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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

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factor-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-

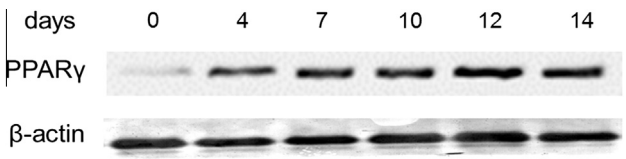

Fig. 1. 3T3-L1 preadipocytes differentiation followed as PPARγ expression. 3T3-L1 preadipocytes were grown and differentiated, as indicated in Section 2, over 14 days. Differentiation into mature adipocytes was followed with Western blotting analysis by evaluating PPARγ expression in cells lysed at different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

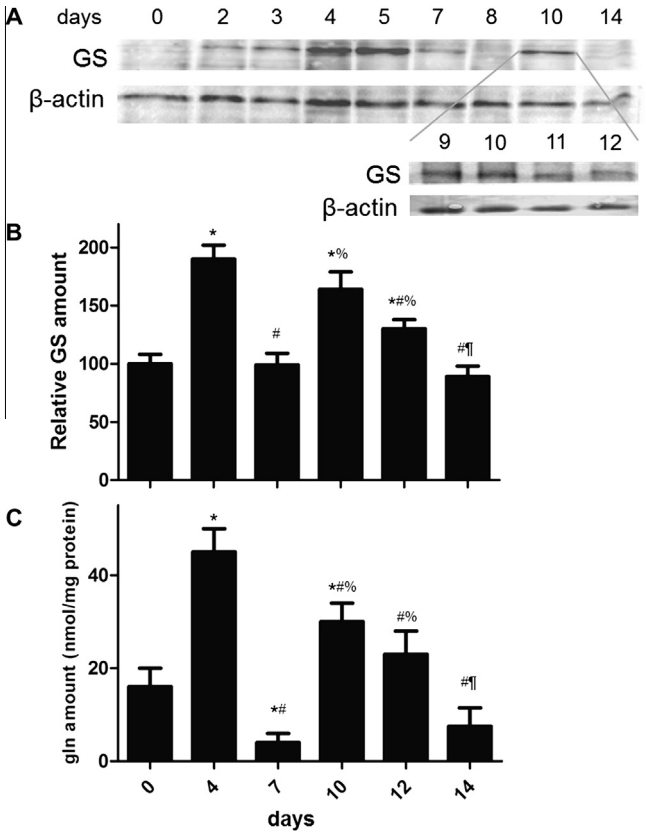

Fig. 2. GS expression and intracellular Gln levels during 3T3-L1 preadipocytes differentiation. (A) 3T3-L1 preadipocytes were grown and differentiated, as indicated in Section 2, over 14 days and GS expression was assayed by Western blotting. (B) GS protein levels are expressed in percentage considering control as 100% and normalized to β-actin densitometric levels. Data are reported as mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ versus day 0, $^{\#}P < 0.05$ versus day 4, $^{\%}P < 0.05$ versus day 7, $^{\P}P < 0.05$ versus day 10). (C) Gln levels were assessed by LC–MS/MS analysis of cellular extracts of differentiating cells and normalized to total protein levels. Data are reported as mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ versus day 0, $^{\#}P < 0.05$ versus day 4, $^{\%}P < 0.05$ versus day 7, $^{\P}P < 0.05$ versus day 10).

cellular Gln levels were significantly higher at day 10 compared to day 7 cells but lower that day 4 (Fig. 2C).

These results show that adipocytes express GS in a dexamethasone-independent fashion at very late differentiation times and this is linked to intracellular Gln accumulation.

3.2. GS expression influences the response of adipocytes to proinflammatory stimuli

Since evidences show that adipocytes are involved in inflammation processes [30,31], we evaluated if GS expression plays a role in regulating adipocytes susceptibility to an inflammatory challenge at different stages of differentiation. 3T3-L1 cells at 4, 7 and 10 days of maturation were treated with LPS in the presence or absence of MSO. Then cells were tested for proinflammatory mediators such as PGE2 and IL-6, GS expression and intracellular Gln levels.

Day 10 cells treated with LPS were insensitive to the proinflammatory stimulus, as shown by the measured PGE2 (Fig. 3A) and IL-6 levels (Fig. 3B) similar to untreated cells. However, in the presence of 1 mM or 5 mM MSO the sensitivity to LPS was rescued, as cells produced much significantly higher levels of PGE2 and IL-6 than LPS-treated and untreated cells (Fig. 3A and B). Consistently, LPS-treated adipocytes displayed an increase of GS protein that was further induced in the presence of MSO (Fig. 3C) compared to untreated cells. A strong increase in intracellular Gln levels was also observed upon LPS stimulation, which were promptly lowered by co-treatment with MSO (Fig. 3D).

Day 7 adipocytes displayed a slight sensitivity to LPS, since PGE2 (Fig. 4A) but not IL-6 (Fig. 4B) levels were significantly higher in LPS-treated compared to untreated cells. MSO did not induce any further significant increase of PGE2 compared to LPS-treated cells (Fig. 4A). In line with the slight release of proinflammatory cytokines, both GS expression (Fig. 4C) and Gln levels (Fig. 4D) were not significantly different in LPS-treated compared to untreated cells. In both cases MSO and LPS co-treatment did not modify GS expression (Fig. 4C) nor Gln levels (Fig. 4D) compared to day 7 LPS-treated cells.

Day 4 cells displayed very high GS basal levels (Fig. 2B), which is probably due to the presence of dexamethasone in the differentiation cocktail, known to induce GS expression [11]. Consistently, basal levels of intracellular Gln were very high (Fig. 2C). LPS-activated day 4 cells displayed a very high level of sensitivity to LPS, as shown by the strong increase of PGE2 (Fig. 5A) and IL-6 (Fig. 5B) in LPS-treated compared to untreated cells. MSO treatment further increased the levels of PGE2 (Fig. 5A) of LPS-treated day 4 cells. GS expression (Fig. 5C) was lower and Gln levels (Fig. 5D) strongly decreased in LPS-treated compared to untreated day 4 cells, and both were unaffected by MSO co-treatment (Fig. 5C-D). These data suggest that sensitivity to proinflammatory stimuli is inversely correlated to GS expression and intracellular Gln levels during LPS activation.

No difference in any of the measured parameters was noticed in differentiating adipocytes treated with 1 or 5 mM MSO alone compared to untreated cells (data not shown).

3.3. Supraphysiological levels of external glutamine influence the response of adipocytes to proinflammatory stimuli

To confirm that the release of PGE2 and IL-6 of LPS-activated and MSO treated-day 10 cells (Fig. 3) is related to intracellular Gln levels, we incubated LPS/MSO treated day 10 cells with very high Gln levels with the purpose of increasing intracellular levels to at least those measured in LPS-treated day 10 cells and reverting the sensitivity of MSO-treated cells to LPS. As shown in Fig. 2C, the intracellular Gln levels of untreated 10 day cells is 30 nmol/mg. LPS activation almost doubles this amount (+90%) whereas MSO concomitant treatment lowers it to about 77% of that measured in untreated day 10 cells (see Fig. 3D). Since 5 mM extracellular Gln is known to double intracellular Gln levels [32], cells were incubated with 10 mM Gln, leading to a more than twofold increase in the intracellular Gln levels in LPS/MSO treated day 10 cells, which should be sufficient to revert the sensitivity of MSO-treated cells to LPS. A similar extracellular Gln concentration is also known to reduce inflammation in many cell systems [33–37]. After a 48 h incubation with LPS/MSO in a 10 mM Gln medium, Gln levels were 200% higher than those measured in similarly treated cells in a medium containing 2 mM Gln, which is the concentration normally used for cell culture (Fig. 6A). The subsequent intracellular Gln accumulation strongly impaired cellular ability to produce PGE2 (Fig. 6B) and IL-6 (Fig. 6C) despite MSO treatment, which was previously shown to sensitize mature adipocytes to LPS treatment (Fig. 3A and B).

4. Discussion

The data above represent the first investigation on the role of GS during adipocyte differentiation. GS expression is strongly dependent on the status of adipocyte maturation. The significant poten-

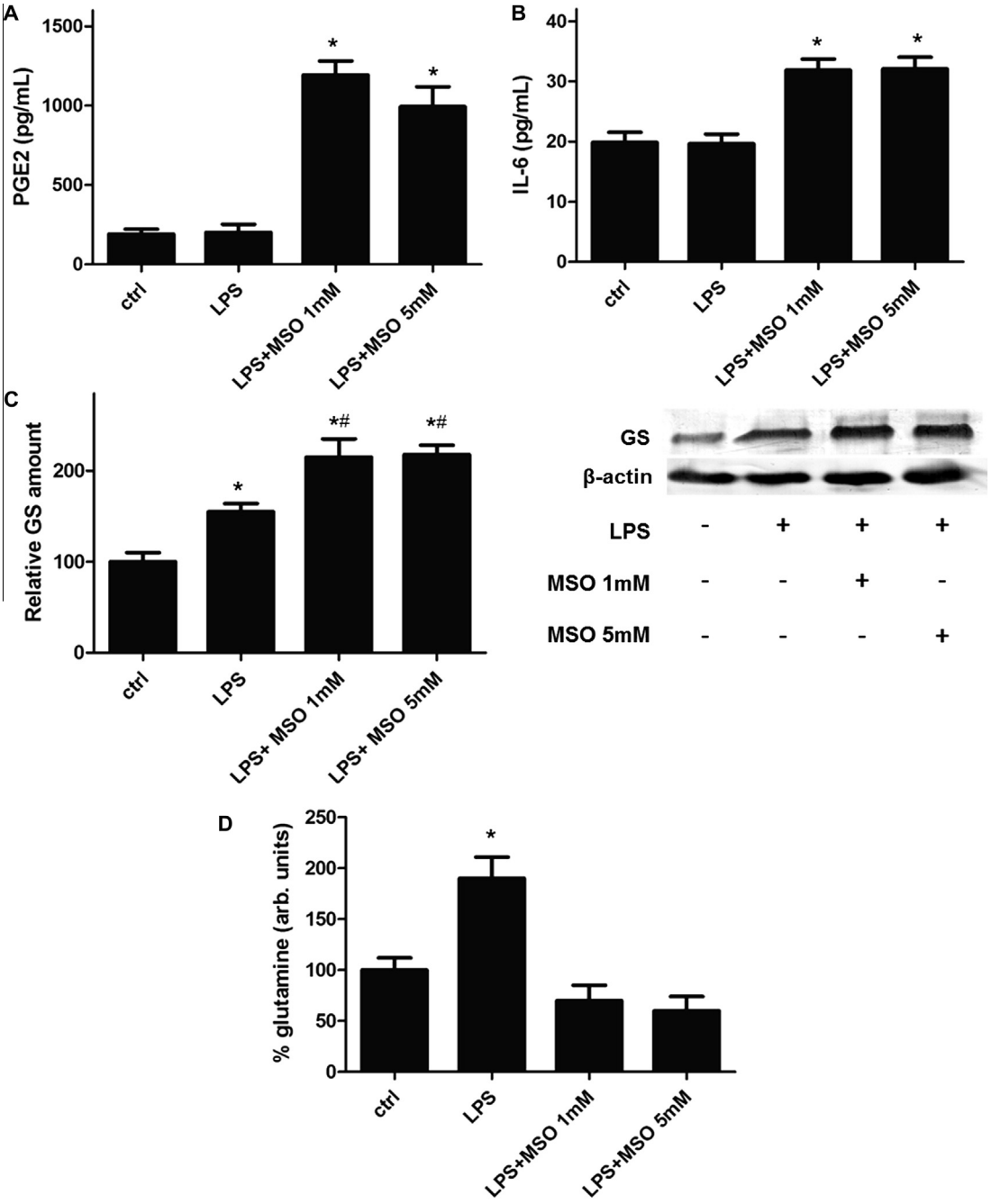

Fig. 3. Response to LPS of adipocytes at day 10 of differentiation. 3T3-L1 cells were grown and differentiated, as indicated in Section 2, over 14 days. LPS challenge was carried out for 48 h on day 10 differentiating cells pretreated or not with 1 and 5 mM MSO. (A and B) Culture media were assessed for PGE2 (A) and IL-6 (B) by ELISA. Data are reported as mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ versus LPS). (C) Cells were pelleted and tested for GS expression, reported in percentage considering control as 100% and normalized to β-actin densitometric levels. Data are reported as mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus LPS). (D) Gln levels were assessed by LC–MS/MS analysis, normalized to total protein levels and expressed in percentage considering control as 100%. Data are reported as mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ versus control).

tial of the adipocyte as an inflammatory cell puts GS regulation in adipocytes at center stage for the pathophysiology of many diseases.

Challenged adipocytes are able to secrete inflammatory cytokines in a NF-κB-dependent fashion, contributing to the systemic inflammation and metabolic dysregulation observed in obesity [20]. Strong evidence correlates inflammation with the extent of adipocyte maturation. Not yet mature adipocytes are potentially able to acquire many macrophage-specific features [31]. LPS induces NF-κB- and MAPK-dependent proinflammatory cytokine/chemokine expression in preadipocytes but not in mature adipocytes, leading to suppression of PPARγ activity and insulin responsiveness [30]. Several studies indicate that in vitro subcutaneous adipocyte differentiation is negatively associated with obesity [38–40]. The status of subcutaneous adipocyte differentiation is correlated to metabolic syndrome in obese women, suggesting that, in the setting of obesity, an increased adipogenic capacity could be protective for metabolic syndrome and that metabolic syndrome could be considered a disorder of dysfunctional preadipocytes [41]. However, the mechanism by which this occurs has never been proposed so far. Our data are contributing to fill this gap by identifying the mechanism responsible for the transition

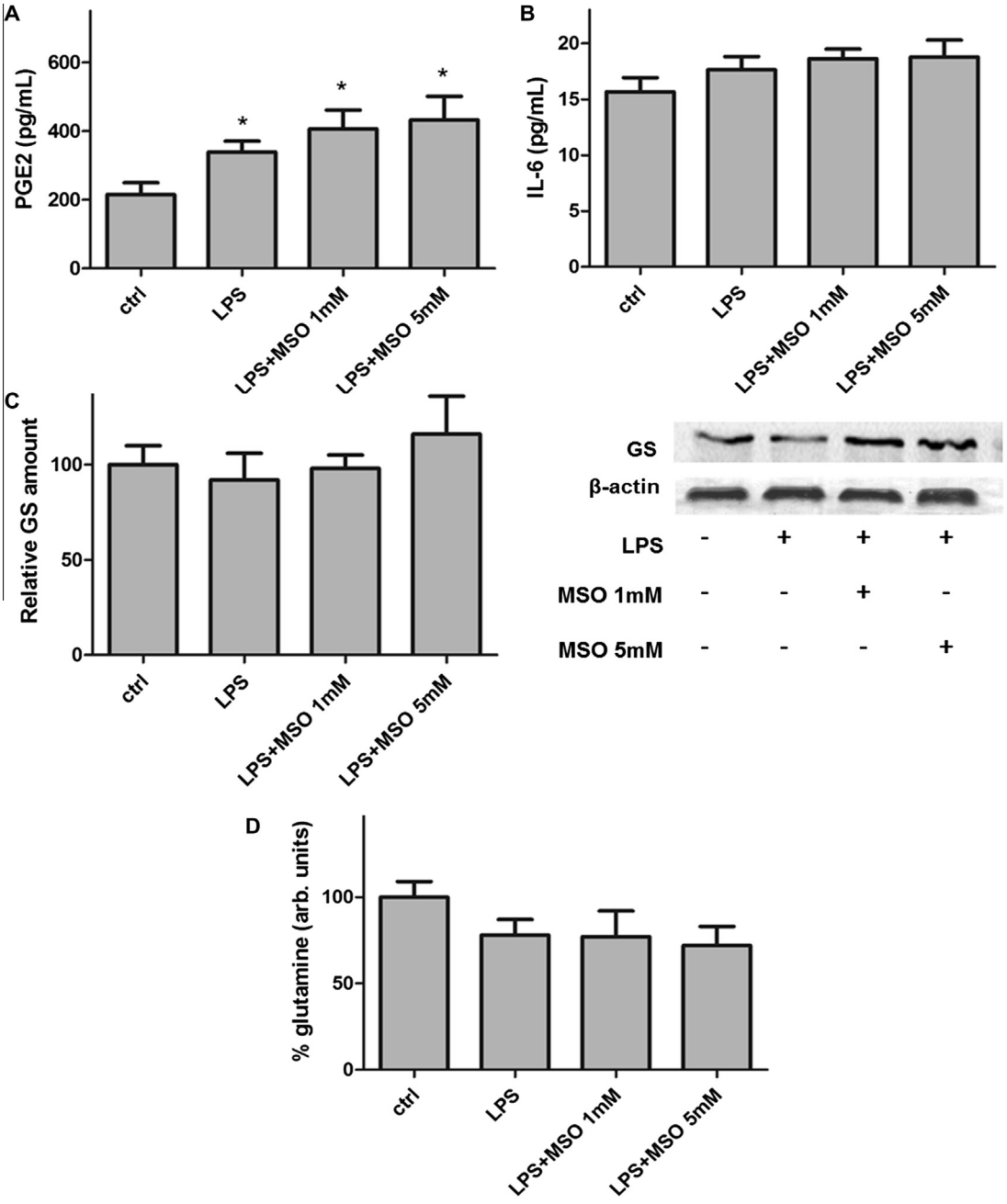

Fig. 4. Response to LPS of adipocytes at day 7 of differentiation. 3T3-L1 preadipocytes were grown and differentiated, as indicated in Section 2, over 14 days. LPS challenge was carried out on day 7 differentiating cells pretreated or not with 1 and 5 mM MSO for 48 h. (A and B) Cells were pelleted and the media was assessed for PGE2 (A) and IL-6 (B) by ELISA. Data are reported as mean ± S.E.M. of four independent experiments (*$P < 0.05$ versus control). (C) Cells were pelleted and tested for GS expression, reported in percentage considering control as 100% and normalized to β-actin densitometric levels. Data are reported as mean ± S.E.M. of four independent experiments. (D) Gln levels were assessed by LC–MS/MS analysis, normalized to total protein levels and expressed in percentage considering control as 100%. Data are reported as mean ± S.E.M. of four independent experiments.

from a proinflammatory non-completely mature to an inflammation-insensitive mature adipocytic state, through a undefined-before function of GS. Since inhibition of GS activity sensitizes differentiated adipocytes to proinflammatory stimuli, we infer that GS expression increases intracellular Gln levels to the critical point where glutamine is no longer an energy metabolite but probably a 'signalling' molecule capable of modulating expression of key inflammatory mediators ultimately leading to suppression of inflammation.

The concept of Gln exerting a regulatory role is not novel to the scientific community, since Gln has been described as a transcriptional modulator in many cases. For instance Gln mediates heat shock transcription factor 1 (HSF1) [42], argininosuccinate synthase [43] and PPARγ [44] genes expression. Furthermore Gln has been reported to increase turnover of the NF-κB p65 subunit [45] and induce autophagy [46]. The role of Gln has been always referred as proinflammatory, as the aminoacid has been widely recognized as an important metabolic fuel for immune cells [47]. However there are many cases in which treatment with Gln is associated to reduction of the proinflammatory response. In the duodenal mucosa of humans Gln decreases the production of IL-1, IL-6 and IL-8 and increases the production of IL-10 in a concentration dependent fashion [33,35]. In human intestine epithelial cells Gln reduces the production of proinflammatory cytokines

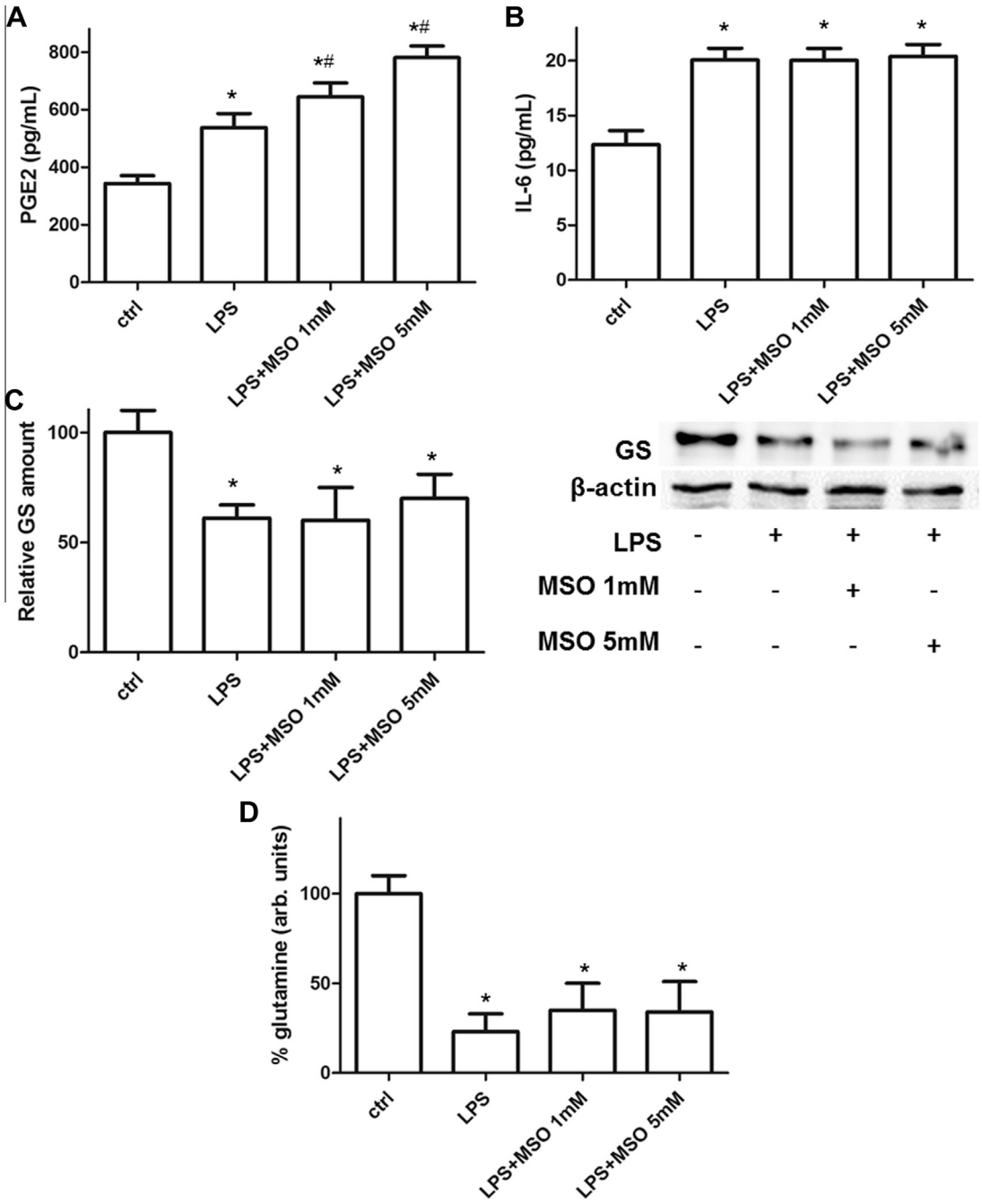

Fig. 5. Response to LPS of adipocytes at day 4 of differentiation. 3T3-L1 preadipocytes were grown and differentiated, as indicated in Section 2, over 14 days. LPS challenge was carried out for 48 h on day 4 differentiating cells pretreated or not with 1 and 5 mM MSO. (A and B) Culture media were assessed for PGE2 (A) and IL-6 (B) by ELISA. Data are reported as mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus LPS). (C) Cells were pelleted and tested for GS expression, reported in percentage considering control as 100% and normalized to β-actin densitometric levels. Data are reported as mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ versus control). (D) Gln levels were assessed by LC–MS/MS analysis, normalized to total protein levels and expressed in percentage considering control as 100%. Data are reported as mean ± S.E.M. of four independent experiments ($^{*}P < 0.05$ versus control).

and at 10 mM concentration decreases ubiquitinated IκBα while increasing free IκBα expression [36]. Strongly supraphysiological (up to 10 mM), but not physiological (2 mM) Gln concentrations lead to lower cytokine production and anti-inflammatory effect in murine peritoneal cell cultures, and this effect is exacerbated by malnutrition [37]. The ability of Gln to degrade p65 in Caco2 cells is maximal at 10 mM and absent at 2 mM Gln [45]. The increase in PPAR-γ DNA binding activity in IE-6 cells by Gln is dose-dependent, with 10 mM displaying the maximal effect [44]. In line with these evidences we show that the proinflammatory effect of GS inhibition is completely abolished by incubating cells with very high levels (10 mM) of Gln, which evidently disrupt Gln homeostasis, abnormally increasing the intracellular Gln concentration and thus mimicking the mechanism by which intracellular Gln levels raise upon GS activation.

However, the exact role of intracellular Gln as regulator of inflammation has never been completely understood because its metabolic role together with the tight control of its intracellular availability have covered up its regulatory role inside the cell, especially in cell culture. Indeed intracellular Gln levels are tightly regulated by the concerted activity of the cellular glutamine transporters and glutaminase, the mitochondrial glutamine

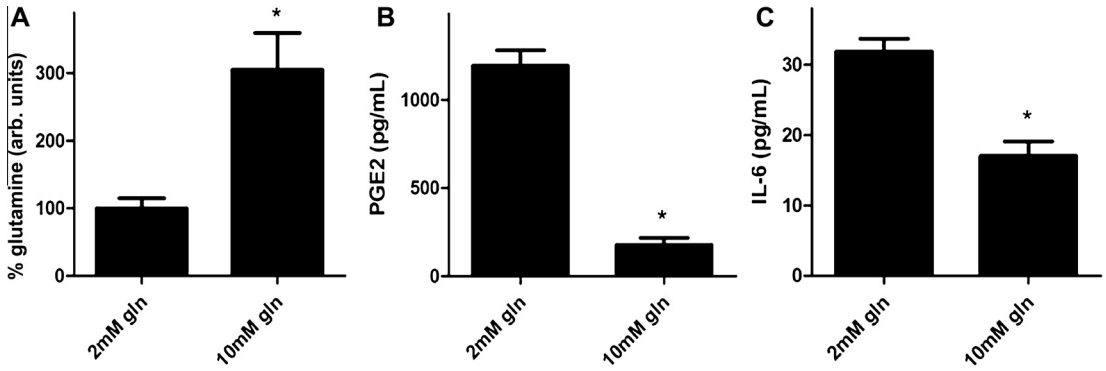

Fig. 6. PGE2 and IL-6 release in LPS/MSO- treated day 10 adipocytes in the presence of supraphysiological Gln concentrations. Day 10 differentiated-adipocytes, as indicated in Section 2, were treated with LPS and 1 mM MSO in a medium containing 2 mM or 10 mM Gln for 48 h. (A) Intracellular Gln levels were assessed by LC–MS/MS analysis, normalized to total protein levels and expressed in percentage considering 2 mM Gln values as 100%. Data are reported as mean ± S.E.M. of four independent experiments (*$P < 0.05$ versus 2 mM). (B) PGE2 was assessed in the media from both cell samples by ELISA. Data are reported as mean ± S.E.M. of four independent experiments (*$P < 0.01$ versus 2 mM Gln). (C) IL-6 was assessed in the media from both cell samples by ELISA. Data are reported as mean ± S.E.M. of four independent experiments (*$P < 0.05$ versus 2 mM Gln).

degrading enzyme active during glutamine utilization as a fuel. Glutamine synthetase and glutaminase appear to be inversely correlated. Glucocorticoids upregulate GS expression [11] while depressing glutaminase activity [48]. The oncogenic transcription factor c-MYC (MYC) stimulates glutamine catabolism to fuel growth and proliferation of cancer cells by upregulating glutaminase (GLS) and the glutamine transporter SLC1A5 [49], and is antagonized by the FOXO3a transcription factor, mediator of growth arrest and apoptosis that conversely upregulates GS [46]. The pro-inflammatory p65 NF-κB subunit activates Gln metabolism through upregulation of glutaminase [50] but is degraded by high Gln levels [45].

Our results demonstrate that adipocytes possess a cellular mechanism capable of controlling the response to a proinflammatory stimulus by raising intracellular Gln. Since GS is also expressed into macrophages [51,52], it is conceivable that the same process may also take place in this cell type to modulate inflammatory response. With this respect, the biological significance of the well known free radical-mediated GS inactivation [14,16,53,54] should be re-considered in the scenario of macrophage activation, in which free radical production is prominent. In particular, GS inactivation due to free radical production could represent a mechanism that potentiates macrophagic activation. Our laboratory is now evaluating this possibility. If this mechanism by which Gln modulates inflammatory response in adipocytes holds true also for macrophages and immune cells in general, then we should re-think about the meaning of Gln metabolism with respect to acute and chronic inflammatory states, metabolic syndrome and cancer. In particular, data on Gln supplementation during disease states should be reinterpreted in light of the intracellular availability of the supplemented amino acid. GS is then a promising therapeutic target for treating diseases in which inflammation plays a role.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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