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Glutamine synthetase desensitizes differentiated adipocytes to proinflammatory stimuli by raising intracellular glutamine levels

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ABSTRACT

The role of glutamine synthetase (GS) during adipocyte differentiation is unclear. Here, we assess the impact of GS on the adipocytic response to a proinflammatory challenge at different differentiation stages. GS expression at the late stages of differentiation desensitized mature adipocytes to bacterial lipopolysaccharide (LPS) by increasing intracellular glutamine levels. Furthermore, LPS-activated mature adipocytes were unable to produce inflammatory mediators; LPS sensitivity was rescued following GS inhibition and the associated drop in intracellular glutamine levels. The ability of adipocytes to differentially respond to LPS during differentiation negatively correlates to GS expression and intracellular glutamine levels. Hence, modulation of intracellular glutamine levels by GS expression represents an endogenous mechanism through which mature adipocytes control the inflammatory response.

1. Introduction

Glutamine synthetase (GS; a.k.a. glutamate ammonia ligase, GLUL, EC 6.3.1.2) is a key enzyme involved in nitrogen metabolism, acid–base homeostasis, and cell signaling across multiple species of prokaryotes and eukaryotes [1,2]. One of the main roles of GS in vertebrates is to produce glutamine (Gln) from glutamate and ammonia, which are toxic to the central nervous system (CNS) [3–5]. Moreover, a continuous supply of Gln is required for several physiological processes, including synthesis of glutamate and GABA, synthesis of proteins, and osmoregulation [6]. Since GS is the only known enzyme in humans capable of synthesizing Gln, alterations in its expression and activity are likely to have significant biological effects. While it is widely distributed among adult mammalian tissues, GS activity is very high in adipose tissue, liver, brain and kidney. GS mRNA is uniformly distributed in adipose tissue, where it is most abundant, and in heart, spleen and skeletal muscle [7]. During hormone-induced adipocyte differentiation of cultured 3T3-L1 cells GS specific activity, cellular content and mRNA are known to strongly increase [8–10]. With this respect a study has identified a glucocorticoid responsive element in rat GS [11]. However, its role in adipocytes has never been clarified. At variance with myotubes and hepatocytes, adipocytic GS is only marginally feed-back inhibited by Gln [12], similarly to brain GS [13], where it holds the important task of removing excitoxic glutamate. Brain GS has been extensively studied because of its susceptibility to oxidative stress. Indeed ROS-mediated loss of function of GS has been demonstrated in many neurodegenerative disorders [14–16].

A close link between inflammation and metabolism control has also been highlighted through studies on adipocytes, suggesting that adipocyte might represent a cellular nexus for the processes of inflammation and metabolic dysregulation by sensing and producing inflammatory mediators. The adipocyte displays a high level of sensitivity to bacterial lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interferon-γ (IFN-γ), and retains the ability to induce nitric oxide synthase (iNOS) under proinflammatory stimulus [17]. Activation of nuclear fac-

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tor-κB (NF-κB) reverses differentiation of cultured adipocytes, which is prevented by the adipogenic transcription factor peroxisome proliferator-activated receptor γ (PPARγ) [18,19].

In this study we investigate the role of GS in differentiating adipocytes. We demonstrate that GS is expressed at late stages of differentiation in a glucocorticoid-independent manner and abolishes adipocytic sensitivity to LPS by increasing intracellular Gln levels. These results demonstrate that raising intracellular Gln levels through GS expression is a novel endogenous mechanism that inhibits the response of mature adipocytes to proinflammatory stimuli. To our knowledge, this is the first study describing a mechanism by which adipocytes regulate, through intracellular production of the signal metabolite Gln, the inflammatory response to an external challenge.

2. Materials and methods

2.1. Materials

Dulbecco modified Eagle medium (DMEM), fetal bovine serum (FBS), insulin, dexamethasone, 3-isobutyl-1-methylxanthine, methionine sulfoximine (MSO), protease inhibitors, phenylmethanesulfonyl fluoride (PMSF) and Gln were obtained from Sigma Aldrich. Bacterial lipopolysaccharides (LPS) from *Escherichia coli* 0.111:B4 was purchased by Calbiochem. Bradford protein assay was obtained from Bio-Rad. Anti-GS primary antibody and the Immobilon Western Chemiluminescent horseradish peroxidase (HRP) substrate were purchased from Millipore. Anti-PPARγ antibody was purchased from Abcam. Anti-actin antibody was purchased from Santa Cruz Biotechnology. The HRP-conjugated secondary antibody was obtained from Thermo. IL-6 ELISA kit was purchased from *USCN* Life Sciences. DetectX High Sensitivity PGE2 Enzyme Immunoassay Kit was purchased from Arbor Assays. 3T3-L1 murine fibroblasts were obtained from the Biological Bank and Cell Factory IRRCS San Martino.

2.2. Cell culture

3T3-L1 murine fibroblasts were propagated in DMEM supplemented with 10% FBS, glutamine (2 mM), penicillin–streptomycin (2 mM) and differentiated according to described protocols [20–22]. In brief, cells were allowed to reach confluence and after 2 days (*day 0*), the medium was changed to differentiation medium, containing and 1 μg/ml insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine. Unless indicated medium Gln concentration was 2 mM. Four days later (*day 4*), the medium was switched to adipocyte maintenance medium containing 1 μg/ml insulin. Then the medium was changed every 48 h until reaching *day 14*. Cells at various stages of differentiation (referred as *day 4*, *day 7*, and *day 10*) were stimulated with 2 μg/ml bacterial LPS for 48 h as described [17,23,24]. To inhibit GS activity 1 or 5 mM MSO was added to cells right before LPS treatment. For experiments with high Gln, 10 day cells underwent LPS/MSO treatment in the presence of 10 mM Gln.

2.3. Western blot analysis

Whole cell lysates were prepared by treating pelleted adipocytes at various stages of differentiation with ice cold RIPA buffer (1% Nonidet P-40, 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 0.5% sodium deoxycholate) containing 1× protease inhibitors and 1 mM PMSF for 30 min at 4 °C. Protein concentration was determined by the modified Bradford protein assay and 10 μg of proteins was electrophoresed in a 12% SDS–PAGE under reducing conditions and transferred to nitrocellulose using standard procedures. Anti-GS and PPARγ primary antibodies were used to immunodetect proteins. Western blots were processed also for actin with a specific antibody as an equal total protein loading control. Immunodetection of proteins was obtained after incubation with a HRP-conjugated secondary antibody.

2.4. Metabolites quantification by LC–MS/MS

For mass spectrometry analysis of Gln 2×10^5 cell pellets were washed twice in PBS and finally resuspended in milli-Q water. The suspension was extracted with phenol/chloroform 1:1 mixture. A Quattro Premier mass spectrometer interfaced with an Acquity UPLC system (Waters) was used for ESI-LC–MS/MS analysis as described [16,25–28]. Calibration curves were established using standards, processed under the same conditions as the samples, at five concentrations. The best fit was determined using regression analysis of the peak analyte area. The multiple reaction monitoring transitions in the positive ion mode was m/z 147.20 > 84.00 for Gln. Chromatographic resolution was achieved as indicated [16,25,27–29] with a flow rate set at 0.3 ml/min.

2.5. Enzyme-linked immunosorbent assays for IL-6 and PGE2

To measure cytokines adipocytes at different stages of differentiation were treated with LPS, LPS plus MSO and LPS plus MSO in the presence of 10 mM Gln as indicated above. After 48 h 100 μL of the cellular media supernatants were assayed for IL-6 with a IL-6 ELISA kit and for PGE2 with a DetectX High Sensitivity PGE_2 Enzyme Immunoassay Kit as indicated [23].

2.6. Statistical analysis

Results are shown as means ± S.E.M. Comparisons between groups were carried out by unpaired Student's *t*-test. *P* values < 0.05 were considered significant.

3. Results

3.1. GS is dynamically expressed in differentiating adipocytes

To study changes in GS expression during differentiation, 3T3-L1 fibroblastic cells were differentiated to lipid-laden fat cells. Differentiation was followed by monitoring triglyceride droplets by microscopy (not shown) and by Western blotting of the differentiation marker PPARγ, peculiar to mature adipocytes [30] (Fig. 1).

Over the 14-days differentiation process, there was a significant induction of GS at day 10 (Fig. 2A). The expression window ranged from day 9 up to day 12, with a peak at day 10 (Fig. 2A). The induction at day 4 is a result of the differentiation cocktail used in the experiments [11] (see Section 2). Indeed at day 7 expression of GS was low, consistent with the day 4 removal of dexamethasone from the medium. Following GS expression levels (Fig. 2B), intra-

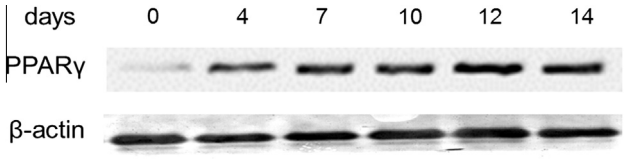

Fig. 1. 3T3-L1 preadipocytes differentiation followed as PPARγ expression. 3T3-L1 preadipocytes were grown and differentiated, as indicated in Section 2, over 14 days. Differentiation into mature adipocytes was followed with Western blotting analysis by evaluating PPARγ expression in cells lysed at different maturation times.

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