Molecular Endocrinology. First published ahead of print May 8, 2007 as doi:10.1210/me.2007-0060

The Molecular Mechanisms Underlying the Pro-Inflammatory Actions of Thiazolidinediones (TZDs) in Human Macrophages

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Running Title: TZDs activate PPARδ

Key Words: PPARγ, PPARδ, TZDs, Inflammation, Macrophages, Monocytes, Cancer

Funding Acknowledgement: National Institutes of Health grant R37 DK048807 (to (D.P.M.); SEED grant from the Duke University Comprehensive Cancer Center and Nicholas School of the Environment (to J.M.H.).

Disclosure Summary: JH has nothing to declare. DPM consults for and has received lecture fees from Wyeth Pharmaceuticals. DPM consults for Ligand Pharmaceuticals. DPM has received research support from GSK.

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ABSTRACT

It is hypothesized that the anti-inflammatory actions of peroxisome proliferator-activated receptors (PPARs) may explain the protective effect of these receptors in diabetes, atherosclerosis, cancer and other inflammatory diseases. However, emerging evidence for pro-inflammatory activities of activated PPARs is concerning in light of new studies which associate PPAR modulators with an increased incidence of both cardiovascular events in humans and the sporadic formation of tumors in rodents. In an attempt to define the role of each PPAR subtype in inflammation, we made the unexpected observation that human PPARδ is a positive regulator of inflammatory responses in both monocytes and macrophages. Notably, TNFα-stimulated cells administered PPARδ agonists express and secrete elevated levels of inflammatory cytokines. Most surprising, however, was the finding that thiazolidinediones (TZDs) and other known PPARγ ligands display different degrees of pro-inflammatory activities in a PPARγ- and PPARα-independent manner via their ability to augment PPARδ signaling. A series of mechanistic studies revealed that TZDs, at clinically relevant concentrations, bind and activate the transcriptional activity of PPARδ. Collectively, these studies suggest that the observed pro-inflammatory and potentially deleterious effects of PPARγ ligands may be mediated through an off-target effect on PPARδ. These studies highlight the need for PPAR modulators with increased receptor subtype-specificity. Furthermore, they suggest that differences in systemic exposure and consequently in the activation of PPARγ and PPARδ may explain why TZDs can exhibit both inflammatory and anti-inflammatory activities in humans.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1). Three subtypes have been identified, PPARα, PPARγ, and PPARδ, each of which mediates the physiological actions of a large variety of fatty acids and fatty acid derived molecules (2-8). Upon binding an agonist, activated PPARs form heterodimer complexes with their partner retinoid X receptor (RXR), enabling them to interact with DNA response elements within target genes and positively regulate gene transcription. The activated PPARs are also capable of transcriptional repression through DNA-independent protein-protein interactions with other transcription factors such as NFkB and AP-1 (9-11).

The tissue distributions of the three PPARs are quite unique, perhaps reflecting the distinct biological roles of the receptors. PPARα expression is highest in the liver, where it is involved in regulating lipid catabolism by promoting free fatty acid uptake, cholesterol trafficking and beta oxidation, whereas PPARγ is enriched in adipose tissue where it functions as a master regulator of adipogenesis. In addition, moderate levels of PPARγ are present in other tissues (i.e. liver, muscle, pancreas) enabling the receptor to regulate insulin secretion and sensitivity (12). PPARδ, while ubiquitously expressed, is perhaps the least understood of the three receptors. However, recent studies suggest that PPARδ has functions both similar to and distinct from those of PPARα and PPARγ, as PPARδ agonists can regulate metabolic homeostasis, promote fat burning and enhance insulin action by complementary effects in distinct tissues (13, 14).

The PPARs are well-validated drug targets that regulate key processes in cellular metabolism. The fibrate class of PPARα agonists are used to treat hypertriglyceridemia, whereas thiazolidinediones (TZDs), PPARγ ligands, increase peripheral insulin-sensitivity and are used to treat type II diabetes (12, 15). Together these agents have had a significant impact on the management of the pathological manifestations of Metabolic Syndrome X (16).

With the discovery that PPARs mediate a variety of biological processes came the realization that these receptors are also involved in the development of several chronic conditions, including diabetes, obesity, atherosclerosis, and cancer (17). Interestingly, a common feature of each of these conditions is systemic inflammation, secondary to elevations in circulating levels of inflammatory cytokines such as intereukin−6 (IL−6), IL−1β, Tumor Necrosis Factor alpha (TNFα) and others. Given that all three PPARs are highly expressed in monocytes, macrophages and endothelial cells, where they can regulate cytokine production, it has been hypothesized that these cells may be the primary targets for the anti-inflammatory activities of fibrates and TZDs (18, 19). This seems to be the case in some instances, as the mechanism by which fibrates reduce atheroma plaque formation was found to occur by activation of vascular PPARα receptors, which inhibit the inflammatory response within the vascular wall (20). Furthermore, the anti-inflammatory actions of PPARγ may be responsible for the insulin-sensitizing properties of TZDs; large populations of macrophages reside in adipose tissue where they produce cytokines that mediate obesity-related insulin resistance (21, 22), yet TZD-activated PPARγ, via suppression of inflammatory cytokine production from macrophages, increases systemic insulin sensitivity (23-25).

The role of PPARδ in inflammation has been more difficult to elucidate. Initially PPARδ was shown to suppress inflammatory cytokine expression from activated macrophages (26), suggesting an anti-inflammatory role for the receptor. However, some reports have suggested otherwise, as both levels of inflammatory cytokines produced by macrophages and size of athlerosclerotic lesions were significantly reduced in mice harboring PPARδ–null macrophages (27). Other studies in human monocyte and macrophages and in mouse keratinocytes have also demonstrated a clear role for this receptor in stimulating a pro-inflammatory response, prompting the suggestion that PPARδ may be involved in

chronic inflammation (28, 29). Thus, despite a wealth of recent attention on this receptor, the role of PPARδ in inflammation to date remains controversial.

Despite the established therapeutic value of PPAR agonists in treatment of several diseases, concern has arisen over the various toxicities demonstrated by these ligands (30). Results from the DREAM study, which assessed the effectiveness of TZDs in preventing diabetes, revealed increased incidence of cardiovascular events in humans administered rosiglitazone; similar concern over cardiac toxicity caused by PPARα/γ pan agonists resulted in removal of Muraglitazar from late stage clinical trials last year (31, 32). Furthermore, agonists for all three PPAR subtypes have been associated with hepatotoxicity and are associated with a higher incidence of tumors in rodents (30). Unfortunately, the mechanisms by which these compounds manifest their deleterious effects are not yet clear. Since many of the therapeutic benefits of PPAR modulators are attributed to their anti-inflammatory effects, an understanding of the role of each receptor in regulating inflammatory responses should allow for future development of safer, yet effective PPAR modulators. Recognizing an urgent need to further define the roles of the PPARs in inflammation, we undertook these studies with the intent to evaluate the contribution of each receptor to the inflammatory response in human monocytes and macrophages.

RESULTS

We initiated these studies by identifying a cell line in which we could detect inflammatory responses and which expressed PPARγ and PPARδ at a level comparable to that observed *in vivo*. In this regard, it was determined that THP-1 cells (human monocytes that can be differentiated in macrophages) produce and secrete substantial levels of cytokines in response to pro-inflammatory stimuli. Furthermore, these cells possess both functional PPARγ and PPARδ that are expressed at a similar level and ratio as detected in *in vivo* murine models (unpublished observation). Using this system, the effect of activated PPARδ on the inflammatory response in THP-1 cells was first assessed. Specifically, we evaluated changes in the expression of the pro-inflammatory cytokines MCP-1, IL-1α, IL-1β, IL-8, eotaxin and others by quantitative real-time PCR in TNFα-stimulated THP-1 cells in the presence or absence of the PPARδ agonist carbaprostacyclin (Carb). As expected, TNFα enhanced expression of all cytokines examined; however, activated PPARδ markedly increased the expression of MCP-1, IL-8 and several other cytokines in untreated cells and synergizes with TNFα to result in super-induction of these cytokines in co-treated cells (Fig. 1A and Suppl. Fig. 1A). This effect was not specific for ligand or cell type, as Carb and other PPARδ agonists tested displayed similar pro-inflammatory actions on cytokine expression in this assay in both THP-1 and U-937 human monocytes and in THP-1 cells that had been differentiated into macrophages (not shown); however, not all inflammation-related genes were regulated in this manner, indicating some specificity in target gene responses (Suppl. Fig. 1B). Furthermore, the PPARδ antagonist GSK660 (33) completely attenuated induction of IL-8 and other cytokines by Carb and TNFα, demonstrating that cytokine induction was receptor-dependent (Fig. 1B). GSK660 displayed no effect on cytokine expression in PPARδ-negative cells induced with TNFα, suggesting that the antagonist effects of this compound in THP-1 cells were not due to toxicity (Suppl. Fig. 2). Thus, activated PPARδ displays substantial pro-inflammatory activity in human monocytes and macrophages.

The effect of activated PPARγ on the inflammatory response in THP-1 cells was assessed next. Specifically, changes in the expression of several pro-inflammatory cytokines were evaluated by quantitative real-time PCR in the presence or absence of TNFα and the PPARγ agonist rosiglitazone (Rosi). We elected to use 1μM of Rosi, which is standard dose used in the field and consistent with that achieved in a physiological context (34). To our surprise, Rosi, rather than exhibiting an anti-inflammatory activity, functioned as an agonist in this system, increasing IL-1α, IL-1β and MCP-1 levels. Furthermore, as seen with PPARδ agonists (Fig. 1), Rosi functioned synergistically with TNFα to enhance the expression of IL-1α, IL-1β, MCP-1, IL-8 and several other cytokines (Fig. 2A and data not shown). This effect was not specific to Rosi, as four different TZDs, rosiglitazone, pioglitazone, troglitazone and ciglitazone (Rosi, Pio, Trog and Cig, respectively) functioned as pro-inflammatory agents, each cooperating with TNFα to increase both expression of IL-8 RNA and protein as well as that of the other cytokines examined (Figs. 2B, 2C and data not shown). Thus, TZDs, when administered at physiologically relevant levels, display substantial pro-inflammatory activities in a manner similar to that of PPARδ agonists.

The results thus far suggest that PPARγ and PPARδ have similar pro-inflammatory activities in human monocytes and macrophages. However, this conclusion is at odds with the wealth of recent reports which suggested that activated PPARγ is anti-inflammatory, one property which has helped to make this receptor an attractive therapeutic target. In an attempt to reconcile our findings with that of others, we evaluated our hypothesis using another cell line, different immune stimuli and drug dosing parameters. When we repeated these studies in U-937 (human monocyte) cells or substituted LPS for TNFα in both THP-1 and U-937 cells, similar results were reproducibly observed (not shown). Furthermore, activation of PPARα by its selective agonist clofibrate resulted in an expected anti-inflammatory response in THP-1

cells, eliminating the possibility that some aspect of the assay was causing all drug treatments to enhance cytokine production in our system (Fig. 2D). Interestingly, when different doses of Rosi or other TZDs were used in the cytokine expression analysis, a biphasic effect was observed: at nanomolar concentrations Rosi displayed anticipated anti-inflammatory effects in the ability to suppress TNFα-stimulated expression of IL-1β, MCP-1 and other cytokines, yet as seen before, agonist activity was manifest at the micromolar concentrations (Fig. 3 and not shown). Thus, surprisingly, TZDs can display both pro- and anti-inflammatory effects in the same cell type, suggesting a more complex mechanism of action than originally anticipated.

Given these observed dose-dependent actions of TZDs, we considered the possibility that at higher doses they could be acting through another receptor. A potential candidate was PPARδ, given that TZDs administered in the micromolar range were functioning similar to PPARδ agonists with regards to effects on cytokine expression. Thus, to explore the possibility that TZDs can interact with more than one PPAR subtype, we next compared their ability to activate PPARγ, PPARα and PPARδ. We elected to use the PPAR negative HeLa cells for these assays, enabling us to measure the transcriptional responses of each receptor in isolation. HeLa cells were transfected with expression plasmids for either PPARγ, PPARα or PPARδ in combination with the DR1-luc reporter. As expected, TZDs displayed a dose-dependent activation of PPARγ, with maximal activity achieved in the micromolar range (Fig. 4A). While no response to TZDs was seen when PPARα was tested in the assay, TZDs were in fact capable of stimulating PPARδ-mediated transcription, functioning as partial agonists of the receptor when compared to the PPARδ full agonist Carb (Figs. 4B and C). In comparison to PPARγ, Rosi and other TZDs showed a 1-2 log lower potency in transactivation of PPARδ, although this concentration was still within the known pharmacological range.

To verify that the partial agonist activity manifest by high doses of TZDs is mediated through PPARδ, we used the specific PPARδ antagonist GSK660 (660). Specifically, the transcriptional activity of the receptor was measured with each ligand in the presence or absence of GSK660, which functions as a competitive inhibitor by interacting with the ligand-binding pocket to displace agonist (33). In HeLa cells transfected with a PPARδ expression vector and DR1-Luc reporter, all TZDs functioned as receptor agonists as expected (Fig. 4D). However, coadministration of GSK660 was able to completely block transcriptional responses of PPARδ to Carb and all TZDs. Furthermore, GSK660 functioned as an inverse agonist, decreasing receptor activity below basal levels; this finding demonstrates that PPARδ possesses a significant amount of constitutive transcriptional activity as shown previously ((35-37) and our unpublished observations). Receptor specificity was demonstrated by the inability of GSK660 to interfere with the ligand-activated PPARγ (Fig. 4E). Taken together, these results provide strong evidence that TZDs can function as PPARδ agonists.

Our next objective was to demonstrate that TZDs could manifest their activity by direct binding to the ligand-binding pocket of PPARδ. Because of the relatively low affinity of the TZDs for PPARδ, it was not possible to use standard ligand-binding assays for these studies. Classical nuclear receptor agonists function by binding to the receptor and inducing an activating conformational change that facilitates recruitment of transcriptional coactivators. Thus, we used a mammalian two-hybrid assay to assess the ability of TZDs to facilitate an interaction between the AF-2 domain of PPARδ and the nuclear receptor interacting domain (NR-box) of the coactivator Activating Signal Cointegrator 2 (ASC-2) (Fig. 5A). HeLa cells were transfected with pM-ASC-2 (NR-Box), containing the yeast Gal4 transcription factor DNA-binding domain fused to the ASC-2 NR-Box, and VP16-PPARδ or VP16-PPARγ, which are chimeras of the strong Herpes Simplex virus VP16 activation domain fused to N-terminus of each PPAR. Transcriptional readout, a measurement of protein-protein interactions in the assay, was obtained by cotransfection of a luciferase reporter vector containing five tandem

Gal4 binding sites (5xGal4-Luc). Notably, a substantial amount of ASC-2 interaction with both PPARδ and γ was observed in the absence of ligand (Figs. 5B and C). This likely reflects the fact that in the absence of an added activating ligand PPARs reside in an active conformation, as discussed above. Despite the high basal level of ASC-2 binding to PPARδ, all four TZDs were able to enhance the interaction in a manner comparable to the full agonist Carb (Fig. 5B). Furthermore, each of these PPARδ interactions was completely antagonized by GSK660, indicating that all compounds were binding in the known ligand-binding pocket of the receptor. As a control, the TZD-induced interaction between PPARγ and ASC-2 was not affected by GSK660 (Fig. 5C). Thus, TZDs function as *bona fide* agonists of PPARδ via their ability to induce an activating conformational change in the receptor that facilitates coactivator recruitment.

Our next objective was to relate our initial observations that TZDs can display pro-inflammatory activity with the finding that they could function as PPARδ agonists through direct activation of the receptor. Based on our studies thus far, we hypothesized that pro-inflammatory activities of TZDs were the consequence of activating PPARδ, whereas the anti-inflammatory actions of TZDs were being mediated through PPARγ. If these predictions are correct, then knockdown of PPARγ should allow the pro-inflammatory activities of TZDs to be manifest while knockdown of PPARδ should allow the anti-inflammatory activities of TZDs to be dominant. Thus, in the following series of experiments we addressed this issue using a set of small-interfering RNAs (siRNAs), which were designed to specifically target each PPAR subtype.

In THP-1 cells transfected with siRNAs to PPARγ, a 90% knockdown of the PPARγ mRNA was achieved without effecting the expression of PPARδ, PPARα or other NRs examined (not shown). Notably, these cells displayed enhanced responsiveness to Carb and Rosi when assayed for expression of IL-1β and MCP-1, and this sensitivity was even more striking in the presence of TNFα (Fig. 6A). Very similar results were observed when a different siRNA to PPARγ was used to confirm these studies (not shown). It was also interesting in both cases to note that cytokine expression in the presence of TNFα alone was greatly elevated in the absence of PPARγ. This finding supports the observation that even in their basal state, PPARs (α/γ/δ) reside in an active conformation and display significant transcriptional activity, which is enhanced by agonist (Fig. 4D, Fig. 5 and (35-37)). The high level of constitutive activity exhibited by the receptor enables the apo-PPAR to activate target gene expression, or as shown previously, to suppress cytokine expression through inhibition of NFkB (38). Thus, having removed PPARγ from the cell, both the constitutive and ligand-mediated anti-inflammatory effects of the receptor are lost, resulting in enhanced pro-inflammatory cytokine expression.

We next tested whether the observed pro-inflammatory effects of TZDs are indeed PPARγ-independent and mediated solely through PPARδ, knockdown of PPARδ should enable TZDs to act through PPARγ alone. Thus, THP-1 cells were transfected with siRNAs to PPARδ and assayed for changes in cytokine expression by real-time PCR. Using three different siRNA sequences separately, 95% knockdown of the PPARδ mRNA was achieved in three independent experiments without effecting the expression of PPARγ, PPARα or other NRs examined (not shown). As expected, in these cells the pro-inflammatory response to Carb is absent, reflecting the loss of PPARδ (Fig. 6B). Notably, however, the agonist activity of Rosi [via PPARδ] is also lost, and the anti-inflammatory activity [via PPARγ] is manifest. The ability of PPARδ to display constitutive pro-inflammatory activity is evident from the observation that IL-1β and MCP-1 expression in the presence of TNFα alone was greatly reduced in the absence of PPARδ (Fig. 6B).

To rule out the possibility that PPARα was a confounding factor or potential target of TZDs, the knockdown of this receptor was also tested in this system (Fig. 6C). Interestingly, a 90% reduction in PPARα expression in THP-1 cells yielded results similar to those of the PPARγ knockdown in that enhanced pro-inflammatory

responsiveness to Carb and Rosi was observed, albeit to a lesser degree (compare Figs. 6A and 6C). This observation eliminated the possibility that PPARα was responsible for the observed pro-inflammatory activity of Rosi. Taken together, the results in Fig. 6 indicate that in our system: (1) PPARγ and PPARδ play opposing roles in regulating pro-inflammatory cytokine production and (2) the pro-inflammatory activities of TZDs require PPARδ, whereas the anti-inflammatory actions appear to be manifested through PPARγ.

DISCUSSION

In probing the roles of the PPARα/γ/δ subtypes in inflammation, we found that PPARα and γ display anti-inflammatory activities in human monocytes and macrophages, which is consistent with previously described functions in both humans and rodents. Surprising, however, was the discovery that human PPARδ in human monocytes and macrophages induces the expression and secretion of key pro-inflammatory cytokines. Furthermore, PPARδ synergizes with either TNFα or LPS to amplify the inflammatory response. These studies also reveal that PPARδ displays constitutive pro-inflammatory activity, suggesting a role for PPARδ antagonists as modulators of inflammatory responses. This activity of PPARδ may have gone undetected in the past as most studies of the role of this receptor in inflammation have been performed in murine macrophages (26). Although mouse and human cells contain relatively equivalent levels of PPARδ and a similar ratio of PPARγ to PPARδ, our studies suggest that there may be some species-specific functional differences (Suppl. Fig. 3). These differences are not entirely surprising given that the molecular mechanisms and biology of several other nuclear receptors (i.e. PPARα, PXR/SXR, ERβ) differ significantly when compared between humans and the rodent models often used to study them. In addition to species-specific differences, it is also possible that pro-inflammatory activity of human PPARδ has been over-looked since, as we found, receptor agonists administered alone have only modest effects on cytokine expression compared to those increases presence of TNFα. However, in our intent to mimic the environment of chronic inflammation, a state characterized by continuous production and exposure to TNFα, we were able to uncover a robust agonist activity of PPARδ on cytokine production. These findings suggest it is most likely that pro-inflammatory activities of PPARδ modulators would be manifest in a physiological setting during circumstances of chronic inflammation, as those associated with obesity, Type II diabetes or cancer. Thus, it will be important to extend our studies to examine PPAR action in primary human macrophages as a means to further assess the biological significance of our findings.

TZDs Manifest Pro-Inflammatory Activities Through PPARδ

One of the most interesting findings in these studies was that TZDs, at clinically relevant doses, display substantial pro-inflammatory activities in human monocytes and macrophages. While still controversial, there exists other evidence demonstrating that TZDs can display pro-inflammatory activities in a wide range of circumstances. Desmet et al. showed that Rosi and Trog potentiate the inflammatory response to TNFα in a series of different epithelial cell types, and this occurs in a manner sufficient to enhance the pro-survival activity of co-cultured neutrophils (39). Interestingly, as we observed in the current study, they found that the inflammatory response occurred only at micromolar concentrations, and the effect was independent of PPARγ. In mice dosed with TZDs and challenged with LPS, rather than an immunosuppressive response, animals developed elevated blood levels of pro-inflammatory cytokines, substantially higher than seen in mice dosed with LPS alone (40). Importantly, this observation provided evidence that sufficient concentrations of TZDs are available *in vivo* to enable these compounds to manifest their pro-inflammatory activities. Finally, studies with mice containing a macrophage-specific knockout of PPARγ indicated that low concentrations of Rosi resulted in PPARγ-dependent immunosuppressive responses, yet at high doses the effects were independent of the receptor (26). Thus, in support of our findings, these collective observations indicate that PPARγ has anti-inflammatory activity, but that TZDs have variable activity due to their ability to act via different PPAR subtypes when present at different concentrations.

We provide here an explanation for the conflicting observations of differing biological activities of PPARγ receptor and PPARγ agonists, namely that activated PPARγ is anti-

inflammatory, yet high doses of its TZD ligands can elicit a pro-inflammatory response by acting through PPARδ. There are in fact some hints in the literature that TZDs can display cross-over effects onto PPARδ, as that was postulated to occur at high doses of Rosi in mouse peritoneal macrophages (26). In biochemical assays and in cells, TZDs were shown to enhance interaction of both PPARγ and PPARδ with an NR-box fragment of the coactivator CREB Binding Protein (CBP), and increase dissociation of the ID (interaction domain) of the corepressor Nuclear Receptor Co-repressor (NCoR) (41). Regardless, the current study is the first to our knowledge to relate all of these previous observations and demonstrate a potential biological outcome for the cross-reactivity properties of TZDs.

Pro-Inflammatory Activity of PPARδ- A Potential Risk Factor In Cancer

A wealth of evidence has emerged indicating that PPARδ plays a key growth regulatory role in cancer (42-46). One likely mechanism is through direct actions of the activated receptor in the epithelial cells of tumors. For example, activation of PPARδ in breast carcinoma cells is associated with upregulation of ERα, CdK2, VEGFα and its receptor (FLT-1); by this means, PPARδ is thought to initiate an autocrine pathway for proliferation (44). Furthermore, epithelial cells of mammary tumors in mice treated with a PPARδ agonist show an increase in both expression and colocalization of PPARδ and activated 3-phosphoinositide-dependent protein kinase (PDK1), the latter of which has known oncogenic activity in epithelium (42). Furthermore it has been observed that (1) PPARδ is highly expressed in monocytes and macrophages, (2) monocyte/macrophages are a major component of the infiltrate of most if not all tumors, (3) macrophages secrete cytokines which are known to increase tumor cell proliferation, stimulate angiogenesis, and promote invasion and metastasis and (4) activated PPARδ significantly increases cytokine production and secretion. Thus, in addition to direct effects, activated PPARδ could impact tumor growth in an indirect manner by stimulating cytokine production and release from macrophages.

It has always been counter-intuitive that PPARγ is protective against cancer, yet exposure to PPAR modulators is correlated with incidence of tumors in rodents. One plausible explanation is that both PPARγ and PPARδ ligands are capable of stimulating growth-promoting pathways in cancer cells by enhancing PPARδ activity. In addition, TZDs acting through PPARδ may promote the inflammatory response in macrophages, resulting in the release of cytokines that alter growth, motility and/or invasiveness of colocalized cancer cells (unpublished observations). Both mechanisms are plausible and could potentially act synergistically. Of note, many of the tumors that have been found in animal carcinogenicity studies for PPAR ligands are sporadic in distribution and nature (47). This observation again correlates more with known PPARδ distribution (moderate to high ubiquitous expression), compared to that of PPARγ, whose expression is more limited and is at low levels in most tissues (12). Thus, it will be important to determine in rodent models whether TZDs and other PPAR modulators can stimulate both cancer cell growth and inflammation by acting through PPARδ. Our data also suggest that there may be a role for PPARδ antagonists as chemotherapeutics for cancer.

PPARγ Specific Ligands- An Unmet Medical Need

Clearly our studies suggest that there exists an unmet medical need for PPAR subtype-selective ligands with improved specificity. Importantly, it appears that cross-reactivity with PPARδ is not a property unique to TZDs, as several other known synthetic PPARγ agonists and selective PPARγ modulators (SPARMs) are capable of binding and activating PPARδ ((41) and our unpublished observations). It is well established that patients receiving TZDs for type 2 diabetes experience severe side effects such as edema, weight gain and bone loss. Thus, it is possible that some of these undesirable physiological effects of TZDs could be alleviated with better receptor-specific ligands.

In summary, in this study we report that at therapeutically relevant levels, TZDs can function as partial agonists of PPARδ and may enhance inflammatory responses by acting through this receptor in human monocytes and macrophages. This discovery provides an explanation for several puzzling observations made previously, such as the ability of TZDs to manifest PPARγ-independent effects and that in some circumstances TZDs can display inflammatory activities. Given the observed pro-inflammatory activity of the human PPARδ, we suggest that PPAR subtype-selectivity ligands with increased specificity may provide safer, more effective therapeutics for metabolic diseases and perhaps other inflammatory conditions.

MATERIALS AND METHODS

Biochemicals

PCR reagents were obtained from BIO-RAD (Hercules, CA). Carbaprostacyclin and rosiglitazone were purchased from Cayman Chemicals (Ann Arbor, MI). Troglitazone, ciglitazone, clofibrate and LPS were purchased from Sigma (St. Louis, MO). GSK660 (Methyl 3-({[2-(methoxy)-4 (phenylamino)phenyl] amino}sulfonyl)-2-thiophenecarboxylate was a gift from Glaxo-Smith-Kline Pharmaceuticals (Research Triangle Park, NC). TNFα was obtained from Roche (Indianapolis, IN). The IL-8 ELISA kit was obtained from Invitrogen (Carlsbad, CA). siRNA oligos were purchased from Amersham Biosciences (Piscataway, NJ) and Invitrogen. PCR oligos were obtained from Integrated DNA Technologies (Coralville, IA).

Plasmids

The luciferase reporter constructs DR1-Luc and 5x-Gal4-TATA-Luc and the pCMV-β-galactosidase normalization plasmid (pCMV-βgal) have been described previously (48). Mammalian expression vectors for human PPARα/δ/γ were constructed as follows: the coding sequence of each receptor was cloned into the pENTR2B Gateway entry vector (Invitrogen). LR Clonase reactions were used to shuttle the receptors into either pcDNA3nV5 or pVP16GWb destination vectors as according to the manufacturer's protocol (Invitrogen); these reactions created the mammalian expression vectors pcDNA3-PPARα, pcDNA3-PPARδ and pcDNA-PPARγ, and VP16-PPARδ and VP16-PPARγ. The pVP16GWb destination vector was constructed by inserting a cassette containing Gateway attL1 and attL2 sites (for site-specific recombination of the entry clone) into the pVP16 expression plasmid (Clontech). pM-ASC-2 (NR) was created by inserting the nuclear receptor interacting domain (NR-box) of the coactivator ASC-2 adjacent to and in frame with the yeast Gal4 DNA-binding domain within the pM parental vector (Clontech).

Mammalian Cell Culture and Transient Transfection Assays

All cell lines were obtained from American Type Culture Collection (Manassas, VA). THP-1 (human acute monocytic leukemia) and RAW 264.7 gamma NO(-) (mouse monocyte/ macrophage) cells were maintained in RPMI 1640 (Invitrogen) supplemented with 8% FBS (Hyclone), 1 mM sodium pyruvate, 10 mM HEPES, and 1.5 g/L sodium bicarbonate (Invitrogen), and 4.5 g/L glucose (Sigma). For THP-1 cells, media also contained 0.05 mM β-mercaptoethanol (Invitrogen). HeLa (human cervical carcinoma) and MDA-MB 231 (human breast adenocarcinoma) cells were maintained in MEM (Invitrogen) supplemented with 8% FBS, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. MCF-7 (human breast adenocarcinoma) cells were maintained in DMEM F12 supplemented with 8% FBS, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. All cell lines were grown in a 37°C incubator with 5% CO_2.

HeLa cells were used for transactivation and mammalian-two-hybrid assays. For transient transfections, cells were plated in 24-well plates 24 h before transfection. Lipofectin (Invitrogen)-mediated transfection has been described in detail previously (49). Briefly, prior to transfection, the media was replaced with phenol-free MEM containing 8% charcoal-stripped serum (Hyclone), 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (Invitrogen). A DNA-Lipofectin mixture containing a total of 3 μg of plasmid for each triplicate sample was added to the cells. For transactivation assays, each triplicate contained 2 μg DR1-Luc, 0.1 μg pCMV-βgal, 0.1 μg pcDNA3-PPAR (α, δ, or γ) and 0.8 μg PBSII filler vector. For mammalian two-hybrid assays, each triplicate contained 1.5 μg 5x-Gal4-TATA-Luc, 0.1 μg pCMV-βgal, 0.7 μg pVP16-PPAR (δ, or γ) and 0.7 μg pM-ASC-2. Receptor ligands were added to cells 4 h following transfection. Cells were assayed 40 h following transfection. Luminescence was measured on a Fusion luminometer (Perkin Elmer) and β -galactosidase activity on a Multiskan MS plate reader (Thermo Labsystems). Results are

expressed as normalized luciferase activity (normalized with β-Gal for transfection efficiency) per triplicate sample of cells in a representative experiment; error bars indicate the S.E.M. of triplicate determinations. Each experiment was repeated at least three independent times with very similar results.

RNA Isolation and Quantitative PCR

For RNA analysis, THP-1 or RAW cells were seeded in 6-well plates. Cells were treated for 24 h +/- 100 nM PMA (duplicate plates) in regular culture media (PMA differentiates cells into macrophages). Cells were then washed, and administered ligands for 24 h in RPMI 1640 supplemented with 0.5% charcoal/dextran filtered FBS and other additives as indicated above. After 24 h, cells were harvested and total RNA was isolated using the RNeasy kit with RNase-free DNase (Qiagen). One μg of RNA was reverse transcribed using the BioRad iScript cDNA synthesis kit. The BioRad iCycler Realtime PCR System was used to amplify and quantitate levels of target gene cDNA. QPCR reactions were performed using 0.1 μl of cDNA, 10 μM specific primers, and iQ SYBRGreen supermix (BioRad). Data are the mean +/-SEM of three biological replicates performed in triplicate.

For siRNA experiments, THP-1 cells ($2x10^6$ cells/sample) were transfected with 0.25 pmol siRNA oligos using the Nucleofector Kit V and Nucleofector electroporation apparatus according to the manufacturer's optimized protocols for THP-1 cells (Amaxa Biosystems, Gaithersburg, MD). After 48 h, cells were administered ligands for 24 h and then processed as described above.

ELISA

THP-1 cells were seeded in 6-well plates and treated for 24 h +/- 100 nM PMA (duplicate plates) in regular culture media. Cells were then washed, and administered ligands for 24 h in RPMI 1640 supplemented with 0.5% charcoal/dextran filtered FBS and other additives as indicated above; triplicate wells were used for each treatment. After 24 h, cells were pelleted and supernatants (spent media) were collected. The IL-8 ELISA was performed in a 96-well format on triplicate samples of spent media, as according to the manufacturer's protocol (Invitrogen). Data are the mean +/- SEM of three biological replicates.

Acknowledgments

The authors would like to thank Drs. Timothy Willson and Andrew Billin (GlaxoSmithKline) for their generous gift of reagents and valuable scientific input. We thank members of the McDonnell laboratory for critical review of the manuscript. This work was supported by National Institutes of Health grant R37 DK048807 (to (D.P.M.) and a SEED grant from the Duke University Comprehensive Cancer Center and Nicholas School of the Environment (to J.M.H.).

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Fig. 1. PPARδ Displays Pro-Inflammatory Activity in Human Monocytes

MCP-1, IL-1α, IL-1β and IL-8 RNA levels in THP-1 cells were measured by real-time PCR. **A,** THP-1 cells were treated with vehicle (Veh), TNFα (50 ng/ml), 10^{-5} M PPARδ agonist carbaprostacyclin (Carb) or Carb+TNFα for 24 h. **B**, THP-1 cells were treated for 24h with Veh, TNFα, Carb or Carb+TNFα in the absence or presence of $1x10^{-6}$ M PPARδ antagonist GSK660. Total RNA was harvested, and cDNA was prepared and used as a template for gene expression analysis. All values were normalized to a 36B4 control. Graphical data is represented as fold induction over vehicle (set at 1). Data points represent the average of triplicate amplification reactions for each condition in a representative experiment. Very similar results were observed in THP-1 cells that were differentiated into macrophages.

Fig. 2. Pro-inflammatory Activity of Rosiglitazone

A, Expression of pro-inflammatory cytokines IL-1α, IL-1β, MCP-1 and IL-8 in THP-1 cells was measured by real-time PCR. THP-1 cells were dosed 24 h with vehicle (Veh), TNFα (50 ng/ml), or 10^{-5} M rosiglitazone (Rosi) or Rosi+TNFα for 24 h. Total RNA was harvested, and cDNA was prepared and used as a template for gene expression analysis. All values were normalized to a 36B4 control. Graphical data is represented as fold induction over vehicle (set at 1). Data points represent the average of triplicate amplification reactions for each condition in a representative experiment. **B**, Pro-inflammatory activity of TZDs. IL-8 expression in THP-1 cells was analyzed by real-time PCR. THP-1 cells were dosed 24 h with vehicle (Veh), TNFα (50 ng/ml), or 10^{-5} M TZDs (+/- TNFα): rosiglitazone (Rosi), pioglitazone (Pio), troglitazone (Trog) or ciglitazone (Cig). Total RNA was harvested and processed as in **A**. Similar pro-inflammatory effects of TZDs were observed when expression of other cytokines was analyzed (not shown). **C**, IL-8 protein production by THP-1 cells was measured by ELISA. THP-1 cells were treated as in **B**. Spent media was collected from cells and used for quantitation of cytokines by ELISA. Data points are the average of triplicate determinations. C1 and C2 refer to signal provided by positive controls (pure IL-8 protein; 4 and 8 pg). **D**, IL-1β expression was analyzed by real-time PCR (see **A**) in THP-1 cells treated for 24 h with Veh, TNFα, 10^{-5} M clofibrate (Clof) or Clof+TNFα.

Fig. 3. Dose-Dependent Regulation of Cytokine Expression by TZDs

A, IL-1β and **B**, MCP-1 expression in THP-1 cells were analyzed by real-time PCR. THP-1 cells were treated with vehicle (Veh), TNFα (50 ng/ml), 10^{-9}-10^{-5} M rosiglitazone (Rosi) or 10^{-9}-10^{-5} M Rosi + TNFα for 24 h. Total RNA was harvested, and cDNA was prepared and used as a template for gene expression analysis. All values were normalized to a 36B4 control. Graphical data is represented as fold induction over vehicle (set at 1). Data points represent the average of triplicate amplification reactions for each condition in a representative experiment.

Fig. 4. TZDs Function as Agonists of Both PPARγ and PPARδ

PPAR transcriptional activity was measured by mammalian cell transfection assays. HeLa cells were transfected with an **A**, PPARγ or **B**, PPARδ expression vector in combination with the DR1-Luc reporter plasmid and a β-galactosidase normalization vector. After transfection, cells were treated with vehicle (Veh) or increasing concentrations (10^{-10}-10^{-4} M) of carbaprostacyclin (Carb), or TZDs: rosiglitazone (Rosi), pioglitazone (Pio), troglitazone (Trog) or ciglitazone (Cig) for 40 h. Cells were harvested and assayed for luciferase activity; all luciferase assay values were normalized to β-gal controls**.** Data points are the average of triplicate determinations in a representative experiment, and the average coefficient of variance for each value is <10%. **C**, HeLa cells were transfected with a PPARα expression vector in combination with the DR1-Luc reporter plasmid and a β-galactosidase normalization vector. After transfection, cells were treated with vehicle (Veh) or increasing concentrations (10^{-5} M) of carbaprostacyclin (Carb), PPARα agonist clofibrate (Clofib), or TZDs: rosiglitazone (Rosi), pioglitazone (Pio), troglitazone (Trog) or ciglitazone (Cig) for 40 h. Cells were harvested and assayed as in **A** and **B**. HeLa cells were transfected with a **D**, PPARδ or **E**, PPARγ expression vector in combination with the DR1-Luc reporter plasmid and a β-galactosidase normalization vector. After transfection, cells were treated with vehicle (Veh) or 10^{-6} M carbaprostacyclin (Carb), or 10^{-6} M TZDs: rosiglitazone (Rosi), pioglitazone (Pio), troglitazone (Trog) or ciglitazone (Cig) in the absence or presence of 10^{-6} M PPARδ antagonist GSK660 (660) for 40 h. Cells were harvested and assayed as described above.

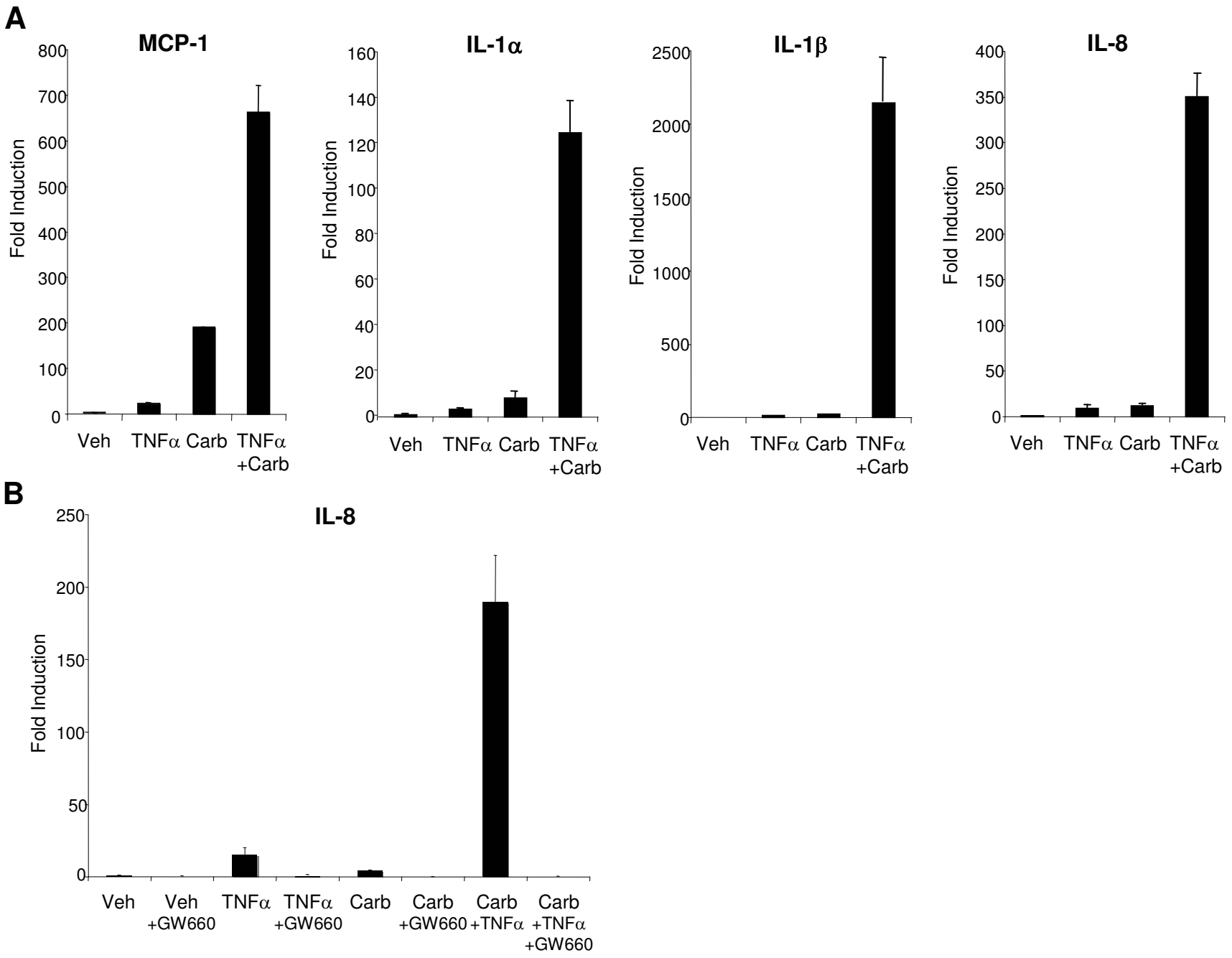
Fig. 5. TZDs induce an active conformation of PPARδ

A, Schematic of mammalian two-hybrid assay. **B**, **C**, HeLa cells were transfected with VP16 and pM control vectors, pM-ASC-2 (NR-box), and VP16-PPARδ **(B)** or VP16-PPARγ **(C)** expression vectors in combination with a 5x-Gal4-TATA-Luc reporter plasmid and a β-galactosidase normalization vector. After transfection, cells were treated with vehicle (Veh), 10^{-6} M carbaprostacyclin (Carb), or 10^{-6} M TZDs: rosiglitazone (Rosi), pioglitazone (Pio), troglitazone (Trog) or ciglitazone (Cig) in the absence or presence of 10^{-6} M PPARδ antagonist GSK660 (660) for 40 h. Cells were harvested and assayed for luciferase activity; all luciferase assay values were normalized to β-gal controls**.** Data points are the average of triplicate determinations in a representative experiment.

Fig. 6. Knockdown of PPARγ Enhances Pro-Inflammatory Effects of Rosiglitazone

A, IL-1β and MCP-1 RNA levels in THP-1 cells were measured by real-time PCR. THP-1 cells were transfected with siRNA for human PPARγ or control (Scramble) siRNA. After 48 h, cells were treated with vehicle (Veh), TNFα (50 ng/ml), 10^{-5} M PPARδ agonist carbaprostacyclin (Carb), 10^{-5} M rosiglitazone (Rosi), or Carb+TNFα or Rosi + TNFα for 24 h. Total RNA was harvested, and cDNA was prepared and used as a template for gene expression analysis. All values were normalized to a 36B4 control. Graphical data is represented as fold induction over vehicle (set at 1). Data points represent the average of triplicate amplification reactions for each condition in a representative experiment. **B**, Knock-down of PPARδ enhances anti-inflammatory effects of rosiglitazone. IL-1β and MCP-1 RNA levels in THP-1 cells were measured by real-time PCR. THP-1 cells were transfected with siRNA for human PPARδ or control (Scramble) siRNA. After 48 h, cells were treated as in **A**. Total RNA was harvested and analyzed as described above. **C**, Knock-down of PPARα enhances pro-inflammatory effects of rosiglitazone. IL-1β and MCP-1 RNA levels in THP-1 cells were measured by real-time PCR. THP-1 cells were transfected with siRNA for human PPARα or control (Scramble) siRNA. After 48 h, cells were treated as in **A**. Total RNA was harvested and processed as described above.

Figure 1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MCP-1
IL-1α
IL-1β
IL-8
Fold Induction
Veh
TNFα
Carb
TNFα +Carb
B
IL-8
Fold Induction
Veh
Veh +GW660
TNFα
TNFα +GW660
Carb
Carb +GW660
Carb +TNFα
Carb +TNFα +GW660

Figure 2

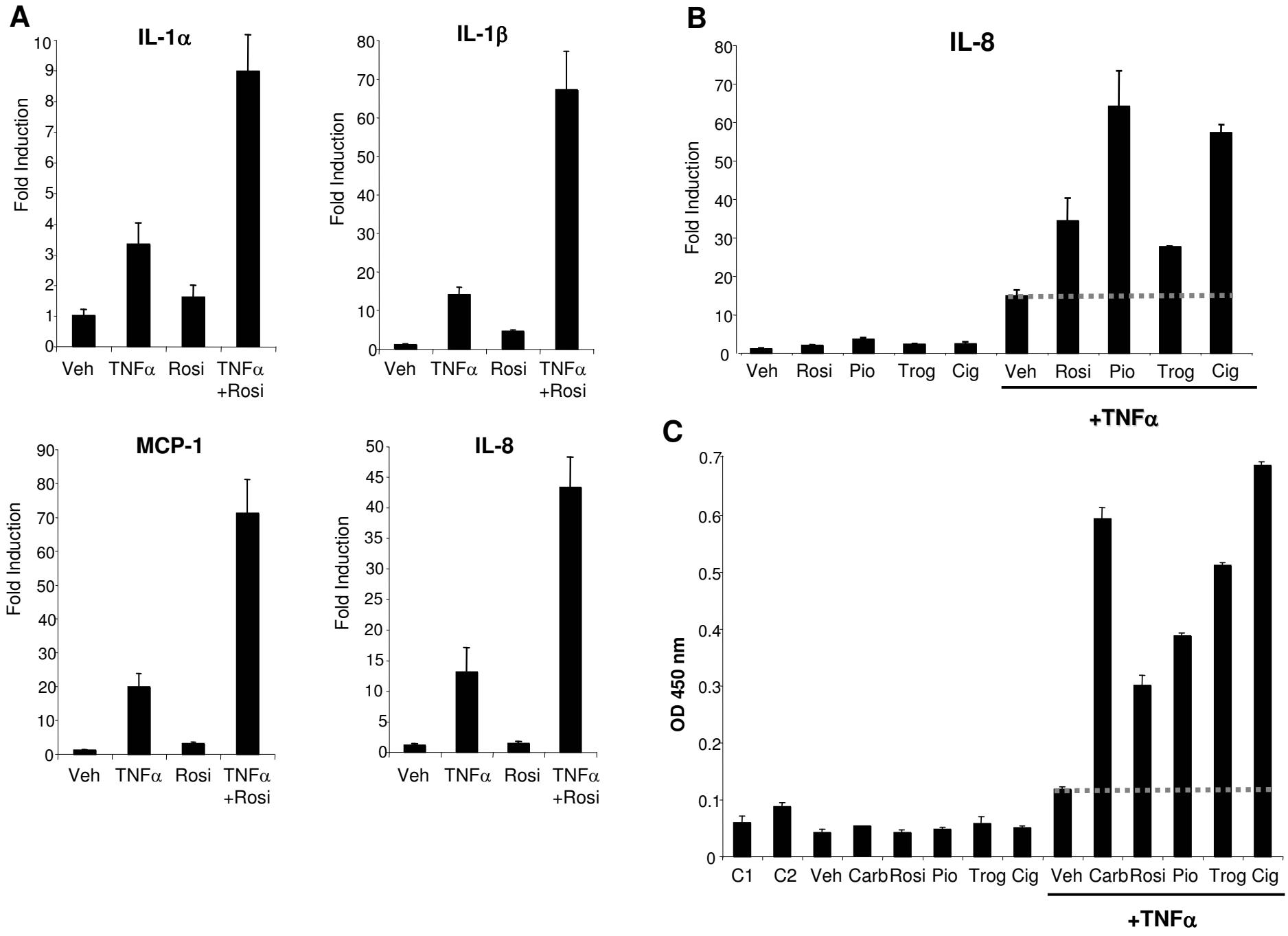
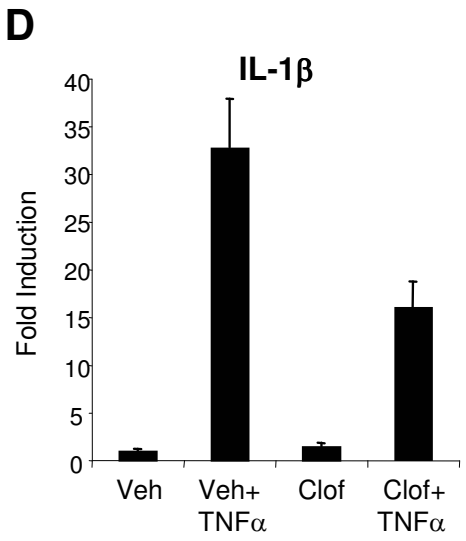

A
IL-1α
Fold Induction
Veh
TNFα
Rosi
TNFα +Rosi
IL-1β
MCP-1
IL-8
B
IL-8
Pio
Trog
Cig
+TNFα
C
OD 450 nm
C1
C2
Carb

Figure 2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IL-1β
Fold Induction
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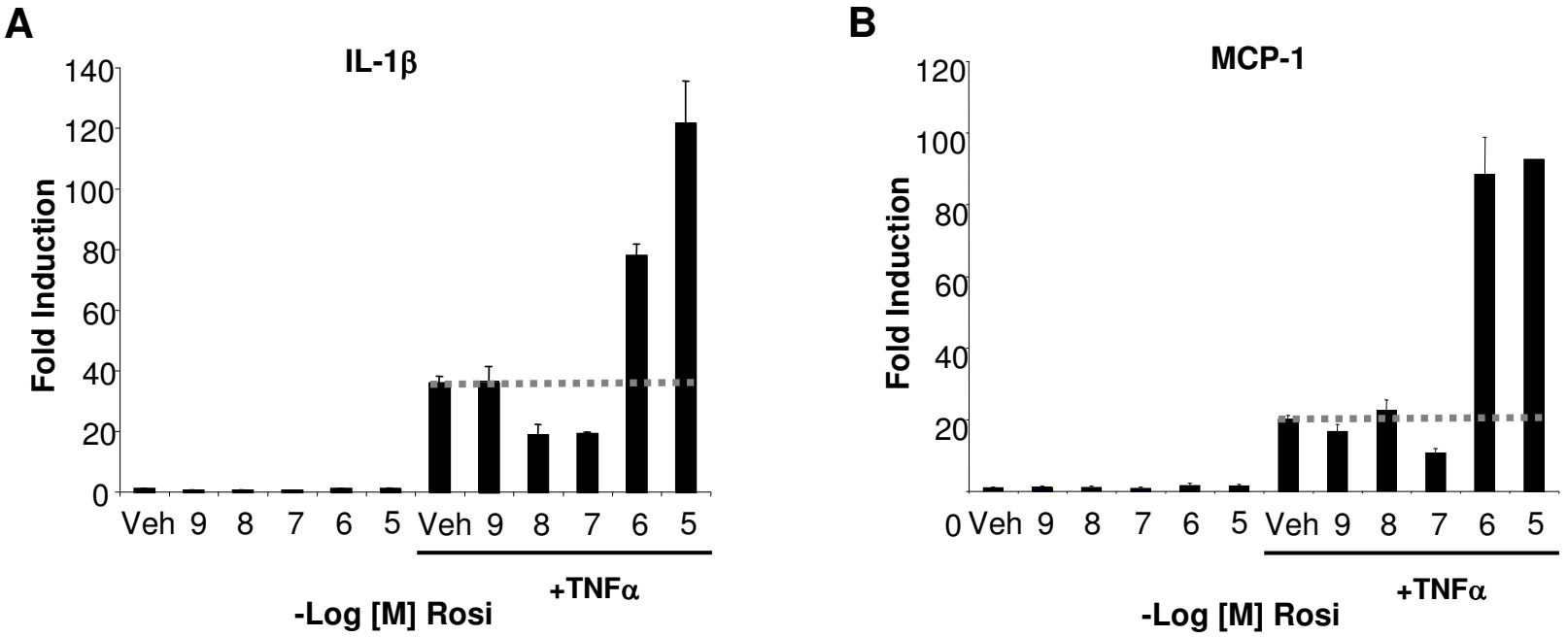
35
30
25
20
15
10
5
0
Veh
Veh+ TNFα
Clof
Clof+ TNFα

Figure 3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Fold Induction
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120
100
80
60
40
20
0
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+TNFα
-Log [M] Rosi
B
MCP-1
Fold Induction
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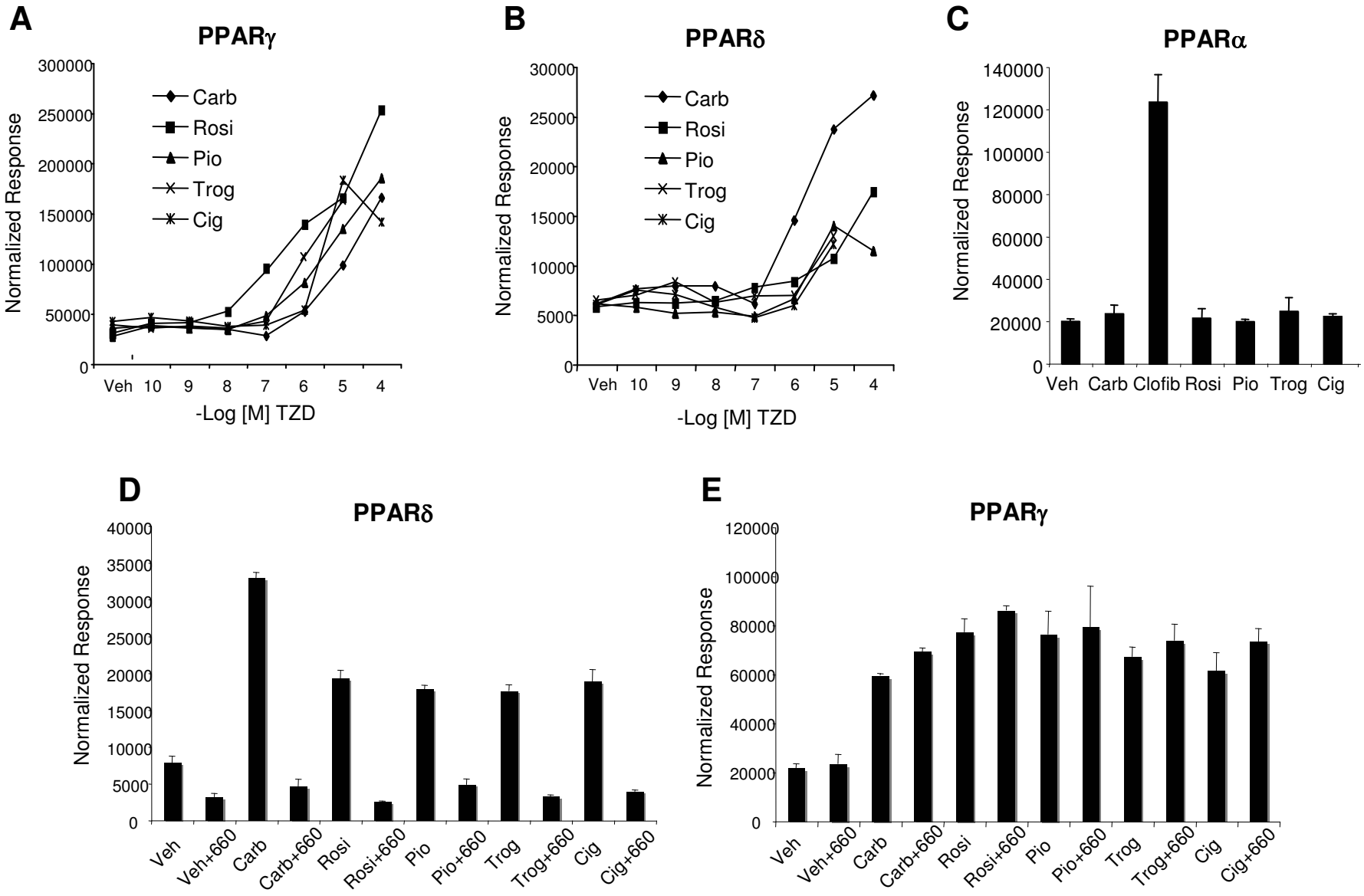
100
80
60
40
20
0 Veh 9 8 7 6 5 Veh 9 8 7 6 5
+TNFα
-Log [M] Rosi

Figure 4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PPARγ
Normalized Response
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250000
200000
150000
100000
50000
0
Carb
Rosi
Pio
Trog
Cig
Veh 10 9 8 7 6 5 4
-Log [M] TZD
B
PPARδ
Normalized Response
30000
25000
20000
15000
10000
5000
0
Carb
Rosi
Pio
Trog
Cig
Veh 10 9 8 7 6 5 4
-Log [M] TZD
C
PPARα
Normalized Response
140000
120000
100000
80000
60000
40000
20000
0
Veh Carb Clofib Rosi Pio Trog Cig
D
PPARδ
Normalized Response
40000
35000
30000
25000
20000
15000
10000
5000
0
Veh
Veh+660
Carb
Carb+660
Rosi
Rosi+660
Pio
Pio+660
Trog
Trog+660
Cig
Cig+660
E
PPARγ
Normalized Response
120000
100000
80000
60000
40000
20000
0
Veh
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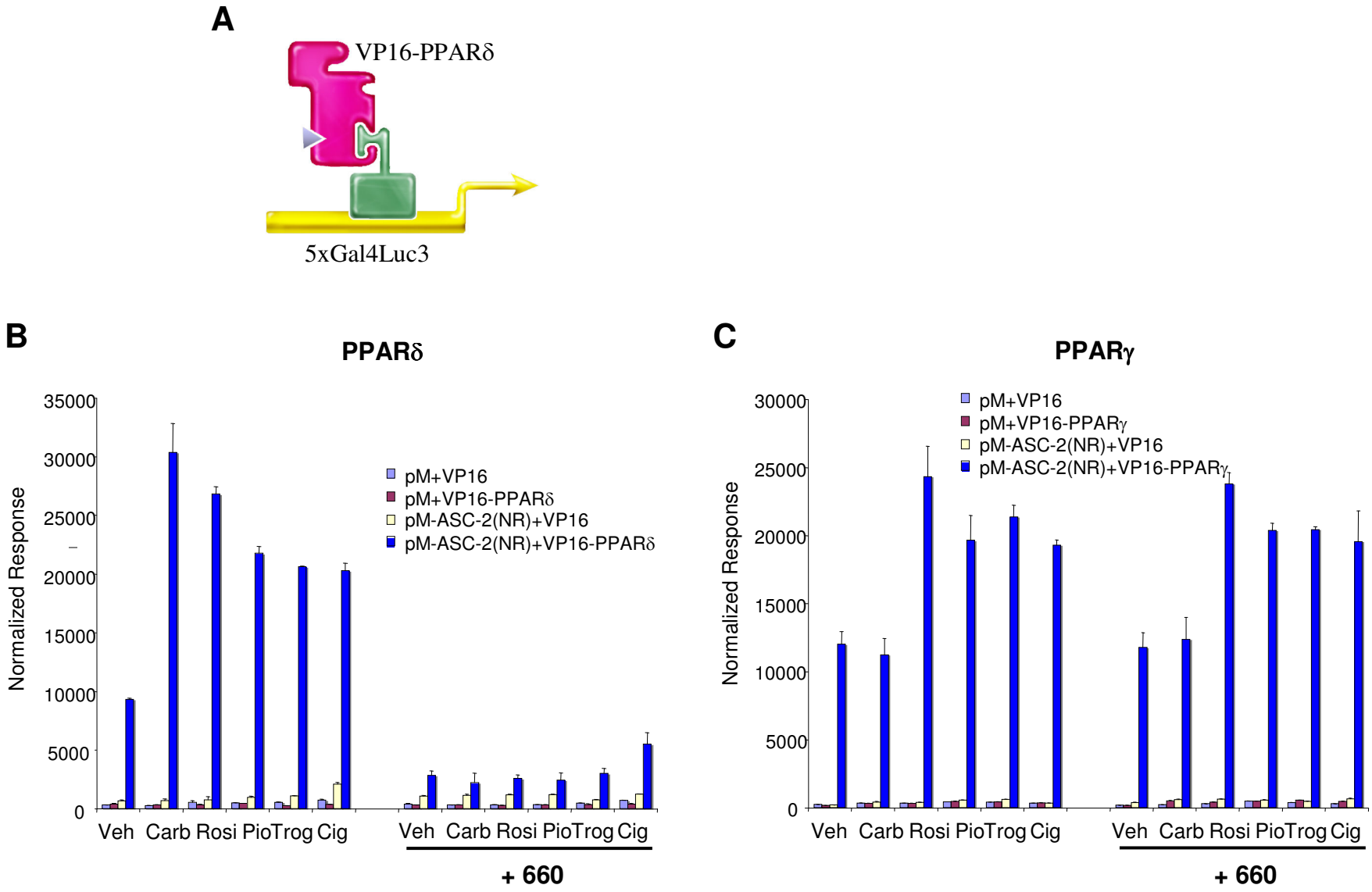
Pio+660
Trog
Trog+660
Cig
Cig+660

Figure 5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5xGal4Luc3
B
PPARδ
Normalized Response
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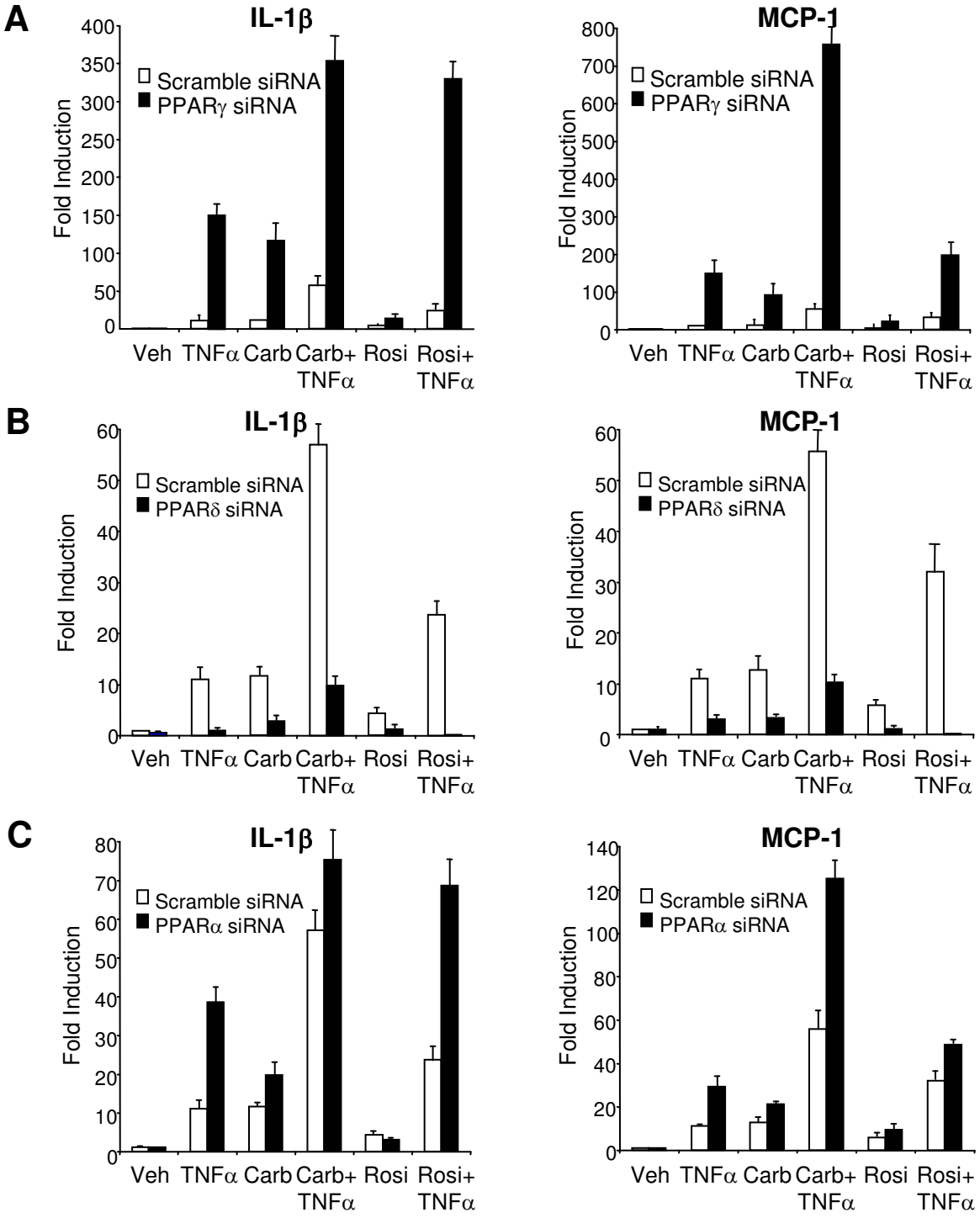
30000
25000
20000
15000
10000
5000
0
pM+VP16
pM+VP16-PPARδ
pM-ASC-2(NR)+VP16
pM-ASC-2(NR)+VP16-PPARδ
Veh Carb Rosi PioTrog Cig
Veh Carb Rosi PioTrog Cig
+ 660
C
PPARγ
Normalized Response
30000
25000
20000
15000
10000
5000
0
pM+VP16
pM+VP16-PPARγ
pM-ASC-2(NR)+VP16
pM-ASC-2(NR)+VP16-PPARγ
Veh Carb Rosi PioTrog Cig
Veh Carb Rosi PioTrog Cig
+ 660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MCP-1
Scramble siRNA
PPARγ siRNA
Fold Induction
Veh
TNFα
Carb
Carb+ TNFα
Rosi
Rosi+ TNFα
B
IL-1β
MCP-1
Scramble siRNA
PPARδ siRNA
C
IL-1β
MCP-1
Scramble siRNA
PPARα siRNA

Figure 6