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Effects of high fat diet on GPR109A and GPR81 gene expression ☆

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ABSTRACT

GPR109A (PUMA-G, NIACR1, HCA_2) and GPR81 (HCA_1) are G protein-coupled receptors located predominantly on adipocytes that mediate anti-lipolytic effects. These cell surface receptors give the adipocyte the ability to "sense" metabolic changes in the environment and respond through lipolytic regulation and release of products including free fatty acids and pro- or anti-inflammatory adipokines. The endogenous ligands for GPR109A and GPR81 are β-hydroxybutyrate and lactate, respectively, both of which are hydroxycarboxylic acids and intermediates of energy metabolism. Circulating β-hydroxybutyrate levels are increased during a 2–3 day fast and prolonged starvation, while lactate levels are elevated during times of intense exercise. Therefore, regulation of expression of these receptors is crucial for the metabolic sensing ability of the adipocyte and ultimately whole body energy homeostasis. We investigated the effects of high fat diet-induced obesity on expression of GPR109A and GPR81. Sixteen male C57BL/6 mice were placed on a control (10% kcal fat; $n = 8$) or a high fat (60% kcal fat; $n = 8$) diet for 11 weeks. Diet-induced obesity significantly reduced GPR109A and GPR81 gene expression in epididymal fat pads. This decrease in GPR109A and GPR81 gene expression was positively correlated with a decrease in adipose tissue PPARγ gene expression. In contrast, acute treatment of both 3T3-L1 adipocytes and RAW 264.7 macrophages with lipopolysaccharide significantly increased GPR109A gene expression, but had no effect on GPR81 expression in 3T3-L1 adipocytes. In conclusion, chronic obesity reduces GPR109A and GPR81 expression in the adipose tissue, while acute *in vitro* LPS treatment increases expression of GPR109A in adipocytes and macrophages.

1. Introduction

Adipocytes express a number of cell surface receptors that contribute to their ability to sense metabolic changes in the surrounding environment, including GPR109A and GPR81. In 2003, GPR109A was identified as the receptor for the beneficial lipid-altering drug, niacin [1–3]. However, under physiological conditions, plasma concentrations of niacin do not reach levels high enough to activate the receptor, making it unlikely to be the endogenous ligand. In 2005, Taggart et al. demonstrated that β-hydroxybutyrate, a ketone body produced by the liver, is an endogenous ligand for GPR109A [4]. β-Hydroxybutyrate activates GPR109A and inhibits adipocyte lipolysis at concentrations seen during a 2–3 day fast, with an EC_{50} of 767 ± 57 μM [4]. β-Hydroxybutyrate may represent a homeostatic mechanism for surviving starvation by acting in a negative feedback manner to inhibit lipolysis. In this manner, β-hydroxybutyrate can regulate its own production by decreasing the serum level of fatty acid precursors available for hepatic ketogenesis [4] and possibly preserving lipid stores during a prolonged fast [5]. GPR109A is predominantly expressed in adipocytes of white and brown adipose tissue, and is expressed to a lesser extent in keratinocytes and immune cells, including dermal dendritic cells, monocytes, macrophages and neutrophils [1–3,6–8].

GPR81 shares a 52% amino acid sequence identity in humans to GPR109A [1–3]. In addition, GPR81 is localized more specifically to the adipose tissue [3]. In 2008, lactate was discovered to be the endogenous ligand for GPR81 [9,10]. Plasma lactate levels reach concentrations capable of activating the receptor during bouts of intense exercise, with an EC_{50} of ~5 mM [10]. Infusion of lactate reduces lipolysis *in vivo* [11–13] as does treatment of adipocytes *in vitro* [14,15]. The effect of lacate to reduce lipolysis is mediated through activation of GPR81 [9,10]. However, these effects are somewhat controversial, as studies have demonstrated that the

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receptor is not involved in the regulation of lipolysis during intensive exercise when lactate concentrations are elevated [16].

Both GPR109A and GPR81 are located on chromosome 12q24 [3] and mediate anti-lipolytic effects through coupling to G_i-type G proteins [17]. It is important to note that with the recent deorphanization of these receptors, there has been a recommendation to the International Union of Basic and Clinical Pharmacology (IUPHAR) to rename GPR81, GPR109A, and GPR109B (a human-specific, low-affinity niacin receptor) as hydroxy-carboxylic acid (HCA) receptor family members, whereby GPR81 is HCA_1, GPR109A is HCA_2, and GPR109B is HCA_3 [18]. Studies have revealed that all three receptors bind hydroxy-carboxylic acids as their endogenous ligands, leading to the proposed nomenclature for this receptor family.

G protein-coupled receptor synthesis is regulated by gene transcription, translation, and posttranslational processing, which may be regulated by the ligand itself and by other hormones and factors [19]. GPR109A gene expression in adipocytes is reduced with age in mice [20]. While the regulation of GPR109A gene expression in the adipose tissue in various disease states (including diabetes or obesity) has not been demonstrated, a down-regulation of GPR81 mRNA expression in mouse adipose tissue has been demonstrated in response to acute treatment with inflammatory stimuli [21]. In addition, *ob/ob* mice, a mouse model of obesity and type 2 diabetes, demonstrate reduced adipose tissue expression of GPR81 [21]. Our objectives were to investigate the effects of high fat diet-induced obesity and inflammation on gene expression of GPR109A and GPR81.

2. Materials and methods

2.1. Animal studies

Sixteen male C57BL/6 mice were purchased at three weeks of age from Charles River Laboratories (Wilmington, MA). After three days of acclimatization, mice were either placed on a high fat diet (HFD; 60% kcal as fat; $n = 8$) or a control diet (10% kcal as fat; $n = 8$) obtained from Research Diets (New Brunswick, NJ) and were maintained on such diets for the duration of the study (11 weeks).

After 11 weeks on the diets, mice were fasted overnight and sacrificed. Epididymal fat pads were excised, flash frozen in liquid nitrogen, and stored at −80 °C until analysis. Trunk blood was collected, processed for serum, and stored at −80 °C until analysis.

2.2. Serum analysis

Serum insulin concentrations were measured using an ELISA kit from Millipore (Temecula, CA). Serum glucose and triglyceride concentrations were measured using kits from Cayman Chemicals (Ann Arbor, MI). Serum non-esterified fatty acids (NEFAs) were measured using a kit from Wako Chemicals (Richmond, VA).

2.3. Cell culture studies

Adipocyte differentiation of 3T3-L1 fibroblasts was induced 48 h post confluence, with DMEM supplemented with 10% fetal bovine serum (FBS), insulin (10 μg/mL), dexamethasone (250 nM), IBMX (500 nM), and rosiglitazone (1 μM) for 4 days. Media was then replaced with DMEM supplemented with 10% FBS and insulin (10 μg/mL) for 3 days, at which point media was replaced with DMEM supplemented with 10% FBS for an additional 3 days. Cells were used at day 10 of differentiation. RAW 264.7 macrophages were cultured in DMEM supplemented with 10% FBS and used when cells reached 70–80% confluence.

Cells were serum starved for 9 h in DMEM supplemented with 0.2% bovine serum albumin (serum free media; SFM). Media was replaced with SFM with or without LPS (50 ng/mL) for 15 h. RNA was collected by the TRIzol method, and was quantified using the NanoDrop1000 spectrophotometer (NanoDrop Products Wilmington, DE).

For conditioned media (CM) studies, 3T3-L1 adipocytes and RAW 264.7 macrophages were serum-starved for 3 h in SFM, then treated with SFM ($n = 5$) or LPS (50 ng/mL; $n = 5$) for 6 h. Treatment media was then removed by aspiration and cells were washed once with PBS, then SFM was placed on all dishes and cells secreted into this media for 15 h. After 15 h, CM media from each treatment group was collected and pooled by treatment group, and stored at −80 °C for less than 1 month. CM was used to treat the opposing cell type (i.e. RAW 264.7 macrophages were treated with CM from 3T3-L1s and vice versa for 6 h). RNA was collected as described above.

2.4. Real-time PCR

RNA was isolated from epididymal white adipose tissue (EWAT) using Qiagen RNeasy Lipid Tissue Mini Kit (Valencia, CA). RNA was isolated from 3T3-L1 and RAW264.7 cells as described above. RNA (1 μg) was reverse transcribed into cDNA using iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA). Analyses were performed on a Bio-Rad iCycler iQ thermocycler. Samples were analyzed in 30 μl reactions using SYBR Green PCR Master Mix (Bio-Rad). Relative changes in gene expression were calculated by the comparative C_T method using the $2^{\Delta\Delta C_T}$ equation with results normalized to the corresponding 36B4 mRNA levels. Primer sequences are shown in Supplementary Table 1.

2.5. Statistical analysis

Data were analyzed by unpaired *t*-test using GraphPad Prism software version 4.0 (GraphPad Software, La Jolla, CA). Significance was accepted at the $P < 0.05$ level.

3. Results

3.1. Effects of HFD on serum and metabolic parameters in mice

As expected, HFD caused significant increases in body and epididymal fat pad weights (Supplementary Table 2). Serum glucose and insulin concentrations were increased in mice fed a HFD, but no significant effect on serum triglycerides was observed (Supplementary Table 2).

3.2. HFD decreases GPR109A and GPR81 gene expression

Eleven weeks of HFD reduced adipose tissue gene expression of both GPR109A and GPR81 in male C57BL/6 mice (Fig. 1). Adipose tissue is comprised of various cell types including adipocytes, preadipocytes, and immune cells, including macrophages. Obesity results in adipose tissue inflammation, characterized by infiltration of inflammatory macrophages and elevated expression of inflammatory cytokines. We demonstrated increased gene expression of the inflammatory cytokines, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) in the epididymal adipose tissue of the obese mice, as well as increased expression of the general macrophage marker, CD68 (Fig. 2). Previous studies have demonstrated that GPR81 is decreased in *ob/ob* mice, an obesity mouse model associated with mild adipose tissue inflammation [21]. Therefore, we investigated

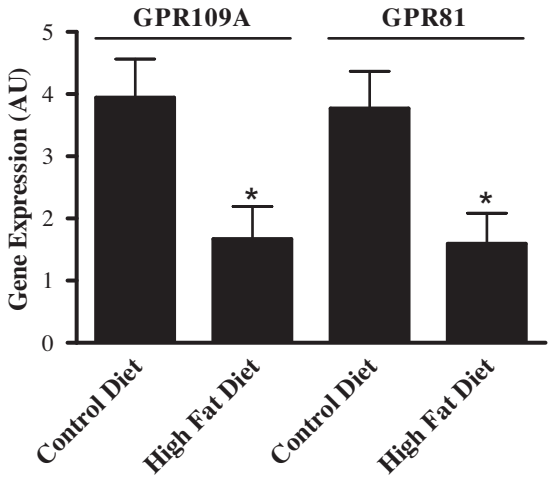

Fig. 1. Effect of HFD on GPR109A and GPR81 gene expression. Effects of 11 weeks of HFD on EWAT GPR109A and GPR81 gene expression. $^{*}P < 0.05$ compared to mice receiving control diet.

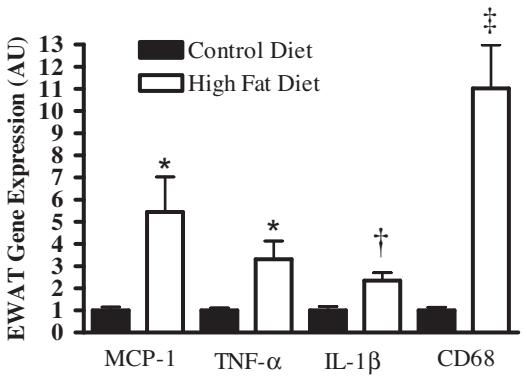

Fig. 2. Effects of HFD on markers of inflammation in EWAT. Effects of 11 weeks HFD on gene expression of inflammatory cytokines in EWAT; $^{‡}P < 0.0001$, $^{†}P < 0.01$, $^{*}P < 0.05$ compared to control.

the effects of acute inflammation on GPR109A and GPR81 expression *in vitro*.

3.3. Effect of LPS on GPR109A and GPR81 gene expression in RAW 264.7 macrophages and 3T3-L1 adipocytes

In order to simulate the adipose tissue inflammation seen in obesity, we treated 3T3-L1 adipocytes and RAW 264.7 macrophages, with the pro-inflammatory molecule, LPS for 15 h and measured receptor gene expression. In contrast to our chronic HFD *in vivo* study, LPS treatment significantly increased the expression of GPR109A in RAW 264.7 macrophages, while GPR81 mRNA was not detectable in RAW 264.7 macrophages (Fig. 3A). Both GPR109A and GPR81 were expressed in fully differentiated 3T3-L1 adipocytes, but GPR109A was expressed at about a tenfold higher level than GPR81 (Fig. 3B). LPS treatment significantly increased GPR109A gene expression in 3T3-L1 adipocytes, but had no effect on GPR81 expression (Fig. 3B).

3.4. Effect of conditioned media on GPR109A and GPR81 gene expression in RAW 264.7 macrophages and 3T3-L1 adipocytes

Conditioned media studies were then conducted to determine if LPS treatment is capable of stimulating the release of factors which could modulate GPR109A and GPR81 gene expression. Treatment of RAW 264.7 macrophages with CM from adipocytes treated with LPS resulted in a significant increase in GPR109A gene expression compared to macrophages treated with CM from untreated adipocytes ($P < 0.001$; Fig. 3C). Since there was no LPS in the CM, the effect of CM from adipocytes to increase GPR109A expression in macrophages is due to a secreted product from the adipocytes in response to LPS treatment. GPR81 was not detected in RAW 264.7 macrophages. Treatment of 3T3-L1 adipocytes with CM from RAW 264.7 macrophages treated with LPS significantly increased the gene expression of GPR109A, but did not affect the expression of GPR81 (Fig. 3D). Again, the increase in GPR109A expression in response to CM from LPS-treated macrophages is due to some product, perhaps an inflammatory cytokine, from RAW 264.7 macrophages.

3.5. GPR109A and GPR81are positively correlated with adipose tissue PPARγ gene expression

While acute LPS treatment did not mimic obesity-induced reductions in receptor expression found *in vivo*, we demonstrated a positive correlation between PPARγ gene expression and GPR109A (Fig. 4A; $R^2 = 0.47$; $P < 0.0001$) and GPR81 gene expression (Fig. 4B; $R^2 = 0.40$; $P < 0.0001$) in the mouse epididymal fat pads. In support of these findings, recent studies have demonstrated that activation of the nuclear receptor PPARγ enhances GPR109A and GPR81 expression in cultured adipocytes [22].

4. Discussion

Here, we demonstrate, for the first time, that HFD reduces adipose tissue GPR109A and GPR81 gene expression. Downregulation of GPR109A and GPR81 during states of obesity may contribute to the disruption of normal regulation of adipocyte lipolytic function often seen in obesity, such as elevated basal lipolysis and reduced catecholamine-induced lipolysis [23,24]. GPR109A and GPR81 give adipocytes the ability to "sense" metabolic changes in the surrounding environment and respond through lipolytic regulation and release of products including free fatty acids and pro- or anti-inflammatory adipokines. Regulation of expression of these receptors is crucial for the metabolic sensing ability of the adipocyte and ultimately whole body energy homeostasis.

Obesity is associated with a chronic low-grade inflammation of the adipose tissue, characterized by increased production of pro-inflammatory cytokines and chemokines including MCP-1, TNF-α, IL-1β, and IL-6. GPR81 downregulation in adipose tissue of ob/ob mice (a mouse model of obesity) has previously been demonstrated [21]. Others have demonstrated that adipose tissue GPR81 expression is downregulated in response to the inflammatory stimuli LPS, zymosan, and turpentine [21]. However, its expression does not appear to be regulated by the usual cytokines that induce metabolic changes during obesity-associated inflammation. Specifically, treatment of adipocytes with the pro-inflammatory cytokines typically elevated in the adipose tissue during obesity, including TNF- α, IL-1 β, IL-6, or interferon γ failed to reduce the expression of GPR81 *in vitro* [21]. Here, we demonstrate for the first time that HFD-induced obesity also reduces GPR81 gene expression. The exact mechanism for the down-regulation of GPR81 in obesity has yet to be fully elucidated, but it does not appear to be mediated through the increased production of pro-inflammatory cytokines produced abundantly in adipose tissue of obese subjects.

To date, no studies have demonstrated any effect of obesity or inflammation on GPR109A expression in adipose tissue. Our findings demonstrate that HFD-induced obesity decreases adipose

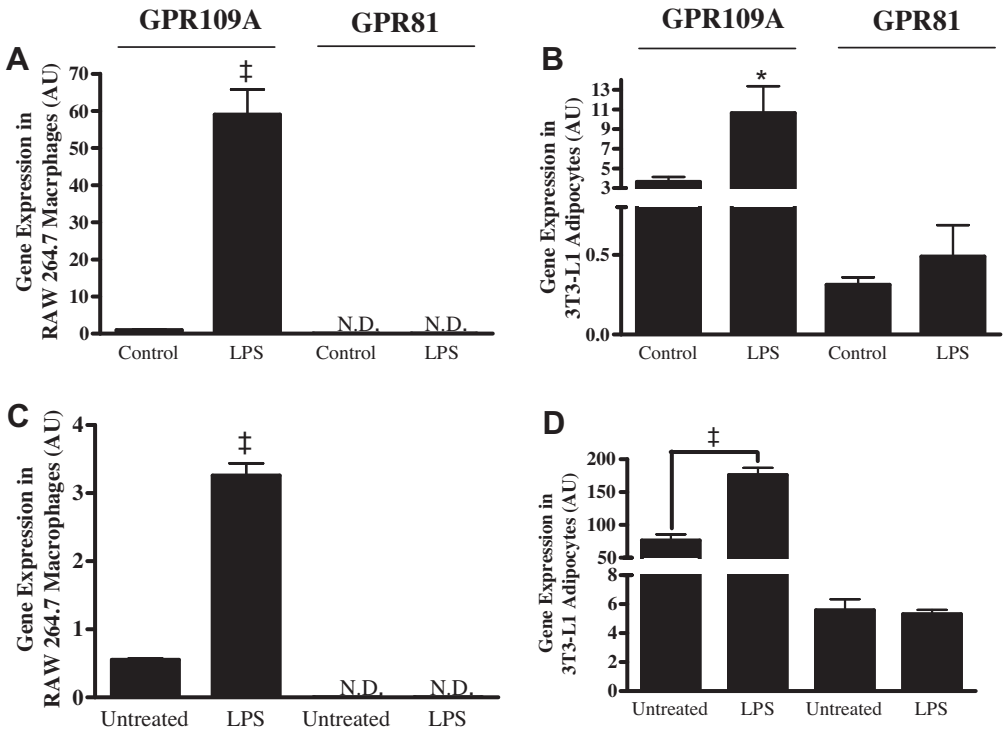

Fig. 3. Effect of LPS on GPR109A and GPR81 gene expression. Effects of 15 h LPS (50 ng/mL) treatment on GPR109A and GPR81 gene expression in RAW 264.7 macrophages (A) and 3T3-L1 adipocytes (B); $^{‡}P < 0.0001$, $^{*}P < 0.05$ compared to control; N.D. – not detectable. (C) RAW264.7 macrophages at ~70% confluence were treated with CM from 3T3-L1 adipocytes treated with serum-free media (untreated) or LPS (50 ng/mL) for 6 h. CM was collected after 15 h secretion and opposing cells were treated with CM for 6 h. (D) Differentiated 3T3-L1 adipocytes were treated with CM from RAW 264.7 macrophages treated with serum-free media (untreated) or LPS (50 ng/mL) for 6 h. CM was collected after 15 h secretion and opposing cells were treated with CM for 6 h; $^{‡}P < 0.0001$ compared to control; N.D. – not detectable.

tissue GPR109A gene expression. Contradictory to our findings in adipose tissue *in vivo*, Digby et al. report that exposure of 3T3-L1 adipocytes to TNF-α (1.0 ng/ml) for four hours up-regulates GPR109A mRNA expression [25]. We demonstrated that HFD-induced obesity results in a marked increase in TNF-α mRNA, concomitant with reduced GPR109A gene expression. However, we demonstrated that treatment with LPS increases GPR109A gene expression in cultured 3T3-L1 adipocytes and RAW 264.7 macrophages. Others have shown an increase in GPR109A expression in retinal pigment epithelial cells from *db/db* mice and streptozotocin-induced diabetic mice compared to normal controls [26]. Additionally, GPR109A expression is increased in macrophages treated with interferon γ [27]. The adipose tissue of the mice on the HFD has been infiltrated by macrophages. Hence, in obese mice there are at least two cell types expressing GPR109A in the adipose tissue: adipocytes and macrophages. Since we examined whole epididymal white adipose tissue, we cannot differentiate the effects of HFD on GPR109A expression in adipocytes and macrophages. We demonstrated a dramatic reduction in GPR109A gene expression in the adipose tissue in response to HFD, yet we found a pronounced increase in GPR109A expression in RAW 264.7 macrophages and 3T3-L1 adipocytes treated with inflammatory LPS. Others may suggest that GPR109A expression in macrophages would increase during HFD owing to a broad array of inflammatory stimuli present. It is possible that inflammation has opposing effects in the isolated adipocytes and macrophages versus the whole adipose tissue.

Gene expression of both GPR109A and GPR81 in adipocytes has been linked to PPARγ activation [22]. Treatment with the thiazolidinedione (TZD) PPARγ agonist, rosiglitazone, increases GPR109A and GPR81 expression, and knockdown of PPARγ suppresses receptor expression in fully differentiated human multipotent adipose-derived stem cells and in 3T3-L1 adipocytes [22]. Increased GPR109A and GPR81 expression in response to TZD treatment may reduce circulating free fatty acid levels and perhaps ameliorate insulin resistance [22]. In support of these cell culture findings, we observed a positive correlation between PPARγ gene expression and GPR109A and GPR81 gene expression in the mouse epididymal fat pads.

There is an obvious discrepancy between the effects we observed for HFD-induced obesity to decrease receptor expression *in vivo* and acute inflammatory stimulus to increase receptor expression *in vitro*. This may be due to the duration of the HFD feeding. Initially, in response to HFD-induced obesity, the adipose tissue becomes very inflamed. While inflammation persists throughout the course of obesity, a remodeling process takes place with prolonged HFD, leading to a state of adipose tissue repair and a partial resolution of inflammation [28]. If the mice had been fed a HFD for a shorter time period, it is possible that we may have seen an upregulation of receptor expression in the adipose tissue due increased adipose tissue inflammation. At 11 weeks, the adipose tissue may begin to adapt to the HFD, and decrease receptor expression in order to prevent excessive lipolysis, when it is in a remodeling stage. The mechanism by which HFD reduces GPR109A and GPR81 expression remains to be elucidated. However, the current and previous investigations indicate that inflammation may play a key role in regulation of receptor expression.

Other highly expressed adipose tissue receptors that give the adipocyte metabolic sensing abilities are GPR43, GPR84, and GPR120, all of which are receptors for endogenous free fatty acids [29–31]. Cornall et al. demonstrated HFD increased GPR43 gene expression in the skeletal muscle and liver, while GPR120 expression was increased in the skeletal muscle and cardiac tissue [32]. They did not examine receptor expression in the adipose tissue. However, others have reported increased adipose tissue expression of GPR43 and GPR120 in response to HFD [33,34]. Additionally,

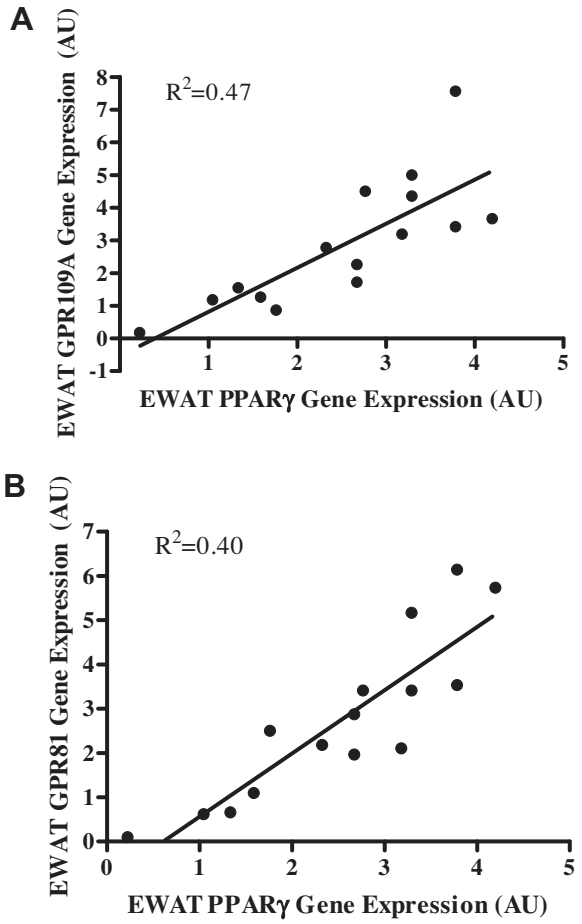

Fig. 4. Correlation between PPARγ gene expression with GPR109A (A) and GPR81 (B) gene expression in EWAT.

Nagasaki et al. reported that GPR84, a receptor for medium-chain fatty acids, is upregulated in the adipose tissue of mice in response to five weeks on a HFD [31]. They also demonstrated that co-culture of 3T3-L1 adipocytes with RAW 264.7 macrophages enhances GPR84 expression in 3T3-L1 adipocytes. We treated RAW 264.7 macrophages and 3T3-L1 adipocytes with CM from the opposing cells and found that treatment with CM from cells treated with LPS significantly increased GPR109A expression in 3T3-L1 adipocytes and enhanced GPR109A expression in RAW 264.7 macrophages, but did not affect GPR81. Thus, the ability of different cell types to affect receptor expression is not dependent on direct contact with the opposing cells, but rather on secreted products. While we did not conduct studies to investigate what that secreted product may be, Nagasaki et al. demonstrated that TNF-α secreted from macrophages enhanced GPR84 expression in adipocytes. Additionally, others have reported that treatment of 3T3-L1 adipocytes with TNF-α increases GPR109A gene expression [25]. TNF-α is secreted by both adipocytes and macrophages, making it a likely candidate for the effects on GPR109A that we observed.

In conclusion, we observed differential effects of obesity-induced chronic, low-grade inflammation and LPS-stimulated acute inflammation on receptor expression. HFD-induced obesity down-regulated adipose tissue gene expression of GPR109A and GPR81, while treatment with LPS had no effect on GPR81 expression, and actually increased GPR109A expression in adipocytes and macrophages. The different effects observed are likely due to the nature and duration of the inflammation. Future studies are needed to elucidate the mechanisms for these observations on receptor expression.

Disclosure summary

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.082.

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