9-cis retinoic acid induces retinoid X receptor localized to the mitochondria for mediation of mitochondrial transcription

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ARTICLE INFO

Article history:
Received 22 September 2008
Available online 7 October 2008

Keywords:
9-cis retinoic acid
Mitochondrial biogenesis
Retinoid X receptor

ABSTRACT

We previously reported that 9-cis retinoic acid (RA) treatment induced an increase in mitochondrial (mt)DNA transcription. In order to extend these results, we tested various concentrations of 9-cis RA were used to treat 143B cells. Cells with low membrane potential treated with 9-cis RA showed significantly lower amounts of RXRα in mitochondria. We also found lower RXRα levels in mtDNA-depleted cells. Treating cells with 9-cis RA significantly increased expression of *ND1*, *ND6*, and *COX I* RNA. However, 9-cis RA-treatment did not appear to induce any significant changes in mtDNA copy number or mitochondrial mass. This study represents that 9-cis RA increases mtDNA transcription but not mtDNA replication, and it suggests that the effects of 9-cis RA on mitochondria are mediated by RXR localization to mitochondria. In addition, this is the first report that 9-cis RA regulation of RXR mitochondrial translocation depends on mitochondrial membrane potential and ATP.

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Mitochondria have long been known to participate in cellular energy metabolism. The contribution of mitochondria to regulation of cellular aging, apoptosis, and developmental processes has recently attracted significant attention [1–3]. Several recent studies have examined whether mitochondria play important roles in cell proliferation and differentiation, independent of their role in ATP production [4,5]. Decreased numbers of mitochondria or reductions in mtDNA transcription and translation result in mitochondrial dysfunction and altered cellular characteristics, as has been observed in various cell types. For example, MIN6 cells treated with ethidium bromide (EtBr) showed a marked decrease in the level of mtDNA and mRNAs transcribed from it as well as impaired insulin secretion in response to glucose [6]. Decreased mtDNA copy number has also been observed in renal cancer [7] and hepatocellular carcinoma [8]. In addition, Lewis [9] showed that a mutated polymerase gamma causes mtDNA depletion and subsequent mitochondrial oxidative stress and cardiomyopathy. Furthermore, several studies have established that many severe diseases are induced by rearrangements of the mitochondrial genome [10,11]. Those reports highlight the need for precise regulation of mitochondrial activity for normal cellular physiology.

Nuclear receptors comprise a superfamily of structurally related transcription factors that regulate a variety of cellular processes. Receptors for glucocorticoids [12], thyroid hormones [13], and the retinoid receptor X alpha (RXRα) [14] have been found in mitochondria. Retinoid receptors, retinoid X receptor (RXR), and retinoic acid receptors (RARs) are members of the steroid/thyroid hormone receptor superfamily. They function as ligand-dependent transcription factors, and are involved in mediating retinoid effects [15,16]. Several studies support the hypothesis that nuclear receptors affect mitochondrial transcription by directly influencing hormones that regulate the mitochondrial transcription machinery [17–20].

In a recent study, we found that RXRα is located not only in the nucleus, but also in the mitochondrial fraction following ligand 9-cis RA-treatment. 9-cis RA significantly induces RXRα expression and translocation into mitochondria. These results prompted us to examine how RXRα translocation into mitochondria contributes to mitochondrial activity.

Materials and methods

Cell culture and drug treatment. Osteosarcoma 143B thymidine kinase-negative (143B TK-) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown for 3 days at 37 °C in a humidified atmosphere with 5% CO_2 before the start of experiments. Cells depleted of mtDNA were created using siRNA targeting the mitochondrial transcription factor A (Tfam) gene, as previously described [21]. This siRNA down-regulates mtDNA transcription and replication. 9-cis RA was

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purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in EtOH according to the literature and the manufacturer's instructions. Cells were seeded in 96-well culture plates with various concentrations of 9-cis RA. Control cells were cultured in an equivalent concentration of carrier (0.1% EtOH), and no significant difference was found between cells grown in medium with or without the carrier.

Determination of mtDNA copy number and mitochondrial mass. Levels of mtDNA were determined by amplification of the *ND1* gene (5′-GGAGTAATCCAGGTCGGT-3′ and 5′-TGGGTACAATGA GGAGTAGG-3′) and the *GAPDH* gene (5′-ATCAAGAAGGTGGTGAAGC -3′ and 5′-CTGTAGCCAAATTCGTTGTC-3′); the latter served as the internal standard. The polymerase chain reaction (PCR) amplification profile was as follows: 1 cycle of 94 °C for 10 min; 35 cycles of 94 °C for 1 s, 62 °C for 5 s, and 72 °C for 10 s; and 1 cycle of 65 °C for 15 s, followed by storage at 4 °C. A LightCycler PCR machine (Roche Diagnostics, Mannheim, Germany) was used to perform quantitative PCR. The DNA content of the *ND1* gene was normalized to that of the *GAPDH* gene to calculate the copy number of mtDNA.

Mitochondrial mass was measured using the fluorescent dye nonyl acridine orange (NAO) (Molecular Probes, Eugene, OR), which binds to cardiolipin in the mitochondrial inner membrane and which accumulates in mitochondria regardless of the mitochondrial membrane potential. Cells grown to 70–80% confluence were trypsinized and resuspended in 0.5 ml of phosphate-buffered saline (PBS, pH 7.4) containing 0.5 μM NAO. After incubation for 15 min at 25 °C in the dark, cells were immediately transferred to a tube for analysis in a FACS Calibur system (BD Biosciences, San Jose, CA, USA). The excitation wavelength was set at 488 nm and the intensity of emitted fluorescence of a total of 10,000 cells at 525 nm was recorded. Data acquisition and analysis were performed using Cell Quest Pro Software.

RNA extraction and real-time reverse-transcription (RT)-PCR analysis. Total RNA was extracted from cells with the Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Five micrograms of total RNA was reverse-transcribed into cDNA using oligo $(dT)_{18}$ (Protech Technology, Taipei, Taiwan) as a primer and MMLV reverse transcriptase (Epicentre Biotechnology, Madison, WI, USA). The cDNA template (5 μl) was subsequently used to amplify the different mRNAs. A LightCycler PCR machine (Roche Diagnostics) was used to perform the real-time PCR. PCR conditions were 94 °C for 5 min, followed by 45 cycles of 94 °C for 10 s, 60 °C for 5 s, and 72 °C for 8 s. Primers were designed using Primer Express (Applied Biosystems). Sequences of the oligonucleotide primers used in this study were: NADH dehydrogenase subunit 1 (ND1) (forward, CCCAACCCTCTCCCTTACA; reverse, ATTTGAGGCTCATCCCG), ND6 (forward, CCAGCCACCAC TATCATTC; reverse, GAGTTGGTAGTGTTCTACTTGT), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, GAGA GGCAATGAAAAGGTA; reverse, ACATTGTTGCATCAGCTCAGGTCT).

Western blot analysis. Total protein was extracted from harvested cells using lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 10% glycerol, and 1% Triton X-100) with protease inhibitors. The cell lysate was cleared of cell debris by centrifugation at 10,000*g* for 5 min. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins were resolved on SDS–polyacrylamide gels, and were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia, Piscataway, NJ, USA). Membranes were blocked for 1 h at 4 °C with 10% skim milk in TBST buffer (1 M Tris–HCl, 100 mM NaCl, and 1% Tween-20). Blots were probed with monoclonal antibodies (mAbs) against COX I (Molecular Probes, Eugene, OR, USA) and a polyclonal antibody against GAPDH. Blots were then incubated with the appropriate horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) antibody. Antibody-bound protein was detected using the Western blotting chemiluminescence luminol reagent (Santa Cruz, Santa Cruz, CA, USA) and exposure to film.

Results

9-cis RA induces RXRα expression and translocation into the mitochondria

To determine whether 9-cis RA-treatment increases RXRα expression and induces its translocation into mitochondria, levels of RXRα in total cellular and mitochondrial fractions was determined. 143B TK- cells were treated with 2, 5, or 10 μM 9-cis RA, and the RXRα expression was determined. We detected elevated RXRα expression in cells treated with all three of these 9-cis RA concentrations. Significantly increased RXRα expression was also observed in the mitochondrial fraction of cells treated with 9-cis RA, regardless of the concentration (Fig. 1A).

RXRα translocation into mitochondria depends on mitochondrial membrane potential

To characterize whether the mitochondrial membrane potential affects 9-cis RA-induced RXRα translocation into mitochondria, RXRα levels in carbonyl cyanide m-chlorophenylhydrazone (CCCP)-treated cells and mtDNA-depleted cells were determined. CCCP is an uncoupling reagent used to decrease the mitochondrial membrane potential. The mtDNA-depleted cells were created by decreasing mtDNA transcription and replication through siRNA knockdown of the *Tfam* gene. Significantly decreased mitochondrial membrane potential and ATP production were observed in both the CCCP-treated and the mtDNA-depleted cells (Table 1). Cells treated with 10 μM 9-cis RA together with 50 μM CCCP showed significantly decreased RXRα translocation into the mitochondria. Cells depleted of mtDNA, which contained 22 ± 18% of the normal amount of mtDNA, were also treated with 10 μM 9-cis-RA and showed reduced RXRα expression (Fig. 1B).

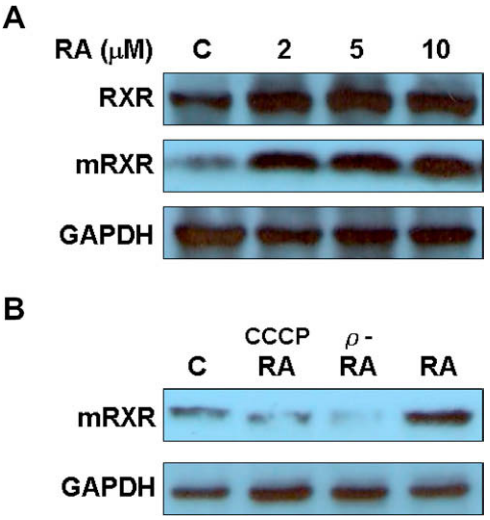

Fig. 1. 9-cis retinoic acid (RA) induces retinoid X receptor (RXR)α expression. (A) 143B TK- cells were treated with 2, 5, or 10 μM 9-cis RA for 24 h. Levels of RXRα in the total cellular and mitochondrial fraction were determined. mRXR, RXRα in the mitochondrial fraction; C, control. (B) Retinoid X receptor (RXR)α translocation into mitochondria. Levels of RXRα were determined in the mitochondrial fraction of cells that had been incubated with 10 μM 9-cis retinoic acid (RA) for 24 h; RA+CCCP, cells treated with 10 μM 9-cis RA and 50 μM CCCP; RA+ρ−, mtDNA-depleted cells treated with 10 μM 9-cis RA.

Table 1
Cellular characteristics and mitochondrial functions of the cells studied.

Cells[a]	mtDNA[b,e]	Growth[c] rate	Morphology[d]	Membrane[e] potential	ATP[e] production
143B	100	+++	R	100	100
mtDNA-depleted cells	22±18*	++	S	32±14*	42±16*
CCCP-treated cells	104±21	+	S	12±11*	8±5*

[a] 143B, 143B thymidine kinase-negative cells; mtDNA-depleted cells, cells with low copy number of mtDNA produced through siRNA treatment; CCCP-treated cells, cells with low membrane potential caused by CCCP treatment.
[b] The mtDNA copy number was determined by real-time PCR analysis.
[c] +++, the growth rate is the same as the wild-type; ++, <60% of the wild-type; +, <30% of the wild-type.
[d] R, rhombus-like; S, spindle-like.
[e] mtDNA copy number, membrane potential, and ATP production are expressed as a percentage of to the values of non-treated control 143B cells. Data are expressed as means±SD of three separate experiments.
* $p<0.05$, significantly different from control cells.

RXRα translocation into mitochondria does not affect mtDNA or mitochondrion replication

To determine whether RXRα translocation into mitochondria affects mtDNA or mitochondrial biogenesis, the mtDNA copy number and mitochondrial mass were determined. Cells depleted of mtDNA had 22±18% mtDNA of control cells. No significant differences in the mtDNA copy number (Fig. 2A) or mitochondrial mass (Fig. 2B) were observed following 9-cis RA-treatment.

Increased mtDNA transcription and translation are induced by RXRα located in mitochondria

To determine whether RXRα translocation into mitochondria affects mtDNA transcription or translation, RNA and protein expression of mtDNA-encoded genes were determined. Significantly increased expression of *ND1* and *ND6* RNA was observed at all three concentrations of 9-cis RA-treatment (Fig. 3A). In addition, 9-cis RA-induced an increase in COX I protein expression (Fig. 3B), but this effect was not observed in CCCP-treated or mtDNA-depleted cells (Fig. 3C).

Discussion

The present study found that 9-cis RA increases RXRα expression in 143B cells. Importantly, RXRα was elevated in the mitochondrial fraction (Fig. 1A). A truncated form of RXRα located in the mitochondrial matrix has been reported [22], and Casas et al. [14] identified a 44-kDa truncated form of RXRα that is cleaved by a mitochondrial calpain-like activity and translocates into mitochondria. Both RXRα and TR3 translocation into mitochondria were observed in MGC80-3 gastric cancer cells stimulated by 9-cis RA [23]. All of these results indicate that RXRα translocation into

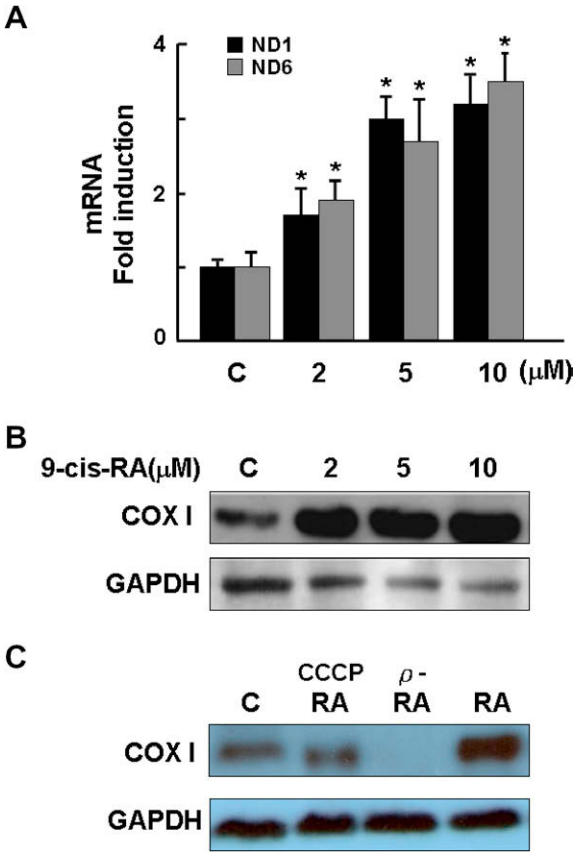

Fig. 3. Expression levels of mitochondrial (mt)RNA and protein. (A) Cells were treated with 2, 5, or 10 μM 9-cis retinoic acid (RA) for 48 h. *ND1* and *ND6* gene expression was determined using real-time RT-PCR analysis. (B) Protein levels of COX I were determined from cells treated with 2, 5, or 10 μM 9-cis RA for 48 h. (C) Protein levels of COX I were determined from cells incubated with 10 μM 9-cis RA for 24 h; RA+CCCP, cells treated with 10 μM 9-cis RA and 50 μM CCCP; RA+ρ−, mtDNA-depleted cells treated with 10 μM 9-cis-RA. Data are expressed as means±SD of three separate experiments. * $p<0.05$, significantly different from control cells.

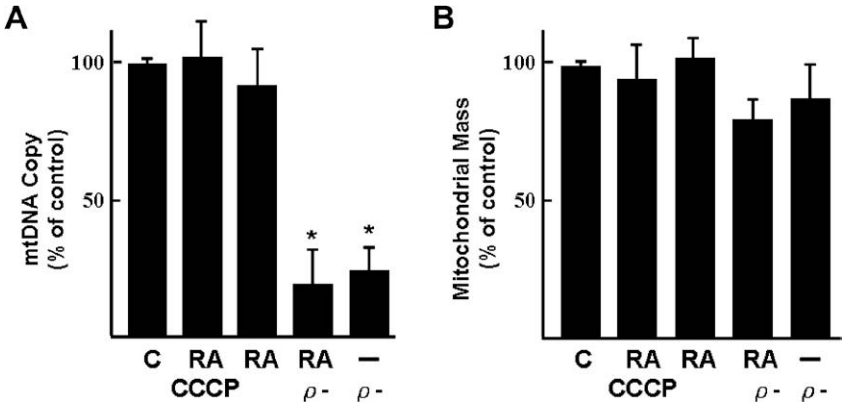

Fig. 2. Determination of the mitochondrial (mt)DNA copy number and mitochondrial mass. The mtDNA copy number and mitochondrial mass were determined from cells incubated with 10 μM 9-cis retinoic acid (RA) for 24 h; RA+CCCP, cells treated with 10 μM 9-cis RA and 50 μM CCCP; RA+ρ−, mtDNA-depleted cells treated with 10 μM 9-cis RA. * $p<0.05$, significantly different from control cells.

mitochondria depends on 9-cis RA, which is consistent with the results of our study.

To evaluate whether RXRα translocation stimulated by 9-cis RA depends on mitochondrial membrane potential, cells were treated with 9-cis RA in combination with CCCP, or they were treated with 9-cis RA in conjunction with mtDNA depletion using siRNA. In cells co-treated with 9-cis RA and CCCP, RXRα translocation into mitochondria was significantly decreased (Fig. 1B). CCCP is a mitochondrial uncoupler and is used to dissipate the mitochondrial membrane potential [24,25]. Loss of mitochondrial membrane potential following CCCP treatment prevented the translocation of RXRα into the mitochondria following treatment with 9-cis RA. Reduced RXRα translocation was also observed in mtDNA-depleted cells treated with 9-cis RA. Using carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), however, a previous study found that this uncoupling agent did not affect mitochondrial import of RXRα [14]. The high dose of CCCP and long incubation time used in this study may explain why our results differ from those of the previous work.

We observed significantly decreased RXRα mitochondrial translocation in mtDNA-depleted cells. These cells contained less than 25% of the original mtDNA content, and their ATP production was compromised as a result of dysfunctional mitochondria (Table 1). In fact, the reduced ATP production appears to have disrupted RXRα translocation in the present study. Consistent with our findings, Lin et al. [23] demonstrated that shuttling of RXRα is energy- and ligand-dependent.

Several experiments in various systems have suggested the presence of nuclear receptors in mitochondria that regulate mitochondrial transcription. For example, glucocorticoid hormone receptors have been found to localize to mitochondria and to modulate mitochondrial gene transcription in Hela cells [26] and in skeletal muscle [17]. In MCF7 cells, ERα and ERβ translocated into mitochondria and increased transcription of mtDNA-encoded genes was reported [18]. In addition, Casas et al. also provided convincing evidence that overexpression of the T3 receptor stimulates mitochondrial biogenesis *in vitro* [19] and *in vivo* [27].

In agreement with previous work, we demonstrated that 9-cis RA induces RXRα translocation into mitochondria and increases levels of mtDNA transcription and translation. However, this translocation of RXRα did not affect mtDNA content or mitochondrial biogenesis, since we found no significant changes in mtDNA copy number or mitochondrial mass in the present study. These experimental data provide convincing evidence that 9-cis RA increases mtDNA transcription but not mtDNA replication, which suggests that RXR mitochondrial localization, mediates the effects of 9-cis RA on mitochondria. Furthermore, to our knowledge, this is the first report indicating that translocation of RXR into mitochondria depends on ATP and on mitochondrial membrane potential.

Acknowledgments

The authors gratefully acknowledge support from grant NSC95-2320-B-036-MY2 from the National Science Council of Taiwan, grant 94TMU-WFH-208 from Taipei Medical University-Wan Fang Hospital, and grant SKH-TMU-93-19 from Shin Kong Wu Ho-Su Memorial Hospital.

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