Effects of Thiazolidinediones on Differentiation, Proliferation, and Apoptosis

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Abstract
Thiazolidinediones induce adipocyte differentiation and thereby limit proliferative potential; hence, early investigations focused on their ability to modulate cellular proliferation and apoptosis. Several lines of evidence indicate significant thiazolidinedione-mediated antitumor activity. An emerging view is that some antitumor effects are totally or partially peroxisome proliferator-activated receptor-γ (PPARγ) dependent, whereas others are PPARγ independent. The aim of this review is to examine the current evidence about the molecular mechanisms by which thiazolidinediones augment cellular differentiation, inhibit cellular proliferation, and induce apoptosis. We first address the role of thiazolidinediones and/or PPARγ on Wnt/β-catenin signaling pathway as it affects cellular differentiation and then discuss other pathways that are also involved in differentiation as well as proliferation and apoptosis. (Mol Cancer Res 2007;5(6):523–30)

PPARγ Signaling
PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily and include PPARα, PPARβ/δ, and PPARγ, each with specific expression patterns (1). On ligand binding, PPARγ heterodimerizes with retinoid X receptor, translocates to the nucleus, and transactivates multiple genes involved in metabolism (2). The activated nuclear PPARγ/retinoid X receptor transcriptional complex binds to PPARγ response elements and recruits coactivator proteins, including p300, SRC-1, and TRAP220, in a ligand-specific manner that is thought to provide biological specificity (3-5). Natural ligands include J-type prostaglandins, whereas thiazolidinediones are synthetic agents that have been widely used in treating type-2 diabetes mellitus and possess cellular growth-inhibiting activity (Fig. 1). In contrast, PPARγ antagonists and Δ2-thiazolidinedione derivatives do not permit transactivation but may retain growth-inhibiting activity (6).

Wnt/β-Catenin Signaling
The Wnt/β-catenin pathway was first discovered in Drosophila embryogenesis (7). An orthologous pathway was found to exist across invertebrate and vertebrate species, including humans (Fig. 2). Nineteen identified human Wnt genes code for secreted proteins that act in a paracrine and autocrine manner (8). Wnt proteins bind to members of the frizzled receptor family of 11 identified human seven-transmembrane domain-containing proteins to facilitate signal transduction through the canonical and noncanonical pathways. In canonical Wnt/β-catenin signaling, Wnt-frizzled recruits low-density lipoprotein-related protein (LRP)-5 or (LRP)-6 to the plasma membrane (9, 10). Intracellular signaling is mediated by Dishevelled (Dvl) as evidenced in mouse NIH3T3, L, and CS7MG cell models, in which Dvl is phosphorylated on Wnt3a treatment, possibly mediated by casein kinase-1 and/or casein kinase 2, or other additional protein kinases (11-13). In the absence of Wnt signaling, glycogen synthase kinase-3β (GSK3β) forms a destruction complex with axin and adenomatosus polyposis coli proteins. GSK3β phosphorylates axin to further stabilize it in the complex. On being recognized by the destruction complex, β-catenin is phosphorylated, ubiquitinated, and proteosomally degraded by the F-box protein β-transducin repeat protein (10, 14). By contrast, when Wnt signaling is activated, Dvl interrupts the axin-GSK3β interaction, possibly by promoting interaction between GSK3β and frequently rearranged in advanced T-cell lymphomas (FRAT) protein on the GSK3β axin-binding site. Dvl ultimately leads to dissociation of the β-catenin destruction complex, resulting in the accumulation of unphosphorylated cytoplasmic β-catenin (10, 12, 15-17). Uncomplexed β-catenin then translocates to the nucleus where it binds to T-cell factor (TCF)/lymphoid enhancer factor transcription complex and transactivates various genes that are involved in differentiation, proliferation, and apoptosis, such as cyclin D1, c-myc, c-jun, and Twist (a comprehensive gene list is available online).4

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4 http://www.stanford.edu/~rnusse/pathways/targets.html
GSK3β was initially discovered to be a negative regulator of glycogen synthesis and protein synthesis by phosphorylating and inactivating glycogen synthase and eIF2B, respectively; in this way, insulin and other growth factors inactivate GSK3β by Akt-dependent phosphorylation at Ser9. Ser9 phosphorylation serves as a "pseudosubstrate" to prevent GSK3β from phosphorylating its true substrate. However, GSK3β is regulated differently in the Wnt/β-catenin signaling pathway. Indeed, GSK3β does not become phosphorylated on Ser9 in response to Wnt signaling activation (16, 17). Moreover, evidence shows a substantive colocalization of the axin- and FRAT-binding sites on GSK3β, suggesting that FRAT and axin binding is competitive, and Dishevelled-mediated FRAT binding prevents axin binding and the resultant formation of the destruction complex (18). The mechanism by which β-catenin is phosphorylated by GSK3β (i.e., a priming phosphorylation on Ser45 is required for subsequent phosphorylation on Ser33/Ser37/Thr41) is similar to the way GSK3β phosphorylates glycogen synthase and eIF2B in that the existence of a priming phosphorylation site is required for further phosphorylation by GSK3β; further suggesting that, although the absence of FRAT is critical for the formation of destruction complex, the Ser9 phosphorylation site on GSK3β may also contribute to the phosphorylating ability of GSK3β within the destruction complex. However, recent evidence examining FRAT-deficient mice suggests that FRAT is not an essential component of the Wnt signaling pathway in mammals (19).

Endogenous Wnt inhibitors include Dickkopf protein (Dkk) that is secreted and binds to lipoprotein-related protein, which promotes internalization of lipoprotein-related protein, making it unavailable for Wnt signaling (10, 20-23). Wnt inhibitory factor-1 and frizzled-related proteins are secreted inhibitors that sequester Wnt proteins from their receptors (24, 25). Inhibitor of β-catenin and TCF4 (ICAT) is a 9-kDa polypeptide that binds to β-catenin in the Armadillo repeat region preventing it from interacting with either E-cadherin or TCF/lymphoid enhancer factor; this destabilizes β-catenin/E-cadherin interaction and inhibits transactivation of Wnt-responsive genes (26, 27). Because ICAT does not prevent β-catenin from binding to the destruction complex, β-catenin preferentially undergoes proteosomal degradation (28).

**Thiazolidinedione Effects on Differentiation**

Emerging evidence suggests thiazolidinedione-mediated cross-talk between PPARγ and the Wnt pathways particularly...
related to induction of adipocyte differentiation via PPARγ activation (Fig. 3). Although thiazolidinediones can also induce differentiation of some tumors (29, 30), the mechanism of thiazolidinedione-induced differentiation via regulation of Wnt/β-catenin is best studied in adipocytes and will be the primary focus of this section.

PPARγ and GSK3β interact through the transcription factors CAAT/enhancer binding proteins (C/EBPs); several lines of evidence indicate that GSK3β phosphorylates C/EBPα and C/EBPβ (31, 32). C/EBPα controls expression of many genes and is required for adipocyte differentiation (11). Ectopic overexpression of PPARγ in C/EBPα-deficient fibroblasts could only moderately induce expression of the adipocyte differentiation marker adiponectin, whereas expression of PPARγ along with intact C/EBPα facilitates adiponectin production (33). Moreover, C/EBPβ is phosphorylated by GSK3β on a consensus extracellular signal-regulated kinase/GSK3β domain (34). An in vitro study using NIH3T3 cells with conditional expression of C/EBPβ (tetracycline inducible) shows that early induction of C/EBPβ along with the exposure of the cells to dexamethasone, insulin, and fetal bovine serum is responsible for the initiation of PPARγ expression and activation; by contrast, PPARγ expression is not possible in the absence of induced C/EBPβ expression (35). In another study using 3T3-L1 preadipocytes, the authors showed that expression of a dominant-negative isoform of C/EBPβ (liver-enriched transcriptional inhibitory protein) did not interfere with the induction of PPARγ1, but C/EBPα, PPARγ2, and adipose protein 2 were inhibited, as was adipogenesis; interestingly, treating the cells with the PPARγ agonist troglitazone rescued the differentiation process (36, 37) It is now believed that C/EBPβ modulates PPARγ expression and activation, and on GSK3β-mediated phosphorylation of C/EBPβ at Thr188 within the consensus extracellular signal-regulated kinase/GSK3β site, C/EBPβ is able to facilitate the expression of C/EBPα (34). However, there is no clear evidence suggesting that PPARγ and C/EBPα interact directly, although both are crucial for adipogenesis; however, it is postulated that phosphorylated C/EBPβ is a coactivator of C/EBPα and that C/EBPα is activated by PPARγ, although this may be indirect. Taken together, these observations suggest that C/EBPβ induces PPARγ, which activates C/EBPα; however, the terminal stages of adipogenesis

**FIGURE 2.** The Wnt signaling pathway. Key events include secretion and binding of Wnt proteins to frizzled/lipoprotein-related protein (LRP), which signal through Dishevelled (Dvl) to inhibit constitutively active GSK3β, which inhibits the destruction complex. This permits nuclear translocation of β-catenin where it binds to TCF4/lymphoid enhancer factor (LEF) to form a transcriptional complex that regulates many target genes. Inhibition of Wnt signaling can occur via elaboration of extracellular inhibitors or intracellular ICAT. Cross-talk with the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway occurs via Akt-induced phosphorylation and inactivation of GSK3β that facilitates Wnt signaling.
will occur only in the presence of C/EBPβ that has been phosphorylated on the consensus extracellular signal-regulated kinase/GSK3β site. Furthermore, C/EBPα is the downstream differentiating factor that leads to production of adiponectin and completion of adipocyte differentiation.

In a 3T3-L1 Swiss mouse cell model with stable inducible expression of the C/EBPβ isoform liver-activated protein, PPARγ was activated on liver-activated protein induction and a time-dependent decrease in β-catenin expression was also observed. Moreover, cells with forced expression of Wnt1 protein not only failed to differentiate into adipocytes but also had significantly higher β-catenin levels and lower PPARγ levels, suggesting a correlation between PPARγ and β-catenin expression (35). Another experiment using 3T3-L1 adipocytes also showed that β-catenin mRNA and protein levels were lower than in fibroblasts, and levels were further decreased by treatment with rosiglitazone; however, it is unclear if this is PPAR-dependent (38). By contrast, expression of mutant β-catenin that is resistant to phosphorylation by GSK3β leads to accumulation of β-catenin, which then blocks adipocyte differentiation even if PPARγ is activated (34).

Direct interactions among PPARγ, retinoid X receptor α, and β-catenin have been found recently. Lu et al. (39) first showed that nonsteroidal anti-inflammatory drugs inhibited β-catenin-mediated transcripational activity in the presence of high-level expression of PPARγ and retinoid X receptor α; they also showed that β-catenin directly interacts with PPARγ and retinoid X receptor α by immunoprecipitation experiments in human kidney embryonic HEK293 cells and in human metastatic prostate cancer LNCaP cells. They further showed a functional interaction between β-catenin and PPARγ that involves the TCF/lymphoid enhancer factor binding domain of β-catenin and a catenin binding domain within PPARγ (40). They suggested that in normal cells, PPARγ can function to suppress Wnt signaling by targeting phosphorylated β-catenin to the proteasome through a process involving its catenin binding domain, whereas mutant β-catenin with Ser37-Ala can resist proteasomal degradation by inhibiting PPARγ activity (40). However, the authors failed to examine the total β-catenin protein levels in response to PPARγ overexpression and further activation by troglitazone. A functional assay would help determine whether the direct interaction between PPARγ and β-catenin plays an important role in differentiation.

The relationship between Wnt signaling and differentiation has also been recognized in myocytes. Adipocytes and myocytes derive from the same precursor fibroblast; induction of Wnt signaling leads to accumulation of β-catenin in the nucleus and enhances binding to the TCF/lymphoid enhancer factor complex, which then increases expression of Myf5, a myocyte-differentiating factor (41). However, overexpression of dominant-negative TCF4 in C2C12 myoblasts led to interruption of downstream of Wnt signaling, causing the cells to differentiate into Oil Red-O-positive lipid-laden adipocyte-like cells rather than myocytes (42).

The above data imply a positive feedback mechanism involving GSK3β, PPARγ, and β-catenin that amplifies the signal for differentiation and inhibits proliferation. Once GSK3β activates PPARγ via C/EBPβ, PPARγ inhibits Wnt signaling, thereby making GSK3β available to form a destruction complex that targets β-catenin for degradation, which is then associated with PPARγ activation.

Importantly, initiation of differentiation is associated with a gradual loss of proliferative potential (i.e., highly differentiated cells typically do not enter the cell cycle again). As thiazolidinediones trigger differentiation of preadipocytes, overexpression of PPARγ and/or C/EBPα can counterbalance the differentiation-inhibiting effect of Wnt signaling through down-regulation of β-catenin and thus inhibit β-catenin–mediated cyclin D1 induction (35). Hence, the thiazolidinedione-induced inhibition of cell proliferation in normal and cancerous cells is thought to allow the cell to proceed to either differentiation or apoptosis.

**Thiazolidinedione Effects on Proliferation and Apoptosis**

Almost a decade ago, Altiok et al. (43) observed that PPARγ mRNA and protein levels in slow-growing adipocytes are
- 3-fold higher than in fibroblasts in culture; furthermore, induction of PPARγ during differentiation is sufficient to induce cell cycle arrest, suggesting that induction of differentiation is associated with growth inhibition. These observations led many groups to hypothesize that thiazolidinediones can induce differentiation of cancer cells and inhibit cancer growth; indeed, thiazolidinediones inhibit cell proliferation and induce apoptosis in developmental and cancer models (44-50). Several mechanisms have been proposed, including inhibition of phosphatidylinositol 3-kinase/Akt signaling, retinoblastoma protein (Rb) dephosphorylation, decreased cyclin D1 and Bcl-xl/Bcl-2 expression, up-regulation of p21 and p27, as well enhanced sensitivity to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced cell death (Fig. 4; refs. 2, 6, 50-54).

Cyclin D1 phosphorylates and inactivates Rb and is a key regulator of cell cycle progression from G1 to S phase; it is required for fibroblast and epithelial cell proliferation and is an important downstream Wnt effector (55-59). Cyclin D1 is overexpressed in many cancers where it promotes G1 phase progression and has been shown to induce tumorigenesis (60). PPARγ and cyclin D1 seem to have a reciprocal relationship. PPARγ ligands inhibit cyclin D1–mediated Rb phosphorylation at Ser807 and Ser811 and thereby maintain Rb in its active form, which prevents G1 to S phase transition (61-63). Moreover, forced overexpression of both PPARγ and C/EBPα can lead to activation of Rb and cell cycle arrest (61, 62).

Chang et al. (50) showed that DNA laddering is accompanied by Rb hypophosphorylation in non–small cell lung cancer cells treated with ciglitazone and PGJ2. However, Wang et al. (64) showed that cyclin D1 inhibits the expression and promoter activity as well as transcriptional activity of PPARγ through a cyclin-dependent kinase (CDK)– and Rb-independent mechanism. Moreover, cyclin D1−/− murine embryonic fibroblasts show more robust adipocyte differentiation in response to PPARγ ligands associated with a reduction in histone deacetylase-1 activity; this phenomenon is reversed by cyclin D1 reintroduction, which increases histone deacetylase-1 activity and blocks PPARγ-mediated adipogenesis (64). Conversely, it is well known that thiazolidinediones decrease cyclin D1 expression both directly via proteasomal degradation and indirectly by promoting proteasomal degradation of β-catenin (51, 65). Yin et al. (66) determined that troglitazone inhibits cyclin D1 expression and CDK2/4 activity as well as Rb phosphorylation in MCF-7 breast cancer cells and that those changes were associated with cell cycle arrest and were abrogated by cyclin D1 overexpression.

Inhibition of Wnt signaling is necessary for 3T3-L1 preadipocytes to gradually cease proliferation and complete differentiation. However, at the beginning of adipogenesis, 3T3-L1 preadipocytes enter the cell cycle for several rounds of cellular proliferation. At this stage, the levels of free E2Fs, transcription factors that regulate expression of genes involved in DNA synthesis, are increased and cellular proliferation...
proceeds (67). Subsequently, PPARγ expression is induced resulting in decreased binding of E2Fs to target genes, partly mediated through down-regulation of protein phosphatase 2A (43). Therefore, thiazolidinediones inhibit multiple G1-S transition proteins, including cyclin D1, Rb, and E2F.

Thiazolidinedione-mediated β-catenin down-regulation might also partially be mediated by phosphatidylinositol 3-kinase/Akt-dependent mechanisms. Han et al. (49) showed that rosiglitazone decreased non–small cell lung cancer cell proliferation, increased phosphatase and tensin homologue protein expression, and reduced phosphorylation of Akt. These effects were blocked or diminished by the PPARγ antagonist GW9662, whereas overexpression of PPARγ restored the effects of rosiglitazone on phosphatase and tensin homologue, Akt, and growth in the presence of GW9662 (49). In the same article, they showed that rosiglitazone increased phosphorylation of AMP-activated protein kinase A, a downstream target of mammalian target of rapamycin (42). GW9662 did not inhibit phosphorylation of AMP-activated protein kinase A nor decrease phosphorylation p70 ribosomal protein S6 kinase; however, the mammalian target of rapamycin inhibitor rapamycin enhanced the growth-inhibitory effects of rosiglitazone, although this effect was partially blocked by AMP-activated protein kinase A siRNA. Hence, rosiglitazone inhibits non–small cell lung cancer proliferation through PPARγ-dependent and PPARγ-independent mechanisms. Moreover, Akt is a key GSK3β kinase, and when Akt phosphorylation is inhibited, GSK3β phosphorylation on Ser9 is also diminished. Once unphosphorylated GSK3β becomes part of the β-catenin destruction complex, Akt is able to recognize the priming site of β-catenin on Ser45 to facilitate upstream phosphorylation on Ser25/Ser37/Thr41. Therefore, rosiglitazone-mediated Akt inactivation may also play a role in the down-regulation of β-catenin and its downstream transcriptional activity.

Recently, Wnt pathway inhibition has been linked to effects on Bcl-2 members. Shou et al. (68) found that hDkk-1—overexpressing U87MG/hDkk brain tumor cells exhibited down-regulated antiapoptotic Bcl-2 expression and up-regulated proapoptotic Bax expression on C2-ceramide treatment. This link may underlie some of the observed thiazolidinedione effects on Bcl-2 members. Elstner et al. (69) showed that troglitazone, combined with either all-trans-retinoic acid or 9-cis-retinoic acid, induced apoptosis in breast cancer cell lines that express the highest levels of Bcl-2, including MCF-7, MDA-MB-231, and ZR-75-1, but decreased Bcl-2 levels only in MCF-7 cells. The ability of thiazolidinediones to induce apoptosis, but variably affect Bcl-2 expression, is likely due to thiazolidinedione effects on Bcl-2/Bcl-XL function (6). Data from a competitive fluorescence polarization analysis suggest that troglitazone, ciglitazone, and their Δ2 counterparts compete with a Bak BH3 domain peptide for binding to Bcl-XL and Bcl-2, and the potency of the thiazolidinediones and their Δ2 analogues in inhibiting the peptide binding correlated with the respective effectiveness in inducing apoptotic death (6). The inability of rosiglitazone and pioglitazone to trigger apoptotic death in these cells was reflected by their lack of potency in disrupting binding of Bcl-XL and Bcl-2 to the Bak BH3 domain, in contrast to troglitazone and Δ2-TG, which perturbed the dynamics of intracellular Bcl-2/Bak and Bcl-XL/Bak interactions and liberated proapoptotic Bak to induce apoptosis by facilitating cytochrome c release and activating caspase-9 (6). Finally, overexpression of Bcl-XL conferred protection against troglitazone- and Δ2-TG–induced apoptosis (6). Because Δ2-TG is devoid of PPARγ agonistic activity, these data suggest that the effect of troglitazone on Bcl-XL/Bcl-2 function is PPARγ independent. Heaney et al. (70, 71) completed two important mechanistic studies of thiazolidinedione-induced apoptosis using pituitary tumor xenografts in nude mice. They found that, depending on the tumor phenotype, rosiglitazone suppressed tumor growth, decreased hormone levels in secretory tumors, and induced apoptosis and that these functional effects are associated with suppression of Rb and Bcl-2 and induction of Bax (70, 71).

The CDK inhibitors p21 and p27 inhibit CDK2/4 and CDK2 activity, respectively, and promote cell cycle arrest. Emerging data reveal that p21 can inhibit Wnt signaling and have a role in differentiation of various cell phenotypes (72). Moreover, Wnt1 has been shown to down-regulate p27 expression (73). Thiazolidinedione effects on p21 and p27 are variable; however, induction of both proteins has been shown in multiple studies. Chen and Harrison (52) noted that ciglitazone induced p27 gene and protein expression and suppressed p27 proteosomal degradation in colon cancer cells. In a follow-up study, the same group noted that ciglitazone effects were mediated by the transcription factor Sp1 and negatively regulated by mitogen-activated protein kinase (53). Using troglitazone, Motomura et al. (74) found increased p27, but not p21, levels in pancreatic carcinoma cells; in their study, antisense p27 abrogated troglitazone-mediated inhibition of cell growth. Furthermore, Han et al. (75) showed that treatment of lung carcinoma cells with PGJ2, ciglitazone, troglitazone, and GW1929 elevated p21 mRNA and protein levels. Transient transfection assays indicated that PPARγ ligands increased p21 gene promoter activity, whereas p21 antisense oligonucleotides inhibited PPARγ ligand-induced p21 protein expression and significantly blocked PPARγ-mediated growth inhibition of lung carcinoma cells (75). In the same study, they use electrophoresis mobility shift assays to show that PPARγ ligands increased the nuclear binding activities of Sp1 and C/EBP, which both have regulatory elements in the p21 promoter (75). In a promising study with potential clinical implications, Copland et al. (76) identified a novel high-affinity PPARγ agonist (RS5444), with an IC50 for growth inhibition of 0.8 nmol/L, which inhibited anaplastic thyroid carcinoma tumor growth 3- to 4-fold in nude mice. RS5444 up-regulated p21, whereas silencing p21 rendered anaplastic thyroid carcinoma cells insensitive to the drug (76). RS5444 plus paclitaxel showed additive antiproliferative activity in cell culture and diminished anaplastic thyroid carcinoma tumor growth in vivo; whereas RS5444 did not induce apoptosis, the combination with paclitaxel doubled the apoptotic index compared with paclitaxel alone (76). Finally, Göke et al. (77) showed that pioglitazone suppressed the growth and induced apoptosis of carcinoid cells. Moreover, pioglitazone significantly enhanced TRAIL–induced cell death associated with up-regulation of p21 expression. Overexpression of p21 in carcinoid cells
sensitized them to tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis, suggesting that pioglitazone inhibits cell growth and sensitizes cells to tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis by induction of p21 (77).

Conclusions

Over the past several years, studies have begun to elucidate mechanisms of thiazolidinediione effects on cellular differentiation, proliferation, and apoptosis. It has become clear that many of these effects are mediated through thiazolidinediione actions on Wnt signaling that rely on both PPARγ-dependent and PPARγ-independent mechanisms. We have enumerated recent findings in this area and have attempted to unify them by focusing on how thiazolidinediiones affect developing cells, such as preadipocytes, where Wnt signaling is appropriately activated, as well as cancer cells where Wnt signaling is inappropriately activated. We anticipate that future studies will build on current knowledge to better understand thiazolidinedione effects on these cellular processes and then leverage those findings to predict synergies with other agents.

References


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