Adipose tissue loss in adjuvant arthritis is associated with a decrease in lipogenesis, but not with an increase in lipolysis

A I Martín, E Castillero, M Granado, M López-Menduiña, M A Villanúa and A López-Calderón
Department of Physiology, Faculty of Medicine, University Complutense of Madrid, 28040 Madrid, Spain

Abstract

Adjuvant-induced arthritis is a model of rheumatoid arthritis that induces cachexia. In other cachectic situations, there is an increase in lipolysis resulting in a loss of adipose tissue mass. The aim of this work was to analyse the effect of chronic arthritis, induced by adjuvant injection, on white adipose tissue (WAT). For this purpose, rats were killed 10 days after adjuvant injection, when the first external symptoms appeared, on days 15 and 22 when the external signs of the illness reach their severest level. As arthritis decreases food intake, a pair-fed group was also included. Serum concentrations of insulin, leptin, adiponectin, glycerol and nitrates, as well as gene expression of leptin, adiponectin, hormone-sensitive lipase (HSL), fatty acid synthase (FAS), tumour necrosis factor α and zinc-α₂-glycoprotein (ZAG) were determined. Arthritis decreased food intake between days 5 and 16, but not during the last 5 days of the experiment. There was a marked decrease in relative adipose tissue weight and in serum leptin and adiponectin as well as in their gene expression in WAT in arthritic rats. Arthritis decreased the gene expression of FAS in the WAT. However, none of these effects was found in pair-fed rats. Arthritis did not increase lipolysis, since arthritic rats have lower serum concentrations of glycerol, HSL mRNA in WAT, as well as liver ZAG mRNA than the pair-fed or control rats. These data suggest that in chronic arthritis the decrease in white adipose mass is secondary to a reduced adipose lipogenesis, and this effect is not mainly due to the decrease in food intake.

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Introduction

Cachexia is characterized by a marked decrease in body weight and a progressive decrease in adipose tissue and skeletal muscle mass. This debilitating response occurs in many chronic diseases, such as cancer, heart or renal failure, diabetes, as well as in inflammatory diseases such as Crohn’s disease, sepsis and rheumatoid arthritis. Rheumatoid arthritis patients have hypermetabolism and accelerated protein breakdown (Roubenoff et al. 1994), and it has been postulated that rheumatoid cachexia is an important contributor in increasing morbidity and premature mortality in those patients (Walsmith et al. 2004).

Adjuvant-induced arthritis is a well-established model of rheumatoid arthritis that can be induced in rats by an intradermal injection of Freund’s adjuvant. Ten days after adjuvant injection, rats start to lose body weight even before the external signs of the illness are manifested (López-Calderón et al. 1999). On day 22 after adjuvant injection, when the external signs of the illness reach their severest level, the decrease in body weight in arthritic rats is associated with a marked decrease in skeletal muscle and white adipose tissue (WAT) mass (Granado et al. 2006).

Muscle wasting in experimental arthritis, as well as in other inflammatory illnesses, seems to be due to enhanced protein breakdown by the ubiquitin–proteasome proteolytic pathway (Lecker et al. 2004, Granado et al. 2005b). However, the mechanism by which chronic inflammation reduces fat stores is not well known. Fat mass loss can be secondary to an increase in lipolysis and/or a decrease in lipogenesis. Lipolysis in WAT is under hormonal control, where the hormone-sensitive lipase (HSL) is the main regulatory pathway in rodent lipolysis (Holm 2003). In cancer cachexia, both lipolysis and HSL gene expression are increased in rodents and humans (Tisdale 2004, Agustsson et al. 2007).

Zinc-α₂-glycoprotein (ZAG) is a lipid mobilizing protein that has also been proposed to be one of the causes of cachexia-induced WAT loss (Sanders & Tisdale 2004). ZAG stimulates adipocyte adenyl cyclase, induces lipolysis and decreases body weight (Russell et al. 2004). In vivo ablation of ZAG induces an increase in body weight that correlates with a reduction in adipocyte lipolysis (Rolli et al. 2007). ZAG is expressed ubiquitously but it is mainly expressed in the rodent liver (Rolli et al. 2007). This protein is overexpressed by several tumours (Todrov et al. 1998), and its mRNA is increased in WAT and in the liver of mice with tumour-induced cachexia (Bing et al. 2004). For these reasons, increased gene expression of ZAG has been related with cachexia.

In the present study, we examined the effect of chronic arthritis on visceral WAT mass regulation. Serum concentrations...
of leptin and adiponectin as well as their gene expression in WAT were measured as they are the main adipokines released by the adipose tissue. As lipolytic factors, HSL and ZAG gene expression as well as serum concentration of glycerol were determined. To evaluate whether decreased lipogenic capacity could play a role in arthritis-induced decrease in body weight, we measured the expression of fatty acid synthase (FAS) in WAT, since this enzyme plays a central role in the novo lipogenesis in mammals.

Materials and Methods

Experimental design

Arthritic and control male Wistar rats were purchased from Charles River (Barcelona, Spain). Arthritis was induced in the rats by an intradermal injection of 1 mg heat-inactivated Mycobacterium butyricum in the right paw (day 0). Control animals were injected with mineral oil. After arriving (day 3 after adjuvant injection), rats were housed 3–4 per cage, and maintained under standardized conditions of temperature (20–22 °C) and light (lights on from 0730 to 1930 h). The procedures followed the guidelines recommended by the EU for the care and use of laboratory animals and were approved by the Complutense University animal care committee.

The effect of arthritis on visceral adipose tissue was studied in different stages of the illness, when rats started to develop external signs of inflammation (day 10 after adjuvant injection) and when the illness was established (days 15 and 22). Body weight, food intake and the arthritis index scores were examined daily. Assessment of arthritis was performed by measuring the arthritis index of each animal, which was scored by grading each paw from 0 to 4. Grading was determined as: 0, no erythema or swelling; 1) slight erythema or swelling of one or more digits; 2) swelling of paw; 3) swelling of entire paw and the ankle; and 4) ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16. On days 10, 15 and 22, eight control and ten arthritic rats were killed by decapitation between 1200 and 1300 h. As arthritis decreases food intake, a control group of eight rats that received the same amount of food (g/100 g bw) eaten by the arthritic rats (pair-fed group) was also included.

Food intake per cage was calculated by measuring the difference between the initial and the remaining amount of pellets in the feeder, and expressed as g per rat per 100 g body weight. The same amount of food consumed by the arthritic rats was given to the pair-fed group the following day. At the end of the experiment, on day 22 after adjuvant injection, pair-fed rats were also killed by decapitation between 1200 and 1300 h. All rats were killed in a separate room, within 30 s after being removed from their cages. Trunk blood was collected in cooled tubes, allowed to clot, centrifuged and the serum was stored at −20 °C until insulin, leptin, glucose and glycerol assays were performed. Immediately after decapitation, spleen and epididymal WAT were weighted. The epididymal WAT and the liver were dissected, frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Real-time PCR

RNA was extracted by the guanidine thiocyanate method using a commercial kit (Ultraspere RNA, Biotecx Laboratories Inc., Houston, TX, USA). The integrity of the RNA was confirmed using agarose gel electrophoresis. For RT-PCR analysis, 1 μg total RNA was reverse transcribed with QuantiScript Reverse Transcription kit (Qiagen GmbH, Hilden). Primers for PCR (Table 1) were obtained from Roche (Madrid, Spain) using the EXIQON Universal Probe Library (leptin, HSL, FAS, ZAG, adiponectin) or from previously published sequences of tumour necrosis factor-α (TNF-α; Dehoux et al. 2004) and hypoxanthine-guanine phosphoribosyltransferase (HPRT; Peinmequin et al. 2004). Primers were designed to span a single sequence derived from two exons (i.e. separated by an intron in genomic DNA and primary RNA transcripts to minimize amplification).

Each real-time PCR consisted of 10 ng total RNA equivalents, 1× Takara SYBR Green Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan) and 300 nM forward and reverse primers in a reaction volume of 25-5 μl. Reactions were carried out on a SmartCycler (Cepheid, Sunnyvale, CA, USA).

Parameters included an initial activation of hotStarTaq DNA polymerase at 95 °C for 15 s, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C and extension at 72 °C for 30 s. Specific amplification was confirmed by the presence of one single peak in the melting

<table>
<thead>
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<th>Gene</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
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curve plots. In addition, the PCR products were analysed by agarose gel electrophoresis. Results were calculated as per cent of control rats, using the cycle threshold \(2^{\Delta\Delta Ct}\) method (Livak & Schmittgen 2001) with the HPRT as reference gene.

**Serum determinations**

Nitrite and nitrate concentrations in serum were measured by a modified method of Griess assay, described by Miranda et al. (2001). Serum was deproteinized to reduce turbidity by centrifugation through a 30-kDa molecular mass filter using a Centrifree Micropartition Device with a YM-30 ultrafiltration membrane (Amicon Division, Millipore Corporation, Bedford, TX, USA), at 15000 r.p.m. for 1 h at 37 °C for 300 μl samples. Mixing of 100 μl filtrated serum with 100 μl vanadium chloride was carried out and was quickly followed by the addition of the Griess reagents. The determination was performed after incubation at 37 °C for 30 min. The absorbance was measured at 540 nm. Nitrite and nitrate concentrations were calculated using a NaNO₂ standard curve and expressed as percentage of the control group.

Serum concentrations of rat leptin and adiponectin were determined by RIA and ELISA using commercial kits from Linco Research Inc. (St Charles, MO, USA) and B-Bridge International Inc. (Otsuka Pharmaceuticals, Japan), following the manufacturer’s instructions. Insulin was measured by RIA that has been previously described (Vara & Tamarit-Rodriguez 1988).

Glycerol and glucose levels were determined according to their respective protocol kits (Sigma; and Infinity Glucose Oxidase, Thermo Electron, Louisville, CO, USA).

**Statistical analysis**

All data are presented as the mean ± S.E.M. Comparisons between means were made by one-way ANOVA and subsequent LSD multiple range test. Because there were no significant differences between data obtained in the control rats killed 10, 15 or 22 days after vehicle injection, they were used as one control group. The TNF-α mRNA data were subjected to log transformation, since variances showed a log-normal distribution. A value of \(P < 0.05\) was considered significant.

**Results**

The arthritis index scores, left paw volume and serum concentrations of nitrite + nitrate are shown in Fig. 1. During the first phase of the illness, until day 10 after adjuvant injection, arthritic rats had a small inflammatory reaction in the injected paw, with arthritis scores between 2 and 3 (Fig. 1A), whereas in the non-injected paw (left paw) the paw volume of the arthritic rats was similar to that of control or pair-fed rats (Fig. 1B). On day 10 the poly-articular inflammation started, reaching the maximum value of arthritis scores (14-7) on days 21 and 22. Coinciding with the increase in the arthritis index score, on days 15 and 22, arthritic rats had higher paw volume (\(P < 0.01\)) than control rats (Fig. 1B). Pair-fed rats had a paw volume similar to that of control rats. Arthritis increased the serum concentrations of nitrates + nitrates (\(P < 0.01\)) having the highest levels on day 10 after adjuvant injection (Fig. 1C). On day 22, the serum concentrations of nitrates + nitrates were between those observed on day 10 and in control rats.
Figure 2 shows body weight and the daily food intake from day 4 to 22 after adjuvant injection, as well as spleen and epididymal WAT weights. Arthritic rats had lower body weight than controls from day 5 (Fig. 2A), but on day 10, when the external signs of arthritis started to dramatically increase, the body weight gain in the arthritic rats was even lower than before. Between days 5 and 15, the arthritic rats ate less food than controls (Fig. 2B). The decrease in body weight in the arthritic rats is not only due to the lower food intake but also due to the fact that pair-fed rats had higher body weight than arthritic rats ($P<0.01$). In addition, during the last 5 days of the experiment (days 18–22), arthritic rats ate similar amounts to control rats, but the increase in body weight was lower in arthritic rats than in control or pair-fed rats (Fig. 2A). The spleen weight increased ($P<0.01$) in the arthritic rats from day 10 after adjuvant injection (Fig. 2C) and continued increasing on days 15 and 22. In contrast, arthritis decreased the relative weight of the epididymal WAT in all the days studied ($P<0.01$) in the arthritic rats, whereas no differences were observed between control and pair-fed rats (Fig. 2D). Epididymal WAT weight, in the arthritic rats, progressively decreased on days 10 and 15, and the values on day 22 were similar to those on day 15.

Arthritis decreased ($P<0.05$) the serum concentrations of insulin on days 10 and 15; however, on day 22, the serum concentrations of insulin were similar to those of control rats (Fig. 3A). The serum concentrations of glucose were not modified by arthritis (data not shown). Arthritis also decreased the serum concentrations of leptin and adiponectin on all the days analysed ($P<0.01$, Fig. 3B and C). The pair-fed rats have similar serum concentrations of insulin and leptin, but higher serum adiponectin than control rats.

In the arthritic rats, TNF-$\alpha$ expression in WAT was already increased from day 10 ($P<0.05$) and throughout the rest of the days studied (Fig. 4A). In contrast, leptin gene expression in the epididymal WAT was decreased in arthritic rats to very low levels, 20–30% of control values, in all the days analysed (Fig. 4B). Arthritis also decreased gene expression of adiponectin and FAS in the epididymal WAT on days 10 and 15 ($P<0.01$, Fig. 4C and D). On day 22, FAS and adiponectin mRNA were higher than before, but still lower...
than in the control rats. The pair-fed rats had lower leptin mRNA in the epididymal WAT than the control rats \((P<0.05)\), but both groups of non-arthritic rats had similar adiponectin and FAS mRNA in epididymal WAT.

HSL mRNA was decreased in arthritic rats \((P<0.05)\), but the decrease was significant only on day 15 after adjuvant injection (Fig. 5A). Arthritis decreased the serum concentration of glycerol, but this decrease was significant \((P<0.05)\) only on day 22 (Fig. 5B). In pair-fed rats, HSL gene expression in WAT and serum concentrations of glycerol were similar to those of control rats. The effect of arthritis on ZAG gene expression was analysed in the epididymal WAT as well as in the liver (Fig. 5C and D). The ZAG mRNA in the epididymal WAT was not modified in pair-fed or arthritic rats on any of the days studied. In contrast, the ZAG mRNA in the liver of arthritic rats was decreased on days 15 and 22 (Fig. 5).

Discussion

Adjuvant-induced arthritis decreased visceral WAT weight as well as food intake. However, the decrease in visceral WAT weight in arthritic rats is higher than the decrease in body weight and food intake. Moreover, the pair-fed rats have lower body weight than the control rats, but had similar relative WAT weight to the control rats. These results indicate that arthritis-induced decrease in WAT weight is not only due to a decrease in food intake, but also related to metabolic alterations that result in a dramatic decrease of adiposity.

Among all factors studied in serum or in WAT, pair-feeding the rats only decreased the gene expression of leptin, although serum concentrations of leptin were not modified. A similar decrease in leptin gene expression in WAT has been recently reported in pair-fed mice of a cancer cachexia model without severe anorexia (Bing et al. 2006). Although the pair-fed and arthritic rats had similar food intake to the control rats between days 18 and 22, their leptin gene expression in WAT is lower than that in the control group. These data suggest that leptin gene expression is very sensitive to the total WAT mass, rather than to the caloric intake.

The decrease in serum leptin and its gene expression in arthritic rats has previously been described in different models of experimental arthritis (Hultgren & Tarkowski 2001, Granado et al. 2005a, Stofkova et al. 2006). A decrease in leptin secretion has also been reported in humans with other chronic inflammatory illnesses, such as tuberculosis or inflammatory bowel disease (van Crevel et al. 2002, Karmiris et al. 2006). Furthermore, long-term incubation with cytokines inhibits leptin mRNA and leptin release from adipocytes (Bruun et al. 2002). The cause of leptin decrease in arthritic rats can be a combination of two factors: the decrease in adipose mass and chronic inflammation. In contrast, the effect of rheumatoid arthritis on leptin is not well known, since contradictory data have been reported. Both a decrease and an increase in serum concentrations of leptin in rheumatoid arthritis patients have been reported (Tokarczyk-Knapik et al. 2002, Otero et al. 2006). An inverse correlation between plasma leptin and the degree of inflammation in rheumatoid arthritis patients has also been reported (Popa et al. 2005). However, most researchers find a correlation between the leptin and the body mass index in
rheumatoid arthritis patients, but not between the leptin and the disease activity (Hizmetli et al. 2007, Wisiowska et al. 2007).

The time course of serum nitrite + nitrate was different to those of arthritis scores and paw volume. As has been described previously (Connor et al. 1995, Conforti et al. 2001), serum concentrations of nitrite + nitrate were higher on days 10 and 15 than on day 22, whereas the highest level of paw inflammation and the highest arthritis scores were observed on day 22 after adjuvant injection. Similarly, arthritis-induced changes in serum concentrations of insulin and adiponectin, and gene expression of FAS, adiponectin and the HSL were more evident on day 15 than on day 22 after adjuvant injection. These data suggest that systemic inflammation, contrary to the external signs of inflammation, might be higher on day 15 than on day 22.

The HSL is a rate-limiting enzyme of lipolysis that has been reported to be increased in other inflammatory situations such as sepsis (Sugawara et al. 2003) and cancer (Tisdale 2004). In addition, caloric restriction induces lipid mobilization through induction of HSL in visceral fat (Li et al. 2003). However, adjuvant arthritis did not increase the gene expression of HSL in WAT. On the contrary, this lipase is significantly decreased on day 15 after adjuvant injection. The decrease in HSL is concomitant with the minimum relative weight of epididymal WAT. It is possible that the decrease in WAT mass in arthritic rats was secondary to an increase in the expression of the lipomobilizing protein ZAG, as it has been described in cancer (Bing et al. 2006). However, ZAG gene expression was decreased in the liver of arthritic rats and not modified in WAT. The decrease in ZAG mRNA in the liver occurred on days 15 and 22, when the epididymal WAT weight values were very low. Similarly, in another inflammatory situation, such as LPS administration, ZAG mRNA expression was also decreased in the liver (authors’ unpublished observation).

Other data against the hypothesis that chronic arthritis increases lipolysis are the low serum concentrations of glycerol in arthritic rats. Glycerol and free fatty acids are derived from

Figure 4 (A) TNF-α, (B) leptin, (C) adiponectin and (D) FAS gene expression in WAT, in control rats fed ‘ad libitum’ (C), pair-fed rats (PF, black triangle) and in arthritic rats 10, 15 and 22 days after adjuvant injection. The mRNAs were measured by real-time PCR as described in Materials and Methods, and results are expressed as percentage of the control rats. Arthritis increased TNF-α mRNA levels in WAT (F_{4,47} = 5.2, P < 0.01), whereas it decreased those of leptin (F_{4,46} = 7.4, P < 0.01), adiponectin (F_{4,48} = 64, P < 0.01) and FAS (F_{4,43} = 11, P < 0.01). Values shown are the mean ± S.E.M. (n = 8–16 rats), one-way ANOVA and LSD multiple comparison test. *P < 0.05, **P < 0.01 versus control rats.
triglycerides when they are hydrolysed by lipolysis. Serum glycerol levels are increased in processes such as cancer (Agustsson et al. 2007). Accordingly, serum glycerol has been measured as an index of in vivo lipolytic activity. As observed with the gene expression of the two lipolytic factors (HSL and ZAG) in arthritic rats, the serum concentrations of glycerol are not elevated, but they are decreased on day 22 after adjuvant injection. All these data suggest that arthritis does not increase lipolysis, on the contrary it seems to decrease it. Similarly, in burned rats, the atrophy of the WAT is associated with a decrease in catecholamines-induced lipolysis (Ikezu et al. 1999), and the alterations in lipid metabolism are concomitant with an increase in adipocyte apoptosis (Yasuhara et al. 2006). Between the possible factors that mediate the apoptosis response, the aforementioned authors postulated that prolonged elevation of cytokines in adipose tissue can be the cause.

Taking into account that insulin increases the gene expression of FAS (Mounier & Posner 2006), the increase in FAS mRNA in arthritic rats on day 22, in relation to day 15, can be secondary to the normalization in serum concentrations of insulin. Food intake is another factor that is much related with FAS gene expression. Food deprivation decreases FAS activity in WAT, whereas refeeding dramatically increases FAS activity over control values (Karbowska et al. 2001, Kochan et al. 2006). However, on day 22, the arthritic rats still had lower FAS mRNA in epididymal WAT than control rats, although their food intake during the last 5 days and the serum concentrations of insulin on day 22 were similar to those of control rats.

It has been suggested that in cancer cachexia, loss of WAT mass is related to anorexia and to a decrease in food intake, whereas skeletal muscle loss is more related with increased

Figure 5  (A) Hormone-sensitive lipase (HSL), (B) serum concentrations of glycerol, (C) ZAG mRNA in the liver of control rats and (D) zinc-$\alpha_2$ macroglobulin (ZAG) mRNA in the epididymal white adipose tissue (WAT) fed ‘ad libitum’ (C), pair-fed rats (PF, black triangle) and in arthritic rats 10, 15 and 22 days after adjuvant injection. The HSL and ZAG mRNA were measured by real-time PCR as described in Materials and Methods, and results are expressed as percentage of the control rats fed ‘ad libitum’. Arthritis decreased liver ZAG mRNA ($F_{4,42} = 2.9$, $P<0.05$). Values shown are the mean $\pm$ S.E.M. ($n=8–16$ rats), one-way ANOVA and LSD multiple comparison test. *$P<0.05$, **$P<0.01$ versus control rats.
proteolysis (Fouladi et al. 2005). This does not seem to be the case in adjuvant arthritis, since pair-fed rats, although they have lower body weight than control ad libitum fed rats, have a relative WAT weight similar to them. Although we cannot exclude that part of the inhibitory effect of arthritis on body and WAT weights can be due to anorexia, inflammatory reactions play an important role. The adiponectin data support this hypothesis. The decrease in serum concentrations of adiponectin and its gene expression in WAT does not seem to be secondary to a decrease in food intake. On the contrary, dietary energy restriction leads to an increase in adiponectin secretion (Escrivá et al. 2007, Martin et al. 2007). The inhibitory effect of arthritis on adiponectin can be due to the inflammatory response, since macrophage-derived factors inhibit adipocyte differentiation as well as adiponectin and FAS gene expression in murine and human preadipocytes (Constant et al. 2006).

A decrease in serum adiponectin in adjuvant-induced arthritis has been previously described (Haruna et al. 2007). An inverse correlation between serum adiponectin and markers of inflammation has been reported in obesity (for review see Fantuzzi (2007)). The role of adiponectin in human arthritis is a paradox, since adiponectin is increased in both synovial fluid and serum of rheumatoid arthritis patients (Schäffler et al. 2003, Otero et al. 2006, Senolt et al. 2006). At the time of blood sampling, the majority of the rheumatoid arthritis patients were treated with anti-rheumatic drugs, corticosteroids or TNF-α antagonist. Thus, it must be taken into account that these treatments might modify adiponectin release from adipocytes. In this sense, anti–TNF-α therapy increases the serum adiponectin levels in patients with rheumatoid arthritis, where adiponectin is negatively correlated with disease activity score (Komai et al. 2007).

Among pro-inflammatory cytokines, TNF-α has been reported to decrease adiponectin production from adipocytes (Kappes & Löfler 2000, Simons et al. 2005). Furthermore, TNF inhibits the differentiation of human adipocyte precursor cells and also promotes the delipidation of mature fat cells (Petruschke & Hauner 1993). This cytokine has been reported to inhibit the differentiation of new adipocytes and suppress the expression of the lipogenic enzymes such as FAS (Zhang et al. 1996, Lacasa et al. 2007). All these data suggest that in the arthritic rats, after cytokine release, adipocytes secrete less adiponectin leading to a decrease in lipid accumulation. This can be reflected in the inhibition of FAS expression and a reduction in WAT weight.

In conclusion, experimental arthritis does not increase lipolysis, but has a marked inhibitory effect on peripheral lipogenesis in visceral WAT, whereas those effects are not observed in pair-fed rats.

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