Erythropoietin signaling: A novel regulator of white adipose tissue inflammation during diet-induced obesity

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Abstract

Obesity-induced white adipose tissue (WAT) inflammation and insulin resistance are associated with macrophage (Mϕ) infiltration and phenotypic shift from “anti-inflammatory” M2-like to predominantly “pro-inflammatory” M1-like cells. Erythropoietin (EPO), a glycoprotein hormone indispensable for erythropoiesis, has biological activities that extend to non-erythroid tissues including anti-apoptotic and anti-inflammatory effects. Using comprehensive in vivo and in vitro analyses in mice, EPO treatment inhibited WAT inflammation, normalized insulin sensitivity and reduced glucose intolerance. We investigated EPO receptor (EPO-R) expression in WAT, and characterized the role of its signaling during obesity-induced inflammation.Remarkably, and prior to any detectable changes in body weight or composition, EPO treatment reduced M1-like Mϕ and increased M2-like Mϕ in WAT, while decreasing inflammatory monocytes. These anti-inflammatory effects were found to be driven, at least in part, by direct EPO-R response in Mϕ via Stat3 activation, where EPO effects on M2 but not M1 Mϕ required IL-4R/Stat6. Using obese ∆EpoR mice with EPO-R restricted to erythroid cells, we demonstrated an anti-inflammatory role for endogenous EPO. Collectively, our findings identify EPO-R signaling as a novel regulator of WAT inflammation, extending its non-erythroid activity to encompass effects on both Mϕ infiltration and subset composition in WAT.
**Introduction**

Macrophage (M\(\phi\)) infiltration to white adipose tissue (WAT) during obesity marks a state of chronic inflammation, an important cause of obesity-induced insulin resistance and glucose intolerance (1-4). This chronic inflammatory response impacts type 2 diabetes pathogenesis (1, 4), and associates with a shift in M\(\phi\) population from alternatively activated F4/80\(^+\)MGL-1\(^+\) (anti-inflammatory) M2 to predominantly classically activated F4/80\(^+\)MGL-1\(^-\) (pro-inflammatory) M1 (5-8), in association with recruitment from circulating “inflammatory” Ly6C\(^{hi}\)CCR2\(^+\) monocytes to M\(\phi\) clusters in WAT via CCL2/CCR2-axis, and not to the conversion of resident M2 M\(\phi\) to M1 (1, 3, 5, 6, 8-10).

M\(\phi\) infiltration and activation state influence inflammation-induced insulin resistance and glucose intolerance during diet-induced obesity (DIO) (11-13). It is suggested that M\(\phi\) may be an initiator in insulin-resistant states, and that with their precursors may contribute to propagation of insulin resistance (11-17). M\(\phi\) infiltration to WAT requires and kinetically follows CD8\(^+\) T lymphocytes recruitment (18). Obesity-induced inflammation and immune cell infiltration elevate cytokines and chemokines such as TNF-\(\alpha\) and CCL2, systemically and locally in WAT, where cells of the stromal vascular fraction (SVF) including M\(\phi\), are known to be the main producers, particularly in visceral fat depots (1, 3, 4, 19-21). Signaling of such inflammatory mediators is adversely implicated in the impairment of systemic glucose metabolism (1, 5).

Erythropoietin (EPO) is a glycoprotein hormone, induced by hypoxia and necessary for erythrocyte production (22-24). EPO is used for treatment of anemia in chronic kidney disease including type 2 diabetes patients (25). Its biological activity extends beyond regulating erythropoiesis, and the non-erythroid expression of its receptor (EPO-R) has been reported (26-
EPO was reported to reduce Mφ infiltration and inhibit inflammation (32). Although, proposed to occur via anti-apoptotic rather than “direct” anti-inflammatory effects on cells of the immune system (32), it can also occur directly via inhibiting Mφ and/or activating immune suppressive lymphocytes (33, 34).

Early treatment of mice with exogenous EPO at the onset of high fat diet (HFD) feeding or EPO transgenic over-expression halts body weight and fat mass gain, and improves glucose tolerance (35-38). Using ΔEpoR mice with EPO-R restricted to erythroid tissue (39), we previously showed EPO-R absence in WAT contributes directly to obesity and glucose intolerance on normal chow (36). EPO protects against diabetes through direct effects on pancreatic β-cells in mouse models of types 1 and 2 diabetes (36,37,40). It remains unknown whether anti-inflammatory EPO effects in WAT contribute to EPO effects on the pre-diabetic state during obesity.

In this study, we hypothesized that EPO/EPO-R signaling can attenuate obesity-induced WAT inflammation. Our findings identify a novel role for EPO in regulating inflammatory monocytes recruitment, and Mφ infiltration and activation during DIO.

**Research Design and Methods**

**Animals and animal care**

Wild type (WT) male C57BL/6 mice were obtained from National Cancer Institute Animal Production Program (Fredrick, MD). Mice with EPO-R expression restricted to hematopoietic tissue (ΔEpoR) were provided by Dr. Masayuki Yamamoto (Tohoku University, Japan). EPO-R expression in ΔEpoR mice arises from transgene expression of EpoR cDNA
driven by the erythroid specific enhancer/promoter of GATA-1 on C57BL/6 EpoR<sup>−/−</sup> background (29). Male Stat-6<sup>−/−</sup> (Stat6tm1Gru) and IL-4<sup>−/−</sup> (ll4tm1Nnt) mice on C57BL/6 background and their age matched WT controls were obtained from the Jackson Laboratory, JAX Mice (Bar Harbor, Maine). Mice were fed control diet (10 kcal% fat) or, to induce DIO, HFD containing 60 kcal% fat (high fat, 5240 kcal/kg, 34.9% crude fat) (Research Diets, Inc, New Brunswick, NJ), starting at 6-8 wk of age for 12 weeks. During the last two weeks of the study period, obese, insulin resistant and glucose intolerant mice were subcutaneously injected with 1000U/kg EPO (EPO dose was chosen based on our previous titration studies; 35) or saline as a vehicle control, every 48hrs while continuing HFD feeding. At the end of week 12, the effects of 2 weeks EPO treatment on several metabolic and inflammatory parameters were assessed as described below. Animals were housed under specific pathogen-free conditions and maintained in a thermo-stated environment under a 12-hour light/dark cycle with free access to food and water. All animal protocols were conducted under the NIH guidelines and approved by the institute’s Animal Care and Use Committee.

**Reagents and antibodies**

Anti-F4/80 and anti-Ly6C antibodies (eBioscience). Anti-MGL1/CD301 and anti-CCR2 (R&D Systems). Fc-block and anti-CD11c antibodies (BD Biosciences). Rat anti-mouse Ki-67 antibody (BioLegend) used in combination with FITC-conjugated goat anti-rat F(ab)2 (anta Cruz Biotechnology Inc). Bromodeoxyuridine (BrdU) (Sigma) and FITC BrdU flow detection kit (BD Pharmingen). Rabbit anti-mouse phospho-Stat5 (Tyr694) (C71E5) and phosphor-Stat3 (Tyr705) (D3A7) monoclonal antibodies (Cell Signaling Technology), mouse anti-mouse phospho-Stat6 (Tyr641) (BD Biosciences). Recombinant human Epoetin alpha or Epogen (EPO)
(Amgen). Vectashield mounting media with DAPI (Vector Labs). Recombinant mouse cytokines GM-CSF, IL-4 and IL-10 (PeproTech).

**Body weight and composition measurements**

Body weight was measured weekly from day 0 to wk 12. Body composition analysis to determine fat and lean body mass was also performed using EchoMRI-100 system (Echo Medical Systems).

**Hematocrit measurement**

Hematocrit was determined for saline and EPO treated animals after centrifugation of blood containing heparin coated capillary tubes, using VIN micro-capillary tube reader (Veterinary Information Network Bookstore).

**Metabolic evaluation**

Glucose tolerance tests were performed upon i.p. injection with 1g/kg dextrose. Insulin tolerance tests were performed after injection with 1U/kg insulin. Total blood and serum glucose measurements were made using AlphaTRAK glucometer (Abbott Animal Health). Serum insulin levels were measured using Sensitive Rat Insulin Radioimmunoassay (RIA) (Millipore). Serum leptin levels were determined by ELISA (Millipore). Hyperinsulinemic-euglycemic clamp studies were performed in restrained mice fasted for 16 hours as described previously (41).

**Stromal vascular fraction cell extraction**
Stromal vascular fraction (SVF) cells were extracted as previously described (18). Briefly, perigonadal WAT were digested by collagenase type 2 (Worthington) at 37°C for 50 min. WAT cell suspensions were spun at 500 g for 5 min to separate floating adipocytes from SVF pellet.

**Gene expression analysis**

Total RNA was extracted from cells or tissues using TRIZol (Invitrogen) and treated with DNase I (Fermentas) according to manufacturer’s protocols. 2 µg of total RNA was then reverse transcribed using MultiScribe Reverse Transcriptase for quantitative real time PCR (qRT-PCR) assays using ABI-7900HT cycler (Applied Biosystems). For EPO-R gene expression analysis, mRNA was quantified using Taqman qRT-PCR, with 16S and β-actin as controls. Relative changes in gene expression levels of inflammatory cytokines, chemokines and receptors, in SVF of epididymal adipose tissue, were analyzed using mouse inflammatory cytokines & receptors RT² profiler PCR (SABiosciences, Qiagen). Briefly, cDNA was synthesized using RT² first strand reaction, and PCR was performed using RT² PCR master mix (SABiosciences, Qiagen). 4-6 replicate 96-well plates were included per group; data analysis was performed using Web based PCR Array Data Analysis Software v3.4 (SABiosciences, Qiagen). Changes in Mφ subtypes signature gene expression levels M1 (iNOS and Il1β) and M2 (Fizz-1, Arg-1, Ppar-γ) were assessed in SVF cells using sybergreen gene expression assays (Fermentas). Relative gene expression levels were determined by normalization to β-actin expression levels using the Delta-Delta Ct method. Primer and probe sequences used are listed in Table-S1.

**WAT and serum cytokine and chemokine protein measurements**
CCL2 levels were analyzed in perigonadal WAT tissue and serum; those for TNF-α, IL-10 and IL-4 were analyzed in serum by ELISA according to the manufacturer’s protocol (PeproTech).

Cell sorting and purification

WAT Mφ were sorted by separation of F4/80^+ (Mφ) and F4/80^- (non-Mφ) fractions of stromal vascular cells using FACSria™ fluorescent cell sorter and FACSDiva software (Becton Dickinson). Sorted WAT Mφ were used for qRT-PCR analysis of EPO-R expression levels or cultured to assess Stat5, 3 and 6 phosphorylation.

EPO treatment of sorted WAT Mφ in vitro

WAT Mφ, FACS sorted from SVF of obese mice (after 12 weeks of HFD feeding), were cultured in saline or EPO (5U/ml) coated 48-well plates at a density of 2x10^5 per well for 15 minutes, then fixed, permealized and tested for Stat3, Stat5a/b and Stat6 phosphorylation by phosphoflow. Stimulation for 15 min with GM-CSF, IL-4 and IL-10 were used as positive controls for Stat5, Stat6 and Stat3 phosphorylation, respectively. In some studies, the sorted WAT Mφ were cultured with EPO (5U/ml) for 24hrs after which their TNF-α, iNOS and IL-10 mRNA levels were quantified using sybergreen qRT-PCR.

Flow cytometry and phosphoflow

SVF of perigonadal WAT were analyzed by flow cytometry as previously described (8). Circulating inflammatory monocytes were quantified using Ly6C and CCR2 expression as previously described (42). Phosphoflow and staining for p-Stat3, p-Stat5a/b and p-Stat6 were
performed as previously described (43). Cell proliferation was assessed using Ki-67 detection and BrdU labeling. *In vivo* labeling with BrdU was achieved by supplementing drinking water with 0.8mg/ml BrdU, starting 48hrs prior to the first EPO injection during weeks 11 and 12 of HFD, for 9 consecutive days followed by a changeover to normal water until the end of week 12. FACSCalibur™ (Becton Dickinson) and CellQuest were used for flow cytometric and phosphoflow analyses.

**Histology and Microscopy**

Frozen sections of perigonadal fat pads were prepared after dissection and fixation in 10% formalin. Hematoxylin and eosin (H&E)-stained sections were imaged for crown-like structures. Immunofluorescence was performed for Mφ detection by incubation en block with directly conjugated primary anti-F4/80 antibodies. All imaging analyses were performed on OLYMPUS IX70 inverted microscope (Optical Elements Corporation). Image capture was performed using SPOTFLEX camera and SPOT Basic imaging software (Diagnostic Instruments Inc), using 15 sections per group (n=5 mice per group).

**Statistical analysis**

Statistical comparisons were performed using Student’s *t*-test, with *P* values <0.05 defined as statistically significant. Results are presented as mean ± standard error of the mean (SEM).


Results

EPO treatment regimen has no effect on body weight or fat mass during DIO

EPO treatment of obese mice reduces body weight and fat mass after 3-4 weeks (36). Considering that body weight and fat mass changes influence WAT inflammation and Mφ infiltration, affecting glucose tolerance and insulin sensitivity (1, 2, 44), we chose short-term EPO treatment regimen (< 3 weeks) after obesity induction. Obese, insulin resistant, glucose intolerant mice, fed HFD for 12 weeks, were EPO treated during the last 2 weeks of the study with no detectable effects on body weight, body weight gain, fat mass, perigonadal fat weight, or serum leptin levels (Figs. 1A-E). Consistent with previous reports (36), prolonged EPO administration (≥ 3 weeks) reduced body weight (Fig. 1F). All subsequent studies were conducted using the 2 week regimen of EPO treatment.

EPO treatment attenuates insulin resistance and glucose intolerance during DIO

We investigated whether EPO treatment can ameliorate existent systemic insulin resistance and glucose intolerance associated with DIO using insulin tolerance test (ITT) and glucose tolerance test (GTT). EPO improved both (Figs. 2A-B), with striking reduction in fasting blood glucose levels (Fig. 2C), and impressive effects on glucose tolerance (Fig. 2B). EPO increased hematocrit (Fig. 2D); however, serum glucose (Fig. 2E) and serum insulin (Fig. 2F) were also reduced showing that total blood glucose reduction is not the result of increased packed red cell volume. To examine insulin sensitivity of individual tissues we performed euglycemic-hyperinsulinemic clamp studies. EPO treated mice required higher glucose infusion rate (GIR) to maintain blood glucose during insulin infusion, and exhibited higher whole body glucose disposal (Rd) and glycogen synthesis rate compared to controls (Figs. 2G-H). Although these differences have not reach statistical significance, EPO significantly increased 2-deoxy glucose
uptake in skeletal muscle but not WAT, Brown adipose tissue (BAT) or heart (Figs. 2I-J). EPO had no effect on basal or clamp endogenous glucose production (EGP) (Fig. 2H), suggesting no changes in liver insulin sensitivity. Taken together these data demonstrate that EPO treatment, which had no effect on body weight, improved whole body insulin sensitivity primarily by enhancing glucose uptake in the skeletal muscles.

**EPO treatment inhibits WAT inflammation and reduces Mφ infiltration**

Unlike WAT with high EPO-R expression in both adipocytes and SVF, EPO-R is not detectable in skeletal muscle (Figs. 3A-B), indicating that EPO indirectly stimulates glucose uptake in skeletal muscle. Although EPO had no effect on WAT insulin sensitivity, we noticed that it dramatically improved inflammation within adipose tissue, as indicated by the lack of crown structure around adipocyte in EPO treated mice (Figs. 3C-D). This novel observation prompted us to study the role of EPO signaling in obesity induced inflammation. We performed comprehensive gene expression profiling of cytokines and chemokines in SVF. EPO reduced Ccl1, Ccl2, Ccl3 and Ccl22 expression levels but not Il10 (Fig. 3E). Protein analysis showed that EPO treatment reduced TNF-α, consistent with the role of TNF-α during obesity-induced insulin resistance (45), and increased IL-10 (Fig. 3F), and down-regulated CCL1, 2, 3 and 22 levels (data not shown).

Mφ are responsible for the majority of EPO-R expression in SVF (Fig. 3G), and EPO treatment reduced total Mφ % and number by ~11.2% and ~30%, respectively (Figs. 3H-I), supporting an inhibitory effect for EPO on WAT inflammation. Mφ are the main responders in SVF, as T-cell and B-cell % showed no detectable changes after EPO treatment (Table-S2). Consistent with results showing Mφ as predominant EPO-R expressing cells in SVF (Fig. 3G),
EPO-treated animals exhibited reduced Mϕ infiltration associated with decreased EPO-R expression in WAT (Fig. 3J), providing a further link between WAT Mϕ and EPO/EPO-R response.

**EPO/EPO-R signaling induces Stat3 phosphorylation in Mϕ and inhibits their inflammatory response in vitro**

To investigate EPO/EPO-R signaling in WAT Mϕ, and its direct effects on their inflammatory responses, Mϕ sorted from obese SVF were cultured in EPO coated plates for 15 min, fixed and analyzed by phosphoflow. Although, p-Stat5 and p-Stat6 were not detected in WT Mϕ upon EPO treatment (Fig. 4A), EPO induced Stat3 phosphorylation (Fig. 4B). These findings were further confirmed by Western blotting (data not shown). EPO-R absence in ΔEpoR Mϕ prevented their response to EPO as shown by the absence of p-Stat3 (Fig. 4B-top right) compared to WT (Fig. 4B-top left). Moreover, treatment of WAT Mϕ with EPO (5U/ml) for 24hrs reduced pro-inflammatory genes expression of TNF-α and iNOS, and markedly increased anti-inflammatory cytokine IL-10 expression (Figs. 4C-D), collectively confirming a direct response by WAT Mϕ to EPO/EPO-R signaling.

**EPO treatment reverses obesity-induced shift in Mϕ population subtypes**

The effect of EPO administration on Mϕ population subtypes in WAT was examined. Using flow cytometry, EPO decreased MGL-1⁻ and CD11c⁺ while increasing MGL-1⁺ Mϕ (Figs. 5A-B). qRT-PCR confirmed phenotypic Mϕ shift (Figs. 5C-D), showing that EPO reduced iNOS and Il-1β, and increased Fizz-1, Ppar-γ and Arg-1. Overall, EPO increased M2-like Mϕ and
reduced M1-like Mφ numbers, thus identifying and confirming its role in the regulation of not only Mφ infiltration, but also local subtype polarization. Whether or not the conversion of M1- to M2-like Mφ, as recently suggested (46), contributes to the observed EPO-mediated increase in MGL-1^+ Mφ remains unknown. Since M1/M2 nomenclature of Mφ is based on in vitro studies, and may not represent in vivo Mφ subtypes (17), we use the designation M1- and M2-like Mφ to describe F4/80^+MGL-1^- and MGL-1^+ cells, respectively.

**Cellular responses driving EPO-mediated reversal of Mφ subtype shift**

M1 Mφ infiltrating the WAT during DIO originate from circulating Ly6C^{hi}CCR2^+ inflammatory monocytes in a CCL2/CCR2 dependent manner (5, 6, 8). EPO lowers Ccl2 expression levels in WAT (Fig. 3C). We predicted EPO-mediated reduction of M1-like Mφ subset (Figs. 5A-B) to be associated with fewer circulating Ly6C^{hi}CCR2^+ monocytes and reduced local WAT and serum CCL2 levels. This was confirmed by flow cytometric analysis of whole blood (Figs. 6A-B), and measurement of local WAT and serum CCL2 protein levels (Fig. 6C).

M2 Mφ have been suggested to self generate via proliferation in the presence of IL-4 in vivo (46, 47). To study how the observed EPO-mediated expansion of M2-like Mφ may occur (Fig. 5), quantitative comparisons of EPO and saline treated obese animals were performed. Ki-67 staining and BrdU uptake showed that EPO promotes MGL-1^+ Mφ proliferation (Fig. 6D) in association with an increase in serum and WAT IL-4 (Fig. 6E). We confirmed this in obese Stat6^{-/-} and IL-4^{-/-} mice, reported previously to have defective M2-like Mφ (48-50). EPO treatment failed to expand M2-like Mφ in these animals (Fig. 6F) unlike controls (Figs. 5B, 6F). These findings highlight a previously unrecognized role for EPO/EPO-R in the regulation of Mφ
polarization in vivo where IL-4/Stat6 axis is indispensable for EPO-mediated M2-like Mφ expansion.

**Endogenous EPO/EPO-R signaling regulates Mφ infiltration and subtype shift**

To investigate the role of endogenous EPO/EPO-R signaling during obesity-induced WAT inflammation, we used DIO in ΔEpoR mice (39). The absence of EPO-R expression in ΔEpoR WAT (adipocytes and SVF) and Mφ were confirmed (Fig. 3B and 7A). ΔEpoR mice maintained on normal chow become obese and glucose intolerant as they age (36). Baseline measurements prior to the onset of HFD feeding showed ΔEpoR mice (6-8 wks old) to have slightly higher body weight and fat mass compared to age matched WT controls (Fig. 7B). However; after 12 wks of HFD feeding there was no significant difference in final body weight or fat mass between ΔEpoR and their age matched WT controls (Fig. 7B).

Mφ express the highest level of EPO-R mRNA among immune cell subsets profiled here (Fig. 7C), and are the only EPO responsive population of immune cells in WT animals (Table-S2). ΔEpoR animals with DIO showed higher circulating inflammatory monocyte numbers (Fig. 7D), suggesting a possible direct role for endogenous EPO/EPO-R signaling in the regulation of WAT inflammation independently from its effect on body weight and fat mass. ΔEpoR SVF cell chemokines and cytokines expression profiles showed elevated levels of Ccl1, Ccl2, Ccl12, Ccl17, Ccl19, Ccl22, Cxcl10 and Cxcl11, with unchanged Ccl3, Tnfa and Il10 (Fig. 7E). Protein profiling confirmed the up-regulation of these chemokines including CCL3 (data not shown). Despite unchanged Tnfa and Il10 mRNA levels, we found higher TNF-α and similar IL-10 protein levels in ΔEpoR animals (Fig. 7F).
CCL2 levels in ΔEpoR exceeded those in WT controls (Fig. 7F), where the difference in local WAT CCL2 but not serum, was significant (p≤0.05). Flow cytometry and histology showed increased total Mϕ, denser inflammatory infiltrates and crown-like structures formation, and more MGL-1^− Mϕ and fewer MGL-1^+ Mϕ in WAT (Figs. 7G-I). The latter was confirmed via qRT-PCR (Fig. 7J). Despite reduced M2-like Mϕ numbers in WAT of ΔEpoR (Fig. 7I), serum and WAT IL-4 levels were not lower than WT controls (Fig. 7K). Based on Ki-67 staining the % of proliferating MGL-1^+ cells in ΔEpoR SVF was lower than their WT counterparts (data not shown).

**Endogenous EPO/EPO-R signaling regulates obesity-induced insulin resistance and glucose intolerance**

To investigate the role of endogenous EPO/EPO-R signaling in regulating glucose metabolism, we compared WT with ΔEpoR mice after 12 weeks of HFD feeding. ΔEpoR mice showed higher insulin resistance, glucose intolerance, fasting blood glucose and serum insulin (Figs. 8A-D). ΔEpoR mice maintained high insulin resistance after 2 wks of EPO administration (Figs. 8E-F), and there was no change in the area under the curve, and no significant difference in glucose intolerance after EPO treatment (Fig. 8G). Not surprisingly, Mϕ infiltration and subtype composition in WAT of ΔEpoR mice remained similar after EPO administration (Fig. 8H).
Discussion

Herein, we characterized the role of EPO/EPO-R during obesity-induced WAT inflammation and insulin resistance. Our observations reveal novel and unexpected role for EPO/EPO-R in regulating M1-like Mφ infiltration, M2-like Mφ expansion in WAT and inflammatory monocytes recruitment (Figs. 3, 5, 6, 7) prior to any body weight or fat mass loss (Fig. 1). We propose that the anti-inflammatory effects of EPO in WAT occur in association with its regulation of systemic glucose metabolism, and that it is not restricted to exogenous high dose EPO treatment, as endogenous EPO is sufficient to regulate WAT inflammation.

We and others have reported the reduction in blood glucose with high dose EPO treatment and transgenic over-expression using mouse models of DIO and insulin resistance (35-38). The improved glucose metabolism is associated with reduced body weight gain and fat mass accumulation, where EPO administration was initiated at the onset of HFD feeding and continued for 3 weeks or longer (35-37). In stark contrast, herein EPO administration was initiated after DIO and its associated insulin resistance and glucose intolerance have already been established, and followed 2 weeks duration regimen. We report improvements in glucose metabolism that precede any detectable reduction in body weight or fat mass (Fig. 1), suggesting the EPO-mediated effects observed here, are independent from its role in body weight and fat mass regulation.

The shift to increased fat oxidation in muscle has been shown to contribute to the observed normalization of glucose sensitivity with EPO (38). These studies are consistence with our finding that EPO increased glucose uptake in skeletal muscle. Moreover, EPO was shown to protect against diabetes through direct effects on pancreatic β-cells in mouse models of types 1 and 2 diabetes not including DIO (36, 37, 40).
Herein, anti-inflammatory effect of EPO is identified and its signaling in Mφ and effect on their activation and infiltration are characterized. Indeed, it is known that obesity-induced WAT inflammation is linked to increased systemic inflammation and insulin resistance (1-4). We demonstrate that EPO/EPO-R signaling in WAT Mφ promotes Stat3 phosphorylation with clear anti-inflammatory effects (Figs. 4B-C), confirming a direct role in the regulation of Mφ response. Interestingly, Stat3 phosphorylation does not appear to be sufficient, in the absence of Stat6, to mediate EPO/EPO-R signaling effect on M2-like Mφ, as EPO treatment failed to expand this Mφ subset in WAT of obese Stat-6<sup>-/-</sup> mice (Fig. 6F), demonstrating a requirement for IL-4/Stat6 axis during EPO-mediated M2-like Mφ expansion. In contrast, EPO treatment markedly reduced total and M1-like WAT Mφ populations in obese Stat-6<sup>-/-</sup> mice and IL-4<sup>-/-</sup> mice (Fig. 6F), indicating that EPO/EPO-R/p-Stat3 axis may be sufficient to inhibit the pro-inflammatory response. Collectively, we conclude that both direct (Figs. 4B-C), and indirect- via IL-4/Stat6 (Fig. 6F) responses of WAT Mφ to EPO/EPO-R are involved in mediating the observed anti-inflammatory effects (Figs. 3, 5-7).

Considering our observations that Mφ are EPO responsive, we propose that a direct contribution of their EPO/EPO-R signaling plays a major role in the inhibition of obesity-induced WAT inflammation. However, involvement of indirect EPO/EPO-R signaling in adipocytes on obesity-induced WAT inflammation cannot be entirely excluded and its further investigation is required. Since EPO/EPO-R signaling appears to regulate TNF-α levels (Figs. 3 and 7), the change in TNF-α may be linked to EPO effects on insulin resistance and glucose intolerance (Figs. 2, 8). The exact molecular networks driving this link, such as the role of JAK/STAT pathway and Akt activation downstream of EPO-R signaling, including the
possibility of insulin sensitivity regulation by directly affecting signaling downstream of insulin receptor require further studies.

Anti-apoptotic and tissue protective activities have been suggested to drive the anti-inflammatory effects of EPO indirectly rather than via direct modulation of immune cell function/response (32). Evidence in support of direct EPO effects on immune cells also exists (33, 34). To this end, we found Mφ to display the highest level of EPO-R expression among SVF cells of WAT (Fig. 3E) and among other immune cell subsets (Fig. 7C). Moreover, the percentage of Mφ appeared to be the only responder to EPO treatment among SVF leukocytes (Supplemental Table-S2), and EPO/EPO-R signaling in Mφ was found to directly regulate their response, in agreement with the recently reported anti-inflammatory role of EPO/EPO-R signaling during bacterial infection and experimental colitis (33).

In summary, our studies demonstrate the involvement of EPO/EPO-R signaling in the reversal of insulin resistance and glucose intolerance, uncouple its role in obesity, body weight and fat mass regulation from its effect on inflammation, and collectively identify and characterize the anti-inflammatory effects of EPO. We reveal a previously unrecognized role for EPO in the expansion of M2-like Mφ population via IL-4/Stat6 axis, and for Stat3 activation in the anti-inflammatory effects of EPO-R in WAT Mφ. These findings highlight a novel EPO/EPO-R signaling-mediated regulation pathway of WAT inflammation in pre-diabetic condition, thus adding to its reported “anti-diabetic” effects shown to act via cytoprotection of pancreatic islet β-cells (40) or muscle fat oxidation (38) in experimental model of diabetes.
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References


Figure Legends

Figure 1. Two weeks EPO treatment regimen does not affect body weight or fat mass. WT C57BL/6 male mice were fed HFD for 12 weeks and treated with or without EPO (1000U/kg) for the final 2 weeks of the study. Lean mice+Saline and Obese mice+Saline were used as negative and vehicle controls, respectively. (A-E) Final body weight (A), body weight gain (B), fat mass (C), perigonadal fat mass (D) and serum leptin (E) were measured for all three groups at the end of week 12. (F) WT C57BL/6 mice with obesity induced by HFD feeding for 13 wks, were treated with or without EPO (1000U/kg) every 48hr during the final 3 weeks of the study. Lean mice+Saline were used as negative controls. Results shown as mean±SEM for n=8 mice per group, representative of three independent experiments with similar results; *, p< 0.05.

Figure 2. EPO treatment attenuates insulin resistance and glucose intolerance during DIO. WT C57BL/6 wild type mice with obesity induced by HFD feeding for 12 wks, were treated with or without EPO (1000U/kg) every 48hr during the final 2 weeks of the study. Lean mice+Saline were used as negative controls. (A) Insulin tolerance test: glucose levels were measured after i.p. injection of 1U/kg insulin. (B) Glucose tolerance test: glucose levels were measured after i.p. injection of 1g/kg glucose. (C-F) Fasting glucose levels (C), % Hematocrit (D), serum glucose levels (E) and serum insulin levels (F) were measured. All measurements were performed at the end of week 12. (G-J) Euglycemic-hyperinsulinemic clamps were performed in DIO mice fasted overnight after 2 weeks of EPO treatment (n=5-8/group). (G) GIR – glucose infusion rate. (H) Whole body glucose fluxes; GIR – glucose infusion rate, EGP – endogenous glucose production, Rd – glucose disposal. (I-J) Tissue 2-deoxyglucose update measured during clamp. Clamp plasma glucose were 209+39 mg/dL and 157+13 mg/dL in saline and EPO treated
mice, respectively. Clamp plasma insulin levels were 6.6±2.3 ng/ml in saline and 5.8±0.8 ng/ml EPO treated mice. Results shown as mean±SEM for n=8 mice per group, representative of three independent experiments with similar results; *, p< 0.05.

**Figure 3. EPO-R expression profiles and the effects of EPO treatment on WAT inflammation and Mφ infiltration.** (A) EPO-R expression in skeletal muscle, liver and WAT of WT mice on normal chow (lean) was assessed using splenocytes and ΔEpoR WAT as positive and negative controls, respectively. (B) EPO-R expression in adipocytes and SVF was assessed in lean versus obese mice. (C-D) Representative hematoxylin and eosin (H&E)-stained sections from perigonadal adipose tissue (C) and Immunofluorescent staining of peridongadal white adipose tissue sections for Mφ (F4/80-red) and nuclei (DAPI-blue) (D), are shown; similar results seen in 12 independent samples. (E) Expression of inflammatory cytokine and chemokine genes in white adipose tissue from perigonadal fat was analyzed by qRT-PCR; expression levels are normalized to β-actin, and fold change in expression are relative to negative control Lean+Saline. (F) Serum TNFα and IL-10 levels were determined for each group (n=5 mice per group). (G) Comparison of EPO-R levels in Mφ (F4/80+) and non-Mφ (F4/80−) fractions, sorted by FACS, from stromal vascular cells of lean and obese WAT. (H-I) Flow cytometry analysis of F4/80 expression in stromal vascular fraction cells of perigonadal WAT depicting total Mφ percentage (H) and number per gram of perigonadal WAT (I) are shown. (J) EPO-R expression levels in SVF of lean and obese mice treated with and without EPO were determined by qRT-PCR. Expression levels were normalized to β-actin. Data are mean±SEM for n=5 mice per group representative of three independent experiments with similar results; *, p< 0.05 and **, p<0.01.
Figure 4. EPO/EPO-R signaling induces Stat3 phosphorylation in Mϕ and inhibits their inflammatory response \textit{in vitro}. (A-B) WAT Mϕ, purified by FACS from obese mice SVF (after 12 weeks of HFD feeding), were cultured with saline or EPO (5U/ml) for phosphoflow analysis. Histogram plots of Mϕ phosphoflow results for p-Stat5a/b (A), and p-Stat3 and p-Stat6 in WT (left two panels) and ΔEpoR (right two panels) (B). (C-D) In some experiments, Mϕ sorted from SVF of obese WT mice were cultured with EPO (5U/ml) for 24hrs after which their TNF-α and iNOS (C), and IL-10 (D) mRNA levels were quantified (C and D). Data represent observations from three independent experiments with similar results plotted as mean±SEM for \( n=4 \) per group; *, \( p<0.05 \).

Figure 5. EPO treatment regulates Mϕ subtype composition in WAT. (A-B) SVF cells from perigonadal fat were used for flow cytometry and analyses. Dot plots depict flow cytometry analysis of MGL-1\(^+\), MGL-1\(^-\) and CD11c\(^+\) Mϕ subsets (A), and their numbers per gram of WAT are shown (B). (C-D) Expression levels of iNOS and IL-1β (C), and Arg-1, Fizz-1 and Pparγ (D) relative to β-actin were assessed. Results shown as mean±SEM for \( n=5 \) mice per group, representative of three independent experiments with similar results; *, \( p<0.05 \) and ** \( p<0.01 \).

Figure 6. EPO treatment decreases circulating inflammatory monocytes and promotes WAT M2-like Mϕ expansion. (A-C) Dot plots for flow cytometry analysis of circulating blood inflammatory monocytes Ly6C\(^{hi}\)CCR2\(^+\) (A), and their numbers per ml of blood (B) are shown, as well as CCL2 levels in serum and perigonadal WAT lysates (C). (D) Flow cytometry results depict the percentage of proliferating MGL-1\(^-\) versus MGL-1\(^+\) Mϕ in perigonadal SVF, based on Ki-67 staining (left) and BrdU uptake (right). (E) Serum and WAT IL-4 levels are shown. (F)
Numbers of total Mφ (bar) and MGL-1\(^{+}\) (top, black) and MGL-1\(^{-}\) (bottom, grey) Mφ subsets per gram of perigonadal WAT from WT, Stat-6\(^{-/-}\) mice and IL-4\(^{-/-}\) mice are shown. Results are presented as mean±SEM for \(n=4\)-6 mice per group, representative of two or three independent experiments with similar results; *, \(p<0.05\) and ** \(p<0.01\).

**Figure 7. Endogenous EPO/EPO-R signaling regulates WAT Mφ infiltration and subtype shift.**

WT C57BL/6 and age matched ∆EpoR male mice with obesity induced by HFD feeding for 12 wks were used. (A) EPO-R expression levels were determined in SVF relative to β-actin. (B-C) Shown are body weight and fat mass before and after DIO (B), and EPO-R expression analysis by qRT-PCR in different immune cell subsets (C) from spleens in which Mφ were FACS purified based on F4/80 expression, and dendritic cells (DC), B-cells and T-cells that were purified by magnetic activated cell sorting through positive selection of CD11c\(^{+}\) (DC), CD19\(^{+}\) (B-cells) and CD3\(^{+}\) (T-cells) cells. (D-K) Shown are percentage and numbers of circulating blood inflammatory monocytes (D), cytokine and chemokine gene expression profile of prigonadal SVF (E), protein levels of TNFα, IL-10 and CCL2 (F), percentage and numbers of total Mφ (G), and representative H&E stained histology sections of perigonadal WAT (H). MGL-1\(^{+}\), MGL-1\(^{-}\) and CD11c\(^{+}\) Mφ subsets percentage (dot plots) and numbers per gram of perigonal fat tissue (I), gene expression determined relative to β-actin (J), and serum and WAT IL-4 levels are shown (K). Results are mean±SEM for \(n=5\) mice per group, representative of three independent experiments with similar results; *, \(p<0.05\) and ** \(p<0.01\) WT versus ∆EpoR.
Figure 8. Endogenous EPO/EPO-R signaling and glucose metabolism during DIO. (A) ITT-InSulin tolerance test, glucose levels were measured after i.p. injection of 1U/kg insulin. (B) For GTT-Glucose tolerance test, glucose levels were measured after i.p. injection of 1g/kg glucose. (C-D) Fasting glucose (C) and serum insulin (D) levels were determined. (E-H) ΔEpoR mice with DIO, induced by 12 weeks of HFD feeding, were injected subcutaneously with saline or EPO (1000U/kg) every 48hrs for the final 2 weeks of HFD feeding and hematocrit (E), ITT (F), GTT (G) and flow cytometry analysis of perigonadal fat SVF cells (H) were determined. All measurements were performed at the end of week 12. Data presented as mean±SEM for n=8 mice per group, representative of three independent experiments with similar results; *, p< 0.05.
Figure 1

(A) Body Weight (g)

(B) Body Weight Gain (g)

(C) Fat Mass (g)

(D) Perigonadal Fat (g)

(E) Serum Leptin (ng/ml)

(F) Body Weight (g)
Figure 2

(A) % Basal Glucose

(B) Blood Glucose (mg/dL)

(C) Fasting Blood Glucose (mg/dL)

(D) Hematocrit

(E) Serum Glucose (mg/dL)

(F) Serum Insulin (ng/ml)
Figure 2

(G) Glucose Infusion Rate

- Saline
- EPO

(H) Bar Graph

- Saline
- EPO

Diabetes
Figure 2

(I) Gastocnemius vs Quadriceps

μmol/kg/h

Saline
EPO

0 50 100 150 200

Gastocnemius Quadriceps Epi WAT Ing WAT

(J) Heart vs BAT

mmol/kg/h

Saline EPO

0 100 200 300 400 500 600 700

Heart BAT
Figure 3

(A) EPO-R Relative Expression (A.U.)

- Muscle
- Liver
- WAT
- Splenocytes
- Lean Adipocytes
- Obese Adipocytes
- Obese SVF
- Splenocytes
- ΔEpoR WAT

(B) EPO-R Relative Expression (A.U.)

(C) H&E

Lean+Saline

Perigonadal White Adipose Tissue

Obese+Saline

Obese+EPO

(D) F4/80

DAPI

For Peer Review Only

Diabetes
Figure 3

(E) Fold Change in Relative Expression

(F) (pg/ml)

(G) EPO-R Relative Expression (A.U.)

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### Table

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<thead>
<tr>
<th>Name</th>
<th>Parameter</th>
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<td>WT ND+PBS Epid SVF.009</td>
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<td>G1</td>
</tr>
<tr>
<td>WT HFD+PBS Epid SVF.010</td>
<td>FL1-H</td>
<td>G1</td>
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<tr>
<td>WT HFD+EPO Epid SVF.011</td>
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<td>Epid SVF unstained cntrl.008</td>
<td>FL1-H</td>
<td>G1</td>
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### Key

- **20%**: Lean+Saline
- **51%**: Obese+Saline
- **40%**: Obese+EPO
- **Unstained control**

### Graphs

**Figure 3**

- **(H)**: Distribution of F4/80+ cells in SVF.
- **(I)**: Relative expression of EPO-R.
- **(J)**: EPO-R Relative Expression (A.U.).

**Legend**

- Blue: Lean+Saline
- Green: Obese+Saline
- Pink: Obese+EPO
- Purple: Unstained control

**Note:**

- The figures illustrate the distribution of F4/80+ cells in SVF and the relative expression of EPO-R under different conditions.
Figure 4

(A) WT Mφ

(B) WT Mφ

(C) 1.5

(D) 0.02
Figure 5

(A) Flow cytometry analysis of F4/80, MGL-1, and CD11c expression in Lean+Saline, Obese+Saline, and Obese+EPO groups. The percentages of F4/80, MGL-1, and CD11c+ cells are shown for each group.

(B) Graph showing the number of F4/80+ SVF cells (x10^6) per g tissue for Lean+Saline, Obese+Saline, and Obese+EPO groups. The graph includes MGL-1+ and CD11c+ cells.

(C) Bar graph showing the relative expression of iNOS in Lean+Saline, Obese+Saline, and Obese+EPO groups.

(D) Bar graph showing the relative expression of IL1β in Lean+Saline, Obese+Saline, and Obese+EPO groups.

(E) Bar graph showing the relative expression of PPARγ in Lean+Saline, Obese+Saline, and Obese+EPO groups.

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Figure 6

(A) 

(B) 

(C) 

Ly6^C^CCR2^+^Monocytes ((x10^4)/ml of Blood)

LFD+Saline HFD+Saline HFD+EPO

Ly6^C^CCR2^+^Monocytes ((x10^4)/ml of Blood)

CCL2 (pg/ml)

Lean+Saline Obese+Saline Obese+EPO

Lean+Saline Obese+Saline Obese+EPO

*
Figure 6

(D) 
**Ki-67**

Gated on F4/80+MGL-1- SVF

% of Ki-67+ cells in MGL-1- MGL-1+ Mφ
- Lean+Saline: 3% 18%
- Obese+Saline: 10% 24%
- Obese+EPO: 9% 32%
- Unstained control:

% of BrdU+ cells in MGL-1- MGL-1+ Mφ
- Lean+Saline: 1.2% 2.3%
- Obese+Saline: 2.5% 9.8%
- Obese+EPO: 3.8% 80%

(E) 

Serum vs WAT lysate

% of Ki-67+ cells in MGL-1- MGL-1+ Mφ
- Lean+Saline: 3% 18%
- Obese+Saline: 10% 24%
- Obese+EPO: 9% 32%
- Unstained control:

% of BrdU+ cells in MGL-1- MGL-1+ Mφ
- Lean+Saline: 1.2% 2.3%
- Obese+Saline: 2.5% 9.8%
- Obese+EPO: 3.8% 80%

(F) 

IL-4 (pg/ml)

Lean+Saline Obese+Saline Obese+EPO

% of MGL-1+ MGL-1- cells compared to control

- MGL-1+ (including MGL-1+ and MGL-1-)
- MGL-1-
Figure 7

(A) EPO-R Relative Expression (A.U.)

- Lean WT (SVF)
- Obese WT (SVF)
- Obese ΔEpoR (SVF)

(B) Body Weight (g)

- WT
- ΔEpoR

-Crude

- After 12 wks of HFD

(C) Fat Mass (g)

- WT
- ΔEpoR

(D) Ly6C<sup>hi</sup> CCR2<sup>+</sup> Monocytes (x10<sup>4</sup>/ml of Blood)

- WT
- ΔEpoR

* indicates statistical significance.
Figure 8

(A) Basal Glucose

(B) Blood Glucose

(C) Fasting Blood Glucose

(D) Serum Insulin

(E) Hematocrit

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Figure 8

(F) % Basal Glucose

- △ EpoR+Saline
- △ EpoR+EPO

Time (min)

(G) Blood Glucose (mg/dL)

- △ EpoR+Saline
- △ EpoR+EPO

Time (min)

(H) F4/80+ Cells in SVF

- △ EpoR+Saline
- △ EpoR+EPO

MGL-1+ MGL-1- (both CD11c+ and CD11c-)

F4/80+ SVF cells (x10^6)/g Tissue

- △ EpoR+Saline
- △ EpoR+EPO

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Supplemental Tables:

Table-S1: Primers and probes sequences

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Table-S2: EPO treatment effects on the percentage of different immune cell subsets in SVF

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<th>Immune Cells Subset</th>
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<th>% SVF Obese</th>
<th>% SVF Obese+EPO</th>
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<td>F4/80⁺</td>
<td>20±5%</td>
<td>51±6.3%</td>
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<tr>
<td>CD3⁺CD4⁺</td>
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<td>CD4⁺CD25⁺Foxp3⁺</td>
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<td>CD19⁺</td>
<td>1.02±0.54%</td>
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* p-value ≤0.05 Obese versus Obese+EPO