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Involvement of ZIP/p62 in the regulation of PPARα transcriptional activity by p38-MAPK

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ABSTRACT

The peroxisome proliferator-activated receptor alpha (PPARα) belongs to the nuclear receptor family and plays a central role in the regulation of lipid metabolism, glucose homeostasis and inflammatory processes. In addition to its ligand-induced activation, PPARα is regulated by phosphorylation *via* ERK-MAPK, PKA and PKC. In this study we examined the effect of p38-MAPK on PPARα transcriptional activity. In COS-7 cells, anisomycin, a p38 activator, induced a dose-dependent phosphorylation of PPARα and a 50% inhibition of its transcriptional activity. In H4IIE hepatoma cells, anisomycin-induced p38 phosphorylation decreased both endogenous and PPARα ligand-enhanced L-CPTI and ACO gene expression. Interestingly, PPARα/p38 interaction required the molecular adapter ZIP/p62. Reducing ZIP/p62 expression by siRNA, partially reversed the inhibitory effect of anisomycin on L-CPTI gene expression. In conclusion, we showed that p38 activation induced PPARα phosphorylation and inhibition of its transcriptional activity through a trimeric interaction between p38-MAPK, ZIP/p62 and PPARα.

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1. Introduction

The peroxisome proliferator-activated receptor alpha (PPARα) is a member of the superfamily of nuclear receptors. It is mainly expressed in cells with high catabolic rates of fatty acids and high peroxisome-dependent activities (hepatocytes, cardiomyocytes, proximal tubules of kidney cortex, intestinal mucosa and brown adipocytes [1]). Natural PPARα ligands are long-chain polyunsaturated fatty acids (especially 20:5n-3 and 22:6n-3 [2]) and their lipooxygenase derived metabolite, leukotriene B4 (LTB4 [3]) whereas hypolipidemic drugs from the fibrate family are specific synthetic ligands (reviewed in [4]). The regulation of target gene expression is due to ligand-binding activation of PPAR that leads to a cascade of molecular events (reviewed in [4]): PPAR heterodimerization with the 9-*cis* retinoic acid receptor (RXRα); binding of heterodimer to a specific DNA consensus sequence, PPRE (peroxisome proliferator response element); dissociation of co-repressors and recruitment of transcriptional co-activators.

In addition to their activation by ligand binding, it was demonstrated that post-translational modification of nuclear receptors could also modify their activities (reviewed in [5]). For instance, PPARα is phosphorylated by mitogen-activated protein kinases (MAPK), cAMP- and calcium-dependent protein kinases (PKA and PKC; reviewed in [6,7]). The physiological consequences of this phosphorylation depend upon the kinase and the cellular context. Indeed, activation of ERK-MAPKs increased PPARα activity in hepatoma cells [8]. Similar data were obtained with PKA [9] and PKC [10]. By contrast, activation of ERK-MAPKs down-regulated PPARα activity in cardiomyocytes [11] whereas phosphorylation of p38-MAPK increased PPARα activity in the same cells [12]. To date, no information is available whether PPARα is regulated by p38-MAPK in hepatic cells.

Thus, the aim of the present work was to investigate the effect of p38 on PPARα phosphorylation and its transcriptional activity in hepatoma cells. Interestingly, p62 protein (also called ZIP for zeta PKC interacting protein) interacts with p38 and co-localize in the nucleus in response to anisomycin, a p38 activator [13]. Therefore we investigated the contribution of such interaction in the regulation of PPARα phosphorylation in response to anisomycin.

2. Materials and methods

2.1. Cell culture

COS-7 cells were cultured in 6-well plates or 60 mm^2 dishes as previously described [14]. Cells were washed twice with PBS, transiently transfected with various constructs in the absence of fetal calf serum and then treated with anisomycin (Sigma) or vehicle.

H4IIE rat hepatoma cells were cultured in 60 mm^2 dishes as previously described [15]. H4IIE cells were washed twice with PBS and then transfected with scrambled or ZIP siRNA. Cells were cultured in the presence of anisomycin, clofibrate (Sigma; dissolved in 0.3% DMSO), or vehicle.

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2.2. Transfection experiments

COS-7 cells were transiently transfected with either pSG-BFE-Luc [16] (kindly provided by Dr. N. Latruffe, laboratoire de Biologie Cellulaire et Moléculaire, Dijon, France), pSG5-PPARα, pcDNA3myc-PPARα (described in [15]), pSG5-RXRα (a gift from Dr. R.M. Evans, The Salk Institute for Biological Studies, Howard Hughes Medical Institute, La Jolla CA USA), pcDNA3-p38-MAPK [17] (a gift from Dr. J. Han, Department of Immunology, Scripps Research Institute, La Jolla CA USA) and pcDNA6c-ZIP (described in [18]) or empty vectors, using lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After 24 h of culture, cells were treated with anisomycin, clofibrate, or vehicle. Cells were washed twice, lysed, and luciferase activity was measured on 20 μl of supernatant using the luciferase assay system (Promega). The protein amount was measured using the Bradford method (Bio-Rad) for normalization.

2.3. Small interfering RNA preparation and transfection protocol

21-nucleotide RNA with 3′-dTdT overhangs were synthesized in the "ready to use" option, in 2′-deprotected, desalted and duplexed form. The siRNA sequence targeting rat ZIP (GenBank accession number. Y08355) was from position 1051 relative to the start codon. The AA-N19 mRNA targets were 5′-GAACUCCAGUCUCUACAGA-3′ for ZIP siRNA (Dharmacon Research Lafayette, CO) and 5′-ACGACGAGCAUCUACUACU-3′ for scrambled siRNA (Eurogentec).

H4IIE cells were transiently transfected with siRNA duplexes as described by Boussif et al. [19]. Briefly, H4IIE cells at 70–80% confluency were incubated in free serum medium with either ZIP, or scrambled siRNAs and polyethylenimine (PEI). Transfection was accomplished by dilution of PEI and siRNA in 50 μl of NaCl (150 mM). In order to increase the efficiency of transfection, 30 pfu/cell of Ad-RSV-nlsLacZ (Rous sarcoma virus promoter driving the *nlsLacZ* gene) was added simultaneously [20]. After transfection (5 h), the medium was changed and cells were treated 20 h later with anisomycin, or vehicle.

2.4. mRNA extraction and expression analysis by real-time quantitative PCR

Total RNAs were extracted using the RNeasy Kit (Qiagen) and 500 ng was reverse-transcribed. RT-qPCR was performed with the LightCycler instrument (Roche Molecular Biochemicals) using SYBr Green I as previously described [21]. The following oligonucleotides primers (Invitrogen) were used: Cyclophilin gene, as an invariant control (GenBank accession no. M19533) 5′-ATGGCACTGGTGGCAAGTCC-3′ and 5′-TTGCCATTCCTGGACCCAAA-3′; L-CPTI gene (GenBank accession no. LO7736) 5′-TCTTGCAGTCGACTCACCTT-3′ and 5′-TCCACAGGACACATAGTCAGG-3′; ACO gene (GenBank accession no. J02752) 5′-GTTCTTGGCCGCTATGATG-3′ and 5′-GGCTTCAAGTGCTTGTGGTA-3′; and ZIP gene (GenBank accession no. Y08355) 5′-GGACCCACAGGGCTGAAGGAAG-3′ and 5′-CTGGTGAGCCAGCCGCCTTCAT-3′.

2.5. Western blotting, nuclear extraction, immunoprecipitation assays and Pro-Q Diamond phosphoprotein analysis

Protein extracts were prepared as previously described [15]. Briefly, cell lysates were then solubilized at 4 °C in lysis buffer (20 mM Tris–HCL, pH 7.5, 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) with gentle rotation at 4 °C for 30 min. Cell lysates were then cleared by a 15,000 g centrifugation at 4 °C for 15 min.

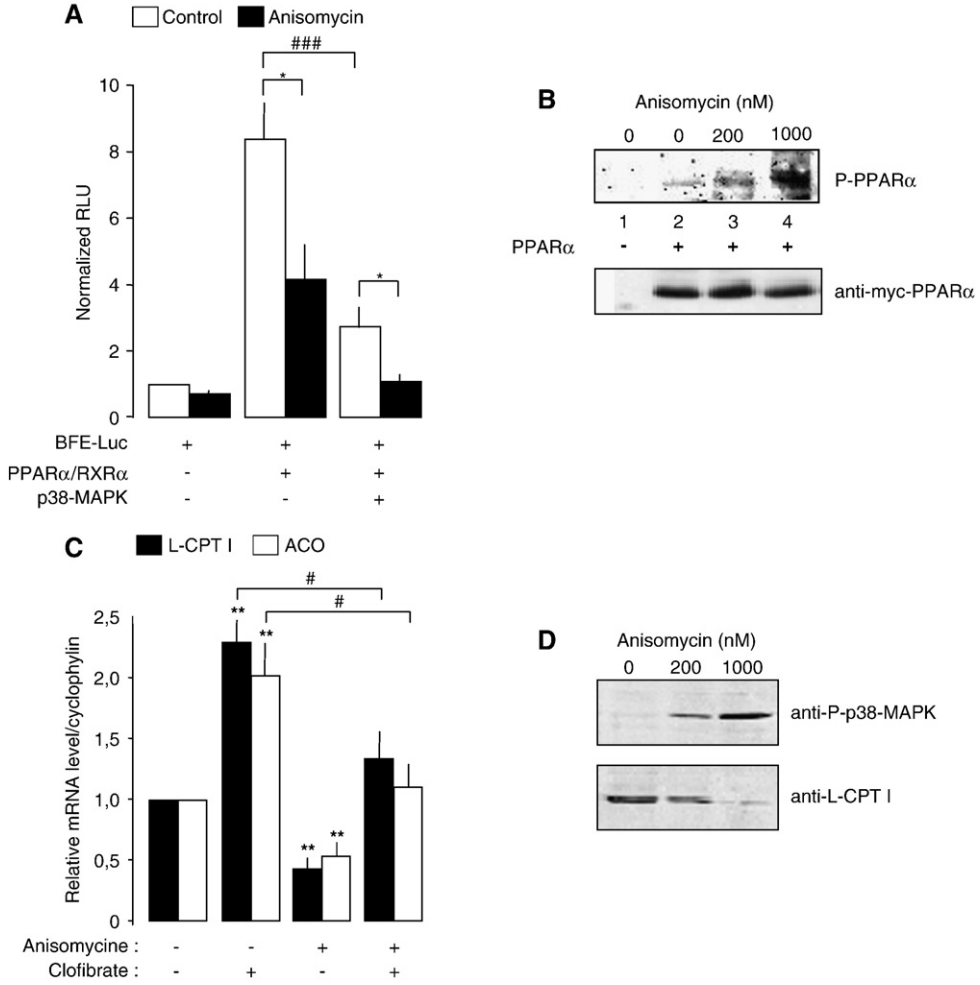

Fig. 1. Effect of p38 activation on PPARα transcriptional activity. A. COS-7 cells were transiently transfected with 500 ng of BFE-Luc reporter gene and 100 ng of the indicated vectors. 24 h after transfection, anisomycin (200 nM) was added for 4 h. Relative Luciferase Units (RLU) were normalized to BFE-Luc under control condition (1.0). Results are means ± S.E.M. from 5 independent experiments performed in triplicate and normalized to the protein amount. *$p<0.05$ when compared to control and $^{\#\#\#}p<0.0001$ when compared to PPARα alone. B. COS-7 cells were transiently transfected with 10 μg of pcDNA3-myc-PPARα or empty vectors. 24 h after transfection, anisomycin was added for 1 h. Cell extracts were immunoprecipitated with anti-myc-PPARα antibody. Immunoprecipitates were separated by SDS-PAGE and phosphoproteins were detected as described in the Material and methods section. Blots and gels are representative of 3 independent experiments. C. H4IIE cells were cultured for 6 h in the absence or presence of 200 nM anisomycin and/or 0.3 mM clofibrate. L-CPT I and ACO mRNA levels were determined by RT-qPCR and normalized to cyclophylin mRNA levels, the reference value (1.0) being mRNA levels under control condition. Results are means ± S.E.M. from 4 independent experiments. **$p<0.005$ when compared to control and $^{\#}p<0.05$ when compared to anisomycin. D. H4IIE cells were cultured for 6 h in the presence of increasing concentrations of anisomycin. Blots are representative of 3 independent experiments.

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