Selected adipokines - plasma concentrations and adipose tissue expressions during 24-hour lipid infusion in healthy men

J. Kopecký, Jr.[1], E. Krušinová[1], M. Klementová[1], L. Kazdová[1], P. Mlejnek[2], M. Pravenec[2], M.Hill[3], T. Pelikánová[1]

[1]Diabetes Center of Institute for Clinical and Experimental Medicine, Prague, Czech Republic;

[2]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

[3]Institute of Endocrinology, Prague, Czech Republic

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Corresponding author:

Terezie Pelikánová MD

Diabetes Center, Institute for Clinical and Experimental Medicine

Vídeňská 1958/9, 140 21 Prague

Czech Republic

Tel: +420 261 364 100

Fax: +420 261 363 183

Email: terezie.pelikanova@medicon.cz

Word count: Abstract 157

        Main text 2619

Abstract

Objective: Our aim was to assess the reaction of TNFα, resistin, leptin and adiponectin to lipid infusion.

Design and methods: Eight healthy subjects underwent a 24-hour lasting infusion of lipid emulsion. Plasma concentrations and expressions of selected cytokines in subcutaneous fat were measured.

Results: TNFα plasma concentration did not change during the first 4 hours of hypertriglyceridaemia, but a significant increase after 24 hours was detected ($p < 0.001$ for 0; 30; 240 min vs. 24 h). Plasma concentration of resistin significantly increased at 30 min of infusion and remained elevated ($p < 0.01$ for 0 min vs. 30; 240 min; $p < 0.001$ for 0 min vs. 24h). Plasma concentrations of leptin and adiponectin did not show any significant changes. Although the expression of resistin in the subcutaneous adipose tissue tended to increase, the change was not significant. Expressions of TNFα, leptin and adiponectin were unaffected.

Conclusion: Our results indicate that acutely induced hyperlipidaemia could influence the secretion of TNFα and resistin.

Key words: resistin, TNFα, leptin, adiponectin, insulin resistance, NEFA, triglycerides, lipotoxicity

Introduction

Hyperlipidaemia of either dietary origin or caused by lipid infusion promotes insulin resistance (IR) in rodents and humans (Boden and Chen 1995; Griffin et al. 1999; Leung et al. 2004). Pathophysiological mechanisms underlying the association between the elevation of plasma non-esterified fatty acids (NEFA) and IR have not been fully determined - for evidence of interplay between glucose and NEFA see review by (Cahova et al. 2007). Some of the pathways that lead to IR might include following cytokines produced by adipose tissue: TNFα, resistin, leptin and adiponectin.

TNFα is a promising candidate for mediating IR, although most of it comes from other sources than adipose tissue (Fain et al. 2003; Gabriely et al.). Plasma concentrations of TNFα are related to obesity and IR (Hotamisligil et al. 1993); TNFα knock-out mice are more insulin sensitive than controls and there is the evidence that TNFα may directly interfere with insulin signaling by inhibiting phosphorylation of the insulin receptor (Hotamisligil et al. 1994). It impairs human preadipocyte differentiation, in mature adipocytes it decreases the activity of markers of differentiation, (Petruschke and Hauner 1993) and increases their apoptosis (Prins et al. 1997). TNFα has been shown to mediate NEFA-induced IR in vitro in 3T3-L1 adipocytes (Nguyen et al. 2005)

Resistin was named for its putative role in mediating IR in obesity. In mice, it is a product of an adipocyte specific gene whose expression is down-regulated by rosiglitazone (Arner 2005). Serum levels of resistin were found elevated in rodent models of obesity and diabetes implicating a dysregulation of resistin in these disease states (Arner 2005). In humans, however, the role if resistin is unclear. The human homologue of resistin is only expressed at very low levels in adipose tissue and in circulating monocytes, and the reports of its function are controversial (Janke et al. 2002; Savage et al. 2001).

Leptin is a cytokine secreted mainly by adipose tissue, its circulating levels are in direct proportion to adipose tissue mass and nutritional status (Maffei et al. 1995). Therefore, it is a

likely candidate for mediating insulin sensitivity. It influences the human energy balance by altering energy intake and expenditure (Hukshorn and Saris 2004). It was shown in experimental animals that leptin infusion could prevent IR induced by acute lipid infusion (Dube et al. 2007). Consistent inverse association between adiponectin and IR as well as the pro-inflammatory state has been established, see (Nedvidkova et al. 2005) for review. Several mechanisms of its metabolic effects have been described (Kadowaki et al.; Whitehead et al. 2006): induction of glucose uptake and NEFA oxidation in muscle, increased insulin sensitivity and NEFA oxidation and a reduced glucose output and NEFA influx in liver.

Our aim was to assess the reaction of TNFα, resistin, leptin and adiponectin to lipid infusion. We have evaulated both the plasma concentrations and expression of their genes in the abdominal subcutaneous adipose tissue in healthy humans.

Subjects and methods

Subjects

We examined eight young healthy males. All of them were euthyroid and none had a concomitant disease. They were not taking any drugs, and none had a family history of diabetes. Clinical characteristics are in Table 1. The study was conducted after local ethics committee gave approval. Informed consent was obtained from all the individuals after the purpose, nature, and potential risks of the study had been explained.

Experimental protocol

The subjects were instructed to adhere to their ordinary lifestyle and avoid changes in food intake, alcohol consumption, and to avoid vigorous exercise on the day before examination. They were examined after overnight fasting with only tap water allowed ad libitum. The study always started between 7:45 – 8:00 AM. Subjects were excluded from the study if the weight change was more than 3 kg in 6 months.

A 24-hour infusion of lipid emulsion (Intralipid 20%; Fresenius Kabi AB, Uppsala, Sweden) was administered through teflon cannula in the antecubital vein. Intralipid 20% contained soya oil 200 g, egg lecithin 12 g and glycerol 22 g per 1 liter. The speed was set to 125 ml.h^{-1} for the first 4 hours, and then until the 24th hour to a constant rate that was calculated to achieve a total dose of 3g of fat.kg body weight^{-1}.d^{-1} This two-step setting of infusion was used to achieve both maximum effect and to stay within the clinically allowed boundaries. 30 minutes before blood sampling a second cannula was inserted retrogradely into a wrist vein for blood collection. To assess the plasma levels of selected adipokines, blood samples were taken at 0 min, 30 min, 240 min and 24 hours of the infusion. 30 minutes before taking each sample the hand was placed in a heated (65$^{°}$ C) box in order to achieve venous blood arterialization. Needle biopsy of abdominal subcutaneous adipose tissue was performed before (0 min), and at the 4th and 24th hour of the lipid infusion. Under local anesthesia (1% trimecain in a field block pattern) an incision (3-4 mm) was made through the skin at the lower abdomen and a subcutaneous fat specimen (200 mg) was obtained by needle (Braunüle MT, no.4; B.Braun, Melsungen, Germany) aspiration. Different place and incision was used each time, same depth in the adipose tissue mass when performing the needle aspiration was attempted. The samples were washed in NaCl 0.9% solution, immediately frozen by liquid nitrogen and stored at -80ºC until used for RNA extraction.

Analytical procedures

Infusion monitoring Plasma glucose concentrations were measured on a Beckman analyzer (Beckman Instruments Inc, Fullerton, CA, USA) using the glucose oxidase method. Hemoglobin A1c was measured by fully automated analyser Tosoh HLC-723 G7 (Tosoh Corporation, Tokyo, Japan). Method was calibrated to IFCC reference procedure (Mosca et al. 2007).

Immunoreactive insulin (IRI) was determined by radioimmunoassay (Insulin IRMA kit, IMMUNOTECH as, Prague, Czech Republic). Plasma concentrations of NEFA were estimated

by Free fatty acids, Half-micro test (Roche Diagnostics GmbH; Penzberg; Germany) Plasma concentrations of triglycerides (TG) by enzymatic assay (BIO-LA-TEST; PLIVA-Lachema, Brno, Czech Republic).

Adipokine assessment in plasma Plasma concentrations of TNFα were measured by immunoassay (Human TNF-α UltraSensitive; BioSource int., Camarillo, CA, USA) and the detection limit was <0.09 pg/ml, intra-assay and inter-assay coefficients of variation (CV) of 5.3 to 6.7% and 8.2 to 9.7% respectively); plasma concentrations of resistin by a Human Resistin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic) the detection limit was 0.033 ng/ml, intra-assay and inter-assay CV of 2.8 to 3.4% and 5.1 to 6.9% respectively; plasma concentrations of leptin by a Human Leptin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic), the detection limit was 0.5 ng/ml, intra-assay and inter-assay CV of 3 to 7.5% and 3.2 to 9.2% respectively; plasma concentrations of adiponectin by a Human Adiponectin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic) the detection limit was 210 ng/ml, intra-assay and inter-assay CV of 6.4 to 7% and 7.3 to 8.2% respectively, all according to manufacturers instructions.

Assessment of expressions in adipose tissue Expression of each adipokine was analysed by the real-time PCR method. Subcutaneous adipose tissue (100 mg) was excised from the biopsy, and homogenized in 1 ml of a QIAzol Lysis Reagent (Guanidin Thiocyanate – Phenol solution) for 2 minutes. The RNA was isolated from the liquid nitrogen frozen biopsy using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Valencia, CA, USA) and QIAzol Lysis Reagent (QIAGEN, Valencia, CA, USA). Possible contamination of RNA with genomic DNA remains was taken off by DNase digestion (RNase-free DNase Set; QIAGEN, Valencia, CA, USA).The cDNA was synthesized using a recombinant Omniscript Reverse Transcriptase (QIAGEN, Valencia, CA,

USA), Ribonuclease Inhibitor from human placenta (SIGMA, St. Louis, MO, USA), and $(dT)_{16}$ oligonucleotides.

The Real-time PCR procedure itself has been carried out on the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). HotStar Taq DNA polymerase and SYBR Green fluorescent dye (QuantiTec SYBR Green PCR Kit, QIAGEN, Valencia, CA, USA) were used for the RT-PCR reaction. To eliminate the influence of primer dimmers, negative controls were used. The human gene cyclophilin was used as a reference. Primers used are shown in Table 2. The data were processed by Q-gene 96 software.

Statistical analysis

The time profile was evaluated using a repeated measures ANOVA model consisting of the time and subject factors. To evaluate the differences between basal values and individual stages of the time profile, the ANOVA testing was followed by least significant difference multiple comparisons. A probability level of $p<0.05$ was considered as statistically significant in all statistical tests. Due to non-Gaussian data distribution in most of the dependent variables, these data underwent a power transformation to attain distributional symmetry and constant variance. Non-homogeneities were detected using residual diagnostics. The experimental points showing absolute values of studentized residuals greater than 3 were excluded from the analysis. With the exception of serum leptin levels (6.3%), the proportions of such data never exceed 5% of the total number. Statistical software Statgraphics Plus v. 5.1 from Manugistics (Rockville, MD, USA) was used for calculations.

Results

Our primary goal was to assess the reaction of selected adipokines to acute lipid infusion and the following results were obtained. TNFα plasma concentration did not change during the first 4 hours of hypertriglyceridaemia, but a significant increase after 24 hours was detected ($p<0.001$

for 0; 30; 240 min vs. 24 h; Fig.1a). The expression of TNFα in subcutaneous adipose tissue did not change (Fig. 2a). Plasma concentration of resistin significantly increased at 30 min of infusion and remained elevated throughout the 24 hours (p<0.01 for 0 min vs. 30; 240 min; p<0.001 for 0 min vs. 24h; Fig.1b). The expression of resistin in the subcutaneous adipose tissue tended to increase, but the change was not significant (Fig.2b). Plasma concentrations of leptin and adiponectin (Fig.1c-d) did not show any statistically significant changes and their expressions were not significantly altered.

To validate the effectiveness of lipid infusion, plasma levels of TG, NEFA, glucose and IRI were monitored. TG increased almost 8-fold and NEFA about 3.5-fold in the 4th hour and then they remained elevated until the end of infusion at more than 3-fold resp. 2-fold (Fig.3a, b). Blood glucose was stable throughout the 24 hours (Fig.3c). After initial modest rise, IRI concentrations were also stable (Fig.3d). Cytokine plasma concentrations did not correlate with IRI concentrations (data not shown).

Discussion

We have characterized the plasma levels of adipose tissue cytokines and their expression in adipose tissue during pharmacologically-induced hyperlipidaemia. This was achieved by an intravenous lipid infusion, in which the effectiveness in rise of plasma TG and NEFA was verified. We have decided not to use heparin infusion alongside as it is not "physiological" – i.e. heparin as a co-factor of a lipoprotein lipase would decrease plasma TG concentrations and increase plasma NEFA concentrations, which was not our aim. It could also add further bias through possible unknown effects. Since heparin was used in some further cited experiments (Garcia-Lorda et al. 2003; Nisoli et al. 2000; Yang et al. 2005), this sole fact could be the reason for differences in results.

Our finding of an increase in TNFα plasma concentration during lipid infusion is novel. It is partly in accordance with the report of Nisoli (Nisoli et al. 2000) who found increase in gene

expression of TNFα in subcutaneous gluteal fat tissue after a 5-hour intralipid/heparin infusion. In our results, the expressions in adipose tissue showed only a tendency to increase but not a significant rise. This can be explained by the differences in expressions in different fat depots. Both in human (Fain et al. 2003) and rodent (Gabriely et al. 2002) studies, it has been reported that production of both of resistin and TNFα is much higher in visceral than in subcutaneous fat. Moreover, most of the free circulating TNFα comes from circulating monocytes and recent findings suggest that significant amounts of resistin, although originally reported as adipocyte specific (Kim et al. 2001), might be of the same source (Savage et al. 2001).

During lipid infusion, plasma concentrations of resistin have increased significantly. This is in accordance with reports from animal models (Yang et al. 2005) where intralipid/heparin infusion induced hepatic and peripheral IR and was associated with elevated plasma resistin levels. In human, it is a novel observation. Since increase in expression of resistin mRNA in subcutaneous adipose tissue was not significant, we can conclude that this adipocyte depot is not responsible for the increase of it's plasma concentration.

Plasma concentration of leptin did not show any statistically significant changes and it's expression was not significantly altered. Previously, although most studies have not shown direct effect of lipid infusion on leptin, as when consistent release from adipose tissue during lipid without heparin infusion (Samra et al. 1998) was reported, some other results were also obtained: no change in plasma levels together with increase in leptin RNA expression in adipocytes in gluteal region during lipid and heparin infusion (Nisoli et al. 2000) and modest decline of it's plasma levels during lipid and heparin infusion by (Garcia-Lorda et al. 2003).

Reaction of adiponectin to acute lipid load was also not statistically significant. The same result of unchanged plasma concentrations was observed during intralipid/heparin infusion (Krzyzanowska et al. 2007).

Lipid infusion resulting in a subsequent increase in NEFA plasma concentrations is a well known method used for inducing IR (Boden and Chen 1995; Griffin et al.; Leung et al.). Therefore, although we have not measured insulin sensitivity, we suggest that changes we have observed might be implicated in the pathogenesis of lipid-induced IR. For TNFα, our results are in agreement with a theory that it is implicated in NEFA induced IR as shown in vitro (Nguyen et al. 2005). For resistin it is an unclear result as it is often found not to be associated with IR (Utzschneider et al. 2005), although the studies are often limited to correlations between resistin and various measures of metabolic syndrome. Rather exception is a report of Sheng (Sheng et al. 2008) where overexpression of resistin in human hepatocytes induced IR most likely by blocking insulin signal transduction pathways of PI-3K/Akt and of CAP/c-cbl.

We are aware that there are limits to our study. Increase in plasma lipid concentrations by intravenous infusion certainly differs from dietary and lifestyle induced hyperlipidaemia observed in real life. Small number of subjects examined might have prevented us from seeing some other possibly significant results and therefore the value of "negative" results is limited. In addition, the lack of placebo control, which was not done due to the potential troublesome interpretation of a 24-hour long fasting, to some extent limits the reliability of "positive" results. Circadian variations were partly assessed by 24-hour duration. Glycerol (a component of intralipid emulsion) fortunately does not affect insulin secretion (Boden and Chen 1999; Pelkonen et al. 1968), glucose effectiveness (Hawkins et al. 2003) or insulin sensitivity (Ferrannini et al. 1983). Effect of glycerol on resistin and TNFα is however not excluded. Resistin could also be affected by hyperinsulinaemia, although this observation was made at several-fold higher insulin concentrations (Krusinova et al. 2007) than observed during our lipid infusion. We have also found significant inter-individual and intra-individual differences of the mRNA content in adipose tissue samples for each adipokine. We must note, however, that this could partly be due to different adipocytes/stroma-vascular cells ratio in each sample. In future

studies, this can be assessed by separation of these fractions by collagenase tissue digestion (Rodbell 1963).

In conclusion, our results indicate that acutely-induced hyperlipidaemia could increase the secretion of TNFα and resistin. This finding supports the hypothesis that these adipokines could be involved in the pathogenesis of lipid-induced IR.

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**Table 1** Characteristics of the study group. Data are expressed as mean ± SEM.SD

(IFCC, International Federation of Clinical Chemistry and Laboratory Medicine)

| n	8
Age (years)	25.8±3.7
Weight (kg)	83.4±4.7
Body mass index (kg.m^{-2})	25±1.7
Waist circumference (cm)	88.5±5.0
Fasting blood glucose (mmol.l^{-1})	4.35±0.7
Blood glucose at 120 min of OGTT (mmol.l^{-1})	4.55±0.7
Fasting IRI (mIU.l^{-1})	4.50±2.85
HbA1c (%) - according to IFCC	2.58±0.61
Albuminuria (µg.min^{-1})	2.52±2.6
Serum creatinine (µmol.l^{-1})	87±10.5
Total cholesterol (mmol.l^{-1})	4.24±0.73
Triglycerides (mmol.l^{-1})	0.87±0.30
HDL-cholesterol (mmol.l^{-1})	1.45±0.34
LDL-cholesterol (mmol.l^{-1})	2.48±0.55

Table 2 Primers used for RT-PCR of the subcutaneous adipose tissue samples taken during the lipid infusion.

Gene	accession number	forward primer	reverse primer
Adiponectin	XM_290602	HACRP30-F : 5´-GGT TCA ATG GCT TGT TTG C -3´	HACRP30-R : 5´-TCA TCC CAA GCT GAT TCT G-3´
Leptin	NM_000230	Hleptin-F : 5´-CCC TAA GCC TCC TTT TGC T-3´	Hleptin-R : 5´-GCT AAG AGG GGA CAA GAC A-3´
TNFα	X02910 X02159	HTNFa-F : 5´-CTA TCT GGG AGG GGT CTT C-3´	HTNFa-R : 5´-TTG GGA AGG TTG GAT GTT C -3´
Resistin	AY207314	HRETN-F : 5´-ATA AGC AGC ATT GGC CTG G-3´	HRETN-R : 5´-TGG CAG TGA CAT GTG GTC T-3´
Cyclophilin	XM_090070	HCLPNa-F: 5´-CAA ATG CTG GAC CCA ACA CA -3´	HCLPNa-R : 5´-TGC CAT CCA ACC ACT CAG TC-3´

Fig.1a-d Plasma levels of selected adipokines during 24-hour lipid infusion. The bars with error bars represent re-transformed means with their 95% confidence intervals. F in the embedded tables represents Fisher's statistics for individual factors. R2 (squared correlation coefficient of the linear model) symbolize the proportion of the total variability in the dependent variable, which is explained by the ANOVA model. Asterisks symbolize significant differences between individual stages of the time profiles and basal values (** for $p<0.01$, *** for $p<0.001$) as detected by least significant difference multiple comparisons.
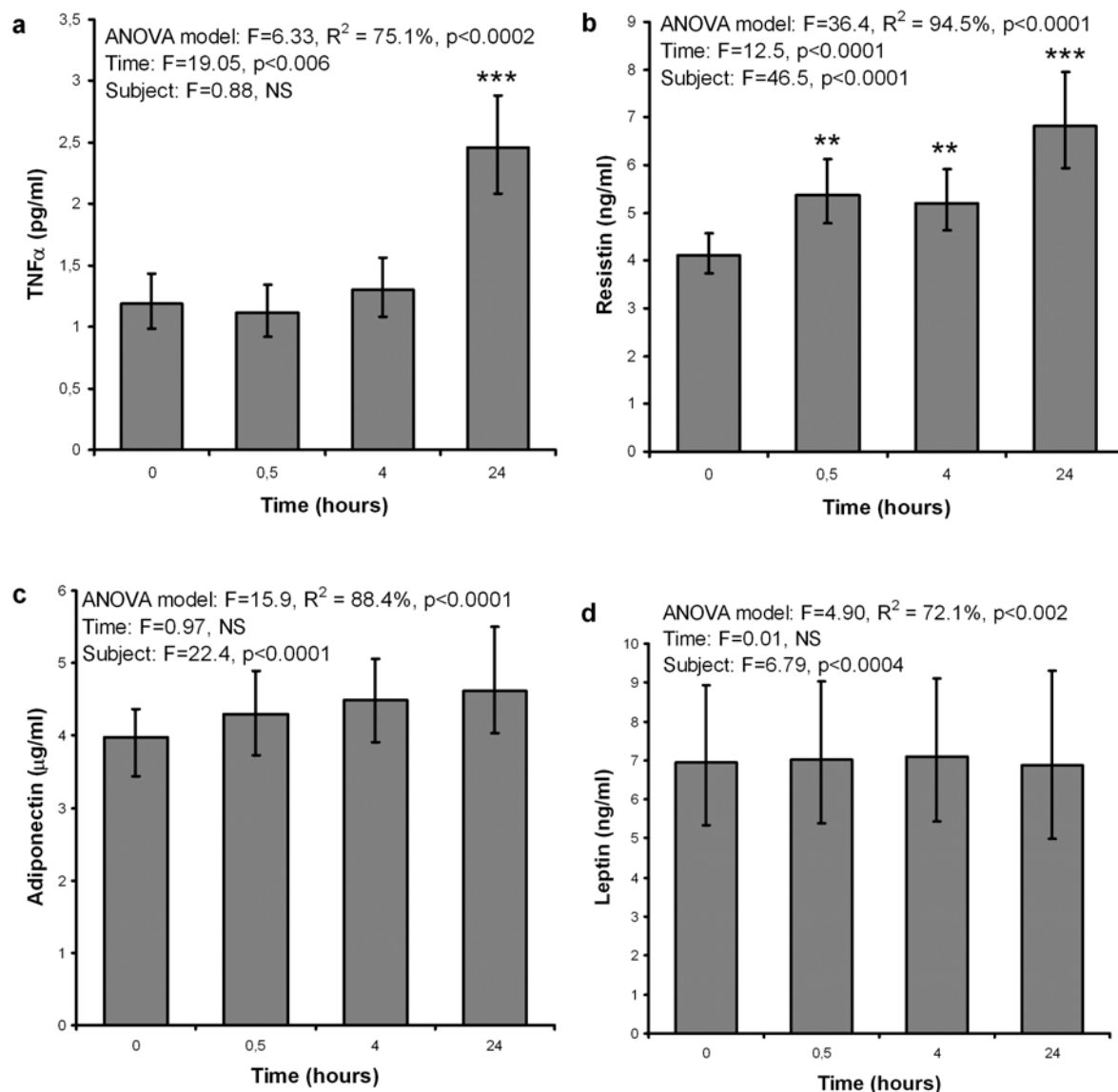
Fig.2a,b Expressions of selected adipokines in subcutaneous abdominal adipose tissue during 24-hour lipid infusion. The bars with error bars represent re-transformed means with their 95% confidence intervals (expression of selected cytokine related to expression of cyclophilin). The changes in time were assessed by ANOVA and are not statistically significant.
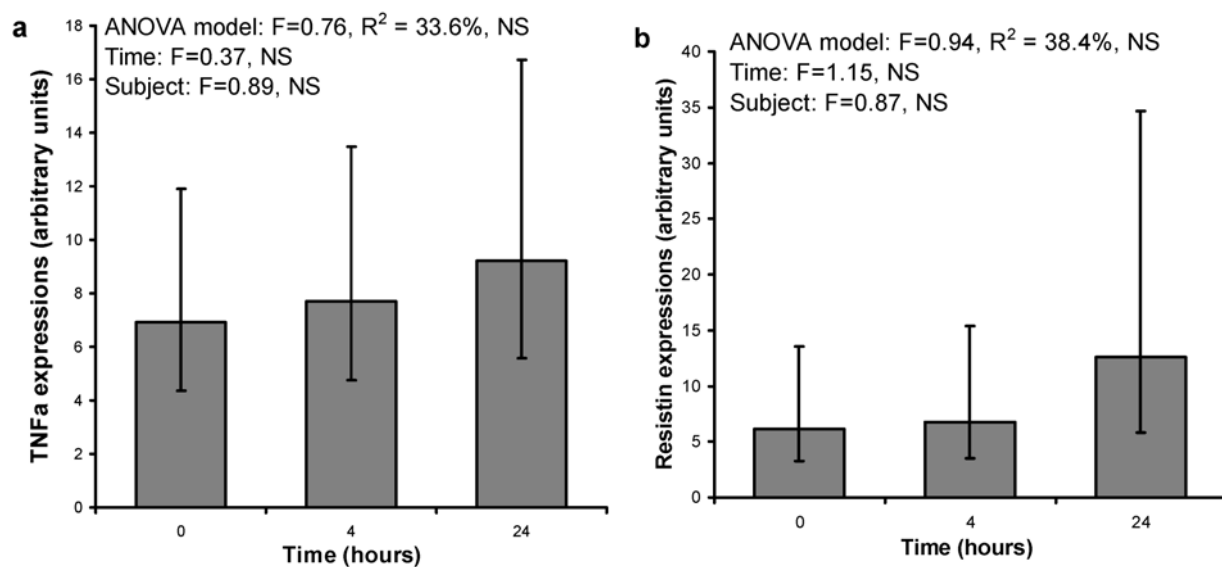
Fig. 3a-d Concentrations of serum triglycerides (●), non esterified free fatty acids (NEFA, □), blood glucose (◊) and immunoreactive insulin (IRI, ▲) during the 24-hour lipid infusion. Data are shown as means with their 95% confidence intervals.