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*Running title: *PGC-1s inhibit NF- κ B activity in muscle*

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Keywords: PGC-1; skeletal muscle; inflammation; NF- κ B

Background: PGC-1 α and PGC-1 β are metabolic coactivators that are dysregulated in muscle in many chronic diseases.

Results: PGC-1 α and PGC-1 β differentially suppress expression of pro-inflammatory cytokines induced by various stimuli.

Conclusion: In muscle cells, PGC-1 α and PGC-1 β modulate the NF- κ B pathway thus profoundly affecting inflammatory processes.

Significance: Targeting PGC-1 α and PGC-1 β in chronic diseases might reduce inflammation and thereby reverse disease progression.

SUMMARY

A persistent, low-grade inflammation accompanies many chronic diseases that are promoted by physical inactivity and improved by exercise. The beneficial effects of exercise are mediated in large part by PGC-1 α while its loss correlates with propagation of local and systemic inflammatory markers. We examined the influence of PGC-1 α and the related PGC-1 β on inflammatory cytokines upon stimulation of muscle cells with TNF α , toll-like receptor agonists and free fatty acids. PGC-1s differentially repressed expression of pro-inflammatory cytokines by targeting NF- κ B signaling. Interestingly, PGC-1 α and PGC-1 β both reduced phosphorylation of the NF- κ B family member p65 and thereby its transcriptional activation potential. Taken together, the data presented here show that the PGC-1 coactivators are able to constrain inflammatory events in muscle cells and provide a molecular link between metabolic and immune pathways. The PGC-1s therefore

represent attractive targets to not only improve metabolic health in diseases like type 2 diabetes but also to limit the detrimental, low-grade inflammation in these patients.

A sedentary lifestyle is a strong and independent risk factor for a large number of chronic diseases including musculoskeletal, metabolic, cardiovascular and neurological disorders. These diseases have been linked to a sterile, persistent, low-grade inflammation with elevated levels of circulating cytokines like interleukin 6 (IL-6), tumor necrosis factor α (TNF α) and IL-1 β that often worsen disease progression (1-3). The nuclear factor κ B (NF- κ B) pathway is a central regulator of inflammatory processes: NF- κ B activation has accordingly been associated with obesity and insulin resistance in different organs (4-6). A wide array of signals including cytokines like TNF α and toll-like receptor (TLR) agonists of pathogenic or dietary origin (e.g. excess free fatty acids as in obesity (7)) is able to boost NF- κ B activity upon cell surface receptor binding. These ligand-receptor interactions trigger the recruitment of adaptor proteins and receptor-proximal kinases ultimately culminating in the activation of the inhibitor of NF- κ B (I κ B) kinase (IKK) complex. IKK subsequently phosphorylates I κ B which is then degraded by the proteasome. Decreased levels of I κ B free NF- κ B, thereby enabling cytosolic-nuclear translocation and ultimately transcriptional induction of a large amount of genes involved in immune function (8). The NF- κ B family comprises the 5 members RelA/p65, RelB, c-Rel, p100/p52 and p105/p50, of which the

heterodimer p65/p50 is the most common form and the target of so-called “classical” NF- κ B activation (8). The transcriptional activity of p65 is further modulated by post-translational modification i.e. inducible phosphorylation events that affect the binding affinity to coactivators and corepressors without altering the recruitment to DNA response elements (9,10).

While physical inactivity clearly has a negative impact on health favoring an inflamed environment, regular, moderate exercise is beneficial against systemic inflammation and counteracts the development of chronic diseases (11). Besides prevention, exercise also is an effective therapeutic strategy to treat obesity, type 2 diabetes, sarcopenia and neurodegeneration (12-14).

At the molecular level, many of the beneficial effects of exercise are mediated by the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) (15). PGC-1 α is transiently induced by a single bout of exercise and chronically elevated in endurance trained muscle (16). Activated PGC-1 α then controls the expression of genes encoding proteins involved in mitochondrial biogenesis, oxidative phosphorylation and other features of oxidative muscle fibers (17). Accordingly, mice with transgenic skeletal muscle-specific PGC-1 α overexpression perform better in endurance tests and display a switch towards oxidative type I and type IIA fibers (18,19). The increased fitness of healthy transgenic animals translates into improvement of symptoms when PGC-1 α is expressed in the context of different muscle wasting conditions as shown for Duchenne muscular dystrophy, sarcopenia, a mitochondrial myopathy and denervation- or lovastatin-induced fiber atrophy (20-22).

Inversely, a skeletal muscle-specific deletion of the PGC-1 α gene facilitates a type IIB and type IIX fiber type switch, reduces exercise performance and promotes muscle fiber damage (23). Furthermore, loss of PGC-1 α results in elevated levels of pro-inflammatory factors locally in muscle as well as systemically (24). These findings suggest an anti-inflammatory role for PGC-1 α : in fact, in skeletal muscle of diabetic patients, PGC-1 α levels negatively correlate with IL-6 or TNF α levels independent of BMI (24).

PGC-1 β , a close related member of the PGC-1 gene family, also exhibits dysregulated

expression in skeletal muscle of diabetic patients and thereby contributes to the mitochondrial dysfunction observed in type 2 diabetes (25). While both PGC-1s share the ability to boost oxidative metabolism, PGC-1 β is not regulated by exercise and primarily drives the formation of type IIX fibers (26). Interestingly, PGC-1 β is required for alternative activation of and mitochondrial ROS production in macrophages (27,28); an immunomodulatory role in skeletal muscle has however not been described so far.

Based on these observations, we now tested the hypothesis that the PGC-1 coactivators exert anti-inflammatory effects in muscle. More precisely, we explored if PGC-1 α and PGC-1 β are able to modify cytokine expression upon exposure of muscle cells to different inflammatory stimuli like TNF α , TLR agonists and free fatty acids (FFAs). We found that the PGC-1s repress the transcriptional activity of p65 and thereby modulate the NF- κ B signaling pathway. These data represent a prime example of crosstalk between metabolic and immune pathways with important implications for skeletal muscle function.

EXPERIMENTAL PROCEDURES

Cell culture and treatments: The mouse skeletal muscle cell line C2C12 was maintained below confluence in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum and 1x Penicillin/Streptomycin (Invitrogen). For differentiation into myotubes, growth medium was exchanged for DMEM supplemented with 2% horse serum (Invitrogen) for at least 3 days. PGC-1 α , PGC-1 β and GFP were overexpressed from recombinant adenoviral constructs 48 h prior to treatment. Stimulation with TNF α (Sigma-Aldrich) and TLR agonists (Invivogen) in growth or differentiation medium lasted for 2 h unless otherwise stated. Concentrations were as follows: TNF α 10ng/ml, PAM3CSK4 1 μ g/ml (TLR1/2 agonist), HKLM 10⁸ cells/ml (TLR2 agonist), poly(I:C) 25 μ g/ml (TLR3 agonist), *E.coli* K12 LPS 1 μ g/ml (TLR4 agonist), *S. typhimurium* flagellin 1 μ g/ml (TLR5 agonist), FSL1 1 μ g/ml (TLR6/2 agonist), ssRNA40 1 μ g/ml (TLR8 agonist) and ODN18266 5 μ M (TLR9 agonist). Free fatty acids (FFA, Sigma-Aldrich) were dissolved in ethanol and further diluted to 1mM final concentration in DMEM containing 2% fatty acid- and endotoxin-free bovine serum albumin (Sigma-Aldrich); FFA treatment lasted for 16 h in serum-free medium.

The protein phosphatase inhibitor okadaic acid (OA, Sigma-Aldrich, 250nM) was present 30 minutes prior to and during treatment with TNF α where indicated, while control samples were incubated with vehicle (DMSO, 0.04%) alone for equal times. The PPAR α inhibitor MK 886 (Tocris Bioscience, 20 μ M) was present 24 h prior to and during treatment with TNF α where indicated, while control samples were incubated with vehicle (DMSO, 0.02%) alone for equal times.

Semiquantitative real-time PCR: RNA was isolated from treated C2C12 cells using Trizol (Invitrogen) and residual DNA contamination was removed by DNase I digestion (Invitrogen). 1 μ g of RNA was reverse transcribed with SuperScript II (Invitrogen) and the resulting cDNA used as template for RT-PCR. To detect relative expression levels, cDNA was amplified with the SYBR Green Master mix (Applied Biosystems) and analyzed on a StepOnePlus RT-PCR System (Applied Biosystems). The respective primer pairs are listed in *Supplemental Table 1*. All values are normalized to the expression of TATA-Box binding protein (TBP) and expressed as fold induction over the untreated control condition.

ELISA: To determine cytokine concentrations in cell culture supernatants, sandwich immunoassays against IL-6 were performed according to the manufacturer's instructions (Quantikine, R&D Systems).

NF- κ B Customized Array analysis: Differentiated C2C12 cells overexpressing PGC-1 α , PGC-1 β or GFP were treated with TNF α for 2h and RNA was extracted (NucleoSpin RNA II, Macherey-Nagel). The custom array analysis was subsequently performed as previously described (29). Briefly, RNA was processed with the Amino Alkyl MessageAMP II aRNA Amplification Kit (Ambion) according to the manufacturer's instructions to yield aminoalkyl-modified aRNA that was further coupled to Cy3 or Cy5 dyes respectively. Biological duplicates were labeled crosswise to control for dye effects. Pairs of samples were hybridized on a total of 14 slides containing probes for 524 genes included in the array as described elsewhere (29, ArrayExpress A-MEXP-1502). Significantly regulated genes as determined by ANOVA ($P \leq 0.01$) were subject to cluster analysis. The 3 resulting clusters were the source of further KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) terms enrichment analysis with FatiGO (30)

considering the adjusted p -value ≤ 0.05 as significant. To predict the relevant transcription factors for the regulation of each cluster, motif search within a region spanning +200bp to -800bp around the transcription start site (TSS) of each gene in the array was performed using MotEvo (31) in combination with a non-redundant set of position weight matrices mainly derived from JASPAR and TRANSFAC. Overrepresented motifs were retrieved by comparing binding site occurrence in each cluster to the occurrence in the whole array and considered significant with a z score ≥ 2 . Microarray data were further analyzed with MARA (Motif Activity Response Analysis (32)) to predict the most important transcription factors that contribute to the changes in gene expression in our set of samples (independent of clustering).

Dual-luciferase reporter gene assays: 90 – 95% confluent C2C12 myoblasts were transfected using Lipofectamin 2000 (Invitrogen) with plasmids containing firefly luciferase under the control of 3 NF- κ B sites (wildtype, wt) or a construct with mutated sites as control in combination with renilla luciferase as internal control. Cells were then either treated with TNF α / TLR agonists for 2h, or co-transfected with p65, increasing amounts of PGC-1 α/β and empty vector (pcDNA3.1) to keep total DNA concentration constant. Cells were assayed 24h post transfection with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Briefly, cells were lysed and firefly and renilla luciferase activities determined on a Centro LB 960 luminometer (Berthold Technologies). Firefly-derived luciferase values were normalized to renilla activity and results expressed as ratio of wt to mutated reporter with the control condition being arbitrarily set to 1.

TransAM NF- κ B DNA binding assays: Nuclear extracts from myotubes were prepared by swelling of the cells in hypotonic buffer (20mM Hepes, pH 7.5, 5mM NaF, 10 μ M Na₂MoO₄, 0.1mM EDTA) and breaking the cytoplasmic membrane with Nonidet P-40 substitute (0.5%). After centrifugation, the residual nuclear pellet was resuspended in Complete Lysis Buffer to dismantle nuclear membrane integrity and obtain the nuclear extract as supernatant of the final, fast centrifugation step. To determine the amount of active NF- κ B in these nuclear extracts, TransAM assays (Active Motif) were performed following

the manufacturer's instructions. In brief, 10 μ g of nuclear extracts were added to wells coated with oligonucleotides containing the NF- κ B consensus sequence (5'-GGGACTTTC-3'). These oligonucleotides trap active transcription factors, which then are detected in an ELISA-like assay with specific primary antibodies for the different NF- κ B subunits and a secondary horseradish peroxidase (HRP)-conjugated antibody. A colorimetric reaction accordingly determines the amount of NF κ B-DNA binding. Results are expressed in arbitrary units minus the respective blank value, and normalized to Ctrl conditions.

Western Blotting: After washing away residual culture medium with PBS, cells were scraped off the dish in lysis buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM Na₄P₂O₇, 1mM β -glycerophosphate, 1mM Na₃VO₄) supplemented with protease- (complete tablets, Roche) and phosphatase inhibitors (phosphatase inhibitor cocktail 2, Sigma-Aldrich) and kept on ice for 10 min with occasional vortexing. Centrifugation at 10'000g yielded the protein lysate, which was subjected to SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane (Whatman) on a semi-dry electroblotting system (Thermo Scientific) with 1.5mA/cm² and transfer confirmed by Ponceau S staining (0.1% in 5% acetic acid). Proteins were detected with the following primary antibodies: anti-p65, anti-Pp65(ser536) (both Cell Signaling Technology), anti-p105/p50 (abcam), anti-p100/p52, anti-RelB, anti-cRel, anti-I κ B α , anti-I κ B β , anti-IKK α , anti-IKK γ , anti- α -tubulin as loading control (all Cell Signaling Technology) and an anti-rabbit-IgG HRP-coupled secondary antibody (Dako). Bands were finally visualized by autoradiography with ECL substrate (Pierce) and density quantified with ImageJ software.

Statistical analysis: Data were analyzed with student's *t* test using $P \leq 0.05$ as the significance threshold.

RESULTS

PGC-1 α and PGC-1 β differentially suppress pro-inflammatory cytokine expression and secretion To delineate the effect of PGC-1s on inflammatory processes in muscle, myotubes overexpressing either adenovirally encoded PGC-1 α or PGC-1 β were compared to GFP-expressing control cells treated with different inflammatory stimuli. PGC-1 coactivator

overexpression was high and functional as evident from the induction of the known target gene medium-chain acyl-coenzyme A dehydrogenase (MCAD, Suppl. Fig. 1A and 1B). Ectopic, recombinant TNF α strongly induced the gene expression of the pro-inflammatory markers IL-6, TNF α and macrophage inflammatory protein-1 α (MIP-1 α /CCL3). While PGC-1 α did not negatively affect basal levels of these pro-inflammatory cytokines, it diminished their induction by TNF α yielding lower levels than in control cells (Fig. 1A). PGC-1 β suppressed both basal and TNF α -induced expression of IL-6, TNF α and MIP-1 α (Fig. 1B). As a consequence of the PGC-1-mediated repression in gene expression, lower levels of secreted IL-6 protein were observed in culture media after TNF α treatment (Fig. 1C and 1D).

Next, we examined the effect of PGC-1s on TLR stimulation in muscle cells. First, we assessed the expression pattern of the TLRs in our experimental system and found that TLR1, TLR2, TLR3, TLR 4, TLR6, and at lower levels TLR5 were detectable in the muscle cells (Fig. 2A). To study the activity of the TLRs, selective agonists for TLR2, TLR3, TLR4, TLR5, TLR8, TLR9, or the TLR1/2 and TLR6/2 heterodimers were applied. Of those, only TLR1/2, TLR4 and TLR6/2 activators consistently elevated expression of IL-6 and TNF α (Fig. 2B). Moreover, the same compounds increased luciferase reporter gene activity controlled by 3 repeats of a minimal NF- κ B DNA-response element (Fig. 2C). For the subsequent experiments, agonists for TLR1/2, TLR4 and TLR6/2, the active TLRs in muscle cells, were utilized and compared to the TLR3 activator that was used as a negative, internal control. TLR1/2, TLR4 and TLR6/2 agonists all induced expression of IL-6 and TNF α in control cells as expected (Fig. 2D). Interestingly, PGC-1 α effectively repressed TLR-mediated TNF α expression by all of the active agonists while IL-6 expression was only reduced by PGC-1 α in the TLR1/2 and TLR4 agonist treatment (Fig. 2D). PGC-1 α furthermore lowered basal IL-6 protein secretion, but not the elevated IL-6 secretion caused by TLR agonist stimulation (Fig. 2E). In contrast, PGC-1 β significantly decreased IL-6 and TNF α gene expression both in the basal state and after activation of TLR1/2 while no effect was observed in the TLR4 or TLR6/2 agonist treated cells (Fig. 2F). Nevertheless however, IL-6 secretion into culture medium was strongly

repressed by PGC-1 β in all of the different experimental conditions (Fig. 2G).

As a third inflammatory stimulus besides TNF α and TLR agonists, the free fatty acids (FFA) palmitic acid (C₁₆H₃₂O₂), oleic acid (C₁₈H₃₄O₂), myristic acid (C₁₄H₂₈O₂), stearic acid (C₁₈H₃₆O₂), linoleic acid (C₁₈H₃₂O₂) and elaidic acid (C₁₈H₃₄O₂) were administered to muscle cells to mimick the lipid overload that is associated with disease progression in the metabolic syndrome. As expected, saturated fatty acids, in particular stearic and palmitic acid and to a lesser extent the shorter-chain species, produced a strong pro-inflammatory response as indicated by the transcriptional induction of the IL-6, TNF α and MIP-1 α genes (Fig. 3A). In contrast, mono- or polyunsaturated fatty acids did not alter pro-inflammatory cytokine expression (Fig. 3A); in fact, oleic acid was even able to reverse the negative effects of palmitic acid (Fig. 3A) as previously described (33). Strikingly, PGC-1 α potently inhibited the increase in IL-6, TNF α and MIP-1 α gene expression mediated by palmitic, myristic and stearic acid (Fig. 3A). Likewise, PGC-1 β also efficiently blocked the FFA-induced elevation of IL-6 and MIP-1 α transcript levels (Fig. 3B). Interestingly however, transcriptional elevation of TNF α by palmitic, myristic and stearic acid was unaffected by overexpression of PGC-1 β (Fig. 3B).

PGC-1 α and PGC-1 β target the NF- κ B pathway to suppress inflammation. To examine the mechanisms behind the repressive effect of the PGC-1 coactivators on TNF α -induced pro-inflammatory cytokines, a customized array designed to represent the most important inflammatory genes and other NF- κ B targets (29) was employed. Out of the 524 genes that are present on the array, 55 genes were found to be differentially regulated by TNF α treatment and/or PGC-1 overexpression ($P < 0.01$) and thus further analyzed. These 55 genes were grouped into 3 clusters (Fig. 4A): cluster 1 contained 21 genes that were upregulated by PGC-1 α and PGC-1 β including vascular endothelial growth factor (VEGF), a known PGC-1 α target (34). The 13 and 21 genes in clusters 2 and 3, respectively, were repressed by PGC-1 α and PGC-1 β . Importantly, genes in cluster 3 were TNF α -inducible while the expression of the genes in cluster 2 was not modulated by TNF α treatment (Fig. 4A). To validate the results of the microarray, three representative genes from each

cluster were chosen and their expression analyzed by real-time PCR. Mitochondrial translational initiation factor 2 (Mtif2), VEGF α and protein arginine methyltransferase 1 (Prmt1) from cluster 1 were indeed induced (Suppl. Fig. 2A) while chemokine (C-X-C motif) ligand 12 (Cxcl12), complement component 2 (C2) and E2F transcription factor 2 (E2f2) from cluster 2 (Suppl. Fig. 2B) and chemokine (C-C motif) ligand 2 (Ccl2), Ccl7 and Cxcl1 from cluster 3 (Suppl. Fig. 2C) were repressed by PGC-1 coactivators confirming the results of the microarray. Furthermore, TNF α -inducibility of genes in cluster 3 was verified.

Functionally, the genes in cluster 1 were enriched in only one Gene Ontology (GO) term compared to 18 significant terms in cluster 2 and 96 terms in cluster 3 (of which only 46 terms with a p -value ≤ 0.01 are shown) (Suppl. Fig. 3). 12 of the GO categories in cluster 2 and 26 categories in cluster 3 are related to inflammation and immunity. Importantly, the inflammation-related GO terms comprise the top 10 and top 15 ranking categories in clusters 2 and 3, respectively (Suppl. Fig. 3). Likewise, all 3 KEGG pathways assigned to cluster 2 and the top 9 KEGG pathways out of 11 of cluster 3 are related to inflammatory signaling (Fig. 4B). These results suggest that the PGC-1s are able to repress inflammatory processes in muscle cells.

To predict which transcription factors are involved in the regulation of each cluster, promoter regions were analyzed in regard to their motif composition. Motifs overrepresented in each of the clusters are listed in Suppl. Table 2A. As background for this analysis, all promoters of the microarray were used, thereby eliminating any putative bias that might have been introduced with the specific choice of genes in the customized microarray. The majority of predicted binding sites were unique to one cluster, i.e. 26 out of 33 motifs in cluster 1, 20 out of 26 motifs in cluster 2 and 21 out of 28 motifs in cluster 3 (Suppl. Table 2B). Intriguingly, one distinct transcription factor binding motif stood out as the clear top ranking candidate in each of the three clusters based on z score, namely NFY{A,B,C} in cluster 1 (z score of 9.74 compared to the second ranking z score of 4.9 for GF11), IRF1,2,7 in cluster 2 (z score of 6.98 compared to 4.46 for FOX{F1,F2,J1}) and NF κ B1_REL_RELTA in cluster 3 (z score of 6.67 compared to 4.93 for AR) (Fig. 4C and Suppl. Table 2A). These predictions indicate that the NF- κ B pathway is a likely target for the PGC-1s

to suppress TNF α -inducible inflammatory gene expression represented in cluster 3. Accordingly, NF- κ B was also the highest scoring motif in the Motif Activity Response Analysis (MARA) of the whole array and thus the most likely transcription factor to modulate TNF α - and PGC-1-dependent gene expression (Fig. 4D).

PGC-1 β reduces p65 and p50 expression levels. To experimentally validate the biocomputational prediction of PGC-1-mediated repression of NF- κ B signaling as the central mechanism for the anti-inflammatory effect of the PGC-1 coactivators on TNF α -inducible genes, reporter gene assays were performed with a construct containing the luciferase gene under the control of 3 NF- κ B DNA response elements. The activity of the reporter gene construct in response to TNF α treatment resembles endogenous IL-6 and TNF α gene expression (Suppl. Fig. 1C and 1D). Increasing amounts of co-transfected PGC-1 α or PGC-1 β progressively inhibited transcription from a NF κ B-responsive promoter that is activated by exogenous p65 (Fig. 5A).

To elucidate the mechanism by which the PGC-1 coactivators repress NF- κ B activity even on minimal NF- κ B response element-driven gene expression, we first examined the expression levels of the different NF- κ B family members before and after TNF α treatment and PGC-1 overexpression, respectively (Fig. 5B and 5C). TNF α led to a significant increase in transcript levels of all 5 NF- κ B isoforms (p65, p105/p50, p100/p52, RelB and c-Rel) in GFP-infected control cells. PGC-1 α did not change the levels of the canonical isoforms p65 and p105/p50 as well as RelB in the basal, vehicle-treated cells while an induction of p100/p52 was observed suggesting a switch towards the noncanonical NF- κ B pathway. In contrast to the first 4 NF- κ B isoforms, c-Rel expression was clearly reduced by PGC-1 α overexpression (Fig. 5B). After TNF α treatment, the levels of all NF- κ B isoforms tended to be lower in PGC-1 α overexpressing cells, however only reaching statistical significance in the case of c-Rel gene expression (Fig. 5B). In striking contrast to the PGC-1 α -mediated effect, PGC-1 β suppressed p65 and p105/p50 as well as RelB and c-Rel gene expression in the basal state (Fig. 5C). The transcript levels of all 5 NF- κ B isoforms tended to be lower in PGC-1 β overexpressing cells after TNF α treatment; similar to PGC-1 α overexpressing cells, this repression was

however only significant for c-Rel gene expression (Fig. 5C). Therefore, while the basal repression of p65 and p105/p50 could contribute to the strong repression of inflammatory gene expression by PGC-1 β in non-stimulated cells, it is unlikely that the small differences in expression levels of the different NF- κ B isoforms after TNF α treatment underlie the profound effects of PGC-1s on pro-inflammatory cytokine expression in stimulated muscle cells. Thus, to further elucidate the molecular mechanism underlying this observation, we next assessed the DNA binding capability of all NF- κ B family members in the context of TNF α treatment and PGC-1 overexpression (Fig. 5D and 5E). Of the 5 NF- κ B isoforms, RelB and c-Rel were undetectable in nuclear extracts from C2C12 myotubes in the TransAM assay and are thus unlikely to play a major role in the regulation of pro-inflammatory cytokine expression in our experimental context of muscle cells as hypothesized in other publications (35). The DNA binding of p50 and p52 was detectable though low (Fig. 5D and 5E). In contrast, recruitment of p65 to DNA response elements was substantial in non-stimulated cells and further elevated after TNF α treatment. Interestingly however, DNA binding of p65 with and without TNF α treatment, respectively, was not changed by PGC-1 α (Fig. 5D) while PGC-1 β strongly inhibited binding of p65 and p50 to DNA in the basal, vehicle-treated muscle cells (Fig. 5E). Comparable to PGC-1 α overexpression, DNA binding of any of the NF- κ B isoforms was not affected by ectopic PGC-1 β in TNF α -stimulated cells (Fig. 5E). It is thus conceivable that both low p65/p50 expression as well as reduced DNA binding account for the PGC-1 β -mediated reduction in pro-inflammatory cytokine expression compared to vehicle-treated control cells. In contrast however, the diminished levels of these cytokines upon TNF α treatment in both PGC-1 α - and PGC-1 β -overexpressing cells can neither be attributed to changes in NF- κ B expression nor to modulation of the NF- κ B protein binding capability to DNA response elements.

PGC-1 α and PGC-1 β diminish the transcriptional activity of p65. Based on our data implying alternative molecular mechanisms distinct from transcriptional regulation or DNA binding of NF- κ B to underlie the repressive action of the PGC-1 coactivators on the activity of this transcription factor, we next studied upstream signaling and the post-translational

modification of p65 that influence the transcriptional activity of NF- κ B. First, we examined the possibility that high levels of I κ Bs after TNF α treatment could account for lowered cytokine expression. However, the relative amount of I κ B β protein was not different between conditions (Fig. 6A). I κ B α , an NF- κ B target gene, was accordingly increased on the protein level by TNF α treatment and reduced in muscle cells overexpressing PGC-1 α or PGC-1 β (Fig. 6A and Suppl. Fig. 4A and 4B), similar to other NF- κ B targets such as IL-6 and TNF α (Fig. 1) excluding the possibility of I κ B-mediated repression. Subsequently, IKK protein levels were assessed: IKK β was not and IKK γ was barely detectable (Fig. 6A). In contrast, IKK α was slightly increased by TNF α treatment; this effect was abrogated in cells overexpressing PGC-1 α or PGC-1 β (Fig. 6A). IKK α is one of the protein kinases that is able to phosphorylate p65 at serine 536. Importantly, the phosphorylation status of p65 at serine 536 affects the transcriptional activity of NF- κ B even when bound to DNA response elements (10). In Western blot analyses of total and phosphorylated p65 protein, a small but significant increase in total p65 protein levels was observed after TNF α treatment in control cells (Fig 6A and 6B). PGC-1 α overexpression did not affect basal levels of p65, while PGC-1 β diminished total p65 protein expression in this context, as expected based on the reduced mRNA expression of p65 in PGC-1 β overexpressing muscle cells (Fig. 5C). Strikingly however, both PGC-1 α and PGC-1 β reduced TNF α -mediated phosphorylation of p65 by about 50% (Fig. 6A and 6B). None of the other NF- κ B family members underwent regulation by TNF α on the protein level. Interestingly, PGC-1 α overexpression resulted in elevated protein levels of p105, p100, p52 and RelB in non-stimulated muscle cells and, to a smaller extent, of p105 and p100 in TNF α -treated cells again suggesting a PGC-1 α -dependent increase in the non-canonical NF- κ B pathway (Fig. 6A and Suppl. Fig. 4A). In contrast, PGC-1 β does not seem to elevate the non-canonical NF- κ B family members like PGC-1 α . Thus, besides the strong increase in c-Rel protein and the more moderate elevation of p105 protein in non-stimulated cells, PGC-1 β significantly reduced RelB protein levels (Fig. 6A and Suppl. Fig. 4B). The discrepancy between the PGC-1-mediated repression of c-Rel gene expression compared to

the elevation of c-Rel protein levels implies post-transcriptional effects in the regulation of this particular NF- κ B family member.

Dephosphorylation and transrepression of p65 are potential molecular mechanisms for diminished cytokine expression. To further substantiate the findings implying that the PGC-1 coactivators modulate NF- κ B activity by preventing p65 phosphorylation, we examined other upstream effectors for this phosphorylation as well as downstream events affecting the DNA-bound NF- κ B transcriptional complex. The protein kinase Akt has been implicated in the regulation of the NF- κ B signaling pathway upstream of IKK α (36), one of the protein kinases to mediate p65 phosphorylation and of which protein levels are reduced by PGC-1 α and PGC-1 β overexpression (Fig. 6A). As expected, based on these findings, PGC-1 α and PGC-1 β diminished Akt activation as evident from diminished phospho-Akt (ser473) levels normalized to total Akt protein (Fig. 7A).

To obtain a more accurate picture of p65 phosphorylation, a time course experiment was performed. It revealed that p65 is phosphorylated at serine 536 after 5 minutes of TNF α treatment even in the presence of PGC-1 α and PGC-1 β (Fig. 7B and 7C) implying the possibility that not only an altered kinase profile but also activity of a protein phosphatase might be involved in the PGC-1-mediated modulation of NF- κ B phosphorylation. We therefore tested whether pharmacological inhibition of protein phosphatase 2A (PP2A) and PP1, two enzymes that dephosphorylate p65 (37), by okadaic acid abolishes the repression of p65 phosphorylation mediated by PGC-1 α and PGC-1 β in muscle cells. As expected, okadaic acid powerfully stabilized phosphorylation of p65 at serine 536 (Fig. 7D and 7F). Strikingly however, PGC-1 α and PGC-1 β overexpression (Fig. 7D and 7E) still reduced p65 phosphorylation, even in okadaic acid-treated cells (Fig. 7D and 7F). Thus, while PP1 and PP2A clearly affect the serine 536 phosphorylation of p65 in our experimental context, these two phosphatases are most likely not involved in the modulation of p65 phosphorylation by the PGC-1s.

Since the phosphorylation status of p65 affects its affinity to cofactors (38), we also determined the expression levels of the coactivator CBP and corepressors NCoR and SMRT. Of those, only SMRT transcript levels were significantly increased by PGC-1 α (Fig 8A and 8B). A well-described mechanism of

inhibition of inflammatory gene expression is mediated by nuclear receptors and termed transrepression (39). We evaluated the gene expression of potential candidates for transrepression and found PPAR α to be strongly induced by both PGC-1 α and PGC-1 β in non-stimulated and TNF α -treated muscle cells similar to previous data (40) (Fig 8A and 8B). PPAR α therefore likely contributes to the PGC-1-dependent reduction in NF- κ B transcriptional activity by impairing the exchange of corepressors for coactivators that is necessary to effectively initiate transcription. In fact, inhibition of PPAR α recovered expression of IL-6 and TNF α in the presence PGC-1 β (Suppl. Fig 5).

DISCUSSION

With an aging population and an increasingly sedentary lifestyle, chronic diseases are on the rise. Obesity and its comorbidities but also some cancers and neurodegeneration have been associated with local and systemic inflammation that worsens disease progression, while exercise has beneficial effects in many of these disorders and even acts preventive (41). PGC-1 α is a major molecular mediator of exercise in skeletal muscle and its loss not only disturbs metabolic processes but also evokes a local and systemic inflammation (24). In the present report we thus tested the idea that PGC-1 coactivators have anti-inflammatory properties.

Indeed, we confirmed such properties as PGC-1 α and PGC-1 β were able to diminish the increase in pro-inflammatory cytokines elicited by different inflammatory stimuli such as TNF α , TLR agonists and saturated FFAs. We identified the NF- κ B pathway as main target of PGC-1-dependent repression. These results are complemented by *in vivo* findings of Brault and coworkers who showed that NF- κ B reporter activity decreases after electroporation of tibialis anterior muscle with PGC-1 α / PGC-1 β in the context of anti-atrophic effects of PGC-1s (42). Similar conclusions were derived from experiments in human aortic smooth muscle and endothelial cells where PGC-1 α suppressed TNF α -induced VCAM-1 and MCP-1 expression that contribute to inflammation in atherosclerosis (43).

Mechanistically, PGC-1 α and PGC-1 β lower phosphorylation of the NF- κ B family member p65 which limits its transcriptional activation potential. This is further substantiated by data from muscle-specific PGC-1 α transgenic

animals that also exhibit reduced p65 phosphorylation (44). Diametrically opposed to this report, another recent publication states that p65 phosphorylation is higher in PGC-1 α transgenic muscle before as well as after injection of TNF α (45). The exact role of PGC-1 α on muscle inflammation *in vivo* thus remains unresolved. In our cellular model, decreased p65 phosphorylation unequivocally corresponds to the loss of IKK α induction by TNF α in PGC-1 overexpressing cells and, interestingly, to diminished Akt activation. Akt-dependent phosphorylation of p65 by IKK is an important mechanism to regulate p65 transactivation potential (36,46) and thus a good candidate to mediate the reduction observed (Fig. 8C). In addition to modulation of kinase activity, the involvement of a phosphatase is suggested by sustained phosphorylation of p65 after TNF α treatment for 5 minutes even in the presence of PGC-1 α and PGC-1 β . As okadaic acid treatment did not abrogate differences between conditions, PP2A and PP1 are however unlikely to account for this effect. Therefore, further experiments are needed to determine the contribution of different phosphatases in this setting.

The transactivation potential of p65 is controlled by its phosphorylation status as it defines the affinity for cofactors important in suppressing or stimulating transcription of target genes (38). Decreased phosphorylation thus favors interaction with corepressors such as SMRT that decreases p65/p50 transactivation (47). Interestingly, SMRT levels are slightly induced by PGC-1 α and accordingly could contribute to lower cytokine expression. Stabilization of corepressor complexes on DNA-bound p65 is also fostered by nuclear receptor-mediated transrepression (39). For example, PPAR α is able to exert anti-inflammatory action by ligand-dependent and -independent transrepressive mechanisms (48,49). We found a marked induction of PPAR α by PGC-1 α and PGC-1 β , which presumably also contributes to negative regulation of pro-inflammatory cytokines by transrepression (Fig. 8C). This claim is substantiated by the reversal of the repressive PGC-1 β effects on pro-inflammatory cytokine expression when PPAR α was inhibited. PPAR α regulates lipid metabolism and FFAs are able to serve as ligands for PPAR α (50). Thus, the very pronounced suppressive effect of the PGC-1s on inflammatory gene expression observed after FFA treatment might reflect an additional ligand-dependent activation of

PPAR α leading to an even stronger transrepression in that experimental context.

Besides the reduction in p65 phosphorylation that is exerted by both PGC-1 α and PGC-1 β , only the latter was further found to repress p65 and p50 transcription in the basal state and accordingly the ability of these proteins to bind to DNA response elements. This offers an attractive explanation for the very low cytokine levels observed in the presence of PGC-1 β . The NF- κ B family members RelB and c-Rel were also suppressed transcriptionally in the basal state which puts PGC-1 β in the position of a broader anti-inflammatory factor in skeletal muscle. Such anti-inflammatory potential was previously described only in macrophages, where PGC-1 β is essential in alternative activation and ROS production (27,28). In contrast, PGC-1 α does not alter expression of the classically activated / canonical NF- κ B isoforms p65 and p50. However, PGC-1 α overexpression induces transcript and protein levels of the alternative isoforms p100/p52 and RelB. This indicates a switch towards noncanonical / alternative NF- κ B signaling. A recent publication outlined that alternative signaling via IKK α and RelB induces an oxidative phenotype in muscle driven by PGC-1 β (51). Furthermore, activation of c-Rel and p50 was suggested to play a role in disuse atrophy (52). Finally, canonical p65 activation was linked to mitochondrial biogenesis in mouse embryonic fibroblasts and liver cells (53,54). All of these findings together with our data suggest a reciprocal, functional link between NF- κ B signaling and oxidative metabolism. A potential induction of the alternative NF- κ B pathway by PGC-1 α in muscle therefore warrants further investigations.

Strikingly, inflammatory gene transcription was often selectively regulated by the two PGC-1 coactivators depending on the stimulus and the specific gene. These findings argue against a general repressive effect of PGC-1 α and PGC-1 β on tissue inflammation in muscle but rather indicate a specific, fine-tuned effect of these coactivators on NF- κ B target genes. For example, while TLR1/2-induced cytokine production was suppressed by both PGC-1 α and PGC-1 β , only PGC-1 α was able to block this production after treatment of the cells with a TLR4-specific ligand. Moreover, TLR6/2-induced TNF α expression was also diminished by PGC-1 α , which was however not the case for

TLR6/2-induced expression of IL-6. This probably reflects the activation of pathways other than NF- κ B downstream of TLRs that are not subject to regulation by PGC-1 or targeted by only one of the PGC-1 isoforms, respectively. Interestingly, while neither PGC-1 α nor PGC-1 β affected expression of TNF receptor 1 (TNFR1) (Suppl. Fig. 6A and 6B) both coactivators lowered mRNA levels of TLR1, TLR4 and TLR6 (Suppl. Fig. 6C and 6D). This downregulation might presumably contribute to the repressive effects observed with some TLR agonists. The expression pattern of TLR1, TLR4 and TLR6 (Suppl. Fig. 6C and 6D) further resembles genes in cluster 2 of the microarray, confirming a broader influence of PGC-1s on inflammatory genes. Moreover, the selectivity of the PGC-1s most likely reflects the distinct expression pattern as well as the distinct functions of PGC-1 α and PGC-1 β in the regulation of skeletal muscle physiology that will have to be further dissected in future experiments. In any case however, the concept that PGC-1s selectively and specifically repress inflammatory processes and thereby avoid the harmful consequences of broad and general cytokine suppression is compelling. Once understood in greater detail, the therapeutic potential of targeting PGC-1s to modulate specific inflammatory responses in muscle would be immense. Obviously, patients suffering from obesity and type 2 diabetes would profit from lower systemic inflammation mediated by rectified gene expression of PGC-1 α and PGC-1 β in skeletal muscle. Intriguingly however, muscle disorders like cachexia, muscular dystrophies, disuse atrophy or inflammatory myopathy also involve an inflammatory component with activation of the NF- κ B pathway (55-58). In fact, chronic stimulation of the classical NF- κ B pathway in muscle is sufficient to induce muscle wasting (5). Accordingly, mice heterozygous for p65 or harboring a genetic ablation of the IKK β gene in the *mdx* background, a model for Duchenne muscular dystrophy, have improved pathology (59). Strikingly, ectopic elevation of PGC-1 α in animal models for some of these diseases resulted in an amelioration of fiber damage and muscle functionality, e.g. in Duchenne muscular dystrophy, sarcopenia, a mitochondrial myopathy and denervation-induced fiber atrophy (20,21,44,60). Our data now suggest that at least part of the therapeutic effect of PGC-1 α in these disease paradigms might stem from the anti-

inflammatory effect. It is, thus, tempting to speculate that elevating PGC-1 α and / or PGC-1 β in muscle would also be beneficial in other conditions of muscle wasting by limiting the detrimental inflammatory component of the disease. In fact, muscle adaptation to endurance training that correlate with increased PGC-1 α expression includes an increased resistance against fiber damage, tissue inflammation and, as a consequence, decreased exercise-induced muscle soreness. In contrast to PGC-1 α , the

implications of the repressive effect of PGC-1 β on inflammatory gene expression remain less obvious until the physiological context of PGC-1 β regulation and the function of this coactivator in muscle tissue have been more clearly delineated. Nevertheless, by virtue of their ability to reduce NF- κ B activation, the PGC-1 coactivators are promising targets to antagonize inflammatory reactions in skeletal muscle associated with a large number of diseases.

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FOOTNOTES

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⁵The abbreviations used are: PGC-1 α/β , peroxisome proliferator-activated receptor γ coactivator 1 α/β ; NF- κ B, nuclear factor κ B; IL-6, interleukin 6; TNF α , tumor necrosis factor α ; TLR, toll-like receptor; FFAs, free fatty acids; I κ B, inhibitor of NF- κ B; IKK, I κ B kinase; MIP-1 α (CCL3), macrophage inflammatory protein-1 α ; BMI, body mass index; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; CBP, cyclic AMP-responsive element-binding protein(CREB)-binding protein; NcoR, nuclear receptor corepressor 1; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; PPAR α , peroxisome proliferator-activated receptor α ; OA, okadaic acid

Accession Numbers: Microarray data have been deposited in the ArrayExpress under accession number A-MEXP-1502 (array design) and E-MEXP-3676 (experimental data).

FIGURE LEGENDS

FIGURE 1. PGC-1 α and PGC-1 β suppress TNF α -induced pro-inflammatory cytokines. **A-D**, Differentiated C2C12 myotubes overexpressing PGC-1 α and GFP (Panels A and C) or PGC-1 β and GFP (Panels B and D) were treated with TNF α for 2h. Expression of pro-inflammatory cytokines was determined by real-time PCR (Panels A and B) and release of IL-6 into the medium quantified by ELISA (Panels C and D). Values represent the mean of at least 3 independent experiments \pm SEM. ## $P \leq 0.01$ GFP TNF versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1 α/β versus GFP.

FIGURE 2. PGC-1 α and PGC-1 β differentially suppress TLR agonist-induced pro-inflammatory cytokines. **A**, Differentiated C2C12 myotubes were tested for mRNA expression of TLR1-9. Expression values are arbitrarily normalized to those of TLR1. **B**, C2C12 cells were differentiated and treated with different TLR agonists for 2h. Expression of IL-6 and TNF α was assessed by real-time PCR. **C**, C2C12 cells were transfected with an NF- κ B reporter construct (or a mutated reporter construct as control) and treated with different TLR agonists for 2h. Luciferase activity was determined and is expressed as ratio of wt to mutated reporter gene values. **D-G**, Differentiated C2C12 myotubes overexpressing PGC-1 α and GFP (Panels D and E) or PGC-1 β and GFP (Panels F and G)

were treated with agonists for TLR1/2, TLR4, TLR6/2 and TLR3 for 2h. Expression of pro-inflammatory cytokines was determined by real-time PCR (Panels D and F) and release of IL-6 into the medium quantified by ELISA (Panels E and G). Values represent the mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ TLR agonist treated versus Ctrl (A, B, D-G) or TLR expression versus TLR1 (C), * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1 α/β versus GFP.

FIGURE 3. *PGC-1 α and PGC-1 β differentially suppress fatty acid-induced pro-inflammatory cytokines.* **A, B,** Differentiated C2C12 myotubes overexpressing PGC-1 α and GFP (Panel A), or PGC-1 β and GFP (Panel B) were treated with different fatty acids (P=palmitic acid, O=oleic acid, M=myristic acid, S=stearic acid, L=linoleic acid, E=elaidic acid) for 16h. Expression of pro-inflammatory cytokines was determined by real-time PCR. Values represent the mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ GFP FFA versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1 α/β versus GFP.

FIGURE 4. *Inflammatory pathways and NF-κB activity are suppressed by PGC-1s.* Differentiated C2C12 myotubes overexpressing PGC-1 α , PGC-1 β and GFP were treated with TNF α for 2h and subsequently subjected to a customized microarray analysis. **A,** Significantly regulated genes ($P \leq 0.01$) were clustered and are depicted as heat map. **B,** KEGG pathways enriched within each cluster (adjusted p-value ≤ 0.05) are shown, the dotted line indicates an adjusted p-value of 0.01. (Abbreviations: cytok. = cytokine, pw = pathway, r. = receptor). **C,** Motifs overrepresented in the promoters of each cluster were identified and their z score distribution plotted. For a complete list see **Suppl. Table 2A.** **D,** Activity plot of NF-κB (top scoring transcription factor motif) over different conditions as predicted by MARA (Motif Activity Response Analysis) and the corresponding sequence logo of the position weight matrix.

FIGURE 5. *PGC-1s suppress NF-κB transcription activation potential without changing DNA binding or affecting NF-κB expression levels.* **A,** C2C12 cells were transfected with a wildtype and a mutated NF-κB reporter construct, p65 and increasing amounts of PGC-1 α or PGC-1 β . Luciferase activity was determined after 24h and is expressed as ratio of wt reporter to mutated reporter gene expression. ## $P < 0.01$ PGC-1 versus Ctrl. **B-E,** Differentiated C2C12 myotubes overexpressing PGC-1 α and GFP (Panels B and D) or PGC-1 β and GFP (Panels C and E) were treated with TNF α for 2h. **B, C,** Expression of NF-κB family members was determined by real-time PCR. **D, E,** DNA binding of NF-κB family members in nuclear extracts was measured by TransAM. Values represent the mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ GFP TNF versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1 α/β versus GFP.

FIGURE 6. *PGC-1s diminish p65 phosphorylation at serine 536.* Differentiated C2C12 myotubes overexpressing PGC-1 α , PGC-1 β and GFP were treated with TNF α for 2h. **A,** Protein abundance of NF-κB family members, pathway components, phospho-p65 (ser536), PGC-1 α , PGC-1 β and α -Tubulin was assessed by immunoblotting. **B,** Quantification of phospho-p65 (ser536) and total p65 protein levels, normalized to α -Tubulin. Values represent the mean of at least 3 independent experiments +SEM. # $P \leq 0.05$, ## $P \leq 0.01$ GFP TNF versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1 α/β versus GFP.

FIGURE 7. *Dephosphorylation of p65 is a potential molecular mechanism for diminished cytokine expression.* **A,** Differentiated C2C12 myotubes overexpressing PGC-1 α , PGC-1 β and GFP were

treated with TNF α for 2h. Protein levels of Akt and phospho-Akt (ser473) were determined by immunoblotting. **B**, Differentiated C2C12 myotubes overexpressing PGC-1 α , PGC-1 β and GFP were treated with TNF α for 5 minutes and 2h, respectively. Protein abundance of Phospho-p65 (ser536) and total p65 was assessed by immunoblotting. **C**, Quantification of Phospho-p65 (ser536) levels from **B**, normalized to α -Tubulin. **D**, Differentiated C2C12 myotubes overexpressing PGC-1 α , PGC-1 β and GFP were treated with TNF α for 2h in the presence or absence of okadaic acid (OA). Protein abundance of phospho-p65 (ser536), total p65, PGC-1 α and PGC-1 β was assessed by immunoblotting **E, F**, Quantification of PGC-1 α and PGC-1 β protein levels from **D**, normalized to α -Tubulin (Panel **E**). Quantification of Phospho-p65 (ser536) protein levels from **D**, normalized to α -Tubulin (Panel **F**). Values represent the mean of at least 3 independent experiments \pm SEM. # $P \leq 0.05$, ## $P \leq 0.01$ GFP OA versus GFP Ctrl, * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1 α/β versus GFP.

FIGURE 8. *Transrepression of p65 is a potential molecular mechanism for diminished cytokine expression.* **A, B**, Differentiated C2C12 myotubes overexpressing PGC-1 α and GFP (Panel **A**) or PGC-1 β and GFP (Panel **B**) were treated with TNF α for 2h. Expression of coactivator and corepressor proteins, and of nuclear receptor PPAR α was determined by real-time PCR. * $P \leq 0.05$, ** $P \leq 0.01$ PGC-1 α/β versus GFP. **C**, Proposed model of PGC-1 α/β interference with NF- κ B signaling. Gene-, activator- and PGC-1-specific repression of NF- κ B target genes is mediated by reduced phosphorylation of p65, transcriptional induction of PPAR α and subsequent transrepression as well as activation of an unknown protein phosphatase by both PGC-1s. Furthermore, PGC-1 β specifically inhibits p65 and p50 expression while PGC-1 α elevates members of the NF- κ B family involved in the alternative, non-canonical activation (not depicted).

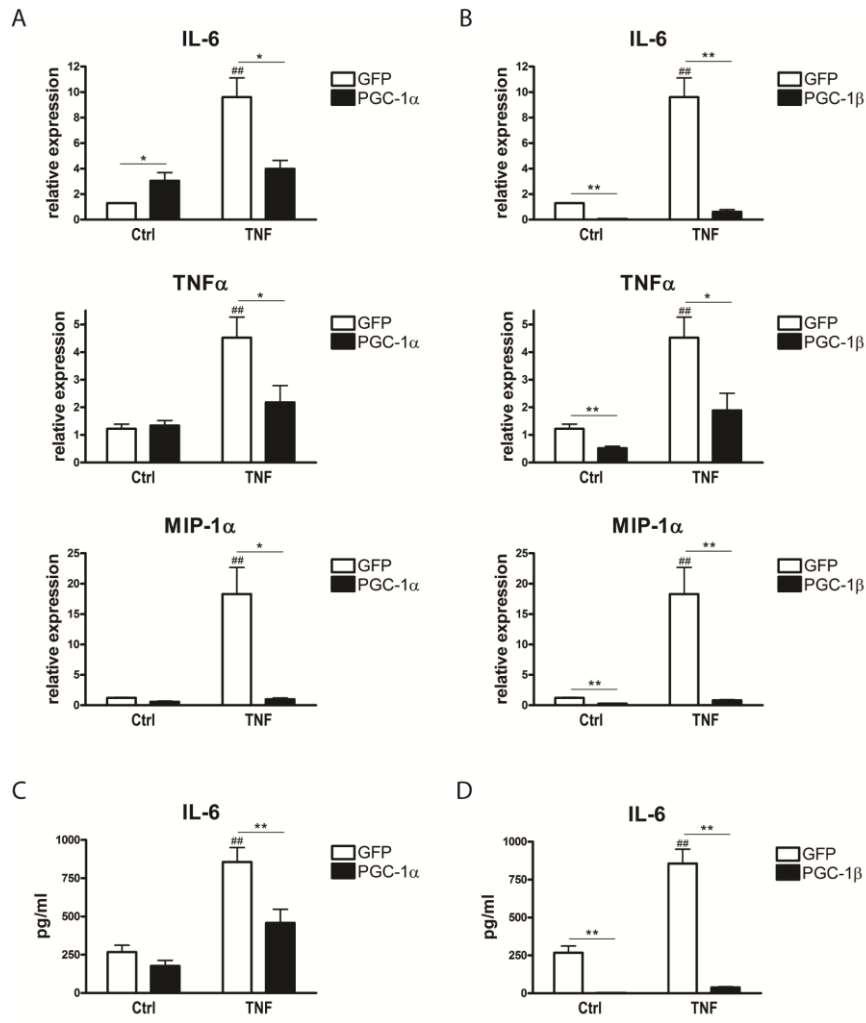


FIGURE 1

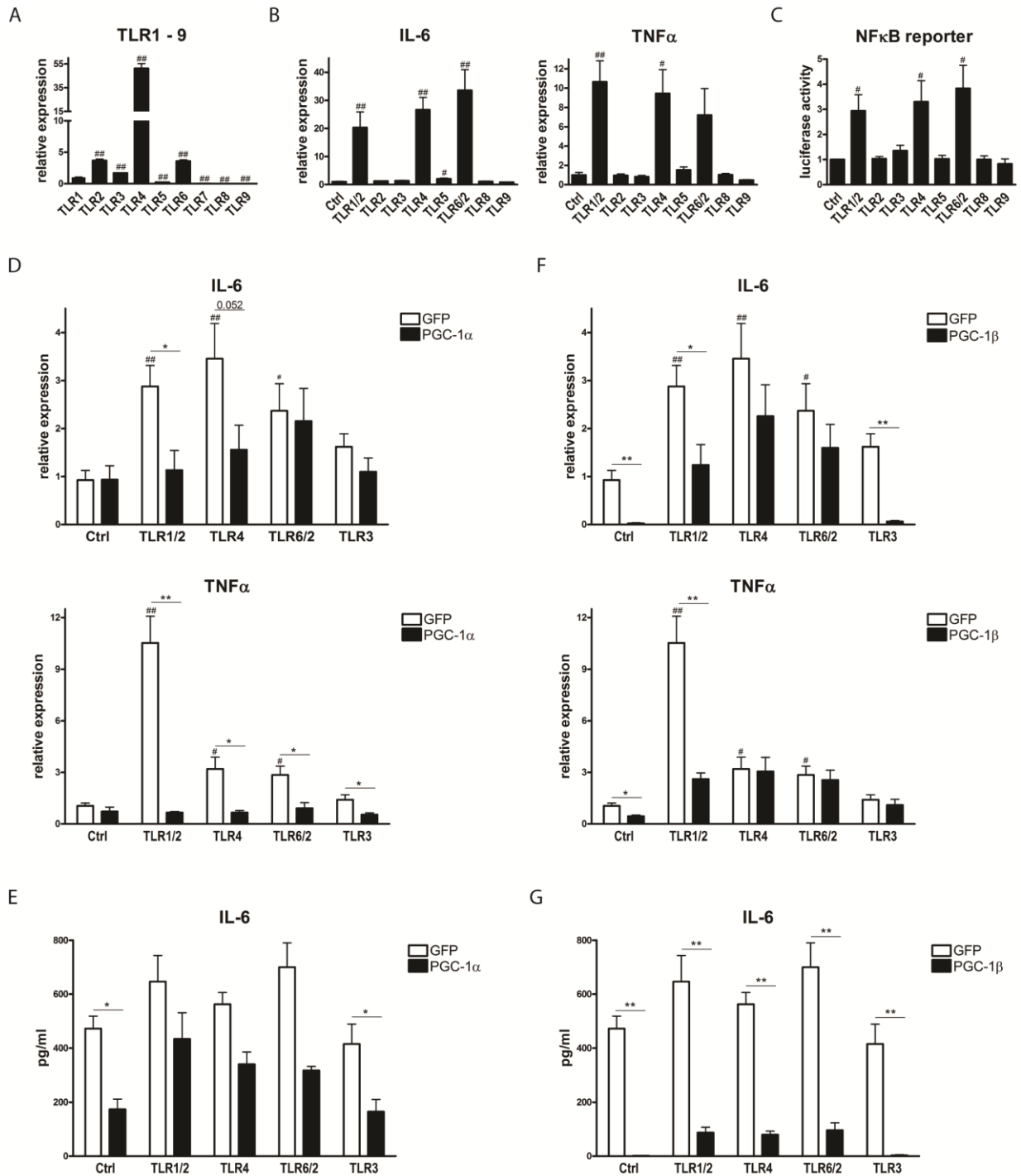


FIGURE 2

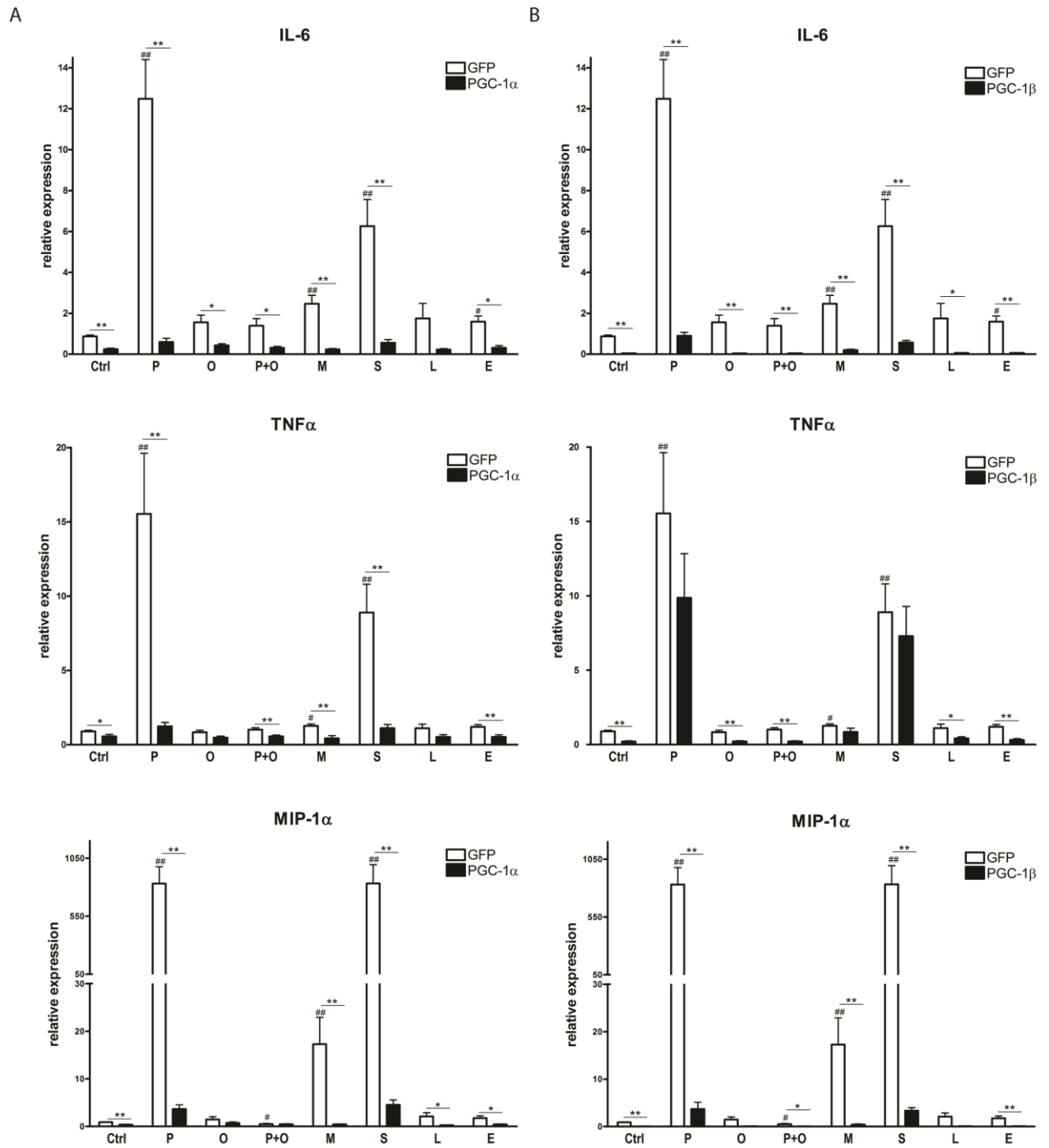


FIGURE 3

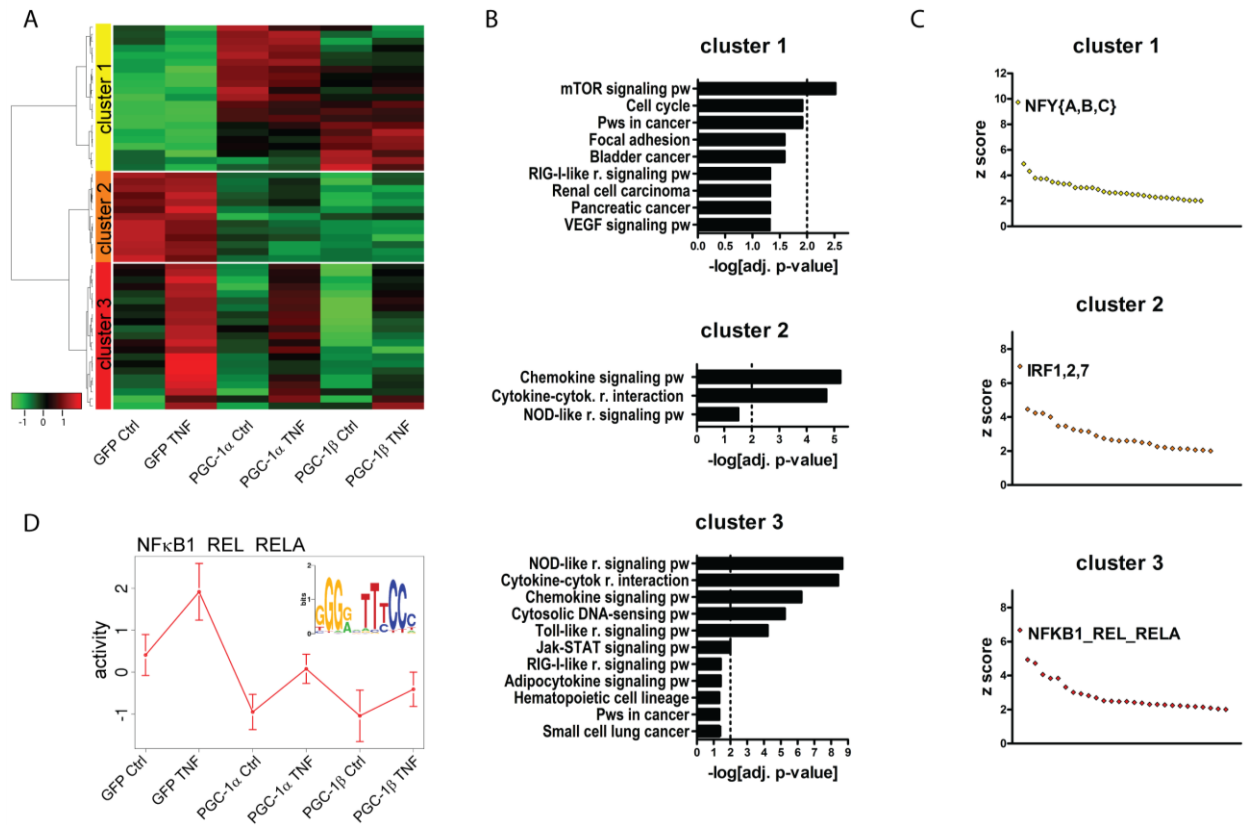


FIGURE 4

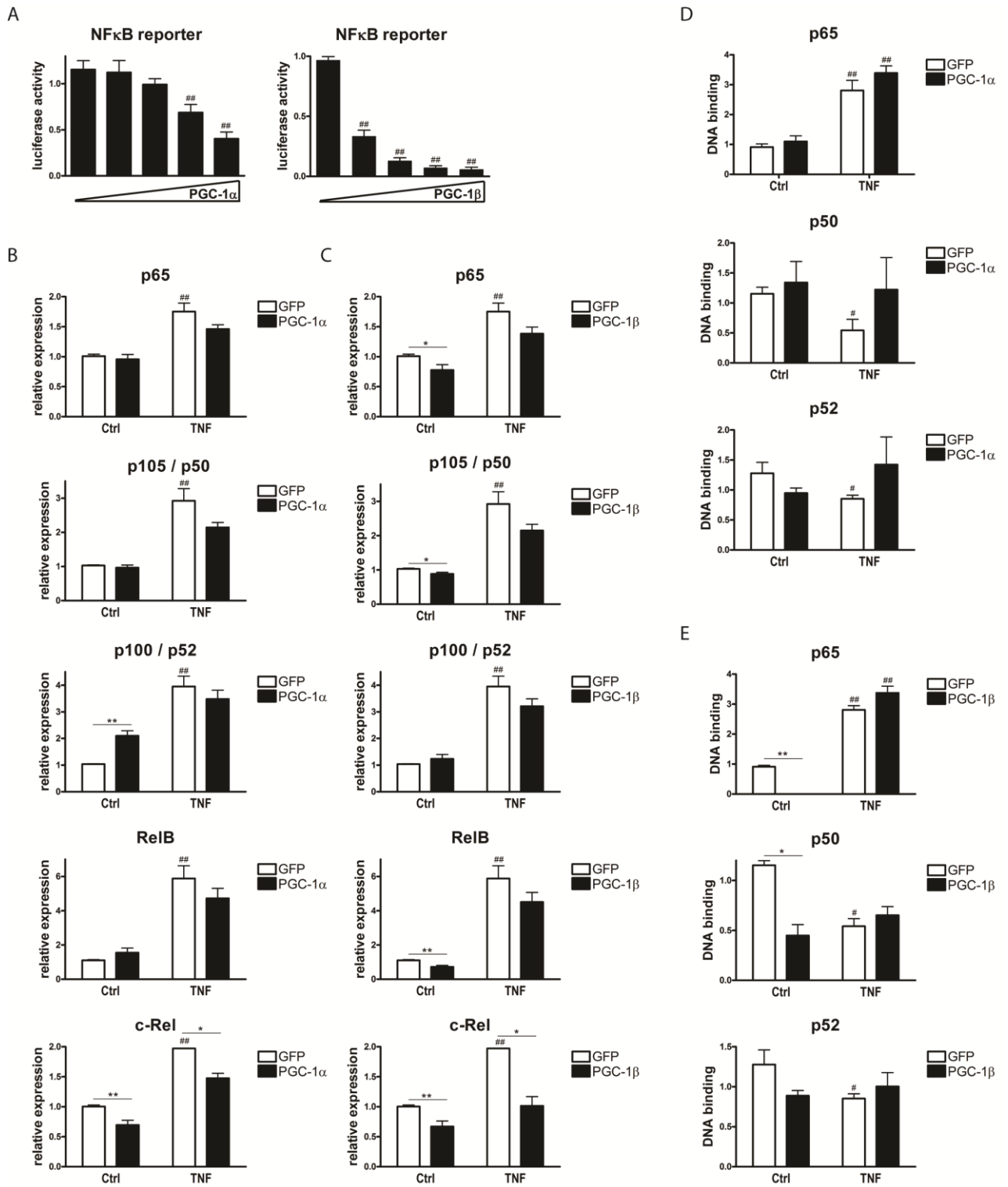


FIGURE 5

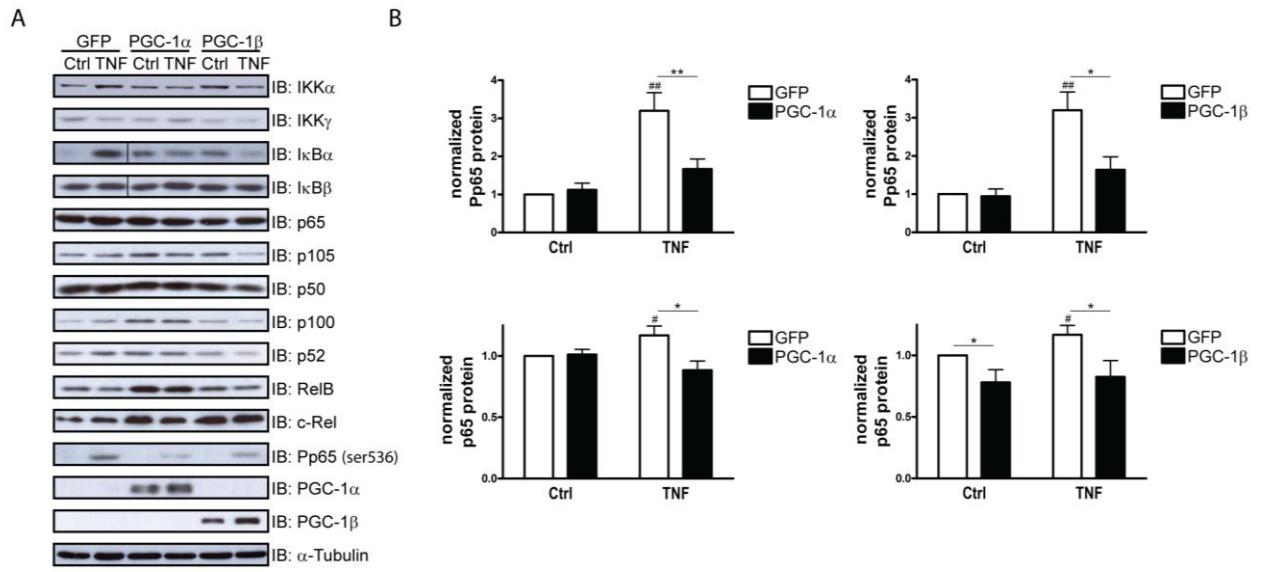


FIGURE 6

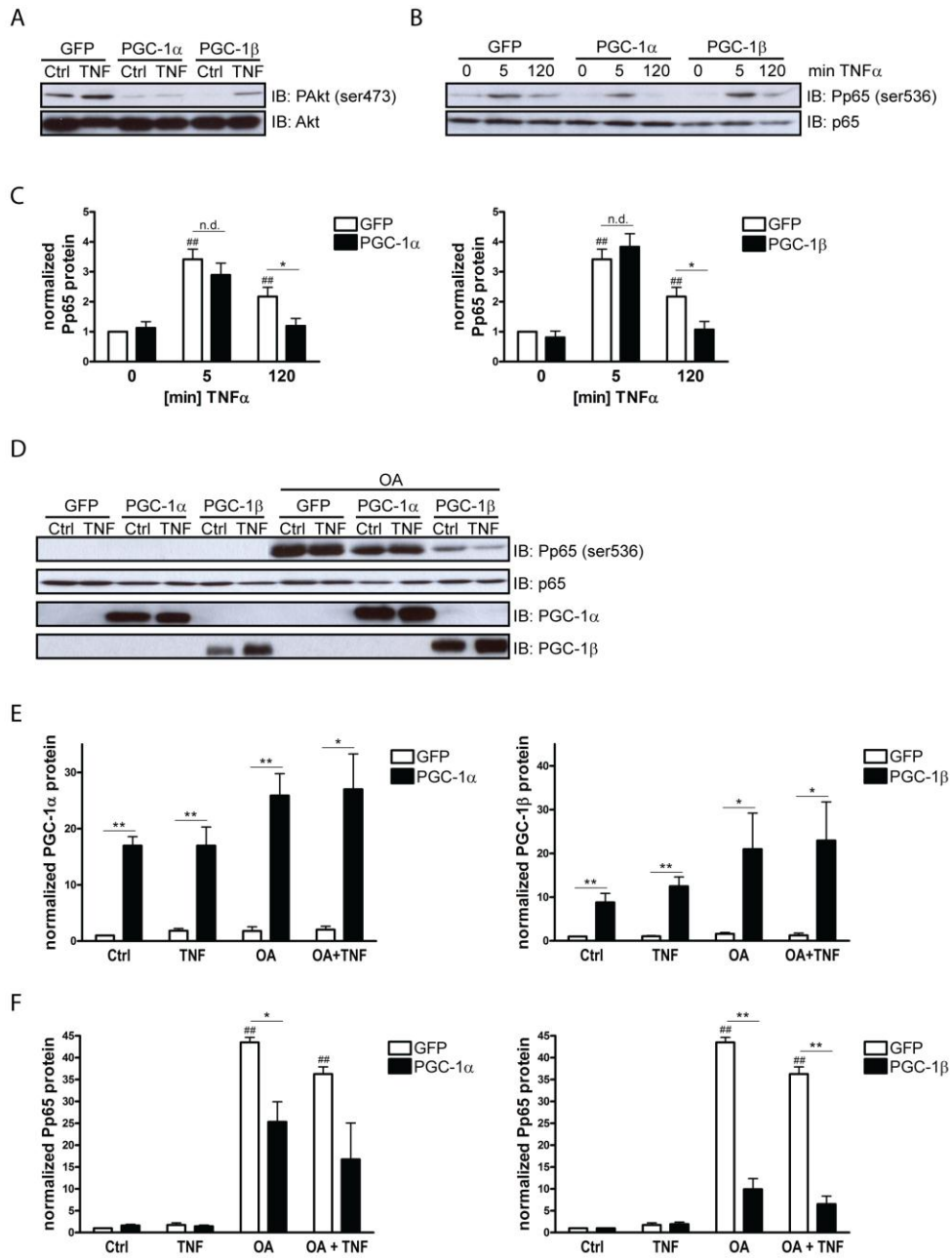


FIGURE 7

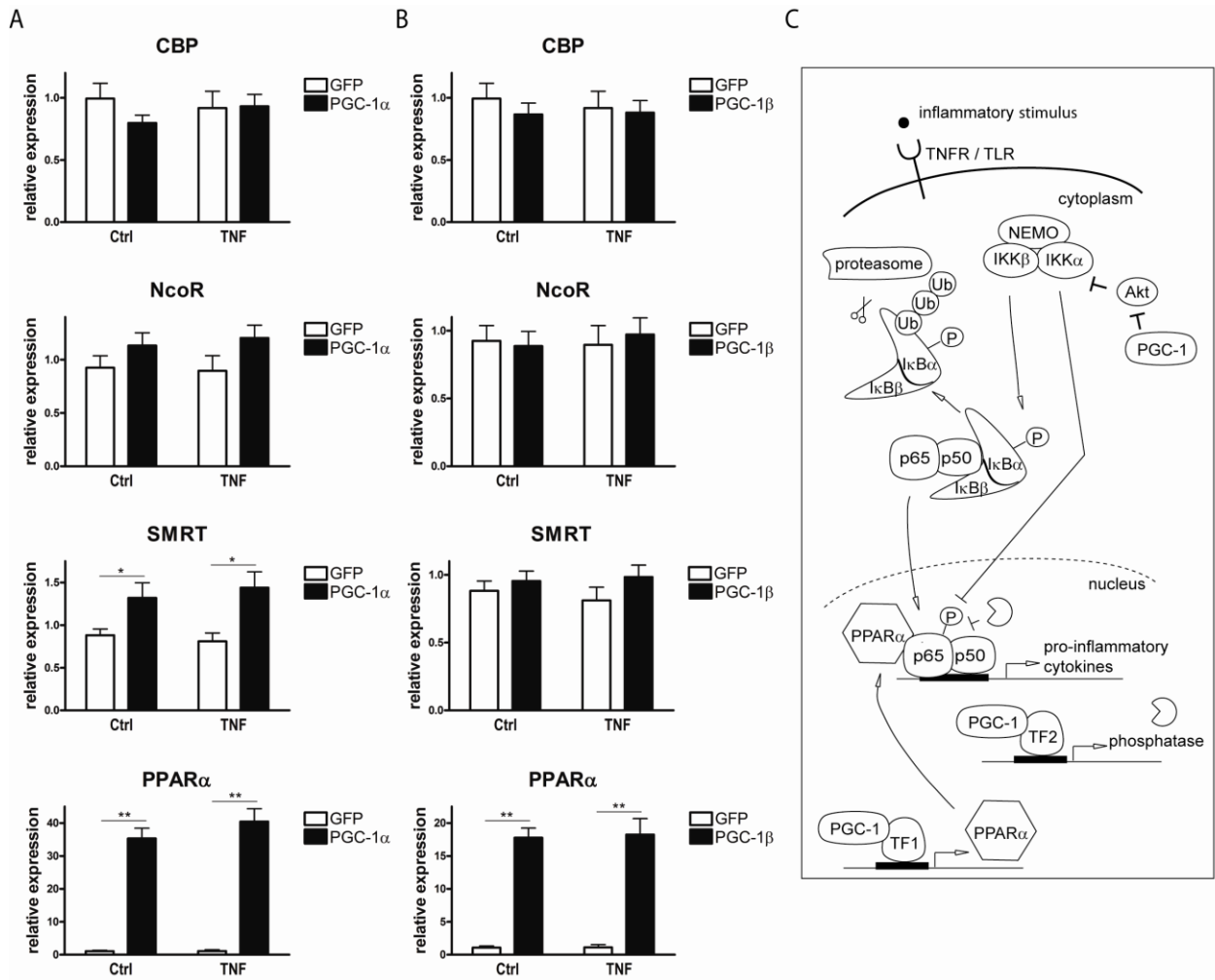


FIGURE 8