Inflammation Stimulates Niacin Receptor (GPR109A/HCA2) Expression in Adipose Tissue and Macrophages

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Short running title: Inflammation Stimulates GPR109A/HCA2 Expression

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Abstract

Many of the beneficial and adverse effects of niacin are mediated via a G protein receptor, GPR109A/HCA2, which is highly expressed in adipose tissue and macrophages. Here we demonstrate that immune activation increases GPR109A/HCA2 expression. LPS, TNF, and IL-1 increase GPR109A/HCA2 expression 3-5 fold in adipose tissue. LPS also increased GPR109A/HCA2 mRNA levels 5.6 fold in spleen, a tissue rich in macrophages. In peritoneal macrophages and RAW cells, LPS increased GPR109A/HCA2 mRNA levels 20 to 80 fold. Zymosan, LTA, and poly I:C, other TLR activators, and TNF and IL-1 also increased GPR109A/HCA2 in macrophages. Inhibition of the MyD88 or TRIF pathways both resulted in partial inhibition of LPS stimulation of GPR109A/HCA2, suggesting that LPS signals an increase in GPR109A/HCA2 expression by both pathways. Additionally, inhibition of NF-kB reduced the ability of LPS to increase GPR109A/HCA2 expression by approximately 50% suggesting that both NF-kB and non NF-kB pathways mediate the LPS effect. Finally, preventing the LPS induced increase in GPR109A/HCA2 resulted in an increase in triglyceride accumulation and the expression of enzymes that catalyze triglyceride synthesis. These studies demonstrate that inflammation stimulates GPR109A/HCA2 and there are multiple intracellular signaling pathways that mediate this effect. The increase in GPR109A/HCA2 that accompanies macrophage activation inhibits the triglyceride accumulation stimulated by macrophage activation.

Supplementary keywords: cytokines, G proteins, DGAT, LPS, triglycerides
**Abbreviations:** GPR109A, G-protein coupled receptor 109A; HCA2, hydroxycarboxylic acid 2 receptor; TLR, Toll-like receptor; LPS, lipopolysaccharide; MPN, mepenzolate bromide; LTA, lipoteichoic acid; poly I:C, polyinosine-polycytidylic acid; PTN, parthenolide; TNFα, tumor necrosis factor alpha; IL-1β, interleukin-1 beta; IL-6, interleukin 6, AcLDL, acetylated low density lipoprotein; MyD88, myeloid differentiation factor 88; NF-kB, nuclear factor kB, AP-1, activator protein-1; TRIF, TIR-domain-containing adaptor protein inducing IFNβ; IRF3, interferon regulatory factor 3; GPAT3, glycerol 3-phosphate acyltransferase 3; DGAT2, diacylglycerol transferase 2.
Introduction

In the 1950s Altschul and colleagues demonstrated that high doses of niacin reduced plasma cholesterol levels and niacin became the first drug available for treating hypercholesterolemia (1). Numerous studies have since shown that niacin decreases plasma triglyceride, VLDL, LDL, and Lp(a) levels, while increasing HDL (1, 2).

The mechanisms by which niacin alters plasma lipid levels are not fully understood (1-3). One of the most rapid effects of niacin is to decrease plasma free fatty acid levels and it has been hypothesized that the decrease in plasma free fatty acids reduces the delivery of fatty acids to the liver resulting in a reduction in hepatic triglyceride synthesis leading to a decrease in VLDL production and secretion (1, 2). This action could contribute to the niacin induced decrease in plasma triglycerides, VLDL, LDL, and Lp(a) (1, 2). The decrease in plasma free fatty acid levels induced by niacin is due to inhibition of lipolysis in adipose tissue mediated by a decrease in cyclic AMP levels (1, 2, 4-6). Cyclic AMP is well known to enhance lipolysis by activating protein kinase A, which phosphorylates hormone sensitive lipase and perilipin, leading to increased triglyceride breakdown (7, 8). Specific binding sites for nicotinic acid are present on adipose tissue and data suggest that niacin binds to a Gi-coupled cell surface receptor (9, 10).

A major side effect of niacin therapy is cutaneous flushing, which is a troublesome side effect that frequently leads to the cessation of niacin treatment (11). The flushing has been shown to be due to increased prostaglandin D production by skin macrophages (Langerhan cells) and prostaglandin E₂ production by keratinocytes leading to cutaneous vasodilation (11-14). Specific binding sites for nicotinic acid are also present in macrophage membranes and niacin has been shown to activate phospholipase A₂ leading to the increased production of arachidonic acid and the increased synthesis of prostaglandins (11, 12, 14). These observations and other data suggest that increased prostaglandin production induced by niacin may be mediated by G coupled cell surface receptors.
In 2003, three groups identified a G-protein coupled receptor (GPR109A, HCA2, HM74A in humans, PUMA-G in mice) that binds nicotinic acid and is expressed predominantly in adipose tissue and immune cells, including macrophages (6, 15, 16). This receptor couples to G proteins of the Gi family and binds not only niacin but other related compounds that have similar effects (for example acipimox) (17, 18). In mice deficient in GPR109A/HCA2, niacin does not decrease plasma free fatty acid or triglyceride levels, suggesting that the niacin induced inhibition of adipose tissue lipolysis is mediated by GPR109A/HCA2 (6, 17). Similarly, studies have also demonstrated that the increase in prostaglandin production and the cutaneous flushing induced by niacin is also dependent on GPR109A/HCA2 in macrophages and keratinocytes (12-14, 17). Thus, many of both the beneficial and the adverse effects of niacin require GPR109A/HCA2.

The factors that regulate the expression of GPR109A/HCA2 have not been rigorously studied. Schaub et al have reported that activation of macrophages with interferon gamma increases GPR109A/HCA2 expression (19) while Wanders et al and Zandi-Nejad et al have recently shown that LPS increases GPR109A/HCA2 expression in RAW 264.7 macrophages (20, 21). Finally, both LPS and TNF alpha have been shown to up-regulate GPR109A/HCA2 in adipocytes (20, 22). In the present study we have defined the effects of other cytokines and Toll-like receptor (TLR) activating stimuli on GPR109A/HCA2 expression in fat cells and macrophages, determined the pathways leading to increased expression, and shown that inhibiting the increase in GPR109A/HCA2 expression or activity leads to increased lipid storage suggesting that the increase in GPR109A/HCA2 acts as a feedback inhibitor.
Materials and Methods

Materials

Lipopolysaccharide (LPS) from Escherichia coli strain O55:B5 was purchased from Difco (Detroit, MI) and diluted in sterile normal saline to the desired concentration. Dulbecco’s Modified Eagle’s Medium (DMEM) and Intralipid were obtained from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Human serum albumin (HSA) was obtained from Bayer (Elkhart, IN). Tri Reagent, concanavalin A (Con A), thalidomide, and mepenzolate bromide (MPN) were from Sigma (St. Louis, MO). Zymosan, lipoteichoic acid (LTA), polynosine-polycytidylic acid (poly I:C), and BX795 were from InvivoGen (San Diego, CA). Parthenolide (PTN) was obtained from EMD Chemicals (Philadelphia, PA). Mouse tumor necrosis factor alpha (TNFα), interleukin -1 beta (IL-1β), and IL-6 were purchased from R&D Systems (Minneapolis, MN). Acetylated low density lipoprotein (AcLDL) was from Intracel (Frederick, MD).

Animal Experiments

Female C57BL/6 mice (8–12 wk of age, ~20 g) were obtained from Charles River Laboratories (Wilmington MA). The animals were maintained in a normal-light-cycle room and were fed Purina mouse chow (Ralston Purina, St. Louis, MO) and water ad libitum. Animals were injected with either saline or LPS (5 mg/kg body weight ip) and food was removed from both control and treated animals following injection. At the indicated time points, mice were rapidly euthanized with an overdose of isoflurane and the spleen and adipose tissue from the peri-uterine/-urinary bladder area were removed and snap frozen in liquid nitrogen, placed in storage tubes in dry-ice bath until the end of experiment, and then stored at –80°C until RNA extraction. All studies involving animals were conducted in conformity with the Public Health Service Policy on humane care and use of laboratory animals. All experimental protocols were approved by the Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center.
**Cell culture**

Murine 3T3-L1 cells (ATCC, Manassas, VA) were grown to confluence and differentiated to adipocytes as described (23). Briefly, preadipocytes were cultured in DMEM and 10% FBS. When cells became confluent, cells were differentiated by treatment with 1.0 µg/ml insulin, 0.5 mM methylisobutylxanthine, and 1 µM dexamethasone in DMEM containing 10% FBS for 2 days. Cells were then maintained in DMEM supplemented with 10% FBS. Experiments were performed 10-12 days post-differentiation. Cells were treated for 24 h with LPS (100 ng/ml), TNFα (10 ng/ml), or IL-1β (10 ng/ml). The doses of LPS and cytokines used in these experiments are similar to those previously shown to induce metabolic alterations in 3T3-L1 adipocytes and other cells (23).

RAW 264.7 cells, a murine macrophage cell line, were from American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% FBS and incubated at 37°C in 5% CO₂. When confluent, cells were washed with serum free medium once and then treated in medium supplemented with 2.5% HSA for indicated times (4–24 h) prior to RNA isolation. For studies with immune stimulators, cells were treated with LPS at 100 ng/ml, zymosan at 500 µg/ml, LTA at 1 µg/ml, or poly I:C at 50 µg/ml for 16 hours. For lipid loading, cells were co-incubated with LPS at 100 ng/ml and AcLDL at 100 µg/ml or Intralipid at 150 µg/ml for 16 h. For treatment with cytokines, cells were treated with TNFα, IL-1β, or IL-6 at 10 ng/ml for 16 h. For inhibitor studies, cells were pre-incubated with thalidomide at 500 µg/ml, PTN at 20 µM, BX795 at 10 µM, or MPN at 100 µM for 1 hour before addition of LPS (100 ng/ml) for 16 h.

**Mouse peritoneal macrophage culture**

Peritoneal macrophages were harvested from C57BL/6 mice three days after the ip injection of 40 µg of Con A in 0.5 ml of phosphate-buffered saline and then cultured as described previously by Tang et al (24). Cells were plated in 12-well plates in DMEM containing 10% FBS and 20% L-cell culture medium.
and allowed to adhere to wells for 1 hour. Cells were washed with serum free medium and then treated in DMEM supplemented with 2.5% HSA with LPS (100 ng/ml) for 16 hours.

**RNA isolation and quantitative PCR**

Total RNA was isolated from tissues and cells using Tri Reagent. First strand cDNA was synthesized from 1µg of total RNA with the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The real-time PCR contained 20 ng of reversed transcribed total RNA, 450 nM forward and reverse primers, and 10 µl of 2 × LightCycler 480 SYBR Green I Master in a final volume of 20 µl in 96-well plates using MyiQ™ Real-time PCR System (Bio-Rad, Hercules, CA). Quantification was performed by the comparative Ct method with 36B4 used for normalization. The following primers were used: GPR109A/HCA2, forward: 5’-TCCAAGTCTCCAAGGTGTT-3’, reverse: 5’-TGTTCCTCCAGCCACGTTGTT-3’; TNF, forward: 5’-CTACTCCAGGTCTCTTCAA-3’, reverse: 5’-GCAGAGAGAGGTGGACTTTT-3’; GPAT3, forward: 5’-GGAGGATGAAGTGACCCAGA-3’, reverse: 5’-CCAGTTTTTGGAGGCTGCTGT-3’; DGAT2, forward: 5’-AGTGGCAATGCTATCATCACGT-5’, reverse: 5’-AAGGAATAAGTGGGAACCAGATCA-3’, 36B4, forward: 5’- GCGACCTGGAAGTCCAACAG-3’, reverse: 5’-ATCTGCTGCATCTGCTTGG-3’.

**Immunocytochemistry**

RAW cells were seeded on chamber glass slides and grown overnight prior to treatment with 100 ng/ml LPS for 16 hours. After fixation with 2% formaldehyde, cells were quenched with 100 mM glycine and then permeabilized in 0.5% saponin in PBS. Cells were then blocked for 1 hour with 10% goat serum and 0.1% saponin in PBS. Slides were then incubated overnight at 4°C with rabbit anti-GPR109A/HCA2 at 1:100 (Novus Biologicals, Littleton, CO), followed by incubation for 1 hour with Alexa 488 goat anti-rabbit IgG at 1:1000 (Invitrogen). All staining was observed with a Zeiss LSM510 Meta confocal microscope.
siRNA Transfection

RAW cells were seeded at 1 X 10^6 cells per well into 6-well plates and grown overnight. The cells were transfected with GPR109A/HCA2 siRNA (ON-TARGETplus SMARTpool purchased from Dharmaco) or nontargeted control using TransIT-TKO Transfection Reagent (Mirus Bio LLC) following the manufacturer’s recommended protocol. After 24 hours of transfection, cells were treated with LPS and harvested for RNA isolation or triglyceride assay.

Triglyceride Accumulation Measurement

Following 24 hour treatment, cells were washed twice with PBS and scraped into 200 µl PBS. The cell suspensions were sonicated and triglyceride levels were assayed using a commercially available enzymatic assay (Sigma Aldrich, St. Louis, MO). The TG accumulation was normalized to protein concentration for each sample.

Statistics

Data are presented as mean±SEM. The Student’s t-test was used for comparisons between groups. A p-value <0.05 was considered significant. When multiple samples were compared, one way ANOVA was used to determine statistical significance.
Results and Discussion

Because the expression of GPR109A/HCA2 is very abundant in adipose tissue we first examined the effect of LPS administration, a model of gram negative infection, on GPR109A/HCA2 expression in mouse adipose tissue. As shown in Figure 1A, LPS administration increased GPR109A/HCA2 mRNA levels approximately 2.7 fold. Similarly, treatment of 3T3-L1 adipocytes with LPS or cytokines (TNFα and IL-1β) also resulted in an approximate 4-5 fold increase in GPR109A/HCA2 mRNA levels (Figure 1B). Thus, inflammatory stimuli produce an increase in GPR109A/HCA2 expression in adipocytes. These results confirm and extend the in vitro studies that previously demonstrated that LPS and TNF increase GPR109A/HCA2 expression in 3T3-L1 adipocytes (20, 21).

Macrophages are another site in which GPR109A/HCA2 is abundantly expressed. Therefore we next determined the effect of LPS administration on GPR109A/HCA2 expression in the spleen, a tissue rich in macrophages. As shown in Figure 2A, LPS administration increased GPR109A/HCA2 mRNA levels ~5.6 fold in spleen. The effect of LPS treatment on isolated mouse peritoneal macrophages is shown in Figure 2B. LPS treatment increased GPR109A/HCA2 mRNA ~19 fold in mouse peritoneal macrophages in vitro. Moreover, LPS increased GPR109A/HCA2 mRNA levels ~30-80 fold in cultured RAW cells, a murine macrophage cell line (Figure 2C). Additionally, as shown in Figure 3, LPS treatment increased GPR109A/HCA2 protein in macrophages as assessed by immunostaining. The increased GPR109A/HCA2 was seen in the plasma membrane accompanied by staining throughout the cytoplasm in macrophages.

Given the marked increase in expression in RAW cells, we next carried out time course and dose response studies in RAW cells. As shown in Figure 4A the increase in GPR109A/HCA2 mRNA occurs rapidly (~20 fold increase by 4 hours), reaches a peak at 16 hours (~60 fold increase) and is sustained for at least
24 hours. The ability of LPS to stimulate GPR109A/HCA2 expression is a relatively sensitive response with the ½ maximal response occurring at ~5 ng/ml and a ~40 fold increase seen at 10 ng/ml and ~65 fold increase seen at 100 ng/ml (Figure 4B).

We next examined the effect of other immune stimulators on GPR109A/HCA2 expression in RAW cells. As shown in Figure 5A, zymosan, a model of fungal infections, LTA, a model of gram positive bacterial infections, and poly I:C, a model of viral infections all increased GPR109A/HCA2 mRNA levels ~8 fold. These increases are considerably smaller than the increase observed with LPS treatment. Additionally, treatment of RAW cells with the cytokines TNF and IL-1 had a smaller effect on GPR109A/HCA2 expression (Figure 5B). The effect was specific as there was little effect of IL-6.

Macrophages in atherosclerotic lesions contain increased quantities of cholesterol esters. To mimic this pathophysiologic state, we incubated RAW cell macrophages with AcLDL, which leads to a marked increase in cholesterol ester levels. As shown in Figure 6, under these conditions AcLDL had little effect on GPR109A/HCA2 expression while LPS treatment still resulted in a marked increase in GPR109A/HCA2 expression, similar to cells not treated with AcLDL, indicating that the LPS stimulation of GPR109A/HCA2 expression occurs even in cholesterol ester loaded macrophages.

LPS signals via TLR4, which has two major intracellular pathways that regulate gene expression (25). The myeloid differentiation factor 88 (MyD88) pathway increases the activity of nuclear factor kB (NF-kB) and activator protein-1 (AP-1) while the TIR-domain-containing adaptor protein inducing IFNβ (TRIF) pathway increases the activity of NF-kB and interferon regulatory factor 3 (IRF3) (25). We therefore employed a number of specific inhibitors to determine the signaling pathways responsible for the LPS induced increase in GPR109A/HCA2 expression. All of these inhibitors, as expected, decreased the ability of LPS to increase the expression of TNFα (data not shown). As shown in Figure 7A, thalidomide, an inhibitor of MyD88 (26) partially inhibited the ability of LPS to stimulate
GPR109A/HCA2 expression suggesting that the LPS effect on GPR109A/HCA2 expression is not solely dependent on MyD88 pathway. BX795, which inhibits the TRIF pathway (27), also resulted in a greater, but still partial inhibition of the ability of LPS to increase GPR109A/HCA2 (Figure 7B), again suggesting that the LPS induced increase in GPR109A/HCA2 expression is not solely dependent on the TRIF pathway. These results suggest that LPS activation of TLR4 signals an increase in GPR109A/HCA2 expression by both the MyD88 and TRIF pathways. PTN, which blocks NF-kB (28), reduced the ability of LPS to increase GPR109A/HCA2 expression by approximately 50% (Figure 7C), suggesting that both NF-kB pathways and non NF-kB pathways are important for the LPS effect on GPR109A/HCA2. It is well recognized that LPS induces cytokines, such as TNF, by multiple pathways, and are not fully blocked by inhibiting one pathway. Similarly, we find that inhibitors of a single pathway do not block the increase in GPR109A/HCA2, suggesting that multiple intracellular signaling pathways mediate the LPS-induced stimulation of GPR109A/HCA2 expression.

Previous studies by our and other laboratories have shown that the activation of macrophages with LPS and other TLR activators increases macrophage triglyceride accumulation (29, 30). We therefore next examined the effect of preventing the increase in GPR109A/HCA2 activity on the ability of LPS to increase triglyceride accumulation. As shown in Figure 8A, using siRNA that targets GPR109A/HCA2, we were able to blunt the LPS induced increase in GPR109A/HCA2. Moreover, as shown in Figure 8B this resulted in a small but statistically significant increase in the ability of LPS activation to increase triglyceride accumulation in the macrophages. A similar increase in triglyceride accumulation with LPS stimulation was observed when an identical experiment was carried out in the presence of intralipid, which enhances macrophage triglyceride accumulation (Figure 8C). Finally, as shown in Figure 8D inhibiting GPR109A/HCA2 activity with MPN also resulted in an increase in triglyceride accumulation with LPS treatment.
Moreover, previous studies have shown that LPS stimulates the incorporation of fatty acids into triglycerides, which is associated with the increased expression of glycerol 3-phosphate acyltransferase 3 (GPAT3) and diacylglycerol acyltransferase 2 (DGAT2), two key enzymes in the synthesis of triglycerides (29). As seen in prior studies this increase in LPS induced triglyceride accumulation is associated with an increase in GPAT3 and DGAT2 expression (Figure 9). Moreover, knocking down GPR109A/HCA2 expression with siRNA or inhibiting GPR109A/HCA2 activity with MPN resulted in LPS treatment increasing the expression of both GPAT3 and DGAT2 to a greater extent than seen with LPS treatment alone (Figure 9). These results indicate that the increase in GPR109A/HCA2 expression that occurs during macrophage activation negatively regulates the increase in expression of GPAT3 and DGAT2 limiting the enhanced accumulation of triglyceride that occurs during macrophage activation.

The precise mechanism by which activation of GPR109A/HCA2 regulates the expression of either GPAT3 or DGAT2 is unknown. Studies by other investigators have also shown that niacin acting via GPR109A/HCA2 can directly affect macrophage lipid metabolism. Specifically, niacin has been shown to suppress the increase in LDL uptake that is induced by LPS treatment in wild type macrophages but not in macrophages deficient in GPR109A/HCA2 (21). Additionally, niacin acting via GPR109A/HCA2 has been shown to stimulate cholesterol efflux by increasing the expression of ABCA1, ABCG1, and CD36 (31-33). These results coupled with our results suggest that activation of GPR109A/HCA2 by niacin, endogenous ligand or enhanced basal activity decreases the ability of macrophages to accumulate lipid.

It should be noted that despite the beneficial effects of the activation of GPR109A/HCA2 by niacin on macrophage lipid accumulation, two recent randomized studies have failed to demonstrate that niacin therapy when added to statins reduces cardiovascular disease events (34-36). This absence of beneficial effect of niacin in combination with statin therapy contrasts with an earlier study demonstrating that niacin monotherapy reduces cardiovascular disease (37). The explanation for the failure of niacin in combination with a statin to reduce cardiovascular disease is unknown. Of note, statin treatment did not attenuate the increase in GPR109A/HCA2 in macrophages induced by LPS treatment (data not shown).
In conclusion the present study demonstrates that multiple infectious and inflammatory stimuli stimulate GPR109A/HCA2 expression in adipose tissue and in macrophages. The increase in GPR109A/HCA2 that accompanies macrophage activation inhibits the triglyceride accumulation stimulated by macrophage activation as demonstrated by blocking the effects of GPR109A/HCA2.

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References


Figure Legends

Figure 1. Effect of LPS or cytokines on GPR109A/HCA2 mRNA levels in adipose. (A) Mice were injected intraperitoneally with LPS (5 mg/kg) and the animals were euthanized at 16 hours after LPS administration. Total RNA was isolated from adipose tissue. (B) 3T3-L1 adipocytes were treated for 24 h with LPS (100 ng/ml), TNFα (10 ng/ml) or IL-1β (10 ng/ml) and total RNA was isolated. GPR109A/HCA2 mRNA levels were quantified by QPCR performed as described in Materials and Methods section. The data are presented as the mean +/- SEM. Data are expressed as fold increase over controls. N = 3 - 4 per group. ** p< 0.01, *** p< 0.001.

Figure 2. Effect of LPS on GPR109A/HCA2 mRNA levels in immune tissue and cells. (A) Mice were injected intraperitoneally with LPS (5 mg/kg) and the animals were euthanized at 16 hours after LPS administration. Total RNA was isolated from spleen. (B) Peritoneal macrophages were harvested from C57BL/6 mice, treated for 16 hours with LPS (100 ng/ml) and total RNA was isolated. (C) RAW cells were treated for 16 hours with LPS (100 ng/ml) and total RNA was isolated. GPR109A/HCA2 mRNA levels were quantified by QPCR performed as described in Materials and Methods section. The data are presented as the mean +/- SEM. Data are expressed as fold increase over controls. N = 3 - 4 per group. *** p< 0.001.

Figure 3. Immunofluorescence analysis for GPR109A/HCA2 in RAW cells. Cells were treated with LPS at 100 ng/ml in serum-free medium for 16 hours. Immunostaining was performed as described in the methods section. Fluorescent GPR109A/HCA2 staining was visualized by confocal microscopy with a 40x oil immersion objective lens. All images were acquired with identical settings.

Figure 4. LPS induction of GPR109A/HCA2 mRNA levels in RAW cells. (A) RAW cells were treated with LPS (100 ng/ml) for indicated times. (B) RAW cells were treated for 16 hours with indicated concentrations of LPS. Total RNA was isolated and GPR109A/HCA2 mRNA levels were quantified by
QPCR performed as described in Materials and Methods section. The data are presented as the mean +/- SEM. Data are expressed as fold increase over controls. N = 3 per group. ** p< 0.01, *** p< 0.001.

Figure 5. Effect of immune stimulators on GPR109A/HCA2 mRNA levels in RAW cells. (A) RAW cells were treated with zymosan (500 µg/ml), LTA (1 µg/ml), or poly I:C (50 µg/ml) for 16 hours. (B) RAW cells were treated with TNFα, IL-1β, or IL-6 at10 ng/ml for 16 hours. Total RNA was isolated and GPR109A/HCA2 mRNA levels were quantified by QPCR performed as described in Materials and Methods section. The data are presented as the mean +/- SEM. Data are expressed as fold increase over controls. N = 3 per group. ** p< 0.01, *** p< 0.001.

Figure 6. Effect of LPS on GPR109A/HCA2 mRNA in AcLDL loaded RAW cells. RAW cells were co-incubated with AcLDL (100 µg/ml) and LPS (100 ng/ml) for 16 hours. Total RNA was isolated and GPR109A/HCA2 mRNA levels were quantified by QPCR performed as described in Materials and Methods section. The data are presented as the mean +/- SEM. Data are expressed as fold increase over controls. N = 3 per group. *** p< 0.001.

Figure 7. Effect of inhibitors on GPR109A/HCA2 mRNA levels in RAW cells. Cells were pre-incubated for 1 hour with (A) thalidomide (500 µg/ml), (B) BX795 (10 µM), or (C) PTN (20 µM) and LPS (100 ng/ml) for 16 hours. Total RNA was isolated and GPR109A/HCA2 mRNA levels were quantified by QPCR performed as described in Materials and Methods section. The data are presented as the mean +/- SEM. Data are expressed as fold increase over controls. N = 3 per group. * p< 0.05, ** p< 0.01, *** p< 0.001.

Figure 8. Effect of inhibition of GPR109A/HCA2 on triglyceride accumulation in RAW cells. Cells were transiently transfected with murine GPR109A/HCA2 siRNA or negative control siRNA, followed by treatment with LPS. (A) Cells were treated with LPS for 16 hours and total RNA was isolated and GPR109A/HCA2 mRNA levels were quantified by QPCR performed as described in Materials and Methods section.
Methods section. Data are expressed as fold increase over controls. (B) Cells were treated with LPS for 24 hours and TG levels were measured as described in Materials and Methods section. Data are presented as µg TG per mg protein. (C) Cells were co-incubated with Intralipid and LPS for 24 hours and TG levels were measured. Data are presented as µg TG per mg protein. (D) Cells were co-incubated with MPN and LPS for 24 hours and TG levels were measured. Data are presented as µg TG per mg protein. The data are presented as the mean +/- SEM. N = 3 per group. ** p< 0.01, *** p< 0.001.

Figure 9. Effect of inhibition of GPR109A/HCA2 on gene expression in RAW cells. Cells were transiently transfected with murine GPR109A/HCA2 siRNA or negative control siRNA, followed by treatment with LPS. (A) Cells were treated with LPS for 16 hours and total RNA was isolated and GPAT3 mRNA levels were quantified by QPCR performed as described in Materials and Methods section. (B) Cells were treated with LPS for 16 hours and total RNA was isolated and DGAT2 mRNA levels were quantified by QPCR. Cells were co-incubated with MPN and LPS for 16 hours and total RNA was isolated and (C) GPAT3 mRNA levels or (D) DGAT2 mRNA levels were quantified by QPCR performed as described in Materials and Methods section. Data are expressed as fold increase over controls. The data are presented as the mean +/- SEM. Data are expressed as fold increase over controls. N = 3 per group. ** p< 0.01, *** p< 0.001.
Figure 1A

GPR109A mRNA levels (fold increase)

Control

LPS

**
Figure 1B

GPR109A mRNA levels (fold increase)

Control  LPS  TNF  IL-1

0  1  2  3  4  5  6

*** *** ***
Figure 2A

GPR109A mRNA levels (fold increase)

Control
LPS

***
GPR109A mRNA levels (fold increase)

Control

LPS

Figure 2B
Figure 2C

GPR109A mRNA levels (fold increase)

Control  LPS

***
Figure 3

Control

LPS
Figure 5A

GPR109A mRNA levels (fold increase)

- Control
- Zymosan
- LTA
- Poly:IC

***
Figure 5B

GPR109A mRNA levels (fold increase)

- TNF
- IL-1
- IL-6

** (statistically significant difference)
Figure 7A

GPR109A mRNA levels (fold increase)

- Control
- LPS
- Thal
- Thal+LPS

* ***
Figure 7C

GPR109A mRNA levels (fold increase)

Control

LPS

PTN

PTN+LPS

0 10 20 30 40 50 60

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Figure 8A

![Graph showing the fold increase in GPR109A mRNA levels for different treatments: Control, LPS, siRNA Con, and siRNA LPS. The LPS group shows a significant increase compared to the other groups, indicated by asterisks.](image-url)
Figure 8B

![Bar chart showing TG/protein (mg/mg) for Control, LPS, siRNA Con, and siRNA LPS groups. The chart indicates significant differences between groups.](chart.png)
Figure 8C

![Graph showing TG/Protein (µg/mg) for Control, LPS, siRNA Con, and siRNA LPS groups with statistical significance indicated by ** and *** symbols.](image-url)
Figure 8D

![Bar graph showing TG/Protein (μg/mg) for Control, LPS, MPN, and MPN + LPS conditions.](image-url)
Figure 9A

[Graph showing the comparison of Gsα mRNA levels (fold increase) across different conditions: Control, LPS, siRNA Con, and siRNA LPS. The graph indicates significant differences between the conditions, marked with asterisks.]
Figure 9B

![Bar graph showing DGAT2 mRNA levels (fold increase) for different conditions: Control, LPS, siRNA Con, and siRNA LPS. The graph includes error bars and statistical significance markers.](image-url)
Figure 9C

[Bar chart showing differences in GPAT3 mRNA levels (fold increase) between Control, LPS, MPN, and MPN + LPS conditions. The chart indicates a significant increase in MPN + LPS compared to the other conditions.]
Figure 9D

![Graph showing DGAT2 mRNA levels (fold increase) for Control, LPS, MPN, and MPN + LPS. The graph indicates a significant increase in MPN + LPS compared to Control, LPS, and MPN, marked with ***.]