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Increased Saturated Fatty Acids in Obesity Alter Resolution of Inflammation in Part by Stimulating Prostaglandin Production

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Extensive evidence indicates that nutrient excess associated with obesity and type 2 diabetes activates innate immune responses that lead to chronic, sterile low-grade inflammation, and obese and diabetic humans also have deficits in wound healing and increased susceptibility to infections. Nevertheless, the mechanisms that sustain unresolved inflammation during obesity remain unclear. In this study, we report that saturated free fatty acids that are elevated in obesity alter resolution of acute sterile inflammation by promoting neutrophil survival and decreasing macrophage phagocytosis. Using a targeted mass spectrometry–based lipodomics approach, we found that in db/db mice, PGE2/D2 levels were elevated in inflammatory exudates during the development of acute peritonitis. Moreover, in isolated macrophages, palmitic acid stimulated cyclooxygenase-2 induction and prostanoid production. Defects in macrophage phagocytosis induced by palmitic acid were mimicked by PGE2 and PGD2 and were reversed by cyclooxygenase inhibition or prostanoid receptor antagonism. Macrophages isolated from obese-diabetic mice expressed prostanoid receptors, EP2 and DP1, and contained significantly higher levels of downstream effector, cAMP, compared with wild-type mice. Therapeutic administration of EP2/DP1 dual receptor antagonist, AH6809, decreased neutrophil accumulation in the peritoneum of db/db mice, as well as the accumulation of apoptotic cells in the thymus. Taken together, these studies provide new insights into the mechanisms underlying altered innate immune responses in obesity and suggest that targeting specific prostanoid receptors may represent a novel strategy for resolving inflammation and restoring phagocyte defects in obese and diabetic individuals. The Journal of Immunology, 2013, 191: 1383–1392.

Obesity has emerged as one of the most important public health concerns of our time. It is a leading risk factor for the development of type 2 diabetes (T2D), and nearly 90% of adults with T2D are obese (1). Obesity is also a risk factor for cardiovascular disease and liver disease, due largely to metabolic disturbances, such as insulin resistance and dyslipidemia (2, 3). Obesity is associated with chronic elevation of free fatty acids (FFAs), which promote insulin resistance and contribute to the development of systemic hyperglycemia (4, 5). High plasma levels of FFAs are also independently associated with hypertension and sudden cardiac death (6, 7). Accumulating evidence indicates that metabolic dysregulation in obesity is intimately linked with the development of chronic inflammation, which in turn contributes to alterations in metabolism in a vicious positive feed-forward cycle (8). Thus, understanding how nutrient excess impacts immune responses is required to identify novel strategies for treatment and management of obesity and its secondary complications.

Effector cells of the innate immune system, including polymorphonuclear neutrophils (PMNs) and macrophages, play key roles in mediating immune responses. These leukocytes also sense nutrient imbalances. Saturated fatty acids, which are elevated in obesity and diabetes (5), initiate inflammatory responses in part via signaling through pattern recognition receptors, that is, TLR2, TLR4, and nucleotide-binding oligomerization domain–like receptors (9–12). Other endogenous mediators of metabolism, such as insulin, leptin, and adiponectin, also regulate leukocyte effector functions, suggesting that the immune system is exquisitely sensitive to the nutrient tone (13, 14). Several studies have shown that low-grade inflammation associated with obesity promotes the development of chronic diseases such as atherosclerosis, cancer, and nonalcoholic fatty liver disease and impairs host defense and wound healing (15–17). Nevertheless, the mechanisms by which metabolic imbalance affects inflammatory processes remain unclear. The initiation and the resolution of inflammatory responses involve different cellular responses that are temporally controlled, in part, by specific lipid mediators (18–21). Upon trafficking to sites of tissue infection and injury, PMNs encounter and attempt to eradicate invading pathogens. Subsequently, they undergo rapid apoptosis and must be effectively cleared by macrophages to prevent inadvertent tissue injury (21, 22). As such, defects in macrophage phagocytosis can give rise to altered clearance of apoptotic cells (23, 24). Our recent studies have shown that in db/db mice, which are a model of human obesity and T2D, defective macrophage-dependent clearance of...
apoptotic cells delays the resolution of acute peritonitis (15). Moreover, we found that restoring defective macrophage phagocytosis with proresolving lipid mediators, such as resolvin D1, enhances diabetic wound healing, suggesting that defects in phagocytosis are causally related to clinically relevant complications of diabetes and obesity (15). Nonetheless, the mechanisms underlying altered resolution of inflammation and phagocyte function in conditions of nutrient excess remain incompletely understood. In this study, we show that resolution of acute peritonitis in obesity is delayed in part because elevated FFAs prolong PMN survival and inhibit macrophage phagocytosis. Our results suggest that defects in macrophage phagocytosis induced by palmitic acid (PA) are due to autocrine actions of PGs E₂ and D₂ and downstream modulation of the cAMP pathway. Overall, this study provides novel insights into the mechanisms by which nutrient excess impacts acute inflammation and its resolution. These findings suggest that targeting prostanooid receptors or downstream signaling mediators may be an effective new means of controlling excessive inflammatory responses and promoting the resolution of inflammation in obese and/or diabetic individuals.

Materials and Methods

Mice and reagents

Zymosan A and acetylsalicylic acid (ASA) were purchased from Sigma-Aldrich (St. Louis, MO). Male leptin receptor–deficient mice (B6.129S4-Leprdb/J; db/db) and their appropriate wild-type (WT) controls (C57BL/6) were purchased from The Jackson Laboratory (Bar Harbor, ME) at 8 wk of age and maintained on normal chow. PGE₂ and PGD₂ were purchased from Cayman Chemical (Ann Arbor, MI). Specific CAMP analogs for protein kinase A (PKA) and Epac-1 activation, 6-Bnz-cAMP and 8-pCPT-2′-O-Me-cAMP, respectively, were obtained from BioLog Life Science Institute (Bremen, Germany).

Measurement of plasma nonesterified fatty acids

Mice maintained on a 60% high-fat diet for 12 wk or 12-wk-old db/db mice and appropriate WT controls were anesthetized according to a University of Louisville Institutional Animal Care and Use Committee approved protocol. Blood was collected by cardiac puncture using EDTA as the anticoagulant. Whole blood was then centrifuged at 4°C for 20 min. Plasma was separated from RBCs and stored at –80°C until use. Nonesterified FFAs were measured using a colorimetric assay (Wako Diagnostics) and a spectrophotometer according to the manufacturer’s instructions.

Acute peritonitis and flow cytometry

Acute peritonitis was initiated in WT and db/db mice by i.p. administration of zymosan A (0.04 mg/g body weight) in 1 ml sterile saline (15). After 6, 24, or 48 h, mice were sacrificed and the peritoneum was lavaged with Dulbecco’s PBS (DPBS) +/− (5 ml; without calcium and magnesium). Leukocytes in the total exudate were enumerated by light microscopy. Specific leukocyte populations and nonapoptotic cells were identified by flow cytometry. For this, peritoneal exudates were suspended in FACS buffer (1% FBS in PBS) and incubated with Fc Block (BD Biosciences, San Jose, CA) for 10 min at 4°C. Cells were then stained with fluorescein isothiocyanate–conjugated anti-F4/80 and PE-conjugated anti-Ly6G; Biolegend, San Diego, CA) or appropriate isotype controls for 30 min at 4°C. For quantification of apoptotic neutrophils, Ly6G+ cells that were negative for annexin V were assessed using FITC–conjugated annexin V (BioLegend). Flow cytometry was performed using a BD LSR II cytometer equipped with FACS Diva version 6.0 (BD Biosciences). FlowJo version 7.6 software (Tree Star, Ashland, OR) was used for gating analysis and quantification. In selected experiments, db/db mice undergoing peritonitis were treated with PGE₂/D₂ dual receptor antagonist, AH6809 (Cayman Chemical; 10 ng/g body weight; i.p.) in 1 ml sterile saline 32 h after zymosan challenge. Peritoneal exudates were then obtained after 16 h and leukocyte populations were assessed by flow cytometry as described above.

Assessment of macrophage phagocytosis

To measure macrophage phagocytosis, resident peritoneal macrophages were isolated from WT, obese-diabetic (db/db), and WT mice fed a low-fat (10%) or high-fat (60%) diet for 12 wk. Macrophages were seeded in 96-well plates at a density of 4 × 10⁶ cells per well in 100 μl DPBS+ and allowed to adhere for 1 h. Macrophages were then washed to remove any nonadherent cells and were incubated with IgG-opsonized FITC–labeled zymosan A (Invitrogen) for 60 min at 37°C in fresh DMEM containing 10% FBS. Trypan blue was used to quench extracellular fluorescence for 1 min, followed by a DPBS+ (pH 7.45) wash. Phagocytosis was quantified using a spectrophotophotometer (PerkinElmer). To determine how saturated prostanooid generation in deficits in macrophage phagocytosis induced by PA, cells were pretreated with ASA (200 μM) or prostanoid receptor antagonist, AH6809 (100 μM), initially and every hour thereafter and phagocytosis of opsonized zymosan was assessed as was. In some experiments, RAW macrophages were incubated with plasma (10% in DMEM, 6 h) collected from 12-wk-old WT and db/db mice and phagocytosis was assessed. In all studies, uptake of FITC–zymosan was confirmed by fluorescence microscopy prior to quantification using an EVOS fluorescence microscope (Advanced Microscopy Group, Bothell, WA).

Assessment of apoptotic thymocyte clearance

WT and db/db mice were treated i.p. with dexamethasone (15 mg/kg body weight in sterile saline). Mice were administered EP/DP prostanooid receptor antagonist AH6809 (10 ng/g body weight) or vehicle (0.1% ethanol in sterile saline) by i.v. injection initially and 4 h later. The mice were euthanized 6 h after dexamethasone administration and the thymus was collected and formalin fixed. Sections of the tissue were stained using the TUNEL+ area was quantified using MetaMorph software (7–11 random fields per animal).

Western blotting

Cell lysates were normalized for protein concentration and boiled in Laemmli buffer, followed by standard SDS-PAGE separation. Gels were then blotted onto Protran BA 85 nitrocellulose membranes (Millipore, St. Louis, MO) and probed for caspase-3 (Cell Signaling Technology, Danvers, MA), perik1/2, or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Alternatively, gels were blotted onto polyvinylidene difluoride membranes and probed for cyclooxygenase (COX)-2 (Cayman Chemical), DP1, EP2, EP4 (Cayman Chemical), or GAPDH (Cell Signaling Technology). Blots were then developed using ECL Plus reagent followed by luminescence detection using a Typhoon 9400 variable mode imager (GE Healthcare, Chalfont St. Giles, U.K.). Quantification of band intensities was performed using ImageQuant TL software (GE Healthcare).

Real-time PCR

For real-time PCR, mRNA was isolated from cells using the RNeasy tissue kit (Qiagen, Germantown, MD), followed by cDNA synthesis. Real-time PCR amplification was performed with SYBR Green qPCR Master Mix (SA Biosciences, Frederick, MD) using a 7900HT Fast real-time PCR system (Applied Biosystems, Foster City, CA) and commercially available primers for PG endoperoxide synthase (Ptgs1), Ptgs2, micromosomal PGE synthase (mPges1), hematopoietic PGD synthase (Pgds), arachidonate 5-lipooxygenase (Alox5), and arachidonate 15-lipooxygenase (Alox15) (SA Biosciences). Relative expression was determined by the 2−ΔΔCt method after internal normalization to hprt.

Measurement of cAMP

Resident peritoneal macrophages were isolated from WT or db/db mice and were plated onto 24-well plates at a density of 7.5 × 10⁵ cells per well for 1 h in DPBS++. Adherent cells were then lysed with 0.1 M HCl for 15 min at room temperature. Levels of cAMP were measured using an ELISA (Cayman Chemical) as per the manufacturer’s instructions.

Mass spectrometry–based targeted lipidomics analysis

Exudates or macrophage supernatants were collected and added to two volumes of cold methanol containing deuterium-labeled PGE₂ (1 ng) as...
Lipid mediators were profiled by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an HPLC system (Shimadzu Prominance) equipped with an Eclipse Plus reverse-phase C18 column (4.6 mm × 50 mm × 1.8 μm; Agilent Technologies) coupled to a triple quadrupole mass spectrometer (API 2000; Applied Biosystems/SCIEX). The instrument was operated in negative ionization mode, and the mobile phase consisted of methanol/water/acetic acid (60:40:0.1 [v/v/v]), which was ramped to 80:20:0.01 during 7.5 min and to 95:5:0.01 in the next 4.5 min at a flow rate of 400 μL/min. The mobile phase was ramped up during the next 6 min to 100:0:0.01 before returning to 60:40:0.1. Lipid mediators of interest were identified by multiple reaction monitoring using established transitions for PGE2/D2 (351 > 271), PGF2α (353 > 193), leukotriene B4 (LTB4; 335 > 195), 15–15-hydroxyeicosatetraenoic acid (HETYE; 319 > 179), 15-HETE (319 > 175), 5-HETE (319 > 115), and thromboxane B2 (369 > 169) (25). Given that PGE2 and PGD2 are not readily separated using these HPLC conditions and that they have nearly identical mass spectral fragmentation patterns, we present data pertaining to PGE2 and PGD2 as PGE2/D2. For quantification, external calibration curves were constructed for each compound using authentic standards (Cayman Chemical) and recoveries were calculated using internal deuterium-labeled PGE2.

Human neutrophil isolation and survival analysis

Blood was obtained from healthy, medication-free human donors on a voluntary basis using standard venipuncture. The study was approved by the University of Louisville Institutional Review Board, and all donors received material regarding the research study and signed consent forms prior to blood donation. Following blood collection, PMNs were isolated using plasma-Percoll gradients (26). After isolation, PMNs were washed and resuspended in Leibovitz’s L15 media (stereile) Krebs-Ringer buffer (with calcium and magnesium) at 5 × 10^6 cells/mL. Neutrophils (0.9 ml/condition) were aliquoted into sterile 1.5 ml Eppendorf tubes and prewarmed at 37°C in a 5% CO2 water bath for 5 min. Neutrophils were left untreated (positive control for constitutive apoptosis) or treated with LPS (a positive control for neutrophil survival), PA-BSA (100 μM), or BSA-control for 10 min followed by addition of 100 μL FBS. Neutrophils were incubated overnight (24 h) at 37°C in a 5% CO2 incubator. The following day, PMNs were spun down in a microfuge and total cell lysates were prepared by resuspending cell pellets in a lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 1% Nonidet P-40, 1% Triton X-100, 20 mM sodium fluoride, and 2 mM sodium orthovanadate. PMSF (4 mM) and protease and phosphatase inhibitor cocktails (Sigma-Aldrich) were added (1:100) to lysis buffer prior to use. Lysed cells were left on ice for 15 min, followed by centrifugation at 13,000 × g for 10 min at 4°C. Supernatants were saved as total lysate and stored at −70°C until analysis. For survival analysis, 50 μg protein in each condition was loaded onto 4–12% gradient gels and proteins were separated by SDS-PAGE, transferred, and probed with cleaved caspase-3 Ab (a marker of cellular apoptosis), pERK (marker of cell survival), and β-actin (as a loading control).

Statistical analysis

Results are presented as mean ± SEM. Statistical significance was assessed using a two-tailed Student’s t test or one-way ANOVA followed by either Bonferroni or Dunnett posttests, as appropriate. In all cases, p < 0.05 was considered significant.

Results

To evaluate how nutrient excess associated with obesity and T2D alters innate immune responses, we used an acute model of sterile peritonitis. Consistent with our recent studies (15), zymosan challenge provoked a large influx of PMNs into the peritoneum 6 h after the injection (Fig. 1A). Maximal infiltration was observed 24 h postinjection (Fig. 1B). As reported before (15), the extent and the time course of PMN infiltration was similar in both WT and db/db mice at 6 and 24 h. However, whereas levels of PMNs declined during the resolution phase (i.e., 48 h) in WT mice, PMNs accumulated to significantly higher levels in db/db mice (Fig. 1C), suggesting that resolution of inflammation was delayed in db/db mice, rather than the initiation of inflammation. No differences in macrophage infiltration were observed throughout the time course of peritonitis.

We previously demonstrated that altered resolution of acute inflammation in db/db mice was associated with the accumulation of apoptotic PMNs, as well as FITC-zymosan, and that these events were associated with defective macrophage-mediated clearance (15). In this study, we found similar changes in high fat (HF)–fed WT mice. Similar to macrophages from db/db mice, resident peritoneal macrophages from WT mice fed a HF diet for 12 wk showed significant defects in their ability to phagocytose IgG-osponized zymosan compared with mice fed a low-fat (LF) diet (Fig. 1D). To determine whether this defect was due to changes in plasmatic factors, macrophages isolated from WT mice were incubated with plasma obtained from WT and db/db mice and macrophage phagocytosis was measured. As shown in Fig. 1E, macrophages incubated with plasma from db/db mice showed diminished phagocytosis relative to macrophages incubated with WT plasma. The extent of this defect was similar to that in macrophages isolated from HF-fed mice (Fig. 1E). These observations indicate that the defective macrophage phagocytosis in diabetes and obesity may be related to the presence of plasmatic factors that suppress phagocytosis.

Because obesity is associated with an increase in circulating FFAs, we measured FFA levels in WT, db/db, and HF-fed mice. As expected, in comparison with WT mice fed a normal chow diet, plasma FFA levels were significantly elevated in both db/db and HF-fed mice (Fig. 1F). To determine whether FFAs can directly suppress macrophage phagocytosis, we incubated WT macrophages with PA, which is one of the major FFAs in the plasma in obese mice (27). For these and following experiments we used PA within the dose range found in the plasma of obese mice (Fig. 1F). As shown in Fig. 1G, we found that PA suppressed macrophage phagocytosis to the extent similar to that observed with plasma isolated from db/db mice, as well as the level of suppression observed in macrophages isolated from HF-fed mice (vide supra). Taken together, these observations, in agreement with previous reports (27), suggest that saturated fatty acids suppress macrophage phagocytosis.

In addition to defective macrophage phagocytosis, altered resolution of inflammation could also be due to prolongation of PMN survival (22, 28, 29). Therefore, we quantified the number of nonapoptotic PMNs (Ly6G+annexin V−) remaining in the peritoneum of WT and db/db mice 48 h after zymosan challenge. As expected, levels of nonapoptotic PMNs were significantly elevated in the peritoneum of db/db mice compared with WT mice (Fig. 1H), indicating that PMN survival is prolonged in db/db mice. To test this directly, we assessed spontaneous apoptosis of human PMNs in vitro. As shown in Fig. 1I, human PMNs readily underwent apoptosis when incubated at 37°C for 24 h, as assessed by caspase-3 cleavage. The extent of apoptosis was markedly diminished when the PMNs were incubated with PA-BSA, but not BSA alone, as assessed by reduced caspase-3 cleavage and enhanced signaling through the prosurvival ERK1/2 pathway. As a positive control, we examined the effects of LPS, which markedly delays apoptosis. We found that incubation with LPS led to a significant decrease in caspase-3 cleavage and increased pERK1/2 levels (Fig. 1I) (30). The extent of increase in PMN survival by LPS was similar to that observed with PA-BSA. Collectively, these results demonstrate that elevated levels of saturated FFAs could potentially alter acute inflammation and its resolution by increasing PMN survival and decreasing macrophage phagocytosis.

Among the mediators of acute inflammation and its resolution, lipid mediators play key roles in regulating leukocyte trafficking and cytokine production, as well as leukocyte effector functions, such as phagocytosis (18–20, 31). To understand how nutrient excess in obesity alters the process of inflammation and its reso-
When resolution was observed, we profiled the production of lipid mediators during the development of acute peritonitis in WT and db/db mice using a targeted LC-MS/MS–based approach. This analysis revealed that 6 h after zymosan challenge, the arachidonic acid–derived proinflammatory lipid mediators LTB4, 12-HETE, and PGE2/D2 were significantly elevated in the peritoneal exudates of db/db mice compared with WT mice, whereas the levels of 15-HETE were significantly decreased (Fig. 2A). By 24 h, the production of LTB4 and 12-HETE largely abated, whereas PGE2/D2 levels remained significantly elevated (Fig. 2B). Interestingly, this pattern persisted for 48 h (Fig. 2C), at a time when more leukocyte accumulation was observed in db/db mice than in WT mice (see Fig. 1). The increase in PGE2/D2 appears to be selective, because other prostans, such as PGF2α and thromboxane (measured as thromboxane B2), were not significantly elevated throughout the time course of peritonitis. Overall, this unbiased approach revealed that, although the levels of several lipid mediators were affected initially, the production of COX-2–derived prostanoids, PGE2/D2, was increased throughout the entire time course and this increase coincided with PMN accumulation. This suggests that the increase in these prostanoids may be related to delayed resolution of inflammation in obese mice.

PGs such as PGE2 and PGD2 have well-defined roles in acute inflammation, such as the regulation of blood flow, cytokine generation, and leukocyte trafficking (20, 32). Additionally, both PGE2 and PGD2 can counterregulate leukocyte phagocytosis (33, 34).

To determine whether the production of these prostanoids is causally related to phagocyte defects induced by nutrient excess, we examined how the production of these mediators was affected by PA in macrophages. Inhibition of phagocytosis by PA was concentration dependent and we found that PA at concentrations $300 \text{ mM (0.3 mEq/l)}$ induced significant defects in the uptake of IgG-opsonized zymosan by RAW macrophages (Fig. 3A, 3B). These observations demonstrate that at concentrations measured in the plasma of obese mice (see Fig. 1F), PA prevents macrophage phagocytosis. To examine how FFAs affect lipid mediator production, we assessed mRNA expression of genes encoding the biosynthetic enzymes of lipid mediators profiled in inflammatory exudates, including Ptgs1, Ptgs2, mPges1, hPgds, Alox5, and Alox15. This analysis revealed that Ptgs2, encoding murine COX-2, and downstream prostanoid synthase, hPgds, were selectively and significantly upregulated in macrophages treated with PA-BSA, but not BSA alone (Fig. 3C). Consistent with this observation, levels of COX-2 protein were also significantly increased by PA-BSA (Fig. 3D).
incubated macrophages with vehicle alone, PGE2, or PGD2 and Therefore, to test this in our experimental conditions, we pre-
sonoid production. Previous studies in alveolar macrophages show 
cating that induction of COX-2 is associated with increased prota-
mediators is sufficient to blunt their effects despite their continued 
Because the results of these studies suggested that elevated pro-
mediators in supernatants of cells stimulated with BSA alone 
Assessed the phagocytosis of IgG-opsonized zymosan. Consistent 
whether increased prostanoid generation in obesity alters macro-
Aspirin is an irreversible COX-2 inhibitor and is considered an 
and DP1/2, respectively, and that activation of these receptors mediates their biological effects (20, 32). In alveolar macrophages, 
inhibition of phagocytosis by PGE2 has been shown to be mediated 
arily by EP2, whereas less is known about the role of the PGD2 
and PGD2 receptors; EP2 and DP1, in macrophage phagocytosis (34). Both EP2 and DP1 
couple to Gαs and activate the cAMP pathway (32). Accumulation 
of cAMP in turn activates downstream kinases, including PKA and 
and the exchange protein activated by cAMP-1 (Epac-1). To test the 
involved in this pathway, we incubated macrophages with cAMP 
alogs that specifically activate either the PKA-dependent 
pathway (6-bnz-cAMP) or the Epac-1 pathway (8-pCPT-2′-O-Me-
cAMP). We found that the selective PKA activator 6-bnz-cAMP 
significantly inhibited phagocytosis in macrophages, whereas the 
Epac-1 selective activator did not (Fig. 4A). To determine whether 
the cAMP pathway is affected by obesity, we isolated resident 
peritoneal macrophages from WT and db/db mice and found that 
cAMP levels were significantly elevated in db/db macrophages 
compared with WT cells (Fig. 4B). Moreover, both EP2 and DP1 
receptors were expressed in macrophages isolated from both WT 
and db/db mice (Fig. 4C), whereas EP4 was not readily detected 
(data not shown). Quantification of band intensities revealed that 
whereas DP1 levels are maintained in WT and db/db macrophages, 
the EP2 levels were significantly decreased (Fig. 4C, lower panel). 
Because our LC-MS/MS analysis revealed that PGE2/D2 are 
significantly elevated in inflammatory exudates isolated from obese-
diabetic mice, we next sought to determine whether blocking PGE2 
and PGD2 receptors would alter the accumulation of leukocytes in 
the inflamed peritoneum. To this end, peritonitis was initiated in 
obese-diabetic mice using zymosan, and leukocytes were allowed to 
accumulate (see scheme in Fig. 4D and results in Fig. 1B). After 
32 h, the EP2/DP1 dual receptor antagonist AH6809 was adminis-
tered by i.p. delivery and leukocyte differentials in inflammatory 
exudates were determined 48 h after the zymosan challenge, the 
time at which PMN accumulation was maximal in db/db mice (see 
Fig. 1C). We found that mice treated with AH6809 had significantly 
less PMNs remaining in the peritoneum as compared with mice 
given the vehicle alone, although the number of macrophages was 
unaffected (Fig. 4E). We also performed LC-MS/MS analysis on 
cell-free exudates to determine whether levels of prostanoids were 
affected by the treatment. As shown in Fig. 4F, levels of PGE2/D2 
were not significantly different in vehicle- and AH6809-treated 
mice, suggesting that blocking downstream receptors for these 
mediators is sufficient to blunt their effects despite their continued 
production.

Because the results of these studies suggested that elevated pro-
duction of prostanoids alters macrophage phagocytosis in obesity 
and that this in turn could alter the clearance of PMNs, we tested 
whether increased prostanoid generation in obesity alters macro-

3D). To determine whether this increase in COX-2 expression 
results in an increase in prostanoid production, we measured these 
lipid mediators in supernatants of cells stimulated with BSA alone 
or PA–BSA. The results of this analysis showed that incubation with 
PA led to a significant increase in PGE2/D2 levels (Fig. 3E), indic-
ating that induction of COX-2 is associated with increased prota-
sonoid production. Previous studies in alveolar macrophages show 
that prostanoids, including PGE2, decrease phagocytosis (34, 35). 
Therefore, to test this in our experimental conditions, we pre-
incubated macrophages with vehicle alone, PGE2, or PGD2 and 
assessed the phagocytosis of IgG-opsonized zymosan. Consistent 
with previous reports, we found that both PGE2 and PGD2 (100 nM) 
significantly decreased phagocytosis at concentrations similar to 
that generated by the cells in the presence of PA (85 nM; Fig. 3F). 
To examine whether PGE2/D2 production is causally related to PA-
induced deficits in macrophage phagocytosis, we tested whether 
hinduction of COX1/2 or antagonism of prostanoid receptors specific 
for PGE2 and PGD2 (EP2 and DP1, respectively) would abolish this 
defect. Indeed, pretreatment with ASA (aspirin) or prostanoid re-
ceptor antagonist AH6809 significantly reversed PA-induced defi-
cits in macrophage phagocytosis (Fig. 3G, 3H), suggesting that 
autocrine actions of prostanoids play a causal role in this process.

Given our results showing that PA stimulates macrophage pro-
stanoid production and that this is related to defects in phagocytosis, 
we examined the downstream signaling pathways that could be 
involved in this process. It has been shown before that both PGE2 
and PGD2 specifically activate the G protein–coupled receptors EP1–4 
and 

FIGURE 2. Altered resolution of inflammation in obesity is associated 
with increased prostanoid generation. (A–C) Peritoneal exudates were 
collected from WT or db/db mice during the time course of acute sterile 
peritonitis. Targeted lipidomics analysis was carried out on cell-free ex-
udates using LC-MS/MS following solid phase extraction (n = 3–4/group/ 
time point). Data are means ± SEM and normalized to exudate volume. 
Recovery was calculated based on internal standard, deuterium-labeled 
PGE2. *p < 0.05 by Student t test. TXB2, Thromboxane B2.
suggest that excessive prostanoid production may be an important underlying reason for the delayed resolution of inflammation under conditions of nutrient excess such as obesity and diabetes.

Discussion

The major findings of this study are that elevated levels of free fatty acids that accompany metabolic diseases such as obesity and diabetes alter resolution of acute inflammation by promoting PMN survival and blocking macrophage phagocytosis. We found that in macrophages, the saturated fatty acid PA induced COX-2 expression and promoted the generation of PGE2/D2, which in turn play a causal role in defective phagocytosis induced by PA. Our results identify cAMP as a critical downstream effector of prostanoids during acute inflammation in obese mice coinciding with the accumulation of PMNs in the inflamed peritoneum, which was decreased by the PGE2/D2 receptor antagonist AH6809. Overall, these studies suggest that nutrient excess alters innate immune cell survival and effector functions, in part by stimulating prostanoid generation, and that targeting specific prostanoid receptors or their downstream mediators may be a novel strategy for controlling inflammation in obese or diabetic individuals.

It is well documented that obesity and diabetes are associated with an increase in circulating levels of FFAs, due in part to increased lipolysis in adipocytes. This increase in plasma FFA levels contributes to insulin resistance in several tissues, including the skeletal muscle, which is the major site of glucose consumption (4, 5). Whereas shifts in glucose metabolism can arise from increased fatty acid flux (i.e., the Randle cycle), it is also becoming apparent that FFAs can initiate inflammatory signaling and that inflammation links obesity with the development of insulin resistance (8). Indeed, insulin resistance induced by direct FFA infusion is largely prevented in mice deficient in Tlr4, a pattern recognition receptor involved in sensing Gram-negative bacteria (9). Moreover, FFAs activate inflammatory signaling, including the NF-kB pathway, and increase the production of inflammatory cytokines in the vasculature and in leukocytes, and these effects are regulated in part by TLR receptor (i.e., TLR2 and TLR4) activation (10, 11). In addition to activating TLRs, FFAs can also induce inflammation by activating the inflammasome, as mice lacking components of the inflammasome, such as Nlrp3 and Pycard, have been shown to be resistant to the inflammatory effects of FFAs (12). Furthermore, other studies have demonstrated that FFAs induce defects in phagocyte function independent of TLR signaling (27). Thus, even though several mechanisms by which FFAs can induce inflammatory signaling in phagocytes have been identified, the downstream mediators of their effects on phagocyte function are not entirely known. The results of our study show that inflammatory signaling induced by FFAs leads to the generation of lipid mediators that regulate phagocytosis and that these mediators might be involved in defective resolution of acute inflammatory events in the context of nutrient excess.

Macrophage phagocytosis is required for the immunologically muted process of apoptotic cell uptake and it is a critical event in the active resolution of inflammation, as failed clearance of apo-
Apoptotic cells can give rise to necrosis and resultant tissue damage (21, 22). Recent results from our group and others show that macrophage-dependent clearance of apoptotic cells is perturbed in obesity and diabetes and that this delays the resolution of an inflammatory insult (15, 36–39). Defects in apoptotic cell clearance are an important feature of delayed wound healing in obesity and diabetes, and such defects are also observed in advanced atherosclerosis (15, 27, 39, 40). Thus, elucidation of the mechanisms by which nutrient excess alters this critical function of macrophages is important for understanding the development of several clinically relevant disease features in obese and diabetic humans.

In addition to phagocytosis of apoptotic cells, macrophages also serve an important role in the clearance of microbial pathogens, a process that is also defective in metabolic disease (41). Our results are concordant with these findings and demonstrate further that nutrient excess induced defects in FcR-mediated macrophage phagocytosis are due in part to enhanced production of PGs that have well-described roles in regulating macrophage phagocytosis. We found that PA suppresses macrophage phagocytosis in part by inducing \( \text{Ptgs2} \) (COX-2) and the downstream production of prostanoids. These findings are consistent with other studies demonstrating that FFAs induce COX-2 in skeletal muscle and that macrophages isolated from obese/diabetic mice, as well as those treated with FFA mixtures, have elevated COX-2 levels (10, 37, 42). Additionally, we found that phagocytosis was compromised in macrophages isolated from mice fed a HF diet, as well as normal macrophages incubated with plasma collected from \( \text{db/db} \) mice. These observations are consistent with a direct, model-independent effect of FFAs on macrophage function. Given that both \( \text{db/db} \) mice and HF-fed mice have elevated FFA levels, these results underscore the importance of nutrient excess in driving acute defects in leukocyte function. Although we cannot rule out effects of other circulating mediators, such as glucose, leptin, and insulin, it is unlikely that these mediators play a major role in the defects in macrophages.

**FIGURE 4.** Role of prostanoid receptors in macrophage phagocytosis, acute inflammation, and apoptotic cell clearance in obese-diabetic mice. (A) Phagocytosis of IgG-opsonized zymosan by RAW macrophages incubated with vehicle, selective exchange protein activated by cAMP 1 (Epac-1) cAMP analog (8-pCPT-2’-O-Me-cAMP), or selective PKA cAMP analog (6-BNZ-cAMP) \((n = 7–9)\). (B) Intracellular cAMP levels measured in resident peritoneal macrophages isolated from WT and \( \text{db/db} \) mice \((n = 3–4)\). (C) Representative Western blots of PGD \(_2\) receptor, DP1, and PGE \(_2\) receptor, EP2, in peritoneal macrophages isolated from WT and \( \text{db/db} \) mice. Quantification of DP1 and EP2 levels in isolated WT and \( \text{db/db} \) peritoneal macrophages normalized to GAPDH is shown in the lower panel. (D) Scheme illustrating treatment protocol for in vivo administration of AH6809 during the time course of acute sterile peritonitis in \( \text{db/db} \) mice. (E) Flow cytometry analysis of PMNs (Ly6G\(^+\)F4/80\(^-\)) and macrophages (Mf; Ly6G\(^-\)F4/80\(^+\)) from peritoneal exudates in vehicle and AH6809-treated \( \text{db/db} \) mice 48 h following zymosan challenge \((n = 4–9/group)\). (F) Quantification of PGE\(_2\)/DP in peritoneal exudates of \( \text{db/db} \) mice undergoing peritonitis and treated without or with AH6809 by LC-MS/MS \((n = 3–5)\). (G) Representative images of TUNEL\(^+\) cells (counterstained with methyl green) in the thymus of WT or \( \text{db/db} \) mice treated without or with AH6809. Scale bars, 200 mm (top panels) and 50 mm (bottom panels) \((n = 5/group)\). Quantification of TUNEL\(^+\) cells as a percentage of the total imaged area is shown in the right panel. Data are means ± SEM. *p < 0.05 by one-way ANOVA (A, G) or Student t test (B, C, E).
observed because in our study glucose was not significantly elevated in mice fed a HF diet (data not shown). Moreover, the plasma used in phagocytosis assays was collected from db/db mice at a time when their fasting glucose values were within the normal range. Additionally, previous studies have shown that defects in macrophage phagocytosis induced by FFAs persist in macrophages isolated from insulin-receptor–deficient mice and were not rescued by leptin treatment (27), indicating that the defect in macrophage phagocytosis may be leptin-independent.

As noted, one of the main findings of our study is that PA suppresses phagocytosis in macrophages by inducing COX-2 and increasing the downstream production of PGs. These PGs play multiple roles in the acute inflammatory response, including the regulation of blood flow, pain sensitization, thrombosis, cytokine production, and phagocytosis (20, 32, 33). At sites of inflammation, inducible COX-2 is commonly upregulated and generates high local levels of specific prostanoids dependent on the expression of downstream synthases, such as mPGES-1 and hPGDS (20). We found that in addition to COX-2, PA stimulated an increase in hPgds mRNA, but not mPges1. Additional studies using improved HPLC conditions to separate PGE2 and PGD2 demonstrated that whereas RAW macrophages produce more PGD2 overall, both PGE2 and PGD2 were significantly increased with PA (data not shown). Moreover, both PGE2 and PGD2 induced defects in phagocytosis, and the receptor antagonist used in these studies blocks both EP2 and DP1 (43). Thus, further studies using genetic manipulation of these prostanoid receptors will be required to fully elucidate the role of each mediator in the context of obesity. Importantly, note that in addition to induction, COX-2 activity may be further stimulated by the increase in FFAs, as it has been shown that saturated fatty acids such as PA, which are not COX-2 substrates, can directly bind one monomer of COX-2 and increase substrate turnover in the other monomer (44, 45). Hence, it is possible that in addition to inducing COX-2 expression, saturated FFAs could also potentiate prostanoid generation by directly activating COX-2.

Prostanoids, such as PGE2 and PGD2, elicit their biological actions by binding to and activating specific G protein–coupled receptors. In leukocytes, PGE2 and PGD2 activate G protein–coupled receptors, such as EP2 and DP1, that couple to G proteins (Gαs) and lead to the activation of adenylate cyclase, resulting in an elevation of cAMP and its downstream effectors, including PKA and Epac-1 (32, 34, 46). Our results show that cAMP analogs specific for PKA activation induce a phagocytosis defect similar to that observed with FFAs and PGE2/D2. Other studies have demonstrated that prostanoids block phagocytosis in a CAMP-dependent manner, although PKA and Epac-1 play differential roles depending on the mode of phagocytosis and the macrophage subset. For instance, Serezani et al. (46) demonstrated that phagocytosis of Candida albicans was blocked by a PKA-dependent pathway, whereas other studies using alveolar macrophages demonstrate that blockade of FcR-mediated macrophage phagocytosis was dependent on Epac-1 (47). There also seems to be some differential involvement of PKA and Epac-1 effectors in cytokine generation and macrophage maturation, highlighting the complexity of cAMP-dependent signaling in macrophages (48). Thus, it is tempting to speculate that cAMP elevation in obesity and diabetes could play a key role in delayed wound healing and inappropriate inflammatory responses, despite leukocyte accumulation in tissues. Further studies are required to understand fully how aberrant cAMP signaling alters innate immunity in the context of obesity and other related chronic inflammatory diseases.

In addition to inducing defects in macrophage phagocytosis, we found that FFAs directly prolong PMN survival. Because delayed apoptosis, or prolonged survival, of PMNs during acute inflammation also delays resolution, our results suggest that the nutrient excess alters resolution of acute inflammation by deranging both PMN apoptosis and their clearance by macrophages. This is important because prolonged accumulation of activated leukocytes in tissues can cause further tissue damage by releasing pro-oxidant species and proteolytic enzymes (21). Indeed, prolonged and heighten ed accumulation of leukocytes is a critical feature of delayed wound healing in obesity and diabetes (49). Activation of MAPK signaling in leukocytes, such as the ERK1/2 pathway, leads to prolonged PMN survival, and our results demonstrate that this pathway is robustly activated by FFAs (30). The extent of activation of this pathway by FFAs was similar to that elicited by LPS and was also associated with decreased activation of caspase-3. Of interest, PGE2 is generated by PMNs and it promotes survival signaling in PMNs, as do other agonists that elevate cAMP (28, 50, 51). We did not observe any increase in PGE2 by FFAs in PMNs at the end of the incubation protocol (data not shown), although we cannot rule out that there was a transient increase in PGE2 during the 24-h incubation. Moreover, because PGE2 was elevated in vivo during the time at which PMNs accumulate, it is likely that PGE2 plays a dual role in promoting PMN survival, as well as blocking macrophage phagocytosis in vivo. That blockade of EP2/DP1 receptors decreased accumulation of PMNs in the inflamed peritoneum when the receptor antagonist was delivered after maximal PMN infiltration adds further support of this dual role for PGE2. Finally, because previous studies have shown that db/db mice have elevated peripheral blood PMNs, it is plausible that sustained infiltration of PMNs gives rise to PMN accumulation in the peritoneum (52). However, as pointed out above, there was no difference in maximal PMN infiltration between WT and db/db mice, although the decrease in PMN levels was much slower in db/db mice, indicating that diet-induced obesity selectively affects PMN clearance.

In this study, we used an unbiased LC-MS/MS approach to profile lipid mediators generated during the initiation and resolution of acute inflammation in vivo because of the well-documented temporal relationships between different classes of lipid mediators in regulating leukocyte trafficking (18, 53). This approach revealed that specific prostanoids, PGE2/D2, are elevated upon initiation of inflammation in obesity and that they persist at high levels throughout the time when inflammation resolves in WT mice. This suggests that normal temporal orchestration of inflammation is perturbed in obesity and that these changes are associated with altered cellular dynamics. Interestingly, previous studies have shown that prostanoids play important roles in both the initiation and resolution of inflammation. Indeed, Gilroy et al. (31) reported that there are two distinct waves of COX-2–dependent production of prostanoids during inflammation and resolution, and additional studies have shown that inhibiting COX-2 delays resolution (54). Moreover, results from Levy et al. (53) show that lipid mediator class switching during inflammation/resolution occurs as PGE2 drives biosynthesis of proresolving lipid mediators in PMNs by stimulating the upregulation of 15-lipoxygenase. Thus, the temporal relationships between lipid mediator classes are important determinants of whether inflammation will resolve or persist. Our results suggest that these temporal relationships are disrupted in metabolic disease and that nutrient excess alters the innate immune response from the very initiation of inflammation. This is demonstrated by our results showing that macrophages isolated from obese mice display defects in phagocytosis and have elevated levels of intracellular cAMP even in the absence of an inflammatory challenge. This observation is consistent with other reports showing that proresolving lipid mediators, such as the lipoxins and resolvins, counterregulate the cAMP pathway in macrophages and reduce the induction of COX-2, indicating that counterregulatory lipid mediators may offer an at-
tractive treatment strategy in the context of obesity and T2D (15, 18, 55, 56). This hypothesis is supported by our recent results showing that resolvin D1 rescues defective phagocytosis in diabetic macrophages and improves wound healing (15).

The findings of our study have significant implications in understanding the dysregulation of innate immunity in diabetes and obesity and its contribution to secondary complications such as delayed wound healing, increased susceptibility to infections, and exacerbation of chronic diseases such as atherosclerosis and cancer. Studies from our laboratory and others have shown that diet-induced obesity is associated with impaired wound healing that could be attributed in part to delayed resolution of inflammation described in this study, and therefore delayed PMN clearance and deficits in macrophage phagocytosis could contribute to deficits in wound healing. Similarly, chronic unresolved inflammation could also contribute to the progression of atherosclerotic lesions, which are comprised of inflammatory cell infiltrates in the vessel wall, or to carcinogenesis, during which inflammation could prime pre-malignant cells for cancerous transformation. Additionally, deficits in resolution of inflammation in diabetes/obesity could also account for increased susceptibility to infection. The effects of obesity and diabetes on susceptibility to infections are more complex. Several studies have shown that obese and diabetic individuals suffer from increased postsurgical nosocomial infections, odontogenic, respiratory, and skin infections, as well as poor wound healing. This susceptibility to infection has been recapitulated in multiple models of obese-diabetic mice (17, 57). However, other investigators have reported that obesity is negatively correlated with mortality in the context of bacterial pneumonia (58, 59). Clearly, additional studies are required to understand the fundamental mechanisms by which metabolic imbalance affects the inflammatory response and contributes to increased susceptibility to infections.

In summary, the results of this study suggest that elevated levels of FFAs in obese-diabetic mice alter inflammation and its resolution in part by prolonging PMN survival and blocking macrophage phagocytosis. Our results indicate that this is in part due to increases in the COX-2–dependent production of PGs and the subsequent activation of the cAMP pathway in leukocytes. These findings add to a growing body of literature demonstrating that nutrient excess alters innate immune responses and identifies new targets that may be promising candidates for therapeutic strategies aimed at treating secondary complications of obesity and related diseases.

Disclosures

The authors have no financial conflicts of interest.

References