

Phytochemicals as Modulators of PPARs and RXRs

Guest Editors: Joshua K. Ko, Susanna S. Lee, and Harry Martin





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PPAR Research

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Editorial

Phytochemicals as Modulators of PPARs and RXRs

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Welcome to this special issue of PPAR Research on “Phytochemicals as modulators of PPARs and RXRs.” Plants have played a crucial role in the prevention and treatment of human diseases for thousands of years. A significant number of contemporary drugs with high efficacy are derived from herbal origin. These natural phytochemicals, including certain nutrients, are therefore of great medicinal value.

Peroxisome proliferator-activated receptors (PPARs) belong to the steroid hormone receptor superfamily that bind to and are activated by fatty acids, eicosanoids, and a group of xenobiotics called peroxisome proliferators. Each of these receptors binds to specific peroxisome proliferator response elements (PPRE) as a heterodimer with a retinoid X receptor (RXR). Binding to these receptors has been proven to control the pathological conditions associated with obesity, aging-related diseases, inflammation, immune disorder, cell cycle regulation as well as cancer. There is a strong mechanistic basis for PPAR and RXR targeting, as these nuclear receptors are transcriptional factors that modulate gene expression relevant to the control of blood glucose and lipids, as well as the processes of inflammation and carcinogenesis. Since PPARs play an important role in lipid metabolism, the search for natural ligands had begun with fatty acids and eicosanoids. In fact, the discovery that many natural dietary compounds, especially phytochemicals, are PPAR activators is of great significance for human health. With metabolic syndrome, diabetes, and chronic systemic inflammatory diseases now reaching pandemic proportions, the importance of the discovery of natural PPAR ligands is self-evident.

The special issue begins with a review of terpenoids as modulators of the PPAR family. To date more than 40,000 terpenoids have been described. Also known as isoprenoids, terpenoids constitute one of the largest and most diverse

group of phytochemicals. Members of this phytochemical family are well known to us as the flavours of cinnamon, ginger, and cloves and the scents of camphor and eucalyptus. The vitamin A precursors known as carotenoids and psychoactive cannabinoids are also forms of terpenoid. In this issue, T. Goto and colleagues review the biosynthesis, metabolism, and plant origins of various dietary terpenoids. The physiological effects of these compounds on carbohydrate and lipid metabolism are described in terms of their PPAR γ and PPAR α activating abilities. This is followed by the research article on the kinetic assessment and therapeutic modulation of metabolic and obesity-associated inflammatory profiles by a high-fat diet, whereas the whole process was found to be mediated by PPAR activation. Another research article has proposed glycyrrhizic acid, the bioactive compound extracted from roots of licorice (*Glycyrrhiza glabra*), as a potent PPAR γ agonist. This phytochemical is capable of ameliorating metabolic syndrome by affecting glucose homeostasis, lipid metabolism, and adipogenesis. The focus is then shifted to the findings on verbascoside, a phytochemical that exerts a strong anti-inflammatory activity against the development of experimental inflammatory bowel disease. This work by the S. Cuzzocrea team has proven that verbascoside is a PPAR α ligand by using a knock-out animal model. S. N. Lewis and colleagues conclude this special issue with a comprehensive review of computational approaches to the discovery of phytochemical ligands for PPAR α and PPAR γ . This timely review is an excellent reference document for the existing 3D structures of PPAR receptors cocrystallized with a variety of ligands and available on-line at the RCSB database. Lewis gives an expert description of the potential for *in silico* screening of phytochemical databases. The challenges of applying computational techniques to the analysis of

a flexible ligand binding to a flexible receptor are described. This review is a most helpful exposition of the state-of-the-art computational methods regarding natural PPAR ligand discovery.

Joshua K. Ko
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Review Article

Various Terpenoids Derived from Herbal and Dietary Plants Function as PPAR Modulators and Regulate Carbohydrate and Lipid Metabolism

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Several herbal plants improve medical conditions. Such plants contain many bioactive phytochemicals. Terpenoids (also called “isoprenoids”) constitute one of the largest families of natural products accounting for more than 40,000 individual compounds of both primary and secondary metabolisms. In particular, terpenoids are contained in many herbal plants, and several terpenoids have been shown to be available for pharmaceutical applications, for example, artemisinin and taxol as malaria and cancer medicines, respectively. Various terpenoids are contained in many plants for not only herbal use but also dietary use. In this paper, we describe several bioactive terpenoids contained in herbal or dietary plants, which can modulate the activities of ligand-dependent transcription factors, namely, peroxisome proliferator-activated receptors (PPARs). Because PPARs are dietary lipid sensors that control energy homeostasis, daily eating of these terpenoids might be useful for the management for obesity-induced metabolic disorders, such as type 2 diabetes, hyperlipidemia, insulin resistance, and cardiovascular diseases.

1. Diversity of Terpenoids in Nature

Nature relies on an intricate network of biosynthetic pathways to produce a lot of small organic molecules required to support life. Terpenoids (also called “isoprenoids”) constitute one of the largest families of natural products accounting for more than 40,000 individual compounds of both primary and secondary metabolisms. Most of them are of plant origin, and hundreds of new structures are reported every year [1–3]. All organisms naturally produce some terpenoids as part of primary metabolism, but many produce terpenoids via secondary metabolism.

Isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the universal five-carbon precursors of all terpenoids. After the discovery of the mevalonate (MVA) pathway in yeast and animals, it was assumed that IPP was synthesized from acetyl-CoA via

MVA and then isomerized to DMAPP in all eukaryotes and some Gram-positive prokaryotes [2, 3] (Figure 1). The origins of archeal terpenoids were unknown until recently, when four of the six enzymes have been identified to be present in sequenced genomes [2]. Recently, a nonstandard MVA pathway involving phosphorylation of isopentenyl phosphate has been discovered in *Methanocaldococcus jannaschii* [4]. Before 1993, the MVA pathway was the only known source of terpenoids. After isotope-labeling studies by Rohmer et al. [5], it has been shown that there is an alternate pathway to terpenoids that do not originate from acetyl-CoA. The complete pathway has been finally elucidated in 2002 [6]. This alternative MVA-independent pathway has been named the methylerythritol phosphate (MEP) pathway (Figure 1), which has been identified in both bacteria and plants [2, 3]. Plants use both pathways although they are compartmentalized: MVA to the cytoplasm and

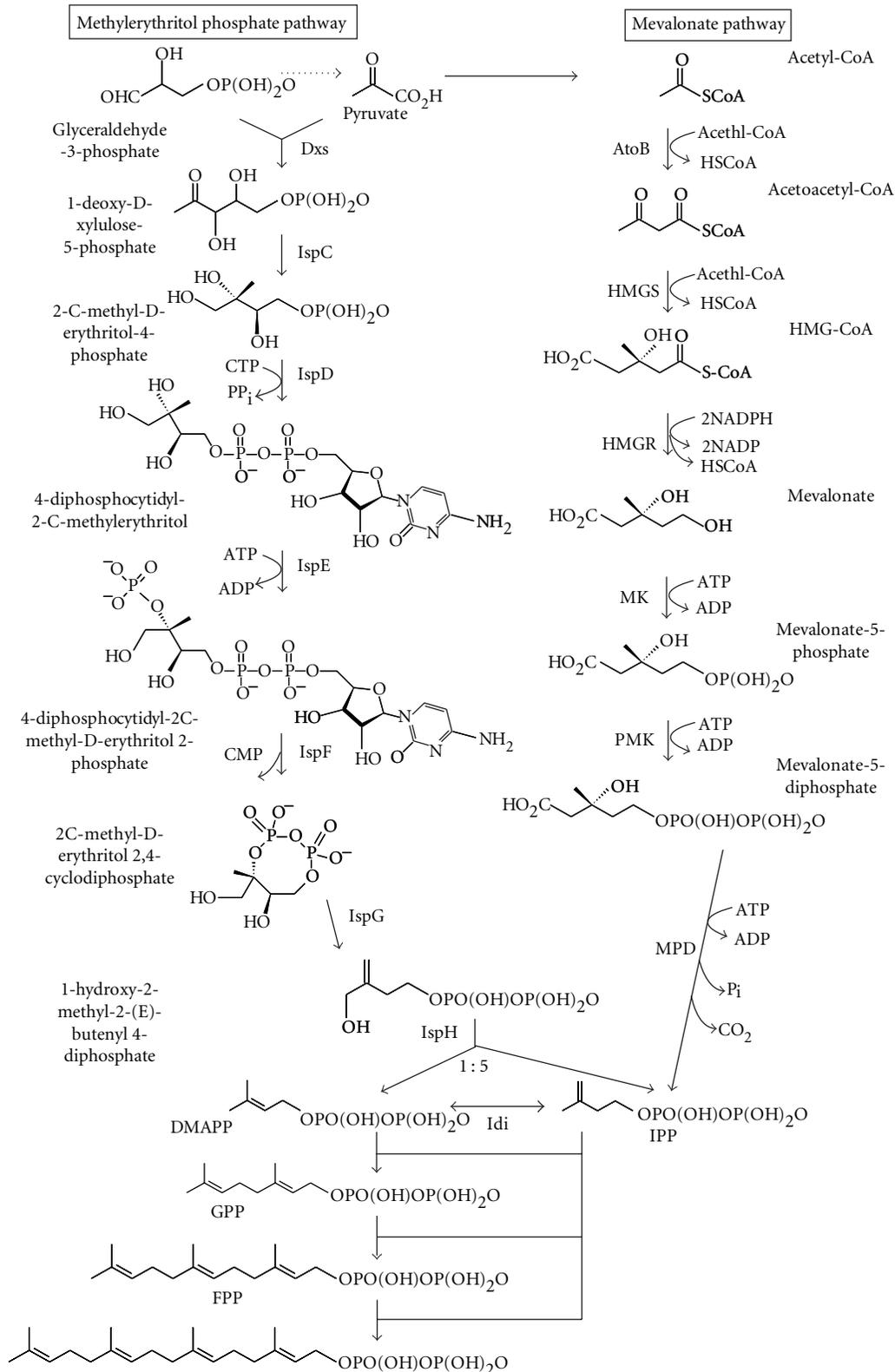


FIGURE 1: Biosynthetic routes to polyprenyl pyrophosphate terpenoid biosynthetic pathways. Dxs: 1-deoxy-d-xylulose-5-phosphate synthase; IspC: 1-deoxy-d-xylulose-5-phosphate reductoisomerase; IspD: 4-diphosphocytidyl-2-C-methyl-d-erythritol synthase; IspE: 4-diphosphocytidyl-2-C-methyl-d-erythritol kinase; IspF: 2-C-methyl-d-erythritol2,4-cyclodiphosphate synthase; IspG: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; IspH: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; AtoB, acetyl-CoA C-acetyltransferase; HMGS: hydroxymethylglutaryl-CoA synthase; HMGR: hydroxymethylglutaryl-CoA reductase; MK: mevalonate kinase; PMK: phosphomevalonate kinase; MPD: mevalonate pyrophosphate decarboxylase; IdI: isopentenyl pyrophosphate isomerase; GPP: geranyl pyrophosphate; FPP: farnesyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate.

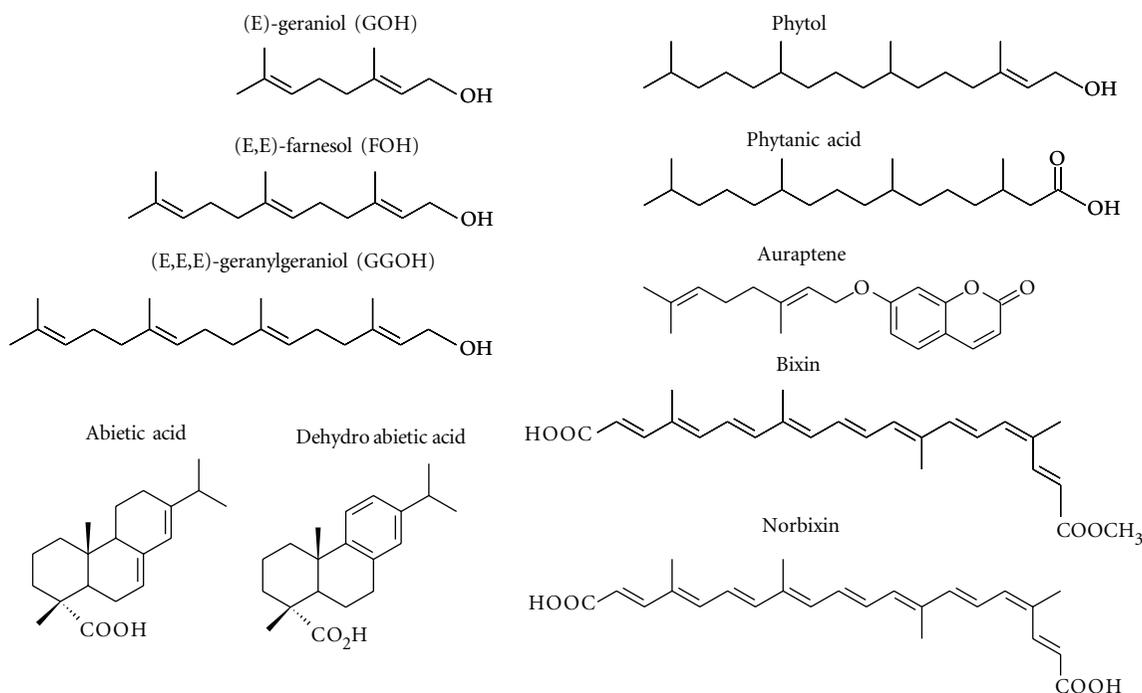


FIGURE 2: Chemical structures described in this paper.

possibly to mitochondria to provide sterols, the side chain of ubiquinone, and sesquiterpenes (C15), and MEP to plastids providing plastidial terpenoids, for example, isoprene (C5), monoterpenes (C10), diterpenes (C20, including gibberellins and the phytol tail of tocopherols and chlorophylls), and carotenoids (C40) [3]. Moreover, there is evidence that a certain degree of crosstalk between the MVA and MEP pathways can occur, implying that these pathways are not completely autonomous [7].

Plants have an enormous capacity to synthesize huge amounts of diverse terpenoids, particularly via the combination of the terpenoid biosynthetic route and other secondary metabolic pathways. For instance, tocopherol biosynthesis occurs as a result of combination of the shikimate and isoprenoid pathways, which lead to homogentisic acid phytol diphosphate, which in combination ultimately lead to the formation of tocopherols (formed by a chromanol head group and a phytol tail) [8].

In addition to universal physiological, metabolic, and structural functions, many specific terpenoids function in various situations, including communication and defense. Members of the isoprenoid group also include industrially useful polymers (e.g., rubber and chicle) and agrochemicals (e.g., pyrethrins and azadirachtin).

It is known that several herbal plants improve medical conditions. Such plants contain many bioactive phytochemicals. In particular, terpenoids are contained in many herbal plants, and several terpenoids have been shown to be available for pharmaceutical applications, for example, artemisinin and taxol as malaria and cancer medicines, respectively. Various terpenoids are contained in many plants for not only herbal medicine use but also dietary use [9].

In this paper, we describe several bioactive terpenoids (Figure 2) contained in herbal or dietary plants, which have the potential to ameliorate metabolic disorders via activation of ligand-dependent transcription factors, namely, peroxisome proliferator-activated receptors (PPARs).

2. PPARs: Therapeutic Targets of Metabolic Syndrome

2.1. Nuclear Receptors. Members of the nuclear receptor superfamily of ligand-dependent transcription factors play a multitude of essential roles in development, homeostasis, reproduction, and immune functions [10–14]. Ligand binding induces a conformational change in nuclear receptors, releasing corepressors in exchange for coactivators. Ligand-activated complexes recruit basal transcriptional machineries, resulting in an enhanced gene expression. Nuclear receptors include the classical steroid hormone receptors (estrogen, progesterone, androgen, glucocorticoid, and mineralcorticoid receptors); “orphan receptors,” which exhibit conserved features of the nuclear receptor family but have not been linked to endogenous ligands; and so-called “adopted orphan receptors,” which were initially identified as orphan receptors but were subsequently linked to endogenous ligands. The adopted orphan receptors include the thyroid hormone receptors, retinoic acid receptors, PPARs, and liver X receptors (LXR). Nearly all members of this family contain a highly conserved DNA-binding domain that mediates sequence-specific recognition of target genes and a C-terminal domain that determines the specific ligand-binding properties of each receptor and mediates ligand-regulated transcriptional activation and/or repression [10].

Nuclear receptors are frequent biological targets of active compounds contained in herbal and dietary plants. This is perhaps not surprising, since nuclear receptors evolved to be regulated by lipophilic molecules derived from diet and the environment [15, 16]. At least ten of these receptors have been shown to be directly activated by compounds purified from herbal remedies [16]. Some compounds have a complex pharmacology; for example, grapeseed-derived resveratrol is a ligand of estrogen receptors and PPARs [17, 18] but has also been suggested to activate sirtuin 1 (SIRT1), an NAD⁺-dependent protein deacetylase enzyme implicated in the biology of aging [19]. Other phytochemicals target multiple nuclear receptors [16].

2.2. PPARs. PPARs are members of the nuclear receptor superfamily, which are activated by fatty acids and their derivatives. PPARs are dietary lipid sensors that regulate lipid and carbohydrate metabolism [20]. In mammals, three subtypes of PPAR, α , δ , and γ , were found [21]. PPARs form heterodimers with retinoid-X-receptors (RXRs) and bind to consensus DNA sites composed of direct repeats (DRs) of hexameric DNA sequences (AGGNCA) separated by 1 bp (DR1). In the absence of ligands, PPAR-RXR heterodimers recruit corepressors and associated histone deacetylases and chromatin-modifying enzymes, silencing transcription by so-called active repression [22–24]. Ligand-binding induces a conformational change in PPAR-RXR complexes, releasing repressors in exchange for coactivators. Ligand-activated complexes recruit the basal transcriptional machinery, resulting in an enhanced gene expression.

PPAR α is highly expressed in the liver, cardiac muscle, and digestive tract, and regulate the expression of target genes involved in lipid catabolism. Activators of PPAR α , such as fibrates, decrease circulating lipid levels and are commonly used to treat hypertriglyceridemia and other dyslipidemic states [25]. PPAR δ is expressed in many tissues including the skeletal muscle and brown adipose tissue. Recently, it has been suggested that PPAR δ activation attenuates obesity and type-2 diabetes [26, 27]. PPAR γ is abundant in adipose tissues functioning as the key transcription factor for adipogenesis. Synthetic ligands for PPAR γ , such as thiazolidinediones, are increasingly used to treat type-2 diabetes [28].

PPARs are involved in not only energy homeostasis but also inflammation. PPAR α regulates inflammatory processes, mainly by inhibiting inflammatory gene expression. In recent years, several molecular mechanisms responsible for the immunosuppressive effects of PPAR α have been uncovered [29]. These include interference with several proinflammatory transcription factors [30]. The number of studies that have addressed the role of PPAR δ during inflammation is limited. So far, an anti-inflammatory effect has been observed in macrophages suggesting a possible role for PPAR δ in the process of atherogenic inflammation. [31]. Similar to PPAR α , PPAR γ is involved in governing inflammatory response, particularly in macrophages. Currently, two different molecular mechanisms have been proposed by which anti-inflammatory actions of PPAR γ are effectuated:

(1) via interference with proinflammatory transcription factors [32] and (2) by preventing removal of corepressor complexes from gene promoter regions resulting in suppression of inflammatory gene transcription [33].

Recently, it has been indicated that obesity is associated with a low-grade chronic inflammation state [34]. The inflammatory condition in obesity is increasingly being recognized as an important contributor to the development of metabolic syndrome and its associated complications. Adipocytes can secrete cytokines involved in inflammation, such as adiponectin, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor- α (TNF- α) [35]. MCP-1, a member of the CC chemokine superfamily, plays a pivotal role in monocyte/macrophage trafficking and activation [36]. Macrophages also produce various proinflammatory factors including MCP-1 and TNF- α . Macrophage-derived TNF- α establishes a vicious cycle that augments inflammatory changes and insulin resistance in obese adipose tissues [37]. Therefore, to prevent obesity-related inflammation, it is important to decrease the production of obese-adipose-tissue-derived proinflammatory factors such as MCP-1 and TNF- α .

Several herbal and dietary plants improve medical conditions including diabetes mellitus, hyperlipidemia, and cardiovascular disease associated with an abnormality of lipid metabolism [38, 39]. To screen for novel natural ligands for PPARs, we have evaluated PPAR ligand activities for various terpenoids in an advanced highly sensitive system with the coexpression of a coactivator for nuclear receptors, the cAMP-response element-binding protein (CREB)-binding protein (CBP), developed by modifying the luciferase reporter assay system [40]. Hereinafter, we describe several terpenoids, identified as novel PPAR ligands, in our PPAR ligand screening.

3. Novel Functions of Dietary Terpenoids as PPAR Ligands

3.1. Isoprenols. We carried out screening for novel PPAR ligands in natural compounds contained in medicinal plants. We used several terpenoids including carotenoids and polyisoprenoid alcohols (isoprenols) for the screening, because these compounds are contained in many medicinal and dietary plants [41]. These terpenoids have multifunctions such as the suppression of tumor proliferation [41–43], apoptosis-inducing activity [9], and cation channel regulation [44]. Some terpenoids, which are intermediates in cholesterol synthesis, regulate the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol synthesis by controlling the degradation of the enzyme [45, 46]. Such functions of dietary terpenoids are significant for the trials to manage disease conditions such as cancers or cardiovascular diseases using food factors.

As shown in Figure 3, several terpenoids activated PPAR γ at a concentration of 50 or 100 μ M [40]. In this assay, we identified that isoprenols, such as geraniol, farnesol, and geranylgeraniol (chemical structures are shown in Figure 2) have a potential to activate PPAR γ as novel ligands. At

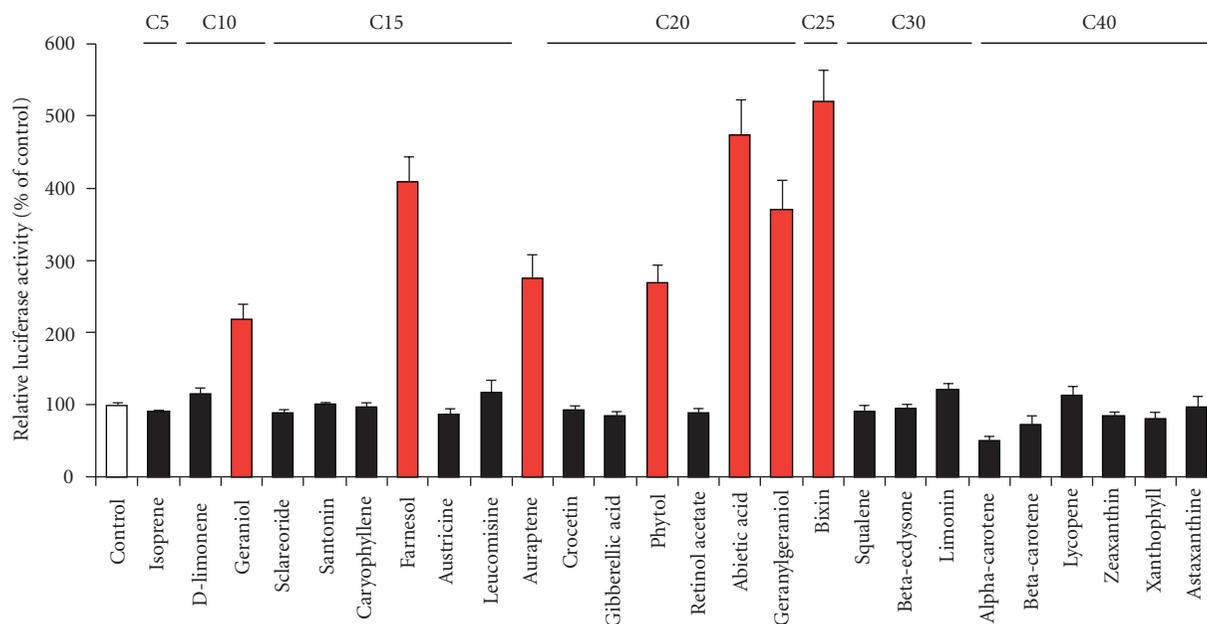


FIGURE 3: Effects of various terpenoids on the activation of PPAR γ ligand and chemical structures of active terpenoids. PPAR γ activity in monkey CV-1 kidney cells was determined by luciferase reporter assay using advanced highly sensitive GAL4/PPAR γ chimera system described in [40]. After 24 hours of incubation with or without each terpenoid at 50 or 100 μ M, luciferase activity was measured. The activity of a vehicle control was set at 100% and the relative luciferase activities are presented as fold induction compared with that of the vehicle control. The values are means \pm S.E.M. of 3-4 replicates. B: Chemical structures of active terpenoids.

100 μ M, the activations by geraniol, farnesol, and geranylgeraniol were 2.2-, 4.1-, and 3.7-fold that by the vehicle control, respectively. On the other hand, squalene had no effect on PPAR γ transactivation. Although geraniol had no effect on PPAR α , farnesol and geranylgeraniol also dose-dependently activated PPAR α (10- and 8.6-fold increases at a concentration of 100 μ M compared with vehicle controls, respectively) in the PPAR α ligand assay system [40]. These activities were so potent and nearly the same as that of 10 μ M fenofibrates, one of the fibrates (antihyperlipidemia drugs) used as a positive control for PPAR α . In this regard, these farnesol and geranylgeraniol isoprenols have the effects of dual activation of PPAR γ and PPAR α .

PPAR γ activation in adipose tissues results in the improvement of insulin resistance [46] and PPAR α activation in the liver induces the lowering of circulating lipid levels [47]. These effects are due to the regulation at mRNA expression levels of target genes of PPARs. The addition of each isoprenol induced mRNA expression of PPAR target genes in 3T3-L1 adipocytes and HepG2 hepatocytes [40]. Therefore, it is possible that isoprenols could regulate insulin resistance and/or circulating lipid levels. The finding of the dual activation of PPAR γ and PPAR α by isoprenols is very important for thinking of the mechanisms understanding the effects of medicinal plants and valuable for the management of diabetic and hyperlipidemic conditions in herbal medicine. Indeed, in our preliminary study, farnesol ameliorated hyperglycemia in high fat diet-fed wild-type mice but not in PPAR α deficient mice (Goto et al., unpublished data). These findings indicate that improvement of obesity-associated metabolic disorders

by farnesol is mainly dependent on PPAR α activation. These results provide not only a significant molecular basis on how herbal plants containing phytochemicals such as isoprenols induce the improvement of diabetes or hyperlipidemia, but also possibilities that phytochemicals might have therapeutic applications in lipid abnormalities, such as obesity, diabetes mellitus, and hyperlipidemia.

3.2. Phytol. Phytol, a diterpene alcohol, which is a carbon side chain of a chlorophyll molecule (Figure 2), is involved in the production of energy from light. Phytol is a plastidial terpenoid and synthesized via the MEP pathway in plastids [48]. Since almost all photosynthetic organisms use chlorophylls, phytol is also abundantly present in nature including various vegetables. It is suggested that chlorophyll molecules are partially digested and the phytol moiety is released in animals [49]. Then, the released phytol is absorbed in the small intestine and converted to phytanic acid in the liver.

Phytanic acid is a branched-chain, terpenoid-derived fatty acid constituent of diet (Figure 2). In surveys of phytanic acid content of a variety of food products, high levels were indeed found in products such as milk, butter, cheese, meat from cows, sheep, and some fish and fish oils, whereas no phytanic acid is present in vegetables [50]. This compound can also be produced from the conversion of dietary phytol in the body [51]. Phytanic acid has been reported to activate PPAR γ and the retinoid-X-receptor (RXR) [52, 53] so that differentiation is stimulated in both white and brown adipocytes [52]. In addition, phytanic acid stimulates PPAR α to regulate lipid metabolism in some types

of cell [54]. Therefore, the intake of phytol as a precursor of phytanic acid may be valuable for the management of lipid metabolism through the activation of PPARs. Indeed, a phytol-enriched diet increased plasma and hepatic levels of phytanic acid, and induced the mRNA expression of PPAR α target genes involved in peroxisomal and mitochondrial β -oxidation and fatty acid metabolism [55].

However, since the conversion of phytol into phytanic acid is not very rapid, a phytol-enriched diet also induced accumulation of phytol in the liver [56]. Moreover, as described previously, several terpenoids, which resemble phytol in structures, activate PPARs in adipocytes and hepatocytes [40]. In this sense, it must be valuable to analyze the effects of phytol itself as an activator of PPARs.

Therefore, we evaluated the effects of phytol on PPAR activity using our advanced highly sensitive luciferase assays (Figure 3). Among the PPAR isoforms, PPAR α was activated the most markedly following the addition of phytol [57]. The effects of phytol on PPAR α activation were larger than those of phytanic acid under our experimental conditions. Phytol induced the mRNA and protein expression of PPAR target genes in a manner dependent on the level of PPAR α expression in HepG2 hepatocytes. In our in vitro coactivator recruiting assay, it was revealed that phytol can activate PPAR α directly [57]. These findings indicate that phytol itself can directly bind to PPAR α as its ligand.

Because the activation of PPAR α is one of the most important factors in lipid metabolism in peripheral tissues including the liver and muscles, the ability of phytanic acid and phytol to activate PPAR α is very important in the management of lipid metabolism using food factors. Induction of PPAR α target gene expression in mice fed a phytol-enriched diet [55] is likely due to not only phytanic acid but also phytol. It is considered that such effects of phytol are valuable for the control of lipid abnormalities in common diseases including obesity, diabetes, and hyperlipidemia through PPAR α activation in the liver.

3.3. Abietic Acid Derivatives. The amount of variety of hydrocarbons and their derivatives used in industrial and commercial activities has been increasing over the years. Abietic acid is a tricyclic-diterpene carboxylic acid (Figure 2), and is the main component of the rosin fraction of oleoresin synthesized by conifer species, such as grand fir (*Abies grandis*) and lodgepole pine (*Pinus contorta*) [58]. Abietic acid is commonly used as a fluxing agent in solder, as a paper sizing agent to make paper more water resistant, and in printing inks, adhesives, and plasticizers [59]. Moreover, it has been reported that abietic acid is a bioactive compound and it has an anti-inflammatory effect. In lipopolysaccharide (LPS)-stimulated macrophages, abietic acid suppresses production of prostaglandin E2 (PGE2) in vitro and in vivo [60].

To investigate whether the activation of PPARs is related to the anti-inflammatory effects of abietic acid and its derivatives, we evaluated the effects of abietic acid and its derivatives on PPAR activity (Figure 2). Abietic acid and dehydroabietic acid, one of major components of colophony (also known as Rosin and pine resin), potently activated

both PPAR α and PPAR γ but not PPAR δ [61, 62]. Similarly to thiazolidinedione, a synthetic PPAR γ ligand, abietic acid suppressed mRNA expressions of TNF- α and cyclooxygenase 2 (COX2), which are induced in inflammatory reactions, in LPS-stimulated macrophages [61]. Dehydroabietic acid stimulated PPAR α and PPAR γ more potently than abietic acid [62]. Dehydroabietic acid significantly suppressed the production of proinflammatory mediators such as MCP-1, TNF- α , and NO in LPS-stimulated macrophages and in the coculture of macrophages and adipocytes [62]. In obese diabetic KK-Ay mice, dietary dehydroabietic acid suppressed obesity-associated elevation of circular MCP-1 and TNF- α levels and their mRNA expressions in white adipose tissues. Moreover, dehydroabietic acid improved carbohydrate and lipid metabolism [63]. These findings indicate that the anti-inflammatory effects of abietic acid and dehydroabietic acid are at least partly due to the activation of PPARs. Additionally, it is suggested that these compounds can be used not only for anti-inflammation but also for regulating carbohydrate and lipid metabolism and atherosclerosis.

3.4. Auraptene. Citrus-fruit-derived compounds have many beneficial bioactivities (e.g., anticarcinogenic, antihypertension, and antiscavenger disease effects) [64, 65]. Through our screening for PPARs ligands (Figure 2), we identified auraptene, a geraniol coumarin ether, as a novel PPAR α and PPAR γ ligand [66, 67]. Auraptene (Figure 2) occurs in a variety of citrus fruits. It has been reported that auraptene has anti-inflammatory and anticarcinogenic activities. In cultured adipocytes, auraptene upregulated an antiatherosclerotic, antidiabetic, and anti-inflammatory cytokine, adiponectin, and downregulated a proinflammatory cytokine, MCP-1. These effects disappeared in the presence of GW9662, a PPAR γ antagonist [66], suggesting that auraptene improves adipocytokine profiles via the activation of PPAR γ . In addition, mRNA expressions of several PPAR α target genes involved in FA catabolism, were also induced in PPAR α -expressing HepG2 hepatocytes by auraptene treatment [67]. It is likely that auraptene regulates the mRNA expressions of both PPAR γ and PPAR α target genes as a dual agonist, and these activities might contribute to the anti-cardiovascular disease effect of citrus fruits.

3.5. Bixin. Annatto obtained from the pericarp of seeds from *Bixa orellana* is a natural pigment extensively used in many processed foods [68, 69]. Bixin and norbixin (Figure 2), which are carotenoids, are the main components of this pigment, and have been reported to possess antioxidative and anticarcinogenic effects [70–72]. Furthermore, it has also been indicated that both annatto extract and norbixin have hypoglycemic effects in nonobese dogs and mice, respectively [73, 74]. In PPAR γ ligand assay, both bixin and norbixin activated PPAR γ (Figure 2), and bixin induced PPAR γ target genes in 3T3-L1 adipocytes, resulting in the promotion of adipocyte differentiation and insulin-stimulated glucose uptake [75]. Therefore, the hypoglycemic effects of annatto and norbixin might be caused by the activation of PPAR γ .

4. Conclusions

In this paper, we mentioned the diversity of terpenoids, functions of PPARs, and several terpenoids activating PPARs. The prevalence of obesity worldwide has progressively increased over the past decades. This unabated rise has spawned proportionate increases in obesity-associated metabolic disorders. Currently, synthetic PPAR agonists are widely used for the treatment of metabolic disorders. Daily intake of dietary terpenoids, which activate PPARs as we described above, may be valuable for the control of carbohydrate and lipid disorders. Dietary patterns rich in vegetables and fruit are associated with a lower prevalence of metabolic syndrome [65, 76]. Because most of the terpenoids are of plant origin and they are contained in vegetables and fruit, dietary terpenoids may contribute to a decrease in risk of metabolic syndrome. Moreover, because the terpenoids constitute one of the largest families of natural products, more potent and useful PPAR activators may exist.

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Research Article

Kinetic Assessment and Therapeutic Modulation of Metabolic and Inflammatory Profiles in Mice on a High-Fat and Cholesterol Diet

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The kinetics of metabolic and inflammatory parameters associated with obesity were evaluated in a murine diet-induced obesity (DIO) model using a diet high in fat and cholesterol. Cellular infiltration and mediator production were assessed and shown to be therapeutically modulated by the PPAR γ agonist rosiglitazone. C57BL/6 mice were maintained on a 45% fat/ 0.12% cholesterol (HF/CH) or Chow diet for 3, 6, 16, or 27 weeks. Flow cytometry was employed to monitor peripheral blood monocytes and adipose tissue macrophages (ATM). Gene expression and protein analysis methods were used to evaluate mediator production from total epididymal fat (EF), stromal vascular fraction (SVF), and sorted SVF cells. To investigate therapeutic intervention, mice were fed a HF/CH diet for 12 weeks and then a diet formulated with rosiglitazone (5 mg/kg) for an additional 6 weeks. A HF/CH diet correlated with obesity and a dramatic proinflammatory state. Therapeutic intervention with rosiglitazone attenuated the HF/CH induced inflammation. In addition, a novel population was found that expressed the highest levels of the pro-inflammatory mediators CCL2 and IL-6.

1. Introduction

In a Western society, where diets are generally high in fat and cholesterol, 33% of adults and 17% of children have been classified as obese [1, 2]. Obesity and its associated disorders, including insulin resistance, type II diabetes, nonalcoholic fatty liver disease, and atherosclerosis, are growing medical concerns [3]. Chronic low-grade inflammation is proposed as an important link between obesity and its associated pathologies [1, 3, 4]. Data from both humans [5, 6] and mice [6–9] have shown a positive correlation between increasing BMI body mass index (BMI) and hyperinsulinemia, and macrophage content in the adipose tissue.

In both human [6, 10, 11] and mouse [6, 8, 9, 12–14], various studies have shown an inflammatory reaction in adipose tissue characterized by macrophage accumulation and increased levels of mediators including TNF α , IL-1 β , IL-6, IL-10, and MCP-1. Macrophages are proposed to be a major source of pro-inflammatory mediators, and the current dogma suggests the existence of two subsets of murine adipose tissue macrophages (ATM). Pro-inflammatory macrophages (PI-ATM) express TNF α , IL-1 β , IL-4, IL-6, CCL2, and CCR2, and the alternatively activated/resident macrophages (R-ATM) express IL-10, ApoE, and arginase [6, 8, 9, 12–14]. In contrast, human ATMs have been reported to have an alternatively activated surface

phenotype, yet secrete both pro- and anti-inflammatory mediators. [11, 15, 16].

Currently, most of the murine models of obesity used to study the inflammatory component of obesity have employed diets rich in fat only. However, humans often consume diets that are rich in cholesterol as well as fat that are associated not only with obesity but also with two comorbidities of obesity such as nonalcoholic fatty liver disease (NAFLD) and atherosclerosis both of which are also associated with underlying chronic low grade inflammation [3, 4, 17–19]. NAFLD is also associated with insulin resistance [17]. It has been reported previously that feeding mice a high-fat and cholesterol (HF/CH) diet has been shown to induce obesity as well as elevate systemic levels of alanine aminotransferase (ALT) and cause liver damage, indicative of nonalcoholic fatty liver disease (NAFLD) [2]. Our goal was to define the impact of a high-fat and cholesterol diet in the DIO model on inflammatory and metabolic parameters by profiling mediator production and by monitoring the modulation of peripheral and adipose tissue immune cells. Although it has been shown in both rodents and humans that diet and/or exercise can modulate certain inflammatory parameters [20–23], in some cases pharmacologic intervention may still be necessary. Therefore, the second aim was to determine the utility of the model for discovery of type II diabetes and metabolic syndrome drugs by evaluating a known clinical glycemic modulating drug, rosiglitazone. Recent studies have shown that in addition to modulating glycemic levels, rosiglitazone also has immunomodulatory activity in adipose tissue [8, 24–27].

The manuscript describes the kinetics of multiple inflammatory parameters associated with the development of obesity. Specifically, we assessed the kinetics of blood monocytes and ATMs from DIO mice, and the modulation of these leukocytes by rosiglitazone. Furthermore, we report on the identification of two novel adipose tissue cellular infiltrates capable of producing inflammatory mediators.

2. Materials and Methods

2.1. Model Establishment. C57Bl/6 mice (Charles River) at 6-weeks of age were placed either on a high-fat diet consisting of 45% fat and 0.12% cholesterol (D0401280, Research Diets Inc, New Brunswick, NJ) or a Chow diet (PicoLab 5053, Lab Diet) (Table 1). The high-fat diet from Research Diets Inc. is built on their basic diet D12451 with the protein content at 20% kcal, the carbohydrates 35% kcal, and the fat 45% kcal. The fatty acid profile of this diet is 36.3% saturated, 45.3% monosaturated, and 18.5% polyunsaturated fat. The diet intake per mouse per day is ~3 grams or 14.16 kcal per day (4.72 kcal/gm * 3 grams). Animals were on a 12:12 light dark cycle and given food ad libitum. Experiments were conducted under our institutional guidelines for the humane treatment of laboratory animals.

Metabolic parameters and flow cytometric analysis were assessed on two separate cohorts of animals from the same study. All animals were analyzed for changes in body weight (BW), epididymal fat pad mass, and total fat and lean mass.

TABLE 1: Composition of rodent diets.

	Chow diet	HF/CH diet
Ingredients (% kcal)		
Protein	24.7	20
Carbohydrates	62.1	35
Fat	13.2	45
Total	100	100
kcal/gm	3.85	4.72
Total cholesterol (% of diet)	0	0.12

Chow: Cat # 5053 PicoLab Rodent Diet 20.

HF/CH: high-fat/cholesterol diet; Cat # D04012801 Research Diets Inc.

Total fat and lean mass were determined 1 week prior to each termination time point using the EchoMRI-100 for mice (ECHOMRI, Echo Medical Systems, LLC Houston TX).

Rosiglitazone was formulated at 5 mg/kg into the diet calculated on the basis of predetermined food intake per mouse (~3 grams). The dose of 5 mg/kg was chosen based on in-house data demonstrating significant improvement in insulin sensitivity as assessed by insulin tolerance test (ITT). Animals were fed an HF/CH diet for 12 weeks before being switched to the HF/CH diet with drug for an additional 6-weeks. Food was available ad libitum for the mice. Insulin and leptin levels were measured prior to initiation of drug dosing and then again at termination.

2.2. Metabolic Parameters. Serum samples were collected into T-MG Capiject tubes (Terumo Medical Corp, Elkton, MD) via cardiac puncture. Serum samples were analyzed using MesoScale Diagnostics assays (Meso Scale Discovery, Gaithersburg, MD): pro-inflammatory mediator kit (Cat # K11012b-2), adiponectin kit (Cat # K112BXC-2), and a custom metabolic multiplex (IL-6, GM-CSF, insulin, leptin, resistin, and TNF α). Insulin and leptin were nonfasting levels measured at termination. ALT was assessed by an ALT enzymatic assay (Catachem # C164-02 Bridgeport, CT).

The epididymal fat pads were collected in KHB buffer (Krebs-bicarbonate-HEPES, pH7.4) (Celsis In Vitro Technologies, Chicago, IL) containing 4% essentially fatty acid-free bovine serum albumin (Sigma-Aldrich, St. Louis, MO) 5 mM glucose (Sigma-Aldrich), and 1 X Penicillin-Streptomycin (Invitrogen Corporation, Carlsbad, CA). The tissue was digested with the described buffer using 1 mg/ml collagenase type 1 (Worthington Biochemical Corp. Lakewood, NJ) with 0.1 mg/ml DNase-1 (Sigma-Aldrich) and then passed through a 250 μ m nylon mesh (Small Parts, Inc., Miramar, FL) and a 100 μ m cell strainer (BD Biosciences, Bedford MA). After centrifugation, the pellet containing the SVF was resuspended in RPMI 1640 (Invitrogen) containing 10% FBS (Gemini Bio-Products, West Sacramento, CA), and penicillin/streptomycin (Invitrogen), and washed twice. 1×10^5 cells were added per well in a 48 well tissue culture plate and incubated at 37°C, 5% CO₂ for 24 hours. Culture supernatants were analyzed using MesoScale Diagnostics multiplex kits (Meso Scale Discovery, Gaithersburg, MD). Statistical analysis was determined by use of the Mann-Whitney *U* test.

2.3. Analysis of Blood and SVF Subpopulations by Flow Cytometry. Epididymal fat was excised and weighed. The SVF was isolated from the epididymal fat using a 45 minute collagenase incubation according to a previously published method [12] and personal communication. Blood was collected from mice into T-MQK Capiject tubes (Terumo Medical Corp) via cardiac puncture. SVF cells or blood leukocytes were blocked with CD16/CD32 (clone 2.4G2, BD Biosciences) and stained with antibodies. For cell sorting, SVF cell suspensions were lysed using RBC Lysis buffer (eBiosciences San Diego, CA) prior to staining; otherwise whole blood and SVF cells were stained and then lysed with BD FACS Lysing solution (BD Pharmingen). Monoclonal antibodies to the following mouse surface antigens were purchased from BD Biosciences CA: Ly6G (1A8), CD11c (HL3), CD3 (145-2C11), B220 (RA-6B2), NK1.1 (PK136), CD45 (30-F11), CD90.2 (53-2.1), CD11b (M1/70), Ly6C (AL-21). Additionally, monoclonal antibodies used were CD62L (clone MEL-14 Invitrogen) and F480 (clone BM8 Ebioscience). Events were acquired on a Becton Dickinson FACSAria and analyzed with FACSDiva Software (BD Biosciences). Sorted cells were collected, pelleted, and stored at -80°C . SVF and blood cells were enumerated with the BD TruCount protocol (Cat # 340498), utilizing the nuclear dye (Control reagent B), and adding antimouse CD45 (BD Biosciences; 30-F11); human antibodies in the kit did not interfere with the detection of mouse CD45 positive leukocytes. Samples were acquired until 4000 beads were collected, and leukocytes were detected by DNA content and CD45.

2.4. RNA Isolation from Epididymal Fat Pad and Quantitative RT-PCR. Epididymal fat tissues were snap-frozen in liquid nitrogen and stored at -80°C . Frozen tissue pieces (<100 mg) were placed in 1 ml of Qiagen QIAzol lysis reagent (Cat # 79306) containing one sterile 5 mm stainless steel ball, and homogenized using two 1-minute cycles at 20 Hz on a TissueLyser (Qiagen, Valencia CA). Total RNA was isolated using Qiagen RNeasy Lipid Tissue mini kit (Cat # 74804) using the additional DNase step according to manufacturer's protocol. RNA was eluted from the column in 60 μl RNase free water and quantified using Nanodrop 1000 v3.3.0 software. cDNA was synthesized using Applied Biosystems High Capacity cDNA reverse transcriptase kit (Cat # 4368813) and quantitated with an 18S standard curve using a TaqMan gene expression assay. Quantitative real-time PCR utilized a custom made TaqMan Low Density Array (TLDA) from Applied Biosystems. 100 ng cDNA was loaded in a 100 μl volume for each port of the TLDA plates. Thermal cycling was performed using an ABI Prism 7900 HT Sequence Detection System and data was analyzed using SDS v2.2 software. The Ct value of each gene is normalized to 18S to obtain ΔCt .

2.5. RNA Isolation and Quantitative PCR for Sorted Cell Subsets. Total RNA was prepared using the RNAqueous-Micro kit (Ambion, Austin, TX) according to the manufacturer's instructions. TaqMan primers and probes (see Supplementary Table 1 available at doi:10.1155/2010/970164) were designed with Universal Probe Library Assay Design software

(Roche, Indianapolis, IN) and purchased from IDT or from Roche, respectively. The PCR reactions were prepared using the components from the iScript Custom One-Step RT-PCR Kit with ROX and assembled according to the manufacturer's instructions (Quanta BioSciences, Gaithersburg, MD). The final concentrations of the primers and probe in the PCR reactions were 200 nM and 100 nM, respectively. The fluorogenic probes were labeled with 6-carboxyfluorescein (6FAM) as the reporter and a nonfluorescent quencher. Each 10 μl PCR reaction contained 2 μl (10 ng) of total RNA. Thermal cycling was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the following protocol: one ten-minute cycle at 50°C , followed by one five-minute cycle at 95°C , followed by forty fifteen-second cycles at 95°C and a final one-minute cycle at 60°C . A eukaryotic 18S rRNA endogenous control probe/primer set (ABI) was used as an internal control for RNA quality. Absolute quantitation of the amount of mRNA in a given sample was determined using a 12-point standard curve generated with 4-fold serial dilutions, starting at 20000 fg, of cDNA containing the gene of interest.

3. Results

3.1. Kinetic Analysis of the Metabolic Parameters in Mice on an HF/CH Diet. DIO mice became obese after consuming a diet containing 45% fat and 0.12% cholesterol (HF/CH), with the body weight (BW) significantly increased by 3 weeks. By 27-weeks the BW was 120% above their original weight (Figure 1(a)). Similar to body weight, total fat mass (Figure 1(c)) increased steadily. Epididymal fat (EF) mass was significantly elevated early and peaked by 6-weeks (Figure 1(b)) but then declined following a trend similar to other reported data [28] and suggestive of a shift in fat depots. No significant change in lean mass (Figure 1(d)) relative to the Chow cohort was detected. Elevated levels of leptin, insulin, and ALT, an indicator of liver damage, were evident by 6-weeks and remained elevated in the DIO mice compared to the control group at each time point. Serum levels of CCL2 and IL-6 were significantly elevated at 16-weeks, while trending upwards at 6-weeks. Nonsignificant increases in IL-10 were also detected from 6-weeks throughout the study. A HF/CH diet had a minimal effect on serum IL-1 β and TNF α levels (Table 2).

3.2. Progressive Infiltration of Pro-Inflammatory ATMs. We employed a detailed flow cytometric gating strategy (Figure 2(a)) to evaluate adipose tissue macrophages. Total leukocytes were identified by CD45. In DIO mice, the proportion of these cells increased to $\sim 30\%$ by 3 weeks (Figure 2(b)). Total macrophages were identified as CD45+F480+CD11b+ cells after electronic removal of lymphocytes identified by cell size and the surface antigens CD3, B220, NK1.1, and Thy1.2. The proportion of macrophages was significantly increased in DIO mice by 6-weeks and peaked by 16-weeks (Figure 2(c)). The absolute numbers per mg of fat of both total leukocytes and macrophages progressively increased in the DIO cohort from 16 to 27-weeks. In control mice, there

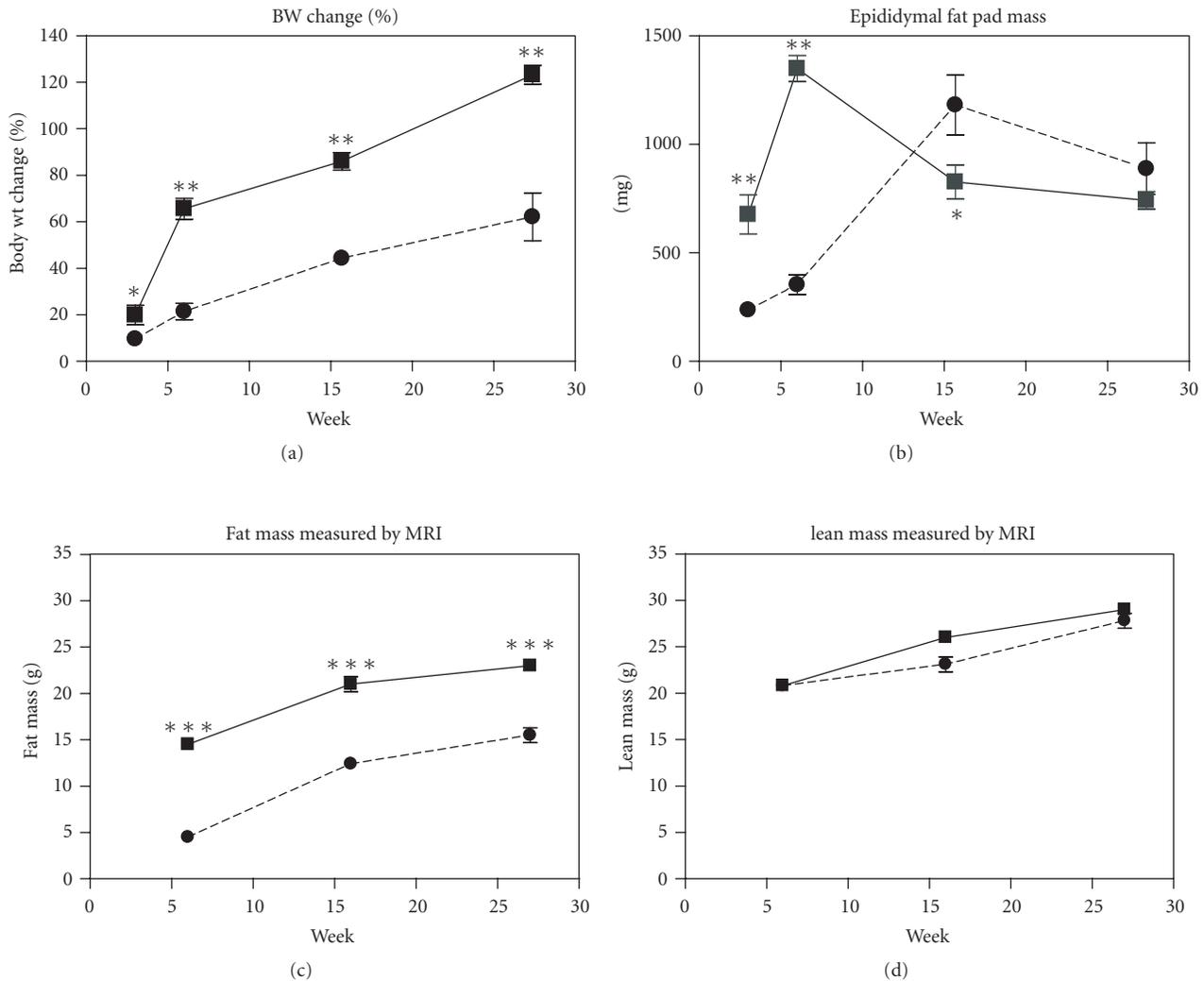


FIGURE 1: An HF/CH diet kinetically induced metabolic obesity. C57BL/6 mice were placed on Chow (circles and dashed lines) or a diet of 45% fat and 0.12% cholesterol (squares and solid line) for 3, 6, 16, or 27-weeks. Groups of $n \geq 7$ were sacrificed at each time point for both chow and HF/CH diets. Body weight (a), mg of epididymal fat (b), total fat (c), and lean (d) mass were all measured. Epididymal fat tissue and blood from these animals were subsequently used for FACS analysis. Values represent the mean and SEM. Unpaired T test was used to compare Chow to DIO (HF/CH diet cohort) at each time point; * $P < .05$, ** $P < .001$, and *** $P < .0001$.

TABLE 2: Elevated serum metabolic parameters and inflammatory mediators in the DIO cohort.

Parameter	6-weeks		16-weeks		27-weeks	
	CHOW	DIO	CHOW	DIO	CHOW	DIO
Insulin, pg/ml	57.8 (41)	383.4 (76)*	710.9 (145)	2901.0 (716)*	1458 (446)	2602.5 (289)*
ALT, U/ml	23.0 (1.1)	29.5 (2.0)*	29.1 (5.3)	174.2 (40.9)*	22.9 (1.9)	82.7 (11.6)*
Leptin, ng/ml	0.69 (0.30)	58.8 (14.6)*	7.1 (1.5)	32.9 (12.5)*	4.6 (1.2)	57.0 (12.6)*
CCL2, pg/ml	0	4.3 (1.1)	18.9 (2.4)	42.1 (3.4)*	168.6 (21)	234.7 (14.4)*
IL-6, pg/ml	0	3.5 (0.6)	9.6 (0.7)	25.5 (7.7) [#]	32.7 (11.2)	54.1 (17)
TNF, pg/ml	15.9 (2.1)	19.9 (2.3)	13.2 (0.4)	13.2 (0.5)	1.6 (0.7)	1.5 (0.3)
IL-1b, pg/ml	15.9 (2.1)	19.9 (2.3)	34.8 (1.4)	37.4 (1.5)	0.9 (0.2)	1.1 (0.2)
IL-10, pg/ml	21.8 (9.2)	33.1 (6.8)	39.1 (1.8)	66.8 (16.4)	22.1 (7.3)	69.8 (33)

At each timepoint, mice from separate groups of animals were terminally bled (Chow $n = 8-13$ mice and the DIO $n = 7-18$ mice). Values represent the mean and SEM. Mann-Whitney U test was used to compare Chow to DIO at each time point; * $P < .05$ and [#] $P < .08$.

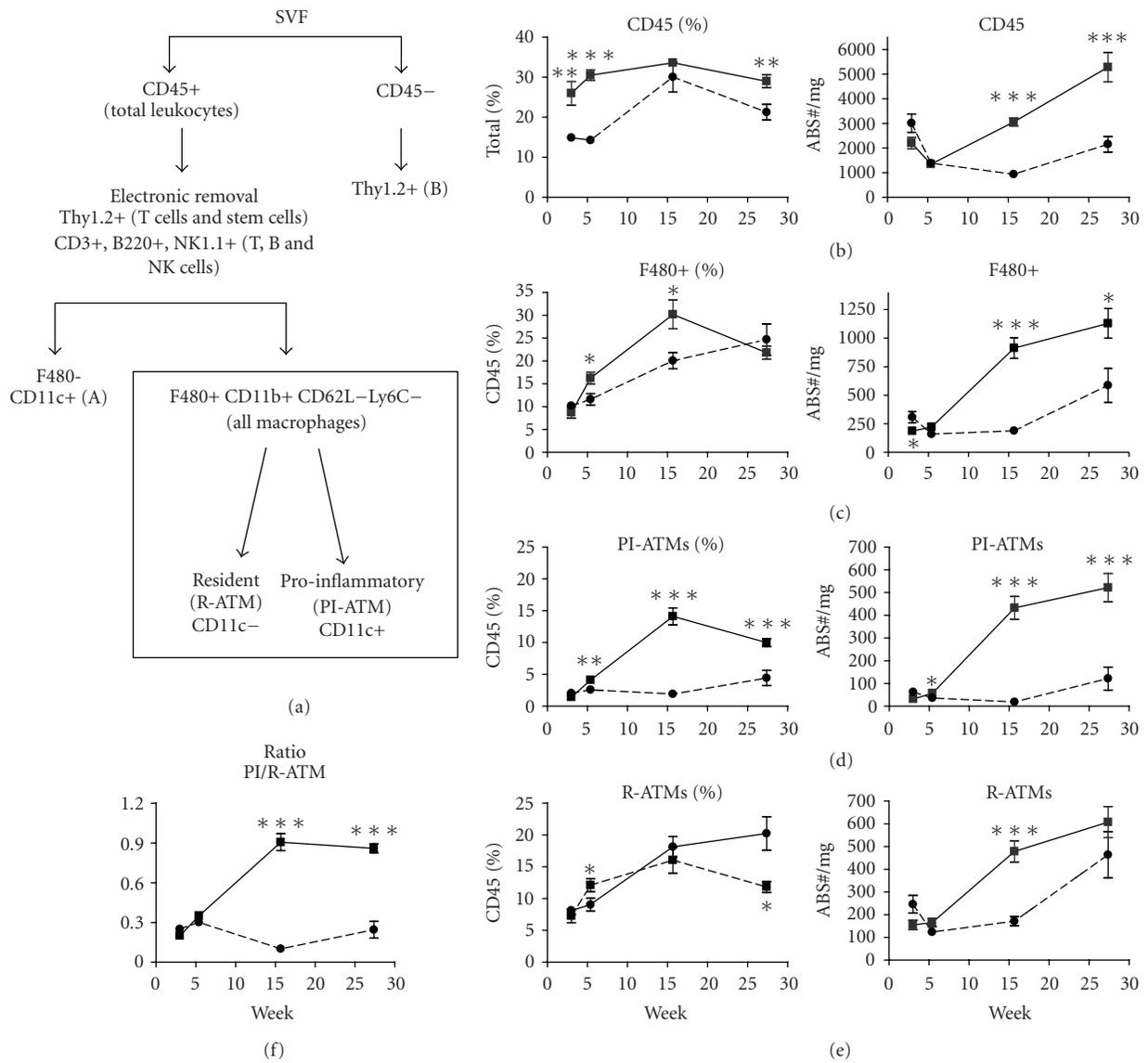


FIGURE 2: Progressive infiltration of pro-inflammatory ATMs assessed by flow cytometry. The epididymal fat pads were isolated from $n \geq 7$ animals per group at each time point. The SVF was isolated and stained to identify total leukocytes (b), macrophages (c), the pro-inflammatory (d) and resident (e) ATM subsets according to the gating strategy (a). The ratio of the pro-inflammatory ATMs over the resident ATMs is a quick indicator of the phenotypic profile of the SVF macrophages. Values represent the mean and SEM. The Chow group is represented by circles and dashed lines and the DIO group on HF/CH diet is represented by squares and a solid line. Unpaired *T* test was used to compare standard chow to DIO at each time point; * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

was a modest increase in the proportion of macrophages at 16-weeks and in the absolute numbers per mg of fat of total leukocytes and macrophages at 27-weeks (Figures 2(b) and 2(c)). In contrast to the macrophages, neutrophils were monitored but constituted a small percentage (<7%) of leukocytes and decreased over time (data not shown), consistent with previously published data [29] and a role for neutrophils in the early onset of disease.

Similar increases in macrophage accumulation were observed in the EF pads by immunohistochemistry using anti-F480, and also by geneexpression analysis of F480

and CD68 (see Supplementary Figure 1 available at doi: 10.1155/2010/970164) . Anti-F480 staining of the EF pad from DIO mice showed an accumulation of macrophages and multinucleated crown structures by 6-weeks which were a predominant characteristic of adipose tissue by 16-weeks. A few crown structures were detected in control mice at 27-weeks (see Supplementary Figure 1(a) available at doi:10.1155/2010/970164). Gene expression analysis of F480 and CD68 showed 2–4 fold induction of macrophages by six weeks; macrophage accumulation peaked (7-8 fold) at 16-weeks. At 27-weeks the fold induction was smaller due

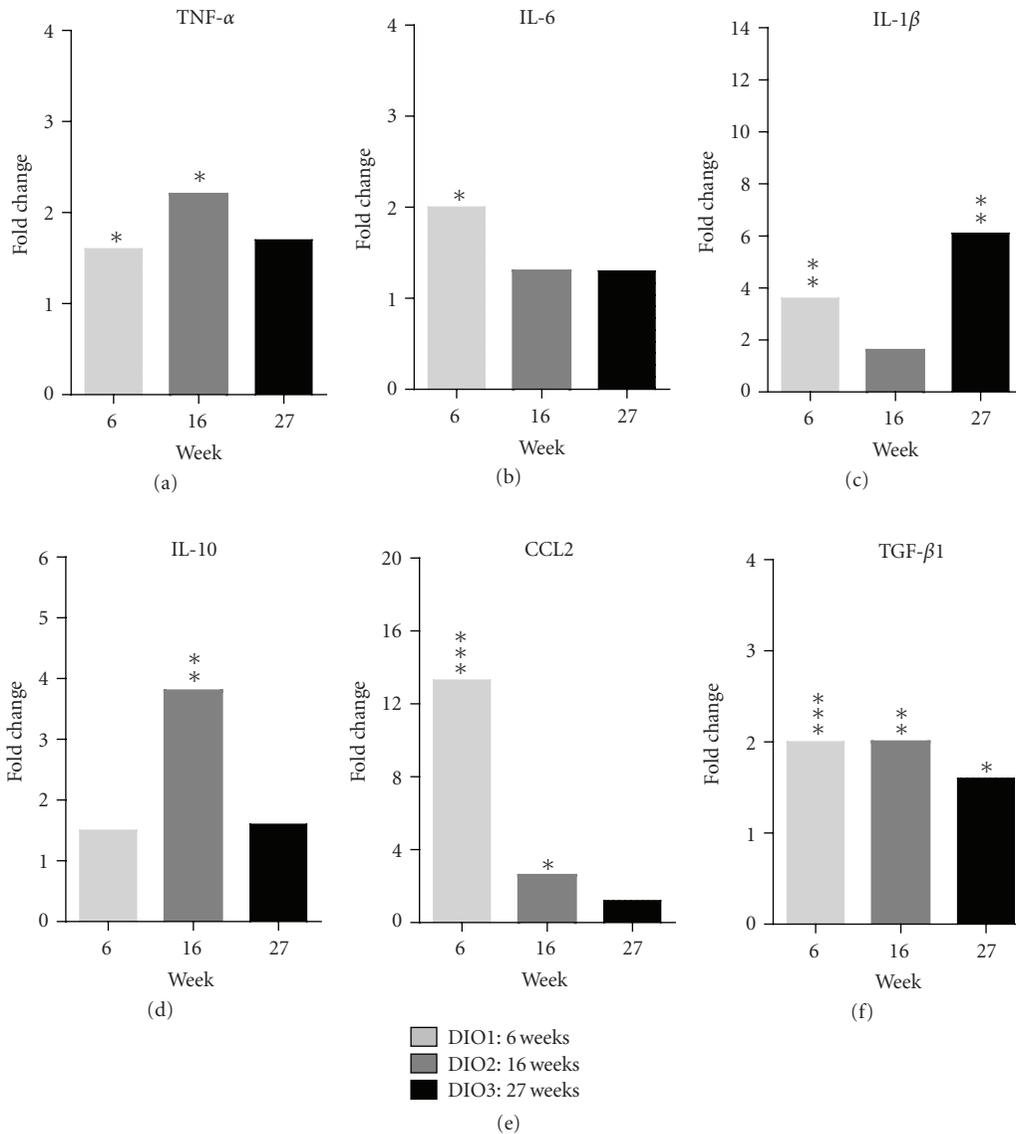


FIGURE 3: Induction of inflammatory mediators in whole EF assessed by gene expression at 6, 16, and 27-weeks. Data represented as HF/CH gene expression fold induction versus the Chow cohort for each time point. ($n = 7-10$ mice for Chow and $n = 10-15$ mice for the HF/CH group). The light gray bars indicate the 6-week timepoint, the dark gray bars indicate 16-week timepoint, and the black bars indicate the 27-week timepoint. Statistical significance was determined by a two tailed Welch t -test; * $P < .05$, ** $P < .01$, and *** $P < .001$.

to the increased number of macrophages in the normal Chow group (see Supplementary Figure 1(b) available at doi:10.1155/2010/970164).

To further phenotype the macrophage content in the HF/CH induced DIO model, we profiled the PI-ATM and R-ATM. The total macrophages were delineated as either PI-ATMs (CD11c+) or R-ATM (CD11c-) by the CD11c marker. There was a modest increase in both the PI-ATM and R-ATMs in DIO mice at 6-weeks, with a dramatic increase in the percentage and number of the PI-ATM at 16-weeks (Figures 2(d) and 2(e)). These data correlated with a dramatic increase in the PI/R-ATM ratio, indicating a preferential accumulation of PI-ATM (Figure 2(f)). In contrast, the normal chow mice exhibited a gradual increase

in their R-ATM (Figure 2(e)) and a consistently low PI/R-ATM ratio (Figure 2(f)).

3.3. Mediator Profile of the Epididymal Tissue, SVF, and Sorted Cells. Similar to the mediator profile in serum, elevations in mRNA for CCL2, IL-6, and IL-10 in EF tissue were observed in DIO mice. Detectable increases in TNF α , IL-1 β , and TGF β expression in EF were observed as well (Figure 3). Spontaneous mediator production from ex vivo DIO SVF cultures also showed elevated levels of IL-6, IL-10, and TNF α , with a minimal increase in IL-1 β (Figure 6(b)). To determine the contribution of ATMs to mediator production, PI-ATM and R-ATM from DIO mice were sorted based on CD11c expression and profiled by gene expression analysis

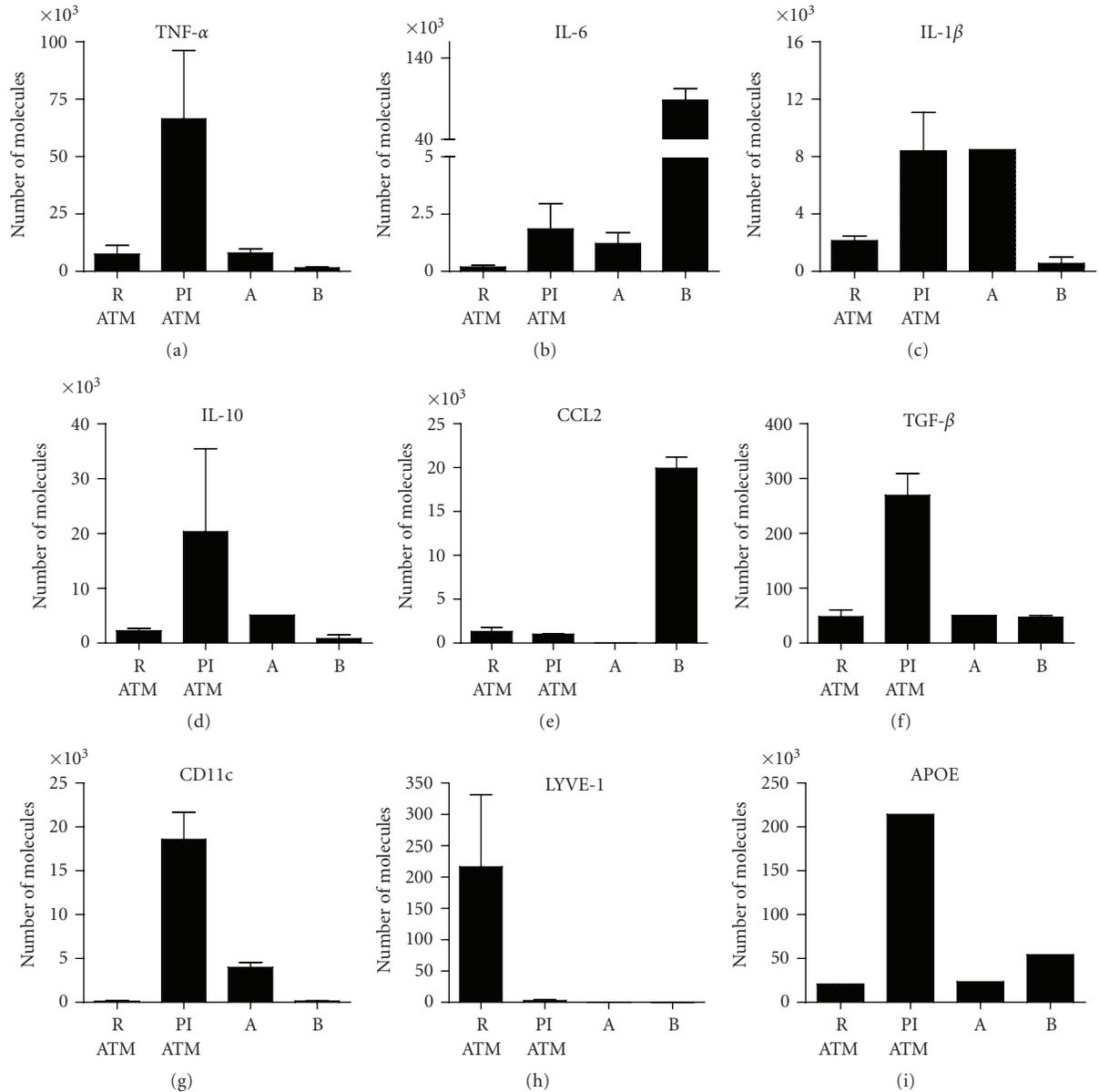


FIGURE 4: Pro-inflammatory ATMs express both pro- and anti-inflammatory mediators as assessed by gene expression. Four cell populations were sorted from the SVF from mice ($n \geq 3$) fed an HF/CH diet for approximately 16-weeks. The pro-inflammatory ATMs (PI-ATM: CD45+F480+CD11c+), resident ATMs (R-ATM: CD45+F480+CD11c-), a CD45+ unknown population (A: CD45+F480-CD11c+), and a CD45- unknown population (B: CD45 Thy1.2+Ly6C+/-CD11b-) were sorted and analyzed for gene expression from two independent sorts.

(Figure 4). In addition to CD11c, PI-ATMs expressed elevated mRNA levels of the characteristic pro-inflammatory mediators, including TNF α , IL-6, and IL-1 β . These cells also expressed elevated levels of ApoE, IL-10, and TGF β . LYVE-1 was the only gene expressed at elevated levels in R-ATMs.

3.4. Novel Cellular Infiltrates in the Epididymal Fat. Besides macrophages, SVF has been proposed to contain several other cell subsets including preadipocytes, epithelial, and endothelial cells [16, 30]. Our gating strategy led to the detection of two additional populations

(Figure 2(a)). The first population, identified as CD45+F480-CD11b+ CD11c+ (A), represented 10–20% of the CD45 population. The second population, identified as CD45-F480-CD11b-Ly6C+/-Thy1.2+ (B), represented 5–10% of total cells of the SVF. Gene expression profiling showed that population A expressed IL-1 β and IL-6 at levels similar to those detected in PI-ATM, whereas population B expressed IL-6 at 48 fold and CCL2 at 20-fold higher levels relative to PI-ATM (Figure 4). Clearly, populations A and B warrant further characterization to determine their role in the described obesity model.

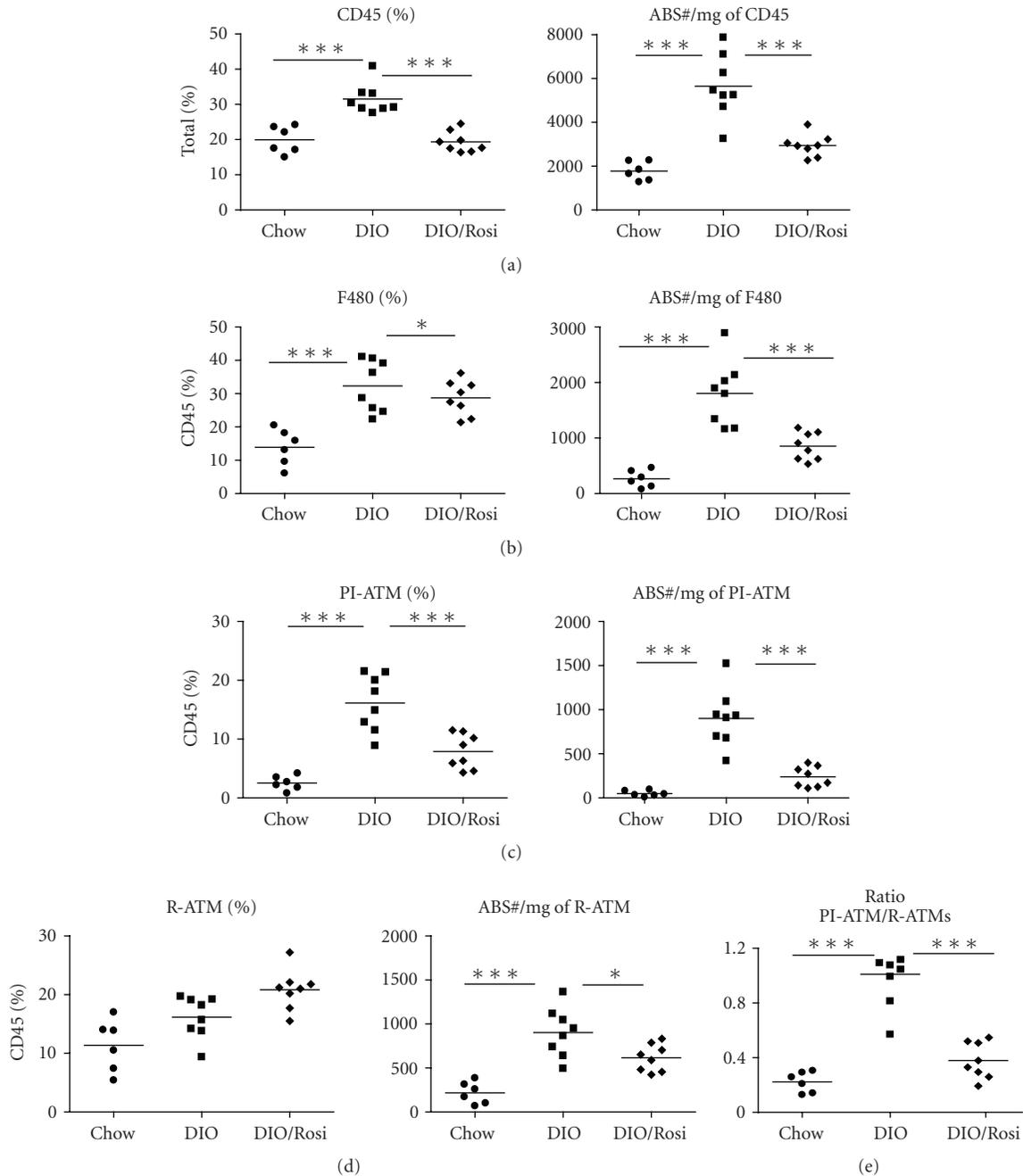


FIGURE 5: Rosiglitazone reduced the pro-inflammatory ATM infiltration in epididymal fat. Animals were put on Chow or an HF/CH diet for 12 weeks. Then Rosiglitazone at 5 mg/kg or vehicle was supplemented in the food for drug treatment the final 6-weeks. (a)–(d). Flow cytometry was performed at the termination of the study at 18 weeks ($n \geq 6$ mice/group). (a) Total leukocyte infiltration was assessed by CD45. (b) Total macrophage infiltration was determined by CD45+F480+CD11b+ cells. Finally total macrophages were subdivided into two groups the (c) pro-inflammatory CD45+F480+CD11b+CD11c+ ATMs and (d) resident CD45+F480+CD11b+CD11c ATMs. (e) The ratio of the pro-inflammatory ATMs to resident ATM populations was reduced with Rosiglitazone. Values represent the mean and SEM. The groups are represented by the following symbols: circles (Chow), squares (DIO), and diamonds (DIO/Rosi). One way ANOVA (Bonferroni multiple comparison) was used to compare Chow to DIO and DIO to DIO/Rosi at each time point; * $P < .05$ and *** $P < .001$.

3.5. Rosiglitazone Attenuated the Inflammatory Mediator Profile of the Epididymal Fat. Rosiglitazone treatment reduced hyperinsulinemia (DIO 4510 \pm 490 pg/ml; DIO-Rosi 2170 \pm 620 pg/ml), serum CCL2 levels (DIO 84.2 \pm

0.18 pg/ml; DIO-Rosi 47.3 \pm 4.6 pg/ml) and SVF mediator production of IL-6, TNF α , IL-1 β and IL-10 (Figure 6(b)). Rosiglitazone treatment significantly decreased the percentage and absolute number of total leukocytes (Figure 5(a))

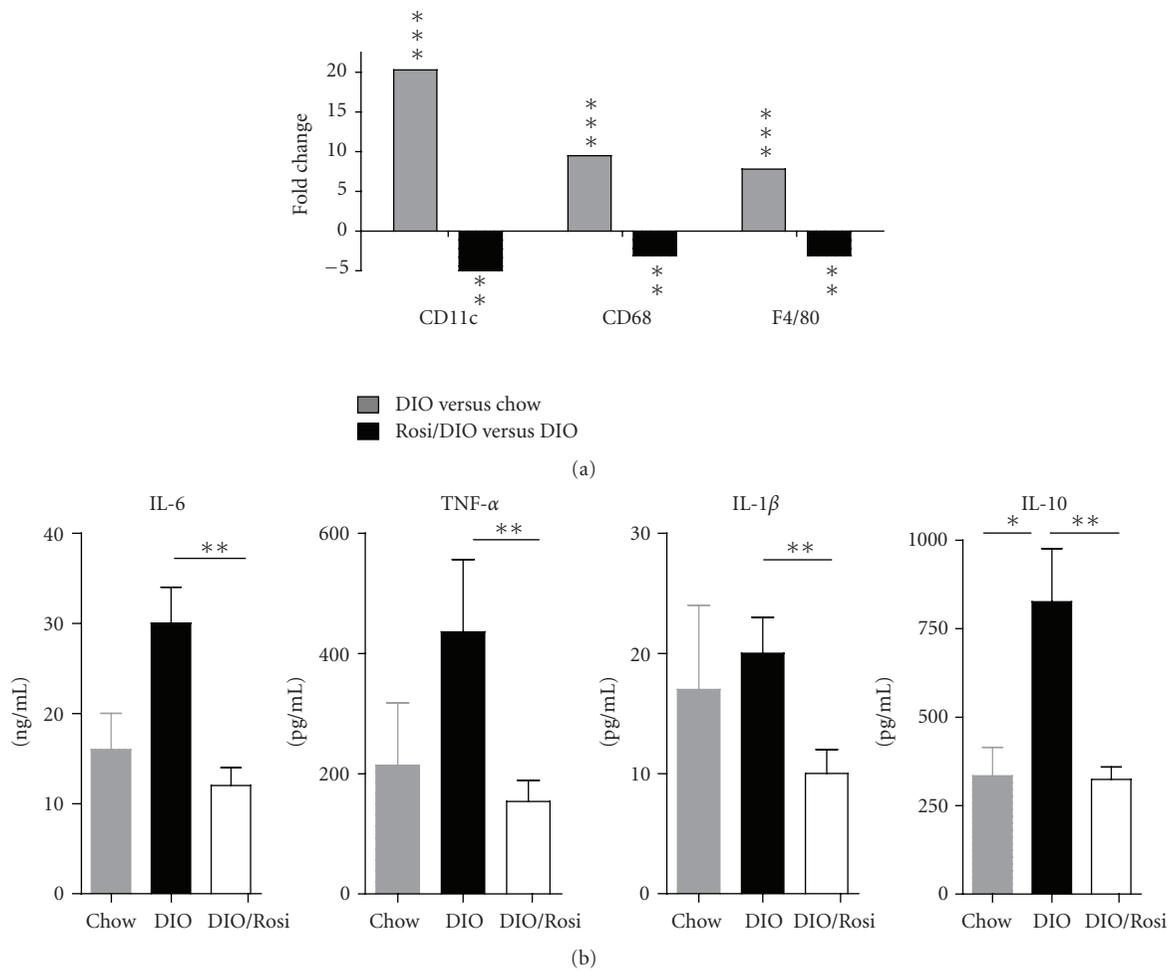


FIGURE 6: Rosiglitazone reduced macrophage and pro-inflammatory markers by gene expression as well as ex vivo spontaneous mediator production. (a) Gene expression of macrophage markers was analyzed from the epididymal fat pads. Data is represented as DIO gene expression fold induction versus the chow cohort or DIO animals treated with Rosiglitazone versus the DIO cohort ($n = 8$ mice/group). Statistical significance was determined by a two-tailed Welch t -test; $*P < .05$, $**P < .01$, and $***P < .001$. (b) Stromal vascular cells were isolated from the EF and incubated ex vivo. Spontaneous mediator production was assessed ($n = 8$ mice/group). Values represent the mean and SEM. Mann-Whitney U test was used to compare standard chow to DIO or DIO to DIO/rosiglitazone; $*P < .05$ and $*P < .01$.

and total F480+ macrophages (Figure 5(b)) in the fat tissue. Of the macrophages, rosiglitazone preferentially decreased the proportion of PI-ATM (Figure 5(c)) and elevated the proportion of R-ATM (Figure 5D), which ultimately decreased the PI/R-ATM ratio (Figure 5(e)). Similarly, decreased macrophage accumulation in the rosiglitazone cohort was supported by decreased mRNA levels of F480, CD68 and CD11c in the epididymal fat (Figure 6(a)). The newly identified populations A and B were not followed in the current rosiglitazone study but will be pursued pending further cell characterization.

3.6. Rosiglitazone Modulates the HF/CH Diet-Induced Blood Monocytes. Blood monocytes were monitored as a potential biomarker [6, 27, 31, 32]. Similar to the gating strategy for the epididymal fat, CD45 was used to delineate the leukocyte population. Subsequently, Ly6G, CD3, B220, NK1.1, and Thy1.2 were used to identify and electronically discard the

neutrophils and T, B, and NK cells (Figures 7(a) and 7(b)). Monocytes were then identified by F480 and Ly6C. Previous reports have classified Ly6C^{Hi} as pro-inflammatory and Ly6C^{lo/int} as anti-inflammatory monocytes [32]. Throughout the kinetic study, there is an upward trend in the proportion of both blood monocyte populations in the DIO cohort compared to chow, although the increase only reached significance at 6-weeks (Figures 7(c) and 7(d)). Assessment of the absolute numbers of blood monocytes revealed no kinetic differences observed between the groups (Figures 7(c) and 7(d)). Despite the very modest alteration in the proportion of circulating monocytes in mice on an HF/CH diet, rosiglitazone attenuated the increase in the proportion of Ly6C^{Hi} and Ly6C^{lo/int} monocytes (Figures 7(e) and 7(f)).

4. Discussion

The goal of this study was to assess the effect of an HF/CH diet on the cellular and molecular parameters in

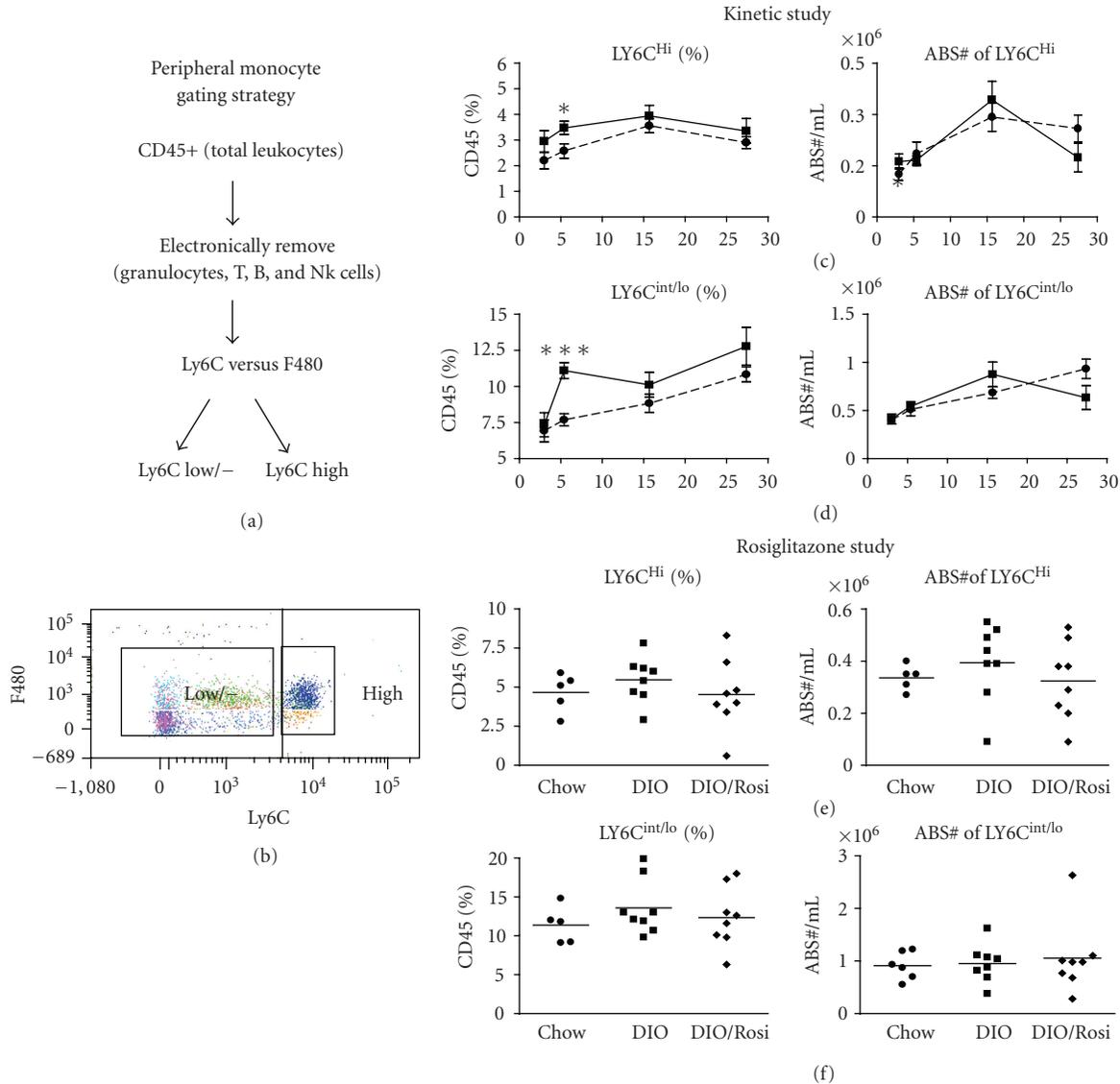


FIGURE 7: Rosiglitazone can modulate a trend for an elevation in the percentage of monocytes. Whole blood was stained and monocytes were assessed in the kinetic timecourse at 3, 6, 16, and 27-weeks, as well as in the Rosiglitazone study terminated at 18 weeks. (a) and (b) show the gating strategy and a representative dot plot that were employed to identify the monocyte populations. (c)-(d). In the kinetic study, results show a trend for an increase in the percentage of both Ly6C^{Hi} and Ly6C^{lo} monocyte populations in the DIO cohort compared to chow, but not in absolute numbers. (e)-(f). Rosiglitazone nonsignificantly reduced the elevation in both the Ly6C high and Ly6C^{lo} monocyte percentages and the Ly6C^{Hi} but not Ly6C^{lo} absolute numbers.

DIO mice and the effect of an insulin sensitizing agent, rosiglitazone, on these parameters. Changes in metabolic parameters occurred as early as 6-weeks in DIO mice as evidenced by hyperinsulinemia, increased leptin levels and modestly increased levels of pro-inflammatory mediators, including CCL2 and IL-6, in the serum and in the EF pad. Despite these early changes, dramatic metabolic changes only occurred at and beyond 16-weeks.

Cell infiltration is one of the hallmarks of inflammation; we observed an increase in total leukocytes within the SVF by 3 weeks in DIO mice. Within the leukocyte population,

the percentage of total macrophages significantly increased by 6-weeks which was corroborated by F480 and CD68 gene expression data and F480 immunostaining of EF tissue. However, the peak of macrophage accumulation occurred at 16 to 27-weeks correlating with maximal insulin levels. In the DIO mice, although there was a modest increase in the proportion of both PI-ATM and R-ATMs at 6-weeks, there was a dramatic increase in the proportion and absolute numbers of PI-ATM at 16 to 27-weeks. This correlated with significant increase in the PI/R-ATM ratio indicative of a pro-inflammatory microenvironment. Thus, as hypothesized,

the HF/CH diet led to an early inflammatory response with kinetics paralleling the peak of the insulin levels and liver damage (ALT).

Analysis of the EF tissue or spontaneous mediator production from cultured SVF cells derived from DIO mice after 16-weeks on the HF/CH diet revealed an induction of the various pro-inflammatory mediators such as CCL2, TNF α , IL-6 or IL-1 β and the pleiotropic mediator, IL-10. To determine if the macrophages were the primary contributors to this mediator production from the SVF, we sorted four populations: PI-ATM, R-ATM and two additional populations and evaluated their mediator profile by gene expression. The two additional populations were CD45⁺ F480⁻CD11b⁺ CD11c⁺ cells (A) and CD45⁻F480⁻CD11b⁻Ly6C^{+/-}Thy1.2⁺ cells (B), which express surface antigens potentially associated with other myeloid or hematopoietic cells.

Our data indicates that both macrophages and novel cell populations within the SVF fraction contribute to the inflammatory state of the adipose tissue in DIO mice. Gene expression profiling of sorted macrophage subsets confirmed that the PI-ATMs had elevated message levels of the pro-inflammatory mediators, including TNF α , IL-6, and IL-1 β , as well as TGF β , a finding consistent with human ATM data [15, 16]. In addition, the PI-ATM also expressed elevated levels of IL-10 and ApoE, which are often perceived as anti-inflammatory mediators. However, both IL-10 and ApoE are pleiotropic molecules and they could be exerting pro-inflammatory effects. For example, overexpression of IL-10 in islet cells in a nonobese diabetic mouse model exacerbates diabetes [33] and, recent data indicate that ApoE can stimulate dendritic cells to present lipids [34]. These mediators could be having pro-inflammatory roles or they could be coexpressed as a mechanism to counter-regulate the pro-inflammatory state. Similar to our data some report the mediator profile of PI-ATMs from mice on HF or Chow diets showed expression of TNF α , IL-6 and/or IL-10 [14] or a subset of these mediators [12, 13, 31]. Overall, from the sorted SVF populations, the PI-ATMs described in this manuscript are highly metabolically active, expressing multiple pro-inflammatory mediators (IL-1 β , IL-6, TNF α), growth factors (TGF β) and pleiotropic mediators (APOE and IL-10). Although the described ATMs could not be definitively classified as pro or anti-inflammatory, these data are consistent with the human reports indicating that human ATMs [10, 11, 16] and cultured human SVF cells showed elevated levels of IL-1 β , TNF α , IL-6 and CCL2 as well as IL-10 [11, 15]. With the exception of LYVE-1, a gene recently associated with tissue angiogenesis [35] or remodeling [16], the R-ATM described herein expressed lower levels of most mediators relative to the PI-ATM, despite reports of elevated levels of IL-10 [13] and ApoE [12, 31] in these cells.

Some of the discrepancies between the ATM subsets described herein and those of previous studies could be due to differences in the diets, activation or differentiation state of the sorted populations, or due to the plasticity of macrophage populations [36, 37]. Alternatively, differences in the stringency of the gating method and sort strategies could account for the discrepancies. Our data clearly indi-

cated that there are additional populations of cells that can be identified in the adipose tissue that could contribute to mediator production.

As stated previously, using our gating strategy, two additional inflammatory cell populations were identified. Population B (CD45⁻Thy1.2⁺) was a prominent population representing 5–10% of the total SVF. It was observed that this population expressed extremely high levels of IL-6 and CCL2 that far exceeded those of the PI-ATMs. Based on this initial characterization, these cells could represent any of several cell types including newly recruited monocytes, cells of endothelial origin which can secrete CCL2 and IL-6 [15, 16], stromal cells as seen in human adipose tissue [30, 38–41], early hematopoietic derived cells, or preadipocytes. [30, 42]. Population A (CD45⁺F480⁻CD11c⁺) represented 10–20% of the CD45 cells of the SVF and expressed levels of IL-6 and IL-1 β similar to those of PI-ATMs. These cells, especially population B, contribute to the pro-inflammatory state of the fat tissue. Clearly, as indicated by the profiling of populations A and B, the PI-ATM and R-ATM are not the only cells producing inflammatory mediators in the SVF. Future studies are being designed to further the characterization and to determine if anti-inflammatory or obesity-related drugs alter these populations.

The insulin sensitizing agent, rosiglitazone, is a PPAR γ agonist used in the clinic to treat type II diabetes and recently has been reported to affect the phenotypic switch of macrophages from a pro to an anti-inflammatory profile as well as to affect macrophage infiltration into the EF pad [27]. Consistent with reports from clinical studies [24], treatment of DIO mice on the HF/CH diet with rosiglitazone increased the body weight and decreased hyperinsulinemia demonstrating the drug's insulin sensitizing effects (data not shown). These changes in metabolic parameters were paralleled by changes in the pro-inflammatory state of the mice. There was a reduction in spontaneous mediator production from cultured SVF cells from rosiglitazone treated DIO mice which correlated with a decrease in macrophage accumulation.

Gene expression analysis indicated a reduction in the macrophage markers, F480 and CD68, as well as CD11c in total adipose tissue from rosiglitazone treated DIO mice. Flow cytometry analysis corroborated and extended these findings by revealing that rosiglitazone treatment significantly reduced the PI-ATMs, lowering the PI/R-ATM ratio to a level close to that observed for the normal Chow cohort. Our data supports the theory that rosiglitazone switches the profile of the ATMs from a pro-inflammatory to a more anti-inflammatory or resting state [43]. Alternatively, rosiglitazone could alter the balance of cell recruitment and migratory clearance of ATMs; in an atherosclerosis model reduced emigration of myeloid derived cells out of plaques contributed to the disease pathology [44].

In addition to the analysis of adipose tissue ATM, peripheral blood monocytes were analyzed as a potential biomarker for obesity and insulin resistance in preclinical and clinical studies. In the kinetic study, we detected a significant elevation in both the pro-inflammatory (Ly6Chi F480^{+/-}) and homeostatic (Ly6Clo⁻ F480^{+/-}) monocyte

populations at 6-weeks. At later time points, there was a trend only for an increase in the percentages but not in number of monocytes consistent with previous reports indicating modest but significant elevations of monocyte populations [27, 32]. Continual emigration from the bone marrow coupled with the simultaneous and continual influx into the fat depots or sites of inflammation may account for the very modest increase in monocytes throughout the treatment period. Despite the limited modulation in monocytes, at 18 weeks post-HF/CH diet, rosiglitazone induced a modest but nonsignificant reduction in the percentage of both monocyte populations. Although it remains possible that rosiglitazone may have had a more dramatic effect on monocyte populations at an earlier time point, 18 weeks represents the peak of the metabolic changes and PI-ATM infiltration. Due to the modest therapeutic window for modulation of monocytes, we concluded that monitoring of monocyte populations in the blood would be inadequate to follow as a biomarker unless a more dramatic modulation of monocytes is observed in human subjects.

In conclusion, an HF/CH-like diet rich in fat and cholesterol induces obesity which correlates with an inflammatory state as exhibited by a rapid increase in epididymal fat pad mass, hyperinsulinemia, macrophage infiltration and mediator production in the fat depots. The increased ratio of PI/R-ATMs and the production of many pro-inflammatory mediators correlated with the peak of disease induced by the HF/CH diet as evidenced by maximal insulin levels at 16-weeks and are indicative of an inflammatory state in DIO mice. At the peak of disease, rosiglitazone attenuates the inflammatory response as exemplified by a reduction in the ratio of PI/R-ATMs and the production of multiple mediators. Lastly, a very intriguing finding was the identification of a novel population in the SVF that expressed very high mRNA levels of IL-6 and CCL2 exceeding those observed in the PI-ATM. This population warrants additional investigation as it could significantly impact the inflammatory state in DIO mice and potentially in human obese and diabetic subjects. This DIO mouse model induced with HF/CH is an appropriate model for characterization of therapeutics targeted not only for the metabolic parameters of the syndrome but also for the low-level chronic inflammatory response that is induced by the diet.

Abbreviations

DIO:	diet induced obesity
HF/CH:	high-fat (45%) and cholesterol (0.12%)
BMI:	body mass index
IR:	insulin resistance
BW:	body weight
PPAR γ :	peroxisome proliferator-activated <i>receptor</i> gamma
EF:	epididymal fat
SVF:	stromal vascular fraction
ATM:	adipose tissue macrophage
R-ATM:	resident adipose tissue macrophage
PI-ATM:	pro-inflammatory adipose tissue macrophage

TZD:	thiazolidinedione drugs
ALT:	alanine aminotransferase
NAFLD:	nonalcoholic fatty liver disease
ITT:	insulin tolerance test
GTT:	glucose tolerance test.

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Research Article

Effects of Glycyrrhizic Acid on Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ), Lipoprotein Lipase (LPL), Serum Lipid and HOMA-IR in Rats

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Studies on ligand binding potential of glycyrrhizic acid, a potential agonist to PPAR γ , displayed encouraging results in amelioration of metabolic syndrome. The regulation of gene cassettes by PPAR γ affects glucose homeostasis, lipid, lipoprotein metabolism and adipogenesis. This study was performed to determine the effects of GA on total PPAR γ and LPL expression levels, lipid parameters and HOMA-IR. Oral administration of 100 mg/kg GA for 24 hours resulted in an increase in insulin sensitivity with decreases in blood glucose, serum insulin and HOMA-IR. Improvement in serum lipid parameters was also observed with a decrease in triacylglycerol, total cholesterol and LDL-cholesterol and an elevation in HDL-cholesterol. GA administration also resulted in up-regulation of total PPAR γ and LPL expression levels in the visceral and subcutaneous adipose tissues, abdominal and quadriceps femoris muscles, as well as liver and kidney, with a significant up-regulation only in the visceral adipose tissue, abdominal and quadriceps femoris muscles. Thus, oral administration of 100 mg/kg GA for 24 hours improved insulin sensitivity and lipid profiles and induced upregulation of total PPAR γ and LPL expression levels in all studied tissues.

1. Introduction

Metabolic syndrome, or more commonly known as syndrome X, encompasses a constellation of risk factors such as visceral (abdominal or central variant) obesity, insulin resistance (IR), glucose intolerance, dyslipidaemia and hypertension [1]. Studies have shown that obesity and insulin resistance are the two predominant underlying mechanisms for the development of metabolic syndrome [2]. Insulin resistance is also a characteristic in most patients with type 2 diabetes mellitus (T2DM) [2].

T2DM involves the impairment of insulin action and secretion from the pancreatic β -cells. Both factors will collectively increase blood glucose levels [3]. Research has proven that impaired insulin secretion together with peripheral insulin resistance is the main regulator behind the incidence of T2DM [4].

Peroxisome proliferator-activated receptor gamma (PPAR γ) gene is a well known susceptibility gene for T2DM due to the presence of polymorphisms within the gene. PPAR γ is a ligand-activated transcription factor originating from the nuclear hormone receptor superfamily [5]. PPARs form heterodimers with retinoid X receptors (RXRs) and bind to the hexanucleotidic PPAR responsive element (PPRE), thereby regulating the expression of target genes involved in lipid and carbohydrate metabolism [6].

Since PPAR γ is a major regulatory transcription factor, it is involved in the regulation of several biochemical pathways through the transcriptional control of target genes [7]. Among the pathways involved through the regulation of gene cassettes by PPAR γ are glucose homeostasis, lipid and lipoprotein metabolism, inflammatory and immune responses as well as adipogenesis [8].

PPAR γ is the master regulator of fat cell function that governs differentiation of pre-adipocyte precursor cells into mature adipocytes capable of lipid filling as well as the mediation of hormone and cytokine expression [9]. In addition to an increase in smaller and more insulin sensitive adipocytes, PPAR γ activation also promotes apoptosis of mature, lipid-filled visceral adipocytes and a redistribution of the free fatty acids towards the subcutaneous adipose tissues [5]. The increase in adipocyte number increases the lipid storage capacity and indirectly confers protection towards non-adipose tissues in the event of excessive lipid accumulation [10].

Lipoprotein lipase (LPL), a water-soluble enzyme, liberates free fatty acids through the hydrolysis of ester bonds of water-soluble substrates such as TAG, phospholipids and cholesterol esters [11]. LPL is synthesized and highly expressed in the adipose tissues, cardiac and skeletal muscle, kidney, and mammary glands while lower levels are present in the liver, adrenal and brain [12]. The expression of LPL is governed by the activation of PPAR γ by cognate ligands as LPL is a downstream gene of PPAR γ . The PPAR γ /RXR complex would bind to the PPRE present in the promoter region of the LPL gene and increases the LPL gene expression [13]. The induction of lipoprotein lipase synthesis by PPAR γ is mainly in the mature adipocytes in order to increase local generation of free fatty acids [14].

It has been reported that Glycyrrhizic acid (Glycyrrhizin or Glycyrrhizinate, GA), the bioactive compound extracted from roots of licorice plants, has anti-diabetic properties [15]. In view of the above, the present study was undertaken to investigate the effects of orally-administered GA on total PPAR γ and LPL expression levels and HOMA-IR in rats.

2. Materials and Methods

2.1. Animals Studies. Male Sprague Dawley rats (*Rattus norvegicus*) (6 weeks old) obtained from University Malaya's Animal House (University Malaya, Malaysia) were housed 1 per cage with free access to food and drinking water. They were maintained on a 12-hour light-dark cycle in a room with controlled temperature ($24 \pm 1^\circ\text{C}$) and humidity ($55 \pm 10\%$). The use and handling procedure of animals had been approved by the Monash University Animal Ethics Committee according to the 2004 NHMRC Australian Code of Practice for the Care and Use of Animal for Scientific Purposes and Relevant Victorian Legislation (Prevention of Cruelty to Animals Act 1986) (AEC: SOBSB/MY/2006/46).

2.2. In-Vivo Assay. The rats were divided into 2 groups (control and treated) [8 rats per group]. The treated group was given 100 mg kg^{-1} of GA (Sigma Chemical Co., St. Louis, MO, USA) orally while the control group was given tap water without GA. The rats were fed ad libitum with Glenn Forest stock-feeder rat chow during the treatment period of 24 hours. Upon completion of the projected treatment period, the rats fasted for 12 hours prior to humane sacrifice under

the influence of anaesthesia via intraperitoneal administration of pentobarbital sodium (120 mg/kg , IP).

Blood was withdrawn from the apex of the heart for measurement of glucose and insulin levels. Tissues (subcutaneous and visceral adipose tissues, abdominal and quadriceps femoris muscles, and liver and kidney) were harvested for measurement of total PPAR γ and LPL expression levels.

2.3. Laboratory Assay. Blood glucose was analyzed by glucose oxidase method employing a Powerwave XS Microplate Scanning Spectrophotometer (BIO-TEK, USA). Enzyme immunoassay (LINCO-Millipore Corp., US) was used to measure serum insulin. For the estimation of insulin sensitivity, the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated (concentration of glucose \times concentration of insulin/22.5) [16].

Total cholesterol and TAG were measured with a Randox CH200 Cholesterol kit (Randox, UK) and a Wako Triglyceride E kit (Wako, Japan). To determine the level of HDL-cholesterol, HDL-cholesterol was first separated from the LDL and VLDL fractions by precipitation of the latter two using the Randox CH203 HDL precipitant, followed by a cholesterol assay using the Randox CH200 Cholesterol kit. The levels of total cholesterol, TAG and HDL cholesterol obtained were used to calculate LDL-cholesterol using the Friedewald formula [17].

2.4. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Quantification of Total PPAR γ and LPL Expression

2.4.1. RNA Isolation and RT-PCR. Total mRNA was obtained from subcutaneous and visceral adipose tissues, abdominal and quadriceps femoris muscles, and liver and kidney of each animal using Qiagen RNeasy Mini Kit and Qiagen RNeasy Lipid Tissue Mini Kit according to the protocol provided by the manufacturer. The concentration of the mRNA was determined by measuring the absorbance at 260 and 280 nm. The quality of the mRNA was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA after electrophoresis on 1.2% agarose gel. $1 \mu\text{g}$ of total RNA was reverse-transcribed with Qiagen Omniscript reverse transcriptase (Qiagen, USA). The expression of total PPAR γ and LPL was assessed by quantitative RT-PCR, using Opticon Monitor 3 (MJ Research Inc, UK) with Locked Nucleic Acid (LNA) Dual-labeled Fluorogenic Probes detection. The forward and reverse primers for the amplifications of total PPAR γ and LPL expression levels are listed in Table 1. The comparison of total PPAR γ and LPL expression levels between control and treated rats was performed using the Comparative Ct ($\Delta\Delta\text{Ct}$) Method, with BAC as reference, treated group as target and control group as calibrator.

2.5. Statistical Analysis. Data were expressed as mean \pm standard error of mean. Statistical analyses of total PPAR γ and LPL expression levels were performed using the Relative Expression Software Tool (REST $\text{\textcircled{C}}$) MCS Beta 2006 while

TABLE 1: Primer sequences used for real-time PCR.

Genes	Primers
Total	Forward 5'-CCCTGGCAAAGCATTGTAT-3'
PPAR γ	Reverse 5'-GGTGATTTGTCTGTTGTCTTTCC-3'
LPL	Forward 5'-CAGCAAGGCATACAGGTG-3'
	Reverse 5'-CGAGTCTTCAGGTACATCTTAC-3'
β -actin (BAC)	Forward 5'-CAGCAAGGCATACAGGTG-3'
	Reverse 5'-CGAGTCTTCAGGTACATCTTAC-3'

that of all other parameters was performed using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL, USA). A P value of $\leq .05$ was considered significant.

3. Results

3.1. Biochemical Analysis. Rats administered with GA showed 65% (control, 9.31 ± 0.40 mmol/L; treated, 3.26 ± 0.23 mmol/L) and 37% (control, 2.71 ± 0.41 ng/mL; treated, 1.53 ± 0.12 ng/mL) decrease in blood glucose ($P < .05$) and serum insulin ($P > .05$) levels, respectively, compared to the control rats (Figure 1). Insulin resistance, calculated as HOMA-IR, was 83% (control, 1.38 ± 0.12 ; treated, 0.62 ± 0.05) lower in the GA-administered rats compared to the control ones (Figure 1) ($P < .05$).

Consistent improvement in all lipid parameters was observed in the GA-administered rats relative to the control (Figure 2). Mean serum TAG, total cholesterol and LDL-cholesterol showed a 28.72%, 21.55%, and 28.60% reduction, respectively, (control, 1.22 ± 0.14 mmol/L; treated, 0.87 ± 0.10 mmol/L; control, 2.04 ± 0.13 mmol/L; treated, 1.60 ± 0.11 mmol/L; control, 0.77 ± 0.08 mmol/L; treated, 0.54 ± 0.05 mmol/L, resp.) ($P > .05$). Rats administered with GA showed an elevation in HDL-cholesterol by 17.95% compared to control rats (control, 1.08 ± 0.10 mmol/L; treated, 1.32 ± 0.06 mmol/L) ($P > .05$).

3.2. Total PPAR γ and LPL Expression in Rat Tissues. Abdominal muscle (MA) displayed the highest fold difference expression of total PPAR γ followed by quadriceps femoris (MT), visceral adipose tissue (ATV), subcutaneous adipose tissue (ATS), liver (L), and kidney (K) (Figure 3). Compared with the control groups, relative expression of total PPAR γ in GA-administered rats was higher in all tissues. The MA, MT and ATV displayed a significant increased fold difference ($P < .05$) of 15.16 ± 5.58 fold, 13.09 ± 3.11 fold and 8.85 ± 3.62 fold, respectively, when compared to the control group. The ATS, L and K also displayed increased fold difference of 2.00 ± 0.81 fold, 5.07 ± 2.17 fold and 2.26 ± 1.08 fold, respectively, albeit an insignificant increase ($P > .05$).

The increased LPL expression in all studied tissues (Figure 3) was similar to the expression of PPAR γ namely the MA, MT, and ATV also displayed a significant increase in fold difference ($P < .05$) and of 11.02 ± 4.06 fold, 8.82 ± 3.01 fold, and 4.53 ± 2.66 fold, respectively. A similar trend of non-significant increase ($P > .05$) in fold difference was observed

in the ATS (1.27 ± 0.85 fold), L (2.76 ± 1.97 fold) and K (2.18 ± 1.44 fold).

4. Discussion

The present study showed significant decrease in blood glucose and HOMA-IR in rats administered with 100 mg/kg of GA for 24 hours. Takii et al. [18] and Nakagawa et al. [19] reported that normal and obese genetically diabetic KK-A Y mice given ethanolic extract of licorice roots displayed suppression of blood glucose levels. Activation of PPAR γ was shown to cause a decrease in blood glucose levels through the inhibition of glucagon gene transcription and secretion by inhibiting the transcriptional activity of PAX6 [20].

The decrease in the serum insulin levels could be attributed to the insulin sensitization properties bestowed upon the activation of PPAR γ . Under a normoglycaemic state, circulatory insulin would oversee the suppression of hepatic glucose production and enhancement of glucose uptake by peripheral organs [5]. Insulin has also been implicated in the biosynthesis of LPL [21], where the insulin-signaling pathway activates PPAR γ , which subsequently binds to the PPRE at the LPL gene promoter region to upregulate LPL gene expression [22]. A significant drop in the serum insulin levels was found in diabetic KK-A Y mice for 7 weeks fed with ethanolic extract of licorice roots [18]. Results from our study exhibited a similar trend but the drop was non-significant probably due to the shorter treatment duration.

A decrease in HOMA-IR in the present study could be due to the lowered insulin and glucose levels as HOMA-IR reflects the product of glucose output and insulin secretion [23]. Therefore, a lower HOMA-IR indicates improved insulin sensitivity. Hanyu et al. [22] demonstrated that plasma LPL activity reflects whole-body insulin sensitivity and is negatively correlated with the HOMA-IR. The results of this study were in agreement with Hanyu et al. [22] where the higher insulin sensitivity (lower HOMA-IR) in the GA-administered rats developed concomitantly with the increase in tissue LPL expression. In addition, the activation of PPAR γ also regulates the adipocyte hormone gene expression to improve insulin sensitivity. Activation of PPAR γ increases the adiponectin expression which potentiates insulin sensitivity in the liver and skeletal muscles [24].

In the present study, increase in tissue LPL expression was consistent with the improvement of serum lipid parameters in the GA-administered rats. A reduction in serum TAG, total cholesterol and LDL-cholesterol together with an elevation in HDL-cholesterol was observed. The decrease in TAG in GA-administered rats may be attributed to the action of GA which causes an increase in its tissue uptake. Berthiaume et al. [25] have demonstrated that inhibition of 11 β -hydroxysteroid dehydrogenase 1 (11 β -HSD1) could reduce hepatic very low density lipoprotein (VLDL) which may have increased the hepatic free fatty acid oxidation. Thus, this could have caused the reduction in TAG as it is required to boost the VLDL secretion. The decrease in LDL-cholesterol

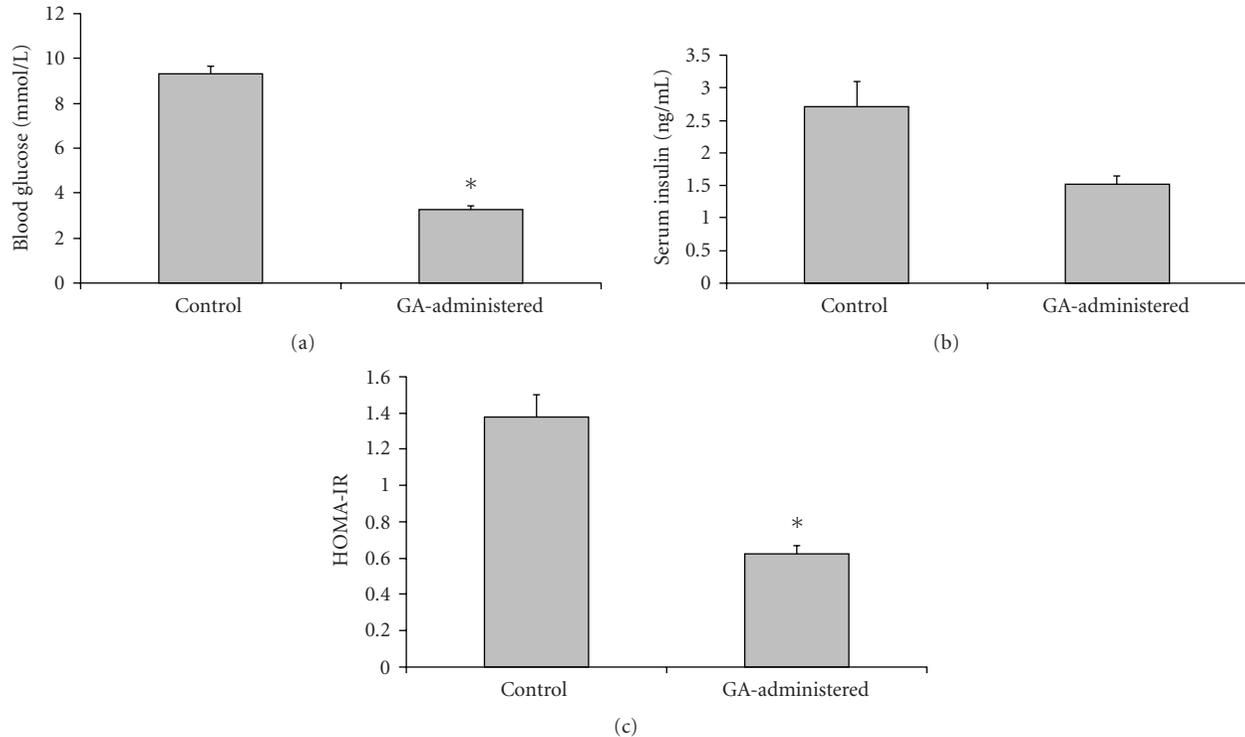


FIGURE 1: (a) Blood glucose, (b) serum insulin, and (c) HOMA-IR levels in GA-administered rats (* indicates $P < .05$).

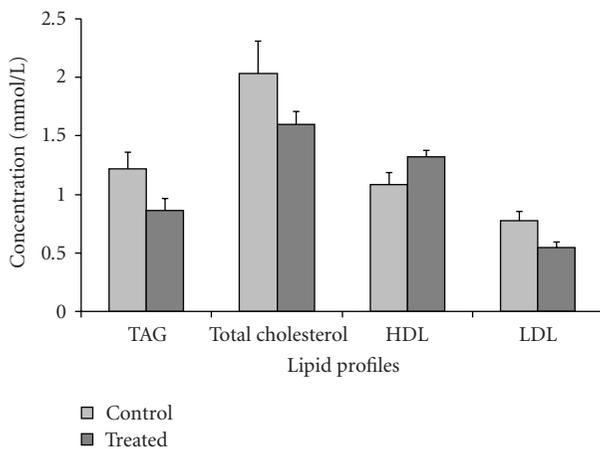


FIGURE 2: Serum lipid profiles in GA-administered rats.

in GA-administered rats may be due the lowering effect of VLDL since LDL is a derivative of VLDL. Elevation of HDL-cholesterol in GA-administered rats may be due to the increased apo A-I production since the rate of HDL synthesis is dependent on the production of apo A-I [26].

With reference to Figure 3, increased LPL expression was also seen in adipose tissue. LPL is a downstream gene that is regulated by the activation of PPAR γ , where an increase in the expression of PPAR γ would lead to an increase in the expression of LPL [27]. Thus, the increase in PPAR γ expression is accompanied by a similar significant increase in

LPL gene expression. LPL is a key enzyme in the metabolism of triglyceride-rich lipoproteins and plays the role of a gate-keeper in energy metabolism by controlling the generation of fatty acids. In adipose tissue, the increase in LPL production could enhance the clearance of plasma triglycerides and provide the (pre-)adipocytes with additional fatty acids, which can further stimulate the transactivation capacity of PPAR [28]. The upregulation of PPAR γ in adipose tissues had been shown to cause an increase in glucose transporter-1 (GLUT4) and c-Cbl associating protein (CAP), pivotal both for the translocation of GLUT4 to the cell surface and for the enhanced glucose uptake [5].

Although the liver is an organ involved in glucose and lipid metabolism, only low levels of PPAR γ were detected in the liver [14]. Although the exact mechanism involved in the activation of PPAR γ in the liver remains unknown, it has been postulated that a circulating factor might be responsible for the stimulation of hepatic total PPAR γ transcription during a state of increased energy availability [29]. The upregulation of hepatic total PPAR γ would in turn activate several genes such as adipocyte fatty acid-binding protein (aP2) and fatty acid translocase (FAT/CD36), previously present only in trace amounts in the liver of lean mice. Activation of aP2 in the liver prevents the detrimental effects of free fatty acids on cells and membranes while that of FAT/CD36 facilitates fatty acid transport which ultimately lowers serum triacylglycerol levels and improves insulin sensitivity [30].

Muscles are pivotal tissues in glucose homeostasis as it is the primary organ for the insulin-stimulated glucose

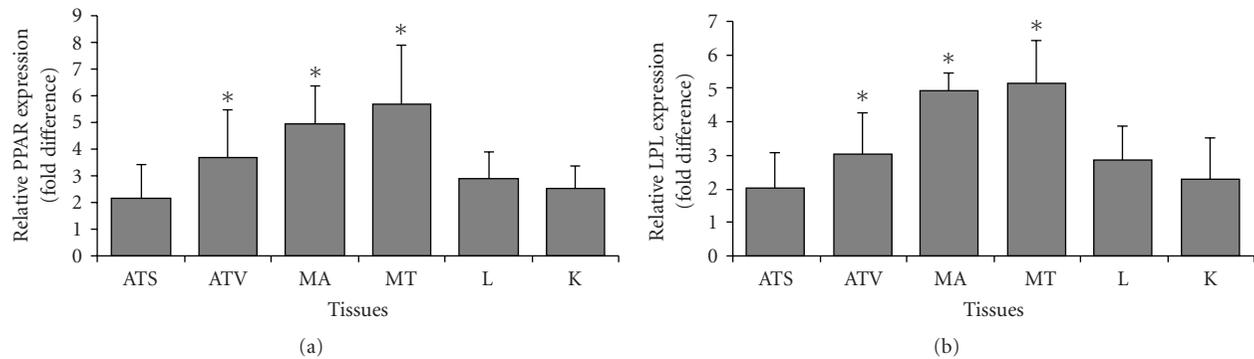


FIGURE 3: Relative expression (fold difference) for (a) total PPAR γ and (b) LPL in tissues with BAC as reference, GA-administered group as target, and control group as calibrator. ATS: Subcutaneous adipose tissue, ATV: Visceral adipose tissue, MA: Abdominal muscle, MT: Quadriceps femoris muscle, L: Liver, K: Kidney (* indicates $P < .05$).

disposal. Significant increases were observed in total PPAR γ and LPL expression levels. Higher fold difference expression was found in quadriceps femoris compared to the abdominal muscle. This could be due to higher physical activity undertaken by the quadriceps femoris muscle and its higher metabolic capabilities [31]. Thus, the increase in PPAR γ expression in the GA-treated rats suggests possible upregulation by GA. Increase in PPAR γ expression correlates with enhanced insulin-stimulated glucose uptake into the muscles mediated by increased insulin-stimulated P13K activity and translocation of GLUT4 towards the cell membrane [32]. Thus, the significant increase in total PPAR γ expression of the quadriceps femoris muscle in the GA-treated rats was probably due to enhancement in insulin-stimulated glucose uptake mechanisms. Wang et al. [33] have reported that a lack of LPL expression in the skeletal muscle could result in insulin resistance in other metabolic tissues (e.g., liver and kidney). This could ultimately lead to obesity and systemic insulin resistance.

The expressions of PPAR γ and LPL in the kidneys are lower compared to those of adipose tissue, complementing the fact that the kidney is not the main regulatory organ involved in lipid and glucose homeostasis [5]. The upregulation of total PPAR γ in the kidney in the present study suggests the potential regulation of renal sodium and water reabsorption [34]. Most importantly, the activation of PPAR γ also renders renoprotective effects on the kidneys through improved glucose metabolism [35].

Both the visceral and subcutaneous adipose tissues displayed the highest expression levels of total PPAR γ and LPL compared to the control tissues, thus substantiating their importance in adipogenesis and adipocyte remodeling. Increased PPAR γ expression levels in the liver and skeletal muscles may indicate an increase in circulatory glucose uptake by glucose transporters. The PPAR γ expression levels were the lowest in the kidney, which plays an important role in the regulation of electrolyte concentration and blood pressure. The upregulation of total PPAR γ and LPL expression levels coupled with a decrease in blood glucose and serum insulin levels as well as HOMA-IR indicated

improvement in insulin sensitivity most likely due to ligand-binding activation of PPAR γ .

In conclusion, we have demonstrated that GA lowered serum insulin, blood glucose and HOMA-IR significantly in rats compared to the control group. Improvements in serum lipid parameters with a decrease in triacylglycerol, total cholesterol and LDL-cholesterol and an elevation in HDL-cholesterol were observed. All six tissues (liver, kidney, abdominal and quadriceps femoris muscles, and visceral and subcutaneous adipose tissue) examined displayed upregulation of total PPAR γ and LPL expression levels. Quadriceps femoris and abdominal muscles and visceral adipose tissue showed a significant increase in total PPAR γ and LPL expression. Increase in expression levels of total PPAR γ and LPL in all the tissues was representative of their functions as regulators of glucose homeostasis.

Acknowledgments

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Research Article

PPAR- α Contributes to the Anti-Inflammatory Activity of Verbascoside in a Model of Inflammatory Bowel Disease in Mice

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The previous results suggest that peroxisome proliferator-activated receptor- α (PPAR)- α , an intracellular transcription factor activated by fatty acids, plays a role in control of inflammation. There is persuasive epidemiological and experimental evidence that dietary polyphenols have anti-inflammatory activity. In this regard, it has been demonstrated that verbascoside (VB) functions as intracellular radical scavenger and reduces the microscopic and macroscopic signs of experimental colitis. With the aim to characterize the role of PPAR- α in VB-mediated anti-inflammatory activity, we tested the efficacy of VB in an experimental model of inflammatory bowel disease induced by dinitrobenzene sulfonic acid, comparing mice lacking PPAR- α (PPAR- α KO) with wild type (WT) mice. Results indicate that VB-mediated anti-inflammatory activity is weakened in PPAR- α KO mice, compared to WT controls, especially in the inhibition of neutrophil infiltration, intestinal permeability and colon injury. These results indicate that PPAR- α can contribute to the anti-inflammatory activity of VB in inflammatory bowel disease.

1. Introduction

The inflammatory bowel diseases (IBDs) has a worldwide distribution, its pathogenesis is not clearly understood [1]. A major advance in the study of IBD has been the discovery and subsequent analysis of a number of models of mucosal inflammation that resemble IBD [2]. Recently, Blumberg and colleagues [3] have indicated that these models fall into four main categories (spontaneous, administration of exogenous agents, gene-targeted knockout or transgenic, and transfer of cells into immunodeficient animals) and each provides unique opportunities to discover insights into the nature of the pathogenesis of IBD. Dinitrobenzene sulfonic acid (DNBS)—induced colitis in experimental animals (e.g., mouse and rats) has proven to be a useful model of IBD, as it possesses many of the cell and humoral immunity characteristics found in human IBD [4].

Peroxisome proliferator-activated receptor (PPAR)- α is an intracellular transcription factor, activated by fatty acids, which play a role in inflammation [5]. PPARs are expressed in the intestine at various levels [6]. Recently, it has been demonstrated that PPAR- α is also expressed in the digestive tract mainly localized in the intestinal mucosa, in the small intestine, and in the colon [6]. In particular, it has been demonstrated that there is a higher expression of PPAR in the more differentiated colonic epithelial cells facing the intestinal lumen as compared to cells in the lower parts of the crypts [7].

In addition, it has been reported that PPAR- α activation can result in inhibition of nuclear factor (NF)- κ B activation and inflammatory genes expression [8]. Our recent results in diseases models of colitis and pleurisy show that mice lacking PPAR- α (PPAR- α KO) develop an increased inflammation as compared to wild type (WT)

mice. Moreover, treatment with appropriate doses of PPAR- α agonists can inhibit inflammatory diseases development [9].

Phenylpropanoid glycosides (PPGs, also synonymous of phenylethanoid glycosides) are water soluble derivatives of phenylpropanoids (PPs), a large group of natural polyphenols widely distributed in the plant kingdom [10]. There is growing evidence that PPGs, like other plant polyphenols in general and PPs in particular, are powerful antioxidants either by direct scavenging of reactive oxygen and nitrogen species, or by acting as chain-breaking peroxy radical scavengers [11]. Recently, PPs have been reported to be effective in the chemoprevention of tumors [10] as well as to have antithrombotic, wound healing, and cardioprotective actions [12]. There is now growing interest in the biotechnological approach to produce plant-derived active substances using nongenetically modified plant cell cultures [13]. Verbascoside (VB), containing a rhamnose unit bound to glucose which acts as a bridge, belongs to the extensive family of PPs, a class of plant-derived organic compounds that are biosynthesized from the amino acid phenylalanine. Recently, it has been demonstrated that VB promotes skin repair and ameliorates skin inflammation due to their ROS scavenging, antioxidant, iron chelating, and glutathione transferase (GST) activity inducing properties [14]. Moreover, we have recently demonstrated that VB from *Syringa vulgaris* IRBSV25/B cell cultures exerted protection on the chronic inflammatory response (colitis) caused by injection of DNBS in the rat [15].

With the aim to characterize the role of PPAR- α in VB-mediated anti-inflammatory activity, we tested the efficacy of VB in an experimental model of inflammatory bowel disease induced by DNBS, comparing PPAR- α KO and WT mice. Results indicate that in a chronic situation like inflammatory bowel disease, VB-mediated anti-inflammatory activity is weakened in PPAR- α KO mice compared to WT controls.

2. Materials and Methods

2.1. Animals. Mice (4-5 weeks old, 20–22 g) with a targeted disruption of the PPAR- α gene (PPAR- α KO) and littermate wild-type controls (PPAR- α WT) were purchased from Jackson Laboratories (Harlan Nossan, Italy). Mice homozygous for the Pparat^{mij}Gonz⁻ targeted mutation mice are viable, fertile, and appear normal in appearance and behavior. Exon eight, encoding the ligand-binding domain, was disrupted by the insertion of a 1.14 kb neomycin resistance gene in the opposite transcriptional direction. After electroporation of the targeting construct into J1 ES cells, the ES cells were injected into C57BL/6N blastocysts. This strain was created on B6,129S4 background, and is maintained as a homozygote on a 129S4/SvJae background by brother sister matings. The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was carried out in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/112/18/1986).

2.2. Plant Cell Line. The *Syringa vulgaris* plant utilized by IRB to originate the cell line derives from the Botanical Garden of the University of Bologna, Bologna, Italy. The stabilized and highly selected cell line with high content of VB was obtained from dissected young *Syringa vulgaris* leaves sterilized by NaOCl and Tween 20. The stabilized and selected cell line was deposited at the Plant Cell Bank (DSMZ, Deutsche Sammlung Von Mikroorganismen und Zellkulturen, Braunschweig, Germany) coded internally IRBSV25/B and internationally DSM 16857. The IRBSV25/B plant cell line was used for the industrial culture fermentors.

2.3. VB Containing Extract Preparation. *Syringa vulgaris* IRBSV25/B cell cultures obtained at the end of the fermentation process were collected and mechanically homogenized by Ultraturrax. The solid residue, mainly cellular debris, was then separated from the aqueous phase containing VB by centrifugation at 1.000×g for 10 minutes. The yield of VB was approx. 3 g/L of the plant cell culture liquid suspension. The VB in the supernatant was recovered by solid phase extraction on XAD4 resin, followed by elution with 80/20 ethanol/water (V/V) mixture. Then, the eluted VB was concentrated under reduced pressure and lyophilized. The final extract, a pale yellow powder, contained VB in an amount over 80% (w/w), together with minor admixture (>10% w/w) of other caffeic acid derivatives. The further purification of VB was performed by repeated column chromatography on C18 silica gel and Sephadex LH20 and subsequent crystallization obtaining a final product with VB content above 97% (w/w). The standard raw extract with a purity of 50 ± 1% (w/w) was obtained from the 80% (w/w) cell extract by addition of maltodextrins.

2.4. HPLC Analysis. The analysis was performed using HPLC system (Agilent, series 1100 DAD, Hewlett-Packard) consisting of an autosampler, high pressure mixing pump, and the column C18 (2) Phenomenex 4.6 × 150 mm. The gradient system was Phase A—water/0.01 N phosphoric acid; and Phase B: acetonitril/0.01 N phosphoric acid.

The reference product used to set up the quantitative method has been internally prepared by IRB. The chemical structure of the product has been determined and confirmed by means of mass spectrometry (MS) and nuclear magnetic resonance (NMR) at the Department of Organic Chemistry of the University of Milan. The NMR spectra were recorded in CD3OD solvent on a Bruker Avance 400 instrument.

The calibration curve of the verbascoside was in good linearity over the range of 20 μ g/mL–150 μ g/mL ($r = 0,9997$) and the average recoveries of verbascoside were 99.2% ($n = 5$, RSD 0.23%).

2.5. Experimental Groups. Mice were randomly allocated into the following groups: (i) PPAR- α WT DNBS + vehicle group. PPAR- α WT mice were subjected to DNBS-induced colitis plus administration of saline ($N = 10$); (ii) PPAR- α KO DNBS + vehicle group. PPAR- α KO mice were subjected to DNBS-induced colitis plus administration of saline ($N = 10$); (iii) PPAR- α WT+VB group. PPAR- α WT were subjected

to DNBS-induced colitis and VB (2 mg/kg dissolved in saline) was given by gavage every 24 hour, starting from 3 hours after the administration of DNBS ($N = 10$); (iv) *PPAR- α KO+VB* group. *PPAR- α KO* were subjected to DNBS-induced colitis and VB (2 mg/kg dissolved in saline) was given by gavage every 24 hour, starting from 3 hours after the administration of DNBS ($N = 10$); (v) *PPAR- α WT Sham + vehicle*. *PPAR- α WT* mice were subjected to the surgical procedures as the above groups except that 50% ethanol were administered to the mice ($N = 10$); (vi) *PPAR- α KO Sham + vehicle*. *PPAR- α KO* mice were subjected to the surgical procedures as the above groups except that 50% ethanol were administered to the mice ($N = 10$), (vii) *PPAR- α WT Sham + VB* group. Identical to *PPAR- α WT Sham + saline group* except for the administration of VB group; and (viii) *PPAR- α KO Sham + VB* group. Identical to *PPAR- α KO Sham + saline group* except for the administration of VB.

2.6. Induction of Experimental Colitis. Colitis was induced with a very low dose of DNBS (4 mg per mouse) by using a modification of the method first described in rats [16]. In preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were anesthetized by enflurane. DNBS (4 mg in 100 μ l of 50% ethanol) was injected into the rectum through a catheter inserted 4.5 cm proximally to the anus. Carrier alone (100 μ l of 50% ethanol) was administered in control experiments. Thereafter, the animals were kept for 15 minutes in a Trendelenburg position to avoid reflux. After colitis induction, the animals were observed for 3 days. On Day 4, the animals were weighed and anaesthetized with chloral hydrate, and the abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and processed for histology and immunohistochemistry. Colon damage (macroscopic damage score) was evaluated and scored by two independent observers according to the following criteria: 0, no damage; 1, localized hyperemia without ulcers; 2, linear ulcers with no significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending >1 cm along the length of the colon; and 5–8, one point is added for each centimeter of ulceration beyond an initial 2 cm.

2.7. Light Microscopy. After fixation for 1 week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, and 1% acetic acid), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, New Jersey). Thereafter, 7- μ m sections were deparaffinized with xylene, stained with haematoxylin-eosin, and observed in a Dialux 22 Leitz (Wetzlar, Germany) microscope. In order to have a quantitative estimation of colon damage, section ($n = 6$ for each animal) was scored by 2 independent observers blinded to the experimental protocol.

2.8. Myeloperoxidase Activity. At 4 days after intracolonic injection of DNBS, the colon was removed and weighed.

The colon was analyzed for myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, as previously described [17]. MPO activity was defined as the quantity of enzyme degrading 1 μ mol of peroxide per min at 37°C and was expressed in milliunits per gram weight of wet tissue.

2.9. Intestinal Permeability. On the 4th day of the experiment, mannitol 5 mg and lactulose dissolved in 0.5 ml of normal saline were gavaged. Individual mice were then immediately placed in metabolic cages to allow for collection of urine for 4 to 5 hours as previously described [18]. Urinary sugar excretion was assessed by gas-liquid chromatography.

2.10. Western Blot Analysis for PPAR- α . Nuclear extracts were prepared as previously described [19] with slight modifications. PPAR- α expression was quantified in nuclear fraction by Western blot analysis. The filters were blocked with 1x PBS, 5% (w/v) nonfat dried milk (PM) for 40 minutes at room temperature and subsequently probed with specific Abs against PPAR- α (1 : 500; Santa Cruz Biotechnology) in 1x PBS, 5% w/v nonfat dried milk, 0.1% Tween-20 (PMT) a 4°C, overnight. Membranes were incubated with peroxidase-conjugated bovine antimouse IgG secondary antibody or peroxidase-conjugated goat antirabbit IgG (1 : 2000, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against lamin A/C protein (1 : 5,000 Sigma-Aldrich Corp.).

2.11. Reagents. Biotin-blocking kit, biotin-conjugated goat antirabbit IgG, and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA, USA). Primary antinitrotyrosine antibody was purchased from Upstate Biotech (Saranac Lake, NY, USA). Primary ICAM-1 (CD54) for immunohistochemistry was purchased by Pharmingen. Reagents and secondary and nonspecific IgG antibody for immunohistochemical analysis were from Vector Laboratories Inc. All other reagents and compounds used were obtained from Sigma Chemical Company.

2.12. Statistical Analysis. All values in the figures and text are expressed as mean \pm SEM of N observations, where n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least 3 experiments performed on different experimental days. Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Student's unpaired t -test. Nonparametric data were analyzed with the Fisher's exact test. A P -value less than .05 was considered significant.

3. Results

3.1. Effects of VB Treatment on PPAR- α Expression in Colon Tissues. Previous studies have demonstrated an important

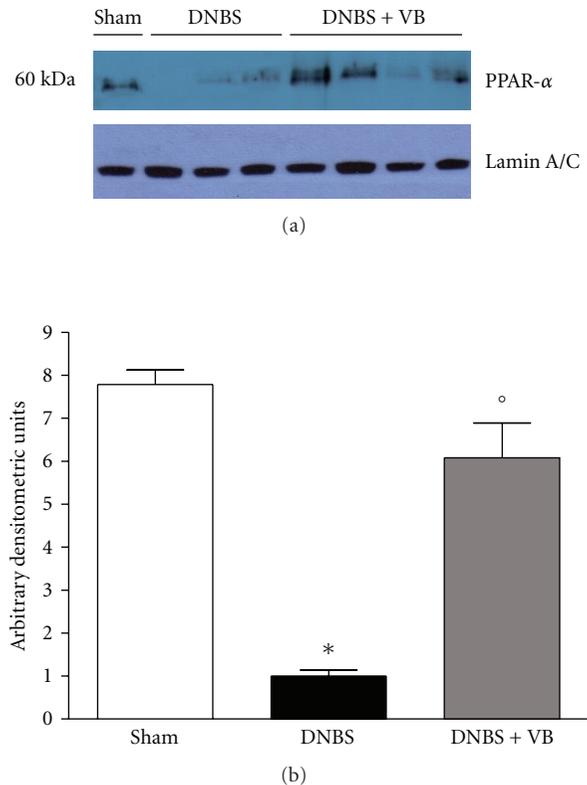


FIGURE 1: Western blot analysis for PPAR- α in nuclear extracts. By Western blot analysis, a basal level of PPAR- α expression was detected in colon tissues from sham-operated mice ((a) and (b)). Four days after DNBS administration, PPAR- α expression was significantly reduced. VB treatments significantly reduced the DNBS-induced inhibition of PPAR- α expression. The relative expression of the protein bands was standardized for densitometric analysis to lamin A/C levels and reported in (b) expressed as mean \pm s.e.m. from $n = 5/6$ colon for each group. * $P < .01$ versus Sham; $^{\circ}P < .01$ versus DNBS-WT group.

role for PPAR- α in experimental colitis [15] and have hypothesized that the ability of VB to reduce inflammation may be dependent on activation of PPAR- α . Thus, we evaluated PPAR- α expression in the nuclear fractions from colon tissue by Western Blot analysis. A basal level of PPAR- α was detected in colon tissue from sham-treated mice, whereas PPAR- α levels were substantially reduced in DNBS-treated mice (Figure 1(a) and 1(b)). The effect of DNBS was significantly reduced by VB administration (Figure 1(a) and 1(b)).

3.2. Role of Functional PPAR- α Gene in the Anti-Inflammatory Activity of VB on the Degree of Colitis. Four days after intracolonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of cecum, colon, and rectum showed presence of mucosal congestion, erosion, and hemorrhagic ulcerations (Figure 2(b), see macroscopic score Figure 2(f)). The histopathological features included a transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon

section from DNBS-treated PPAR- α WT mice (Figure 3(b), see histological score Figure 3(f)). The observed inflammatory changes of the large intestine were associated with an increase in the weight of the colon (Figure 4(a)). The absence of PPAR- α gene significantly increases the extent and severity of the macroscopic signs (Figure 2(c), see macroscopic score Figure 2(f)), histological indications of colon injury (Figure 3(c), see histological score Figure 3(f)), as well as the colon weight (Figure 4(a)). Four days after colitis induced by DNBS treatment, all mice had diarrhea and a significant reduction in body weight (compared with the control groups of mice). Absence of a functional PPAR- α gene in PPAR- α KO mice resulted in a significant augmentation of lost of body weight (Figure 4(b)). No macroscopic (Figure 2(a), see macroscopic score Figure 2(f)) or histological (Figure 3(a), see macroscopic score Figure 3(f)) alteration was observed in the colon tissue from vehicle-treated PPAR- α WT and PPAR- α KO mice. On the other hand, the treatment of PPAR- α WT with VB resulted in a significant decrease in the extent and severity of the macroscopic (Figure 2(d), see macroscopic score Figure 2(f)), histological signs of colon injury (Figure 2(d), see macroscopic score Figure 2(f)), the colon weight (Figure 3(a)), and the lost of body weight (Figure 3(b)). The genetic absence of the PPAR- α receptor significantly blocked the beneficial effect of VB (Figures 2 and 3, see macroscopic score Figures 2(f) and 3(f)).

3.3. Role of PPAR- α in VB-Induced Inhibition of PMN Infiltration. The colitis caused by DNBS was also characterized by an increase in myeloperoxidase activity, an indicator of the neutrophils' accumulation in the colon (Figure 5). This finding is consistent with our observation made with light microscopy that the colon from vehicle-treated DNBS-rats contained a large number of neutrophils. In PPAR- α KO mice, colon myeloperoxidase activity was markedly increased in comparison to those of PPAR- α WT animals (Figure 5). On the contrary, VB significantly reduced the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon. The genetic absence of the PPAR- α receptor significantly blunted the effect of the VB on the neutrophil infiltration (Figure 5).

3.4. Intestinal Permeability. For assessing baseline small bowel permeability, permeability probes (lactulose and mannitol) were administered by gavage, and urinary lactulose and mannitol excretion were measured in urine collected over a 4- to 5-hour post gavage period and L/M ratios were calculated. As indicated in Figure 6, the L/M ratios were unchanged in the sham PPAR- α WT and in sham PPAR- α KO mice. On the contrary, in the urinary samples from PPAR- α WT mice injected with DNBS L/M ratios were significantly higher in comparison to the sham animals. The absence of PPAR- α in mice (animals with the PPAR- α KO phenotype) resulted in a pronounced increase of L/M ratios (Figure 6). On the contrary, VB significantly reduced the increase in L/M ratios. The genetic absence of the PPAR- α receptor significantly blocked the effect of the VB on L/M ratios (Figure 6).

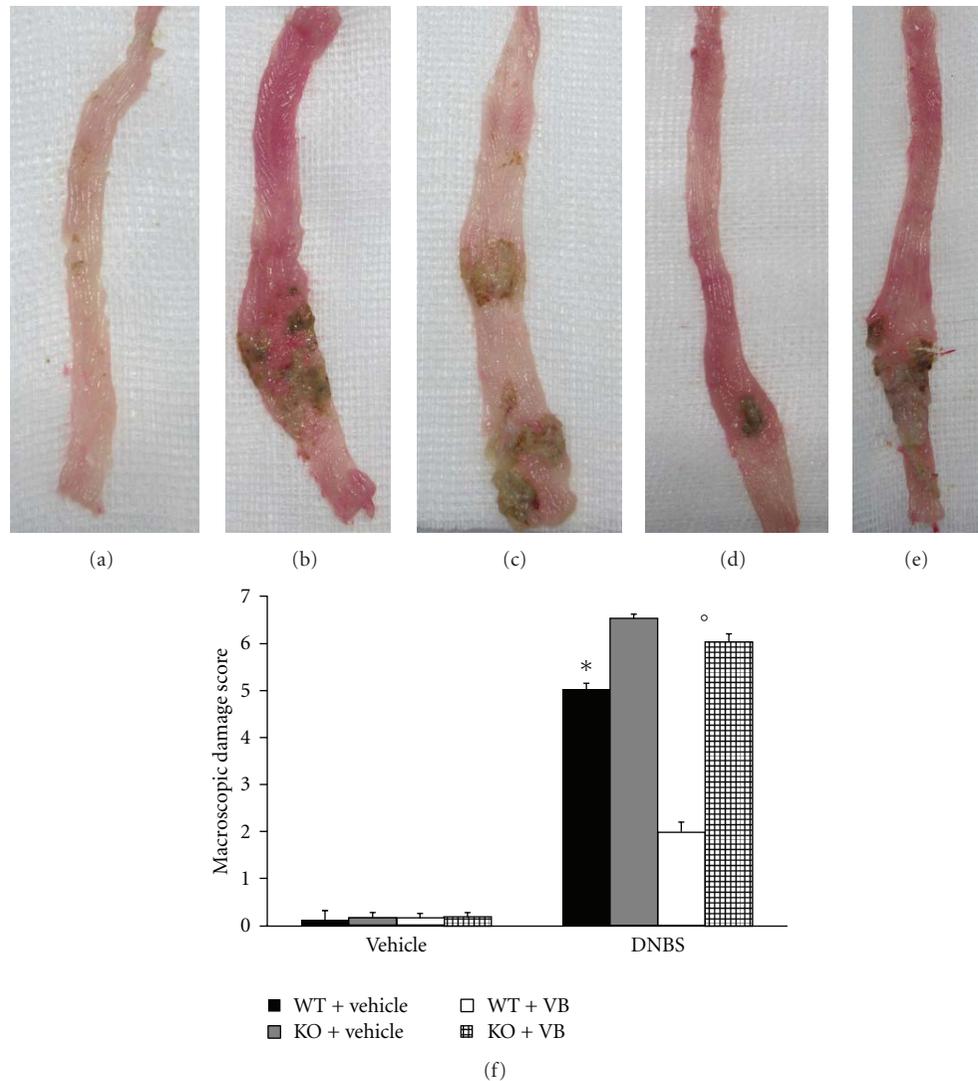


FIGURE 2: Role of PPAR- α in the anti-inflammatory property of VB on clinical expression of DNBS-induced colitis and macroscopic damage score. Colon tissues from sham-treated mice (a), colon tissues from DNBS-treated PPAR- α WT (b) or DNBS-treated PPAR- α KO mice (c) from mice at 4 days post DNBS administration, and the colon tissues collected from DNBS-treated mice which have received VB treatment (d). The treatment of PPAR- α WT with VB resulted in a significant decrease in the extent and severity of the macroscopic signs (d). The genetic absence of the PPAR- α receptor significantly reduced the effect of the VB treatment (e). The macroscopic damage score was made by two independent observers (f). Data are means \pm SEM of 10 mice for each group. * $P < .01$ versus Sham; $^{\circ}P < .05$ versus DNBS-WT group.

4. Discussion

In the present paper, we show that the absence of PPAR- α in mice results in a reduced anti-inflammatory response to VB treatment in an IBD model. These results are in agreement with our previous observations indicating that PPAR- α KO mice are more susceptible to induction of inflammatory bowel disease [20].

There is growing interest in the role of complementary and alternative medicine (CAM) in health and disease. There are a myriad of CAM treatments that may prove beneficial for patients with IBD. Unfortunately, the scientific basis for the use of these modalities frequently is lacking, and safety has not been assessed. Recently, we have demonstrated that

VB from *Syringa vulgaris* IRBSV25/B cell cultures attenuates DNBS-induced colitis in the rats, a well-established model of acute intestinal inflammation with some shared features of Crohn's disease [15].

Various studies have regarded polyphenols as PPAR ligands [21]. In particular they have demonstrated that genistein increases the expression of genes involved in lipid catabolism in a PPAR α -dependent mechanism [22]. Moreover, it has been also demonstrated that epigallocatechin-3-gallate (EGCG) binds PPAR- α [21]. Therefore, other studies have also demonstrated that amentoflavone upregulated PPAR- γ expression in A549 human lung cell as well as that curcumin dramatically induced the expression of PPAR- γ at levels of transcription and translation [23].

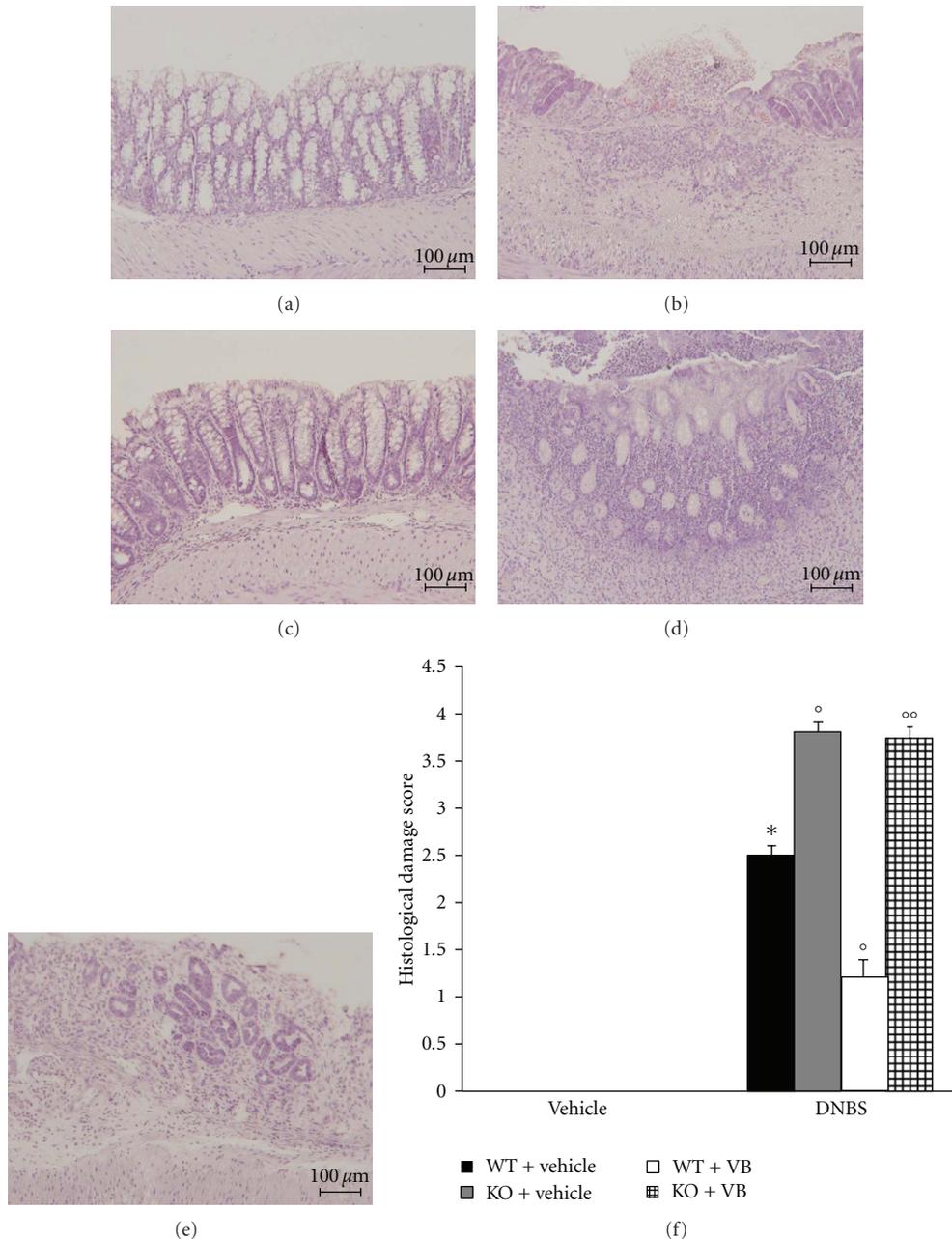


FIGURE 3: No histological alteration was observed in the colon section from sham-operated mice (a). Mucosal injury was produced after DNBS administration characterized by absence of epithelium and a massive mucosal and submucosal infiltration of inflammatory cells (b). Treatment with VB corrected the disturbances in morphology associated with DNBS administration in PPAR- α WT (c). The absence of PPAR- α gene significantly increases the extent and severity of the histological signs of colon injury (d). The genetic absence of the PPAR- α receptor significantly reduced the effect of the VB treatment (e). The histological score (f) was made by two independent observers. Figure 3 is representative of at least 3 experiments performed on different experimental days. Data are means \pm SEM of 10 mice for each group. * $P < .01$ versus Sham; ^o $P < .05$ versus DNBS-WT group.

These findings implied a potential therapeutic value of PPARs ligands in treatment of inflammatory disease [24, 25].

PPAR- α itself is also able to directly mediate some anti-inflammatory effects and it has been shown that agonist-induced activation inhibits a number of inflammatory parameters including TNF- α production, iNOS, COX-2, and

adhesion molecule expression as well cell infiltration in the tissues [5].

Based on these observations, we performed studies in the attempt to determine whether the presence and/or the stimulation of PPAR- α could enhance the VB anti-inflammatory efficacy. For this purpose, we used an experimental model of

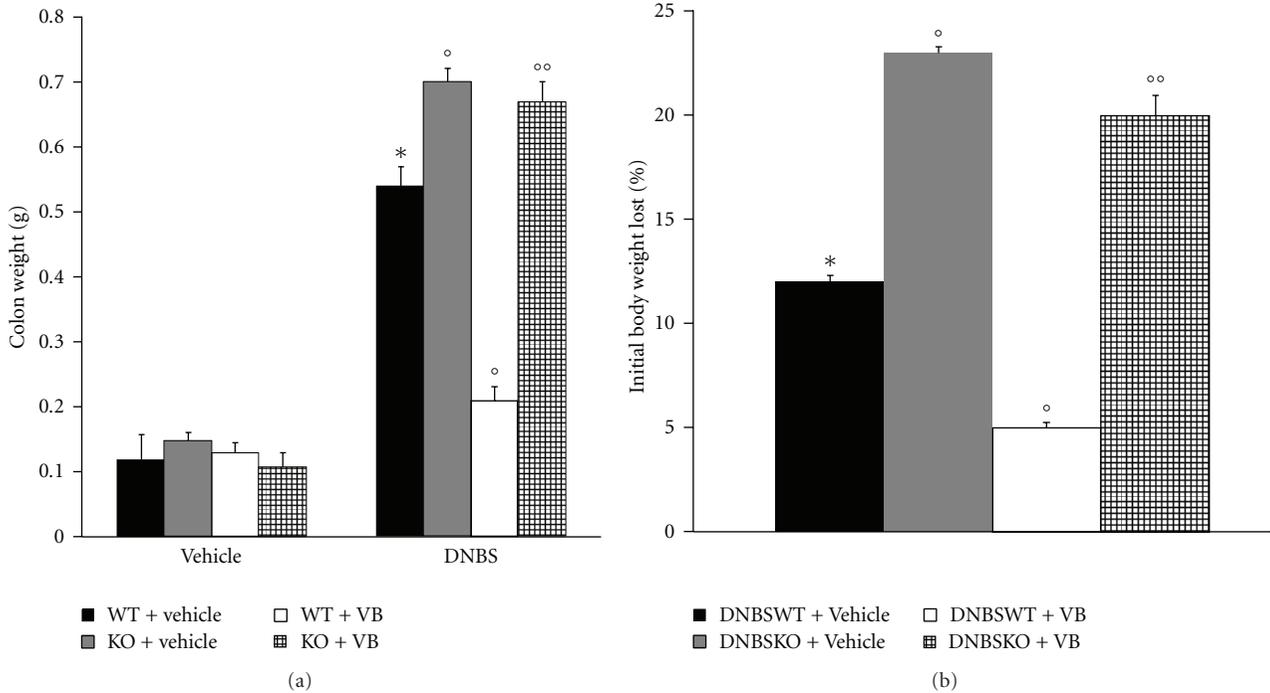


FIGURE 4: Role of PPAR- α in the anti-inflammatory property of VB on colon weight and body weight lost. Four days after DNBS administration a significant increase in colon weight (a) and body weight lost (b) was observed. Treatment with VB significantly reduced the increased colon weight and lowered body weight lost. The absence of PPAR- α gene significantly abolished the effect of VB. Figure 4 is representative of all the animals in each group. Data are means \pm SEM of 10 mice for each group. * $P < .01$ versus Sham; ° $P < .01$ versus DNBS-WT group.

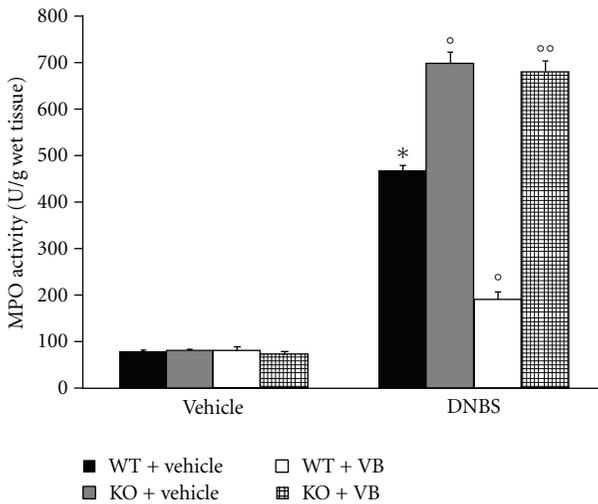


FIGURE 5: Effect of endogenous PPAR- α on neutrophil infiltration. Myeloperoxidase (MPO) activity was significantly increased in DNBS-treated PPAR- α WT mice in comparison to SHAM. MPO activity was significantly enhanced in DNBS-treated PPAR- α KO mice. Treatment with VB significantly reduced the colon MPO activity. The genetic absence of the PPAR- α receptor significantly blocked the effect of VB treatment. Data are means \pm SEM of 10 mice for each group. * $P < .01$ versus sham; ° $P < .01$ versus DNBS WT.

inflammatory bowel disease induced by DNBS using PPAR- α KO mice compared to WT.

VB-type phenolic ester glycosides showed various biological activities, from antibiotic and antifeedant to immunodepressive. Moreover, VB exhibits antioxidative effects [26] and antibacterial [27]. VB has been also shown to modulate nitric oxide (NO) production and the expression of inducible nitric oxide synthase (iNOS) in activated macrophages [28, 29]. It also inhibits histamine, arachidonic acid release, and prostaglandin E2 production in RBL-2H3 mast cells suggesting a possible application of the compound as anti-inflammatory remedy [30].

In the present study, we have clearly confirmed that the increased colon injury in the colon tissue from DNBS-treated PPAR- α KO mice correlated with the enhanced leukocyte infiltration, as assessed by the specific granulocyte enzyme myeloperoxidase. These observations are in agreement with a previous study [20]. Moreover, in the present study we clearly demonstrate that when WT and PPAR- α KO mice were treated with VB, a significant inhibition of neutrophils infiltration as well as tissue injury was observed in WT but not in PPAR- α KO mice.

Furthermore, intestinal permeability reflects the integrity of the intestinal mucosal barrier, which restricts the passive permeation of luminal substances [31]. It is well known that altered intestinal permeability has been reported in

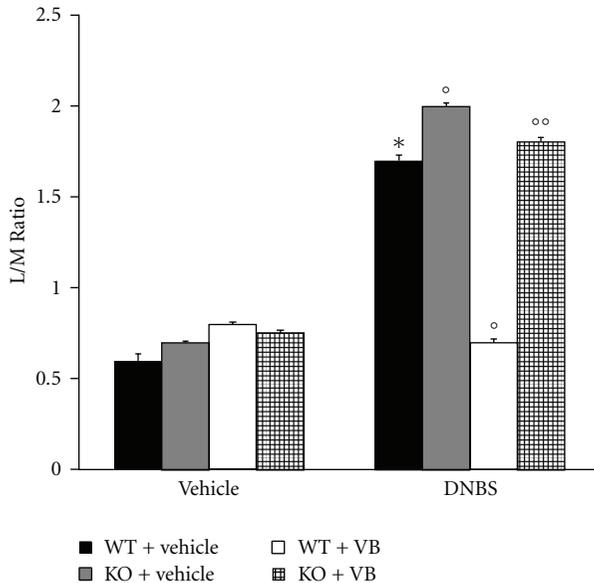


FIGURE 6: Effects of PPAR- α on intestinal permeability. Permeability probes (lactulose and mannitol) were administered by gavage, and urinary lactulose and mannitol excretion were measured in urine collected over a 4- to 5-hour postgavage period, and L/M ratios were calculated. The L/M ratio was unchanged in the sham-treated mice. On the contrary, at 4 days after colitis, the L/M ratios were significantly higher in the urinary samples from DNBS-treated PPAR- α WT mice in comparison with the sham animals. The absence of a functional PPAR- α gene in PPAR- α KO mice resulted in a more pronounced increase of L/M ratios. Treatment with VB significantly reduced the increased L/M ratios, while the absence of PPAR- α gene significantly abolished the effect of VB. Data are means \pm SEM of 10 mice for each group. * $P < .01$ versus sham; ° $P < .01$ DNBS WT.

various intestinal conditions associated with diarrhea such as celiac disease [32], IBD [33], infectious gastroenteritis [34–36], and food intolerance or allergy [37, 38]. It is possible to assess intestinal permeability *in vivo* by measuring the urinary excretion of hydrosoluble and not degradable probes given orally. The lactulose/mannitol (L/M) test uses a mixture of two unmetabolized sugars, minimizing the influence of variables such as gastric emptying, intestinal motility, and renal function, which affect both markers equally. Moreover, it is a nontoxic, noninvasive test and the laboratory procedure is simple and inexpensive [39]. The L/M test is used in the diagnosis and follow-up of celiac [40–42] and Crohn's disease [43, 44].

As clearly demonstrated in this study, the absence of PPAR- α in mice (animals with the PPAR- α KO phenotype) resulted in a pronounced increase in L/M ratios observed at 4 days after DNBS administration in comparison with PPAR- α WT mice. Moreover, when WT and PPAR- α KO mice were treated with VB, a significant inhibition in L/M ratios was observed in WT but not in PPAR- α KO mice. It is also important to point out that the genetic inhibition of PPAR- α did not change the physiological L/M ratios in the sham-operated mice.

The results here described clearly indicate that the anti-inflammatory efficacy of VB treatment is favored by the presence of PPAR- α . Moreover, previous studies showed that PPAR- α agonists exert some anti-inflammatory activity [45]. The efficacy of VB treatment in inflammatory and autoimmune diseases is an important therapeutic subject and while some patients obtain clinical benefits from treatment, others are not responsive or even resistant to therapy. Results discussed here suggest a new mechanism contributing to determine the full VB efficacy and suggest future studies aimed to analyze the possible relevance of PPAR- α in other human inflammatory disease models, such as sepsis.

In conclusion, our results clearly indicate that PPAR- α can contribute by enhancing the anti-inflammatory activity of VB in DNBS-induced inflammatory bowel disease model. These observations suggest that VB could use the same pathway of PPAR- α agonists in inflammatory diseases for its beneficial effects.

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Review Article

Virtual Screening as a Technique for PPAR Modulator Discovery

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Virtual screening (VS) is a discovery technique to identify novel compounds with therapeutic and preventive efficacy against disease. Our current focus is on the *in silico* screening and discovery of novel peroxisome proliferator-activated receptor-gamma (PPAR γ) agonists. It is well recognized that PPAR γ agonists have therapeutic applications as insulin sensitizers in type 2 diabetes or as anti-inflammatories. VS is a cost- and time-effective means for identifying small molecules that have therapeutic potential. Our long-term goal is to devise computational approaches for testing the PPAR γ -binding activity of extensive naturally occurring compound libraries prior to testing agonist activity using ligand-binding and reporter assays. This review summarizes the high potential for obtaining further fundamental understanding of PPAR γ biology and development of novel therapies for treating chronic inflammatory diseases through evolution and implementation of computational screening processes for immunotherapeutics in conjunction with experimental methods for calibration and validation of results.

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1. Introduction

Transdisciplinary research has become a common means of addressing the most pressing societal problems. Past discoveries of scientific hallmarks have favored exploring the depths of established ideas across scientific disciplines to better understand biological systems and processes. This is possible because the wealth of scientific knowledge has only scratched the surface of how biological systems work, and often exploring the unknown intricacies of biological networks requires knowledge of more than one scientific realm.

The extreme amounts of information readily available to the scientific community present a valuable and perpetually renewing resource. However, this overabundance also poses a problem. There is simply too much information within too many areas of science for one person with expertise in a single field to rapidly make novel advances. Take, for example, the question of what factors determine whether an individual suffers from a particular disease. When designing

a treatment, one can look at the symptoms, the cause of the symptoms, genetic differences between healthy and afflicted individuals, genetic differences between individuals with the same disease but slightly different symptoms, methods for treating the symptoms, methods for controlling or correcting the disease, and methods for screening for the disease. This list includes, but is not limited to, disciplines such as genetics, bioinformatics, biochemistry, pharmacology, and medicine, and it is the combination of all these disciplines that facilitates the development of effective preventive and therapeutic approaches.

In a more general sense, there is also an increasing need for integrating computational and experimental approaches. Computers have become a large and vital part of scientific exploration and serve to simplify and expedite processes that could take months to years for an individual to complete. First, computers allow for organization of scientific knowledge. Second, they allow for sharing of ideas and discoveries in an effective and timely fashion. Third, computers allow individuals to better analyze experimental

results and develop more efficacious test methods. The fourth and ultimate benefit of computer technology to science is improved efficiency due to a reduced necessity for time, money, and resources.

Peroxisome proliferator-activated receptor (PPAR) research is one of many areas that may benefit from advances in computational biology and other transdisciplinary approaches. Mixtures of computational and experimental studies have given insight into characteristics of PPARs, particularly PPAR-gamma (PPAR γ) and its modulators, as well as the role of these proteins in treating type 2 diabetes (T2D), gastrointestinal diseases, and genetic disorders associated with glucose homeostasis and lipid uptake.

2. Characteristics of PPAR γ and the Activation Process

PPAR γ is one of three known PPAR isoforms (α , δ , and γ). PPARs belong to the nuclear hormone receptor superfamily and have been found to regulate inflammation, immunity, and metabolism [1, 2]. Members of this superfamily are structurally and functionally conserved transcription factors that regulate both target gene expression and repression after ligand binding occurs [3]. A diverse set of natural and synthetic molecules is classified as ligands that can induce activation and expression of PPARs. These ligands include nutrients, nonnutrient endogenous ligands, and drugs such as thiazolidinediones (TZDs) and fibrates [1, 2, 4]. Known endogenous and dietary agonists include conjugated linoleic acid (CLA), 9-(S)-hydroxyoctadecadienoic (9-HODE), 13-(S)-hydroxyoctadecadienoic (13-HODE) acid, and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15d-PGJ2) [1, 5].

A great deal of literature focuses on increasing insulin sensitivity by controlling PPAR γ interactions and altering gene expression of various transcription factors. PPAR γ is a component of an extensive group of controls for adipogenesis and glucose homeostasis, and both of these processes directly affect obesity and T2D [6]. PPAR γ is located in high concentrations in adipocytes, and has also been found in significant amounts in the retina, cells of the immune system, and colonic epithelial cells [1, 7]. Functionally, PPAR γ downregulates the expression of proinflammatory cytokines by antagonizing the activities of transcription factors such as AP-1 and NF- κ B, and favoring the nucleocytoplasmic shuttling of the activated p65 subunit of NF- κ B [2]. As a consequence of the important roles PPARs play in controlling metabolic homeostasis and inflammatory processes, they are all well recognized as molecular targets for drugs against metabolic diseases, such as T2D [8–10], and treatment of immunoinflammatory disorders.

Structurally, PPAR γ is composed of a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD). The first step in PPAR γ activation is disassociation of corepressors after binding of retinoic acid (RA) to a single retinoid X receptor (RXR) subunit. This step is an essential part of numerous endocrine system pathways [6]. The ligand-bound RXR then associates with ligand-bound PPAR γ . To become fully active, the PPAR γ -RXR α

heterodimer requires association of coactivator molecules [6]. Agonist binding to PPAR γ regulates activity by causing conformational changes to the LBD, which is composed of approximately 250 amino acids near the C-terminal end of the protein [11]. Mediation of activity is a direct result of changes to the transcription activation function-2 (AF-2) domain [6, 12]. These changes vary depending on the type of ligand that binds to the LBD. Changes to AF-2 allow for coactivator recruitment, followed by transcriptional activation.

Co-activator recruitment is based on a LXXLL binding motif (nuclear receptor box) found on both PPAR γ and coactivators like steroid receptor coactivating factor-1 (SRC-1) that associate for transcription induction after the conformational change of the AF-2 region [3, 13, 14]. The DNA binding domains of PPAR γ -RXR α interact with PPAR response elements (PPREs) found within the genome [15]. Such elements include 5' regions for aP2 and PEPCK genes as part of adipogenesis, which suggests PPAR γ plays a major role in fat cell-specific gene function [15]. Though PPAR γ is typically known to interact with DNA, it can also interact directly with other proteins to induce activity. For example, as preadipocytes differentiate, expression of C/EBP β and C/EBP δ directly activate PPAR γ and C/EBP α , which promote further differentiation and full insulin sensitivity [15]. Alternatively, binding by specific ligands can induce activity as well. The use of TZDs in the treatment of T2D improves insulin resistance by increasing GLUT-4 levels and decreasing the levels of cytokines that induce insulin resistance, such as TNF- α and IL-6 [15] by antagonizing the activity of proinflammatory transcription factors [2]. Therefore, it is important to note that understanding the interactions involved in coactivator recruitment is crucial for predicting activity after ligand binding, and ultimately treatment of insulin insensitivity and inflammation.

3. Agonists and the Ligand-Binding Domain of PPAR γ

Fatty acids and lipid metabolites have been found to be endogenous ligands for PPAR γ . A recent study by Waku et al. [16] gives insight into how these ligands bind covalently to Cys285, thereby modifying PPAR γ conformations. In particular, these covalent modifications induce rearrangement of the side-chain network around the created covalent bond in order to generate different transcriptional strengths. This attenuation of strength is specific to the ligand type and conformation. Waku et al. also mention that Ile267 and Phe287 are two key residues repositioned by covalent binding of fatty acids [16]. It is also important to note for some fatty acids, formation of a complex containing two fatty acid units is necessary for binding within the LBD of PPAR γ [5].

Synthetic ligands that can interact with PPAR γ can be divided into at least three classes: full agonists, partial agonists, and antagonists. Full agonists bind and alter the conformation of the AF-2 domain allowing for coactivators to bind for activation of genes for both adipogenic and insulin sensitivity processes. Partial agonist binding leads to

a change that allows for recruitment of coactivators responsible for insulin sensitivity without affecting adipogenesis. Antagonists show high affinity, but do not activate PPAR γ , suggesting the conformational change to AF-2 is either not enough to allow coactivator association or is similar to that of the inactive conformation. A study conducted by Kallenberger et al. showed that the dynamics of the AF-2 region plays a major role in the genetic regulation capabilities of PPAR γ . Binding of a ligand reduces AF-2 mobility and allows for regulation of gene expression. Furthermore, the AF-2 region of PPAR γ can undergo natural mutations, which result in severe insulin insensitivity and cause noticeable changes in dynamics of that AF-2 region [12].

PPAR γ agonists typically possess a small polar region and a hydrophobic region that form hydrogen bonds and hydrophobic interactions, respectively, within the LBD. Hydrogen bonding typically occurs between His323, Tyr473, and His449 of the PPAR γ LBD and carbonyl oxygens of the ligand (Figure 1) [6, 13, 17]. Hydrogen bonding of the ligand to Tyr473 is key to the stabilization of the AF-2 region [13, 18]. The hydrophobic moiety interacts with other residues in the cavity, such as Leu465, Leu469, and Ile472, establishing hydrophobic interactions to stabilize the domain (Figure 2) [6, 13, 17].

In the case of partial agonists, key interactions are different, which result in lesser degrees of AF-2 stabilization and differential stabilization of distinct regions of the LBD [5]. Either of these events leads to activation as a result of a shift of the ligand polar group away from the hydrogen-bonding residues. This shift can prevent hydrogen bonding or lead to a different hydrogen-bonding network. Changes in the hydrophobic interactions between the ligand and residues within the LBD also exist. The combination of these events results in conformational changes different enough from those caused by full agonist binding to elicit only partial activation and recruitment of different coactivators [5, 17].

Antagonists for PPAR γ have not received the same amount of research interest as the full and partial agonists. Therefore, little information is available on the binding of this type of ligand to the γ isoform. Antagonists for PPAR α , however, have provided insight into how ligands of this class might interact with PPAR γ due to the conservation of the mode of corepressor binding. Typically, corepressors bind to PPAR α in the absence of ligand. The complex is then stabilized by antagonists, which disrupt any potential interactions with coactivators, and thereby prevent the initiation of transcription [19].

The LBD of PPAR γ is a large, T-shaped cavity [17] with a volume of approximately 1440 Å³ [6, 17], which can easily accommodate many different ligands due to the dynamics of the ligand-binding pocket [20]. It is important to note that the type of ligand determines which coactivator associates with the PPAR γ -RXR α heterodimer. The coactivator then determines the target gene for regulation and the direction of regulation (up or down). Thus, knowing the final conformation of the LBD that is necessary to elicit a specific activity is crucial for therapeutic development [3].

Until recently, available crystal structures for PPAR γ generally were composed solely of the PPAR γ LBD with

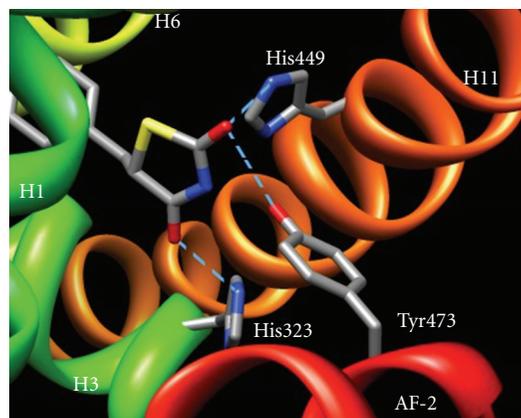


FIGURE 1: Rosiglitazone bound to LBD of PPAR γ . Helices are labeled with H, followed by a number. Key residues involved in hydrogen bonding are labeled. Blue dashed lines represent bonding interactions between the hydrogens of the residue and the oxygens of the ligand. (PDB ID 3DZY).

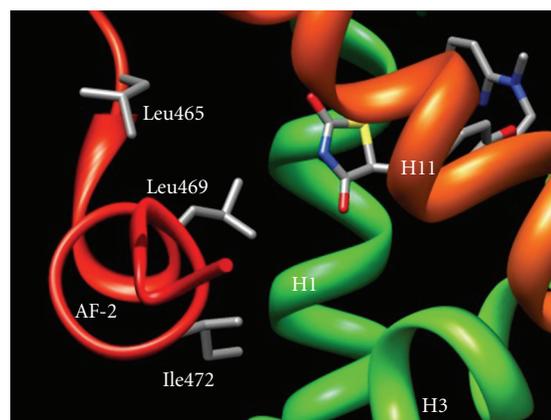


FIGURE 2: Rosiglitazone bound to LBD of PPAR γ . Helices are labeled with H, followed by a number. Some of the key residues involved in hydrophobic interactions are labeled. (PDB ID 3DZY).

a ligand bound, a RXR α LBD heterodimerized to PPAR γ , and a short segment of a coactivator protein. Chandra et al. have published three new crystal structures (3DZU, 3DZY, and 3E00) for PPAR γ composed of the DBD, the hinge region, and the LBD with ligand bound [21]. These structures are in complex with RXR α , polypeptides that mimic the LXXLL motif for coactivator binding, and a short DNA segment representative of a PPRE. Observations related to heterodimerization of PPAR γ and RXR α , as well as activation of response elements are reported in this study. The LBD and DBD of PPAR γ are positioned closely together, which aids in coupling of the PPAR γ LBD to the relatively wide space between the LBD and DBD of RXR α [21]. The study also discusses the polarity of the PPAR γ -RXR α heterodimer, which is determined by the (C)-terminal extension of PPAR γ and the DBD interactions of the two subunits. Table 1 contains a list of all currently available structures for PPAR γ , which can be found in RCSB's PDB online database [22] <http://www.pdb.org/>.

TABLE 1: PDB IDs of published crystal and NMR structures for PPAR γ with various ligands bound. Resolution values are in Angstroms (Å). The “Reference number” columns list references for each PDB ID. The citations for these are present in the reference section. All PDB IDs list *Homo sapiens* as the protein source except 1ZGY (*Rattus norvegicus*). Access date: 23 March 2009.

Structure ID	Resolution (Å)	Release date	Reference number	Structure ID	Resolution (Å)	Release date	Reference number
1FM6	2.1	02/16/01	[6]	2Q6S	2.4	10/23/07	[23]
1FM9	2.1	02/16/01	[6]	2Q8S	2.3	10/14/08	[24]
1I7I	2.3	03/09/02	[25]	2QMV	n/a (NMR)	09/02/08	[26]
1K74	2.3	12/05/01	[27]	2VSR	2.0	08/19/08	[5]
1KNU	2.5	12/19/02	[28]	2VST	2.3	08/19/08	[5]
1NYX	2.7	07/15/03	[29]	2VV0	2.5	08/19/08	[5]
1PRG	2.2	01/13/01	[13]	2VV1	2.2	08/19/08	[5]
1RDT	2.4	11/09/04	[30]	2VV2	2.8	08/19/08	[5]
1WM0	2.9	09/07/04	[31]	2VV3	2.8	08/19/08	[5]
1ZEO	2.5	04/25/06	[32]	2VV4	2.3	08/19/08	[5]
1ZGY	1.8	07/26/05	[33]	2ZK0	2.4	2/24/09	[16]
2ATH	2.3	08/25/06	[34]	2ZK1	2.6	2/24/09	[16]
2F4B	2.1	02/14/06	[35]	2ZK2	2.3	2/24/09	[16]
2FVJ	2.0	05/16/06	[36]	2ZK3	2.6	2/24/09	[16]
2G0G	2.5	05/16/06	[37]	2ZK4	2.6	2/24/09	[16]
2G0H	2.3	05/16/06	[37]	2ZK5	2.5	2/24/09	[16]
2GTK	2.1	09/26/06	[38]	2ZK6	2.4	2/24/09	[16]
2HFP	2.0	09/19/06	[39]	3B3K	2.6	10/28/08	[40]
2HWQ	2.0	08/07/07	[41]	3BC5	2.3	11/18/08	[42]
2HWR	2.3	08/07/07	[41]	3CDP	2.8	1/13/09	[43]
2I4J	2.1	04/17/07	[17]	3CDS	2.7	12/30/08	[40]
2I4P	2.1	04/17/07	[17]	3CS8	2.3	06/03/08	[44]
2I4Z	2.3	04/17/07	[17]	3CWD	2.4	07/08/08	[45]
2OM9	2.8	04/24/07	[46]	3D24	2.1	06/10/08	[47]
2P4Y	2.3	01/08/08	[48]	3D6D	2.4	12/30/08	[40]
2POB	2.3	03/18/08	[49]	3DZU	3.2	10/28/08	[21]
2PRG	2.3	07/19/99	[13]	3DZY	3.1	10/28/08	[21]
2Q59	2.2	10/23/07	[23]	3E00	3.1	10/28/08	[21]
2Q5P	2.3	10/23/07	[23]	3ET0	2.4	2/17/09	[50]
2Q5S	2.0	10/23/07	[23]	3ET3	2.0	2/17/09	[50]
2Q61	2.2	10/23/07	[23]	3PRG	2.9	08/30/99	[3]
2Q6R	2.4	10/23/07	[23]	4PRG	2.9	05/27/99	[51]

4. Docking

Docking can be defined as predicting both ligand conformation and orientation within a targeted binding site [52]. Experimentally derived crystal and NMR protein structures are used as the basis for docking, and the physics involved is based on what is known about atomic and molecular interactions, as well as laws of thermodynamics. All docking methods must include sampling ligand conformations, generating poses of the ligand within the receptor binding site, and scoring the poses.

Before beginning a docking study, one must select from three conformational searching methods: systematic, random, and simulation. The systematic method explores the degrees of freedom possessed by the torsional bonds of a molecule. To achieve this goal, the ligand parts are introduced incrementally in order to obtain an energetically

favorable conformation. Random searching, as the name implies, is based on generating random torsional variations of an initial conformation to test against the target. Simulation methods utilize molecular dynamics and energy minimization, and serve best when coupled with one or both of the above searching methods [52].

A large number of docking and dynamics software packages and online servers exist (Table 2), many of which are freely available for academic research. The variations in calculation methods and results make each program slightly different. Therefore, the researcher must pick which docking programs are ideal for his or her study. Studies have been performed to assess which programs are ideal for specific screening approaches or particular protein families. For instance, Kellenberger et al. published a comparative evaluation of eight widely used docking programs for screening accuracy in 2004 [53]. Of the eight docking programs tested,

TABLE 2: List of some commonly used molecular dynamics and docking software packages with developer URL. This is not intended to be a comprehensive list of all available dynamics and docking programs available. Available programs are typically free to download for academic use, but some require the purchase of a license for use.

Dynamics		Docking	
Program	Developer Site	Program	Developer site
Amber	http://ambermd.org/	AutoDock	http://autodock.scripps.edu/
AMMP	http://www.cs.gsu.edu/~cscrwh/progs/progs.html	FlexX	http://www.biosolveit.de/FlexX/
BALLview	http://www.ballview.org/	FRED	http://www.eyesopen.com/
CHARMM	http://www.charmm.org/	GLIDE	http://www.schrodinger.com/
GROMACS	http://www.gromacs.org/	GOLD	http://www.ccdc.cam.ac.uk/
LOOS Library	http://membrane.urmc.rochester.edu/Software	Sculptor	http://sculptor.biomachina.org/
YASARA	http://www.yasara.org/	SLIDE	http://www.bch.msu.edu/
YUP	http://rumour.biology.gatech.edu/YammpWeb/	SURFLEX	http://www.tripos.com/
ZMM	http://www.zmmsoft.com/	UCSF DOCK	http://dock.compbio.ucsf.edu/

GLIDE, GOLD, and SURFLEX provided the best docking and ranking accuracy within a 2.0 Å cutoff for root-mean squared deviation (RMSD), whereas QXP showed promising docking accuracy but reduced ranking performance. For ranking, FlexX outperformed QXP with percent scoring errors of 15% and 55%, respectively. Efficacy in screening of a compound database was found with SURFLEX, with 8 hits for ligands that bind to a difficult target out of 50 total compounds. GLIDE, GOLD, and FlexX were deemed good programs for virtual screening with hit values of 5, 4, and 4, respectively. Regarding docking times, FRED, which did not perform as well with scoring and docking accuracy, took the least amount of time to perform docking calculations of the eight programs tested, followed by DOCK and FlexX. No single program was deemed the best docking software, but the study demonstrated that the characteristics of the ligand and the target have a significant effect on the efficiency of the docking program used [53].

5. Virtual Screening

Because the process of finding a novel compound showing bioactivity can be time-consuming and expensive, structure-based drug design has been established as a vital first step to therapeutic development [54]. Screening for ligand conformations can be performed using a ligand-based or a structure-based approach [55, 56]. Ligand-based design uses known active and inactive compounds to generate a pharmacophore [55], which is often used in conjunction with quantitative structure-activity relationship (QSAR) analysis to determine ligand-protein interactions. Receptor-based design requires the availability of the receptor structure, which is used to examine the interactions that occur with any members of a large database of ligands [56]. Computational screening of large databases of molecules against the three-dimensional structure of a protein has the potential to provide rapid and accurate prediction of the binding modes and affinities of possible hits for lead optimization. One can prescreen a database of thousands of compounds and narrow the field of ligands to two or three potential hits in a significantly reduced amount of time compared to

laboratory experimental methods. This smaller group would increase the efficiency of experimental assays and new agonist discovery. Virtual Screening, which incorporates high-throughput docking techniques, is a means to explore the LBD of a protein and make predictions about ligand binding. This technique categorizes ligands that bind to the protein of interest and allows predictions to be made about activation or inhibition of the protein.

Development begins with creating an algorithm that can be followed to set up the testing, run the testing, and finally analyze the results. Schneider and Böhm define these three issues that must be addressed when performing iterative structure generation respectively as, the construction problem, the docking problem, and the scoring problem [57]. Deciding which protein crystal structure to use for all ligands, establishing a set of test parameters, and deciding which ligands to include in the test library make up the first part of the process. Typically, a crystal structure with the highest resolution and fewest missing atoms and residues will be selected. Setting parameters involves re-docking of published structures to reproduce experimentally observed docking conformations [52]. The compound database, which can contain numbers of compounds in the thousands [58], should contain small molecules that, based on known chemical interactions between residues of the binding site and known ligands, will bind in varying degrees to the protein of interest and potentially yield the desired effect (e.g., conformational change and activation or inhibition of protein). Protein flexibility is also an important and necessary part of predicting orientations and interactions for many protein families [54], and therefore time should be taken to consider how to incorporate receptor flexibility as well as the binding site microenvironment (i.e., water and/or ions in the binding site). Once the conditions for docking have been established, docking, which is the second step, is relatively straightforward.

The final step, analysis, can often be the most daunting due to the variety of ways output can be interpreted and analyzed. The type of program used to perform simulations has a significant effect on analysis methods because of the information returned. Some programs may be better

suited for calculating inhibition constants and free energy of binding estimations than others, whereas still other programs may provide more variables for consideration. There are many different approaches to analyzing results that one can take for scoring the results of a docking study, and these approaches involve examination of interactions on either a fragment or atomic level [57].

6. Limitations of Virtual Screening

Because VS is not, as of yet, a stand-alone process, ligand-binding and reporter assays are essential processes for validating *in silico* results. Docking predicts ligands that may elicit the desired activity, and assays further refine the group of viable candidates to a select group of hits, which, at a specific concentration, will activate the protein of interest.

Further research into lesser-understood biochemical processes is necessary to improve upon the reliability of VS as a stand-alone process. These processes include protein flexibility and induced-fit adaptations, the role of water in solvation, desolvation and ligand binding, and the involvement of electrostatics [52, 59]. Though these unknowns can prove to be problematic when looking at a single computational method, combining strategies is a way to improve upon successful hit rates. Overall, VS saves time and resources when searching libraries of compounds to narrow candidates down to a handful of potential hits that can then be tested experimentally. There is also potential to find hits that may not have been discovered using experimental processes alone.

Another factor that can limit VS productivity is the amount of information available when building a compound library. An information-rich environment is available when considering natural compounds for the treatment and prevention of diseases. Natural plant extracts typically contain a vast number of components that one would need to sift through in order to find the one compound or multiple synergistic compounds that elicit a desired mechanistic affect (i.e., activation of PPARs). VS would prove useful after fractionation of natural extracts and chemical elucidation of key peaks to aid in identifying which compounds within a library are the bioactive compounds. This has the potential to minimize the need for serial HTS when testing for a lead candidate. It is important to note that fractionation is not a necessary step for VS, but can be useful for guiding database building when examining natural extracts for bioactivity.

7. Docking and Virtual Screening Successes

Despite the present inability of VS to replace HTS, the two can be complementary approaches to candidate pharmaceutical and nutraceutical searching because of the potential for one method to find activators or inhibitors for which the other method does not show results [59]. Klebe [59] mentions in a review of VS strategies a comparison study performed by two groups searching for *Escherichia coli* dihydrofolate reductase inhibitors from a database of approximately 50,000 compounds. The VS portion of the study revealed a number of compounds previously unknown

as inhibitors due to insufficient concentrations of the compounds being used during experimental testing [59–62]. Klebe also provides a list of targets that have previously been addressed by virtual screening. These targets include nuclear receptors such as retinoic acid receptor and thyroid hormone receptor [59].

8. Relevance to PPAR γ Agonist Discovery

Docking techniques would prove useful in the development of new PPAR-based therapeutics, including *in silico* screening of synthetic agonists and natural compounds from plant extracts (i.e., botanicals), all of which have shown promise in the treatment and prevention of immunoinflammatory diseases through PPAR γ agonism. Docking and simulation techniques provide a means to prescreen for and enrich compounds with PPAR γ agonism and thereby increase the efficiency of HTS. Docking also allows for structure-based searches for analogues and derivatives of known agonists.

To date, there have been several studies utilizing standard docking methods [63–66] and VS methods [60, 63, 67–70]. Most of these studies focus on derivatives or analogues of a particular compound showing high affinity for PPAR γ . Studies of this nature can serve two purposes: identify hits for therapeutic development and provide insight into ligand-protein interactions and ligand selectivity.

Xu et al. [64] published a study in 2003 in which docking methods were used to look at interactions between PPAR γ and eighteen known synthetic and natural agonists in order to determine the pharmacophore of PPAR γ agonists. The group determined that PPAR γ agonists must have a polar head group and hydrophobic tail in order to form necessary hydrogen bonding and hydrophobic contacts with the LBD, respectively [64].

In another study, Lu et al. [63] conducted a structure-based VS search for PPAR γ partial agonists as candidates for treatment of T2D with fewer side effects than full agonists. The search revealed a class of ligands that could then be used to test against PPAR γ . Two compounds of the class were identified as partial agonists with selectivity among the three PPAR subtypes, and would serve as candidates for further testing. Using VS, they were able to suggest determinants in ligand specificity. The computational results were coupled with X-ray crystallography and assessment of *in vitro* and *in vivo* protein activity [63].

A study regarding natural products identified as PPAR γ agonists conducted by Salam et al. [68] also utilized structure-based VS to identify 29 potential agonists for experimental testing. Of those compounds, 6 were found to induce PPAR γ transcriptional activity *in vitro*. The study also provided insight into the mechanism underlying the flavonoid-induced conformational change and activation of PPAR γ [68].

9. Future Directions

Naturally occurring compounds with preventive or therapeutic activity (nutraceuticals) represent a widely used

Complementary and Alternative Medicine (CAM) modality and are an alternative to pharmaceuticals (i.e., TZDs) for treating various chronic diseases such as T2D. These natural compounds can modulate gene expression [71] and are typically safer than synthetic counterparts. In the case of T2D, nutraceuticals have the potential to decrease the risk of myocardial infarction, weight gain, and edema associated with current synthetic PPAR γ agonist treatments [10, 72]. Unfortunately, finding a compound that elicits a desired activity is not always easy because isolating a single compound from a bioactive extract is time consuming and expensive [73] and the mechanism by which the compound works is often unknown [74]. VS, in combination with conventional experimental methods, has the potential to put the discovery of bioactive botanical constituents in a better competitive position with mainstream pharmaceutical research by reducing time and costs. For instance, in a study published by Rollinger et al. [75], a chemical feature-based pharmacophore modeling VS technique, in combination with ethnopharmacology, was utilized to identify inhibitors for cyclooxygenase (COX) I and II. Of the thousands of compounds listed in the four databases used (WDI, NCI, NPD, and DIOS), the success rate of finding known inhibitors within these three-dimensional databases was enhanced through the use of VS techniques [75].

A preliminary comparison of a small group of PDB PPAR γ structures shows an overall conservation of backbone conformation across the available structures. This is relevant to the selection of a single macromolecule for large-scale automated testing. These findings suggest it is possible to select one macromolecule for all ligand types with a limited degree of error. It is important to note that though there is a relative consensus position for all key residues, some variation in the positions of key residues due to ligand interactions are present. Therefore, this issue must be considered and several structures must be examined when deciding on a single macromolecule crystal structure for VS.

Another computational method that may prove useful is molecular dynamics (MD), which involves the use of computational chemistry to predict the dynamics of complex molecular systems and the macroscopic properties of those systems based on detailed atomic knowledge [76]. Implementing MD would prove useful for examination of conformational changes and molecular interactions, which would allow for expansion upon what is known about how PPARs interact with ligands and other macromolecules.

To discover potential nutraceutical/CAM hits, further assessment of PPAR γ and ligand characteristics is necessary to determine the best screening approach and which scoring functions compare for analysis. If the components of an extract are known or if one can speculate as to which compounds are present, a database of chemically related compounds could be created to test against PPAR γ , and a smaller hit group can be identified for experimentation. Another necessary element is collecting experimentally proven properties for comparison to computationally derived data. Future work could also encompass finding coagonists and pan-agonists for PPAR subtypes.

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