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Estrogen-related receptor α is essential for the expression of antioxidant protection genes and mitochondrial function

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Abstract

Estrogen-related receptor α (ERRα) is an important mediator of mitochondrial biogenesis and function. To investigate the transcriptional network controlling these phenomena, we investigated mitochondrial gene expression in embryonic fibroblasts isolated from ERRα null mice. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) stimulated mitochondrial gene expression program in control cells, but not in the ERRα null cells. Interestingly, the induction of levels of mitochondrial oxidative stress protection genes in response to increased PGC-1α levels was dependent on ERRα. Furthermore, we found that the PGC-1α-mediated induction of estrogen-related receptor γ and nuclear respiratory factor 2 (NRF-2), was dependent on the presence of ERRα. Basal levels of NRF-2 were decreased in the absence of ERRα. The absence of ERRα resulted in a decrease in citrate synthase enzyme activity in response to PGC-1α overexpression. Our results indicate an essential role for ERRα as a key regulator of oxidative metabolism.

Keywords: Estrogen-related receptor; Mitochondrial gene expression; Peroxisome proliferator-activated receptor γ coactivator-1α; Oxidative metabolism; Type 2 diabetes; Nuclear respiratory factor 2

The members of the estrogen-related receptor (ERR; NR3B1-3) family of orphan nuclear hormone receptors, ERRα, ERRβ and ERRγ, show constitutive, ligand-independent transcriptional activity [1]. ERRα and ERRγ are expressed in tissues with high energetic demand, such as the heart, skeletal muscle and the brain, while the β isoform is prevalent during embryonic development [2]. These proteins are coactivated by the PGC-1 family of transcriptional regulators; PGC-1α is often referred to as a protein ligand for the ERRs [3]. Recent evidence shows that the ERRs partner with PGC-1α to regulate mitochondrial gene expression, thus implicating these nuclear receptors as important modulators of mitochondrial function and metabolism [4,5].

Studies in human subjects have demonstrated a strong correlation between reduced mitochondrial number/function and the incidence of type 2 diabetes [6,7]. Microarray analysis of muscle biopsies from type 2 diabetic individuals displayed a decreased expression of OXPHOS genes regulated by PGC-1α in muscle [8,9]. Studies *in vitro* and *in vivo* suggest that ERRα is necessary for the activation of mitochondrial genes as well as increased mitochondrial biogenesis in response to elevated PGC-1α levels; this

Abbreviations: ERR, estrogen-related receptor; PGC-1, peroxisome proliferator-activated receptor γ coactivator-1; WT, wildtype; NRF-2, nuclear respiratory factor 2; OXPHOS, oxidative phosphorylation; MEFs, mouse embryonic fibroblasts; COX-4, cytochrome *c* oxidase-4; UCP-3, uncoupling protein 3; CPT-1b, carnitine palmitoyl transferase 1b; SOD2, superoxide dismutase 2; TXN2, thioredoxin 2; PRDX-3, peroxiredoxin 3; PRDX-5, peroxiredoxin 5; PPAR, peroxisome proliferator-activated receptor; B2M, beta-2 microgobulin; UQCRb, ubiquinol c reductase b; ROS, reactive oxygen species.

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occurs through a direct interaction of ERRα with promoter regions of mitochondrial genes [4,10]. Additionally, NRF-2 and ERRα co-regulate the expression of each other; however, the hierarchical nature of the interaction between these transcription factors is unclear.

Although ERRα null mice do not exhibit an increased susceptibility to weight gain or lipid accumulation in response to a high fat diet, they do have an impairment in adaptive thermogenesis [10,11]. We used mouse embryonic fibroblast cells (MEFs) from these mice as a tool to demonstrate that the loss of ERRα results in a significant diminution in PGC-1α-driven mitochondrial gene expression. We find that ERRα, through its partnership with PGC-1α, is a pleiotropic regulator of mitochondrial pathways and plays a central role in controlling multiple aspects of mitochondrial metabolism.

Materials and methods

Animals. All mice used in this study were maintained on 12 h light/dark cycle and in accordance with regulations under an approved institutional Animal Care and Use Committee protocol in the Novartis animal facility. ERRα null mice were obtained from Deltagen (San Carlos, CA). The endogenous ERRα gene locus was targeted such that amino acids 92–119 in the DNA binding domain of the protein were deleted. Genotyping analysis was performed as described using the following primers. For the wildtype allele, the primers 5′-TCA TGG AAT CCT GCT CTC CCT TTC C-3′ and 5′-GTG CTC ACC CTC CTT GAG CAT GC-3′ generate a 263-base-pair band. For the knockout allele, the primers 5′-GGG TGG GAT TAG ATA AAT GCC TGC TCT-3′ and 5′-GTG CTC ACC CTC CTT GAG CAT GC-3′ generate a 447-base-pair band. The primary rabbit anti-mouse ERRα antibody for immunoblotting was kindly provided by Dr. Vincent Giguere, McGill University, Montreal, Canada [11].

MEF studies. Mouse embryonic fibroblast cells (MEFs) were isolated from ERRα null mice. Briefly, timed heterozygote matings were set up. On day 13.5 *post coitum*, pregnant females were euthanized and the embryos isolated in PBS. Each embryo was individually dissected and homogenized in PBS using 3 ml syringes and 18.5 gauge needles. These crude suspensions were then plated in 10 cm cell culture dishes. At the second passage, similar genotypes were pooled, and cryopreserved.

Adenoviral transduction and gene expression analysis. MEFs were plated in a 6-well plate at 0.5×10^6 cells per well. Cells were then transduced with adenovirus expressing either (1) GFP, (2) mouse PGC-1α, (3) human ERRα or (4) human ERRα in combination with mouse PGC-1α. For each adenovirus, MEFs were transduced at a viral titer of $12–20 \times 10^9$ viral particles per well. When using two adenoviruses in combination, the adenoviral titer was doubled, resulting in a viral titer of $24–40 \times 10^9$ viral particles per well.

Total RNA was extracted from MEFs using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis performed with Superscript III RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA). QPCR was performed using Taqman (Applied Biosystems, Foster City, CA) as described by the manufacturer. Relative mRNA expression levels were calculated comparing the level of expression of target genes to a control transcript (B2M); further, data were expressed as a fold change relative to cells transduced with adenoviral GFP. Samples were assayed in triplicate and expressed as means ± SEM of the fold change relative to the control (set at 100%).

Total RNA was analyzed using the Affymetrix whole-genome microarray, MOE430 Plus 2.0. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and cRNA synthesis and hybridizations were performed as per the manufacturer's instructions (Affymetrix, Santa Clara, CA). For each condition, the RNA from each of three wells was hybridized to individual chips. Raw expression data were normalized using the RMA procedure [12] with default settings. Gene Set Enrichment Analysis (GSEA) was performed to identify pathways that underwent coordinated modulation as previously described elsewhere [13,14]. Briefly, probe sets were sorted for differential expression as estimated by the LIMMA moderated *t*-test [15]. One thousand two hundred eighty-two gene sets were then projected onto the sorted list of probe sets and evaluated for coordinated modulation with the Wilcoxon ranked sum test. These gene sets were culled from several sources including: Jubilant/Pathart (717), Celera Panther (232), Celera Public Pathways (59), KEGG (128), and the literature [13]. Finally, the False Discovery Rate correction [16,17] was applied to all 1283 *p*-values to account for multiple hypothesis testing.

Citrate synthase activity. Citrate synthase (CS) activity was determined in MEF lysates at 72 h post-transduction as previously described [18]. Each sample was assayed in triplicate. CS activity was normalized for protein content and expressed as means ± SEM of the fold change relative to the control, which was set at 100%.

Experimental setup and data analysis. In each experiment, the transduction and analysis was performed in three independent wells of cells. Each well was assayed in triplicate for gene expression and enzyme activity. Each experiment was independently repeated a minimum of two times, to ensure reproducibility.

Data analyses were performed using Microsoft Excel and GraphPad Prism 4 software (GraphPad Software, San Diego, CA). Data are presented as means ± SEM. Statistical analysis was performed using either a Student's *t*-test for direct comparison of two groups or a one way ANOVA followed by Bonferroni or Newman–Keul's multiple comparison post tests for more than two groups. A *p*-value of <0.05 was considered to be significant.

Results and discussion

ERRα is required for PGC-1α-mediated induction of mitochondrial gene expression

Mitochondrial number and function are regulated by the transcriptional coactivator, PGC-1α. One class of transcription factors coactivated by PGC-1α are the NRFs, which are nuclear encoded proteins that control mitochondrial gene expression [19]. Bioinformatic analysis of promoters of genes upregulated by PGC-1α identified the ERRs as transcriptional regulators of the mitochondrial gene expression program [5]. These authors showed that Gabpa (NRF-2) and ERRα function in an integrated fashion to form a feed-forward loop, inducing the expression of each other as well as that of PGC-1α. Numerous approaches in cell culture systems have reported that a reduction in the expression and/or the function of ERRα, using either genetic or pharmacological tools, results in the reduction of mitochondrial gene expression. We obtained a strain of ERRα null mice from Deltagen (San Carlos, CA). These mice are healthy and viable and are indistinguishable from their control littermates on a normal chow diet (data not shown), in agreement with data reported by Luo et al. [11]. In order to investigate the transcriptional factors that cooperate with PGC-1α to control mitochondrial gene expression, we transduced wildtype and ERRα null embryonic fibroblasts from these mice with PGC-1α adenovirus. Equivalent levels of PGC-1α expression were obtained in these cells (data not shown). As previously reported, ERRα levels were increased in response