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Analysis of nuclear glucocorticoid receptor–DNA interaction in aged rat liver

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Abstract: In order to contribute to the understanding of mechanisms by which regulatory proteins recognize genetic information stored in DNA, analyses of their interaction with specific nucleotides are usually performed. In this study, the electrophoretic mobility shift assay (EMSA) was applied to analyze the interaction of nuclear proteins from the liver of rats of different age *i.e.*, young (3-month-old), middle-aged (12-month-old) and aged (24-month-old), with radioactively labelled synthetic oligonucleotide analogues, corresponding to GRE. The levels of GRE binding activity were assessed by quantitative densitometric scanning of the autoradiograms. The results showed statistically significant decreasing values of up to 78% and 49% in middle aged and old animals, respectively, compared to young animals ($p < 0.05$). The specificity of the nuclear proteins-GRE interaction was demonstrated by competition experiments with unlabelled GRE. In a supershift assay, using the antibody BuGR2, it was shown that the GR proteins present in nuclear extracts have a high affinity for the GRE probe. The stabilities of the protein-DNA complexes were analysed and it was concluded that they changed during ageing.

Keywords: ageing, liver, glucocorticoid receptor, GRE, EMSA.

INTRODUCTION

Ageing of humans and animals may be characterized by changes in the responsiveness of tissues and cells to certain hormonal signals.[1] Glucocorticoid hormones, which are involved in the regulation of various physiological processes and are essential for the maintenance of vital functions, act *via* a glucocorticoid receptor (GR). Age-related changes of the properties and functioning of the glucocorticoid receptor have been observed in numerous tissues.[2,3] The structure and

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activities of the GR have been extensively characterized and reviewed.[4,5] The glucocorticoid response element (GRE) consensus sequences are represented by imperfect inverted repetition of GGTACAnnnTGTTCT in which a trinucleotide spacer separates two hexanucleotides.[6] The GR in the nucleus dimerizes and binds as a homodimer to the GRE.[7]

Protein–DNA interactions are characterized by very high specificity and stability (K_d of the order of $10^{-9} – 10^{-12}$ M).[8] Although electrophoretic mobility shift assay (EMSA) is usually performed with very little modification to the original technique,[9] different factors which affect complex formation and stability are still studied extensively.[10] In this study, the interaction of GR with synthetic oligonucleotide analogues (corresponding to GRE from the rat GR gene[11]), was analyzed using EMSA to determine the GRE binding activity in nuclear extracts from rat livers of three age groups: young (3-month old), middle aged (12-month old) and aged (24-month old) animals.

EXPERIMENTAL

Chemicals

All the chemicals used were of analytical grade and purchased from Sigma Chemical Co, except [α^{32}P]deoxy-CTP (3000 Ci/mmol) which was purchased from the Radiochemical Centre, Amersham, UK and antiglucocorticoid receptor antibody IgG2a (clone BuGR2) which was from Affinity Bioreagents Inc.

Preparation of nuclear extracts

Male Mill Hill hooded rats, aged 3, 12 and 24 months at the time of arrival, were reared under standard laboratory conditions (22 °C, 12:12 hours light/dark cycle with food and water *ad libitum*). The animals were sacrificed by decapitation. The livers were rapidly removed and used for the preparation of the nuclear extracts.[12] The tissue was homogenized in 4 volumes (w/v) of homogenization buffer (20 mM HEPES, pH 7.5, 10 % glycerol, 25 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM EGTA, 2 M sucrose). The homogenate was layered over a 2 mL cushion of the same buffer and centrifuged for 60 min at 25,000 g. The pellet (containing the nuclei) was washed with washing buffer (20 mM HEPES, pH 7.5, 10% glycerol, 25 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM EGTA, 1 mM DTT) by centrifugation for 10 min at 4,000 g. Finally, the pellet was resuspended in 1 mL of lysing buffer (20 mM HEPES, pH 7.5, 10% glycerol, 0.5 M KCl, 1.5mM $MgCl_2$, 0.4 mM EDTA, 1 mM EGTA, 20 mM Na_2MoO_4, 1 mM DTT, 147 μg/mL PMSF, 5 μg/mL antipain, 5 μg/mL leupeptin, 5 μg/mL aprotinin). The extraction was performed for an hour at 4 °C by vortexing from time to time. The supernatant obtained by centrifugation at 14,000 g for 30 min was used as the nuclear extract. The obtained nuclear extracts were frozen in 25 μL aliquots and stored at −70 °C.

Protein determination

The protein concentration in the nuclear extracts was determined by the method of Bradford,[13] using a bovine serum albumin (BSA) as the standard and BioRad Protein Assay Reagent. The protein concentrations in these extracts ranged from 2.5 to 5 mg/mL.

DNA probe labelling

The single-stranded synthetic oligonucleotide GRE (Santa Cruz Biotechnology, 50 ng/μL ≈ 1.75×10^{-6} M) was annealed to its complementary sequence by denaturation for 10 min at 100 °C and, subsequently, by cooling in an ice bath. The double-stranded oligonucleotide was ^{32}P-labelled

by the Klenow enzyme using a Random Primed DNA Labelling Kit (Boehringer Manheim) and 5 μL = 50 μCi [α^{32}P]deoxy-CTP (3000 Ci/mmol). The labelled oligonucleotide was then purified on a Sephadex G-50 minicolumn by collecting the fractions with radioactivity over 100,000 cpm (Cerenkov's).

Electrophoretic mobility shift assay (EMSA)

The binding conditions for the EMSA were: 50 mM Tris pH 7.5, 20 % glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl in 20 μL reaction mixture with an addition of 10 μg poly(dI-dC) to inhibit non-specific binding of the labelled probe to nuclear extract proteins. Different concentrations of nuclear proteins (2.5, 5, 10, 15 and 20 μg per reaction) were added and incubated with 100,000 cpm (approximately 0.5 ng) of a [^{32}P]-labelled GRE oligonucleotide for 20 min at room temperature. In the competition binding experiments, 50 ng, 100 ng and 200 ng of unlabelled oligonucleotides were added to the reaction mixtures concurrently with the labelled oligonucleotide. In the supershift experiments, the anti-GR antibody (1 μg) was preincubated with nuclear extract for 1 h at 4 °C prior to the addition of the radiolabelled probe. After incubation of reaction mixtures, 2 μL of the solution (0.01% bromophenol blue, 0.05% xylene cyanol, 5% ficoll) was added and the protein-DNA complexes were separated by 5% polyacrylamide gel electrophoresis in 0.045 M TBE buffer, pH 8.0 (0.045 M Tris-borate, 0.001 M EDTA), using a Hoefer SE600 Vertical Slab Units (run gel 16 cm), 150 V, 1.5h. Controls without protein samples were run on all gels.

Autoradiography and data analysis

After electrophoresis, the gels were vacuum dried, autoradiographed on Fuji Medical X-ray films 13 × 18 cm in Hypercassettes™ (Amersham) with an intensifying screen at −70 °C overnight. The films were scanned densitometrically using a GelDoc 1000 image analyzer and Multi-Analyst/PC software, BioRad Laboratories. The quantities of the protein-DNA complexes were determined by measuring the average gray/pixel level in the area of the bands corresponding to the protein-DNA complexes. The results are expressed as the mean ± SE of three independent experiments. The statistical significance was assessed using the analysis of the variance and significant differences were confirmed when the probability was less than or equal to 5 %. The data for GR-GRE binding affinities were plotted and analyzed by non-linear regression, GraphPad Prism, version 3.00 (GraphPad Software Inc., San Diego, USA).

RESULTS

A double-stranded oligonucleotide 5'-AGAGGATCTGTACAGGATGTTCTAGAT-3' (containing the underlined consensus GRE element) was radioactively labelled and the DNA binding activity of nuclear GR in rat liver was studied using the electrophoretic mobility shift assay (EMSA) in dependence on age. Initial studies were performed to estimate the relative change in the integrated gray level (determined by densitometric scanning of the autoradiograms) with increasing quantities of nuclear protein loaded on the gel, Fig. 1A. The nuclear extracts were obtained from the liver of rats of different age: young (3-month-old), middle aged (12-month-old) and aged (24-month-old) animals. Incubation of these nuclear extracts with a consensus GRE binding sequence resulted in a significant retardation of the migration of the labelled oligonucleotide and the formation of specific protein–DNA complexes, demonstrated by the presence of retarded bands at the top of the gel, whereas free probe was at the bottom (Fig. 1). Retardation was not seen in the absence of protein (lanes 0 on Fig. 1A).

The relationship between the signal intensities of the specific band and amount of nuclear proteins was analyzed by non-linear regression for all age groups, Fig. 1B. The protein-dependent increase in the densitometric units saturated at about 10 μg protein. The obtained results verified the utility of this quantity of protein for the EMSA detection of the relative changes in GRE binding during ageing. Subsequent densitometric analysis of the EMSA data revealed a significant effect of age on the GRE binding activity in nuclear extracts (the values decreased to 78.46 ± 7.11 % in middle-aged and to 49.34 ± 2.86 % in old-aged animals, compared to young animals (p <0.05), Fig. 1C). The extracts from young animals exhibited a statistically significant greater GRE binding activity than those from either middle-aged or old animals (using the one-way ANOVA test, F = 10.65, p = 0.01).

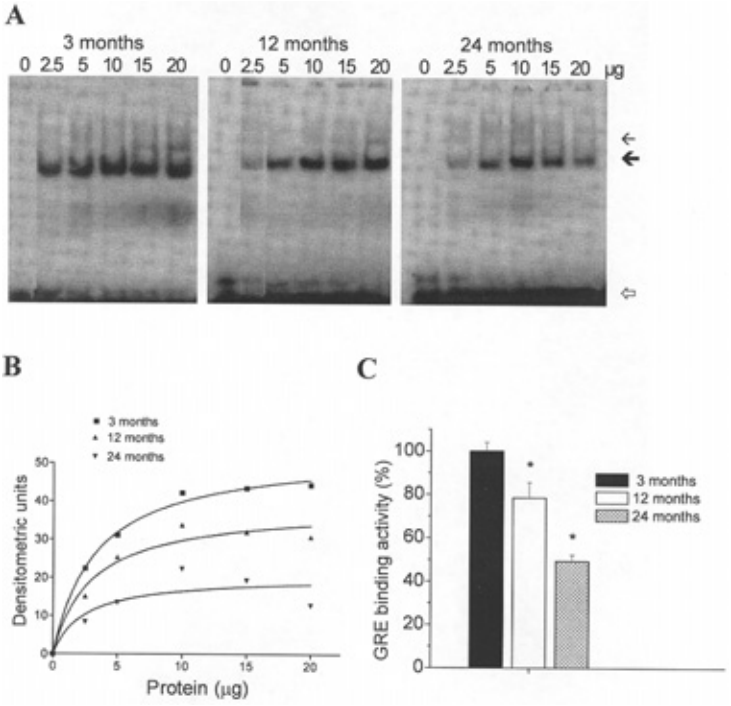

Fig. 1. Relationship between the intensity of the EMSA bands and the amount of protein (μg) loaded in the EMSA reactions, using nuclear proteins from the liver of young (3-month-old), middle-aged (12-month-old) and old (24-month-old) rats. A - Autoradiograms, the arrows indicate the GRE-GR complex, open arrow indicates free radioactively labelled GRE oligonucleotide. B - Analysis of the data of the EMSA experiment by non-linear regression. C - GRE binding activity in rat nuclear extracts from variously old animals, expressed as percentages of the 3-month-old group. The results are represented as the mean ± S.E. of three experiments; *, significantly age-related differences by the one-way ANOVA test (F = 10.65, p = 0.01 at the 0.05 level).

To investigate the nature of the components involved in the protein–DNA interactions, the specificity of the retarded bands was determined by competition studies and by supershift assays.

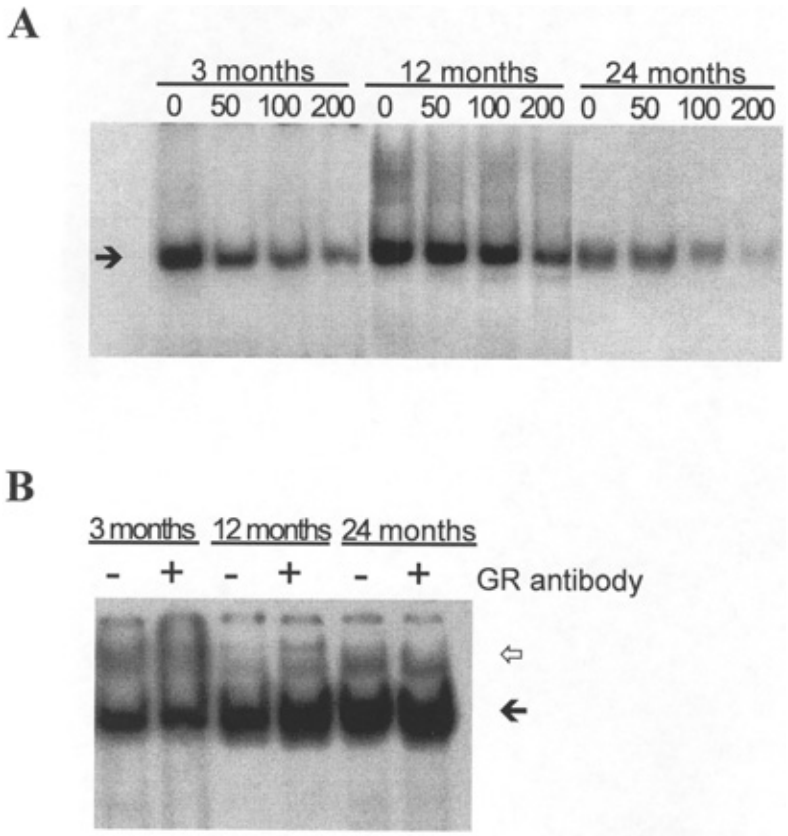

Fig. 2. Specificity of EMSA reactions using nuclear proteins from the liver of young (3-month-old), middle aged (12-month-old) and old (24-month-old) rats. A - competition experiment, the binding of nuclear proteins to GRE had to compete with increasing amounts of unlabelled GRE oligonucleotide (50, 100 and 200 ng corresponding to 100, 200 and 400-fold molar excess relative to the ^{32}P-labelled GRE oligonucleotide, respectively); B - Supershift assay of the nuclear extracts with antibody against GR; the black arrow indicates the bands of the GRE-GR complex, the open arrow indicates supershifted bands.

As shown in Fig. 2A, the retarded bands were inhibited, in a dose-dependent manner by unlabelled GRE in all age groups. The intensity of the bands diminished with increasing amount of unlabelled GRE from 0 ng (incubation mixture without competitor) to 200 ng of competitor, indicating the specificity of the GRE binding activity.

In the supershift assay, preincubation of nuclear extracts from all age groups with GR antibody resulted in a supershifted band, as a consequence of the binding of GRE oligonucleotides with antigen/antibody complexes (Fig. 2B). The addition of GR antibodies to the GRE binding reaction mixture produced a supershift that was consistent with the presence of GR in the GRE–protein complex.

DISCUSSION

In a recent work, the effects of ageing on the DNA binding activity of GR in rat brain[14] were shown. In the present work, the objective was to study the effect of ageing on the DNA binding activity of GR in rat liver, considering the metabolic role of this organ in the maintenance of life.

Young animals exhibited a greater GRE binding activity in the nuclear extracts than either the middle-aged or old animals (the values were significantly different at the level $p < 0.05$, by ANOVA test), Fig. 1. These results are consistent with previously reported data, where it was found that the number of GR changed during ageing[15] and, consequently, there were changes in the quantity of sequence specific DNA binding. Decrements in the protein levels of nuclear GR and DNA binding were observed in the hippocampus of old rat.[16] Moreover, it is known that GR exhibits protein–protein interactions with numerous transcription factors.[17,18] Thus, it is possible that modulation of other nuclear protein factors may interfere with the GR signaling pathway with age, which is an explanation for the age-related decrease in the ability of GR to bind DNA.

Using the EMSA method, the GRE binding activity was quantified densitometrically in the area of a single band designated as specific and corresponding to the protein–DNA complexes. As shown in Fig. 1A, another band (marked by a small arrow) was observed when increasing amounts of nuclear protein were added to a fixed concentration of GRE probe. The obtained result indicates that at low concentration of nuclear proteins, GR predominantly binds GRE as a homodimer complex, as expected in Tris-borate gels.[5] However, at higher concentrations of nuclear proteins, the EMSA allows the resolution and quantification of other complexes which are formed. This observation correlates well with published data which indicate that protein–protein interactions are involved in the formation of the heterodimer of native GR.[19,20] In the case of old-age rats, the appearance of upper band was a smear rather than a band. This indicates that the protein–DNA complexes in nuclear extracts of old animals have a different stability and some of them might dissociate during the EMSA.

Competition experiments with the addition of excess of unlabelled GRE oligonucleotide to the binding mixture (Fig. 2A) demonstrated a specific interaction between nuclear proteins and GRE sequences. In the experiments, a large excess of a specific competitor (amounts of 50, 100 and 200 ng correspond to 100-, 200- and 400-fold molar excess, respectively) was used to prevent reassociation of the protein with a labelled specific DNA fragment, as suggested by others.[21] The observed pattern of the blocking of the specific band was the same in all groups.

The nature of these proteins was demonstrated by antibodies directed against GR, Fig. 2B. The supershifted antibody–GR–GRE complexes showed some differences of the bands, when 3-month-old rats were compared to 12-month-old and 24-month-old animals, indicating that the modification of these complexes which

occurs during rat liver ageing may be the consequence of the modification of GR itself or of GR protein–non-receptor protein–DNA interactions.[20]

The presented results show that the potential of nuclear GR interactions in rat liver change during ageing. EMSA demonstrated that the efficiency of interaction of GR with its GRE is reduced during ageing, as are the stabilites of GR–GRE complexes. The glucocorticoid receptor is a critical regulator of cellular homeostasis and homeostasis of the organism. Thus, the obtained results for an aged liver represent a contribution to the understanding of the attenuated nuclear signalling of this nuclear transcription factor, which have been observed for the ageing process in different tissues.

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ИЗВОД

АНАЛИЗА ИНТЕРАКЦИЈЕ ЈЕДАРНОГ ГЛУКОКОРТИКОИДНОГ РЕЦЕПТОРА И ДНК У ЈЕТРИ ПАЦОВА ТОКОМ СТАРЕЊА

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У циљу доприноса разумевању механизама помоћу којих регулаторни протеини препознају генетичку информацију коју носи ДНК, анализирају се њихове интеракције са специфичним нуклеотидима. У овом раду је методом EMSA анализирана интеракција једарних протеина из јетри пацова из три старосне групе (млади - 3 месеца, средње доба – 12 месеци и стари – 24 месеца) са синтетичким, радиоактивно обележеним, олигонуклеотидним аналогом GRE. Ниво везујуће активности GRE је одређиван квантитативно денситометријском ауторадиографијом. Резултати су показали да постоји статистички значајан пад вредности GRE-везујуће активности до 78% код животиња средњег старосног доба и до 49 % код старих животиња, у поређењу са вредностима добијеним за младе животиње ($p < 0.05$). Специфичност интеракције једарних протеина и GRE је одређена експериментима компетиције са необележеним GRE. Коришћењем антитела BuGR2 показано је да је глукокортикоидни рецептор протеин који у једарном екстракту има највећи афинитет за GRE пробу. Анализирана је стабилност комплекса протеин-ДНК и закључено је да се мења током старења.

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