Arterial Epidermal Growth Factor Receptor Expression in Deoxycorticosterone Acetate–Salt Hypertension

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Abstract—Epidermal growth factor (EGF) causes contraction in arteries from deoxycorticosterone acetate (DOCA)–salt hypertensive rats but not in normotensive sham rats. We hypothesized that an increase in the number of EGF receptors (EGFRs) in arteries from DOCA-salt rats enables the observed contraction to EGF to occur. DOCA-salt rats had a systolic blood pressure >170 mm Hg, whereas all sham rats had a systolic blood pressure <125 mm Hg. Thoracic aorta were removed for measurement of isometric force, EGFR mRNA levels, and EGFR protein levels. EGF caused a significant contraction in endothelium-denuded aorta from DOCA-salt rats (38±7% of maximal phenylephrine-induced [10 μmol/L] contraction) compared with aorta from sham rats (4±2%). The EGFR tyrosine kinase–specific inhibitors 4,5-dianilinophthalimide (10 μmol/L) and AG1478 (250 nmol/L) reduced contraction in aorta from DOCA-salt by 85±14% and 65±10%, respectively. EGFR mRNA in DOCA-salt aorta was increased 4.2-fold compared with that in sham aorta. However, Western analyses of membrane-enriched and whole-tissue lysate of aorta from sham and DOCA-salt revealed no statistical difference in the density of EGFR protein between sham and DOCA-salt aorta. These data refute our hypothesis and suggest that a change downstream of EGFR is responsible for enabling EGF-induced contraction in hypertension. (Hypertension. 2001;38:1337-1341.)

Key Words: EGF ▪ mineralocorticoid ▪ artery ▪ tyrosine kinase

Epidermal growth factor (EGF) is a 6-kDa protein derived from a larger propeptide found in platelets, kidneys, and salivary glands. EGF binds 1 of 4 recognized receptors—ErbB1 (EGF receptor [EGFR], Her-1), the orphan receptor ErbB2 (Neu, Her-2), ErbB3 (Her-3), and ErbB4 (Her-4)—to cause receptor dimerization and intermolecular autophosphorylation through activation of the cytoplasmic tyrosine kinase integral to these receptors.1-2 The autophosphorylated dimer recruits adaptor proteins to lead to activation of multiple signaling pathways, including phosphoinositide 3-kinase, Janus kinase/signal transducer, and activator of transcription (Jak/STAT) and mitogen-activated protein kinase (MAPK) cascades.3 Dimerization is necessary for receptor activation and thus physical clustering of receptors through increased expression or reorganization could potentially facilitate activation of these receptors.

Growth factors such as EGF are vascular smooth muscle cell mitogens.4 Recently, growth factors have been discovered to modulate smooth muscle contractility.5-7 Inappropriate vascular growth or remodeling and enhanced contractility are commonly observed in forms of human and experimental hypertension.8 We previously presented the novel finding of a significant contraction to EGF in arteries from hypertensive but not normotensive rats9 and herein test the hypothesis that contraction to EGF is enabled in arteries of hypertensive animals because of an increased expression of the primary receptor for EGF, EGFR. It is becoming increasingly evident that the EGFR serves as a relay station for signaling of crucial vasoactive hormones such as angiotensin II and endothelin-1,10 The reason is that these hormones, through activation of their unique G protein–coupled receptor, use the EGFR in their signal transduction, a process called transactivation of the EGFR. Thus, it is important to determine the means by which EGFR functions and how its function changes in hypertension.

Methods

Surgery and Blood Pressure
Male Sprague-Dawley rats (250 to 300 g; Charles River Laboratories, Portage, Mich) underwent uninephrectomy and implantation of deoxycorticosterone acetate (DOCA; 200 mg/kg) under pentobarbital anesthesia (50 mg/kg IP).9 DOCA-treated rats drank water supplemented with 1.0% NaCl and 0.2% KCl; sham animals received tap water. Systolic blood pressures were measured with the tail-cuff method.

Isolated Tissue Bath
Thoracic arteries were removed from anesthetized (pentobarbital 60 mg/kg IP) rats, cleaned, cut into endothelium-denuded strips (1.0×10.0 mm), and mounted into 50-mL isolated organ baths filled with buffer for measurement of isometric contractile force.9 EGF was added in a cumulative fashion at 10-minute intervals. Once
maximal contraction reached a plateau, either vehicle (0.1% to 0.2% dimethyl sulfoxide) or inhibitor was added, and the response was followed for 30 minutes.

Reverse Transcriptase–Polymerase Chain Reaction
RNA was extracted by use of a kit (Qiagen); 1 H9262g RNA was used for first-strand cDNA synthesis with oligo(dT) used as a primer. Standard amplifications were performed on a portion of cDNA with a GeneAmp thermal cycler and Taq DNA polymerase. Reactions, performed in triplicate, contained 5 pmol/L of each primer, 200 μmol/L dNTP, 0.2 U Taq, 1.5 μmol/L magnesium chloride, and 1 μCi of [32P] dCTP in the manufacturer’s buffer. cDNA was resolved on an 8% polyacrylamide gel, and the amount of DNA was quantified by phosphorimage analysis with Multi-Analyst software (Bio-Rad) and normalized to the expression of the constitutively expressed gene GAPDH. Oligonucleotide primers used were as follows: EGFR upstream: GAC AGC AGA AGG GAT CAG TCA, downstream: CTG GAA GTT TGC AGA TGC CAA; GAPDH upstream: TCC CTC AAG ATT GTC AGC AA, downstream: AGA TCC ACA ACG GAT ACA TT.

Western Protocol
Membrane Isolation
Thoracic aorta from sham and DOCA-salt rats were cleaned, removed of endothelial cell layer, and placed into Dounce buffer on ice. Tissues were homogenized and samples were taken through a series of standard high-speed centrifugations to obtain a membrane-enriched fraction. The BCA method for protein measurement (Sigma Chemical Co) was used, and samples were stored at −80°C.

Western Blotting
Equivalent amounts of aortic protein from sham and DOCA-salt rats (4:1 in denaturing sample buffer boiled for 5 minutes) were separated on 5% sodium dodecyl sulfate–polyacrylamide gels and transferred to Immobilon-P membrane for standard Western analyses using EGFR antibody raised against amino acids 1005 to 1016 of EGFR (1 μg/mL; Santa Cruz Technologies). Quantification of Western blot analyses was performed on computer-scanned images of developed films with the program NIH Image (version 1.62).

Results
Four weeks after surgery, the systolic blood pressure of all DOCA-salt rats was >170 mm Hg and that of all sham rats was <125 mm Hg. Aorta from DOCA-salt rats contracted in a concentration-dependent fashion to EGF (Figure 1, top) and aorta from sham normotensive rats showed virtually no response to EGF. In contrast, aortas from both sham and DOCA-salt rats contracted to the β1-adrenergic agonist phenylephrine (10 μmol/L) and maximal phenylephrine contraction were similar between DOCA-salt (988±95 mg) and sham (903±112 mg; P=0.05) arteries. The −log EC50 value for EGF in contracting aorta from DOCA-salt hypertensive rats was 9.18±0.14 mol/L, and maximal contraction was 38±7% of maximal phenylephrine-induced contraction (range, 22% to 66%; n=22). Maximal contraction to EGF was mostly steady but sometimes began to wane slightly (Figure 1). Contraction stimulated by EGF in aorta from DOCA-salt rats was significantly reduced by the EGFR.
tyrosine kinase inhibitors AG1478 (250 nmol/L: 65±10% reduction, n=5; 1 μmol/L: 95±3% reduction, n=4) and 4,5-dianilinophthalimide (10 μmol/L, 85±14% reduction, n=4; Figure 1, bottom). Vehicle for these compounds had a minor effect on EGF-induced contraction (2.0±1.2% reduction, n=9).

Reverse transcriptase–polymerase chain reaction (RT-PCR) analyses of aorta from sham and DOCA-salt rats demonstrated a 4.2-fold increase in EGFR mRNA in DOCA-salt rats (Figure 2, top). Surprisingly, we did not observe the expected increase in EGFR protein in Western analyses in either the membrane-enriched lysate or whole-tissue lysate (Figure 2, bottom). Thus, these findings suggest a change downstream of the EGFR protein itself that enables arterial response to EGF.

Growth factor receptors such as EGFR have classically been associated with mitogenesis in vascular smooth muscle, and members of the EGF family are vascular smooth muscle cell mitogens. Activation of the Erk-MAPK pathway is the best-described growth pathway associated with stimulation of the EGFR. Only in the past 15 years have growth factors been described as also having the ability to alter smooth muscle tone. In 1985, EGF was observed to contract vascular smooth muscle from normotensive rats with significant variability and low efficacy. Since then, EGF has been shown to elicit contraction in the rabbit aorta, rat superior mesenteric artery, and dog mesenteric artery. Moreover, arterial contraction stimulated by other agonists such as KCl, norepinephrine, and PGF₂α can be potentiated by EGF. The tissue in which EGF-induced contraction is best studied is the isolated guinea pig longitudinal stomach muscle; contraction to EGF in this gastric smooth muscle can be reduced by the cyclooxygenase inhibitor indomethacin and inhibitors of tyrosine kinase(s) such as the tyrphostins. Such findings, in combination with those presented here, warrant an evaluation of growth factors as hormones that should be considered vasoactive. EGF-induced mitogenesis and contraction in arterial smooth muscle occur within a similar concentration range (10⁻¹⁰ to 10⁻⁷ mol/L). Important here is the finding that the function of growth factors is significantly altered in hypertension.

**Involvement of EGFR in EGF-Induced Contraction**

The present studies demonstrate an “enabled” contraction to EGF in arteries from DOCA-salt hypertensive rats. In aorta from sham normotensive rats, EGF does not cause an appreciable contraction. However, a concentration-dependent contraction followed by the general tyrosine kinase inhibitor genistein and inhibitor of MAPK–Erk kinase (MEK) PD 098,059. Here, we further these studies and demonstrate that activation of the EGFR is necessary to EGF-induced contraction because 2 chemically distinct inhibitors of EGFR—AG1478 and 4,5-dianilinophthalimide—significantly reduced EGF-induced contraction. Collectively, these pharmacological studies demonstrate that the pathway used by EGF to activate vascular smooth muscle growth is the one that also mediates EGF-induced arterial contraction. This is an important point because it raises the possibility that in hypertension signaling through the EGF pathway has the potential to be different at many different levels—the EGFR receptor itself or somewhere in the Erk-MAPK pathway.
EGF is uniquely mediated by the EGFR, we initially tested the hypothesis that it was at the receptor level that changes occur in hypertension.

**Molecular Analyses of EGFR**

RT-PCR experiments support the hypothesis that EGFR may be increased in arteries of the DOCA-salt arteries because there was a modest elevation in EGFR mRNA in aorta from DOCA-salt rats compared with that of the sham rats. However, this increase in mRNA was apparently insufficient to drive an increase in receptor protein because Western analyses revealed no differences in EGFR density between sham and DOCA-salt samples when either a membrane or whole-tissue preparation was used. We have no evidence for a change in EGFR mRNA stability. The finding of an unchanged EGFR protein density was somewhat unexpected because previous immunohistochemical studies in our laboratory indicated that in at least some DOCA-salt tissues, EGFR immunoreactivity was increased compared with that observed in the sham tissues. Immunohistochemical results are historically difficult to quantify; thus, we turned to performing the Western analyses described in this report.

How EGF causes a contraction in the DOCA-salt arteries but not in the sham arteries is yet another question. EGFR is clearly present in arteries from normotensive rats because both EGFR mRNA and EGFR protein was identified. However, this protein does not serve a contractile function in the normotensive artery. Something has enabled the receptor to serve the function of contraction in hypertension. EGFR or ErbB receptors, in general, must dimerize to be activated and stimulate their signaling pathways. A greater density of receptors—either in shear number or location—increases the probability that a stimulated receptor can dimerize and activate a pathway. Importantly, Jones et al have demonstrated that EGFR activation can be facilitated through the receptor clustering caused by the extracellular matrix proteins such as tenascin-C in pulmonary arteries from pulmonary hypertensive rats. This does not, however, appear to be occurring in this tissue. It is possible that in the immunohistochemical results, we unwittingly chose spots of EGFR clustering that could be viewed as an enriched microdomain for EGFR; this could occur in the absence of any total increase in EGFR. However, because of the relatively global staining of the smooth muscle with EGFR antibody, we could not identify true areas of punctate or clustered staining in the DOCA-salt artery. With the finding of an enhanced contraction to PDGF as well as EGF in aorta of DOCA-salt rats, this suggests that signaling elements common to the functioning of these 2 receptors and downstream of the receptors themselves may be responsible for enhanced growth factor–induced contraction. This could include association with integrins, changes in the Erk-MAPK pathway, etc; this is currently being investigated.

**Impact of EGFR in Hypertensive Disease**

The idea that the EGFR signaling pathway is enhanced in hypertension was previously supported by the findings that cultured vascular smooth muscle cells from animals with several different forms of hypertension display enhanced growth sensitivity to EGF; EGF was more potent in stimulating [3H]thymidine uptake in cells from hypertensive rats. This is true even in vascular smooth muscle cells from prehypertensive spontaneously hypertensive rats. Studies did not, however, agree as to whether the density of EGFR is increased; thus, this was important for us to determine. In noncultured cells, compared with the appropriate control, an increase in EGF/ErbB1 was measured in aortic membranes from the Lyon hypertensive rat, placental and fetal membranes from pregnancy-complicated pregnancies, and aorta of angiotensin II hypertensive rats. Thus, these studies support the need for measuring EGFR in the DOCA-salt model of hypertension. We extend these observations to give EGFR a new function in hypertension, that serving arterial contraction.

The finding of a dramatically enhanced contraction to EGF, in particular, has significant implications. First, growth factors must be considered substances that are acutely vasoactive. Many growth factors, including EGF and heparin binding EGF-like growth factor, are stored either on the plasma membrane or on heparin sulfate proteoglycans in the extracellular matrix of the artery and are released on activation of proteases and matrix metalloproteases. It is unlikely that the upregulated response to EGF is a result of chronically lower-than-normal vascular concentrations of growth factor because this typically results in an upregulation of receptor density, which was not found in the present study. Second, an upregulation of EGF signaling may have significant potential to control arterial tone independently of growth factors themselves because of the ability of other hormones to activate the EGFR and the tyrosine kinase intrinsic to the receptor. This process has been called transactivation of the EGFR. A group of stimuli, including angiotensin II, endothelin-1, oxidative stress, arachidonate, lysophosphatidic acid, integrins, IL-8, thrombin, and unsaturated fatty acids, transactivate the EGFR because this typically results in an upregulation of receptor density, which was not found in the present study. Even in vascular smooth muscle cells, the mechanism by which this occurs has not been elucidated, but this occurs predominantly in the absence of growth factors. At least 1 report suggests that enzymatic release of growth factors such as heparin binding growth factor or epidermal-like growth factor through stimulation of matrix metalloproteinases is necessary for transactivation of the receptor. The result is activation of those pathways linked to EGFR, including the MAPK pathway. Zwick et al recently published an article describing the EGFR as a “crucial relay station” because so many important stimuli can activate this receptor and thus have a profound effect on numerous cellular functions. Here, we propose that EGF plays such a role in the vasculature and the dramatic upregulation of this receptor signaling, in the absence of a total increase in receptor expression, has significant implications to arterial function in hypertension. Physiologically, there is indirect evidence to support overactivation of an Erk-MAPK–dependent pathway in hypertension. Studies have demonstrated that the inhibitor of MEK activation PD098059 can reduce blood pressure of DOCA-salt hypertensive rats. Although these studies do not directly implicate activation of the EGFR in supporting the hypertension expe-
rienced by these animals, they lend physiological relevance to this idea.

Conclusions
In summary, we present evidence that arterial response to EGF is significantly modified in DOCA-salt hypertension. EGF induces a concentration-dependent contraction of isolated arteries from DOCA-salt hypertensive but not sham normotensive rats and does so in a manner dependent on activation of EGFR. This contraction does not appear to be facilitated through a significant increase in the expression of EGFR protein in arterial tissue, thus, other proteins downstream of the EGFR must be considered candidates responsible for the observed upregulated response to EGF and PDGF.

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References
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