Regulation of ANP clearance receptors by EGF in mesangial cells from NOD mice

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Regulation of ANP clearance receptors by EGF in mesangial cells from NOD mice. Am J Physiol Renal Physiol 281: F244–F254, 2001.—Mesangial cells from nonobese diabetic (NOD) mice (D-NOD) that develop diabetes at 2–4 mo express an increased density of atrial natriuretic peptide (ANP) clearance receptors [natriuretic peptide C receptor (NPR-C)] and produce less cGMP in response to ANP than their nondiabetic counterparts (ND-NOD). Our purpose was to investigate how both phenotypic characteristics were regulated. Epidermal growth factor (EGF) and heparin-binding (HB)-EGF, but not platelet-derived growth factor (PDGF) nor insulin-like growth factor I, inhibited 125I-ANP binding to ND-NOD and D-NOD mesangial cells, particularly in the latter. NPR-C density decreased with no change in the apparent dissociation constant, and there was also a decrease in NPR-C mRNA expression. The EGF effect depended on activation of its receptor tyrosine kinase but not on that of protein kinase C, mitogen-activated protein kinase and the transcription factor activator protein-1 (AP-1). Moreover, EGF potentiates the ANP-cGMP response, suggesting its possible implication in the biological effects of this hormone.

We showed recently that atrial natriuretic peptide (ANP)-clearance receptors (natriuretic peptide C receptor (NPR-C)) were overexpressed in cultured mesangial cells from nonobese diabetic (NOD) mice that developed diabetes at 2–4 mo of age (D-NOD) in contrast to mesangial cells from mice of the same strain that remained normoglycemic (ND-NOD). Overexpression of NPR-C mRNA and protein in D-NOD cells was associated with a decreased cGMP response to ANP and natriuretic peptide C, which could be attributable to a lesser availability of intact natriuretic peptides in the medium due to their more marked degradation (4). NPR-C expression is submitted to a complex regulation, in which are implied growth factors (31, 19), β2-adrenergic agonists (22), the natriuretic peptides themselves (43), other vasoactive peptides (11), and steroids (3). Of note, heparin-binding epidermal growth factor (HB-EGF) is expressed by mesangial cells and has been shown to be involved in mesangial cell proliferation in experimental glomerulonephritis (36). Furthermore, EGF receptors (EGF-R), which also recognize transforming growth factor-α and HB-EGF (40), are overexpressed in experimental diabetes (21). For all these reasons, we thought it was of interest to examine the effects of growth factors, especially EGF, on NPR-C expression in mesangial cells from diabetic mice. Our data demonstrate that EGF, but neither platelet-derived growth factor (PDGF) nor insulin-like growth factor I (IGF-I), downregulates NPR-C mRNA and protein via a mechanism implicating EGF-R tyrosine kinase and the transcription factor activator protein-1 (AP-1). Moreover, EGF potentiates the ANP-dependent cGMP response, suggesting its possible implication in the biological effects of this hormone.

METHODS

Mesangial cell culture. Two lines of NOD mesangial cells were studied that were propagated from glomeruli of 4-mo-

RENAL VASODILATION AND HYPERFILTRATION occur early in insulin-dependent diabetes mellitus (17). Several factors contribute to hyperfiltration, among which increased renal plasma flow plays the major role. However, at any given renal plasma flow, glomerular filtration rate is greater in diabetic patients than in control subjects, indicating that the parameters of the glomerular microcirculation, essentially the glomerular capillary pressure and the ultrafiltration coefficient, are also modified (28). The latter parameter is considered to largely depend on the contractile status of mesangial cells (26). In addition, altered mesangial cell growth is one of the early abnormalities detected after the onset of diabetes with an initial cell proliferation and a subsequent glomerular hypertrophy that can, in turn, increase the available surface area for filtration (41).
old female diabetic (i.e., D-NOD) and nondiabetic (i.e., ND-NOD) mice. These cell lines were kindly provided by L. and G. Striker (Miami, FL) and were studied between passages 15 and 35. They were cultured as previously described (6) in dishes precoated with fibronectin in defined medium (basal medium); DMEM-Ham’s F-12 (1:2, vol/vol) supplemented with 20 mM HEPES, 20% fetal calf serum, and 2 mM glutamine and containing 6 mM glucose. Cells were maintained in a 95% O2-5% CO2 atmosphere.

Binding studies. After having reached confluence, cells were deprived of serum during 24 h and either exposed or not exposed during this period to EGF (5 or 10 ng/ml), PDGF (10 ng/ml), IGF-I (10 ng/ml), or phorbol 12-myristate 13-acetate (PMA; 0.1 μM). The effect of 20% serum was also studied. In addition to these studies, the effects of the incubation time and of the concentrations of EGF or HB-EGF were also examined.

Binding was performed at the end of the incubation period in Hanks' solution supplemented with 10 mM HEPES, pH 7.4, containing the following concentrations of protease inhibitors: 30 μg/ml aprotinin, 1 μg/ml leupeptin, 0.1 μg/ml pepstatin A, 10 μg/ml thiorphan, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 1 mg/ml BSA. Binding experiments were performed in the presence of 125I-ANP as the ligand at 4°C for 4 h. At the end of the incubation period, the medium was discarded, and cells were washed three times with 1 ml of ice-cold 0.16 M NaCl. Then, cells were solubilized in 1 M NaOH, and cell-associated radioactivity was counted in a gamma counter. Binding experiments were performed by either saturation analysis with increasing concentrations (20–500 pM) of 125I-ANP or other studies were done with a fixed (50 pM) concentration of 125I-ANP.

In competitive binding experiments, increasing concentrations (from 1 pM to 0.5 μM) of ANP or 4–23 C-ANP, a specific ligand of NPR-C, were added. Nonspecific binding was determined in the presence of 1 μM unlabeled ANP and was <10%.

Specific binding was calculated as the difference between total and nonspecific binding. Results of the saturation-binding experiments were analyzed by Scatchard’s transformation of the data using the Ligand software program (27). This allowed the apparent dissociation constant (Kd) and the number of sites (Bmax) to be calculated.

Results of the competitive inhibition studies were analyzed by Hill’s transformation of the data to precisely determine the concentration corresponding to IC50. Results were expressed as femtomoles of 125I-ANP bound per milligram of protein. The amount of protein per well was measured by the Bradford method using BSA as the standard (9).

cGMP determination. After either pretreatment or no pre-treatment with EGF (5 ng/ml) during 24 h in serum-free medium, cells were incubated with increasing concentrations of ANP over 5 min at 37°C. At the end of the incubation period, the medium was collected and put aside. Then, cell cGMP was extracted with ethanol-formic acid (5:95, vol/vol). After a 30-min extraction, the supernatant was collected and pooled with the medium. The pH of the resulting solution was adjusted to 6.0, and cGMP was measured by radioimmunoassay as previously described (5). To increase assay sensitivity, standards and samples were acetylated using tritylamine and acetic anhydride (2:1, vol/vol). Results are expressed as femtomoles of cGMP per milligram of cell protein.

Cell proliferation studies. After they had reached confluence, cells were incubated for 24 h at 37°C in serum-free medium. Thereafter, cells were exposed for 24 h to EGF (5 ng/ml) or to 20% serum as a proliferation control and then incubated with 0.1 μM ANP for 1 h before [3H]thymidine (2 μCi/well) was added for an additional period of 6 h. At the end of the incubation, cells were washed successively twice with ice-cold 0.15 M NaCl and three times with 10% cold trichloroacetic acid. Cells were then lysed with cold 1 M sodium hydroxide for 18 h. Radioactivity of the lysates was measured by liquid scintillation spectrometry after pH was adjusted to 7.0 with 11.2 M hydrochloric acid, and [3H]thymidine uptake was expressed as counts per minute per milligram of cell protein.

Preparation of complementary RNA probes. NPR-C, HB-EGF, EGF-R, and β-actin probes were prepared from cells from NOD mice by RT-PCR using specific primers that were designed according to the respective published sequences of their genes (16, 1, 7, 2). These primers were as follows: CAAGCATCTGCTCCCTCAAAACA (NPR-C, upstream) and CTCTTGGTGGCCTGTCTAGTCGTC (NPR-C, downstream); AAAATCCGCAAGCTCGAGAA (HB-EGF, upstream) and GATCCCTGACTTGACCAT (HB-EGF, downstream); ATGTCTCTATGGCCCTCAAC (EGF-R, upstream) and GCGAGTTGCTCCTCTCTCTCT (EGF-R, downstream) and CAAGGCTGTAGTGGAGGAGG (β-actin, upstream) and GTCA- TCTGGTTCACATGTGGGC (β-actin, downstream). The corresponding expected fragment lengths were 350, 378, 360, and 220 bp for NPR-C, HB-EGF, EGF-R, and β-actin, respectively. PCR products were purified by using a Quiagick PCR purification kit and sequenced. Ten to twenty-five nanograms of PCR products were then ligated using a Lign’scribe kit (Ambion, Austin, TX) with T7 promoter, T4 DNA ligase, and gene-specific primers. A second PCR was prepared. Synthesis of an antisense complementary RNA probe was carried out using an in vitro transcription kit (Ambion). Incubation of 50 ng of probe with T7 RNA polymerase and [α-32P]UTP (30 TBq/mmol, 1.85 MBq for NPR-C, HB-EGF, and EGF-R, 0.92 MBq plus 6.25 μM unlabeled UTP for β-actin) was performed for 1 h at 37°C. Probes were then purified by electrophoresis on 5% polyacrylamide gels containing 8 M urea.

RNase protection assay. NOD cells were incubated or not incubated with EGF (5 ng/ml) during 2 or 4 h inting in serum-free medium. Whenever needed, pharmacological inhibitors, including PD-98059 (15), a selective inhibitor of mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK)-1, tyrphostin AG-1478 (24), a potent and specific inhibitor of EGF-R tyrosine kinase, GF-109203X (38), a selective inhibitor of protein kinase C (PKC), curcumin (32), a blocker of AP-1 activation, or wortmannin (39), an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), were added to the medium 15 min before EGF and maintained in the medium with EGF for 2 h. At the end of the incubation period, RNase was extracted by using TRIzol reagent (GIBCO-BRL, Grand Island, NY). Five to twenty micrograms of RNA were hybridized with 2 × 106 counts per minute (cpm) NPR-C, HB-EGF, or EGF-R probes and 1 × 106 cpm β-actin probes using an RNA assay kit (Ambion). The mixture was incubated at 50°C for 16 h. Nonanucleic acid was digested with RNase A/T1. The protected fragment was electrophoresed on 5% polyacrylamide gels containing 8 M urea at 300 V. Gels were exposed for 8–48 h to Fuji X-ray film. Quantitation of the bands was performed by densitometry (Imager Appligene, Pleasanton, CA) using β-actin as a control.

Immunoprecipitation and immunoblotting. After incubation for 10 min with EGF (5 ng/ml) and curcumin (20 μM) separately or in combination, the degree of phosphorylation of EGF-R was evaluated according to Iwasaki et al. (20). In brief, mesangial cells were lysed in an appropriate lysis buffer and incubated overnight at 4°C in the presence of a rabbit polyclonal anti-EGF-R antibody. Immunocomplexes were adsorbed onto protein G-Sepharose and then washed.
three times. The proteins were resolubilized in the loading buffer, and an amount equal to 20 μg was submitted to immunoblotting. The membrane had been initially treated with either the EGF-R antibody or a mouse monoclonal phosphotyrosine antibody and then with the peroxidase-coupled rabbit IgG or mouse IgG antibody, respectively, the immunoreactive proteins were detected with the enhanced chemiluminescence system. The degree of phosphorylation of EGF-R was expressed as the ratio of the phosphorylated EGF-R to the total EGF-R signal.

Preparation of nuclear extracts and gel mobility-shift assays. Nuclear extracts were prepared according to the procedure of Dignam et al. (14) from NOD-D mesangial cells that had been treated by EGF (5 ng/ml) with or without curcumin (20 μM) for 1 h at 37°C. Double-stranded oligonucleotide corresponding to the AP-1 site (GGCCTGTAGCACTACCGGAA) and labeled with [α-32P]dATP deoxynucleoside-5′-triphosphate was used as a probe in the gel-shift assay. Nuclear extracts were mixed with binding buffer, glycerol, Igepal, poly(dI-dC), and [32P]dATP deoxynucleoside-5′-triphosphate according to the specifications of the band-shift assay kit. After incubation at room temperature for 30 min, the reaction mixtures were loaded on 6% polyacrylamide gels in Tris-borate-EDTA buffer and electrophoresed at 150 V for 3 h. Gels were then exposed to X-ray film with intensifying screens.

Materials. Materials and their sources are as follows: rat ANP(1–28) and 4–23 C-ANP (Peninsula Laboratories, Belmont, CA); PD-98059, GF-109203X, and AG-1478 (Calbiochem, La Jolla, CA); (3-[125I]iodotyrosyl28) ANP (rat, 74 TBq/mm); [α-32P]dUTP (30 TBq/mm); and [125I]-cGMP (74 TBq/mm) (Radiochemical Center, Amersham, Little Chalfont, Buckinghamshire, UK); anti-cGMP antibody (Institut Pasteur, Paris, France); rabbit polyclonal anti-EGF-R antibody (Santa Cruz Laboratories, Santa Cruz, CA); mouse monoclonal anti-phosphotyrosine antibody (Chemicon, Temecula, CA); an in vivo transcription kit (Maxiscrypt, RNase protection assay (RPA III), and Lind’scribe kit (Ambion); a band-shift assay kit for characterization of DNA-binding proteins and enhanced chemiluminescence kit for immunoblotting (Amersham Pharmacia Biotech); oligo-dT primers and random primers, 2′ deoxyxynucleoside-5′ triphosphate, Taq DNA polymerase, and Moloney murine leukemia virus RT (GIBCO Life Technologies, Cergy-Pontoise, France); oligonucleotide primers specific for NPR-C, HB-EGF, EGF-R, and actin (Genset Oligos, Paris, France); and a Quiagen PCR purification kit (Quiagen, Courbevoie, France). The sequencing of PCR products was made by Euro Sequences Gene Service (Montigny le Bretonneux, France). Cell culture media and cell culture supplies were from GIBCO Life Technologies. All other reagents including curcumin and wortmannin were from Sigma (St. Louis, MO).

Statistics. Experiments comparing ND-NOD and D-NOD cells were done in parallel on the same day with identical protein contents and at the same passage. Results are expressed as means ± SE. Statistical comparison of means was performed by using Student’s t-test for unpaired values and analysis of variance.

RESULTS

Effects of EGF, HB-EGF, PMA, PDGF, IGF-I and serum on NPR-C receptors in D-NOD and ND-NOD mouse mesangial cells. [125I]-ANP binding was considered to reflect essentially NPR-C density in the following experiments because we demonstrated previously that this receptor type represented 90% of the total NPR in NOD mesangial cells (4). We first confirmed our previously published finding that NPR-C density is much greater in D-NOD (617 ± 124 fmol/mg protein) than in ND-NOD (138 ± 41 fmol/mg protein) mesangial cells (Fig. 1). Pretreatment of the cells during 24 h with PMA (0.1 μM), serum (20%) or EGF (5 ng/ml) inhibited [125I]-ANP binding in D-NOD and ND-NOD cells (P < 0.01). However, the effect was much more marked in D-NOD cells whatever the mode of expression selected (absolute or relative difference). Inhibitions amounted to 82, 86, and 70% with PMA, serum, and EGF, respectively, in D-NOD cells whereas they were limited to 37 and 34% with PMA and EGF, respectively, in ND-NOD cells. Only the inhibitory effect of serum was close in both cell types (86 and 76% in D-NOD and ND-NOD cells, respectively). Because of this more marked effect of EGF in D-NOD cells, the subsequent binding experiments were all performed using this cell type. Our first goal was to verify whether the inhibitory effect of EGF was specific for this growth factor or was merely associated with a status of dedifferentiation caused by EGF-induced proliferation. Therefore, we examined the effects of other mitogenic cytokines such as PDGF and IGF-I. Both agents (10

Fig. 1. Effect of phorbol 12-myristate 13-acetate (PMA; 0.1 μM), serum (20%), and epidermal growth factor (EGF; 5 ng/ml) on the specific binding of [125I]-labeled atrial natriuretic peptide (ANP) to mesangial cells from nonobese diabetic (NOD) mice after 24-h treatment. Cells from nondiabetic (ND-NOD; A) and diabetic (D-NOD; B) mice were studied. Each bar represents the mean ± SE of 4–6 values. Two-way (cell type, treatment) analysis of variance showed a significant effect of each of these 2 factors and a significant interaction between them. Note the difference in scale between A and B. C, control. *P < 0.05 and **P < 0.01 vs. control.
ng/ml) did not significantly modify $^{125}$I-ANP binding (904 ± 28 fmol/mg for PDGF and 985 ± 38 fmol/mg for IGF-I vs. 956 ± 31 fmol/mg for control). We then analyzed more thoroughly the influence of EGF on $^{125}$I-ANP binding. The inhibitory effect of EGF increased with time ($P < 0.01$) when expressed as a percentage of the control value (Fig. 2). Indeed, $^{125}$I-ANP binding under control conditions progressively increased over the period of study (24–72 h) whereas it persisted at the same low level when cells had been treated with EGF (5 ng/ml). Of note, the control binding value at 24 h corresponded to a longer period of serum deprivation (48 h) because the cells studied had been previously incubated in a serum-free medium during 24 h. This explains why this control value was higher than that observed in the other experiments. The inhibitory effect of EGF increased as a function of the dose studied, from 1 to 5 ng/ml ($P < 0.01$). It was the same or slightly less marked when EGF concentration was increased up to 10 ng/ml. HB-EGF was studied at the same molar concentrations as EGF. It also produced a dose-related inhibitory effect on $^{125}$I-ANP binding over the range of concentrations studied (1.5–7.5 ng/ml). However, HB-EGF was less potent than EGF on a molar basis. For example, 0.8 nM of EGF (5 ng/ml) produced a decrease in $^{125}$I-ANP binding of 75% whereas the same molar concentration of HB-EGF (7.5 ng/ml) produced only a decrease of 52% (Fig. 3).

To evaluate whether EGF modified the NPR-C density ($B_{max}$) or the apparent dissociation constant value ($K_D$) of ANP for its receptor, saturation binding experiments were performed, and the data obtained were transformed according to Scatchard. The results observed under control conditions were in accordance with our previously published findings (4). There was an apparent single group of receptor sites with a $B_{max}$ of 1,411 ± 287 fmol/mg and a $K_D$ value of 365 ± 138 pM. Pretreatment of the cells by EGF (5 ng/ml) during 24 h did not significantly modify the $K_D$ value (208 ± 38 pM) whereas there was a marked decrease ($P < 0.05$) in $B_{max}$ (351 ± 59 fmol/mg) (Fig. 4). Competitive inhibition studies confirmed these results. In the absence of unlabeled ANP, $^{125}$I-ANP binding was three times greater under control conditions than after treatment with 5 ng/ml EGF. Such a decrease in binding in EGF-treated cells was also observed at increasing concentrations of unlabeled ANP up to 1 nM (Fig. 5). Hill’s transformation of the data showed that both transformed straight lines were almost superimposed, suggesting that the calculated $IC_{50}$ were close. The latter values were in the same range as the $K_D$ values derived from the saturation binding experiments (360 vs. 365 pM under control conditions; 114 vs. 208 pM in EGF-treated cells). C-ANP (4–23) was also a potent inhibitor of $^{125}$I-ANP binding. However, the residual binding in the presence of 1 μM of unlabeled peptide was slightly greater with 4–23 C-ANP than with ANP. This difference, roughly amounting to 10% of ANP maximum binding, represented the fraction corresponding to NPR-A receptors that recognize ANP but not 4–23 C-ANP. This fraction was not apparently modified by EGF (results not shown).

We also verified that the decrease in NPR-C density was not associated with a decrease in cell viability by measuring $[3H]$thymidine incorporation in D-NOD mesangial cells under control conditions and in the presence of EGF (5 ng/ml for 24 h). We found
expected marked increase in $[^3]$H]thymidine incorporation (22.5 ± 2.1 vs. 6.4 ± 0.6 cpm/μg protein with and without EGF, respectively, where cpm is counts/min; $P < 0.001$), indicating a normal proliferative response of the cells.

**Effect of ANP on cGMP production by NOD mesangial cells pretreated or not pretreated with EGF.** ANP stimulated cGMP production in untreated D-NOD mesangial cells in a dose-dependent manner over the range of concentrations studied (100 pM-10 nM). The increase at 10 nM was 10 times the basal value. A similar pattern was observed with EGF-treated cells. EGF treatment of D-NOD cells did not modify the basal production of cGMP. However, the cGMP response to increasing concentrations of ANP was more marked when cells had been pretreated with EGF. Analysis of the data shown in Table 1 indicated that the effect of EGF was significant ($P < 0.01$). There was also a significant interaction ($P < 0.01$) between EGF and ANP concentration effects showing that the degree of EGF effect changed with the concentration of ANP studied. Indeed, it was high (+39.5%) at 1 nmol/l of ANP but attenuated (+12.4%) at 10 nmol/l. To confirm this view, we also studied the effect of a much higher concentration of ANP (500 nmol/l). At this concentration, there was no significant effect of EGF on the cGMP response to ANP (results not shown).

**Effect of EGF on NPR-C mRNA.** In accordance with our previously published study (4), NPR-C mRNA was detected by RNase protection assay in D-NOD mesangial cells. Pretreatment of the cells by EGF (5 ng/ml) during 2 or 4 h markedly diminished the expression of NPR-C mRNA ($P < 0.05$). There was a decrease of ~40% of the ratio of NPR-C to β-actin mRNA at the two times studied (Fig. 6). This inhibitory effect of EGF was unchanged in the presence of PD-98059 (50 μM), an inhibitor of the MAP kinase cascade, GF-109203X (1 μM), an inhibitor of PKC, and wortmannin (0.1 μM), an inhibitor of phosphoinositide 3-kinase. In contrast, the effect of EGF was reduced or abolished in the presence of AG-1478 (250 nM), an inhibitor of EGF receptor tyrosine kinase ($P < 0.01$), and curcumin (20 μM), a blocker of activation of the transcription factor AP-1 ($P < 0.01$). None of these inhibitors had a significant effect on NPR-C mRNA when studied alone (Fig. 7). To be certain that the inhibition observed with curcumin was not related to an effect of this drug on EGF-R tyrosine kinase that has been previously de-
scribed (23), we examined whether curcumin utilized under similar conditions modified EGF-R tyrosine kinase phosphorylation by using the Western blot technique. No change was observed compared with control (results not shown).

Effect of EGF on the transcription factor AP-1. The electrophoretic mobility gel-shift assay demonstrated that EGF (5 ng/ml) increased the binding activity of the transcription factor AP-1, a heterodimer made of c-fos and c-jun, as shown by the increased density of the complex of AP-1 present in the nuclear proteins with its labeled oligonucleotide binding site. Addition of curcumin (20 \mu M) to the incubation medium attenuated the effect of EGF. The binding of nuclear proteins to the AP-1-responsive element was specific because the decreased mobility of the radioactive complex was reversed by incubation with a molar excess (200 \times) of unlabeled AP-1 binding site (Fig. 8).

Comparison of HB-EGF and EGF-R mRNA expression in D-NOD and ND-NOD mesangial cells. Because EGF or HB-EGF treatment diminished NPR-C density at the surface of D-NOD mesangial cells, we raised the hypothesis that the greater expression of NPR-C in D-NOD mesangial cells compared with ND-NOD mesangial cells could be due to the lower expression, in the former, of HB-EGF or EGF-R, which both are constitutively expressed in mouse mesangial cells (34, 10). To examine this issue, we measured by RNase protection assay the mRNA expression of these two products.

Table 1. Effect of 5 ng/ml EGF on cGMP response to ANP in D-NOD mesangial cells

<table>
<thead>
<tr>
<th>ANP Concentration, nmol/l</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>528.0 ± 56.1</td>
<td>536.4 ± 46.9</td>
<td>976.6 ± 110.2</td>
<td>4,809.5 ± 617.3</td>
</tr>
<tr>
<td>EGF</td>
<td>508.0 ± 46.3</td>
<td>622.0 ± 36.9</td>
<td>1,362.8 ± 163.5</td>
<td>5,407.5 ± 570.4</td>
</tr>
</tbody>
</table>

Results are means ± SE of 12 values (4 experiments, with 3 preparations in each). The statistical analysis was performed by using 3-way ANOVA (experiment, EGF, and ANP concentration) with repeated values. The effects of EGF and ANP concentration were found to be significant (P < 0.01). There was also a significant interaction between both (P < 0.01), indicating that the magnitude of the EGF effect varied with the concentration of ANP studied.

that EGF (5 ng/ml) increased the binding activity of the transcription factor AP-1, a heterodimer made of c-fos and c-jun, as shown by the increased density of the complex of AP-1 present in the nuclear proteins with its labeled oligonucleotide binding site. Addition of curcumin (20 \mu M) to the incubation medium attenuated the effect of EGF. The binding of nuclear proteins to the AP-1-responsive element was specific because the decreased mobility of the radioactive complex was reversed by incubation with a molar excess (200 \times) of unlabeled AP-1 binding site (Fig. 8).

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There was no difference in HB-EGF mRNA as shown by the identical values of the ratio of HB-EGF to β-actin mRNA found in both cell types (0.64 ± 0.38 and 0.65 ± 0.48 in ND-NOD and D-NOD cells, respectively). In contrast, EGF-R mRNA expression was significantly greater (P < 0.01) in D-NOD cells than in ND-NOD cells. The ratio of EGF-R to β-actin mRNA was two times more elevated in D-NOD mesangial cells (0.97 ± 0.04 vs. 0.51 ± 0.04). Of note, this difference was not in the direction that could have supported our hypothesis (Fig. 9).

**DISCUSSION**

These studies demonstrate for the first time that NPR-C mRNA and receptor number are downregulated by EGF and the apperated peptide HB-EGF, which shares the same receptor with EGF (40) and is synthesized in cultured mesangial cells (36). In contrast, other growth factors such as PDGF and IGF-I were inactive. D-NOD mesangial cells were considered to be an ideal preparation because they overexpressed NPR-C constitutively, which facilitated the study of their regulation. The mechanism of the inhibitory effect of EGF implicates activation of its receptor tyrosine kinase and of the transcription factor AP-1. A secondary goal of our studies was to examine whether there were phenotypic changes in HB-EGF production...
or EGF-R expression in D-NOD mesangial cells that could explain NPR-C upregulation found in these cells compared with their ND-NOD counterpart.

Cultured D-NOD mesangial cell incubation with EGF or HB-EGF produced time- and concentration-dependent decreases in \(^{125}\)I-ANP binding. Decreased binding was not due to a decrease in cell viability, as shown by the normal proliferative response to EGF, but to a marked reduction in the density of the receptor sites with unchanged affinity. EGF was efficient from a concentration of 1 ng/ml, which lies in the range of normal plasma concentrations in humans (10), suggesting that such an event could occur physiologically and also that EGF is one of the inhibitory factors present in serum. The role of serum in the regulation of NPR-C expression previously reported by Paul et al. (31) in rat mesangial cells was confirmed in this study. Serum-inhibitory influence was also apparent from the results of the time course studies, which showed a progressive increase with time of \(^{125}\)I-ANP binding to D-NOD mesangial cells after these cells were deprived of serum. Because EGF modified \(^{125}\)I-ANP \(B_{\text{max}}\) but not \(K_{D}\), and incubations of several hours were necessary to alter ANP binding, it was likely that the EGF effect occurred via a decrease in protein synthesis. To test this hypothesis, we measured NPR-C mRNA levels by a nuclease protection assay. There was a 40% decrease in NPR-C mRNA expression after 2- or 4-h incubation of D-NOD cells with EGF, thus confirming that this growth factor affected NPR-C synthesis. This time course is in accordance with that reported by Kishimoto et al. (22) in vascular smooth muscle cells. Our data did not allow us to decide between an effect of EGF on the transcription rate as shown in the latter study or on the stability of NPR-C mRNA. However, it excluded a translational effect, as reported previously for dexamethasone (3) and thrombin (44). We did not study a possible effect of EGF on NPR-A and NPR-B mRNA which we thought to be unlikely, first because EGF did not modify the maximum ANP-dependent cGMP production and, second, because the fraction of ANP binding corresponding to the NPR-A receptors did not change in the presence of EGF. In addition, the marked decrease (≥70%) in ANP binding after treatment of D-NOD cells with EGF could only be explained by the predominant effect of this growth factor on NPR-C, which represents 90% of the total amount of ANP receptor sites in mouse mesangial cells (4).

EGF-dependent NPR-C downregulation was mediated by EGF-R because HB-EGF reproduced the same effect, although with a lower affinity than EGF and also because pretreatment of D-NOD mesangial cells with tyrphostin AG-1478, a potent and specific inhibitor of EGF-R tyrosine kinase, counteracted the influence of EGF on NPR-C mRNA expression. The latter result was in accordance with the well-known tyrrosine kinase activity of EGF-R (40). In contrast, GF-109203X, a potent and selective inhibitor of PKC, did not modify the EGF-dependent downregulation of NPR-C mRNA. This indicates that PKC did not mediate the EGF effect. A similar conclusion was reported by Zlock et al. (44) for the effect of thrombin on NPR-C in cultured bovine endothelial cells. However, this does not exclude a possible role for PKC in the signal transduction of other agents also controlling NPR-C expression. The latter hypothesis is supported by our finding of the inhibitory role of PMA, a classic agonist of PKC, on \(^{125}\)I-ANP binding to D-NOD mesangial cells, in agreement with the previous report by Paul et al. (30). In addition, PMA could stimulate HB-EGF production in D-NOD cells as shown in rat mesangial cells by Tan et al. (37).

Investigation of the MAP kinase cascade provided negative results, as shown by the lack of effect of PD-98059. This differs from several other studies in which EGF signal was transduced via this pathway (40). We could also exclude the stimulation of PI3-kinase, which has been shown as a downstream effector of EGF-R (33) because wortmannin, considered to be a unique probe for this enzyme, did not counteract the inhibitory effect of EGF. Because a putative AP-1-responsive element has been identified in the 5'-flanking regulatory region of the mouse gene encoding NPR-C (42), we tested the possibility that the transcription factor AP-1, an heterodimer of c-fos and c-jun, was implicated in the EGF-dependent NPR-C downregulation. Curcumin, an inhibitor of several transcription factors such as AP-1, nuclear factor-κ-B, and Egr-1, restored the NPR-C mRNA signal in the pres-
ence of EGF. However, the meaning of this result could be questioned due to the broad specificity of curcumin, which includes EGF-R tyrosine kinase (23). Therefore, we also searched for a direct effect of EGF on AP-1. Using the gel retardation assay, we could show that EGF increased the binding of nuclear proteins to the labeled AP-1-responsive element. Moreover, we verified, using the Western blot technique that curcumin, when utilized under similar conditions of concentration and time of exposure of the cells, did not modify the degree of phosphorylation of EGF-R. Taken together, these results suggest what could be the pathway leading from EGF-R activation to DNA transcription. Another pathway from those of MAP kinases, PI3-kinase, or PKC, but one capable of activating AP-1, must be implicated. This could be the jun NH2-terminal kinase pathway, which leads to the expression of c-jun, one of the subunits of the AP-1 complex. This pathway shunts the ERK-1/ERK-2 step and has been shown as mediating the EGF-induced expression of human 12-lipoxygenase (12). Because AP-1 mediates the long-term effects of vasoconstrictor peptides in mesangial and vascular smooth muscle cells and ANG II acts in part through transactivation of the EGF signaling pathway, one can hypothesize a similar mechanism for the downregulation of NPR-C by ANG II (11).

NPR-C have been postulated to play an essential role in the local removal of ANP from the circulation, thus modulating its local concentration (25). Such a mechanism should affect, in parallel, its biological effects. In our study, EGF did not change the cGMP response to high stimulatory concentrations of ANP (10 nM) probably due to the saturation of both subtypes of receptors, NPR-A and NPR-C, by ANP under such conditions. In contrast, EGF stimulated cGMP production by D-NOD mesangial cells exposed to lower doses of ANP (0.1 and 1 nM). These results suggest that EGF increased cGMP production by downregulating NPR-C expression, thus allowing a greater availability of ANP for its binding to NPR-A. A similar mechanism has been proposed for bFGF in ovine fetoplacental artery endothelial cells (19) and for β2-adrenergic stimulation in vascular smooth muscle cells (22). Supplementary studies are needed to better analyze the physiological importance of this EGF-induced modulation of NPR-C in mesangial cells. EGF synthesis has been shown to be upregulated in experimental diabetes (21, 35) and could thus facilitate the glomerular effects of ANP, in particular the increase in GFR that is characteristic of
the early stages of diabetes mellitus (29). Reciprocally, Dhaunsi and Hassid (13) observed that ANP and C-type natriuretic peptide amplified EGF activity in aortic smooth muscle cells (13). These data strongly suggest that EGF and natriuretic peptides are interregulated. This is particularly important to consider in diabetes mellitus, where both systems are activated simultaneously (21, 35). It is possible that the intrarenal levels of growth factors and, especially of EGF, in association with vasoactive peptides contribute to the pathogenesis of glomerular microvascular lesions in this disease.

Another goal of this study was to examine whether a phenotypic change in HB-EGF synthesis or in EGF-R expression in D-NOD mesangial cells could be involved in the mechanism of the overexpression of NPR-C observed in these cells. Our results confirm that HB-EGF is synthesized by mouse mesangial cells, as shown by HB-EGF mRNA expression. Similar findings were reported by Takemura et al. (36) in rat mesangial cells. However, there was no difference between HB-EGF mRNA expression in D-NOD and ND-NOD mesangial cells. Thus it was not possible to associate diminished production of HB-EGF with the upregulation of NPR-C in the same cells. We also provided evidence for the presence of EGF-R in mouse mesangial cells, which is in accordance with previously published reports of biological effects of EGF (8, 18) and of EGF-R expression (36) in mesangial cells. Of note, there was an upregulation of EGF-R mRNA in D-NOD cells compared with ND-NOD cells. Such a finding excludes the possible implication of modified EGF-R expression in the overexpression of NPR-C in D-NOD cells. However, it is in accordance with other studies reporting the upregulation of EGF-R in various preparations from several diabetic animal models (21, 35).

Thus we have shown that EGF downregulated NPR-C expression in mouse mesangial cells and, more particularly, in D-NOD mesangial cells, where this NPR subtype is overexpressed through a mechanism implicating EGF-R and AP-1. The direct consequence of this effect is to stimulate cGMP formation in the same cells via a better availability of natriuretic peptide at concentrations close to those observed under normal or pathological conditions. On the basis of these findings, regulation of NPR-C could have an important involvement in second messenger pathways and the resulting biological effects of ANP on mesangial cells including contractility and rate of proliferation (18).

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