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Peroxisome Proliferator-Activated Receptor (PPAR) α and γ Ligands Differentially Affect Smooth Muscle Cell Proliferation and Migration

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DHA, docosahexaenoic acid; FBS, fetal bovine serum; MEM, minimal essential medium ; PCA-SMC, porcine coronary artery smooth muscle cell; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; SMC, smooth muscle cell;

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Abstract

Peroxisome proliferator-activated receptors (PPAR) α and γ are expressed in smooth muscle cells (SMC). This study was designed to compare the effects of PPARα and PPARγ on SMC proliferation and migration, and to determine how they operate. Treatment of SMCs from porcine coronary artery revealed that mitogen-stimulated DNA synthesis was blocked by the PPARα ligand WY14,643 and 15d-PGJ_2 (a putative PPARγ agonist), but not by the PPARγ agonist rosiglitazone or the PPARβ/δ ligand GW501516. Inhibition of DNA synthesis by clofibrate and GW7647 confirmed that SMC proliferation is affected by PPARα. This conclusion was supported by the fact that WY14,643 also inhibited the proliferation of H4IIE hepatoma cells (expressing only PPARα) but not A10 SMCs (expressing only PPARγ1). In contrast, the effective inhibition of all cell types with 15d-PGJ_2 indicated this compound likely operates via a PPARγ-independent mechanism. Interestingly, rosiglitazone did not inhibit DNA synthesis of either H4IIE or A10 cells, suggesting activation of PPARγ does not influence cell proliferation. Phosphorylation of cdk2 and expression of PCNA were inhibited by WY14,643, but not rosiglitazone or 15d-PGJ_2, indicating that PPARα prevents progression into S phase. Although rosiglitazone did not block SMC proliferation, it (like WY14,643) reduced neointimal hyperplasia *in vitro*. This observation can be rationalized by the fact that both WY14,643 and rosiglitazone inhibit SMC migration, likely through MMP9. Our study therefore shows that selective interference with mediators of cell cycle progression and cell migration via activation of PPARs may prevent growth-related vascular diseases such as restenosis and atherosclerosis.

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Introduction

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-activated transcription factors that regulate lipid metabolism and homeostasis (Evans et al., 2004). To date, three distinct PPARs, designated PPARα (NR1C1), PPARγ (NR1C3) and PPARβ/δ (NR1C2), have been identified. It is now apparent from numerous studies that the PPARs can indirectly influence vascular disease (Lee et al., 2003). More intriguing, however, are two reports that suggest these proteins may affect vascular smooth muscle cells (SMCs) directly (Hu et al., 2002; Zahradka et al., 2003).

Although PPAR levels are much lower in vasculature tissue compared to hepatic and adipose tissues, each of the three PPAR isoforms has been detected in vascular SMCs (Marx et al., 2004). PPARα agonists have been shown to suppress the NF-κB-dependent induction of cox-2 and interleukin-6 (IL-6) in vascular SMCs (Staels et al., 1998). Since these observations suggest PPARα participates in the inflammatory response of SMCs to cytokines in addition to lipid and lipoprotein metabolism, there is speculation that PPARα agonists may be able to suppress progression of atherosclerotic lesions (Marx et al., 2004). In agreement with this prospect, PPARγ agonists have been shown to inhibit the activation of NF-κB in SMCs, and consequently decrease both chemokine secretion and matrix metalloproteinase expression (Chinetti et al., 2001). As well, PPARγ agonists can decrease both SMC migration and proliferation (Miwa et al., 2000; Gouni-Berthold et al., 2001). PPARγ has therefore been associated with both atherogenesis and the response to injury. Although PPARβ/δ is expressed in SMCs, its function has yet to be established. Oliver et al (2001), however, have reported PPARβ/δ may influence reverse cholesterol transport through its ability to regulate ABCA1 transporter expression.

There is support for the concept that PPARs are important factors in atherogenesis, and this view is substantiated by the results of a limited clinical study which showed that

troglitazone, a PPARγ agonist, reduced the thickness of the carotid artery following administration to patients with type 2 diabetes (Minamikawa et al., 1998). Furthermore, the inhibition of SMC proliferation and migration by PPARγ agonists has led to speculation that these compounds may prevent intimal hyperplasia following revascularization (Bishop-Bailey et al., 2002). Similar data are now accumulating for PPARα, since the PPARα agonist WY14,643 inhibits mitogen-induced DNA synthesis (Hu et al., 2002; Zahradka et al., 2003). In contrast, expression of PPARβ/δ may promote SMC proliferation (Zhang et al., 2002).

In an earlier study, we observed that both WY14,643 (a PPARα agonist) and 15d-PGJ_2 (a putative PPARγ agonist) inhibited DNA synthesis following mitogen stimulation of human SMCs (Zahradka et al., 2003). These results led to the conclusion that these compounds might prove effective in preventing neointimal proliferation following vascular injury. However, a mechanism to explain the growth-inhibitory actions of PPARα agonists was lacking. We therefore examined the effect of PPAR agonists on several mediators of cell cycle progression in SMCs. Both retinoblastoma (Rb) protein and cdk2 were studied, based on evidence that Rb phosphorylation by cdk2 is required for the release of the E2F transcription factor (Andres, 2004) and subsequent expression of genes coding for proliferating cell nuclear antigen (PCNA) and cyclins E and A (Stevens & La Thangue, 2003). In addition, cyclin D levels and IKK (IκB kinase) phosphorylation were monitored, since both have been shown essential for SMC proliferation (Andres, 2004; Zahradka et al 2002). In the current investigation, we present data that confirms the anti-proliferative activity of PPARα agonists, and identify a target for their behaviour. Furthermore, we demonstrate 15d-PGJ_2 does not operate via PPARγ. Finally, we establish that both PPARα and PPARγ agonists block neointimal hyperplasia, but that they operate via different mechanisms.

Materials and Methods

Cell Culture: Primary cultures of SMCs from porcine coronary arteries (PCA-SMCs) were prepared by migration from free-floating explants as described by Saward and Zahradka (1997). PCA-SMCs were propagated in Dulbecco's MEM (DMEM; Invitrogen) containing 20% FBS (Invitrogen). When 75% confluent, the growth medium was replaced with DMEM supplemented with 5 μg/mL transferrin, 1 nM selenium, 20 mM ascorbate and 10 nM insulin for 5 days. A10 SMCs and H4IIE hepatoma cells were cultured as previously described (Saward and Zahradka, 1996; Yau et al., 1998). Quiescence was achieved by placing A10 and H4IIE cells into serum-free media for 72 hours, with A10 cells receiving the same supplement as the PCA-SMCs. Quiescent cells were used for all growth assays, and PPAR agonists were added 60 min prior to mitogen stimulation. Specific agents employed in these studies include WY14,643 (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid, Cayman), rosiglitazone (5-((4-(2-(methyl-2-pyridinylamino)ethoxy)phenyl)methyl)-2,4-thiazolidinedione, generously provided by SmithKline Beacham), 15d-PGJ_2 (15-deoxy-$\Delta^{12,14}$ prostaglandin J_2, Cayman), GW501516 (2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)phenoxy acetic acid, Calbiochem), GW7647 (2-(4-(2-(1-cyclohexanebutyl-3-cyclohexylureido)ethyl)phenylthio)-2-methylproprionic acid, Calbiochem) and clofibrate (2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester, Calbiochem).

DNA Synthesis: Triplicate sets of quiescent cells, prepared in 24-well dishes, were treated with mitogen ± PPAR agonist in the presence of 1 μCi [^{3}H]-thymidine (Perkin Elmer Life Sciences). The labelling period employed for each cell type was established previously (Yau et al., 1998; Saward and Zahradka, 1996). Incorporation of radiolabel into DNA was monitored by trichloroacetic acid precipitation.

Migration Assay: Migration of PCA-SMCs through polycarbonate filters with 5 μm pores was measured with a Boyden chamber (48-well unit) as previously described (Yau et al., 2003).

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Serum-free DMEM containing 0.1 μg/mL PDGF was placed in the lower compartment, while inhibitors were added to the upper compartment. After 48 hours in a standard CO_2 incubator, the cells on the underside of the membrane were visualized with Giemsa stain and quantified.

Reverse transcription-polymerase chain reaction amplification: Total RNA was isolated from cells in 6-well culture dishes or frozen white adipose tissue with TRIzol (Invitrogen). The RNA was resuspended in RNase-free water, and concentration determined by spectrophotometric absorbance at 260-nm. Reverse transcription of 1 μg of RNA was conducted (after removal of possible genomic DNA contamination with DNase I) according to the protocol (62°C annealing temperature) recommended for the Access RT-PCR System (Promega). The number of amplification cycles was empirically determined for each primer pair to identify the logarithmic phase. The specific forward and reverse oligodeoxynucleotide primers employed were: GAPDH (s) 5'-CGCTGTGAACGGATTTGGCCGTAT-3', GAPDH (as) 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; rat PPARα (s) 5'-AAGACGCTTGTGGCCAAGAT-3', rat PPARα (as) 5'-ATGTCGCAGAATGGCTTCCT-3', porcine PPARα (s) 5'-CGTGGCACTGAACATCGAAT-3', porcine PPARα (as) 5'-CGGTCTCGGCATCTTCTAGG-3', rat PPARγ1 (s) 5'-ACAAGACTACCCTTTACTGAAATTACC-3', rat PPARγ1 (as) 5'-GTCTTCATAGTGTGGAGCAGAAATGCT-3'; porcine PPARγ1 (s) 5'-CAGATTTGGTGGAAGCCAACT-3' porcine PPARγ1 (as) 5'-CGTTTAAGGAAACAACCTTCCTG-3', rat PPARγ2 (s) 5'-TACAGCAAATCTCTGTTTTATGCTGTT-3', rat PPARγ2 (as) 5'-GTCTTCATAGTGTGGAGCAGAAATGCT-3', porcine PPARγ2 (s) 5'-GTTCCATGCTGTTATGGGTGAA-3' porcine PPARγ2 (as) 5'-GCATCGCTTTCTGGGTCAAT-3' (Marx et al., 1998; Tanaka et al., 1999; Zahradka et al.,

1995; Benson et al., 2000). Amplification products were analyzed by electrophoresis in 2% agarose gels and the intensity of the Vistra Green-stained bands was quantified with a Molecular Dynamics Storm 850 Imaging System and ImageQuant software. Control reactions (minus RNA, minus RT and minus primers) were used to demonstrate the specificity of the PCR reaction.

Western Blot Analysis: Western blotting of cellular proteins (10 μg) separated by SDS/polyacrylamide gel electrophoresis in a 7.5% gel and transferred to PVDF membrane (Roche) was conducted as previously described (Yau et al., 2003). Membranes were probed with antibodies to Thr-160 phospho-cdk2 (Cell Signaling), Ser-780 phospho-Rb (Cell Signaling), PCNA (Dako), Ser-180/Ser-181 phospho-IKKα/β (Cell Signaling), cyclin A (NeoMarkers), cyclin D (Upstate), cyclin E (Santa Cruz), β-tubulin (Sigma-Aldrich) and horseradish peroxidase-(HRP)-conjugated secondary antibody (1:10,000 diluted) was detected using the ECL chemiluminescent system (Amersham).

Gelatin Zymography: The procedure was conducted with media samples recovered from organ culture after a 48 hour incubation period as previously described (Zahradka et al., 2004).

Coronary Artery Organ Culture: Segments of porcine coronary artery, injured by inflation of an angioplasty catheter (3.5 mm × 20 mm) for 1 minute, were cultured in 24-well dishes containing 20% FBS in DMEM as previously described (Wilson et al., 1999). Media, including treatments, were changed every second day. Vessels harvested from culture were embedded, sectioned and stained in Lee's methylene blue. Digital images were captured with a DAGE-MTI CCD camera and analyzed with StainPoint software (Lynx Graphics Ltd., www.lynxgl.com) to quantify the neointimal index: intimal area/medial area.

Data measurement and statistical analysis: Radiotracer assay data and densitometric scans of Western blots were quantified and plotted as means ± SEM of individual experiments (n=3). Morphometry was performed with 8 replicates per treatment. Treatment means were compared using one-way ANOVA, whereas all other data were analyzed with the unpaired Student's t-test.

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Statistical significance was set at $P < 0.05$.

Results

Differential inhibition of SMC proliferation by PPAR agonists: The proliferation rate of quiescent smooth muscle cells derived from porcine coronary artery (PCA-SMCs) is increased over 10-fold upon treatment with 0.1 μg/ml PDGF-BB as determined by thymidine incorporation assays (Figure 1). Pre-treatment of these cells with 250 μM WY14,643, a PPARα agonist, or 15d-PGJ_2, a putative PPARγ agonist, significantly reduced the growth-stimulatory actions of PDGF, in accordance with data published for human vascular SMCs (Zahradka et al., 2003). In contrast, neither rosiglitazone nor GW501516 were capable of inhibiting DNA synthesis. Although these results were not surprising for a PPARβ/δ agonist such as GW501516, they were unexpected for the PPARγ agonist rosiglitazone given the potency demonstrated by 15d-PGJ_2. We therefore explored in more detail the relationship between PPAR activation and cell proliferation.

Activation of PPARα blocks SMC proliferation: The presence of PPARα in human and rodent SMCs (Zahradka et al., 2003; Diep et al., 2000) suggests porcine SMCs should be responsive to PPARα agonists, and the results shown in Figure 1 support this premise. Since WY14,643 has been described as a specific PPARα agonist (Lee et al., 1995), we examined its ability to block cell proliferation over a range of concentrations and with two distinct mitogens. Quiescent PCA-SMCs were stimulated with either PDGF-BB (0.1 μg/ml) or FBS (2% v/v). Both mitogens significantly increased DNA synthesis, with FBS being considerably more potent than PDGF (2798% ± 660 vs 1072% ± 76, respectively). In both cases, however, addition of WY14,643 produced a concentration-dependent reduction in thymidine incorporation (Figure 2A,B). Interestingly, the effectiveness of WY14,643 varied with the two mitogens, since 250 μM was able to reduce DNA synthesis to near basal levels for PDGF-treated cells (with 100 μM producing a significant reduction) while a concentration of 500 μM was required for FBS-

stimulated PCA-SMCs. This difference in efficacy was clearly evident upon comparison of the respective EC_{50} values for PDGF (120 μM) and FBS (275 μM) stimulated cells.

To verify this apparent link between PPARα and SMC proliferation, we tested two structurally distinct PPARα agonists. Like WY14,643, both clofibrate and GW7647 produced a concentration-dependent reduction in thymidine incorporation (EC_{50} 245 μM and 3.2 μM, respectively) when added to PCA-SMCs stimulated with PDGF (Figure 2C,D). These data support the view that activation of PPARα can block SMC proliferation.

Effect of PPAR agonists on A10 SMCs and H4IIE: *hepatomas*: To determine whether inhibition of cell proliferation was a general property of PPAR agonists, we extended our study to include A10 SMCs and H4IIE hepatoma cells. Each cell type was stimulated with a mitogen (A10 cells with PDGF, H4IIE cells with insulin) that significantly increased thymidine incorporation by about 8-fold and 2-fold, respectively (Figure 3). Addition of WY14,643 had no inhibitory effect on DNA synthesis after mitogen stimulation of A10 SMCs (Figure 3A), but 250 μM WY14,643 significantly inhibited H4IIE cell proliferation (Figure 3B). In contrast, 5 μM 15d-PGJ_2 blocked the proliferation of both A10 SMCs and H4IIE hepatomas (Figure 3C,D). Interestingly, as was seen with the PCA-SMCs (Figure 1), addition of 10 μM rosiglitazone had no effect on the proliferation of either A10 or H4IIE cell type (Figure 3E,F).

Distribution of PPAR isoforms in PCA-SMCs, A10 SMCs and H4IIE hepatomas: The disparate results seen with WY14,643 on SMC-PCA and A10 SMCs, as well as the opposite actions of 15d-PGJ_2 and rosiglitazone, led us to examine the distribution of the PPAR isoforms in these cells. PPAR expression was assessed in PCA-SMCs, A10 SMCs and H4IIE hepatomas by RT-PCR amplification of total RNA with primers capable of distinguishing PPARα, PPARγ1 and PPARγ2. It was observed that PPARα was present in PCA-SMCs (Figure 4A) and H4IIE hepatomas (Figure 4B), but not in A10 SMCs (Figure 4C). In contrast, neither PPARγ1 nor PPARγ2 was expressed in H4IIE cells, although PPARγ1 (but not PPARγ2) was detected in

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both PCA and A10 SMCs (Figure 4). RNA isolated from rat white adipose tissue was used as a positive control for PPARγ2 (Figure 4D). These results indicated there is a correlation between PPARα expression and inhibition of cell proliferation by WY14,643, however, the divergent actions of 15d-PGJ_2 and rosiglitazone could not be traced to differences in PPARγ expression.

PPARα activation interferes with cell cycle progression: A possible link between the putative PPARα agonist DHA (docosahexaenoic acid) and cell cycle progression via cdk2 has been reported (Terano et al., 1999). We therefore examined the effect of PPAR agonists on the cell cycle mediators in quiescent PCA-SMCs stimulated with PDGF. It was observed that cdk2 phosphorylation was prevented by addition of WY14,643 (Figure 5A), while neither rosiglitazone nor 15d-PGJ_2 inhibited this event. Similarly, the increase in PCNA elicited by PDGF was blocked by WY14,643, but not rosiglitazone or 15d-PGJ_2 (Figure 5B). In contrast, these compounds had no effect on PDGF-dependent phosphorylation of either Rb (Figure 5C) or IKK (Figure 5D). Similarly, PDGF-stimulated expression of cyclins D and A was unaffected by the PPAR agonists (Figure 5E), however, cyclin E levels were reduced upon treatment with PGJ_2 but not WY14,643 or rosiglitazone (Figure 5F).

WY14,643 and rosiglitazone inhibit neointimal formation: Vascular injury typically results in formation of a lesion that partially or completely blocks the vessel lumen (Newby and Zaltsman, 2000). SMCs make a significant contribution to neointimal lesion formation subsequent to their transformation to the synthetic phenotype which allows proliferation and migration. Based on the data presented above, WY14,643 would be expected to prevent neointimal hyperplasia following injury. Also, since it as has previously been reported that PPARγ agonists can prevent neointimal hyperplasia (Law et al., 1996), rosiglitazone would be expected to perform similarly. We therefore employed an organ culture model of balloon angioplasty (Wilson et al., 1999) to evaluate the actions of these compounds. Segments of porcine coronary arteries injured by balloon inflation were cultured for 14 days in the presence or absence of either 250 μM

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WY14,643 or 10 μM rosiglitazone. Control vessel segments that had not been injured were cultured in parallel. Morphometric analysis showed that the lesion of balloon-injured vessels was 3.8-fold larger than the control (Figure 6). In the presence of WY14,643, the neointima was only 1.6-fold larger than control, a decline of 76%. Likewise, rosiglitazone decreased the neointimal area by 65%. These results confirm that both WY14,643 and rosiglitazone can inhibit neointimal formation following vascular injury.

PPARα and PPARγ agonists inhibit SMC migration: Injury-induced neointimal proliferation requires both SMC proliferation and migration. Since our data suggest rosiglitazone does not operate by inhibiting cell proliferation (Figure 1), we investigated its ability to interfere with migration. Furthermore, since migration is dependent upon activation of specific cell cycle mediators, including cdk2 (Andres, 2004), we concurrently explored the possibility that WY14,643 could also impede SMC migration. PDGF-induced migration of PCA-SMCs was therefore quantified 48 h after seeding into a Boyden chamber. In the absence of a PPAR agonist, PDGF stimulated cell migration to the lower chamber of the apparatus, however, inclusion of WY14,643, 15d-PGJ_2 or rosiglitazone significantly inhibited migration under these conditions (Figure 7). In contrast, 10 μM GW501516 was ineffective. These results establish that agonists of PPARα and PPARγ are potent inhibitors of SMC migration, but it is unlikely that PPARβ/δ has a role in this process.

To investigate the possible mechanism by which migration is hindered, we examined the effect of the PPAR agonists on MMP2 and MMP9 production in organ culture. Injured vessels were placed into culture for 48 h in the presence of various agonists and release of MMP2 and MMP9 into the medium was measured by gelatin zymography (Zahradka et al., 2004). The levels of both latent and active MMP2 were unchanged relative to control except in the presence WY14,643, whereas MMP9 levels were reduced by both WY14,643 and rosiglitazone (Figure 8B,C). Interestingly, although 15d-PGJ_2 was a potent inhibitor of migration, it did not affect

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MMP production. These results confirm that rosiglitazone and 15d-PGJ_2 operate via distinct mechanisms and that 15d-PGJ_2 likely does not function through PPARγ.

Discussion

This study shows that activation of PPARα with WY14,643 inhibits the proliferation of SMCs, and extends these observations to cell migration. These results are supported by our finding that the PPARα ligands clofibrate, which is clinically used to reduce serum triglyceride levels, and GW7647 also prevent DNA synthesis by SMCs. We further demonstrate that the anti-proliferative effects of these PPARα agonists are restricted to cells expressing PPARα, presumably by inhibiting cdk2 phosphorylation and PCNA expression. Although the PPARγ agonist rosiglitazone was unable to block the proliferative response of SMCs to mitogens, it did inhibit cell migration, which is sufficient to prevent neointimal hyperplasia after vascular injury. Finally, our data support the view that the anti-proliferative effect of 15d-PGJ_2 is likely mediated via cyclin E, but independent of PPARγ. These results provide some clarification concerning the distinct physiological actions of PPARα and PPARγ on vascular tissues.

In this study, we have established that activation of PPARα inhibits DNA synthesis, cell migration and neointimal hyperplasia (Figures 2,6,7), employing three distinct PPARα agonists for this purpose. This approach was necessitated by the fact that WY14,643, has been reported to both function as a highly selective PPARα agonist (Jiang et al., 1998, Seimandi et al., 2005) and to activate PPARγ (Lehmann et al., 1997) when used at 100 μM. However, the similar response seen with the different PPARα ligands, as well as the ineffectiveness of rosiglitazone, supports the conclusion that was reached. Nonetheless, we did note that mitogenic potency apparently influences the degree of inhibition obtained with WY14,643, since a higher concentration was required to block the actions of FBS than PDGF. This observation may explain the variability in WY14,643 potency described in different publications.

While there are few examples of a role for PPARα in SMC proliferation, Terano et al (1999) reported the putative PPARα agonist DHA blocks the phosphorylation of cdk2, a key

event in the progression from G1 to S phase. We have observed that WY14,643 also inhibits cdk2 phosphorylation (Figure 5A), which suggests this process may be the primary target for PPARα. Cdk2 activation mediates trans-activation of genes coding for proteins involved in DNA synthesis, including PCNA which is responsible for recruiting DNA polymerase δ to the prereplication complex (Waga and Stillman, 1998). Since WY14,643 also suppressed PDGF-dependent induction of PCNA (Figure 5B), inhibition of DNA synthesis by WY14,643 likely results from the absence of PCNA. Evidence of a relationship between cdk2 and PCNA (Starkel et al., 2005; Sever-Chroneos et al., 2001) supports this argument. Furthermore, the inability to phosphorylate cdk2 in the presence of WY14,643 suggests this compound may operate through cdk-activating kinase, a mechanism previously identified for mevastatin (Ukomadu and Dutta, 2003). The latter possibility would also explain why WY14,643 had no effect on cyclin expression (Figure 5E,F).

Recognition that PPARs are expressed in vascular SMCs (Marx et al., 2004) led to studies that examined whether PPAR agonists could positively affect vascular function directly, independent of their ability to decrease serum lipid levels. Benson et al (2000) showed that activation of PPARγ with troglitazone inhibited both SMC growth and migration. Similar data have been obtained for 15d-PGJ_2 and other agonists belonging to the thiazolidinedione class (Law et al., 2000), thus providing a plausible mechanism to explain how troglitazone treatment prevented neointimal hyperplasia following balloon angioplasty of the rat carotid artery (Law et al., 1996). Based on these findings, it has been generally accepted that PPARγ can modulate the proliferation of SMCs. On the other hand, the results of our study with rosiglitazone and 15d-PGJ_2 suggest PPARγ may not influence SMC proliferation. This conclusion is based on the fact that H4IIE cells (chosen for comparative purposes because PPARα is strongly expressed in the liver) do not express PPARγ1 (Figure 3), yet still are sensitive to the actions of 15d-PGJ_2. Consequently, the anti-proliferative actions of 15d-PGJ_2 cannot be mediated by PPARγ, an

argument that has been made by other investigators (Jozkowicz et al., 2001; Lennon et al., 2002). At the same time, none of the cell types we employed exhibited sensitivity to rosiglitazone, a ligand that shows a high degree of specificity for PPARγ (Seimandi et al., 2005). Accordingly, it is unlikely that PPARγ activation inhibits cell proliferation. Although this finding is not consistent with the conclusions reached in other studies, it should be recognized that many of the formative studies of PPARγ function employed 15d-PGJ_2 and other agonists with uncertain specificity. On the other hand, it must be noted that the cells used for this investigation do not express detectable amounts of PPARγ2 (Figure 4), whereas it is strongly expressed in white adipose tissue where the bulk of PPARγ action has been reported. It is therefore possible that PPARγ2 may be responsible for modulating cell proliferation in these tissues.

Although the role of PPARβ/δ in SMC proliferation has not been extensively examined, Zhang et al (2002) have reported that PPARβ/δ activation promotes SMC proliferation. Our results with the selective PPARβ/δ agonist GW501516 (Seimandi et al., 2005), however, have shown that PPARβ/δ has no effect on SMC proliferation. Furthermore, GW501516 was unable to prevent PDGF-mediated SMC migration. These results clearly establish that PPARβδ activation is not a factor in the vascular response to injury.

Since neointimal hyperplasia requires both cell proliferation and migration (Newby and Zaltsman, 2000), and inhibition of either process is sufficient to prevent neointimal hyperplasia (Zahradka et al., 2004), the reduction in intimal lesion formation produced by WY14,643 (Figure 6) is not surprising. On the other hand, the selective PPARγ agonist rosiglitizone had no effect on DNA synthesis. This result was unexpected considering the general view that PPARγ can modulate SMC proliferation (Marx et al., 2004). Nevertheless, the ability to prevent cell migration (Figure 7A) could explain why rosiglitazone is an effective inhibitor of neointimal hyperplasia, regardless of its effect on proliferation, as we previously observed with the MMP

inhibitor GM6001 (Zahradka et al, 2004). Interestingly, rosiglitazone and WY14,643 both reduced MMP9 production by vessels that had been subjected to injury by balloon inflation, as previously reported by Shu et al. (2000) for monocytic cells, yet only WY14,643 affected MMP2 (Figure 7B,C). These data support the argument that PPARγ agonists impede neointimal hyperplasia by blocking cell migration, and suggest that PPARγ and PPARα likely operate via distinct pathways. As well, the fact that 15d-PGJ_2 has no effect on MMP levels, in conjunction with its unique action on cyclin E (Figure 5), is strong evidence that this compound does not influence SMC proliferation and migration via PPARγ.

The availability of cell lines that differentially express PPARα and PPARγ has made it possible to distinguish the contribution of these isoforms to SMC proliferation. A10 SMCs may be a particularly interesting system for examining the details of PPARα function without the need to isolate cells from PPARα-null animals. The inhibitory effect of PPARα agonists on SMC proliferation sheds new light on the possible role of this receptor in vascular disease processes. Furthermore, we have identified a target, cdk2, which may be used to investigate the mechanism by which PPARα modulates both SMC proliferation and migration.

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Footnotes

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Figure Legends

Figure 1: *Effect of PPAR agonists on mitogen-stimulated proliferation of porcine coronary artery smooth muscle cells.*

Quiescent PCA-SMCs, grown in 24-well culture dishes containing supplemented serum-free DMEM for 5 days, were treated with platelet-derived growth factor (PDGF-BB, 0.1 μg, PeproTech) in the presence of various PPAR agonists. Agonists for PPARα (250 μM WY14,643), PPARγ (10 μM rosiglitazone, 5 μM 15d-PGJ_2), and PPARβ/δ (5 μM GW501516) were added 1 hour prior to mitogen stimulation. After 24 hours, [^{3}H]thymidine (1 μCi/mL) was added to the medium and the cells were incubated an additional 48 hr before harvest. Incorporation of thymidine into trichloroacetate-precipitable material was measured as described in Materials and Methods. The data are plotted as means ± sem (n=3). Statistically significant differences (P<0.05) relative to control (no agonist) are indicated (*).

Figure 2: *PPARα agonists inhibit DNA synthesis by porcine coronary artery smooth muscle cells in response to mitogen stimulation.*

Quiescent PCA-SMCs were stimulated with either 0.1 μg PDGF-BB (A,C,D)or 2% FBS (B) in the presence or absence of WY14,643 (A,B), clofibrate (C) or GW7647 (D). Thymidine incorporation was measured as described in Materials and Methods. The data are presented as means ± sem (n=3). Significant differences (P<0.05) from control (no agonist) are indicated (*).

Figure 3: *Effect of PPAR agonists on mitogen-stimulated proliferation of A10 smooth muscle cells and H4IIE hepatoma cells.*

Quiescent A10 SMCs (A,C,E) and H4IIE hepatoma cells (B,D,F) were prepared as described in Materials and Methods. The cells were subsequently treated with either 0.1 μg PDGF (A,C,E) or

10^{-7} M insulin (B,D,F) in the presence of the PPARα agonist WY14,643 (A,B) or the PPARγ agonists 15d-PGJ_2 (C,D) and rosiglitazone (E,F). Incorporation of [^{3}H]thymidine (1 μg/mL) over 48 (H4IIE) or 72 (A10) hr was measured as described in Materials and Methods. The data are presented as means ± sem (n=3). Statistically significant differences (P<0.05) from control samples (cells stimulated in the absence of agonist) are indicated (*).

Figure 4: *Expression of PPARα and PPARγ in smooth muscle and hepatoma cells.*
Total RNA was extracted from PCA-SMCs (A), H4IIE cells (B), A10 SMCs (C) and rat adipose tissue (D) with TRIzol and 1 μg was amplified by reverse transcriptase-polymerase chain reaction as described in Materials and Methods with primers specific for PPARα, PPARγ1 and PPARγ2. Differences between porcine and rat sequences were taken into account during primer design. Amplification products were stained with ethidium bromide after agarose gel electrophoresis, and photographed. Lanes for PPARα, PPARγ1 and PPARγ2 are indicated, as is the GAPDH control. The figure represents one of three independent experiments.

Figure 5: *Western blot analysis of cell cycle regulatory proteins.*
Quiescent PCA-SMCs were stimulated with 0.1 μg/ml PDGF for 6 h (A-C,E,F) or 10 min (D) in the presence or absence of PPAR agonists. The cells were subsequently harvested for Western blot analysis. Proteins were separated on resolving gels of 7.5% or 10%, transferred to PVDF membrane and probed with antibodies to phospho-cdk2 (A), PCNA (B), phospho-Rb (C), phospho-IKK (D), cyclin D1 (E), cyclin A (E), β-tubulin (E) and cyclin E (F). Representative blots are presented. Band intensity was quantified by densitometry and the data plotted as means ± sem (n=3) relative to β-tubulin. Statistically significant differences (P<0.05) from control quiescent cells (*) as well as from PDGF treated cells incubated in the absence of agonist (#) are

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indicated.

Figure 6: *Effect of WY14,643 and rosiglitazone on neointimal formation in vitro following injury.*

Porcine coronary arteries were injured by inflation of a balloon angioplasty catheter and vessel segments were cultured for 14 days in the absence or presence of 250 μM WY14,643 and 10 μM rosiglitazone. Vessel segments were subsequently sectioned, stained with Lee's methylene blue and visualized by microscopy. Morphometry was used to quantify neointimal size (neointimal index) as described in Materials and Methods. The data are plotted as means ± sem (n = 8) in panel A. Statistically significant differences (P<0.05) from uninjured control (*) and from vessels subjected to balloon angioplasty (#) are indicated. Representative sections are shown in panel B. NI=non-injured, BI=balloon-injured, m=media, n=neointima, l=lumen.

Figure 7: *Effect of PPAR agonists on SMC migration.*

Panel A: PCA-SMCs were seeded in a Boyden chamber with PDGF (0.1 μg/ml) in the lower compartment. PPAR agonists (250 μM WY14,643, 10 μM rosiglitazone, 5 μM 15d-PGJ_2, 5 μM GW501516) were subsequently added to the upper compartment. Cell migration to the underside of the membrane after an incubation period of 48 h was measured as described in Materials and Methods. The data are presented as means ± sem (n=6). Statistically significant differences (P<0.05) from cells incubated in the absence of agonist are indicated (*). Panels B,C: Injured coronary artery vessel segments were placed into culture for 48 hours in the presence or absence of PPAR agonists as described for panel A. Media samples were analyzed by gelatin zymography (representative gels are shown in B) and the relative amounts of active and latent MMP7 (68 & 72 kDa, respectively) and MMP9 (86 & 92 kDa, respectively) were quantified by scanning densitometry. The transmittance for both latent and active bands was pooled. The data

(C) are plotted as means ± sem (n=6). Statistically significant differences (P<0.05) from cells incubated in the absence of agonist are indicated (*), with MMP2 and MMP9 compared seaparately.

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Figure 1

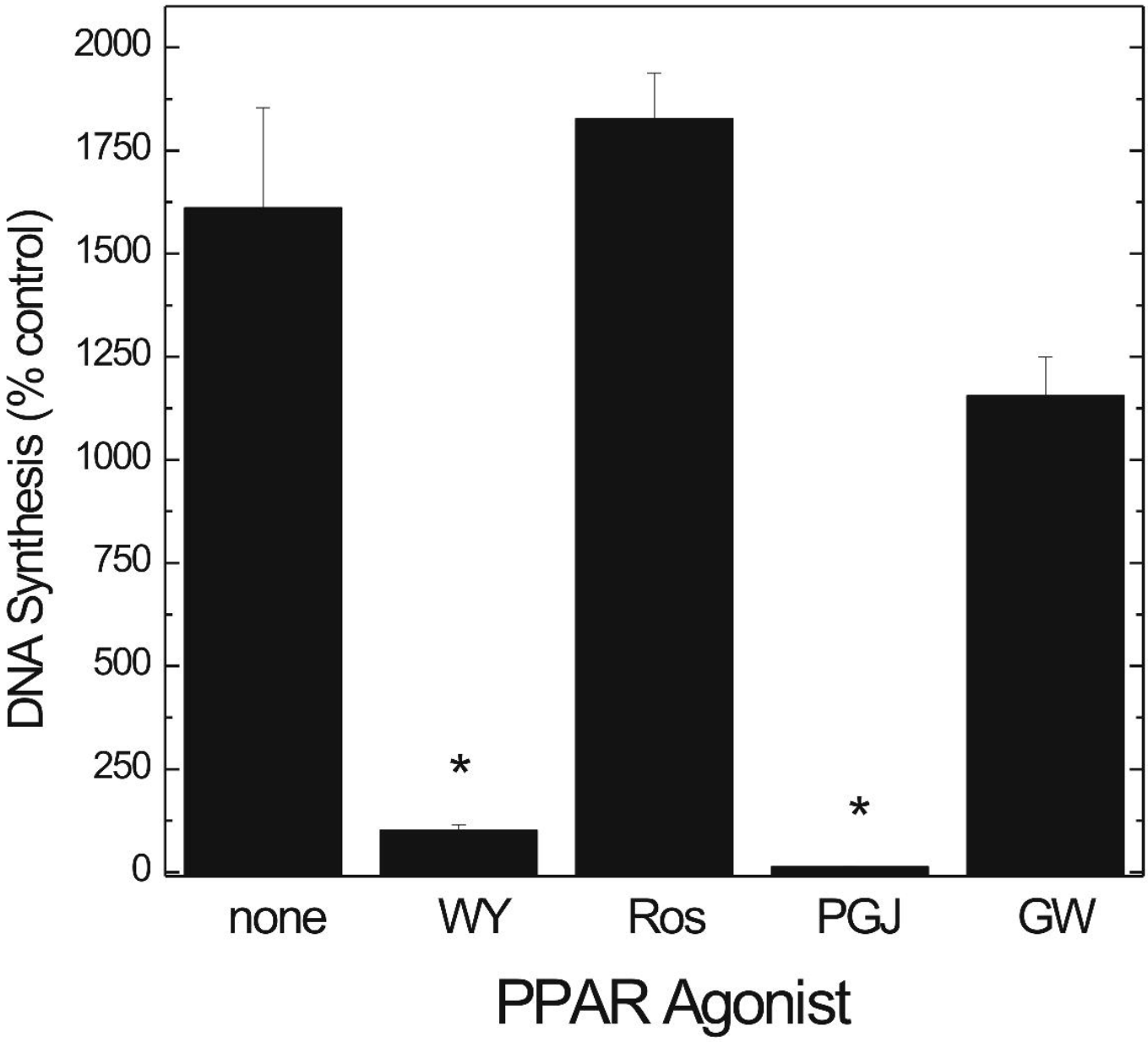

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Figure 2

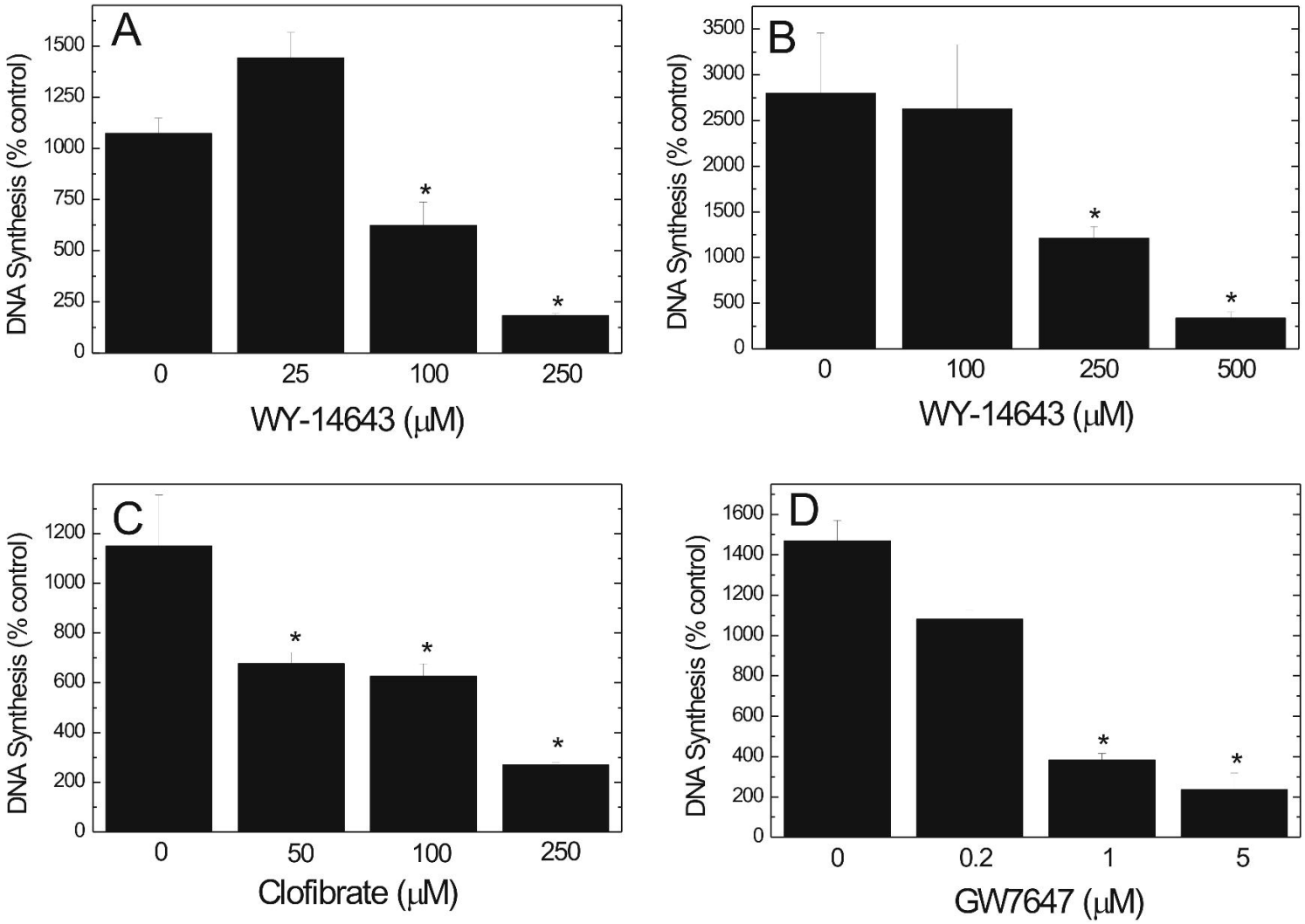

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Figure 3

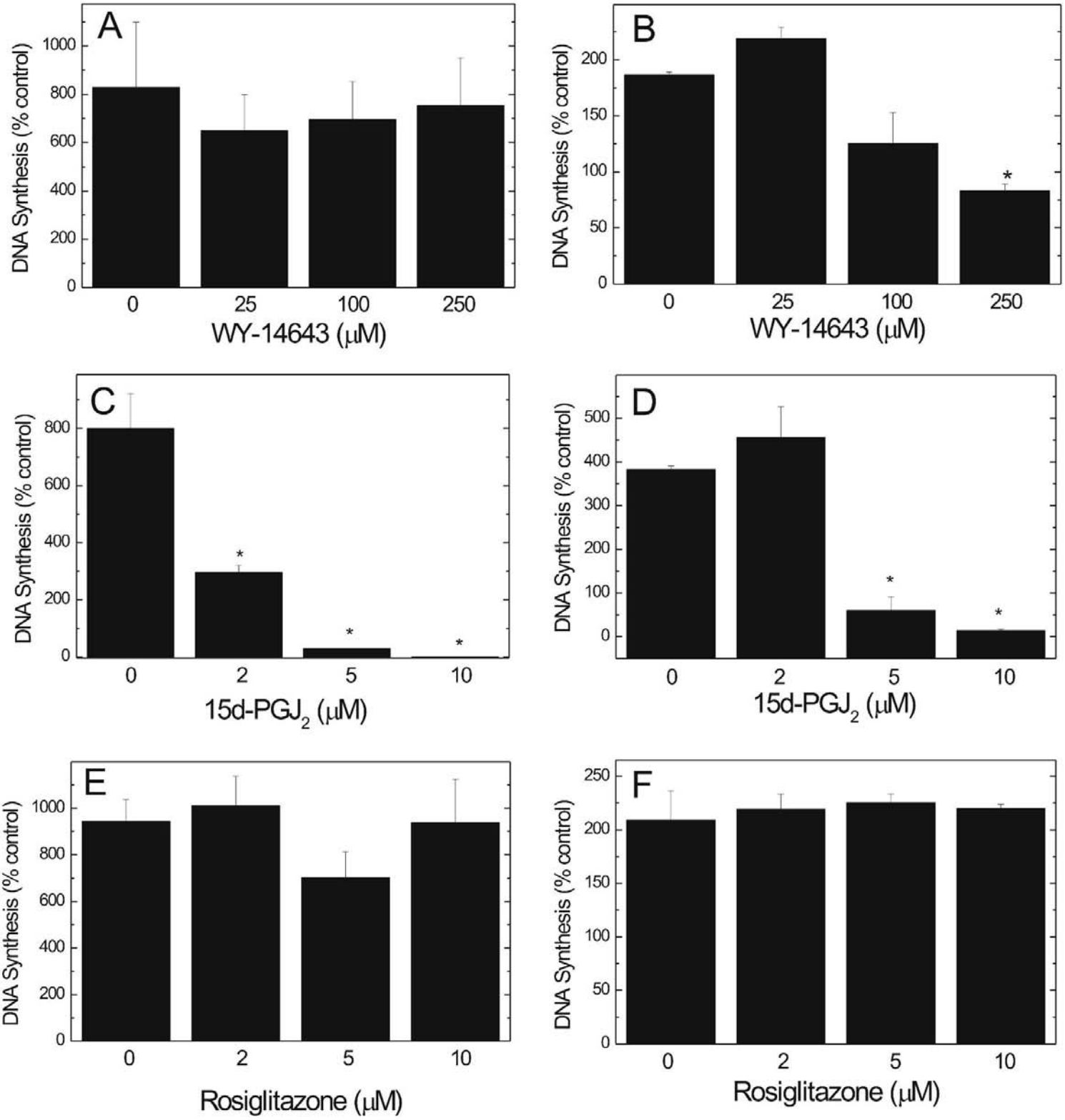

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Figure 4

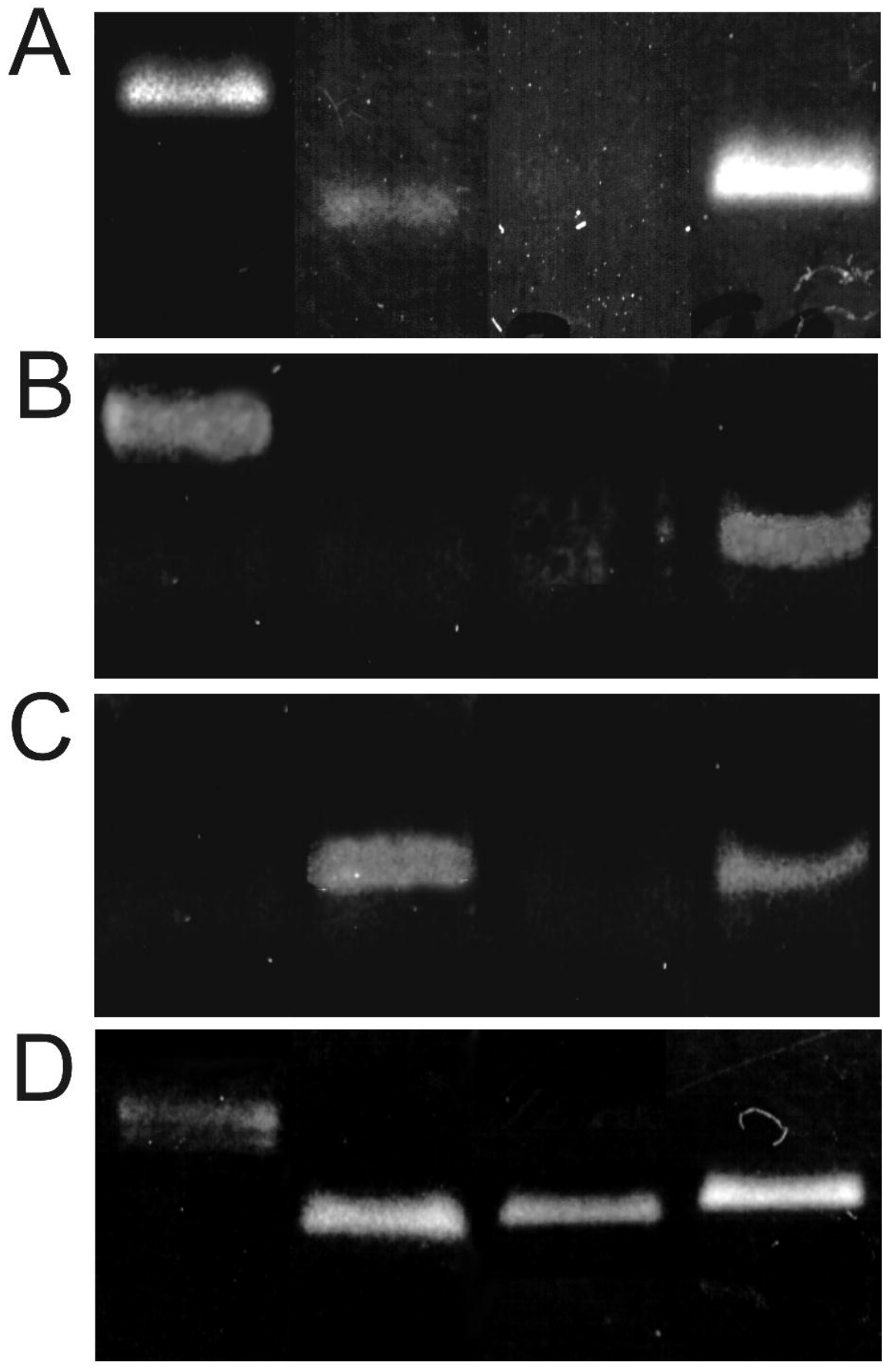

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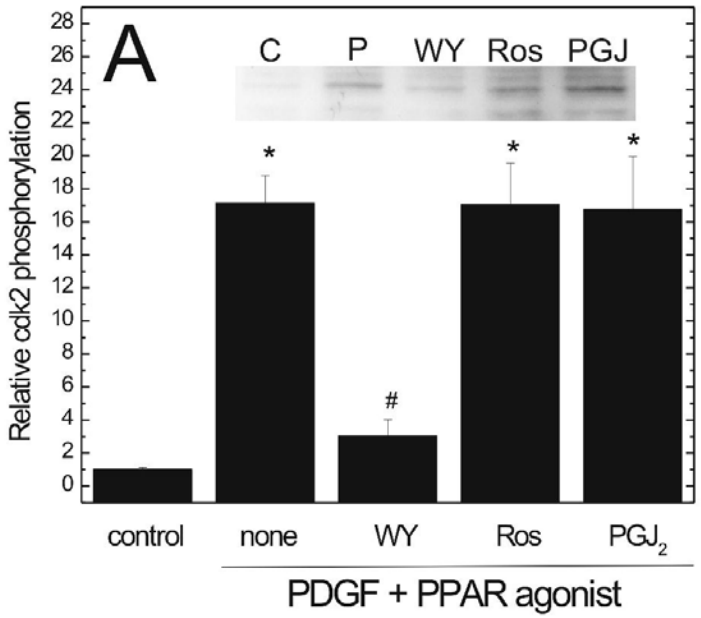
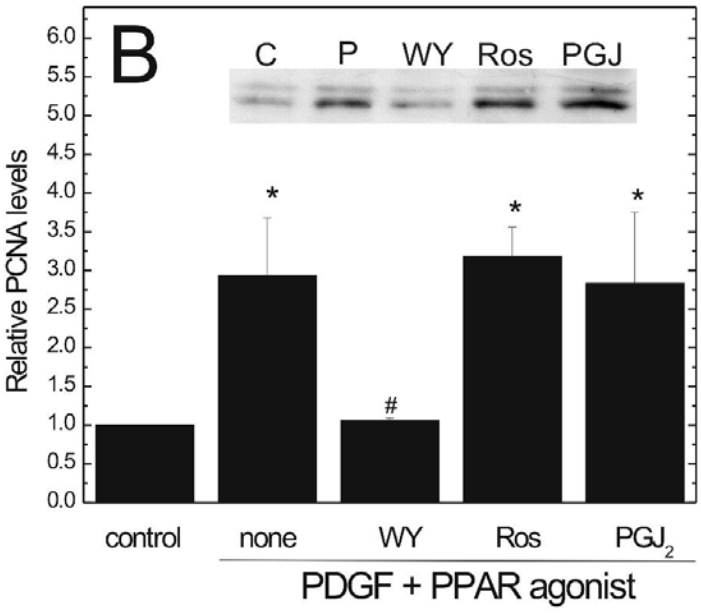
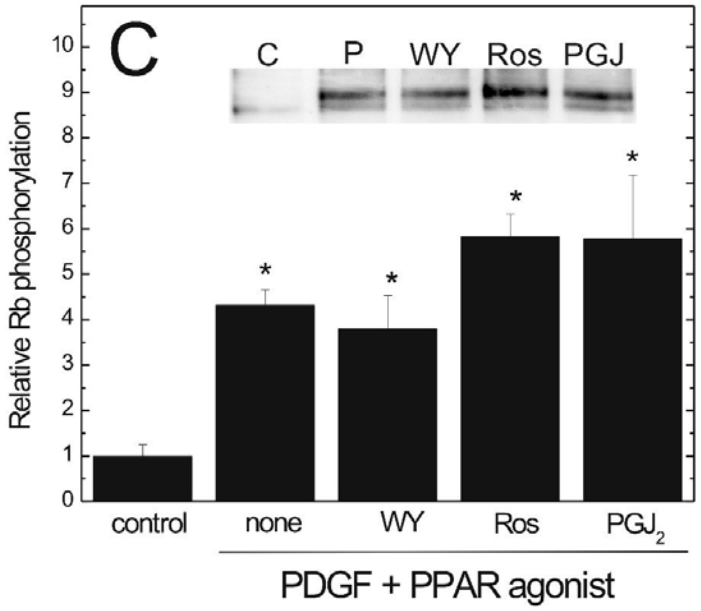
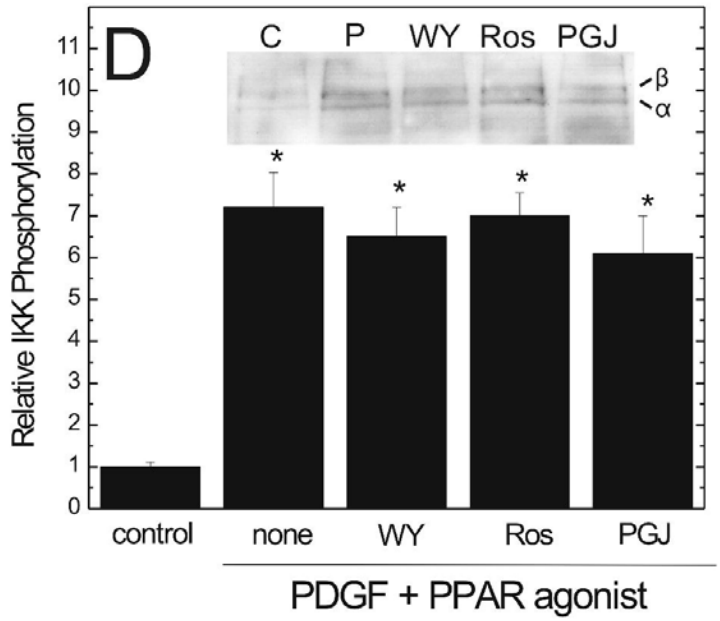
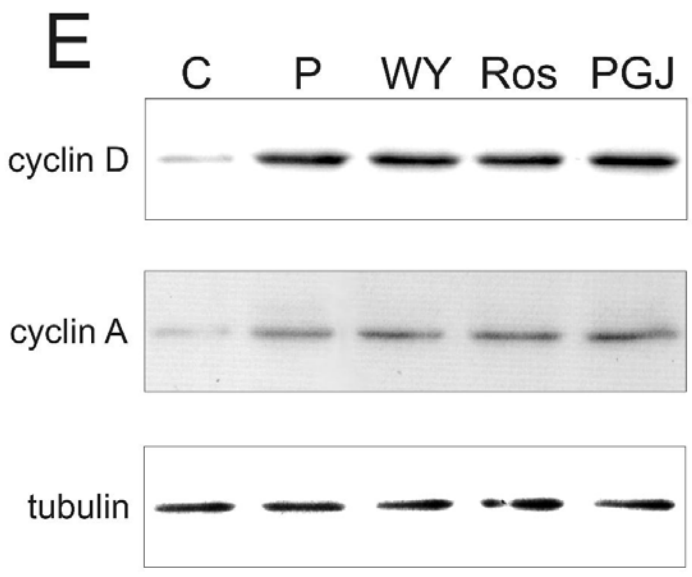
Figure 5











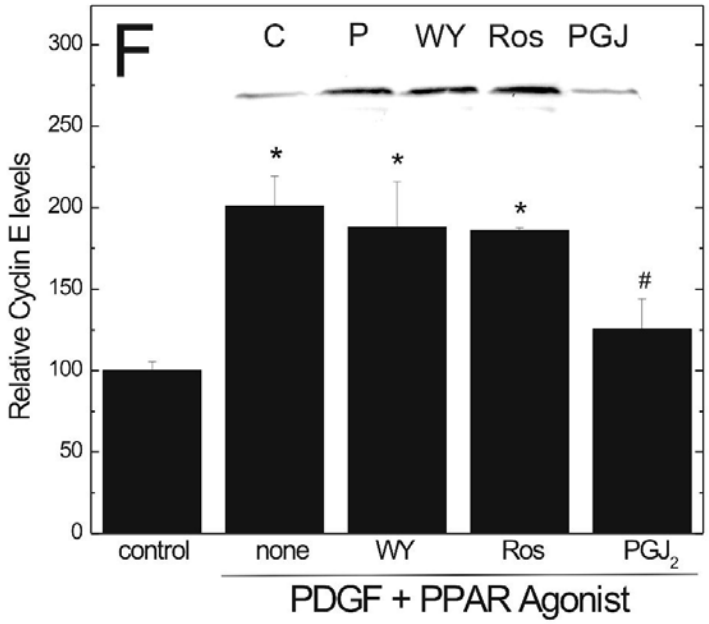


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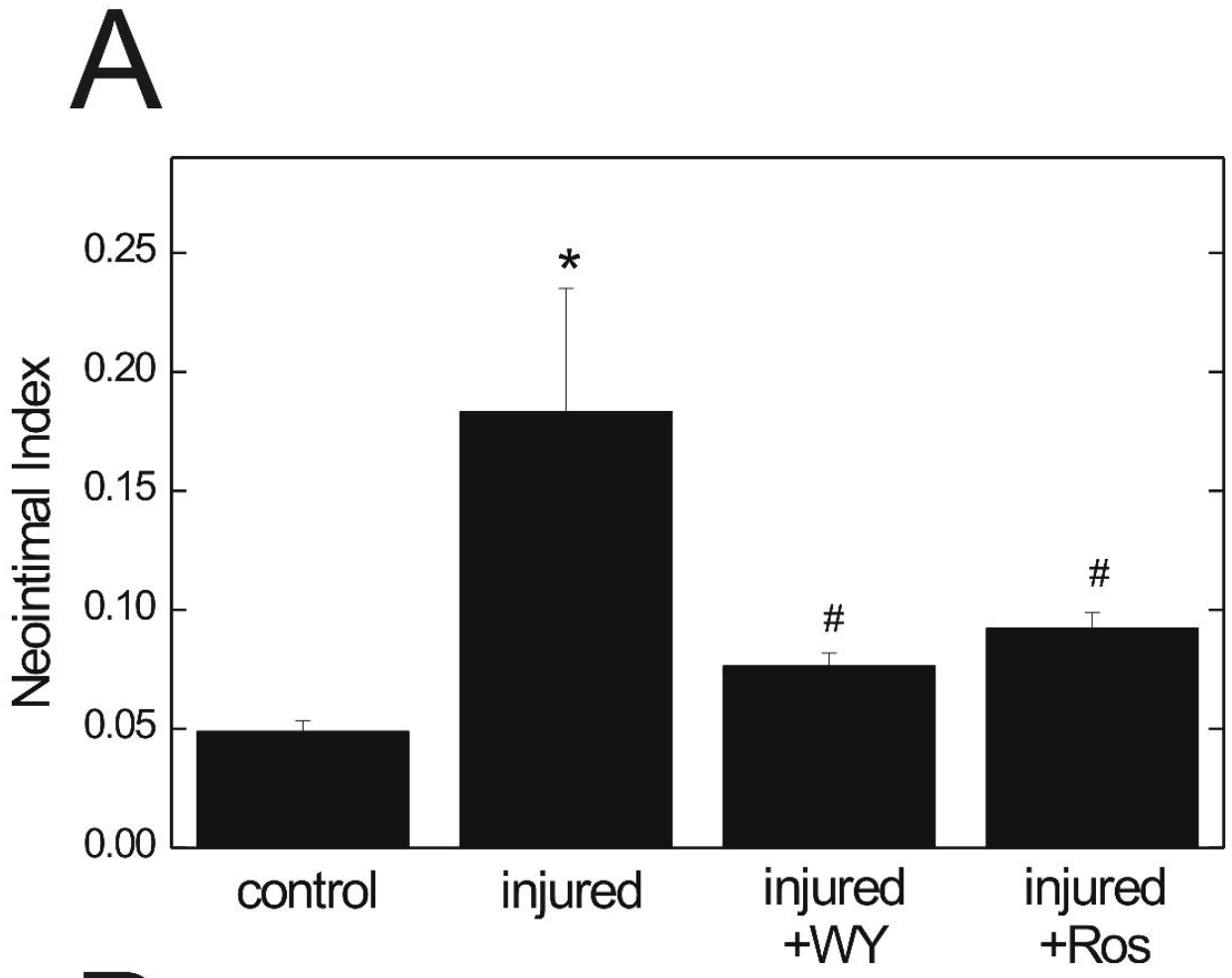
Figure 6



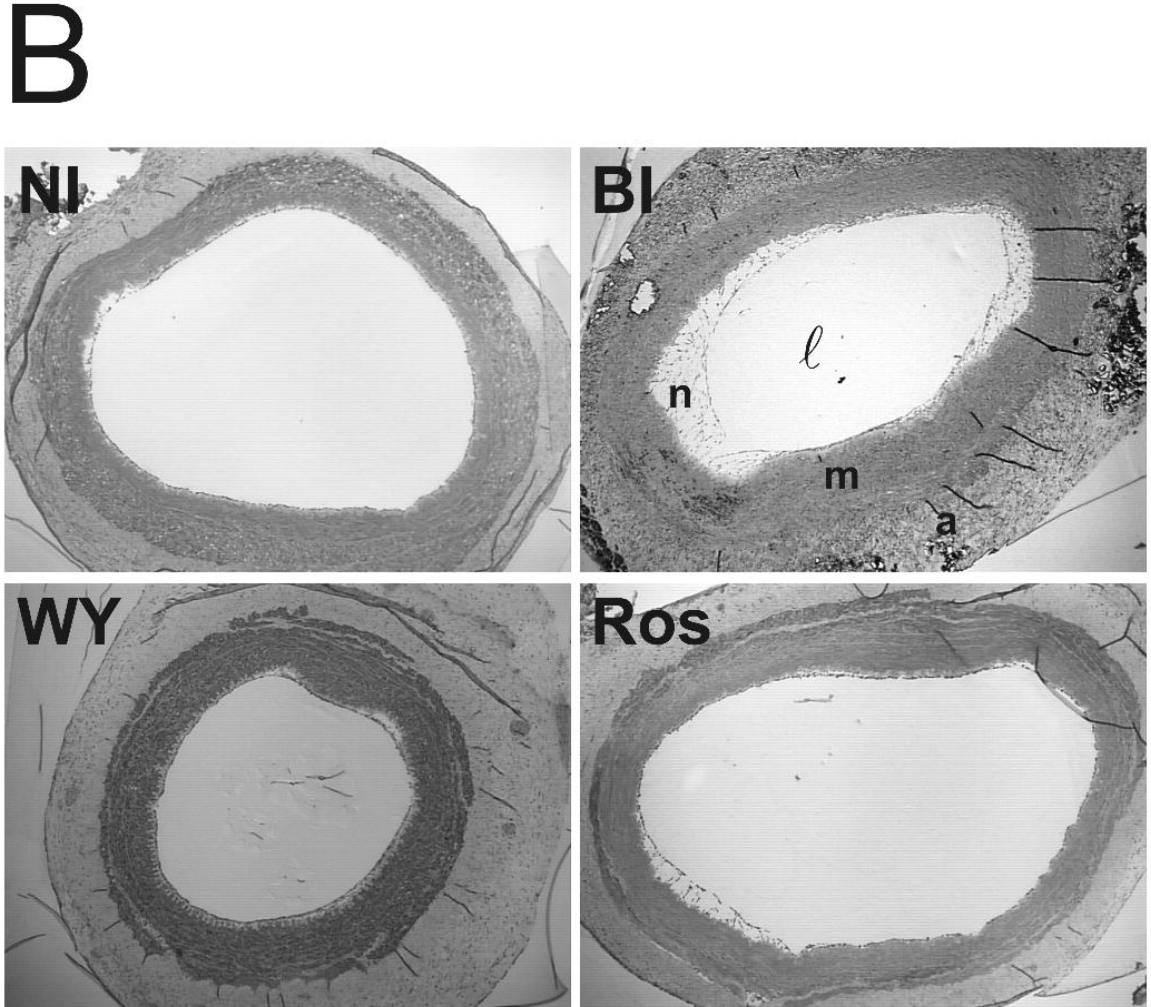


Figure 7