 000 transcripts. The microarrays were scanned with an Affymetrix confocal laser scanner (GeneArray scanner GCS3000) and visualized using GeneChip Operating Software (Affymetrix).

2.4. Analysis of microarray data and bioinformatics

Gene expression levels were calculated by the robust multiarray average method [9]. To identify differences in gene expression between time points, weighted analysis of paired microarray experiments was used [10]. Here, unequal precision and shared sources of variation are estimated and used to weight samples accordingly in calculation of (geometric) signal means and P values for differential gene expression. Thus, arrays of decreased quality are automatically detected and down weighted accordingly. Comparing 3′and 5′expression levels of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase and β-actin assessed cDNA synthesis quality and in vitro transcription.

Adipokines and acute-phase proteins regulated at least ±1.5-fold and with P less than .05 between intensive care and recovery were identified. Selected genes should also be regulated in the same direction during both time points during intensive care as compared with recovery. Gene Ontology (http://www.geneontology.org/) was used to classify genes according to function.

2.5. Real-time polymerase chain reaction analysis of gene expression

Reagents for real-time polymerase chain reaction (PCR) analysis of phospholipase A2, group IIA (PLA2G2A); zink-alpha2 glycoprotein (ZAG); and low-density lipoprotein (LDL) receptor–related protein 10 (Assays-on-Demand, TaqMan Reverse Transcriptase reagents, and TaqMan Universal PCR Master Mix) were purchased from Applied Biosystems (Foster City, CA) and used according to the manufacturer's protocol. Complementary DNA was synthesized from 500 ng of total RNA in a total reaction volume of 50 μL. Complementary DNA corresponding to 10 ng of RNA per reaction was used for real-time PCR amplification. Specific products were amplified and detected with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using default cycle parameters. A standard curve

was plotted for each primer-probe set with a serial dilution of adipose tissue cDNA. Based on our previous report [11] and expression profiles in the present study, human LDL receptor–related protein 10 was used as reference to normalize the expression levels between samples. All standards and samples were analyzed in triplicate.

2.6. Laboratory analyses

Blood chemistry analyses were performed at the Department of Clinical Chemistry, Sahlgrenska University Hospital, accredited according to ISO/IEC17 025. Concentrations of serum amyloid A (SAA) were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, CA) according to the manufacturer's instructions. Plasma concentrations of adiponectin and retinol-binding protein 4 (RBP-4) were determined using the Quantakine ELISA kit (R&D Systems Europe, Abingdon, United Kingdom) and the EIA Kit (Phoenix Peptides, Karlsruhe, Germany), respectively, according to the manufacturer's instructions. Plasma level of ZAG was determined using an ELISA kit (BioVendor Laboratories Medicine, Tumova, Brno, Czech Republic).

2.7. Statistical analysis

Statistical analysis of real-time PCR data and biochemistry analysis were performed using paired *t* tests to compare IC1, IC2, and recovery. *P* values not exceeding .05 were considered significant. Values are expressed as mean ± SD, unless otherwise indicated.

3. Results

3.1. Characteristics of study subjects

Biochemical variables from patients with subarachnoidal hemorrhage during intensive care and at recovery are shown in Table 1. Body mass index was unchanged during intensive care compared with recovery.

Table 2
Adipokines available on the microarrays

mRNA	IC1 vs R	IC1 vs R (*P* value)	IC2 vs R	IC2 vs R (*P* value)
	FC		FC	
Adipokines				
Leptin	1.4	NS	1.1	NS
Adiponectin	1.1	NS	−1.0	NS
Adipsin	1.0	NS	−1.1	NS
RBP-4	−1.0	NS	−1.2	NS
IL-6	1.1	NS	1.1	NS
IL-8	−1.0	NS	1.2	.047
IL-1B	1.1	NS	−1.0	NS
IL-1A	1.1	NS	−1.0	NS
IL-10	−1.1	NS	−1.1	NS
IL-12A	1.0	NS	1.0	NS
IL-12B	1.0	NS	1.1	NS
TNF-α	−1.0	NS	−1.0	NS
MCP1	−1.1	NS	−1.1	NS
Resistin	1.1	NS	−1.0	NS
PAI-1	−1.2	NS	−1.1	NS
ACE	−1.2	.038	−1.2	.038
ZAG	1.6	.012	1.9	.002
Acute-phase proteins				
SAA1/2	1.5	.0004	−2.0	.002
CRP	−1.0	NS	1.1	NS
PLA2G2A	3.4	.00009	3.0	.0004

mRNA indicates messenger RNA; FC, fold change; R, recovery; IL, interleukin; TNF-α, tumor necrosis factor–α; MCP1, monocyte chemoattractant protein–1; PAI-1, plasminogen activator inhibitor type I; ACE, angiotensin-converting enzyme.

Plasma levels of insulin were higher at both time points during intensive care compared with recovery. Total cholesterol, LDL cholesterol, high-density lipoprotein cholesterol, apolipoprotein A-1 (apo A-1), and apo B were lower at both time points during intensive care compared with recovery; and triglycerides (TG) were unchanged. The apo B/apo A-1 ratio increased during the ICU stay. Free fatty acids (FFA) were lower at IC1 compared with recovery. Serum levels of the acute-phase response proteins

Table 1
Characteristics of study subjects

	IC1	IC2	Recovery	IC1 vs R (*P* value)	IC2 vs R (*P* value)
BMI (kg/m^2)	27.1 ± 3.6		26.7 ± 4.4	NS	
kcal/(kg 24 h)	12 ± 6	22 ± 8	NA		
Infusion of insulin[a] (IU/24 h)	37.6 ± 25.8	47.1 ± 45.3	0		
s-Insulin (mU/L)	19.6 ± 14.2	44.1 ± 34.1	13.5 ± 13.8	.03	.05
s-C-peptide (mmol/L)	2.1 ± 3.7	0.77 ± 0.22	0.70 ± 0.17	NS	NS
p-Glucose (mmol/L)	5.8 ± 1.3	5.7 ± 0.9	5.0 ± 0.4	NS	NS
s-Cholesterol (mmol/L)	3.0 ± 0.72	3.5 ± 0.98	5.2 ± 0.90	.0002	.0005
s-TG (mmol/L)	0.9 ± 0.51	1.4 ± 0.53	1.3 ± 0.86	NS	NS
s-LDL-C (mmol/L)	1.4 ± 0.57	2.1 ± 0.73	3.0 ± 0.70	.0002	.007
s-HDL-C (mmol/L)	1.3 ± 0.29	0.7 ± 0.19	1.6 ± 0.42	.002	.0001
s-Apo B (g/L)	0.48 ± 0.23	0.79 ± 0.32	0.87 ± 0.27	.0003	NS
s-Apo A-1 (g/L)	0.99 ± 0.12	0.73 ± 0.16	1.3 ± 0.18	.000006	.00006
Apo B/apo A-1	0.49 ± 0.08	1.09 ± 0.13	0.67 ± 0.09	.008	.0007
p-FFA (mmol/L)	0.33 ± 0.12	0.42 ± 0.15	0.53 ± 0.19	.04	NS

R indicates recovery; NS, not significant; S, serum; P, plasma; LDL-C, LDL cholesterol; HDL-C, high-density lipoprotein cholesterol.
[a] Actrapid.

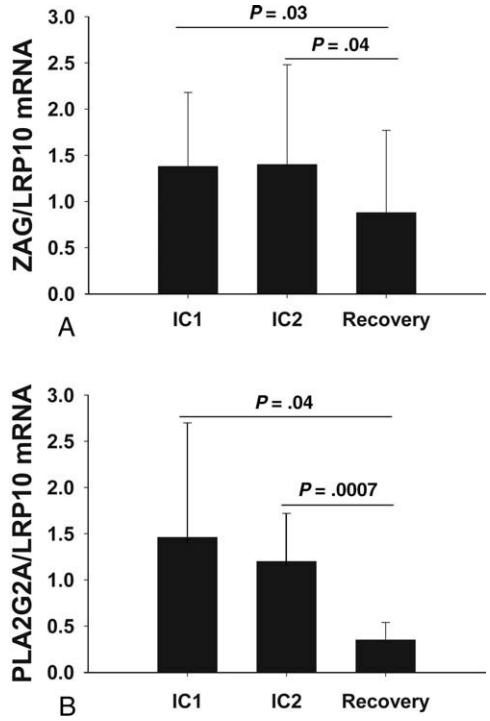

Fig. 1. The expression of adipose tissue (A) ZAG and (B) PLA2G2A analyzed by real-time PCR (mean ± SEM).

C-reactive protein (CRP) and A-SAA were highest at IC1 and lowest at recovery.

3.2. Identification of differentially expressed genes in adipose tissue during intensive care compared with recovery

Of the adipokines available on the microarrays [12], including adiponectin, RBP-4, and leptin, only ZAG was significantly changed (IC1 vs recovery fold change: 1.6, $P = .012$; IC2 vs recovery fold change: 1.9, $P = .002$) (Table 2).

The acute-phase protein PLA2G2A, an enzyme involved in the regulation of the phospholipid metabolism in biomembranes, displayed the largest difference of the genes that were higher during intensive care vs recovery. The changes in expression of ZAG and PLA2G2A were confirmed by real-time PCR (Fig. 1).

3.3. Changes in plasma adipokine levels during intensive care compared with recovery

Plasma levels of ZAG were higher at IC2 compared with IC1, and recovery and plasma levels of adiponectin and RBP-4 were lower at IC1 compared with recovery (Fig. 2).

4. Discussion

In summary, the adipokine ZAG and the acute-phase protein PLA2G2A were differentially expressed in adipose tissue from patients with subarachnoidal hemorrhage during intensive care compared with recovery. Furthermore, we observed higher plasma levels of insulin and ZAG and lower plasma levels of adiponectin and RBP-4 during intensive care compared with recovery. The serum levels of the acute-phase response proteins CRP and SAA were higher during intensive care compared with recovery.

For this study, only ICU patients with subarachnoidal hemorrhage were selected. Subarachnoidal hemorrhage is a severe but, by intensive care standards, homogenous condition where the patients become insulin resistant but usually do not develop serious infections or multiple organ failure. As no direct measurement of insulin resistance was available and homeostasis model assessment calculations [13] in an intensive care setting were judged unreliable, *reduced insulin sensitivity* had to be defined as the need for exogenous insulin to maintain normal glucose levels.

The design of the study, using the recovery phase as control in each patient rather than using unaffected matched controls, was chosen because of the well-known large interindividual variation in adipokine expression [14].

Zink-alpha2 glycoprotein, which was up-regulated during critical illness both in adipose tissue and in the circulation, is a novel adipokine that is present in plasma and other body fluids [15]. The biological function of ZAG remains largely unknown, but it is thought to be responsible for increased lipolysis in the adipose tissue of patients with cancer cachexia and in mice bearing a cachexia-inducing tumor [16]. Increased lipolysis may contribute to the development of insulin resistance [17]. High levels of TG and FFA are known to play a key role in the development of insulin resistance in individuals with the metabolic syndrome [18]. The generally low systemic lipid levels in this study, especially at IC1, were probably due to a combination of the continuous insulin infusion and a low caloric intake. Initially, the caloric intake was about 50% of energy expenditure, approaching resting energy expenditure after 1 week. A dyslipidemic state might still be suspected from the apo B/apo A-1 ratio. This ratio increased dramatically during the first week; and at IC2, the ratio reached a level corresponding to almost a 100% increase in the risk of myocardial infarction [19]. However, the low caloric intake, mainly via total parenteral nutrition, in combination with exogenous insulin infusion makes the comparison of ICU lipid levels with fasting levels at recovery difficult. Thus, ZAG may be involved the development of insulin resistance either through a local elevation [20] of FFA in adipose tissue or by mechanisms unrelated to lipolysis.

Adiponectin is an adipokine with beneficial effects on glucose and lipid metabolism [21]. Adiponectin levels are decreased in diseases associated with insulin resistance (visceral obesity, nonalcoholic fatty liver disease, atherosclerosis, and type 2 diabetes mellitus), and adiponectin is one of the best markers of decreased insulin action in vivo [22]. To our knowledge, the reduced adiponectin level during critical illness is a novel finding and may indicate that adiponectin plays a key role in the development of insulin resistance in such patients. We did not observe a parallel change in adiponectin gene expression in adipose tissue, but

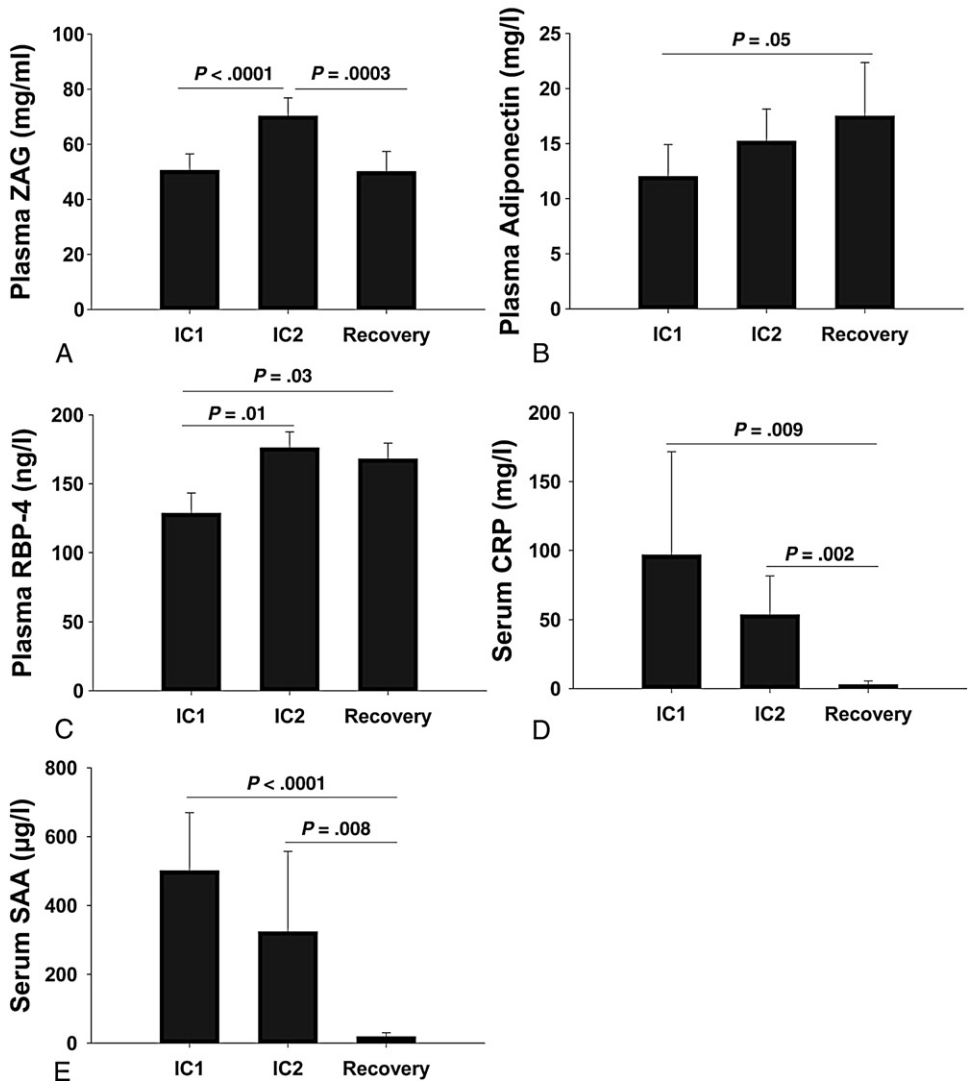

Fig. 2. Plasma and serum levels of adipokines and acute-phase proteins. Plasma levels of (A) ZAG, (B) adiponectin, and (C) RBP-4. Serum levels of (D) CRP and (E) SAA.

this is in line with our previous finding that it is difficult to relate adiponectin expression to circulating levels [23].

Retinol-binding protein 4 is a recently discovered adipokine that is thought to mediate systemic insulin resistance [24]. Elevated plasma RBP-4 levels indicate overt or impending insulin resistance in both lean and obese individuals with type 2 diabetes mellitus [24]. However, although insulin resistance was clearly evident during intensive care in our patients, RBP-4 levels were reduced. This discrepancy could be explained by the very low energy intake of our patients during intensive care, as severe calorie restriction has been shown to promote a reduction in adipose tissue and plasma levels of RBP-4 [25]. The metabolism of critical illness is characterized by a combination of starvation and stress [26], and decreased RBP-4 levels might therefore reflect the effects of calorie restriction during intensive care rather than insulin resistance. We did not observe a parallel decrease in RBP-4 gene expression in adipose tissue. This is in line with a previous report showing that RBP-4 gene expression in adipose tissue correlates poorly with plasma levels [27], and it is possible that plasma RBP-4 is produced mainly by the liver [28].

C-reactive protein and SAA are acute-phase proteins; and their concentrations increase markedly within hours of trauma, infection, or inflammation [29]. As expected, we observed high serum levels of CRP and SAA during intensive care. Increased serum levels of CRP are associated with atherosclerotic disease and are independent predictors of type 2 diabetes mellitus [30]. Furthermore, a recent study indicated that increased CRP levels might lead to decreased adiponectin levels and thereby regulate insulin sensitivity [31]. Thus, it is possible that the high levels of CRP observed during intensive care play a role in the development of insulin resistance.

Serum amyloid A is a proinflammatory and lipolytic adipokine in humans [32], and it may be involved in the

regulation of insulin sensitivity. In our study, the increase in serum levels of SAA during intensive care was not matched by parallel changes in adipose tissue gene expression. Although human adipocytes are primary sources of SAA during nonacute phase, SAA probably originates from the liver during acute illness [33].

Phospholipase A2, group IIA was first discovered in the synovial fluid of patients with rheumatoid arthritis [34] and has subsequently been shown to be associated with a variety of inflammatory diseases. Serum levels of the secreted enzyme can rise 1000-fold during severe sepsis, and the enzyme has been recognized as an acute-phase protein under the transcriptional control of proinflammatory cytokine signaling [35]. The DNA microarray analysis showed up-regulation of PLA2G2A during intensive care, which is interesting because it has been suggested that this enzyme may be involved in the cellular link between inflammatory pathways, lipid metabolism, and insulin resistance [36].

In conclusion, our data suggest that high levels of ZAG and the acute-phase proteins PLA2G2A, SAA, and CRP together with low levels of adiponectin may contribute to the development of insulin resistance during critical illness.

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