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Review Article

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Differential distribution of glucocorticoid and estrogen receptor isoforms: localization of GRβ and ERα in nucleoli and GRα and ERβ in the mitochondria of human osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cell lines

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Abstract

The localization of glucocorticoid and estrogen receptors α (GRα, ERα) and β (GRβ, ERβ) in osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cells was studied by immunofluorescence labelling and confocal laser scanning microscopy, as well as by subcellular fractionation and immunoblotting of the proteins of the fractions with respective antibodies[1,2]. In HepG2 and SaOS-2 cells GRβ and ERα were localized mainly in the nucleus, particularly concentrated in nuclear structures, which on the basis of their staining with antibody against C23-nucleolin, were characterized as nucleoli. A faint, diffuse GRβ and ERα staining was also observed in the cytoplasm. GRα and ERβ were specifically enriched at the site of cell mitochondria, which were visualized by labelling with the vital dye CMX. Immunoblotting experiments corroborated the immunofluorescence labelling distribution of glucocorticoid and estrogen receptor isoforms in the cell lines studied. These findings support the concept of a direct action of steroid/thyroid hormones on mitochondrial functions by way of their cognate receptors and also suggest a direct involvement of GRβ and ERα in nucleolar-related processes in HepG2 and SaOS-2 cells.

Keywords: Glucocorticoid Receptor, Estrogen Receptor, Mitochondrion, Nucleolus

Introduction

Steroid hormones are principal regulators of metabolic, growth and developmental processes[3,4], therefore their mode of action at the cellular and molecular level is at the focus of interest. A major way of their action is by binding and activating cognate receptor proteins, which represent ligand-activated transcription factors. Upon activation, the receptors interact, as dimers, with specific binding sites – the hormone responsive elements – at the regulatory regions of the genes, initiating, together with polymerase II, other transcription factors and regulatory proteins, a series of events, culminating in activation or repression of transcription of respective gene(s)[5]. Alternative mechanisms of steroid hormone action, some non-genomic[6], have also been demonstrated. Action through receptors localized in cell membrane[7,8] and through mitochondria[9] are now at the focus of interest.

In the context of delineating mechanisms of glucocorticoid and estrogen hormone action, we have studied the intracellular distribution of the two glucocorticoid receptor isoforms, GRα and GRβ and the two estrogen receptors,

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ERα and ERβ, in human hepatocarcinoma HepG2 and osteosarcoma SaOS-2 cells. The aim of our study was to explore the possible distinct roles of the GR and ER isoforms. GRα and GRβ are splicing variants of the same glucocorticoid receptor gene, differing only in the last exon[10], whereas ERα and ERβ are encoded by two different genes[11].

The HepG2 and the SaOS-2 cell lines are target cells for glucocorticoids and estrogens. In hepatocytes glucocorticoids stimulate synthesis of enzymes involved in energy production[12], whereas in osteoblasts they initiate events leading to apoptosis[13]. Estrogens in rat liver, in cultured rat hepatocytes and HepG2 cells increase the levels of several mRNAs of genes encoding enzymes of oxidative phosphorylation[14]. They also stimulate the expression of genes involved in bone formation, promote apoptosis of osteoclasts and prevent apoptosis of osteoblasts and osteocytes[15-17].

Our data demonstrate a predominant mitochondrial localization of GRα and ERβ in both target cells and a nuclear localization of GRβ and ERα, with marked accumulation in nucleoli, implicating discrete mitochondrial and nucleolar action of the differentially localized receptors[1-2].

Materials and methods

Cells and culture conditions

Human hepatocarcinoma HepG2 and osteosarcoma SaOS-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

Antibodies

Rabbit polyclonal anti-ERα (G-20), anti-ERβ (H-150), anti-GR (M20 - against an N-terminus GR epitope) antibodies and blocking peptides were commercially provided by Santa Cruz Biotechnology and anti-GRβ antibody was purchased from Affinity Bioreagents. For double-immunolabeling experiments, mouse monoclonal antibody against C23-nucleolin (Santa Cruz Biotechnology) was used. Secondary FITC- and HRP-conjugated antibodies were also purchased from Jackson Laboratories.

Immunostaining

Cells grown on coverslips were incubated for 30 minutes at 37°C with 400 nM MitoTracker Red CMXRos (CMX; Molecular Probes Inc), washed 3x5 with PBS, fixed for 7-10 minutes in ice-cold methanol and transferred in ice-cold acetone for 2 minutes. After rinsing in PBS, cells were blocked at room temperature with control serum (5% in PBS) for an hour, to reduce non-specific binding. For immunofluorescence microscopy, specimens were incubated with primary antibodies (1:20 dilution for ERα and ERβ, 1:50 for GR (M-20), 1:600 for GRβ and 1:50 for C23-nucleolin) for 2 hours in a moist chamber at room temperature. Following 3 washing steps with PBS, anti-rabbit FITC-conjugated secondary antibody diluted 1:50 was added for 1 hour. The specimens were washed 3x3 min in PBS and mounted in a 50% glycerol solution. For double immunolabeling experiments, two primary antibodies (against ERα/GRβ and C23-nucleolin) were added simultaneously. After rinsing in PBS, ERα/GRβ were visualized by anti-rabbit FITC-conjugated secondary antibody (1:50 dilution) and C23-nucleolin by anti-mouse IgG coupled to Rhodamine (Santa Cruz; 1:200 dilution). Cell specimens were subjected to Confocal LASER Scanning Microscopy[1-2].

Subcellular fractionation and Protein (Western Blot) Analysis

Nuclei, mitochondria and cytosol were isolated from SaOS-2 and HepG2 cells by sucrose gradient centrifugation of cell homogenates[1-2,18]. Cells were homogenized at 4°C in an isotonic buffer and the homogenate was centrifuged for 5 minutes, at 1000 g, in a Sorvall centrifuge using the SS-34 rotor. The pellet was further processed to yield purified nuclei. The supernatant was centrifuged at 12,000 g for 20 minutes, in a Sorvall centrifuge using the SS-34 rotor, to give the crude mitochondrial pellet. The obtained supernatant was further centrifuged at 100,000 g, for 1 hour, in a Beckman L-8 ultracentrifuge using the 50 Ti rotor. The resulting supernatant (cytosol) was aliquoted and kept at -70°C. Purified mitochondria were obtained from the crude mitochondrial pellet[1-2,18].

The mitochondrial and nuclear pellets were subsequently lysed for 30 minutes in TAPS buffer (25 mM TAPS, 1 mM EDTA, 10% glycerol; pH 8.8), in which protein inhibitors were added (1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, aprotinin 5 μg/ml, pepstatin 2.5 nM, antipain 20 μg/ml and leupeptin 1 mM), sonicated twice with an ultrasonicator, at level 40 for 20 seconds and centrifuged at 12,000 g for 15 minutes at 4°C. The resulting supernatants, which represent the soluble mitochondrial (mitosol) and nuclear (nucleosol) fractions, respectively, were aliquoted and kept at -70°C.

Cytosol, nucleosol and mitosol were then western blotted[1-2,18].

Results

Immunofluorescence localization of glucocorticoid and estrogen receptors in HepG2 and SaOS-2 cells

The anti-GR M-20 (Figure 1) and the anti-ERβ (Figure 2) antibodies stained the nucleus, the cytoplasm and the mitochondria of both cell types. In the nucleus, the staining was faint, particularly in nuclei of HepG2 cells, and diffuse, often with some brighter foci surrounding the nucleoli, which were not stained. The cytoplasm showed diffuse staining. An intense fluorescence was observed on numerous, dot-like structures, which were defined as mitochondria, using the mitochondrial specific marker dye CMX (bright red fluorescence). Many of the mitochondria were perinuclearly arranged.

Applying the anti-GRβ (Figure 3) and anti-ERα specific antibodies (Figure 4), fluorescence labeling was restricted almost exclusively to the nucleus, in the form of aggregates,

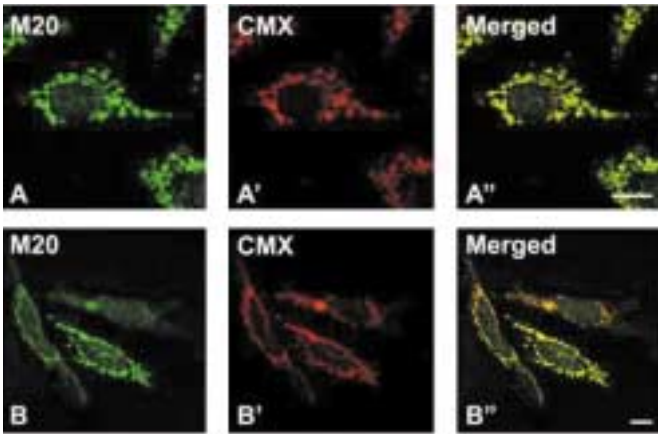

Figure 1. Localization of GR, using the anti-GR (M20) antibody, in HepG2 (**A**) and SaOS-2 (**B**) cells. Cells were incubated with CMX, fixed in methanol-acetone and treated with anti-GR (M20) antibody (**A, B**), followed by incubation with FITC-conjugated secondary antibody. CMX-staining (**A', B'**). Merged pictures (**A", B"**). Bars indicate 10 μm.

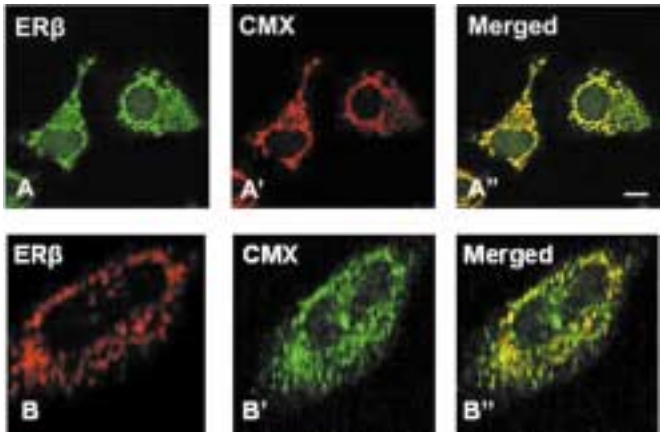

Figure 2. Localization of ERβ in HepG2 (**A**) and SaOS-2 (**B**) cells. Cells were incubated with CMX, fixed in methanol-acetone and treated with anti-ERβ antibody (**A, B**), followed by incubation with FITC-conjugated secondary antibody. CMX-staining (**A', B'**). Merged pictures (**A", B"**). Bars indicate 10 μm.

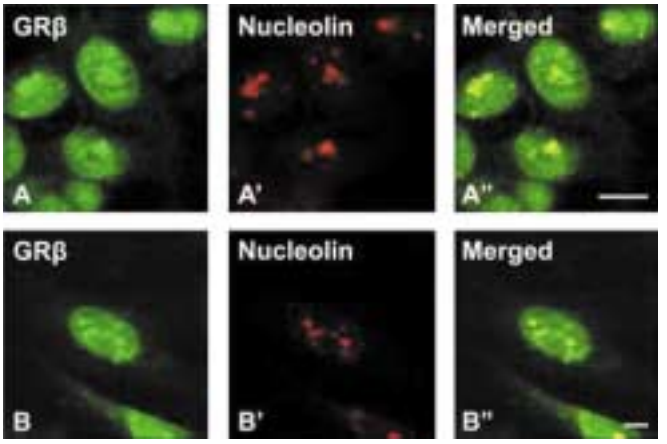

Figure 3. Localization of GRβ in HepG2 (**A**) and SaOS-2 (**B**) cells. Cells were incubated with CMX, fixed in methanol-acetone and treated with anti-GRβ antibody (**A, B**), followed by incubation with FITC-conjugated secondary antibody. CMX-staining (**A', B'**). Merged pictures (**A", B"**). Bars indicate 10 μm.

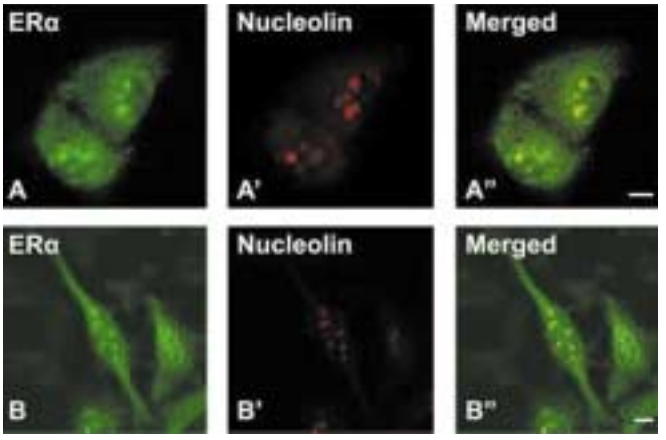

Figure 4. Localization of ERα in HepG2 (**A**) and SaOS-2 (**B**) cells. Cells were incubated with CMX, fixed in methanol-acetone and treated with anti-ERα antibody (**A, B**), followed by incubation with FITC-conjugated secondary antibody. CMX-staining (**A', B'**). Merged pictures (**A", B"**). Bars indicate 10 μm.

with only occasional faint staining of the cytoplasm and nucleoplasm. These structures in the nucleus proved to be nucleoli, since they co-localized with nucleolin, a nucleolar protein involved in nucleolar RNA biosynthesis.

Biochemical identification of glucocorticoid and estrogen receptors in subcellular fractions in HepG2 and SaOS-2 cells

All subcellular fractions of HepG2 and SaOS-2 cells treated with the GR (M20) antibody showed in Western Blots a band of approximately 95kDa, corresponding to the molecular mass of GRα (Figure 5). In the mitochondrial fraction, a 95kDa protein and a 90kDa band were detected, which did not react with antibody to GRβ. These bands were not observed after preincubation of the fractions with GR-specific blocking peptides. In all subcellular fractions, lower molecular weight polypeptides reacting with the GR (M20) antibody were occasionally found, which also represent GR species, as demonstrated by the use of specific blocking peptides. Such species were also previously observed in mito-

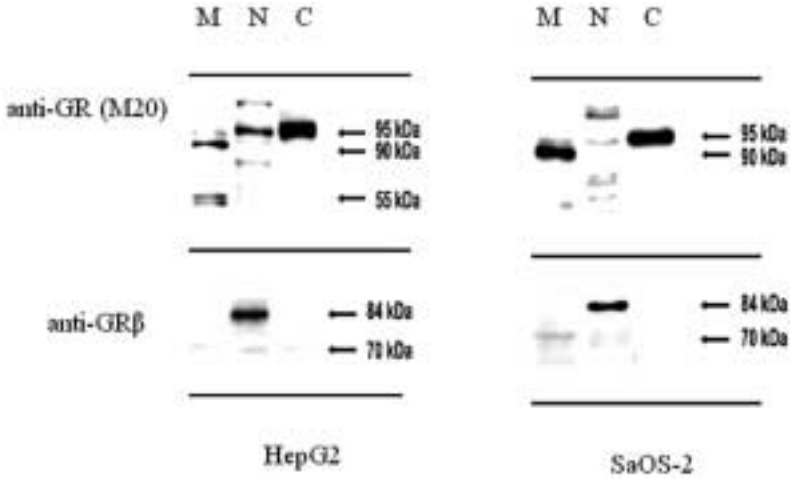

Figure 5. Detection of GR isoforms by Western Blotting in HepG2 and SaSO-2 subcellular extracts. Mitosol (M), Nucleosol (N), Soluble Cytosol (C) probed with the GR (M20) and the GRβ antibodies (Modified from Psarra et al., 2005).

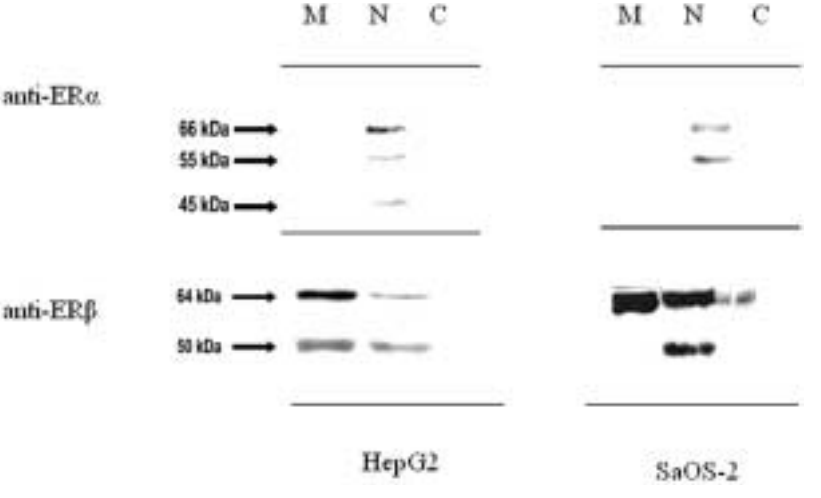

Figure 6. Detection of ERα and ERβ by Western Blotting in HepG2 and SaSO-2 subcellular extracts. Mitosol (M), Nucleosol (N), Soluble Cytosol (C) probed with the anti-ERα and the anti-ERβ antibodies (Modified from Solakidi et al., 2005).

chondria of other cell types[18,20-28].

Applying the GRβ specific antibody in Western Blots of subcellular fractions from HepG2 and SaOS-2 cells, a band of approximately 85kDa was detected in the nucleosol, representing GRβ. In addition, in all fractions, faint bands of lower molecular weight were present. No GRβ could be detected in mitochondria, conform to the immunofluorescence data (Figure 5).

Using antibodies against ERα and ERβ, subcellular fractions from HepG2 and SaOS-2 cells were also western-blotted. In the nuclear fraction of both cell types, applying the anti-ERα antibody, a band of 66kDa representing ERα was demonstrated (Figure 6), in accord with the immunofluorescence data. Two other faint bands with molecular weights of 55 and 45 were also observed in HepG2 cells. The 55kDa band was also detected in the nucleosol of SaOS-2 cells.

As shown in Figure 6, in all subcellular fractions of the two cell populations, particularly in the mitochondrial fraction, a 64kDa band corresponding to ERβ was found. These results conform to the immunofluorescence data. In the nucleosol of both cell types and in the mitosol of HepG2 cells, a second protein of 50kDa was also demonstrated, which could represent degraded ERβ produced either during the cell fractionation procedure or resulting from a physiological processing mechanism.

Discussion

Our data demonstrate that glucocorticoid and estrogen receptors, in addition to the well established nuclear and cytoplasmic localization, are also found in two other cell organelles, mitochondria (GRα and ERβ) and nucleoli (GRβ and ERα). The presence of steroid receptors in mitochondria was suggested years ago[19] and has been proved by several studies[18,20-28]. The presence of steroid receptors in mitochondria has been correlated with a direct effect of the respective hormones on mitochondrial transcription or apoptosis[9,29]. Glucocorticoids and estrogens affect energy production in target cells by increasing the availability of enzymes of oxidative phosphorylation. Well documented are the effects on muscle, where glucocorticoids induce the transcription of some oxidative phosphorylation and mitochondrial biogenesis genes[30]. Similar results have been obtained by adding dexamethasone to C2C12 mouse myotubes, leading to upregulation of mitochondrial gene transcription[30]. Estradiol also enhances mitochondrial gene transcription in MCF-7 and HepG2 cells, mediated by ERβ[27].

The presence of steroid receptors in mitochondria and the demonstration of mitochondrial DNA sequences showing high similarity to HREs[31,32] interacting with the receptors, point to direct effects on mitochondrial transcription. This has been verified in the case of the thyroid hormone receptor, which belongs to the family of nuclear receptors, in a mitochondrial *in organello* system[33-35].

Mitochondria are involved in a series of other basic functions, such as ROS production and apoptosis, and a role of mitochondrial receptors in these functions is now emerging[36-37].

The presence of GRβ and ERα in nucleoli raises the question of the role of these receptors in nucleolar function. Well documented are the effects of steroid hormones in regulating ribosomal RNA synthesis[38-42]. GRβ does not bind glucocorticoids, therefore its possible role on nucleolar function could be indirect, by interaction with a glucocorticoid-activated molecule. ERα binds estrogens, so that the known effects of estrogens on ribosomal RNA synthesis could be mediated by induction of the activated receptor, together with nucleolar DNA and/or with nucleolar proteins involved in ribosomal biogenesis, e.g., transcription, processing and trafficking[43].

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