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Ryan W. Grant  
*College of Health and Human Sciences*

Jacqueline I. Boudreaux  
*College of Health and Human Sciences*

Jacqueline M. Stephens  
*Pennington Biomedical Research Center*

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2-Deoxyglucose Inhibits Induction of Chemokine Expression in 3T3-L1 Adipocytes and Adipose Tissue Explants

Ryan W. Grant1, Jacqueline I. Boudreaux1, and Jacqueline M. Stephens2,3

Objective: To determine the influence of glycolytic inhibition on the adipocyte inflammatory response.

Methods: To determine the effect of 2-deoxyglucose (2-DOG) on the inflammatory response, mature 3T3-L1 adipocytes were co-treated with 2-DOG and LPS or TNF. To determine the effect of endoplasmic reticulum stress on TNF-induced induction of chemokines, adipocytes were pretreated with thapsigargin or salubrinal. Chemokine mRNA levels were determined using quantitative real-time PCR, and secretion of CCL2 was determined by Western blot.

Results: 2-DOG treatment reduced the ability of LPS and TNF to induce CCL2 mRNA levels and reduced secreted CCL2 protein levels in a dose-dependent manner. A similar pattern of mRNA regulation was observed for other chemokines. The attenuation of TNF-induced CCL2 mRNA levels occurred regardless of whether glucose or pyruvate was present in the media, suggesting that mechanisms other than glycolysis might mediate the observed effects. Treatment with the endoplasmic reticulum stressor thapsigargin and the endoplasmic reticulum signaling activator salubrinal reduced chemokine mRNA levels similarly to 2-DOG.

Conclusions: Collectively, our data indicate that 2-DOG suppresses inflammatory chemokine induction in adipocytes. The effects of 2-DOG do not seem to be linked to glycolysis but correlate with endoplasmic reticulum stress activation.

Introduction

Obesity is characterized by a substantial increase in adipose tissue-associated immune cells that contribute to systemic inflammation. For a 100 kg person with 50% body fat, this represents approximately $64 \times 10^9$ cells, which make up a large immunologically active organ (1). Recruitment of immune cells is a key component of the inflammatory response and is controlled by the secretion of recruitment signals (e.g., chemokines) from various tissues. Adipocytes are the most abundant cell type in adipose tissue and are capable of secreting numerous chemokines that are elevated during obesity.

A growing literature suggests that macronutrient metabolism and metabolic pathways control inflammatory responses. Recent findings have suggested that M1 polarized macrophages rely on glycolysis (2), while M2 macrophages rely on fatty acid oxidation (3). In addition, 2-deoxyglucose (2-DOG) is a glucose analog that functions as a glycolytic inhibitor and is known to have anti-inflammatory properties (2). In macrophages, 2-DOG modulates intracellular metabolites, particularly succinate, that link glycolysis to LPS-induced expression of several inflammatory gene transcripts such as IL-1β (2). Interestingly, the anti-inflammatory properties of 2-DOG appear to be shared across immune cells, since 2-DOG treatment has been shown to inhibit the development of Th17 cells while promoting the generation of T<sub>reg</sub> cells (4). Although studies using 2-DOG have demonstrated that it has potent effects on inflammation, the precise mechanisms that couple glycolytic inhibition to gene expression remain unknown. There is also evidence that 2-DOG influences glycosylation and has the ability to activate NF-κB, albeit in a longer time frame (3 h) than immunological stimuli (5).

Evidence suggests that glucose metabolism controls the metabolic response of adipocytes to inflammatory stimuli. Adipocyte lipolysis is responsive to glucose concentrations, with higher glucose concentrations promoting lipolysis and glucose analogs inhibiting lipolysis (6). These data suggest that glucose influences the metabolic response to TNF and other inflammatory mediators in adipocytes. However, the ability of glucose and glycolytic inhibitors to control

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1 Department of Nutrition Science, Purdue University, West Lafayette, Indianapolis, USA. Correspondence: Ryan Grant (rwgrant@purdue.edu)
2 Adipocyte Biology Laboratory, Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA
3 Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, USA.

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the inflammatory response in adipocytes is not known. Because adipocytes secrete a wide variety of chemokines that control immune cell recruitment, we sought to determine the role of glycolytic inhibition on chemokine mRNA levels and secretion in adipocytes challenged with inflammatory stimuli.

Methods

Cell culture

Murine 3T3-L1 preadipocytes, obtained from Dr. Green’s laboratory (7), were grown, maintained, and induced to differentiate using a standard protocol (8). Fully differentiated adipocytes were maintained in DMEM (Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS (Sigma-Aldrich) until 2 days before experimentation when cells were fed with 10% calf serum (Sigma-Aldrich). Prior to treatments, media was changed to low-glucose (5.5 mM) DMEM (Sigma-Aldrich) and 1% calf serum overnight. For shorter treatments, cells were pretreated with 2-DOG (Sigma-Aldrich), thapsigargin (Sigma-Aldrich), or salubrinial (R&D Systems, Minneapolis, MN) for 30 min and then treated with TNF (R&D Systems) for 1 to 4 h.

siRNA-mediated GLUT1 knockdown

Knockdown of GLUT1 was accomplished using a lipid-based transfection system (Dharmafect, GE Dharmacon, Lafayette, CO) and performed as described previously (9). Briefly, 3T3-L1 adipocytes were differentiated, and 7 to 10 days after differentiation, cells were trypsinized and plated into 24-well plates using low-glucose DMEM and 10% FBS. After replating, cells were treated with siRNA for 48 h and then challenged with 0.5 nM TNF. After 4 h of treatment, cells were collected and analyzed for mRNA levels.

Animals and adipose tissue explants

Ad libitum chow-fed (Teklad 2018S) C57BL/6 female mice 5 to 6.5 months of age were used for epididymal adipose tissue explant studies for proof of concept that 2-DOG can modify the TNF-induced inflammatory response in whole adipose tissue. Mice were euthanized in the fed state by CO2 asphyxiation. Tissues were collected in 30 mL of nuclease-free water then RNA extraction and qRT-PCR

Following treatment, cells were collected in 350 μL of RLT Buffer (Qiagen, Germantown, MD) and frozen at −80°C. Cell suspensions were thawed and RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer instructions. RNA was eluted from the mini columns in 30 μL of nuclease-free water then assessed for concentration and purity using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA), then real-time PCR was performed with the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). RNA was analyzed using a mouse chemokine and receptors pathway array (SAB Biosciences, Valencia, CA), and primer sets for CCL2 (F: GCAGAGAGCCAGGGAAGGA, R: TGGGGCGTAACTGCACTCTGG), CCL5 (F: GGTAACCATGAAGATCTCCTGC, R: TCTAGGAGAGGTAGGCAAG), CCL8 (F: AGGGATGAGAGGAGGTAG, R: GGTGACTGAGGCTTATCTG), CXCL10 (F: TCAGACCATGAAACCCTAA, R: CTATGGCTCTATTCTCAGT), and adiponectin (F: AAAAGGGCTTCAGGATGCTACTG, R: TGGCCAGATTAGAGGAACA) were also used to measure mRNA transcript levels. PPIA was used as the housekeeping gene (all primers from Integrated DNA Technologies, Coralville, IA). Samples were run in triplicate.

Western blotting

Protein concentration was measured using BCA protein assay (Thermo Scientific™ Pierce™ Protein Biology, Waltham, MA). Proteins were separated in 10% polyacrylamide gels containing SDS and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) in 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol. Nitrocellulose was blocked in casein blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h and incubated with primary antibody overnight. A secondary anti-rabbit antibody (LI-COR) labeled with an infrared label that emits signal at a wavelength of 680 nm was used. Results were visualized and captured on an Odyssey CLx (LI-COR).

Statistical analysis

SAS statistical software (SAS Institute, Cary, NC) was used for all statistical analyses. Cell culture experiments were analyzed using t-tests. Adipose tissue explants were analyzed using a 2 × 2 factorial design for the effects of 2-DOG and TNF. Results were considered significantly different at P < 0.05.

Results

2-DOG inhibits LPS-induced CCL2 secretion in mature adipocytes

The influence of glycolytic inhibitors on adipocyte chemokine gene expression and secretion in response to proinflammatory stimuli is not known. To determine the influence of glycolytic inhibition on the response to proinflammatory stimuli, LPS-induced chemokine mRNA levels were measured in 3T3-L1 adipocytes. Mature adipocytes were treated with LPS alone or in the presence of 1.0 mM or 2.0 mM 2-DOG for 20 h. These doses of 2-DOG have previously been shown to reduce inflammatory gene expression in macrophages (2). LPS-induced CCL2 secretion into the cell culture media was inhibited by 2-DOG in a dose-dependent manner with the greatest inhibition occurring at the highest 2-DOG concentration (∼70%, P < 0.05, Figure 1A, B). Differences in CCL2 secretion were mirrored by dose-dependent decreases in CCL2 mRNA in adipocytes treated with LPS and 2-DOG (∼93%, P < 0.05, Figure 1C). Based on CCL2 secretion in response to LPS, preadipocytes were relatively unresponsive to 2-DOG, while the inhibitory effect of 2-DOG on CCL2 secretion was larger in mature adipocytes (Figure 1D). These data indicate that 2-DOG reduces CCL2 mRNA levels and CCL2...
secretion in response to LPS, and that modulation of mRNA may be a critical target of 2-DOG in mature adipocytes.

2-DOG reduces TNF-induced CCL2 mRNA levels in mature adipocytes

Although LPS is known to drive proinflammatory gene expression in adipocytes, its role in driving adipocyte dysfunction during obesity and its associated metabolic diseases is less established. Hence, we examined the actions of TNF, a well-established mediator of adipose tissue inflammation and dysfunction during obesity and type 2 diabetes (11). We tested the capacity of 2-DOG to regulate TNF-induced CCL2 mRNA levels. Mature adipocytes were treated with TNF, or co-treated with TNF and 2-DOG (1.0 and 2.0 mM) for 20 h (Figure 2A). Similar to the experiments using LPS described above, TNF significantly increased CCL2 mRNA levels compared with control adipocytes. TNF-induced CCL2 mRNA levels were decreased by co-treatment with 2-DOG in a dose-dependent fashion with the greatest inhibition occurring at 2.0 mM (~83%, P < 0.01). We also used a targeted mouse chemokine PCR array and observed that the mRNA levels of 10 chemokines were regulated in a similar fashion (Table 1). These data clearly demonstrate that 2-DOG reduces the expression of several chemokines in 3T3-L1 adipocytes in response to TNF and LPS treatment.

We performed additional experiments to understand the time frame in which 2-DOG is effective. Induction of CCL2 by TNF occurred after a 1-h treatment and increased over an 8-h treatment. However, the most dramatic increases were observed after 1 and 2 h (Figure 2B). To determine the acute effects of 2-DOG, adipocytes were pre-treated for 30 min with 2-DOG and then stimulated with TNF for 1 h. 2-DOG significantly reduced CCL2 mRNA levels (~52%, P < 0.05) at 1 h, indicating acute inhibitory effects of 2-DOG (Figure 2C). To further assess whether the effects of 2-DOG were reliant on glycolysis, we used an experimental paradigm where the cell culture media contained either 10.0 mM glucose (substrate of glycolysis) or 10.0 mM pyruvate (end product of glycolysis). Surprisingly, 2-DOG was effective at reducing (P < 0.05) CCL2 mRNA levels in either glucose or pyruvate containing media (Figure 2D). No differences were observed between cells in glucose or pyruvate containing media in control and TNF-stimulated conditions.

GLUT1 knockdown modestly increases CCL2 mRNA

In adipose tissue, macrophage-derived TNF acts on adipocytes to increase basal glucose transport and reduce insulin sensitivity (12). GLUT1 is a basal glucose transporter whose expression is increased in 3T3-L1 adipocytes by proinflammatory factors including TNF (12). To determine if glucose transport via GLUT1 plays a role in TNF-induced CCL2 mRNA, we knocked down the GLUT1 gene using siRNA in mature adipocytes. Knockdown of GLUT1 protein was successful (~70%, Figure 3A, B). At 4 h of TNF treatment,
differences in CCL2 protein were not detected in cell extracts (Figure 3A, B); however, TNF-treated adipocytes had modestly increased secretion of CCL2 compared with unstimulated cells, and this was unaffected by GLUT1 knockdown (Figure 3C, D). CCL2 mRNA was modestly increased \((P < 0.01)\) in adipocytes treated with TNF and GLUT1 siRNA (Figure 3E). Although differences in mRNA expression were detected, differences detected at the protein level likely take longer than 4 h to develop because of the modest difference in mRNA and the time necessary for transcription and translation to occur. These results indicate that a reduction in

![Diagram](image)

**Figure 2** 2-DOG decreases TNF-induced CCL2 mRNA in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were maintained in 5.5 mM glucose media and were co-treated with 0.5 nM TNF and 1 or 2 mM 2-DOG. (A) CCL2 mRNA was measured in control, TNF, and 1 and 2 mM 2-DOG with TNF co-treatment after 20 h. (B) Time course of CCL2 mRNA response to 0.5 nM TNF from 0 to 8 h. (C) CCL2 mRNA was measured after 30 min of pretreatment with 2-DOG and 1 h of treatment with 0.5 nM TNF in control, 2-DOG, TNF, and 2-DOG followed by TNF. (D) The influence of media containing 10.0 mM glucose or 10.0 mM pyruvate on the suppressive effect of 2-DOG on TNF-induced CCL2 mRNA. Data are presented as the mean ± SEM. Experiments on the acute effects of 2-DOG were performed independently three times.

**Table 1** 2-DOG co-treatment reduces adipocyte chemokine mRNA levels

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<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>TNF 2-DOG</th>
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GLUT1 expression modestly increases TNF-induced gene expression of CCL2 and does not protect 3T3-L1 adipocytes from TNF-induction of CCL2.

Pretreatment with thapsigargin or salubrinal inhibits the induction of chemokines in response to TNF

2-DOG was effective in reducing mRNA levels of inflammatory genes even when the end product of glycolysis was provided, suggesting that its effects are likely mediated through nonglycolytic pathways. 2-DOG is also known to interfere with the formation of glycoproteins by inhibiting the transfer of dolichol oligosaccharides to glycoproteins and induces the unfolded protein response (UPR) (13). To determine the influence of pretreatment with a UPR activator on TNF-induced chemokine expression mature 3T3-L1 adipocytes were pretreated with 1 µM thapsigargin for 30 min, and then treated with 0.5 nM TNF for 4 h. TNF significantly increased mRNA expression of CCL2, CCL5, CCL8, and CXCL10 (Figure 4A-D). However, pretreatment with thapsigargin blunted the TNF response in a manner similar to 2mM 2-DOG (Figure 4A-D). These data confirm mRNA alterations in genes identified by the targeted
chemokine array (Table 1) and also indicate that the effects of 2-DOG on chemokine expression are similar to those induced by the UPR activator thapsigargin. To further understand the contribution of pathways downstream of the UPR, cells were pretreated with either 2-DOG or salubrinal, which causes the accumulation of phosphorylated eIF2α by inhibiting its de-phosphorylation. Interestingly, salubrinal dose-dependently decreased TNF-induced expression of CCL2, CCL5, CCL8, and CXCL10 (Figure 5). These data indicate that UPR activation and UPR signaling pathways have the capacity to significantly regulate the secretion of chemokines in adipocytes.

2-DOG reduces chemokine mRNA levels in adipose tissue explants

2-DOG effectively reduces LPS and TNF-induced chemokine mRNA levels in 3T3-L1 adipocytes in vitro. Hence, we determined whether 2-DOG would be effective in reducing TNF-induced chemokine levels in whole adipose tissue. Adipose tissue explants from female mice were pretreated with 2-DOG for 30 min and then treated with TNF. 2-DOG alone reduced CCL2 mRNA levels (−24% $P < 0.05$, Figure 6A). This is likely because basal levels of TNF and other proinflammatory cytokines maintain CCL2 gene expression in adipose tissue. Our ex vivo analysis also showed that TNF increased CCL2 mRNA levels and pretreatment with 2-DOG reduced TNF-induced CCL2 mRNA to basal levels (−28%, $P < 0.01$, Figure 6A). These results are consistent with our experiments in 3T3-L1 adipocytes (Figure 2). Similarly, TNF-induced CCL5 mRNA levels were reduced by pretreatment with 2-DOG in ex vivo conditions (−54%, $P < 0.01$, Figure 6B). Adiponectin mRNA levels did not vary with 2-DOG or TNF treatment (Figure 6C). Adiponectin mRNA is very stable (14), and unlikely to change due to the short TNF treatment time. These results indicate the 2-DOG can act on whole adipose tissue to reduce chemokine mRNA levels. These results should be interpreted with caution because other cell types in adipose tissue are known to be targets of 2-DOG and may be contributing to this ex vivo response.
Discussion

Our novel observations reveal that 2-DOG inhibits TNF-induced increases of chemokine mRNA in cultured adipocytes and adipose tissue explants. Surprisingly, these results were not affected by media glucose concentrations, and GLUT1 knockdown increased CCL2 mRNA levels. These results indicate that the effects of 2-DOG on TNF-induced mRNA levels in the adipocyte are likely not...
related to glycolytic inhibition. Instead, the anti-inflammatory effects of 2-DOG on adipocytes may be related to the activation of endoplasmic reticulum (ER) stress pathways and downstream signaling events. Thus, 2-DOG not only influences metabolism, but also modulates other pathways that regulate the adipocyte inflammatory response.

Glycolytic inhibition by 2-DOG has previously been linked with improvements in metabolism and aging. Initial studies demonstrated that 2-DOG can improve markers associated with caloric restriction including improved blood glucose homeostasis, reduced heart rate, and reduced blood pressure (15,16). Other studies have demonstrated that 2-DOG impacts pathways associated with stress response, particularly in models of neuronal damage including iron (17), amyloid-B-peptide (17), and 1-methyl-4-phenyl-1,2,3,5-tetrahydropyridine (18). 2-DOG has been shown to induce GRP78 and HSP70 in many of these models (17,19,20). Initially the data on 2-DOG as a caloric restriction mimetic was positive. However, long-term studies using 2-DOG demonstrate that it can cause heart failure in multiple strains of rats (21). Interestingly, our results suggest that the effects of 2-DOG are not solely related to glycolysis, but suggest that ER stress signaling pathways may contribute to the effects of 2-DOG on inflammation and metabolism. Determining the contribution of glycolysis versus ER stress signaling to the functions of 2-DOG and their tissue specificity may yield important information on the biology of aging.

Activation of the UPR contributes to cellular homeostasis by regulating protein expression, stabilization and degradation; however, prolonged UPR activation can lead to apoptosis. In this series of experiments, acute activation was able to block the inflammatory response to TNF; however, chronic stimulation may produce different effects. This study indicates that activation of the signaling factor eIF2α may be responsible for reductions chemokine mRNA by ER stress. Recent work by others has demonstrated that overexpression of the ER signaling protein XBP-1 is sufficient to down regulate metabolic enzymes in a manner similar to TNF (22). Given that multiple signaling pathways arising from the ER influence autophagy (23), future work will be needed to examine pathway specificity. However, the effects of salubrinal indicate that eIF2α phosphorylation is critical to UPR regulation of the inflammatory response.

The extent to which chemokine expression can modify obesity-induced adipose inflammation has been investigated primarily through the use of global knockout mice. Targeting the production of single chemokines has been problematic with some studies indicating strong protection of CCL2/−/− mice from macrophage accumulation (24) and overexpression of CCL2 has been demonstrated to drive adipose tissue macrophage accumulation (25). While other studies demonstrate only modest protection from inflammation in CCL2 knockouts fed a high-fat diet (26,27). CCR2, the receptor for CCL2 and other MCP family chemokines, are protected against insulin resistance (28,29). Designing approaches that target multiple chemokines and their response to inflammatory stimuli may be a way forward in this regard. 2-DOG and the pathways that it activates may be favorable in this respect because it targets multiple chemokines.

Overall, our studies show that 2-DOG has the capacity to suppress TNF-induced chemokine mRNA levels. Although the function of 2-DOG is oftentimes attributed to its effects on glycolysis, our data strongly suggest the involvement of other cellular processes. Our results suggest that 2-DOG can function to suppress induction of a specific subset of chemokines and it may do this through the induction of ER stress in adipocytes. Future studies in this area will be needed to determine the responsible signaling pathways and whether they can be specifically targeted for reducing metabolic inflammation.

Acknowledgments
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