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Enhanced Activation of the Mineralocorticoid Receptor in Genetically Hypertensive Rats

M. Mirshahi, C. Nicolas, and M. K. Agarwal
CNRS and Inserm U-86, Centre Universitaire des Cordeliers, Paris, France

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The relative abundance and availability of the mineralocorticoid receptor (MCR) appeared to be similar in the heart, kidney and ocular tissues of the genetically hypertensive SHR and normotensive WKY rats by a number of criteria including Western blotting, immunoprecipitation, dot blot analysis, and immunohistochemistry. On the other hand, the activation of the MCR, as judged by binding to DNA cellulose, was significantly enhanced in the hearts and kidneys of 14 week-old, hypertensive, SHR rats compared to the normotensive WKY animals. The activation of the renal MCR was elevated in the SHR strain even at the age of six weeks when the tail arterial pressure was statistically identical to that of the WKY strain. Thus, precocious receptor activation may represent a primary lesion leading to hypertension in the SHR strain, thereby providing a new model to elucidate the hypertensive state.

The hypertensive disease in man stems from a set of multifactorial determinants whose primary tenets remain largely unknown (1–4). The disease imparts increased risk of myocardial infarction, stroke, renal failure and blindness (5,6). The physiopathological mechanisms apparently do not differ from population to population and even from continent to continent. Since increased fluid retention is a general feature of hypertension, it was logical to assume that an etiological search may most fruitfully be directed to delineating the key aspects controlling this complex process.

The regulation of ion and water balance in the mammal is accomplished via a whole variety of ion channels, exchangers and pumps in the cell membrane that are regulated by different hormones, metabolites and cofactors (7–10). Schematically, the vectorial Na^+ transport provides an electrical driving force for an anion, generally Cl^-, and the net salt movement drives water flow to maintain isotonicity (7–10).

In a number of tight epithelia, the rate limiting step of sodium entry at the apical membrane, and of sodium extrusion at the basal membrane, proceeds via specific proteins whose activity and/or synthesis is regulated by several cell signalling mechanisms, including the mineralocorticoid hormones (7–10). In this model, the steroid binds to its specific receptor in the cell and the complex thereafter bahaves as a transcription factor to modify the expression of selected, organ-specific, genes (7). Thus, qualitative and/or quantitative changes in the mineralocorticoid receptor (MCR) may alter the activity of proteins resposnible for cellular sodium exchange, thereby leading to hypertension.

We tested this hypothesis by studying various aspects of the MCR in selected organs of the genetically hypertensive SHR and the corresponding normotensive WKY rat strains. The data reported here show that the activation of the MCR was significantly and persistently enhanced in the SHR strain despite the fact that the abundance of the receptor appeared to be identical in both the SHR and the WKY animals. Thus, altered mineralocorticoid receptor function may well form an etiology of genetic hypertension.

MATERIALS AND METHODS

Animals and tissues. Male, hypertensive SHR/Nico and normotensive WKY/Nico rats (Iffa-Credo, France) were housed in a climate controlled animal house and maintained on laboratory food and water *ad libitum*. The blood pressure at 14 weeks of age, determined by the tail cuff method, was recorded as 230 mm and 141 mm, respectively, but it was comparable in both strains at the age of six weeks. Male, Wistar rats (Iffa-Credo) were uninephrectomized and maintained on 1 mg per day aldosterone and high salt per os for six weeks.

The animals were sacrificed under ether anesthesia, perfused with ice-cold saline, and processed either for histochemistry or the preparation of a cell extract. For cell cytosol, the organs were weighed and homogenized (w/v) in 20 mM Tris-HCl pH 8 containing 12 mM MTG - 10 mM KCl-10% glycerol-10 mM PMSF. A cell free extract was finally obtained by centrifugation at 105,000 × g (1 h, 4° C). Protein was quantitated by the method of Bradford (11–13).

Western blots. Tissue extracts (60 μg protein) were subjected to SDS-PAGE on 15% polyacrylamide gels and electrotransferred to nitrocellulose membranes. After 1 h at 37° C in presence of 15% fat free powder milk, the membranes were flooded for two h at 4° C with the anti-MCR serum diluted 1:250 in phosphate buffered saline (PBS) containing (per liter) 0.25g potassium dihydrogen phosphate,

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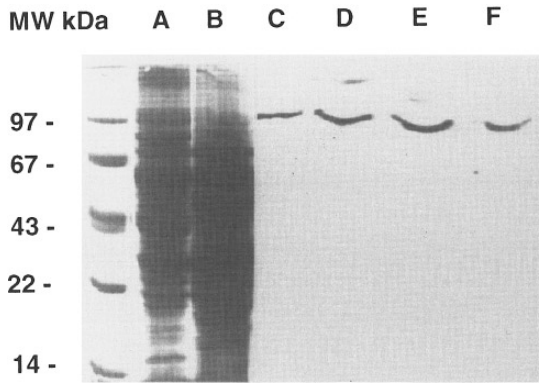

FIG. 1. Western blot analysis of the mineralocorticoid receptor from the hypertensive SHR and the normotensive WKY rat strains. Organ cytosol (60 μg protein) was denatured and electrophoresed by SDS-PAGE. The gels were colored with the Coomassie blue (A,B) or electrotransferred to nitrocellulose membranes for Western blotting with the anti-MCR antibody (C-F). A, D = WKY kidney; C = SHR kidney; B, F = WKY heart; E = SHR heart.

1.38g disodium phosphate, 0.25g KCl, 9g NaCl and supplemented with 1% bovine serum albumin (BSA). The blots were saturated with sheep anti-rabbit biotinylated antibody followed by streptavidin biotinylated horseradish peroxidase complex (Amersham) for 90 min each. After repeated washes with PBS, the membranes were developed for 10-20 min in dark with a mixture of methanol (10%), hydrogen peroxide (0.01%) and 4-chloro-1-naphthol (0.05%). The gels were calibrated with molecular weight markers (Biorad) and aligned with the blots (11–13).

The MCR content was quantitated on 2-fold serial dilutions of various cytosols (4.7 mg/ml) deposited as dots (2 μl/dot) on nitrocellulose membranes and developed by the anti-MCR antibody using the immunoperoxidase method (11–13).

Immunoprecipitation. Organ cytosol (0.3 ml) was incubated (2 h, 4° C) with 20 nM ^{3}H-RU 26752 alone or in presence of 2 mM of the radioinert ligand and unbound steroids were absorbed by the dextran-charcoal method. The samples (in triplicate) were brought to 0.3 M KCl and incubated for 3 h with 10 μg control or anti-MCR IgG, followed by 3 h incubation in presence of 100 μl of goat anti-rabbit IgG-agarose (Sigma), all at 4° C. The precipitates at 3000 × g were washed thrice with ice cold PBS and eluted once for 30 min with 1 ml 0.1 M glycine-0.15 M NaCl, pH 2.4. The eluate was counted in 10 ml Picofluor (Packard) as described before (11-15).

Receptor activation. Organ cytosol (0.3 ml) was incubated (2 h 4° C) with 20 nM ^{3}H-RU 26752 and charcoal treated to remove free steroids. Following 45 min in either melting ice or at 25° C, the samples were incubated with 0.3 ml DNA-cellulose (7 mg/g; Sigma) for 45 min at 4° C. After three washes (3000 g, 4° C) with the Tris buffer the pellets were extracted (40 min, 4° C) with 1 ml Tris buffer, containing 1 M KCl, and counted in 10 ml Picofluor (11-13).

Immunocytochemistry. Organs were fixed in Bouin's solution for 18 h followed by standard paraffin embedding. After removal of paraffin with toluene, 7 μm thick sections were hydrated in graded series of ethanol (100%-70%) and washed with PBS. Thereafter, the slides were covered sequentially with anti-MCR antiserum or the control serum(1/100), sheep antirabbit IgG biotinylated antibody (1/200), and streptavidin-FITC (Amersham), all diluted in PBS + 1% BSA. Each incubation step (60 min at room temperature) was followed by extensive washing with PBS and the slides were finally mounted in Fluoprep. Sections were photographed on a Kodak 400 ASA film under a Nikon fluorescent microscope (11-13).

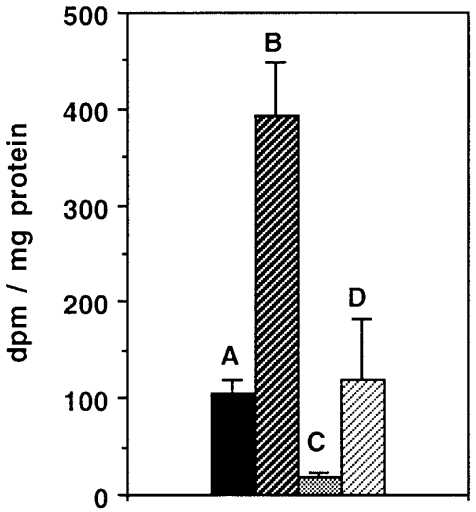

FIG. 2. Immunoprecipitation of the mineralocorticoid receptor from the organs of the hypertensive SHR rats. Immunoprecipitation of the MCR was carried out in the presence of control (A,C) or immune (B,D) IgG from SHR kidneys (A,B) and hearts (C,D) as described in Methods and (11-13). All values are average of three individual determinations ± the standard error.

Chemicals and reagents. The MCR was purified biochemically from rat kidney in the presence of RU 26752 that binds specifically to the MCR. This homogeneous antigen, resolved as a single band by SDS-PAGE, was used to generate a polyclonal antiserum in fawn Burgundy rabbits and its specificity for the MCR was assessed by a number of criteria including immunoprecipitation, macroaggregation, ELISA, Western blots, and cytochemistry. Rabbit serum, fractionated on a DEAE-trisacryl column to obtain the IgG fraction, exhibited the same MCR-specificity as the whole serum (14,15).

Sheep anti-rabbit biotinylated IgG (lot 21) and streptavidin biotinylated horseradish peroxidase complex (lot 82) were purchased from Amersham. Goat antirabbit IgG-Agarose (lot 77F-8985) and DNA cellulose (lot 54H7095) were obtained from Sigma. Tissues from uninephrectomized rats, maintained on aldosterone and high salt, were kindly provided by Inserm U-199. ^{3}H-RU 26752 (7 α propyl spironolactone: 50 Ci/mM, lot 3025 A) and the corresponding radioinert steroid were furnished by Roussel-Uclaf, Romainville.

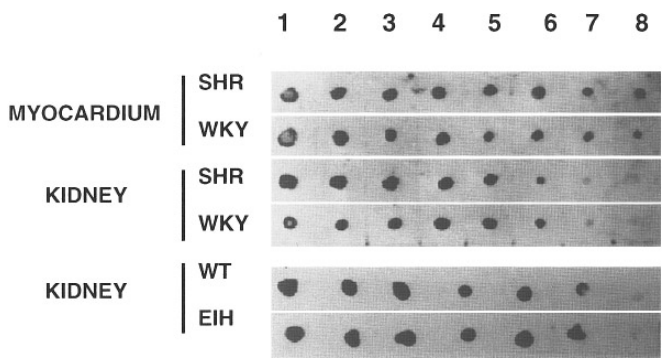

FIG. 3. Semiquantification of the mineralocorticoid receptor by the dot blot procedure. The MCR content was quantitated on 2-fold serial dilutions of various cell extracts (4.7 mg/ml protein) deposited (2 μl per dot) on nitrocellulose membranes and revealed by the anti-MCR antibody using the immunoperoxiade method. SHR = genetically hypertensive, WKY = genetically normotensive; EIH = experimentally-induced hypertensive and WT = sham operated Wistar rats.

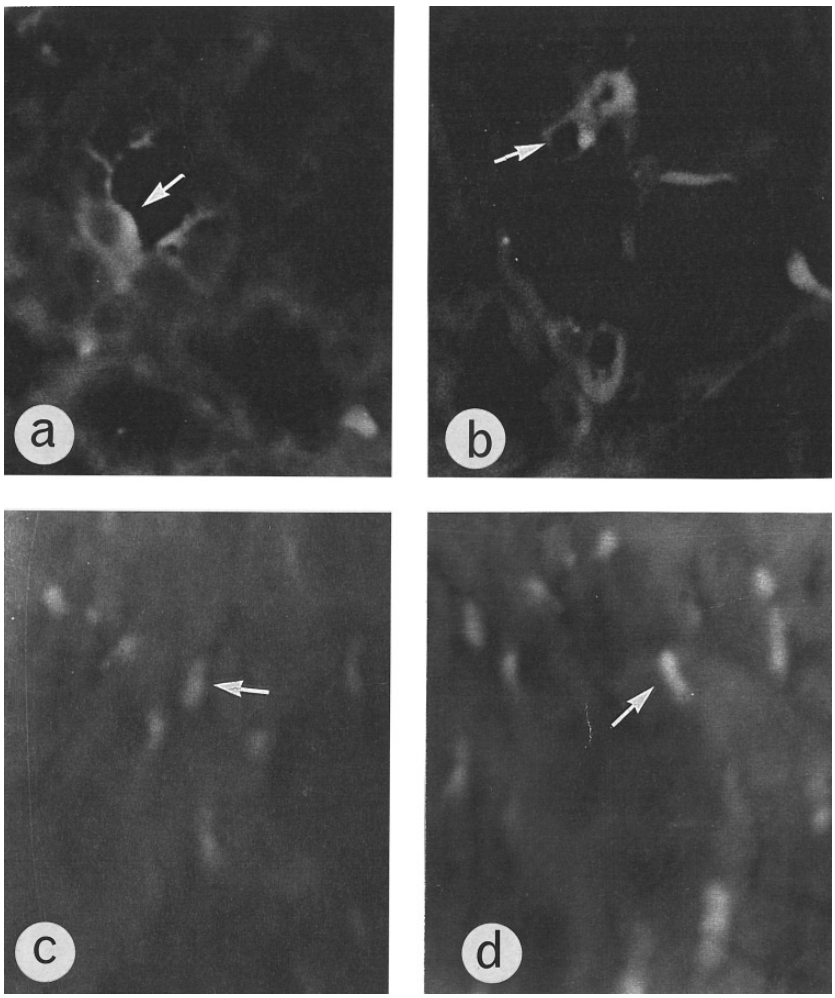

FIG. 4. Analysis of the mineralocorticoid receptor by immunofluorescence from the hypertensive SHR and the normotensive WKY rats. The MCR was revealed by immunofluorescence as in Methods and (11-13). SHR kidney (a); WKY kidney (b); SHR myocardium (c); WKY myocardium (d).

RESULTS

Data in Fig. 1 show a single band in the 98-102 kDa region whose abundance appeared to be similar in the hearts and kidneys from SHR/Nico and WKY/Nico rats (mean arterial pressure at 14 weeks = 230 mm and 141 mm, respectively). This molecular weight corresponds to that of the MCR as detailed in previous studies (7,11–13). Immunoprecipitation of the ^{3}H-RU 26752-MCR complex from kidneys of 14 week-old animals further confirmed the identity of the renal MCR in the hypertensive SHR strain (Fig. 2). In the presence of the immune IgG, the amount of radioactivity in the precipitate increased to 393 and 117 dpm/mg protein compared to the control level of 107 and 19 dpm/mg protein from the kidneys and hearts, respectively, of the SHR rats ($p < 0.001$ in all cases). similar results were obtained with the normotensive WKY rats (not shown).

Semiquantitation of the MCR by the dot blot procedure suggested that the abundance of the MCR was nearly identical in the hearts and kidneys from normotensive and hypertensive rats (Fig. 3). Similar results were obtained in the kidneys of rats rendered hypertensive by uninephrectomy and maintained on aldosterone and a high salt diet (Fig. 3). Thus, the hypertensive state is apparently not associated with quantitative modifications in the organ MCR. This was confirmed by immunohistochemistry since the MCR-specific immunofluorescence in the epithelial cells of the renal distal tubule and the cardiac myocytes was largely cytoplasmic in both strains, and qualitatively very similar (Fig. 4). Thus, enhanced nuclear uptake of the MCR could not be held responsible for genetic hypertension. Similar results were obtained with the ocular tissues (data not shown) despite their marked sensitivity to the hypertensive disease (12,13). Data in Fig. 5 show considerable cardiac sclerosis in uninephrectomized

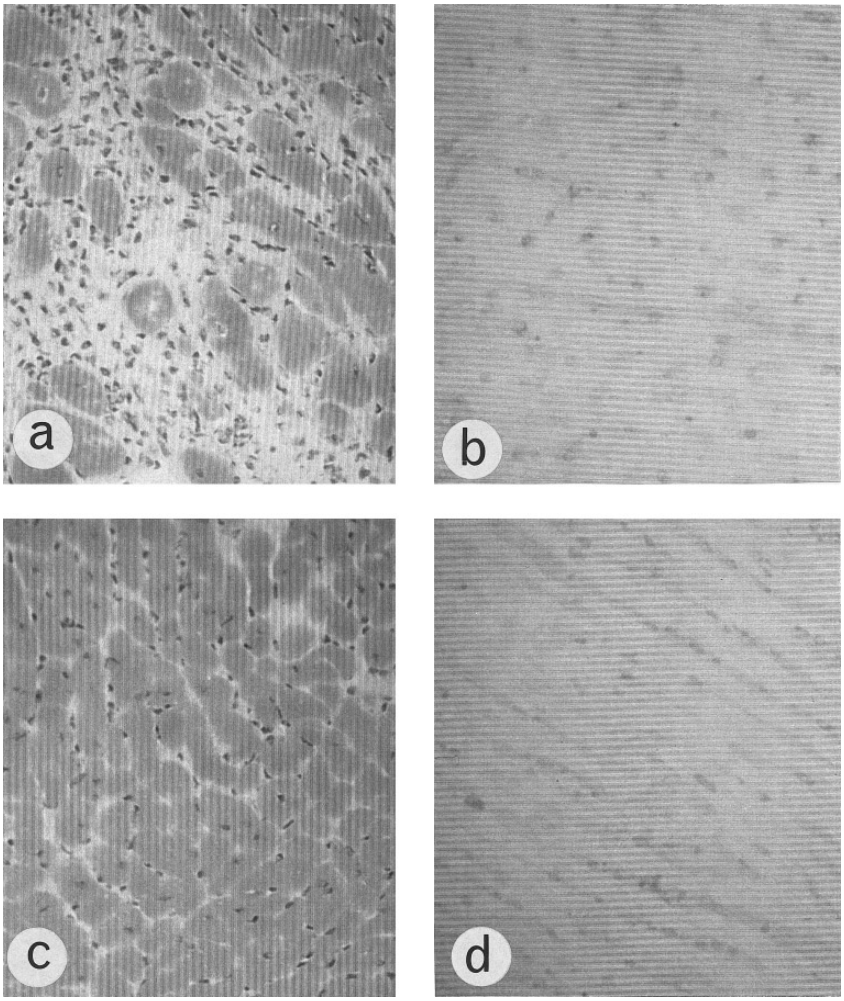

FIG. 5. Immunohistochemical analysis of the mineralocorticoid receptor in experimentally hypertensive rats. Immunofluorescence was used to reveal the MCR in the myocardium of Wistar rats following sham operation (d) or uninephrectomy (b); necrosis was clearly evident after Giemsa staining in hypertensive rats (a) compared to the sham control (c).

rats maintained on aldosterone and high salt per os, compared to the sham counterparts. Again, the MCR-specific immunoreactivity in experimentally hypertensive and control rats appeared to be very comparable despite a disruption of cell architecture in the former series; similar results were obtained with the kidney (not shown).

Functional analysis of the MCR was finally attempted by quantitating the binding of the radioligand-receptor complexes to the DNA cellulose resin in vitro—a technique believed to reflect the binding of the ligand-receptor complex to the proximal promoter in vivo (7). In 14 week old hypertensive rats, the amount of radioactivity bound to the DNA cellulose increased significantly ($p < 0.001$) after exposure of ^{3}H-RU 26752-SHR kidney cytosol mixtures to 25° C for 45 min (Fig. 6) compared to those left at < 4° C (127 and 43 dpm/mg protein, respectively). Heat activation was also observed with kidney cytosol from the normotensive WKY strain (19 and 57 pm/mg protein at < 4° C and 25° C, respectively; $p < 0.001$). However, the extent of activation was much greater with SHR kidney cytosol compared to the WKY kidney cytosol (127 and 57 dpm/mg protein = $p < 0.004$) such that the activated level in the WKY kidney (57 dpm/mg) was statistically similar to the constitutive level in the SHR kidney (43 dpm/mg). Similar results were obtained with cardiac cytosol (not shown). In six week old WKY rats (Fig. 7), the renal MCR could not be activated significantly (222 and 287 dpm/mg at < 4° C and 25° C, respectively) contrary to the situation in the SHR strain where the activation of the kidney MCR progressed satisfactorily (333 and 590 dpm/mg = $p < 0.001$), despite the fact that the tail arterial pressure in both strains was comparable at this age. Indeed, the basal, constitutive level (< 4°C) of the MCR binding in the SHR kidney (333 dpm/mg) was statistically similar to the activated level (25° C) in the WKY kidney (287 dpm/mg). Thus, precocious

and persistent activation of the MCR may well lead to the hypertensive state in SHR rats.

DISCUSSION

In genetically hypertensive rats, BP/SP-1 and BP/SP-2 genes are believed to be responsible for blood pressure regulation (16). The onset of rodent hypertension is associated with increased production of the mineralocorticoid deoxycor-ticosterone (DOCA) and its metabolites (17,18), is blocked by the antiminera-locorticoid RU 28318 (19), and may stem from altered hepatic metabolism of aldosterone (20). Similarly, the pathogenesis of human endocrine hypertension has been linked to at least three genes whose mutations may result in (1) ectopic production of the endogenous mineralocorticoid aldosterone by the synthase; (2) constitutive activation of the β subunit of the apical sodium channel (Liddle syndrome); (3) angiotensin dysfunction or essential hypertension (21,22). Aldosterone overproduction from adrenocortical adenoma (Conn's syndrome) and idiopathic nodular hyperplasia account for 65% and 34% clinical incidents of hypertension, respectively. Biochemically, increased plasma aldosterone correlates well with the urinary excretion of the corresponding metabolites (23).

In one experimental model of hypertension, the antimineralocortcioid RU 26752 reversed the increased saline consumption, urine output and decreased urinary excretion obtained in response to aldosterone (24); this was confirmed by another MCR-specific antagonist ZK 91587 (25). On the other hand, the antiglucocorticoid RU 38486 prevented dexamethasone-induced hypertension but did not influence the disease produced by DOCA (26,27). These and numerous other considerations suggest that the receptor mediated action of both glucocorticoids and mineralocorticoids is apparently important in the general control of blood pressure and the maintenance of the vascular tone.

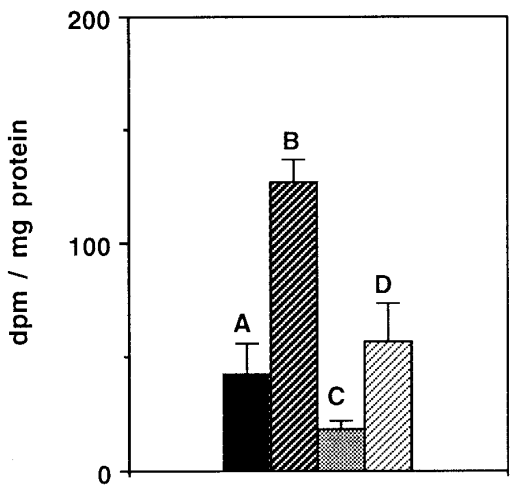

FIG. 6. The activation of the mineralocorticoid receptor during hypertension in 14 week old SHR rats is significantly increased over that in the WKY rats. The binding of ^{3}H-RU 26752-renal MCR complex to DNA-cellulose was assessed at $< 4°C$ (A,C) and after 45 min at 25° C (B,D); A,B = SHR; C,D = WKY. All values are average of three individual determinations ± the standard error.The significance of difference between mean values was determined by the *t* test. A vs B and C vs D = $p < 0.001$; B vs D = $p < 0.004$; A vs D = not significant.

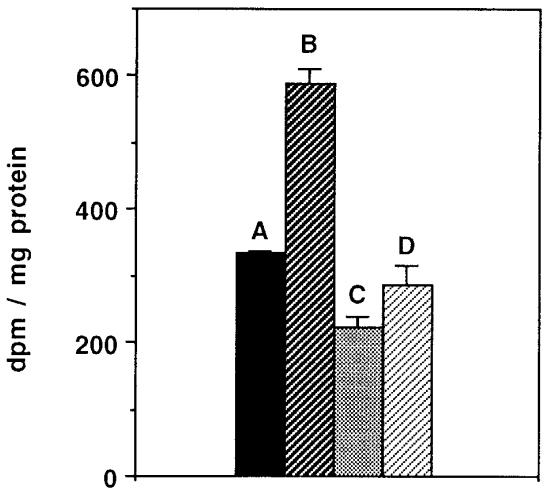

FIG. 7. The mineralocorticoid receptor in 6 week old SHR rats is hyperactive even in the absence of elevated tail blood pressure. The binding of ^{3}H-RU 26752-MCR complex to DNA cellulose from SHR (A,B) and WKY (C,D) kidneys was assessed at either $< 4°$ C (A,C) or 25° C (B,D). All values are average of three individual determinations ± the standard error. The significance of difference between mean values was determined by the *t* test. A vs B and B vs D = $p < 0.001$; A vs D = $p < .057$; C vs D = $p < .033$.

The studies reported here provide experimental support for the notion that MCR function may indeed be important in the genesis of hereditary hypertension. A constitutively active MCR since the age of six weeks or earlier (when the tail pressure is normal) may progressively exacerbate those mechanisms (channels, pumps) that would favor salt retention, leading to the hypertensive state within the next 6-8 weeks (about 14 weeks after birth). Thus, superactivation of the MCR should be kept in mind as a possible etiology of certain forms of hypertension. Further work is obviously required to delineate the molecular basis of this sort of MCR hyperfunction. If such a receptor mutation were to exist in the human disease, it would be important to look for a convinient marker for timely diagnosis and possible prevention of an adverse prognosis by MCR-specific steroid antagonists (7). These open a new lead to delineate the physiopathology of the hypertensive disease.

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