Enhanced expression of EGF receptor in a model of salt-sensitive hypertension

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Nephrology Research and Training Center, Comprehensive Cancer Center, and Cell Adhesion and Matrix Research Center, Division of Nephrology, Departments of 1Medicine and 2Physiology and Biophysics, University of Alabama at Birmingham, and Department of Veterans Affairs Medical Center, Birmingham, Alabama

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Ying, Wei-Zhong, and Paul W. Sanders. Enhanced expression of EGF receptor in a model of salt-sensitive hypertension. Am J Physiol Renal Physiol 289:F314–F321, 2005. First published April 12, 2005; doi:10.1152/ajprenal.00003.2005.—Chronic kidney disease in the Dahl/Rapp salt-sensitive (S) rat is related to an arteriolopathic process that occurs following the onset of hypertension and involves vascular smooth muscle cell (VSMC) hyperplasia and luminal constriction. Because previous studies have shown that activation of the epidermal growth factor receptor (EGFR) produces a mitogenic stimulus in VSMC and the EGFR participates integrally in the vasconstrictor responses of renal arterioles, the present study analyzed the expression of EGFR in these animals. Compared with Sprague-Dawley (SD) rats, renal cortical expression of EGFR was increased in both prehypertensive and hypertensive S rats. Immunohistochemistry using a polyclonal antibody to EGFR demonstrated that EGFR expression was prominent in the renal vasculature, particularly in the media of afferent and efferent arterioles and the aorta of S rats. When examined, primary cultures of VSMC from S rats showed increased expression of EGFR, compared with VSMC from SD and Dahl/Rapp salt-resistant rats. Following addition of EGF, autophosphorylation of the EGFR was enhanced in cells from S rats, as was the downstream signaling events that included activation of p42/44 MAPK and Akt pathways. Thus in vivo and in vitro studies demonstrated augmented expression and functional activity of the EGFR in S rats.

vascular smooth muscle; chronic kidney disease

CHRONIC KIDNEY DISEASE is one of the severe complications of arterial hypertension. It is estimated that 5.6 million individuals in the US population have elevated serum creatinine concentrations and 70% of these are hypertensive (11). Unfortunately, the incidence of end-stage kidney disease attributed to hypertension continues to increase (31). The risk of progressive renal failure is directly related to the degree of blood pressure elevation (11) and blood pressure reduction appears to decrease the rate of loss of kidney function (23), but other factors appear to play important roles in disease progression. For example, the risk of development of end-stage renal disease is greater in black, compared with non-black, hypertensive patients (23, 25). Recent evidence from animal models of hypertension supports a genetic basis for susceptibility to end-organ kidney damage (2, 9, 10, 12, 24). While the genes responsible for development of hypertensive renal disease have not been elucidated, the pathological changes that typically occur represent an arteriolopathic process, and it is logical to hypothesize that the responsible genetic alterations affect the renal vascular response to hypertension.

The Dahl/Rapp salt-sensitive (S) rat is an interesting genetic model of salt-sensitive hypertension. When fed a diet high in salt content, these rats rapidly and uniformly develop hypertension. They are also exquisitely sensitive to end-organ kidney damage from hypertension. Within 4 wk of development of salt-sensitive hypertension, S rats demonstrated severe reductions in glomerular filtration rates; prevention of hypertension preserved renal function (8). Analysis of the vasculature demonstrated progressive luminal narrowing and thickening of the medial layer of the interlobular arteries and preglomerular arterioles of S rats made hypertensive by a high-salt diet. In addition to a progressive increase in the numbers of nuclei in the medial layer, immunohistochemical analyses showed nuclear accumulation of proliferating cell nuclear antigen (PCNA) and 5-bromo-2'-deoxy-uridine (BrdU) in smooth muscle cells of the medial layer of the kidney resistance vessels, compared with the groups of Sprague-Dawley (SD) rats on 0.3 and 8.0% NaCl diets and S rats maintained on 0.3% NaCl diet. Associated with luminal narrowing was an increase in markers of tissue hypoxia in the kidney parenchyma (34). These data demonstrated a disorder of the vascular remodeling process with proliferation of vascular smooth muscle cells temporally followed by development of tissue hypoxia in the hypertensive nephropathy of S rats on 8.0% NaCl diet.

This laboratory recently performed a mini-array analysis of steady-state mRNA in the kidneys of hypertensive S rats and observed a striking increase in expression of the epidermal growth factor receptor (EGFR). The purpose of the present study was to analyze expression of EGFR before and during the development of hypertension.

METHODS

Animal preparation. The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved the project. Studies were conducted using 46 male SD, 46 S, and 4 Dahl/Rapp salt-resistant (R) rats. The rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) and were 28 days of age at the start of study. The protocol that was followed has been standardized in our laboratory (36–38). The rats were housed under standard conditions and given formulated diets (AIN-76A, Dyets, Bethlehem, PA) that contained 0.3 or 8.0% NaCl. These diets were prepared specifically to be identical in protein composition and differed only in NaCl and sucrose content. The rats were studied at baseline and days 7, 14, and 21 of the study. The rats were anesthetized by intraperito-
Day 0

Northern

S (0.3%)  SD (0.3%)

EGFR

MW ($10^3$)

250  150  100  121  79  41

GAPDH

Western

Fig. 1. Northern and Western analyses of epidermal growth factor receptor (EGFR) expression in kidney cortex of Dahl/Rapp salt-sensitive (S) and Sprague-Dawley (SD) rats maintained on 0.3% NaCl diet before entry into the study ($n = 6$ rats in each group). Both steady-state mRNA and protein levels were increased ($P < 0.05$) in S rats compared with SD rats.

Vascular smooth muscle cell culture. Primary cultures of vascular smooth muscle cell (VSMC) were established by pooling thoracic aortas from four prehypertensive, 28-day-old rats in each group (SD, R, and S) using standard enzymatic digestion techniques and culture conditions (5, 40, 42). The cells were grown in DMEM (Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum in a humidified 5% CO$_2$-95% air atmosphere. VSMCs were used between subpassages 4 and 6.

Northern blot analysis. Total RNA from kidney cortex or from isolated VSMC was obtained by the single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction. Fifteen micrograms of total RNA from each sample were electrophoresed in 1.2% agarose gels containing 2.2 M formaldehyde and 0.2 M MOPS, pH 7.0, then transferred to a nylon membrane. EGFR was detected using a cDNA probe, which was labeled with digoxigenin-11-dUTP using a kit (DIG-High primer, Roche Applied Science, Indianapolis, IN). The cDNA for rat EGFR was produced by subcloning a PCR product obtained using the primer pairs 5'-CCGGAATTCTCATC-CAGTGCCATCCAGGATG-3' (upstream) and 5'-CCGCTCGAG-TGCCAATGCTCCTGAACCC-3' (downstream); the DNA sequence of the amplified product was confirmed. Membranes were hybridized in DIG Easy Hybridization Buffer with DIG-labeled probes at 54°C overnight. After hybridization, membranes were finally washed in 0.1x SSC/0.1% SDS. Bound probes were detected using alkaline phosphatase-conjugated anti-DIG antibody and CDP-
Western blot analysis. Tissue was placed in chilled lysis buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.0% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM PMSF) and homogenized using a tissue homogenizer (Omni-Mixer 17105, Omni, Waterbury, CT). After several passages through a 26-gauge needle, the homogenates were centrifuged at 20,000 g for 45 min and the
supernatant fractions were collected. VSMCs were pelleted and the proteins were solubilized in Laemmli sample buffer. Total protein concentration was determined using a kit (Micro BCA Protein Assay Reagent Kit, Pierce, Rockford, IL). Western blot analysis proceeded in standard fashion, using samples that contained 60 μg of total protein. Proteins were separated by SDS-PAGE under reducing condition and were blotted onto nitrocellulose membranes. The membranes were blocked in 10% fat-free milk before incubation with antibodies to EGFR, phospho-EGFR (at Y845, Y992, and Y1068), Akt, phospho-Akt (at position S473), p42/44 MAPK, and phospho-p42/44 MAPK (all from Cell Signaling Technology, Beverly, MA), and β-actin (Sigma, St. Louis, MO), which was used to confirm loading of comparable amounts of protein in each lane. After incubation with horseradish peroxidase-conjugated secondary antibody, bands were detected using chemiluminescence (Pierce).

Immunohistochemistry. Immunohistochemistry was performed in standard fashion (34, 39, 41) using paraffin-embedded aortic and kidney tissues from 12 S and 12 SD rats fed diets containing either 0.3 or 8.0% NaCl (n = 6 in each group) for 21 days. EGFR was detected using a commercial antibody (EGF Receptor Antibody, Cell Signaling Technology) and Vectastain ABC Kit (Vector Laboratories, Burlingame, CA).

FACS analysis. Following treatment with EGF (100 ng/ml) or vehicle, VSMCs were detached using PBS containing 2.2 mM EDTA and 0.2% BSA. After being washed with staining buffer consisting of PBS containing 2% BSA and 0.2% NaN₃, cells were fixed and permeabilized in a Cytofix/Cytoperm solution (Becton-Dickinson, Franklin Lakes, NJ). For immunostaining, 1 × 10⁶ cells were incubated with antibodies directed against EGFR, phospho-EGFR (Y845, Y992, Y1068), Akt, phospho-Akt (at position S473), p42/44 MAPK, and phospho-p42/44 MAPK in a final concentration of 1–5 μg/ml for 30 min at 4°C, then washed and stained with propidium iodide or FITC-conjugated secondary antibodies. Fluorescence was acquired using a FACS Calibur Flow Cytometer (Becton, Dickinson and Co.). Data were analyzed with Cell Quest software.

Detection of EGFR activity in culture. Subconfluent VSMCs were incubated in serum-free DMEM for 14 h, then human recombinant EGF (100 ng/ml) was added to medium for 10 min. Cells were washed and harvested for Western analysis to detect phospho-EGFR at Y845, Y992, and Y1068 (Cell Signaling Technology). To determine the effect of EGF on intracellular signaling events in the Akt and MAPK pathways, cell lysates were also used for Western blot analyses using antibodies to phospho-Akt (S473), Akt, phospho-p42/44 MAPK, and p42/44 MAPK for up to 6 h following addition of EGF. In some experiments, VSMCs were pretreated with 10 μM LY-294002, a phosphatidylinositol 3-kinase inhibitor (13), for 1 h just before incubation with the EGF (100 ng/ml).

Statistical analysis. All data were presented as means ± SE. Significant differences among data sets were determined by unpaired t-test or by ANOVA with standard post hoc testing (Statview, version 5.0, SAS Institute, Cary, NC), where appropriate. A P value < 0.05 assigned statistical significance.
RESULTS

Renal cortical expression of EGFR was increased in both prehypertensive and hypertensive S rats. Previous studies demonstrated that young S rats maintained on 0.3% NaCl are normotensive but rapidly develop hypertension when placed on a diet that contains 8.0% NaCl; mean blood pressures of SD rats did not change with an increase in dietary salt intake (6–8, 34, 38). Before the development of hypertension, both steady-state mRNA and protein levels of EGFR of the kidney cortex of S rats were greater ($P < 0.05$) than levels observed in the
kidney cortex of SD rats (Fig. 1). Compared with tissue from SD rats, the approximate twofold increased ($P < 0.05$) amounts of EGFR in the kidney cortex of S rats persisted over the course of the experiment (Figs. 2 and 3). While dietary salt did not affect renal cortical expression of EGFR in SD rats, the development of salt-sensitive hypertension promoted further increases in cortical EGFR in S rats (Fig. 3).

Vascular smooth muscle of S rats expressed more EGFR than SD rats. By light microscopy, kidney morphology of SD rats on both diets and S rats on 0.3% NaCl diet for the duration of the experiment demonstrated no significant abnormalities. In contrast, renal morphological changes were prominent in the hypertensive S rats. Tubular atrophy with tubular epithelial cell dropout, dilated tubular lumens, and cast formation was present. The media of the small arteries and particularly the arterioles, which were indistinct in the kidneys of SD rats, were thickened with constricted lumens in the kidneys of untreated, hypertensive S rats. These findings were consistent with previous studies (8, 34). Immunohistochemistry using a polyclonal antibody to EGFR demonstrated that EGFR expression was prominent in the renal vasculature, particularly in the afferent and efferent arterioles of S rats on either diet (Fig. 4). EGFR was also detected in tubular epithelial cells, which have been shown to possess receptors for EGF (1, 17, 19, 20), and in the extraglomerular mesangium, but not in the glomerulus, using this technique (Fig. 4E). Immunohistochemical analysis of aortic tissue demonstrated expression of EGFR particularly in smooth muscle cells in the media of S rats on both diets (Fig. 5, A and B). The increased expression was confirmed using Western blotting (Fig. 5). Mean densities of the EGFR band relative to β-actin of S rats maintained on 0.3% NaCl (0.47 ± 0.02) and S rats on 8.0% NaCl (0.54 ± 0.01) were greater ($P < 0.05$) than mean densities of SD rats on either 0.3% NaCl (0.25 ± 0.01) or 8.0% NaCl (0.32 ± 0.02).

Primary cultures of VSMC from aortic tissue of S, SD, and R rats were established in standard fashion and examined between subpassages 4 and 6. Mean density of mRNA of EGFR relative to GAPDH of VSMC from S rats (0.60 ± 0.04) was greater ($P < 0.05$) than the mean relative density of EGFR of VSMC from SD rats (0.30 ± 0.04; Fig. 6). Using Western blotting and FACS analysis, the amount of EGFR in VSMC from S rats was also greater ($P < 0.05$) than EGFR present in VSMC from SD rats (Fig. 6). By Western blot analysis, the amount of EGFR in VSMC from R rats did not differ from VSMC from SD rats and was less ($P < 0.05$) than that seen in S VSMC (data not shown).

Signal transduction events induced by EGF were amplified in VSMC of S rats. Ligand binding promotes EGFR homodimerization, which is required for autophosphorylation of the receptor and subsequent generation of intracellular signaling events (21, 28, 29). Autophosphorylation of the EGFR was examined using Western blotting and FACS analysis following administration of EGF. Using both techniques, increases ($P < 0.05$) in tyrosine phosphorylation at multiple sites (Y845, Y992, and Y1068) in the EGFR were observed in VSMC from S rats, compared with VSMC from SD rats (Fig. 6). By Western blot analysis, tyrosine autophosphorylation of the EGFR in response to EGF did not differ between VSMC from SD and R rats (data not shown). Activation of the p42/44 MAPK pathway, which was demonstrated by phosphorylation of p42/44 MAPK, was prolonged in VSMC from S rats following addition of EGF (Fig. 7). Akt activation, which was demonstrated using antibodies that specifically recognized the phosphorylated form of Akt, was similarly prolonged in VSMC from S rats following addition of EGF (Fig. 8).

DISCUSSION

A sustained increase in blood pressure in S rats results in progressive increases in wall thickness of arteries and arterioles of the kidney; intraluminal narrowing of interlobular arteries and preglomerular arterioles occurs early in the course of the process (34). Renal ischemia ensues and coincides with the decline in glomerular filtration rate and tubular epithelial cell apoptosis, producing tubular atrophy and dilatation with occasional intraluminal cast formation (8, 27, 34, 35, 41). These previous studies emphasize the prominent role of a vascular disorder in the development of hypertensive renal disease in S rats and is a focus of the present study. In a brief report, Swaminathan and Sambhi (30) examined Dahl salt-sensitive (DS) and salt-resistant (DR) rats maintained on a high-salt diet for 4 wk. At a time when hypertension was severe, these investigators (30) observed increased binding of radiolabeled epidermal growth factor to homogenates of kidney and aortic tissue from DS, compared with DR, rats. The present study...
demonstrates some of the similarities between Dahl rats used in that publication and Dahl/Rapp rats used in the present study and provides additional insights into the time course and sites of expression of EGFR in the kidney and aorta. The major findings of the current in vivo and in vitro experiments include 1) expression of EGFR was enhanced in the kidney cortex and particularly the resistance vessels of S rats even before the onset of hypertension and 2) expression and activity of the EGFR were increased in VSMC from S rats. Renal vascular expression of EGFR increased in S rats over the course of the study as hypertension and hypertensive renal injury developed. The effect appeared to be independent of salt intake, as expression did not increase in SD rats on the 8.0% NaCl diet.

The identification of enhanced expression of EGFR in the renal vasculature and aorta of prehypertensive S rats along with demonstration of increased expression and functional activity of EGFR in VSMC from these animals are important observations. VSMCs in culture express high-affinity receptors for EGF and addition of EGF to the culture medium induces a dose-dependent increase in proliferation (26). In addition, recent studies have suggested a potential role for the EGFR in hypertension and progressive renal failure. EGFR is trans-activated by GPCR ligands that promote vasoconstriction, including ANG II (3, 4, 14, 33), endothelin-1 (15), and α1b-adrenergic agents (18). Inhibition of EGFR activity attenuates ANG II-induced hypertension and cardiac hypertrophy (22), lessens endothelin-1-mediated hypertension in vivo and induction of the α2-chain gene of collagen I (15), prevents renal vascular and glomerular fibrosis in N1/nitro-l-arginine methyl ester-induced hypertension (16), and inhibits α1b-adrenergic-mediated arterial vasoconstriction (18). Transforming growth factor-β (TGF-β) promotes the release of HB-EGF, which trans-activates the EGFR; EGFR activation appears to be required for TGF-β-mediated fibronectin synthesis in mesangial cells (32). These studies suggest that the EGFR is a master regulator of vascular tone and has a role in renal matrix protein production.

Expression of EGFR was especially prominent in the preglomerular arterioles of S rats. Carmines et al. (3) demonstrated an important role for the EGFR in vasoconstrictor responses of the afferent and efferent arterioles, in part, by contributing to the intracellular calcium mobilization in response to ANG II. In the context of observations reported in the literature, the present data indicate that the combination of the previously described impairment of nitric oxide production (5–7) and the potential for augmented vasoconstrictor and fibrogenic responses mediated through the EGFR, which was particularly increased in the resistance vessels of the kidney, may be responsible for the subsequent abnormal renovascular responses to hypertension observed in S rats.

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DISCLOSURES

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REFERENCES


