Adipose Tissue Tumor Necrosis Factor-α and Interleukin-6 Response to Glucocorticoid Treatment during Inflammation*

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The study was performed to examine the actions of glucocorticoids on cytokine (TNF-α and IL-6) concentrations in blood plasma, adipose tissue and cytokines gene expression during acute (streptozotocin, STZ treatment) and chronic inflammation (overweight) in Swiss mice. The experiment was carried out on 6-week-old animals divided into two groups: I – non-obese (fed with a commercial food) and II – overweight mice (fed with a high-fat diet). In each group mice were divided into 4 experimental subgroups: I – control, II – acute inflammation (STZ), III – treated with glucocorticoids (DEX), and IV – STZ with DEX. After injections the animals were decapitated, blood and white adipose tissue (WAT) was quickly removed and directed to measure the plasma levels, tissue concentrations and gene expression of the cytokines (TNF-α, IL-6). Three weeks of treatment with a high-fat diet resulted in increased body weight gain and plasma level of cytokines, whereas did not change TNF-α and IL-6 mRNA gene expression in control animals. STZ, administered once, changed the TNF-α and IL-6 concentrations in a different manner according to the diet. The TNF-α and IL-6 actions in mice white adipose tissue are down-regulated after glucocorticoids treatment only in overweight animals. The obtained results suggest that glucocorticoids' effects on adipose tissue immune response, both in a pro- and an anti-inflammatory manner, depend on the nutritional status.

Key words: adipose tissue, inflammation, streptozotocin, glucocorticoids, mice.

White adipose tissue plays an important role in maintaining systemic energy balance by acting as a reservoir for excess energy, and it is a significant target for both natural and synthetic glucocorticoids (AHIMA & FLIER 2000). Glucocorticoid receptors are involved in metabolic regulation and distribution of body fat (JOYNER et al. 2000). In addition to their effects on energy metabolism, GCs play a crucial role in regulating inflammatory processes. Depending on concentration and timing, GCs enhance or suppress immune responses and promote differentiation of anti-inflammatory macrophages during inflammation (EHRCHEN et al. 2007). Glucocorticoids are used as antagonistic regulators of cytokine-induced inflammation. Some data suggest that GCs might antagonize the majority of pro-inflammatory cytokines by regulating their genes expression (HERMOSO et al. 2004).

However, due to GC’s effects on systemic energy balance, chronic use results in side effects including obesity, dyslipidemia, metabolic syndrome and steroid-induced diabetes (RHEN & CIDLOW-SKY 2005). The white adipose tissue (WAT) undergoes molecular and cellular alterations, affecting systemic metabolism and inflammatory states (PICKUP 2004; BERG & SCHERER 2005). It is recognized that adipose tissue produces multiple peptides, called adipokines, which not only influence the local function but also affect much of the metabolic pathway through the bloodstream (CHALDAKOV et al. 2003). Several adipokines are linked to inflammation and immune response (TILG & MOSCHEN 2006). The inflammation-related adi-

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pokines include cytokines (e.g., Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), chemokines (MCP-1) and acute phase proteins (CRP). TNF-α and IL-6 are the most important adipocyte derived pro-inflammatory factors. TNF-α increased in the adipose tissue and plasma of obese patients and has been related to obesity-associated complications such as insulin resistance (HOTAMISLIGIL et al. 1993). Indeed, TNF-α inhibits the transcription of many adipocyte-specific genes, such as those involved in glucose uptake, insulin responsiveness and lipogenesis (ZECHNER et al. 1988; RUAN et al. 2002). Like TNF-α, the level of the major inflammatory cytokine IL-6 correlates with body mass index and is released by visceral adipose tissue (VAT); however, in adipose tissue the greater portion of IL-6 is produced by preadipocytes, endothelial cells and macrophages rather than by mature adipocytes (FRIED et al. 1998). One of the main effects of this cytokine is the induction of hepatic CRP production, which is known to be a major risk marker of cardiovascular complications (RIDKER et al. 2003). In addition IL-6 has been proposed to play a central role in the link between obesity, inflammation and coronary heart disease (YUDKIN et al. 2000).

Taken together, adipose tissue is an important source of inflammatory proteins and could therefore actively participate in the initiation/ regulation of immune response/inflammation. Moreover, adipocytes can be modulated by glucocorticoids, which affect their development, metabolism and endocrine function. Thus, in the present study we plan to examine the effects of glucocorticoids on TNF-α and IL-6 plasma levels, adipose tissue concentration and gene expression of these cytokines during acute and chronic inflammation in mice. In particular, in order to observe this activity in the pre-obesity (overweight) state, at the beginning of pathological changes.

Material and Methods

Animals and experimental procedure

All procedures were approved by the Local Animal Ethics Commission in Kraków, Poland (No. 14/2009).

The experiment was carried out on 6-week-old Swiss mice (female, n=48) housed under a 12L:12D cycle in a temperature (23±1°C) and humidity (55±5%) controlled environment. They were housed in groups of 3 per cage. Animals with initial weight of 23±1.2g were randomly divided into two groups (n=24): I – non-obese (fed with a commercial food) and II – overweight mice (fed with a high-fat diet). On a caloric basis, the high-fat diet consisted of 58% fat from lard, 25.6% carbohydrate, and 16.4% protein (total 23.4 kJ/g), while the regular diet contained 11.4% fat, 62.8% carbohydrate, and 25.8% protein (total 12.6 kJ/g). Diets with more than 55 kcal% fat are commonly used to induce obesity in rodents (JOHNSTON et al. 2007).

After 3 weeks of the experiment, the mice of group II weighed more than the mice of group I. Reaching this stage, animals in each part were divided into 4 subgroups (n=6). Mice from the control subgroups received an intraperitoneal (i.p.) injection of 0.2 ml 0.9% NaCl. In order to develop acute inflammation, the animals received injections (i.p.) of streptozotocin (STZ, 2-deoxy-2-(methyl-3-nitrosoureido)-D-glucopyranose, Sigma Aldrich). The mice were weighed prior to injection, and the STZ was freshly dissolved in dilution buffer (0.1 M sodium citrate, pH 4.5, with HCl, stored at 4°C). The STZ was given at a dose of 0.2 ml (single i.p. injections of 100 mg/kg b.w.), 24 hours before decapitation. Mice from another subgroup were treated with dexamethone (DEX, synthetic glucocorticoid – dexamethason, Jelfa S.A.) at a dose of 100 µg/kg b.w., (0.2 ml, i.p.) two times – 24 h and 1 h before decapitation. Animals from the IV subgroup received STZ and glucocorticoid given in the same manner as the subgroup with STZ or DEX alone.

Blood was taken to heparinized tubes after decapitation, centrifuged for 20 min with 3000xg, at 4°C and stored at -20°C for further analysis. White adipose tissues were quickly removed and frozen in RNA Later for further analysis.

Cytokine measurements

The plasma level and tissue concentrations of the cytokines TNF-α and IL-6 were measured using commercial ELISA kits for the mouse proteins (Diaclone, France). The assays were conducted in 96-well microplates according to the manufacturer’s instructions. The sensitivity (minimal detectable dose) of each of the ELISAs were: TNF-α < 25 pg/ml and IL-6 < 6 pg/ml. The intra- and inter-assay CV for the TNF-α ELISA were 5.1 and 8.1% and for IL-6 ELISA 7.3 and 7.2%, respectively. Absorbance of the experimental and control samples was measured 20 min after termination of the reaction at 450 nm, using an ELISA plate reader (BioTek, USA).

Metabolic parameters measurements

Plasma glucose, triglycerides and cholesterol levels were measured by commercial kits (Pointe Scientific INC, USA), using an enzymatic method.
RNA extraction and cDNA synthesis

Total RNA was extracted from WAT using TRIzol Reagent (Life Technologies, USA) with our own modification. Concentration and purity of the RNA samples were determined by UV spectroscopy at 260/280 nm, and integrity was confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide. The first strand cDNA was transcribed from 1 µg RNA with MultiScribe Reverse Transcripase (50 U/µl, Life Technologies, USA) using random primers at 25°C for 10 min, and then at 37°C for 120 minutes and 85°C for 5s. The cDNA was reconstituted in 50 µl of sterilized water and 100 ng of the cDNA solution was used as a template.

Real time PCR

Relative quantification of gene expression was measured by real-time PCR on the StepOnePlus Real-Time PCR System (Life Technologies, USA) with the Universal Master Mix and TaqMan chemistry (Life Technologies, USA). The conditions for amplification were as follows: an initial step of 50°C for 2 min, which is the required optimal AmpErase UNG enzyme activity, and then at 95°C for 10 min, to activate the AmpliTaq Gold DNA polymerase and to deactivate the AmpErase UNG enzyme, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of analyzed genes (IL-6, TNF-α, Table 1) was related to the expression of reference gene 18S rRNA (Hs99999901_s1, RefSeq: X03205.1; Life Technologies, USA). The data were calculated according to the ΔΔCt method (LiVAK & SCHNITTGEN 2001), using the expression in the white adipose tissue of the control subgroup as the calibrator (relative quantity; RQ=1) and presented as RQ±SEM.

Statistical analysis

Results were analyzed statistically using analysis of variance (ANOVA) followed by Student’s t-test. Results are presented as means ± SEM. Data with different superscript letters are significantly different (P<0.05).

Results

The effect of the high-fat diet on weight gain

All mice gained weight throughout the study, either on the control or the experimental diet, although weight gain was significantly higher in groups fed the high-fat diet (10.1±0.7g) compared to the group on the control diet (4.2±0.3g), P<0.05. Body weight gain was determined as the difference between final and initial weight.

The effect of high calories diet on the inflammatory status (Table 2)

Three weeks treatment with a high calories diet significantly increased the plasma level of all pro-inflammatory tested cytokines. Plasma levels of TNF-α, CRP, IL-1β, and IL-6 was higher in overweight mice by 224%, 63%, 54% and 89%, respectively (P<0.05).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lean mice</th>
<th>Overweight mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (ng/ml)</td>
<td>8.93±1.61</td>
<td>28.91±5.21*</td>
</tr>
<tr>
<td>CRP (ng/ml)</td>
<td>2367.57±426.16</td>
<td>3864±695.69*</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>34.41±5.16</td>
<td>53.07±9.56*</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>5.13±0.94</td>
<td>9.68±1.71*</td>
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</tbody>
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*P<0.05 – compared to mice fed a standard diet
The effect of high calories diet on the metabolic parameters (Table 3)

Our experiment shown that after three weeks treatment with high calories nutrition no statistical differences was observed in the level of glucose, triglycerides and cholesterol in the blood plasma, compare to animals fed with standard diet.

Cytokines plasma level (Fig. 1 a, b)

TNF-α. In the control subgroup the plasma level of TNF-α was statistically higher in overweight than in standard mice (28.91±5.21 pg/ml vs 8.93±1.61 pg/ml, respectively, P<0.05). Single STZ injection strongly increased the level of TNF-α in lean animals (by 166%), whereas it decreased in overweight mice by 43%. The response to synthetic glucocorticoids treatment also depended on the type of diet. Unexpectedly, dexaven raised this cytokine by 530% in lean mice and decreased it in overweight mice by 32% (P<0.05). Treatment with Streptozotocin and glucocorticoids together showed that in animals fed with the standard diet STZ inhibits DEX effect on the TNF-α (26.46±3.96 pg/ml, P<0.05). No effects of a combination of these factors in overweight mice were observed (28.33±5.09 pg/ml).

IL-6. In control animals treatment with a high-calorie diet significantly increased the plasma level of IL-6 (9.68±1.71 pg/ml) compared to standard-fed mice (5.13±0.94 pg/ml), P<0.05. In lean mice streptozotocin injection decreased the level of IL-6 by 41%, whereas glucocorticoids increased by 54% (P<0.05). Treatment with STZ and DEX together set the IL-6 results at the level of on 8.06±1.01 pg/ml. Surprisingly, no effects of injections in overweight animals were observed.

Cytokines concentration in adipose tissue (Fig. 2 a, b)

TNF-α. Tissue concentration of TNF-α was significantly higher in overweight mice (20.71±3.3 pg/mg, compared to lean group 13.77±2.1 pg/mg, P<0.05). Single STZ injection increased by 41% (independently of the diet used) while DEX treatment decreased this cytokine concentration by 48% (in lean) and by 75% (in overweight) in mice adipose tissue (P<0.05). STZ and DEX given together induced higher concentrations of TNF-α (46.02±5.9 pg/mg) in the group fed with the high-fat diet, but lower concentrations (0.97±0.2 pg/mg) in the group fed with the normal diet (P<0.05).

IL-6. No differences in IL-6 adipose tissue concentration after the high-fat diet were observed in the control subgroup. Also single STZ injection not changed IL-6 concentration. DEX treatment decreased cytokine concentration by 49% in lean mice and by 95% in overweight animals, P<0.05. STZ and DEX given together had an inhibiting ef-
pect on the IL-6 adipose tissue concentration, intense in the standard-fed group (by 93%) compared to the high-fat fed group – 54%, P<0.05.

Cytokines mRNA gene expression (Fig. 3 a, b)

**TNF-α.** The 3-week treatment with a high-fat diet had no effect on the expression of TNF-α mRNA in mice adipose tissue compared to animals fed a standard diet. STZ treatment did not change this expression. The treatment with synthetic glucocorticoid stimulated TNF-α mRNA expression only in mice fed a standard diet (by 165%, P<0.05). STZ treatment significantly reduced DEX-stimulated TNF-α mRNA expression in this group (P<0.05).

**IL-6.** The obtained results indicate that a short-term high-fat diet did not change IL-6 mRNA gene expression in control animals. Increased expression of IL-6 in STZ-treated animals was observed both in lean and overweight mice, 2-fold and 33-fold, respectively (P<0.05). DEX increased the IL-6 mRNA expression in both experimental groups, independently of the diet used (to 3.12±0.97 RQ in animals fed a standard diet and 4.06±1.25 RQ in overweight mice, P<0.05). DEX and STZ given together increased cytokine expression in all the animals, dependent on the diet used, to 32.1±7.56 RQ after the standard diet and to 4.05±1.37 RQ after the high-fat diet, P<0.05.

**Discussion**

In this study we demonstrated that a high-fat diet has an important role in the maintenance of immune functions in white adipose tissue in mice. Several studies have reported that obesity induced by feeding mice with a high-fat diet above 10 weeks can be considered as an adequate model of human obesity (Lin et al. 2000; Gavia et al. 2001). It has been demonstrated that the long-term supplementation of a diet containing 40-60% fat promotes metabolic alterations, proinflammatory cytokine production, and, consequently, obesity, insulin resistance, and hypertension (Flanagan et al. 2008). In the present study, we found that three weeks of treatment with a high-fat diet resulted in increased body weight gain by approximately 23% (P<0.05) and caused obesity in mice. Additionally,
the analyzed plasma levels of pro-inflammatory factors confirmed developing inflammation in experimental animals. Numerous studies have found that compared to healthy lean individuals, obese individuals have higher pro-inflammatory cytokines and lower anti-inflammatory cytokines, which established obesity as a chronic inflammatory disease (DIXIT 200; NATHAN 2008). In fact, several studies have reported that adipocytes can synthetize and secrete the pro-inflammatory factors (WAKI & TONTONÖZ 2007). In our study the increase in inflammatory cytokines concentration (such as TNF-α and IL-6) is probably associated with an increase in body fat. Interestingly, after this short time, the high-fat nutrition affected the response of the immune system with unchanging metabolic parameters (a growing trend but no statistical differences in the levels of glucose and triglycerides in blood plasma compared to animals fed with a standard diet, data not shown). The first major finding of the study is that the high caloric diet induced an early phase of inflammation (up to 3 weeks) independently of the metabolic state. Taken together, this can lead to the low-grade inflammation characterized by the over-production of pro-inflammatory cytokines. Furthermore, the effects of a different immune system activity on adipose tissue responses was investigated. Firstly, we tested the applicability a STZ to induce acute inflammation. It is already known that the induction of experimental diabetes mellitus (DM) using chemicals which selectively destroy pancreatic B cell is very convenient. Streptozotocin is synthesized by Streptomyces achromogenes and is used to induce both insulin-dependent and non-insulin-dependent DM. It has been reported that STZ is capable of producing mild or severe types of diabetes according to dosage used when it is given to animals by either intravenous or intraperitoneal injection. STZ may be given in single or multiple doses and cause the activation of immune mechanisms (CHATTOPADHYAY et al. 1997; ITO et al. 1999). Some studies indicate that the type of diabetes and characteristics differ with the administered dose of STZ, and the animal or species used (ARORA et al. 2009). In the present data STZ, administered once (at a dose of 100 mg/kg b.w.), changed the TNF-α and IL-6 concentrations in a different manner according to the diet. As a result of STZ treatment a certain degree of inflammatory response was observed, determined by the systemic and/or tissue levels of the two pro-inflammatory cytokines. Unexpectedly, while STZ increased the TNF-α level in animals fed with the standard diet and decrease in high-fat feeding reverse reaction was observed in relation to the IL-6. These results suggest that food rich in fat modulates the STZ-dependent production of inflammatory cytokines selectively.

Chronic stress leads to various diseases, mainly through the activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system (SNS) axis, with up-regulation of glucocorticoids and catecholamines. Several studies have reported that that GCs can directly and indirectly by their receptors regulate many genes involved in suppressing the immune response (REVOLLO & CIDŁOWSKI 2009; BASCHANT & TUCKERMANN 2010). Synthetic glucocorticoids (such as dexamethasone) are commonly used for treatment of many inflammatory diseases. Glucocorticoids inhibit many of the initial events in an inflammatory response by anti-inflammatory and immunosuppressive actions (ASHWELL et al. 2000). More recently, identified many genes co-regulated (rather than anti-correlated) by GCs and TNF-α which indicating that glucocorticoids and cytokines can both regulate genes involved in inflammation (LIBERMAN et al. 2007; LANGILAILIS et al. 2008). Microarray studies revealed the surprising result that many of the genes regulated by both pro- and anti-inflammatory factors were co-regulated rather than anti-correlated. However, it was determined that many of the genes co-regulated by dexamethasone and TNF-α are involved in inflammation, are biologically diverse, and perform functions currently unrelated to inflammation (or their functions are unknown) (LANNAN et al. 2012).

In our experiment we observed that dexamethasone given once prior to determination of the level of cytokines has different effects depending on the prior stimulation of the immune system (lean vs overweight mice). We observed pro-inflammatory effects (rise in plasma levels in non-obese animals), whereas in overweight (chronic inflammation) mice anti-inflammatory influence by reduction of these cytokines. Our results indicate that the TNF-α and IL-6 actions in mice white adipose tissue (at the protein level) are down-regulated after glucocorticoids treatment. It is possible that interaction between these signaling pathways may be responsible for some of the negative side effects of long-term glucocorticoid usage. At the same time we observed stimulatory effect of GCs on the TNF-α and IL-6 mRNA gene expression only in non-obese mice. The transition from acute to chronic inflammation occurs only in selected circumstances. It is possible that our cytokines gene expression is up-regulated in responses to acute phase inflammation. Suggestions have been made that co-regulation by glucocorticoids and cytokines in vivo in adipose tissue occurred, depending on the metabolic status.

Additionally, in recent years the classic view that glucocorticoids are universally anti-inflammatory has been challenged at a variety of levels. Some
studies suggest that the type of exposure to glucocorticoids and the basal state of the immune system are important factors influencing the effects of GCs. While chronic exposure to GCs seems to be immunosuppressive, acute exposure enhances the peripheral immune response (DHABHAR 2002). Still the mechanism by which the same hormone directs opposite responses are not well understood.

Glucocorticoids are potent pharmacological agents used to treat a number of immune conditions and also can contribute to the development of type-2 diabetes mellitus. It is known that elevations of GCs promote whole body insulin resistance (PECKETT et al. 2011). The present study proposed investigating the combined immunological effects of elevated GCs (anti-inflammatory agent) with STZ (proinflammatory drugs) after normal and high-fat feeding. The obtained data showed an increase in plasma levels of TNF-α and IL-6 in lean animals. Unexpectedly, different effects of co-administration of STZ and DEX on cytokines action was observed in WAT. Summarizing the effect of both pro- and anti-inflammatory factors (streptozotocin and dexamethasone) can clearly indicate that their activity is strictly dependent on the stimulation of the immune system, and their role in the development of inflammation is not clear.

In conclusion, the obtained result showed that 3 weeks of a high-fat diet modulated proinflammatory cytokines in white adipose tissue and that observed changes in protein are not always accurately reflected in mRNA changes. We have shown that streptozotocin (24 hours after a single injection) can cause activation of the immune system by release of pro-inflammatory agents in a normal-weight organism (especially TNF-α). Additionally, the results suggest that glucocorticoids affect adipose tissue immune response both in pro- and anti-inflammatory manners, dependent on the nutritional status and that the effect of GCs are more complex than previously recognize. In pathologi cal situations (overweight mice) glucocorticoids may function as anti-inflammatory factors, in contrast, under normal physiological condition (non-obese animals) GCs may play a pro-inflammatory role. Future studies are needed to characterize the tissue-specific effects of the pro- and anti-inflammatory roles of glucocorticoid receptor signaling.

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