Effects of Age and Dexamethasone Treatment on Glucocorticoid Response Element and Activating Protein-1 Binding Activity in Rat Brain

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The effect of dexamethasone (DEX) on glucocorticoid receptor (GR)-mediated gene expression was examined in the brain of young and aged rats. Electrophoretic mobility shift assays showed that DEX treatment led to an increase of glucocorticoid response element (GRE) binding activity in aged rats, whereas in young animals GRE binding activity was decreased. Western blot analysis and reverse transcriptase polymerase chain reaction confirmed that, in aged animals, the GR mRNA and the GR protein levels were increased on DEX treatment. The binding activity of GRE activating protein-1 (AP-1) site and cross-competition analysis demonstrated specific pattern of expression during the ageing and DEX treatment, suggesting that GR modulates the activity of transcription factors AP-1 (Fos/Jun proteins) through protein–protein interaction. On the basis of these results, it can be concluded that the composition of transcriptional complexes that bind to GRE and AP-1 regulatory elements changes upon DEX treatment in an age-specific manner.

GLUCOCORTICOID hormones (GCs) are known to have widespread effects in the brain, such as modulation of both energy metabolism and the expression of numerous genes responsive to stress and adaptation (1,2). They also influence behavior, learning, memory, mood, and affect. The involvement of neuroendocrine factors in age-related alterations of brain functioning was hypothesized many years ago (3,4). In particular, ageing is often associated with an altered function of the hypothalamic–pituitary–adrenal (HPA) axis (4–6). Attenuation of HPA regulation, associated with elevated cortisol concentration, hippocampal atrophy, and memory impairment, is symptomatic for several neurological disorders such as Alzheimer’s disease, depression, and schizophrenia (7–10). GCs exert their regulatory effects on the HPA axis via two types of corticosteroid receptors: a high-affinity mineralocorticoid receptor (MR) and a lower-affinity glucocorticoid receptor (GR) (11). GR is widely distributed throughout the brain, being the most abundant in hypothalamic corticotropin-releasing hormone (CRH) neurons and pituitary corticotropes. In contrast, the highest concentration of MR is found in the hippocampus, whereas the lower concentrations are found in hypothalamic sites (12). MR and GR act as ligand-activated transcription factors, which, in the hormone-bound state, modulate the expression of target genes by binding to cis-acting DNA sequences known as glucocorticoid responsive elements (GREs) (13,14). The GRE consensus sequences are represented by an imperfect inverted repeat of GGTA-CAnnnTGTTCT (15), in which a trinucleotide spacer separates two hexanucleotides. More than 200 GREs have been discovered in over 50 hormone-responsive genes, and their number is increasing. The new level of complexity in steroid hormone actions arose from the observation that the GR gene expression is down-regulated by glucocorticoids (16), possibly through binding of GR to GRE in the 3′ flanking region of the rat GR gene (17).

Finally, GR-mediated transcription is regulated by numerous trans-acting factors, including the AP-1 transcription factor (14). For example, activated GR directly interacts with the members of Jun/Fos protein families to attenuate the activation of the AP-1 transcription factor (18–21). Moreover, selective interaction between the GR and different components of the AP-1 family negatively or positively modulates transcription depending on the composition of AP-1 subunits (22). It has been reported that the presence of a potential AP-1 site is located at –899 to –893 of the human GR promoter, which modulates the GR gene expression through Jun/Fos activity (23). Although cross-talk of GR and AP-1 transcription factors has been observed by several researchers (24,25), the role of a possible mutual interaction between these signaling pathways during brain ageing has not been studied in molecular details.

In the present study, we examined the effects of age on the GRE and AP-1 DNA binding activity in the brain of rat males, under physiological conditions and after dexamethasone
(DEX) treatment. We also explored whether the composition of transcription complexes, which bind to these regulatory elements, is age-related. In addition, the GR mRNA and protein levels were estimated to assess the role the GR plays in the regulation of its own gene expression during ageing.

**METHODS**

**Animals and Treatment**

Mill-Hill hooded rat males of different age (3,12, and 24 months old), reared under standard laboratory conditions (22°C, 12/12 light/dark cycle), with food and water ad libitum, were used. Each age-matched group was divided into two groups: (i) Group 1: rats injected intraperitoneally with DEX 4 mg/kg (Sigma; Germany), and (ii) Group 2: controls injected with physiological saline (17). All animals were decapitated 18 hours after the injections; whole brains were rapidly isolated and used for the preparation of nuclear extracts.

**Preparation of Nuclear Extracts**

Nuclear extracts were prepared from the pools of three whole brains as described previously (26). Briefly, the brains were homogenized with a Dounce homogenizer (Cole-Parmer; Austria) in 4 volumes (w/v) of ice-cold buffer (0.25 M sucrose, 15 mM Tris-HCl, pH 7.9, 16 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.15 mM spermine, and 0.15 mM spermidine) and supplemented with the following protease inhibitors: 0.1 mM PMSF, 2 mM leupeptin, 5 mM aprotinin, and 2.5 U of murine leukemia virus RT (MuLV RT) in 100,000 cpm of [32P]deoxy-CTP-labelled double-stranded oligonucleotide was then added, and the incubation was continued for 20 minutes at room temperature. The specificity of DNA–protein binding was determined by competition with excess of nonradiolabelled oligonucleotides. Protein–DNA complexes were separated on 5% polyacrylamide gels in 0.045M TBE buffer, pH 8.0 (0.045 M Tris-borate, 0.001 M EDTA), 150 V, 1.5 hours. After electrophoresis, the gels were vacuum-dried and autoradiographed overnight on radiograph films with intensifying screens at –70°C. The density of the detected bands was determined using image analyzer GelDoc 1000 Multi-Analyzer/PC software (BioRad Laboratories; CA, U.S.A.).

For the super shift assay, 1 μg of the polyclonal antibodies against BuGR2 (Antigliucocorticoid Receptor Antibody IgG2a), c-Fos (K-25), and c-Jun/AP-1 (N) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were preincubated with nuclear extracts for 14–16 hours at 4°C prior to addition of the radiolabelled probe, and the same procedure as described above was followed.

The following double-stranded oligonucleotides (Santa Cruz Biotechnology) were used as probes in gel shift assay: GRE probe corresponds to consensus binding site for glucocorticoid receptor

5’-AGAGGATCTGTAGAGCTTCTCATGAT-3’

and reverse complement,

3’-TCTTCTAGATGTCTTACAGATCTTA-5’,

with the core consensus element underlined.

GRE AP-1 probe corresponds to binding site for AP-1 Jun homodimers and/or Jun/Fos heterodimers in “composite” GRE element (28):

5’-CGTAGATTGCATGATCGTCA-3’ and

3’-GACATGTAATCGATCTCAGT-5’.

Consensus AP-1 corresponds to consensus binding site for AP-1 Jun homodimers and/or Jun/Fos heterodimers:

5’-GCTTGTAGTACTAGCACCAGGA-3’ and

3’-GGCAACTACGTAGGGCGCTT-5’.

Probes were radiolabelled with [32P]deoxy-CTP using Klenow fragment of DNA polymerase I employing Random Primed DNA Labelling Kit (Boehringer Mannheim, Germany). Labelled oligonucleotides were then purified by Sephadex G-50 minicolumn chromatography (Sigma; Germany).

**RNA Extraction and Reverse Transcription**

RNA was isolated by the guanidine isothiocyanate (GTC)/cesium chloride (CsCl) centrifugation method (29). Each brain was homogenized in 7 ml of GTC buffer (4 M GTC, 4% N-lauryl sarcosine, 50 mM Na acetate, pH 5.5, and 1% β-mercaptoethanol). Dry CsCl, 0.5 g/ml, was added to the homogenates and layered on 3 ml of CsCl, density of 1.7 g/cm³, for ultracentrifugation (24 hours, 38,000 rpm in a Ti-50 rotor [Beckman; CA, U.S.A.]). The resulting pellets were suspended in precipitation buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5% SDS). After butanol/chloroform extraction, the pellets were suspended in 1 volume of 3 M Na acetate, pH 5.0, and 2 volumes of absolute cold ethanol, and kept overnight at –20°C.

For the synthesis of cDNAs, 5 μg of total RNA were reverse-transcribed in reverse transcriptase (RT) buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 100 μM DTT, 2.5 μM Oligo (dT)₆, dNTPs 0.5 mM each, 1 U RNase inhibitor, and 2.5 μ of murine leukemia virus RT (MuLV RT) in a final volume of 10 μl. The reaction cycle...
was 1 hour at 42°C, 10 minutes at 95°C, and cooling to 5°C. The cDNAs were kept at –20°C. To minimize errors in pipetting among the samples, master mixes of cDNA synthesis buffer containing the dNTPs, RT buffer, oligo(dT), DTT, and the enzymes were prepared and used for RT of all RNA samples.

Polymerase Chain Reaction

For polymerase chain reaction (PCR) amplification, appropriate dilutions of cDNA samples representing 100–200 ng of total RNA were mixed with PCR buffer containing 100 μM dNTPs, 1.5 mM MgCl₂, Stoffel Buffer (50 mM KCl and 10 mM Tris-HCl), 0.8–1 μM each of the primers, and 1.25 U Stoffel Taq polymerase in a total volume of 25 μl. PCR primers designed for GR amplification (30) were used with housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) (31). The primers were designed in the region located 5' to the DNA binding domain, where the homology between various steroid receptors is the lowest.

The primer sequences and PCR product size were as follows:

**GR upstream** 5'-TGC AAA CCT CAA TAG GTC GAC CAG-3'

**GR downstream** 5'-TAA ACT GGG CCC AGT TTC TCT TGC-3'

**GAPDH upstream** 5'-AAG GTG AAG GTC GGA GTG AAC G-3'

**GAPDH downstream** 5'-GGC AGA GAT GAT GAC CCT TTT GGC-3'

GR and GAPDH amplification products were 522 bp and 350 bp long, respectively. The samples were denatured initially at 94°C for 2 minutes and amplified. Cycle parameters in all cases were 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute (30 cycles), followed by 72°C for 8 minutes. PCR-amplified products were analyzed by electrophoresis in 2% agarose gel. All PCRs were repeated at least three times. The levels of GR mRNA were compared and the correction was made for the differences in GAPDH mRNA.

Western Blot Analysis

Nuclear extract proteins (4 mg) were diluted with 2× RIPA buffer (300 mM NaCl, 20 mM HEPES pH 7.5, 0.2% SDS, 2% Na deoxycholate, 2% Triton X-100) to a final volume of 200 μl and incubated with 1 μg of BuGR2 (antigliucocorticoid receptor antibody IgG2a) on a rotating wheel for 60 minutes at 4°C. Protein A sepharose was added and the sample was incubated for an additional 2 hours and centrifuged as above. The pellet was then washed 4 times, first with cold 1× RIPA buffer and then with 0.1 mM Tris. Finally, the pellet was resuspended in an equal volume of 2 × Laemmli sample buffer and subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

Samples immunoprecipitated proteins were resolved according to Laemmli (32) on 10% SDS-polyacrylamide. Proteins were transferred to nitrocellulose membranes in a semidyry transblot apparatus. The blots were incubated in 2% nonfat dry milk in TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 30 minutes. The primary antibody diluted 1:5000 (BuGR2, ABI) was added to the membrane and incubated for 2 hours. Biotinylated antirabbit secondary antibody (horseradish peroxidase-conjugated IgG) and ABC (avidin-biotin complex) reagents were added for sequential 30-minute incubations. Detection of proteins was performed by diaminobenzidine-nickel chloride substrate solution.

Statistical Analyses

The data were analyzed for statistical significance using analysis of variance (ANOVA). All results are presented as means ± SEM. Group differences were assessed by two-factorial analysis of variance (two-way ANOVA), where age and treatment were two between-subject factors. When significant age × treatment effects were found, the post hoc Tukey test was subsequently performed. The level of significance was set at p < .05. All statistical testing procedures were run using SigmaStat software (Jandel Scientific, Erkrath, Germany).

RESULTS AND DISCUSSION

GRE Binding Activity and Effects of DEX Treatment During Ageing

In common with other tissues, the responses to glucocorticoid hormones in the brain are diverse, ranging from the permissive to the suppressive. A decrease in glucocorticoid responsiveness during ageing could lead to a further loss of homeostasis and thus to increased susceptibility to neurodegeneration (33). This prompted us to study the effects of ageing on GR-dependent DNA binding and on GR mRNA and protein expression, since the changes at molecular level could result from an overexposure of brain cells during ageing to glucocorticoid hormones.

In order to examine the effect of age on hormone-mediated action, GRE binding activity was measured using the electrophoretic mobility shift assay (EMSA) in brain nuclear extracts of rats of different ages (3, 12, and 24 months old). As shown in Figures 1A and 1B, the GRE binding activity was diminished in the brain of 12-month-old control rats as compared with young (3-month-old) animals (3 vs 12 months, p < .05), whereas no significant changes were observed between the binding activities in the brains of 3- and 24-month-old rats. To determine the effect of DEX treatment on GRE binding activity, nuclear extracts from the brains of the same age groups were prepared following DEX administration (Figure 1A). Quantitative analysis revealed that DEX produced 30% inhibition of the GRE binding activity in 3-month-old rats when compared with the controls (Figure 1B). However, DEX treatment caused a statistically significant increase (p < .01) in the binding of protein complexes to the GRE consensus sequence in the brain of 12-month-old rats as compared with the corresponding control. This change at the transcription level after DEX treatment remained almost constant until 24 months of age. Two-way ANOVA indicated that the effects of the hormone were significantly influenced by age (F = 6.397, p < .001). Due to the specific interaction effect, post hoc analysis within each treatment and within each age group was per-
formed. Possible composition of protein–DNA complexes on GRE elements was investigated by cross-competition experiments. Both unlabelled probes for GRE and GRE AP-1 were similarly effective in competing for the GRE consensus sequence in a concentration-dependent manner (Figure 1C). In contrast, binding of unlabelled nonspecific poly (dI:dC) oligonucleotides did not reduce the intensity of the bands (Figure 1C). In addition, the content of transcriptional complexes in GRE sequences was determined by the super-shift assay, using specific antibodies to the GR, and Fos. An anti-Fos antibody induced a marked super-shift/shift inhibition of the probe/protein complex for the GRE binding in the brain of middle-aged 12-month-old rats (Figure 1D), thus confirming that the Fos protein represents one of the components in the GRE transcription complex.

Reverse transcriptase-polymerase chain reaction assay was applied to measure the effect of age and DEX treatment on the level of the GR mRNA in the rat brain (Figure 2A and B) (treatment vs control, \( p < .001 \)). Densitometric analyses revealed a significant difference between control and DEX-treated animals (Figure 2B). There was a contrast between aged DEX-treated rats, in which the GR mRNA showed a 65% increase, and young animals (3 months old), where it was down-regulated by DEX. No significant changes in the GR mRNA levels were observed in age-matched control animals. Western blot analysis with anti-GR antibody was subsequently performed to determine protein levels of the GR in the brain cells isolated from young and old rats (3, 12, and 24 months old). Progressive ageing led to an increase in total GR protein level in brain nuclear extracts of control animals as shown in Figure 2D (12 vs 3 months, \( p < .05 \); 24 vs 3 months, \( p < .05 \)). Analysis of variance indicated statistically significant age-related changes (\( p < .01 \)). On the other hand, administration of DEX reduced the level of the GR protein in all age groups when compared with the corresponding controls (Figure 2D). However, no significant differences were found in the expression of the GR protein between 12- and 24-month-old rats after DEX treatment (Figure 2D).

Experiments investigating age-related changes in hormone responsiveness have been performed that aim to provide evidence that GRE binding activity might be involved in the age-related brain dysfunction. The present data indicate increased GRE binding activity in the brains of young (3 months) and aged (24 months) rats in contrast to a significant age-related decline in the brain of 12-month-old rats. The
GRE binding activity after DEX administration was decreased by 30% only in the group of young 3-month-old animals as compared with the age-matched controls. Unlike the control group, the GRE binding activity was increased in the group of middle-aged (12 months) and aged (24 months) rats after DEX treatment, indicating that aged animals are more susceptible to this hormone in terms of impaired glucocorticoid-negative feedback. At molecular and cellular levels, these alterations are compatible with the excessive activation of the HPA axis and hypersecretion of glucocorticoids, resulting in the morphological changes such as loss of neurons, following alternation of GR gene transcription and leading to further damage by elevated glucocorticoid hormone concentration (34,35).

Moreover, the analyses of the GR mRNA and protein levels were consistent with the levels of GRE binding since GR mRNA and protein also increased in aged animals after DEX treatment. Following glucocorticoid treatment, an increase in GR mRNA was observed during aging (12 and 24 months), which is most probably mediated via post-transcriptional mechanisms, such as an increase in mRNA stability (36). The results obtained by Western blot analyses are not in full agreement with the data obtained from both DNA binding and mRNA analysis. This difference was more obvious in DEX-treated animals, where the level of GR protein was lower compared with the control rats of all age groups. Our results were in accordance with earlier studies reporting that 5% of the GR mRNA and 80% of the GR protein were decreased upon chronic DEX treatment, either due to an inefficient translation of the GR mRNA or a decrease in the half-life of the GR protein (17,37).

AP-1 Binding Activity and the Effect of DEX During Ageing

Activating protein-1 is one of the best-characterized interaction partners of GR (25,38). It represents a heterodimeric transcription factor consisting of two subunits, Jun and Fos, and is activated upon phosphorylation. The transcription of GR-driven genes is in some cases induced by GR and inhibited by AP-1. This regulation is based on a direct physical interaction, as shown by immunoprecipitation of activated GR and AP-1 (25). In the present study, two different oligonucleotide sequences were used to evaluate AP-1-binding activity in the brain nuclear extracts; the first one contained the consensus AP-1 sequence present in promoter region of numerous genes, while the second probe corresponds to the AP-1 element reported in composite GRE elements (28).

For this purpose, gel shift analysis using brain nuclear extracts were performed on both AP-1 sequences. Analysis of the GRE AP-1 site (Figure 3A and B) demonstrated a maximum binding activity in the brain of 24-month-old rats. Almost negligible activity was observed in the brain of
12-month-old animals (24 vs 12 months, \( p < .01 \); 3 vs 12 months, \( p < .01 \)) (Figure 3B). Administration of DEX did not significantly affect the binding of proteins to the GRE AP-1 element (Figure 3B), regardless of animal age. Similar to the GRE element, the cross-competition experiments were performed to evaluate binding specificity of GRE AP-1 probe. Brain nuclear extracts of 24-month-old rats were examined for GRE AP-1 binding activity in the presence of radio-inert GRE or radio-inert GRE AP-1 sequences. As shown in Figure 3C, the retarded band was inhibited by unlabelled AP-1 oligonucleotide in a dose-dependent manner. However, the competition was much stronger in the presence of GRE oligonucleotides than that of the GRE AP-1 alone.

Electrophoretic mobility shift assay studies performed with consensus sequences showed that the AP-1 binding was constitutively present in nuclear brain extracts of both young and old animals, with inclination to be slightly elevated in aged rats (data not shown).

Together, these findings suggest that GR AP-1 transcriptional activity may be drastically reduced in middle-aged animals because of alterations in the level of constituent Fos and Jun family of proteins, as well as in the composition of AP-1 complexes (39). Our results also indicate that DEX treatment exerted no effect on the binding of the AP-1 transcription factor to the specific DNA sequence, but the possibility of a direct protein–protein interaction between GR and AP-1 cannot be excluded (19). In cross-competition experiments, GRE binding activity from brain nuclear extracts competed similarly with nonlabelled GRE and GRE AP-1. This result suggests that AP-1 may be one of the components of the transcription complex bound to the GRE sequence. The same results were obtained in cross-competition experiments for GRE AP-1 sequences, when the addition of a nonlabelled GRE probe at the lowest concentration led to a complete loss of the AP-1-specific binding. In super-shift assay, the addition of an anti-c-Fos antibody caused elimination of the transcriptional complex with GRE binding activity. Some researchers reported mutual transrepression of GR and Fos (18, 21); however, the exact mechanism is still unclear. One of the possible mechanisms could be that the activated transcription factors compete for a common interacting protein, probably for cyclic AMP response element binding protein (CREB) co-activator CREB-binding protein (40).

Conclusions

In summary, our results show that, in rat brain cells, there are age-specific alterations in DNA binding activity of different nuclear proteins. The diverse effects of DEX on GRE binding activity or GR mRNA and protein expression may help to answer the question of how glucocorticoids can enhance neuronal differentiation and survival, or, on the
other hand, how they can make neurons more vulnerable to insults with advancing age. This data emphasizes the importance of AP-1 activity in the regulation of GR gene expression during aging and provides evidence for age-related alterations of constituent proteins forming GRE AP-1 binding complexes. An understanding of GR regulation and GR interaction with other transcription factors is important for better comprehension of the mechanism of glucocorticoid hormone action and its role in neuronal plasticity, cognition, and neuroendocrine homeostasis during aging.

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REFERENCES