Glucocorticoid receptor polymorphism in genetic hypertension

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ABSTRACT

The Milan hypertensive strain of rat (MHS) displays abnormalities in both renal function and adrenocortical activity. While the pressor role of the former has been studied in detail, the role of the latter has not yet been clearly evaluated. In the present study, glucocorticoid receptor (GR) binding characteristics in liver cytosol from adult MHS and Milan normotensive controls (MNS) have been investigated. Dexamethasone, aldosterone and corticosterone were bound with lower affinity to cytosol of MHS rats compared with that of MNS rats. This pattern of binding could explain the raised plasma corticosterone concentrations and adrenocortical hypertrophy previously noted in MHS.

The coding sequence of MHS and MNS GR genes have been determined. The MHS gene differed in four respects from that of MNS: three

silent point mutations and a polymorphic micro-satellite region in exon 2. The latter polymorphism has been used in cosegregation studies of F₂ hybrids of MHS × MNS. The MHS GR genotype was associated with hypercalciuria and lower blood pressure in female rats and lower body weight in male rats. Although the effect on blood pressure is small, it is consistent with the affinity data. MHS GR genotype cosegregated with lower blood pressure in F₂ rats and displayed a lower affinity in binding studies.

In conclusion, GR polymorphism may be responsible for differences of adrenocortical function between MHS and MNS. This may lead to a reduction in the blood pressure difference between the two strains.

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INTRODUCTION

The Milan hypertensive strain of rat (MHS) presents altered renal function (Bianchi *et al.* 1986) and increased adrenocortical activity (Ferrari *et al.* 1985) when compared with its normotensive control strain (MNS). A significant portion of the MHS hypertension is sustained by the kidney (Bianchi *et al.* 1984) as demonstrated by a long series of observations, starting from studies of renal cross transplantation between the two strains (Bianchi *et al.* 1986). Recently, genetic studies have demonstrated that a point mutation within the α -subunit of a heterodimeric cytoskeleton protein, adducin, increases Na⁺/K⁺ ATPase activity when transfected into renal epithelial cells (Tripodi *et al.*

1996). This mutation also affects blood pressure, accounting for 40% of the difference in blood pressure between MHS and MNS, when interacting with a mutated β -adducin subunit (Bianchi *et al.* 1994).

The present study has considered whether the residual gap in blood pressure difference between MHS and MNS could be due to abnormal aspects of adrenocortical function. Previous studies have established that MHS exhibit adrenocortical hypertrophy with higher steroid secretory rates and raised plasma corticosterone concentrations compared with MNS (Stewart *et al.* 1993, Fraser *et al.* 1994, Ferrari *et al.* 1985). There are also signs of mineralocorticoid excess in MHS with extracellular volume expansion, plasma renin

suppression and increased exchangeable body sodium content (Fraser *et al.* 1994). Taken together, these observations might suggest that the normal negative feedback regulation of corticosterone synthesis is reduced because hormone access to the glucocorticoid receptor (GR) is impaired. This in turn raises plasma corticosterone concentrations causing activation of mineralocorticoid receptors. Clinically similar situations arise because of defects in steroid metabolism or because of intrinsic abnormalities of the GR (Lamberts *et al.* 1992, Arai & Chrousos 1994). Steroid metabolism has been shown to be different between MHS and MNS but not in a way which would impair corticosterone binding to adrenocorticosteroid receptors (Stewart *et al.* 1993). The present study has considered an alternative possibility, that GR function is affected. First, we have compared MHS and MNS glucocorticoid binding characteristics in liver cytosol extracts. Secondly, we have compared the sequences of cDNA for the GR genes of MHS and MNS. Thirdly, we have screened DNA from F₂ hybrids of crosses of MNS and MHS to determine whether an expressed microsatellite marker in the GR gene shows association with blood pressure.

MATERIALS AND METHODS

Animals

Rats used in receptor binding studies and for GR gene sequencing were obtained from the Field Station, University of Sheffield, UK and were maintained at a constant temperature on a 12 h light:12 h darkness cycle with free access to food and water. All F₂ rats used for genetic analysis were bred in our own facilities in Milan and maintained as described elsewhere (Bianchi *et al.* 1994). F₁ hybrids were produced by crossing MNS with MHS. No maternal or paternal effects were observed in average systolic blood pressures in F₁ hybrids. By intercrossing F₁ hybrids, an F₂ population was obtained, consisting of equal numbers of offspring from each reciprocal cross (251 total individuals, 121 male and 130 female).

GR binding

On separate occasions, pairs of MHS and MNS rats (female: 200–250 g; male 300–350 g) were stunned and decapitated. Livers were perfused with ice-cold isotonic saline via the hepatic portal vein to remove residual blood. Once blanched, livers were excised and minced in three volumes of ice-cold buffer (10 mM Tris-HCl, 2 mM dithiothreitol, 1.5 mM EDTA, 0.1 M sodium molybdate, 10% glycerol)

and homogenised using a Polytron homogeniser (Kinematic, Lucerne, Switzerland). The homogenate was centrifuged (20 000 *g*, 20 min) and the supernatant was centrifuged again (105 000 *g*, 1 h). Preparations were maintained at <5 °C throughout. The protein content of the final supernatant was determined by the method of Lowry *et al.* (1951).

Binding constants for dexamethasone, corticosterone and aldosterone (Sigma Chemical Company, Poole, Dorset, UK) were calculated by measuring homologous and heterologous competition for [³H]dexamethasone binding sites. Aliquots of cytosol (final protein concentration 2 mg/ml) were equilibrated overnight at 4 °C (except when investigating the effects of temperature) with 3 nM [1,2,4-³H]dexamethasone (1.44 TBq/mmol; Amersham International plc, Amersham, Bucks, UK) and various concentrations of non-radioactive ligand. Steroids were dissolved in ethanol at a concentration of 10 mM and serially diluted with buffer to give appropriate final concentrations. The total volume of incubation was 0.25 ml. Non-specific binding was measured by incubating cytosol with a 500-fold excess of non-radioactive dexamethasone. Free and bound fractions were separated by the addition of 0.25 ml of a charcoal suspension (5 g activated charcoal and 0.5 g Dextran T70/l buffer). After vortexing and centrifuging (1500 *g*, 10 min), the supernatants were removed and their radioactivity counted. Dissociation constants (*K_d*) and binding capacity (*B_{max}*) were calculated from heterologous competition curves with the curve fitting program, Ligand (Biosoft, Cambridge, UK), using information from equivalent homologous competition with dexamethasone.

The effects of temperature on receptor binding were assessed in two experiments. Receptor binding constants were estimated for dexamethasone after cytosol had been equilibrated at 37 °C for 30, 60, and 120 min with radioactive and non-radioactive dexamethasone as described above. To investigate the thermostability of the bound [³H]dexamethasone–receptor complex, cytosol was equilibrated at 4 °C overnight with [³H]dexamethasone (± 0.5 µM non-radioactive dexamethasone) and then transferred to 24 °C and 37 °C waterbaths. Aliquots were removed at intervals for separation of free and bound radioactivity.

GR genotype determination

Genomic DNA from F₂ rats was extracted from the tail according to a standard procedure (Laird *et al.* 1991). MNS and MHS GR microsatellites were amplified using a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT,

USA) in a 20 µl reaction volume. Genomic DNA (250 ng) was combined with 10–20 pmol of each primer and 1 U Taq polymerase (Bioline, Glasgow, UK) in a mixture containing 2 mM MgCl₂, 67 mM Tris–HCl (pH 8.0), 16 mM (NH₄)₂SO₄, 0.01% Tween-20 and 125 µM of each dNTP. Reactions were cycled 30 times at 94 °C for 50 s, 53 °C for 50 s and 72 °C for 90 s with a final extension at 72 °C for 5 min. DNA templates were initially denatured at 94 °C for 3 min. Primers for GR were based on published sequences (Miesfeld *et al.* 1986) and were designed to flank the N-terminal polyglutamine microsatellite producing overall PCR product sizes of 116 bp (MNS) and 113 bp (MHS). Sequences of PCR primers were 5'-AAAGGCTCCACAAGC AATGTG-3' and 5'-GACAGTGAAACGGCTT TGG-3' respectively.

PCR products were resolved on 6% polyacrylamide sequencing gels (19:1, acrylamide: bisacrylamide) at 50 W for 3 h in 1 × TBE buffer (0.09 M tris-borate buffer with 2 mM EDTA, pH 8.0). GR-specific sequences were identified after blotting onto a positively charged nylon membrane (Pall Biosupport Division, Portsmouth, Hants, UK) and hybridisation with appropriate PCR fragments which had been labelled with ³²P using a Random Primer Labelling Kit (Stratagene, La Jolla, CA, USA). Hybridisations were carried out in 10–20 ml sodium phosphate buffer (0.5 M pH 7.2) containing 7% SDS and 10 mM EDTA for 2–6 h at 55 °C. Membranes were washed with 100–125 mM sodium phosphate buffer containing 0.1% SDS at 55 °C and then autoradiographed.

GR sequencing

The coding sequence of MHS and MNS GR was determined by RT-PCR methods using total RNA extracted from 100 mg fresh liver tissues with RNazol (Biogenesis Ltd, Bournemouth, UK) according to the manufacturer's protocol B.

RNA/DNA hybrids used as templates in PCR reactions were generated using MMLV reverse transcriptase (RT) (Stratagene, Cambridge, UK). RNA (5–10 µg) was reverse transcribed from a GR 3'UT gene-specific primer, 5'-GTTGAACCA CATGGACTTGG<3', under conditions recommended by the supplier of the transcriptase. After 1 h at 39 °C, reactions were terminated by extraction with phenol/chloroform; aliquots of the supernatant were used for PCR.

PCR primers, based on the sequence of rat GR cDNA (Miesfeld *et al.* 1986) were designed to produce overlapping templates covering the entire GR coding sequence. Primer pair sequences were as follows:

rGR19-BIO, 5'>BIO-ATGTTTGACAGCTTA GGATCTTGCCAATGG ACTCCA AAGAA<3' (sense), rGR21, 5'>GTTTCAGAGCCCCCAAGG AAGT<3' (antisense); rGR31-BIO, 5'>BIO-TTC CTTCTCGAAGGGGACACG (sense), rGR28, 5'>TTGCCAGTTGTGACTGGAGTT<3' (antisense); rGR5-BIO, 5'>BIO-ATTTCAGCAAGC CACTGCAGGA<3' (sense), rGR2, 5'>CTATAA ACCACATGTACTGCG<3' (antisense).

Amplifications were carried out using an Omni-gene Thermal Cycler (Hybaid, Basingstoke, Hants, UK) in 25 µl reaction volumes by combining 2 µl RT product with 20 pmol of each primer and 2 U Taq DNA polymerase (Promega, Southampton, Hants, UK) in a mixture containing 1.5–2.0 mM Mg²⁺ (depending on the primer pair) and 0.01 mg/ml acetylated BSA. Reactions were cycled at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s for 30 cycles with a final extension at 72 °C for 5 min. RNA/DNA hybrid templates were initially denatured at 95 °C for 2 min prior to the addition of Taq polymerase.

Sequencing of PCR products was carried out directly using Dynabeads (Dynal Ltd, Merseyside, UK). Briefly, purified PCR product (250–500 ng DNA with 5'-biotin in a volume of 50 µl) was combined with Dynabeads and immobilised and denatured according to the manufacturer's specifications. Denatured single-stranded templates were sequenced from the reverse primers: 5'>GGG GGAGCAAAGTTCAATGAA<3', 5'>GGAGCA AAGCAGAGCAGGTT<3' and 5'>TACAAGA CAAATTGATAAGTT<3', using the Sequenase kit version 2.0 (Amersham International). Sequencing reactions were run on standard 6% polyacrylamide 8 M urea gels and exposed to Hyperfilm-MP for 16–18 h at room temperature.

Blood pressure measurements

At 3 months of age, under light halothane anaesthesia, a cannula was inserted into the carotid artery of F₂ rats and exteriorised at the back of the neck through a subcutaneous tunnel. The animals recovered within 5 min and 4 h later the cannulae were connected, without restraint, to a Gould BS 3200 blood pressure recorder. For each rat, average values for systolic and diastolic blood pressures and heart rate were calculated from simultaneous measurements taken at 1 min intervals over a 1 h period. The body weight was recorded just before surgery.

Urinary volume and calcium excretion

Rats were acclimatised to metabolism cages over 2 days; urine was collected for the following 24 h

FIGURE 1. Comparison of the effects of competing ligands for specific [^3H]dexamethasone binding sites in liver cytosol from MHS and MNS rats. Cytosol was equilibrated for 16 h at 4 °C. Values are means $\pm$ S.E.M., $n=7$.

and the total volume recorded. Urinary calcium concentration was measured by atomic absorption spectrophotometry (Perkin Elmer 1100B), in the presence of 0.2% LaCl₃; results are expressed as mmol calcium/24 h.

Statistical analysis

Results are expressed as means $\pm$ standard error of the mean (S.E.M.).

Data from receptor binding studies from MHS and MNS rats were compared using ANOVA for binding capacity and Kruskal–Wallis tests for K_d values; P values of <0.05 were considered significant.

Data from cosegregation studies were analysed by one-way ANOVA with Neumann–Keul's correction test for multiple comparisons (SPSS statistical package).

RESULTS

Competition for dexamethasone binding sites

All ligands tested in cytosol from both strains of female rat competed for specific [^3H]dexamethasone binding sites. Ranking, in terms of affinity (dexamethasone > corticosterone > aldosterone) was not different between strains. Competition curves for all three of the ligands are shown in Fig. 1. Both aldosterone and corticosterone appeared to compete less effectively in cytosol from MHS than in that from MNS rats. The competition curves for MNS are similar to those we have reported for other normotensive strains of rat (Panarelli *et al.* 1995,

FIGURE 2. Differences between MHS and MNS cytosol in binding affinities of various GR ligands (dissociation constant, left panel) and comparison of GR binding capacities ($B_{\max}$, right panel). Values are means $\pm$ S.E.M., $n=7$.

Soro *et al.* 1995). Scatchard analysis of dexamethasone binding indicated that, for both MHS and MNS cytosol, the data best fitted a one-site model with K_d and $B_{\max}$ values of 1.39 ± 0.15 and 1.0 ± 0.11 nM and 434 ± 33 and 382 ± 43 fmol/mg protein respectively. For all ligands, K_d values for MHS were greater than for MNS (Fig. 2). Differences between strains were greatest for the weaker ligands; compared with MNS K_d values, those for MHS were 1.39-, 2.19- and 4.12-fold greater for dexamethasone, corticosterone and aldosterone respectively; in respect of corticosterone this difference of affinity would be of physiological significance. In cytosol from male rats, strain differences in binding properties for dexamethasone were not apparent (Fig. 3) but the difference in affinity between MHS and MNS (6-fold; $P<0.05$) for corticosterone was similar. Pre-treating cytosol with charcoal extract did not significantly affect steroid binding properties in either strain; a 6-fold difference between strains in binding affinities for corticosterone ($P<0.05$; Fig. 3).

Effects of temperature on dexamethasone binding

When cytosol, pre-equilibrated overnight with [^3H]dexamethasone, was incubated at 24 °C, specific binding in buffer containing sodium molybdate remained constant; binding in MNS and MHS cytosol were both unaltered after 3 h (Fig. 4). When pre-equilibrated cytosol was incubated at 37 °C, specific binding declined rapidly, reaching a minimum after 90 min. The rate and extent of the decrease were not significantly different between strains. To test whether this decline in binding

FIGURE 3. Glucocorticoid binding constants in cytosol from male MHS and MNS rats: effects of pre-extraction with charcoal suspension. Values are means $\pm$ S.E.M., $n=4$. * $P<0.05$.

reflected a change in affinity and/or receptor concentration, binding characteristics were measured during long-term incubation at this higher temperature. Preliminary time course studies with rat liver cytosol (data not shown) indicated binding of [³H]dexamethasone had achieved equilibrium by 30 min. Differences between MHS and MNS binding constants at this time were not statistically significant (Fig. 5). Binding capacities after 30 min at 37 °C were similar to those after overnight incubation at 4 °C but the affinities were less. Figure 5 shows that the decreases in specific binding with prolonged incubation at 37 °C were due to both a decrease in affinity and a decrease in capacity. After 2 h, capacity of both MHS and MNS cytosol was reduced by more than 50% and K_d values were 6- to 7-fold higher. Decreases in

FIGURE 4. Thermostability of specific [³H]dexamethasone binding to cytosolic fractions from MHS and MNS livers. After overnight pre-equilibration at 4 °C with [³H]dexamethasone (± 0.5 mM non-radioactive dexamethasone) cytosolic extracts were warmed to 24 °C or 37 °C. Values are means $\pm$ S.E.M., $n=7$.

capacity could represent denaturation of the receptor although Western blotting studies (R P Heeley & C J Kenyon, unpublished data) indicate that the receptor is stable under these conditions. It is well established that heat shock proteins (HSP) are necessary for high affinity binding of GR ligands (Pratt 1993). Decreased affinity with prolonged incubation at 37 °C probably represents dissociation of HSP from the receptor complex. There were no significant differences between MHS and MNS in either capacity or affinity at any time point.

GR sequence

As noted previously (Heeley *et al.* 1998) the sequence of the cDNA of GR from a rat hepatoma

FIGURE 5. The effects of prolonged incubation at 37 °C on dexamethasone binding constants of cytosol fractions from MHS and MNS liver. Values shown are mean dissociation constants (K_d , left panel) and binding capacities (B_{max} , right panel) $\pm$ S.E.M., $n=7$.

cell line (6·10·2) differed in ten different places from that originally reported by Miesfeld *et al.* (1986). The sequence of cDNA of the GR gene from MNS was the same as that of the cDNA clone but the sequence of the GR gene of MHS differed in several respects. The trinucleotide CAG repeat in exon 2 that encodes the polyglutamine tract contained 20 glutamine codons in MHS and 21 in MNS. The MHS GR gene also showed three silent mutation nucleotides at positions, C198T, C531T and T708C corresponding to Phe66, Phe177 and Asp237 in the modulatory domain of the GR protein.

Associations between GR genotype and phenotypes

The trinucleotide repeat difference between MHS and MNS was used to genotype F_2 progeny of an MHS $\times$ MNS cross. There was no segregation of GR genotype with systolic pressure when the total F_2 population was analysed (ANOVA; Table 1). Comparing only homozygous animals, rats of MNS-GR genotype had higher systolic blood pressures than those of MHS genotype (MNS $\times$ MNS $150\cdot1 \pm 1\cdot8$ mmHg, $n=59$ versus MHS $\times$ MHS $145\cdot4 \pm 1\cdot3$ mmHg, $n=54$; $P=0\cdot04$). There was no significant association between GR genotype and diastolic blood pressure.

Interestingly, when the F_2 population was analysed by sex, female F_2 progeny, homozygous for the MNS GR allele, had significantly higher systolic blood pressures than either heterozygotes or those homozygous for the MHS allele ($P=0\cdot02$; Table 1). The body weight of male, but not female F_2 rats, was associated with GR genotype; MNS homozygotes were 11% heavier than MHS homozygotes ($P<0\cdot01$). In female, but not male F_2 progeny, MHS GR homozygotes excreted more calcium than MNS homozygotes; strain differences in calcium metabolism in Milan rats have been described elsewhere (Cirillo *et al.* 1989). Heart rate and urine volume were not affected by GR genotype (data not shown).

DISCUSSION

Previous studies of phenotypic differences between MHS and MNS indicated the primacy of renal

TABLE 1. Association of GR genotype with blood pressure, body weight and urinary calcium excretion (mean $\pm$ S.E.M.) in F_2 (MNS $\times$ MHS) rats

		GR genotype			P value
	Sex	MNS $\times$ MNS	MNS $\times$ MHS	MHS $\times$ MHS	
Systolic blood pressure (mmHg) (n)	Both	150·1 $\pm$ 1·8 (59)	147·7 $\pm$ 1·0 (132)	145·4 $\pm$ 1·3 (55)	NS
	Male	147·9 $\pm$ 2·7 (22)	149·1 $\pm$ 1·6 (68)	146·2 $\pm$ 1·9 (29)	NS
	Female	151·4 $\pm$ 2·4 (37)	146·3 $\pm$ 1·2 (64)	144·5 $\pm$ 1·7 (26)	0·02
Body weight (g) (n)	Male	439 $\pm$ 11 (21)	420 $\pm$ 5 (65)	406 $\pm$ 7 (29)	<0·01
	Female	276 $\pm$ 4 (36)	276 $\pm$ 3 (63)	279 $\pm$ 5 (26)	NS
Urinary calcium (mmol/day) (n)	Male	29·6 $\pm$ 4·8 (21)	24·0 $\pm$ 2·2 (59)	31·4 $\pm$ 3·8 (25)	NS
	Female	55·6 $\pm$ 7·6 (35)	63·8 $\pm$ 6·0 (63)	89·7 $\pm$ 8·9 (24)	<0·005

function in the control of blood pressure (Baer & Bianchi 1978, Bianchi & Ferrari 1983, Persson *et al.* 1985). Point mutations in the genes encoding a membrane skeleton protein, adducin, were identified, which appear to explain part of the difference in blood pressure between MHS and MNS (Bianchi *et al.* 1994). The possibility that an abnormality of GR function could account for the remaining difference in blood pressure and/or provide the genetic basis for other phenotypic differences between MNS and MHS is the subject of the present investigations.

Here we demonstrate: (i) evidence of impaired GR function in MHS liver cytosol; (ii) sequence differences in the genes encoding GR; and (iii) cosegregation of GR genotype with systolic pressure and body weight. These observations are discussed below.

Increased adrenocortical activity with hypertrophy of the adrenal cortex, increased secretory activity and raised plasma corticosterone concentrations have been identified in MHS compared with MNS (Mantero *et al.* 1983, Ferrari *et al.* 1985, Stewart *et al.* 1993, Fraser *et al.* 1994). Normally, feedback inhibition by corticosterone of pituitary adrenocorticotrophin (ACTH) secretion would be expected to correct overactivity of the cortex. However, if GR function is impaired, then higher than normal concentrations of corticosterone are required to maintain homeostasis. Thus in rodents and primates, naturally occurring or genetically engineered differences in receptor function are associated with abnormal patterns of hormone secretion (Mantero *et al.* 1983, Brandon *et al.* 1991, Hurley *et al.* 1991, Cole *et al.* 1993, Stewart *et al.* 1993, Keightley & Fuller 1994). We compared both ligand specificity and thermolability of GR, as well as binding capacity, in hepatic cytosol of MNS and MHS. Binding capacities were similar but affinities for dexamethasone, corticosterone and aldosterone were lower in MHS than MNS liver cytosol. The differences in affinity were least for the strongest ligand (dexamethasone) and greatest for the weakest ligand (aldosterone). It seems unlikely that the presence of endogenous steroids could account for differences in specificity since the difference remained after pre-extraction of cytosol with charcoal to remove endogenous steroids. Specificity and sensitivity of glucocorticoid hormone actions are controlled by a variety of factors including: (i) access of hormone to receptor, which is regulated by enzymes and by cell membrane steroid hormone transporters; (ii) a number of secondary proteins and other factors which, when complexed with the receptor, maintain a high affinity binding state and facilitate translocation to the nucleus or interaction

with glucocorticoid response elements on DNA; (iii) tissue-specific receptor expression; and (iv) the primary structure of the receptor protein. Most of these factors can be excluded as likely explanations of the present observations.

Lower hepatic 11 β -hydroxysteroid-dehydrogenase (11 β HSD) activity has been observed in MHS compared with MNS (Stewart *et al.* 1993). The liver isoform of 11 β HSD (type 1) favours the reduction of 11-dehydrocorticosterone (biologically inactive) to produce corticosterone (Jamieson *et al.* 1995). Reduced 11 β HSD in MHS liver would therefore limit access of corticosterone to receptor. However, reduced 11 β HSD activity is unlikely to be a cause of impaired receptor binding in MHS for two reasons. First, 11 β HSD is a microsomal enzyme which would not be active in the cytosolic extracts used here, particularly in the absence of added cofactors. Secondly, the ranking of the differences in affinity between MHS and MNS for the ligands tested (aldosterone > corticosterone > dexamethasone) does not match substrate specificity for 11 β HSD. For similar reasons, the involvement of plasma membrane steroid hormone transporters in the regulation of receptor binding in cytosol is unlikely. Previous studies (Stewart *et al.* 1993) and present observations of binding capacity do not indicate strain differences in tissue-specific expression of GR.

A common observation in clinical studies of steroid hormone resistance is that receptor binding is thermolabile (Werner *et al.* 1992). In these cases, affinity of the receptor is markedly decreased by prolonged incubation at high temperatures. This effect is probably due to dissociation of the receptor from HSP; high affinity binding is only observed when the receptor is complexed with other proteins including hsp90 and hsp70 (Pratt 1993). For example we have previously observed greater thermostability of the receptor from spontaneously hypertensive (SHR) compared with normotensive Wistar Kyoto (WKY) rats (Panarelli *et al.* 1995). In the present study, no such difference between MHS and MNS receptors has been observed.

The MHS GR cDNA sequence differs in four respects from that of MNS cDNA. There are three point mutations which are silent and therefore unlikely to be of phenotypic importance. The fourth difference concerns a trinucleotide (CAG) repeat region in exon 2 which encodes a polymeric glutamine region close to the N terminus of the GR. We have shown that this region is highly variable in wild and inbred strains of laboratory rats with CAG repeat lengths varying from 7 to 23; the MHS GR gene has one CAG repeat less (20) than the MNS gene (21). When expressed in CV-1 cells, full length

GR cDNAs with either 20 or 21 CAG repeats resulted in GR proteins which showed no difference in binding affinity for dexamethasone or corticosterone (Heeley *et al.* 1998). In some respects the MHS rat is similar to the New World primates, which generally show evidence of glucocorticoid resistance *in vivo*; white blood cells of the Squirrel monkey show impaired steroid receptor binding properties. However, cloned GR from this species shows no difference in binding properties when compared with cloned human GR despite evidence of several differences in gene sequence (Reynolds *et al.* 1997).

Mutations in the N-terminal region of steroid hormone receptors affect other receptor properties. For example, changing the amino acid sequence of rat GR from polyglutamine to a polyalanine region renders the receptor incapable of transactivation (Lanz *et al.* 1995). *In vivo* studies have also demonstrated that polymeric regions of genes are of functional importance in various diseases (Han *et al.* 1994). In Kennedy's disease, for example, a CAG repeat region in the gene encoding the androgen receptor (AR), a member of the superfamily of steroid/thyroid hormone receptors, is greatly expanded. This polyglutamine tract in AR, which is homologous with that in rat GR, causes androgen insensitivity in patients with Kennedy's disease due, in part, to subnormal transactivation and possibly also to reduced affinity for endogenous steroid hormones (Warner *et al.* 1992, Mhatre *et al.* 1993).

The presence of a microsatellite polymorphism in the GR gene of Milan rats represents a useful marker for analysing blood pressure and other phenotypes in cosegregation studies. GR genotype cosegregated with three variables, blood pressure, calcium excretion and body weight. The association of GR genotype with body weight might indicate a quantitative trait locus on chromosome 18. In SHR $\times$ WKY F₂ hybrids a gap junction protein locus on chromosome 18 has also been shown to cosegregate with body weight (Katsuya *et al.* 1995). Further studies with close flanking markers are required to show that the maximum effect is determined by GR polymorphism.

As has been described in patients with primary cortisol resistance (Lamberts *et al.* 1992, Arai & Chrousos 1994), an impaired GR might cause an increase in blood pressure secondary to raised plasma corticosterone concentrations acting on mineralocorticoid receptors. Given that MHS exhibit signs of excess mineralocorticoid activity (Fraser *et al.* 1994), our preliminary hypothesis was that MHS represented a similar genetic mutation of GR. Counter to our expectations, MHS GR genes were associated with lower not higher blood pressure. Although this observation effectively

excludes a role for GR in the aetiology of hypertension in MHS, questions remain as to whether and how the GR locus is a determinant of blood pressure and why females and not males are affected.

As with all association studies, the possibility that the GR locus is in linkage disequilibrium with another gene unrelated to GR function cannot be dismissed. Alternatively, given that glucocorticoid hormones affect blood pressure by a mineralocorticoid-independent mechanism (Tonolo *et al.* 1988), GR impairment could affect blood pressure more directly. A subtle impairment of GR properties, insufficient to raise plasma corticosterone levels beyond the capacity where mineralocorticoid receptors are protected from endogenous glucocorticoid hormones, might nevertheless reduce net glucocorticoid hormone and hence lower blood pressure. Against this hypothesis is evidence that GR impairment, at least in parental strains, is corrected through reduced feedback control of the hypothalamo-pituitary-adrenal axis. As discussed above, however, mutations in homologous regions of other steroid receptors affect transactivation as well as binding functions. Recent studies have suggested that interactions of GR transactivation domains with secondary proteins and other non-GR transcription factors can influence GR properties in a tissue-specific manner (Chrousos *et al.* 1993, Adcock *et al.* 1995, De Lange *et al.* 1997). The mechanisms of glucocorticoid-induced hypertension and relevant target tissues are not precisely known (Kenyon & Morton 1994). Altered GR interaction with other proteins may account for blood pressure and other phenotypes associated with MHS GR genes.

Phenotypic associations with MHS GR alleles differ in male and female rats; body weight is affected in males whereas variations of urinary calcium excretion and low blood pressure are traits which are more obvious in females. In clinical cases, sex-dependent variations in manifestations of primary glucocorticoid resistance are thought to be a consequence of increased adrenal androgen synthesis due to raised plasma ACTH (Arai & Chrousos 1994). In rats, however, adrenal androgen production is not as great and female sex steroid hormones are perhaps more important. Circulating levels of corticosterone, ACTH and corticosterone-binding globulin and responses to stress are markedly higher in female rats (McCormick *et al.* 1995). It is significant that female rats assume more male-like glucocorticoid-dependent variables after ovariectomy with lower corticosterone-binding globulin, altered steroid metabolism, lower plasma corticosterone, reduced adrenal weight and increased thymus and body weight (C J Kenyon,

J Noble, A E King & J Melville, unpublished observations). It follows that expression of impaired GR characteristics may also be differentially influenced by sex hormones which, in turn, could account for gender differences in the present association study.

In conclusion, we have sequenced the GR gene in MHS and MNS rats and demonstrated a polymorphism in a polyglutamine tract of the coding region of exon 2. Compared with MNS, MHS have impaired GR function such that the endogenous ligand, corticosterone, is bound with less affinity in hepatic cytosol fractions. It is not known whether GR polymorphism affects transactivation properties of the receptor. Cosegregation studies in a cross between MHS and MNS rats indicated that GR polymorphism is associated with differences in a number of phenotypes including systolic blood pressure. Further studies are needed to establish whether differences in the GR gene cause phenotypic differences or whether genes, in close linkage disequilibrium with the GR locus, are involved.

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