EGF Receptor as a Drug Target in Arterial Hypertension

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Abstract: Drugs which inhibit epidermal growth factor receptor (EGFR) are used in the treatment of cancers. EGFR may contribute to the development of hypertension by regulating vascular tone and renal Na^+ handling. Synthetic EGFR inhibitors reduce blood pressure in some experimental models of hypertension suggesting that this receptor is a novel target for antihypertensive therapy.

Key Words: Epidermal growth factor receptor, arterial hypertension, receptor transactivation, extracellular signal-regulated kinases, receptor tyrosine kinase inhibitors, leptin, metabolic syndrome.

INTRODUCTION

Epidermal (or epithelial) growth factor (EGF) is produced in different tissues and exerts stimulatory effects on cell growth, proliferation and survival. Because abnormal activation of the EGF receptor (EGFR) is observed in many human cancers, this receptor has been studied mainly as a target for antitumor therapy. However, studies performed during the last decade indicate that abnormal EGFR-mediated signaling plays an important role in cardiovascular pathology. EGFR is abundantly expressed in the vascular wall and myocardium, and is activated not only by EGF itself but also by other mediators such as angiotensin II, endothelin-1 and norepinephrine; the phenomenon referred to as "transactivation". Enhanced EGFR signaling is observed in experimental models of arterial hypertension and myocardial hypertrophy. In particular, EGFR may contribute to abnormal regulation of vascular tone and renal sodium handling – two major factors which regulate blood pressure in the long run. The purpose of this article is to provide a brief overview of EGFR signaling, its role in the regulation of blood pressure, and to discuss perspectives of anti-EGFR therapy in arterial hypertension. Role of EGFR in other cardiovascular pathologies such as congenital heart defects, atherogenesis, myocardial hypertrophy, ischemia-reperfusion injury and hypertension-induced renal damage has been recently described in excellent reviews [1-3].

EGFR AND ITS LIGANDS

EGF receptor was identified as a plasma membrane protein tyrosine kinase in 1980 [4]. In 1984, it was demonstrated that *v-ErbB* oncogene of avian erythroblastosis virus encodes a truncated form of EGFR [5]. Currently, EGF receptor is classified as a first member of the ErbB family of receptor tyrosine kinases, and thus is also referred to as ErbB1 or HER1 (from Human Epidermal Growth Factor Receptor 1). In 1984, Schechter *et al*. identified *neu* oncogene in ethylnitrosourea-induced neuroblastoma, and demonstrated its sequence similarity with *v-ErbB* [6]. The respective protein encoded by a cellular protooncogene was termed $p185^{her2/neu}$, now referred to as HER2 or ErbB2. Two other members of the family, ErbB3 and ErbB4, were identified later. Overexpression or oncogenic mutations of ErbB receptors (in particular ErbB1 and ErbB2) are observed in many solid tumors [7-9]. Members of the ErbB family and their ligands are listed in Table **1**.

EGFR is a single-chain transmembrane proteins with its N-terminus outside and C-terminus inside the cell. Upon ligand binding, the tyrosine kinase (TK) domain within its intracellular portion is activated and phosphorylates the receptor at several autophosphorylation sites including Tyr^{992}, Tyr^{1068}, Tyr^{1086}, Tyr^{1148} and Tyr^{1173}, which subsequently bind various adaptor proteins containing Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains. Activated EGF receptor triggers many signaling pathways including: (1) extracellular signal-regulated kinases (ERKs), (2) phosphoinositide 3-kinase (PI3-K) – protein kinase B/Akt, (3) phospholipase Cγ.

Extracellular signal-regulated kinases-1 and -2 (ERK1/2), are serine/threonine kinases belonging to mitogen-activated protein kinases (MAPKs). The mechanism linking EGFR and ERK is depicted in Fig. (**1**). ERKs are activated through the phosphorylation cascade including Ras protein, Raf kinase, and two specific mitogen activated protein kinase kinases-1 and -2 (MAPKKs or MAP2Ks) also referred to as MAPK/ERK kinase-1 and -2 (MEK1 and MEK2), which finally phosphorylate ERK1 and ERK2, respectively. Phosphorylated EGFR recruits Growth hormone receptor-binding-2 (Grb2) adaptor protein either directly or through Src homology-containing protein (Shc); in the latter case, Shc first binds to EGFR and then recruits Grb2. Under resting conditions, Grb2 is constitutively bound to "Son of sevenless" (Sos) guanine nucleotide exchange factor. Relocation of Grb2-Sos complex to the plasma membrane allows the interaction of Sos with membrane-bound Ras protein; consequently, Sos catalyzes the exchange of GDP for GTP thus converting Ras from its inactive to active form. GTP-bound Ras activates Raf kinase, which phosphorylates MEK1 and MEK2. To become active, Ras proteins have to

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Table 1. ErbB Receptors and their Ligands

Gene	Receptor	Ligands
c-ErbB (ErbB1)	EGFR (ErbB1, HER1)	EGF, TGFα, HB-EGF, AR, EPR, BTC
neu	$p185^{her2/neu}$ (ErbB2, HER2)	-
ErbB3	ErbB3	NRG-1, NRG-2
ErbB4	ErbB4	NRG-1, NRG-2, NRG-3, NRG-4 HB-EGF, EPR, BTC

TGFα – transforming growth factor-α, HB-EGF – heparin-binding epidermal growth factor, AR – amphiregulin, EPR – epiregulin, BTC – betacellulin, NRG – neuregulins.

be farnesylated, e.g. bound to the farnesyl group (isoprenoid group produced from mevalonate) by protein farnesyltransferases. Ras possesses an intrinsic GTPase activity; this activity is enhanced by GAP (GTPase-activating proteins). Hydrolysis of Ras-bound GTP to GDP inactivates Ras and terminates Raf-MEK-ERK signaling.

The second pathway triggered by EGFR is PI3-K, which phosphorylates phosphoinositides at 3-position of inositol ring thus converting phosphatidylinositol 4, 5-bisphosphate to phosphatidylinositol 3,4,5-triphosphate, or phosphatidylinositol 4-phosphate to phosphatidylinositol 3,4-bisphosphate. These phosphoinositides activate phosphoinositide-dependent protein kinases-1 and -2 (PDK-1 and PDK-2), which then phosphorylate and activate protein kinase B (Akt). Phospholipase Cγ (PLCγ) is phosphorylated by TK domain of EGFR and hydrolyzes membrane phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG), which activates protein kinase C (PKC), and to inositol 1,4,5-triphosphate (IP_3), which releases Ca^{2+} from intracellular stores.

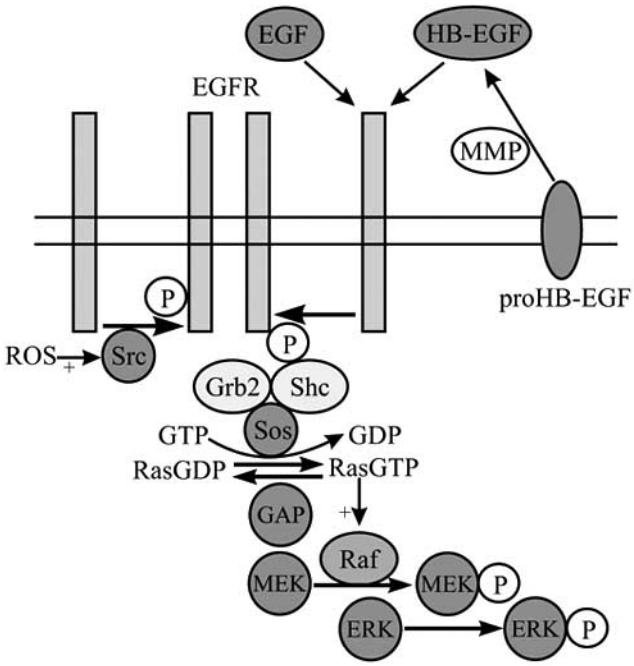

Fig. (1). Mechanisms of EGFR activation, and activation of ERKs by EGFR. MMP – matrix metalloprotease, P – phosphate group.

All ErbB receptor ligands are synthesized as plasma membrane-bound precursors from which mature soluble growth factors are released. Ligands of ErbB receptors may be classified into three groups (Table **1**):

- epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and amphiregulin (AR), which bind only to EGFR
- heparin-binding epidermal growth factor (HB-EGF), epiregulin (EPR) and betacellulin (BTC), which bind EGFR and ErbB4
- neuregulins (NRG), which bind either ErbB3 and ErbB4 (NRG-1, NRG-2) or only ErbB4 (NRG-3 and NRG-4)

EGF was first isolated from mice submandibular gland and identified because of its effect on tooth eruption and eyelid opening in newborns [10]. Its human equivalent was called β-urogastrone, because was isolated from urine and demonstrated to inhibit gastric acid secretion [11]. EGF is detected in blood at picomolar concentrations, most of it being associated with platelets. TGF-α is synthesized in many tissues such as decidua, brain, keratinocytes, kidneys and macrophages. HB-EGF is widely expressed in various tissues including vascular endothelial and smooth muscle cells [12]. It contains a characteristic heparin-binding domain; its interaction with heparan sulfate on the cell surface facilitates binding of HB-EGF to its receptors. Other ErbB receptor ligands have more restricted tissue distributions and their role is less well recognized [13-16].

Soluble ErbB receptor ligands are cleaved from their membrane-bound precursors by either soluble matrix metalloproteases (MMPs) or membrane-bound metalloproteases belonging to "A Disintegrin and Metalloprotease" (ADAM) family. The identity of cleaving enzymes was recognized only in some experimental systems. However, it is generally appreciated that ADAMs are most important sheddases. Gene knockout experiments revealed that tumor necrosis factor-α converting enzyme (TACE or ADAM17) plays an important role in the cleavage of ErbB receptor ligands; other enzymes implicated in this process are ADAM9, ADAM10 and ADAM12 [17].

That inhibiting EGF receptor may be a useful therapeutic option in arterial hypertension is suggested by the following observations: (1) EGFR is activated by many vasoconstricting mediators known to contribute to hypertension such as

angiotensin II, catecholamines and endothelin-1, (2) signaling mechanisms downstream from EGFR induce vasoconstriction and enhance renal tubular Na^+ reabsorption – two abnormalities known to occur in arterial hypertension, (3) enhanced EGFR signaling has been observed in several models of hypertension, (4) EGFR inhibitors reduce vascular tone and/or decrease blood pressure in some of these models.

TRANSACTIVATION OF EGFR BY VASOACTIVE MEDIATORS

In 1996 Daub *et al*. [18] observed that several agonists of G-protein coupled receptors (GPCR) rapidly activated EGFR and ErbB2 in cultured fibroblasts. The phenomenon was too rapid to be mediated by stimulation of ligand synthesis *de novo*, and therefore was named "transactivation", i.e. ligand-independent activation. The term "transactivation" is now commonly used to describe activation of EGFR by factors other than peptide ligands listed in Table **1**, although in many cases this phenomenon is in fact ligand-mediated. The most common mechanism of transactivation was first characterized in 1999, when Prenzel *et al*. [19] observed that many GPCR ligands activate EGFR by stimulating HB-EGF cleavage from its membrane precursor; the mechanism referred to as "triple membrane spanning signaling" which is shown in Fig. (**1**).

Several other mechanisms of EGFR transactivation have been described. Non-receptor tyrosine kinase, c-Src, phosphorylates EGFR at Tyr^{845} within its TK domain and stimulates receptor kinase activity [20]. Moreover, growth hormone and prolactin activate EGFR by inducing its phosphorylation at Tyr^{1068} by cytosolic tyrosine kinase, Jak2 [21]. A nonreceptor Ca^{2+}-dependent tyrosine kinase, proline-rich tyrosine kinase-2 (Pyk2) has also been implicated in EGFR activation. Reactive oxygen species (ROS) may activate EGFR through multiple mechanisms. First, ROS inactivate protein tyrosine phosphatases and thus enhance EGFR activity by inhibiting its dephosphorylation. Second, ROS activate intermediate kinases which subsequently phosphorylate EGFR, such as c-Src or Jak2. Third, ROS activate ADAMs and MMPs and stimulate ligand shedding [22].

Angiotensin II

Angiotensin II (AII) – the major active peptide of the renin-angiotensin-aldosterone system (RAAS) – is one of the principal mediators regulating vascular tone. Most effects of AII are mediated by AT_1 receptor, a GPCR which stimulates phospholipase C to produce DAG and IP_3 from membrane phosphoinositides. DAG/PKC and IP_3/Ca^{2+} pathways mediate fast contraction of vascular smooth muscle cells in response to angiotensin II. However, AII also activates many other signaling mechanisms including ERKs, which play an important role in hypertrophic effect of this peptide. Currently available evidence suggest that EGFR is a central link between AT_1 receptors and many protein kinases such as ERK, c-jun N-terminal kinase (JNK), p38 MAPK, c-Src, etc.

Various mechanisms of EGFR activation by angiotensin II have been described in the vascular wall including: (1) Ca^{2+}-dependent activation of c-Src or Pyk2 [23-26], (2) Ca^{2+}-induced activation of MMP/ADAMs and subsequent HB-EGF cleavage [27], (3) ROS-induced activation of c-Src [28], (4) ROS-dependent activation of ADAMs [29]. In addition, angiotensin AT_1 receptor may directly interact with EGFR and activate it [30, 31]. Stimulation of angiotensin AT_2 receptors opposes AT_1-induced EGFR phosphorylation in vascular smooth muscle cells; the effect mediated by tyrosine phosphatase SHP-1 which dephosphorylates EGFR [32].

Catecholamines

Two studies reported transactivation of EGFR by catecholamines in the vascular wall. Phenylephrine stimulates EGFR phosphorylation and ERK activity in isolated rat thoracic aorta [33]. This effect is abolished by EGFR inhibitor, AG1478, HB-EGF inhibitor, CRM197, anti-HB-EGF neutralizing antibody, MMP inhibitors and HB-EGF gene knockout, suggesting that phenylephrine stimulates MMP-dependent proHB-EGF shedding. In addition, Hao *et al*. [34] have demonstrated that phenylephrine stimulates phosphorylation of EGFR at Tyr^{1173} in rat mesenteric arteries in AG1478-sensitive manner.

Endothelin-1

Endothelin-1 (ET-1) is a potent vasoconstrictor, growth factor and vascular cell mitogen. In smooth muscle cells isolated from the rat aorta, ET-1-induced DNA synthesis was inhibited by AG1478 [35]. ET-1 stimulated EGFR phosphorylation, its association with Grb2, and ERK phosphorylation in these cells, which was abolished by AG1478 [36]. The precise mechanism of ET-1-induced EGFR activation was not established, however, it was demonstrated that ET_A receptor and increase in intracellular Ca^{2+} were involved [36]. In smooth muscle cells isolated from rabbit internal carotid artery, ET-1-induced EGFR phosphorylation was sensitive to deficiency of extracellular Ca^{2+} but not to nifedipine, indicating that Ca^{2+} influx from extracellular space by voltage-independent (nifedipine-insensitive) Ca^{2+} channels mediated this effect [37]. Flamant *et al*. [38] have shown that ET-1 increases EGFR and ERK phosphorylation level in isolated mice aorta, and that these effects are abolished by EGFR inhibitor, PD153035, as well as by endothelin receptor antagonist, bosentan. In smooth muscle cells isolated from rabbit carotid artery, ET-1 increased ERK phosphorylation in AG1478-sensitive manner, suggesting the involvement of EGFR [39].

Aldosterone

Recent studies indicate that aldosterone not only regulates renal Na^+ handling, but also has many direct effects in the cardiovascular system, including stimulation of ROS formation and myocardial fibrosis [40]. Some of the injurious effects of aldosterone are mediated by EGFR. Aldosterone exerts two effects on EGFR: (1) increases receptor density by stimulating its gene expression in mineralocorticoid receptor (MR)-dependent manner, (2) transactivates EGFR by rapid non-genomic mechanism [41]. Aldosterone infused by osmotic minipumps increases EGFR expression in aorta and myocardium of adrenalectomized rats, but has no effect on receptor level in adipose tissue and liver [42]. The similar effect of aldosterone was observed *in vitro* in human aortic smooth muscle cells [43]. Mazak *et al*. [44] have demonstrated that aldosterone rapidly augments angiotensin II-

induced EGFR transactivation in rat aortic smooth muscle cells, and that this effect is abolished by mineralocorticoid receptor antagonist, spironolactone, superoxide scavenger, tiron, and reduced glutathione. Similarly, aldosterone augmented mitogenic effect of angiotensin II on rat aortic SMCs by increasing EGFR phosphorylation level [45].

EGFR AND VASCULAR TONE

Many signaling pathways triggered by EGF receptor, such as IP_3-induced increase in intracellular Ca^{2+} and PKC are well-known stimulators of vascular smooth muscle cell contraction. Moreover, ERK and PI3-K have also been implicated in vasoconstrictor response. However, the effect of EGF itself on vascular tone is controversial. In some studies vasodilating and/or blood pressure-lowering effects of EGF have been reported [46, 47], whereas in others no effect or increase in blood pressure after EGF administration have been observed [48, 49]. *In vitro*, EGF constricted rat aortic rings [50], and augmented vasoconstrictor effect of angiotensin II on isolated rat pulmonary artery [51]. Similarly, EGF itself constricted rabbit aortic rings [52] and augmented constricting effect of bradykinin B1 receptor agonist [53]. Despite many studies reporting transactivation of EGFR by vasoactive mediators, only few of them addressed its role in the regulation of vascular tone by these mediators. Hao et al. [34] have demonstrated that phenylephrine induces sharp and long-lasting contraction of isolated rat mesenteric artery, and that EGFR inhibitors, AG1478 and PD153035, abolished the late phase of vasoconstriction. This effect was mimicked by ERK inhibitors, suggesting the major role of ERK in vasoconstriction. In addition, vasoconstriction induced by α_1-adrenergic agonists was abolished by CRM197 and by anti HB-EGF antibody, as well as by MMP inhibitor, GM6001 [34]. Subsequently, the same group has demonstrated that EGFR transactivated by phenylephrine increases ROS formation in mitochondria. Combined pharmacological and genetic approaches have revealed that inhibiting ROS formation specifically in the mitochondria attenuates vasoconstrictor effect of phenylephrine [54]. Flamant *et al*. [38] have demonstrated that ET-1-induced contraction of mice aortic rings is abolished by EGFR inhibitor, PD153035. In addition, increase in blood pressure induced by bolus intravenous ET-1 in anesthetized mice is reduced by pretreatment with either PD153035 or AG1478, whereas neither of these inhibitors has any effect on BP in animals not receiving ET-1 [38]. Moreover, ET-1-induced increase in blood pressure is markedly impaired in *waved-2* mice, which bear spontaneous mutation in the *egfr* gene. Similarly, BP-elevating effect of ET-1 is attenuated in HB-EGF$^{-/-}$ mice [55]. It has been demonstrated that these mutations, as well as pharmacological blockade of EGFR, PI3-K, HB-EGF or MMP in wild-type mice attenuate ET-1-stimulated Ca^{2+} influx to vascular smooth muscle cells [55]. Moreover, AG1478 inhibited ET-1-induced EGFR phosphorylation and contraction of rabbit basilar artery rings in a dose-dependent manner both *in vitro* and *in vivo* [56].

Carmines *et al*. [57] have demonstrated that AII-induced constriction of isolated rat renal afferent and efferent arterioles was abolished by AG1478 but not by EGFR-inactive analogue, AG9. In contrast, Escano *et al*. have recently demonstrated that AII stimulates ERK in SMCs isolated from rat renal microvessels in EGFR-independent but Src-dependent manner, whereas stimulation of ERK in SMCs isolated from thoracic aorta is EGFR-dependent [58]. These data suggest that EGFR is involved in vascular effect of angiotensin II only in large conduit vessels which are not involved in the regulation of peripheral resistance and blood pressure. Consistently with this, AG1478 had no effect on blood pressure either in control or in angiotensin II-infused rats [58]. Finally, Lucchesi *et al*. [59] have demonstrated that MMP2 and/or MMP9-mediated HB-EGF shedding and subsequent EGFR transactivation contribute to the development of myogenic tone (vasoconstriction induced by increase in perfusion pressure) in mouse mesenteric artery. Taken together, these data suggest that EGFR mediates vasoconstriction in response to various humoral and mechanical factors, at least in some vascular beds.

EGFR AND RENAL SODIUM HANDLING

EGF is synthesized in the medullary thick ascending limb of the loop of Henle and in the distal convoluted tubule of various mammalian species [60, 61]. ProEGF is detected in the apical membrane of tubular cells, and mature EGF is cleaved to the tubular fluid and is excreted in urine at relatively high levels (10^{-7}-10^{-8} M *vs*. 10^{-11}M in plasma). Removal of salivary glands (a major source of plasma EGF) has no effect on EGF excretion in urine, suggesting that the kidney is a predominant source of urinary peptide [62]. Interestingly, EGF receptors are localized in the basolateral membranes of tubular cells making it unlikely that locally generated EGF binds to these receptors [63]. However, other EGFR ligands such as TGF-α and HB-EGF are also abundantly expressed in the kidney [64].

Infusion of EGF to the renal artery of anesthetized rat induces reversible decrease in glomerular filtration rate (GFR) due to constriction of pre- and postglomerular vessels (reduced renal blood flow), and mesangial cell contraction (decrease in filtration fraction). These effects result in the decrease in sodium and potassium excretion [65]. However, intravenously infused EGF increased urine output and Na^+ excretion in conscious sheep, suggesting that renal effect of EGF may be species-dependent [66, 67]. Effect of EGF on tubular sodium transport is also controversial. EGF inhibits Na^+ reabsorption by rabbit cortical collecting duct [68], most likely by inhibiting amiloride-sensitive epithelial sodium channels (ENaC) [69]. Prolonged incubation of collecting duct cells with EGF or TGF-α resulted in ERK-dependent inhibition of ENaC mRNA level [70]. Tong and Stockand have demonstrated that EGF reduces ENaC open probability without affecting the number of channels in the plasma membrane [71]. However, stimulatory effect of EGF on Na^+ transport has also been observed. EGF stimulates Na^+-dependent phosphate reabsorption in microperfused rabbit proximal tubule [72] and in cultured porcine proximal tubular cells [73]. Moreover, EGF stimulates HCO_3^-- dependent Na^+ uptake by rabbit proximal tubular cells [74]. Gekle *et al*. [75] have demonstrated that EGF stimulates Na^+/H^+-exchanger (NHE) in tubular cells; NHE is a major pathway of apical Na^+ influx in the proximal tubule. Interestingly, this effect of EGF is reproduced by aldosterone, which transactivates the EGF receptor in these cells [76].

Sodium reabsorption by tubular cells is a two-step process. First, Na^+ passively enters the cell through the apical membrane, and then in actively extruded across the basolateral membrane by Na^+,K^+-ATPase. Na^+,K^+-ATPase plays a pivotal role in active Na^+ reabsorption and is regulated by most, if not all, mediators involved in the control of Na^+ balance [77]. Ten years ago, it was demonstrated that EGF increased Na^+,K^+-ATPase activity in rat proximal tubular cells at a rate-limiting Na^+ concentration but not under V_{max} conditions, i.e. increases enzyme's affinity for sodium but does not change its catalytic rate [78]. In contrast, we have demonstrated that EGF infused into the renal artery stimulates, in ERK-dependent manner, catalytic activity of renal Na^+,K^+-ATPase measured under V_{max} conditions [79]. These studies suggest that EGF and possibly other intrarenally generated EGFR ligands stimulate sodium pump in tubular cells.

Angiotensin II transactivates the EGF receptor in rabbit and mouse proximal tubular cells [80-82]. Moreover, H_2O_2 transactivates EGFR in native and cultured tubular cells [83-86]. In rat cultured tubular cell line, NRK-52E, endothelin transactivates EGFR in a reactive oxygen species-dependent manner [87]. Alpha 2B adrenergic receptor agonists transactivate EGFR in various tubular segments, partially *via* ROS-dependent mechanism [88]. It is possible that a part of antinatriuretic effect of angiotensin II, norepinephrine and endothelin-1 is mediated by EGFR. In addition, ROS stimulate tubular Na^+ transport and therefore, enhanced intrarenal oxidative stress may contribute to the development of arterial hypertension [89]. Hydrogen peroxide stimulates Na^+ transport in amphibian A6 tubular cell monolayers by activating EGFR [90]. We have shown that H_2O_2 infused to the renal artery increases renal cortical and medullary Na^+,K^+-ATPase activity in ERK-dependent manner [91]. Subsequently, we have demonstrated that H_2O_2 increases phosphorylation level of c-Src, ERK 1/2 phosphorylation, and EGFR phosphorylation at Tyr^{845} and, to a lesser extent, at Tyr^{1068}, in the renal cortex and medulla [79, 92]. The effect of H_2O_2 on c-Src phosphorylation was abolished by c-Src inhibitor, PP2, but not by AG1478 or ERK inhibitor, PD98059, its effect on EGFR phosphorylation was abolished by PP2 and AG1478, and effect on ERK phosphorylation and Na^+,K^+-ATPase activity by both these inhibitors as well as by PD98059. These data indicate that H_2O_2 stimulates renal sodium pump by the mechanism involving sequential activation of c-Src, EGF receptor and ERK [92]. Moreover, GM6001, CRM197 and anti-HB-EGF antibody had no effect on H_2O_2-induced Src-EGFR-ERK pathway, suggesting that metalloprotease-dependent shedding of proHB-EGF is not involved in the stimulation of renal EGFR by H_2O_2. These results indicate that EGFR and ERK-dependent stimulation of Na^+, K^+-ATPase mediates, at least in part, antinatriuretic and prohypertensive effects of intrarenal oxidative stress.

EGFR IN EXPERIMENTAL HYPERTENSION

EGFR signaling is enhanced in various animal models of hypertension. Spontaneously hypertensive rat (SHR) is a model of essential hypertension which develops independently of salt intake. In contrast, Dahl salt-sensitive rats (DSSR) develop hypertension only if dietary sodium intake is high. Hao *et al*. [34] observed that MMP-7 activity was enhanced in mesenteric arteries of SHR, which was accompanied by accelerated processing of proHB-EGF. Interestingly, this was observed at the onset of hypertension (at the age of 5 weeks), suggesting that HB-EGF/EGFR signaling contributes to the development of hypertension rather than is secondary to blood pressure elevation. Moreover, the effect of angiotensin II on EGFR and ERK phosphorylation was enhanced in aortic smooth muscle cells isolated from SHR [93]. Consistently with these observations, EGF constricted endothelium-denuded thoracic aorta isolated from SHR but not from WKY [94].

Swaminathan and Sambhi [95] observed a 3-fold higher density of EGF receptors in the kidney and 2-fold higher receptor density in aorta of Dahl salt-sensitive rats kept on high salt diet in comparison to Dahl salt-resistant animals. Moreover, affinity of EGFR to its ligand was greater in aorta (but not in the kidney) of salt-sensitive rats receiving high-salt diet. EGFR mRNA and protein levels are greater in the renal cortex of DSSR than in salt-resistant rats even before the development of hypertension, suggesting that enhanced EGFR signaling is not secondary to blood pressure elevation. Interestingly, high-salt diet had no effect on EGF receptor abundance in the kidney of normotensive Sprague-Dawley rats, but further increased receptor density in the kidney of Dahl salt-sensitive rats [96]. Moreover, EGF-stimulated phosphorylation of EGFR at multiple sites (Tyr^{845}, Tyr^{992}, Tyr^{1068}) in vascular smooth muscle cells is greater in Dahl salt-sensitive rats than in either Dahl salt-resistant or Sprague-Dawley rats, which is accompanied by prolonged ERK and PKB/Akt activation [96]. Finally, increased density of EGF receptors was observed in the kidney and aorta of Lyon hypertensive rats compared to Lyon normotensive rats [97].

Controversies exist about role of EGFR in angiotensin II-induced hypertension. Kim *et al*. [98] observed that although acute infusion of angiotensin II increased EGFR phosphorylation level in the rat aorta, AII infusion for 3 days had no effect on EGFR phosphorylation, but stimulated phosphorylation of platelet-derived growth factor receptor. In the other study, hypertension induced in the rat by 4-week infusion of AII was associated with increased EGFR mRNA and protein level in aorta, but phosphorylation of the receptor was not examined [99]. Lautrette *et al*. [100] studied the role of renal EGFR in the development of angiotensin II-induced hypertension and renal damage in mice. It was demonstrated that infusion of angiotensin II for 2 months increased blood pressure by about 40 mmHg, which was associated with higher renal TGF-α protein (but not mRNA) level, greater ADAM17/TACE activity, and enhanced EGFR phosphorylation (but not absolute EGFR level) in the kidney. However, expression of a dominant-negative EGFR specifically in the proximal tubule, TGF-α gene knockout, or treatment with ADAM17/TACE inhibitor did not reduce BP in this model, although these treatments prevented renal damage. Although these data suggest that renal EGFR is not involved in AII-induced hypertension, it should be noted that neither of these approaches completely eliminated EGFR signaling. Dominant-negative EGFR was expressed only in the proximal tubules, TGF-$\alpha^{-/-}$ genotype did not eliminate other EGFR ligands, and TACE inhibitor did not block ligand processing by other MMP/ADAMs [100].

Several studies examined the role of EGFR in arterial hypertension induced by mineralocorticoid deoxycorticosterone acetate (DOCA) and high-salt diet. Florian *et al*. [101] observed that EGF and TGF-α constricted aortic rings isolated from DOCA-salt rats but had no effect in normotensive animals. Constrictor response to EGF was observed after 14 days of DOCA-salt treatment when blood pressure was already elevated. These findings suggest that EGFR does not drive the development of hypertension in this model, but may contribute to the maintenance of high blood pressure. Increased vasoconstrictor potency of EGF results most likely from excess of mineralocorticoids rather than from blood pressure elevation, since DOCA-salt treatment induces a similar phenomenon in Wistar-Furth rats in which blood pressure only marginally increases. Although EGFR mRNA level is increased in aorta of DOCA-salt rats, the level of receptor itself is unchanged, suggesting that enhancement of vasoconstricting effect of EGF is a postreceptor phenomenon [102]. Enhanced constrictor response to EGF in DOCA-salt rats is mediated by ERK, PI3-K, and partially by PKC and Rho-dependent kinase [103].

Metabolic syndrome is a commonly observed cluster of related abnormalities which result from central obesity. Metabolic syndrome is a major cause of arterial hypertension. Indeed, it is estimated that in developed countries about 2/3 cases of arterial hypertension are directly associated with excess body weight [104]. Recent studies suggest an important role of adipose tissue-derived mediators, or "adipokines", in obesity-associated hypertension [105]. In particular, leptin is secreted by adipose tissue and regulates energy balance by inhibiting food intake and stimulating energy expenditure. Several lines of evidence suggest the role of leptin in obesity-associated hypertension: (1) plasma leptin concentration is increased in obese subjects reflecting greater amount of adipose tissue and resistance to anorectic effect of this hormone, (2) chronic leptin administration or transgenic overexpression increase BP in experimental animals, (3) high leptin level correlates with increased BP in humans independently of body weight [106]. We and others have examined the model of hypertension induced in healthy rats by central or peripheral administration of exogenous leptin. Although this model does not reproduce all abnormalities associated with obesity, it allows studying specific consequences of leptin excess [107]. We have demonstrated that leptin stimulates renal Na^+,K^+-ATPase by increasing ROS formation, which in turn activate c-Src ultimately leading to EGFR stimulation and ERK-dependent activation of the sodium pump [92]. In addition, leptin stimulates ROS-c-Src-EGFR-ERK pathway also in the abdominal aorta [92]. EGFR or ERK inhibitors attenuate blood pressure elevation induced by chronic leptin administration. These data suggest that EGFR signaling may contribute to the pathogenesis of hypertension associated with hyperleptinemic states such as obesity and type 2 diabetes.

EGFR AS A TARGET FOR ANTIHYPERTENSIVE THERAPY

Drugs targeting EGF receptors currently available in clinical practice are classified into two groups: (1) antibodies specific for extracellular receptor domain, (2) receptor tyrosine kinase (TK) inhibitors. Chemistry, mechanisms of action and clinical application of these drugs in the treatment of malignancies have been extensively characterized in many comprehensive reviews [see e.g. 108,109] and thus will be described here only briefly.

Antibodies specific for EGFR, cetuximab (Erbitux) and panitumumab (Vectibix), are used in patients with advanced metastatic colorectal cancer. These antibodies not only block the interaction between EGFR and its ligands but also may induce cell-mediated or complement-mediated cytotoxicity, leading to the destruction of target cells – the phenomenon beneficial from oncological point of view. In addition, binding of antibody to extracellular domain of EGFR triggers receptor internalization, leading to the decrease in receptor density in the plasma membrane. Antibodies may be effective against EGFR mutants resistant to TK inhibitors but do not block ligand-independent receptor activity (e.g. triggered by ligand-independent transactivation) until membrane receptor density is decreased. High costs, need of parenteral administration and possible side effects associated with the administration of exogenous protein makes antibodies unlikely candidates for antihypertensive therapy, at least in the long run. In addition, antibodies may have limited ability to penetrate tissues in comparison to small-molecule TK inhibitors.

Receptor tyrosine kinase inhibitors are categorized as reversible or irreversible. Reversible inhibitors compete with the ATP binding site within the kinase domain. Currently available reversible inhibitors are quinazoline derivatives. Two closely related compounds, AG1478 and PD153035 (AG1517), are commonly used in experimental studies; their structures are presented in Fig. (**2**). Unlike antibodies, most kinase inhibitors are not completely specific toward EGFR but target also other ErbB receptors. In addition, since TK inhibitors interact with ATP-binding site, they have some affinity also to other tyrosine or even serine/threonine protein kinases. The disadvantage of currently available inhibitors is also that they have to compete with high intracellular ATP concentration and thus must be used at relatively high amounts, raising concerns about possible side effects in conjunction with their limited selectivity. Substrate-based inhibitors interfering with kinase binding to protein which is to be phosphorylated, would be devoid of these disadvantages, however, because substrate-binding domain is more open and less structurally defined, it is highly difficult to design such compounds [110]. It should be noted that TK inhibitors do not block signaling through EGFR independent of its ty-

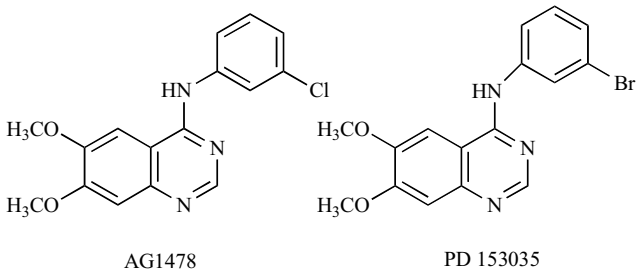

Fig. (2). Structure of EGFR TK inhibitors commonly used in experimental studies.

rosine kinase domain. For example, Jak2 kinase may phosphorylate the intracellular domain of EGFR which thus becomes a docking site for intracellular signaling proteins [21]; this pathway does not require EGFR's kinase activity and thus is not inhibited by TK inhibitors. Similarly, EGFR activation by c-Src is much less sensitive to AG1478 than EGF-induced activation of the receptor [92].

Gefitinib (Iressa, ZD1839) and erlotinib (Tarceva, OSI-774) are orally available, more soluble anilinoquinazoline derivatives selective for EGFR tyrosine kinase. Both are about three orders of magnitude less effective in blocking ErbB2 than EGFR. In addition, gefitinib exhibits low affinity toward several other kinases including cyclin G-associated kinase (GAK), mitogen-activated protein kinases -9 and -10 (MAPK9, MAPK10), lymphocyte specific tyrosine kinase (LCK), STE20-like kinase (SLK) and Src, whereas erlotinib targets Abl, GAK and MAPK9 kinases [111]. Both gefitinib and erlotinib are approved for the treatment of non-small cell lung cancer. Lapatinib (Tykerb, GW572016), approved in 2007 for the treatment of advanced breast cancer, binds both EGFR and ErbB2 with comparable activity but does not target other protein kinases [111]. Vamdetanib (Zactima, ZD6474) is a dual inhibitor of EGFR and vascular endothelial growth factor (VEGF) receptor tyrosine kinase. Structures of clinically applicable EGFR tyrosine kinase inhibitors are shown in Fig. (**3**).

EGFR inhibitors are in general well-tolerated. The most frequent side effects include skin rash, diarhoea, nausea/vomiting, asthenia and allergic reaction (toward antibodies only). Trastuzumab (Herceptin), the anti-ErbB2 antibody used in the treatment of breast cancer, is cardiotoxic. Congestive heart failure is observed in adult ErbB2-knockout mice, whereas knockout of this receptor at the embryonic stage induces congenital heart defects [112]. Interestingly, in contrast to cardiotoxic trastuzumab, low molecular-weight dual EGFR/HER2 inhibitor, GW2974, protected cultured cardiomyocytes against tumor necrosis factor-α-induced apoptosis by stimulating AMP-activated kinase, increasing fatty acid oxidation and elevating intracellular ATP concentration [113]. These data indicate that low molecular weight EGFR/HER2 inhibitors may be less cardiotoxic than antibodies and, at least in the case of lapatinib, more selective toward ErbB family *vs*. other kinases [111, 113].

Irreversible inhibitors such as canertinib (CI-1033) inactivate all members of the ErbB family and are effective toward some tumor cells resistant to reversible inhibitors. Irreversible inhibitors also interact with ATP-binding site but they covalently modify Cys^{773} to permanently inactivate the kinase. Currently, no irreversible inhibitor is approved for therapy, but some compounds from this group are in phase II clinical trials for the treatment of advanced breast cancer and other solid tumors.

Can EGFR inhibitors be useful in the treatment of hypertension? Until now, little is known about effect of these drugs on blood pressure in humans. However, several attempts have been made to inhibit EGFR in experimental hypertension. These attempts may be divided into two groups. The first group includes more "specific" therapies such as

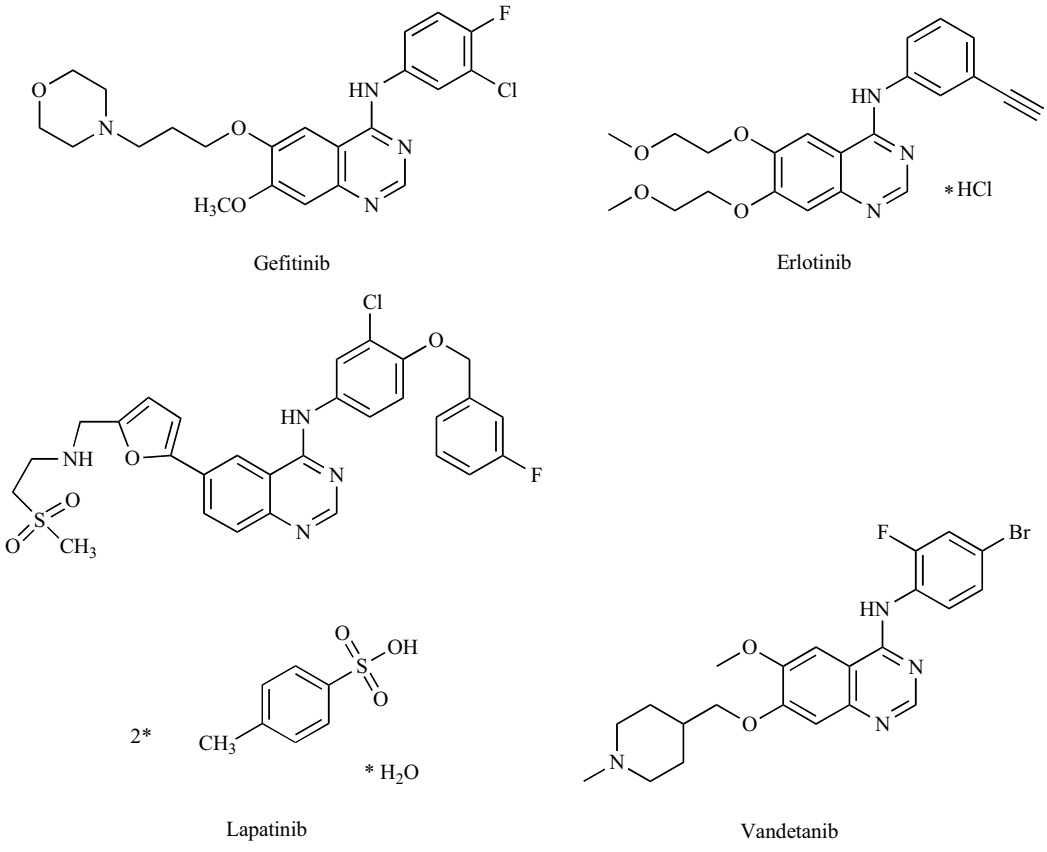

Fig. (3). Structure of EGFR TK inhibitors currently used in humans.

Table 2. Targeting EGFR in Experimental Hypertension

Experimental Model	Species	Treatment	Effect of Treatment on BP	Other Outcomes	Ref.
Angiotensin II infusion for 14 days	rat	EGFR-AS (2 mg/kg total dose) in continuous i.v. infusion	↓*	↓ total and phosphorylated EGFR in the left ventricle	[114]
SHR	rat	EGFR-AS (1.5 mg/kg once a week for 2 months)	↓**	↓ total and phosphorylated EGFR in the left ventricle	[115]
L-NAME induced	rat	gefitinib	No change	↓ EGFR phosphorylation in the kidney ↓ renal lesions	[116]
Leptin-induced hypertension	rat	AG1478 PP2	↓	↓ EGFR phosphorylation in aorta and kidney	[92]
Angiotensin II infusion for 2 months	mice	WTACE2 (ADAM17/TACE inhibitor)	No change	Amelioration of renal lesions and EGFR phosphorylation in the kidney	[100]
Phenylephrine or Angiotensin II infusion for 7 days	mice	KB-R7785 (ADAM12 inhibitor)	No change	Amelioration of left ventricular hypertrophy	[117]
SHR	rat	Doxycycline (MMP inhibitor) for 5 weeks	↓	↓ HB-EGF shedding in mesenteric artery	[34]

*Moderate decrease in comparison to AII-treated group, but not complete normalization.

**Reduction of SBP by 10% only in young but not in adult animals.

EGFR kinase inhibitors, as well as inhibitors of transactivation pathways (e.g. Src or MMP/ADAM inhibitors) (Table **2**). As can be seen, the effect of these treatments on BP is controversial. EGFR-specific antisense oligodeoxynucleotides were used in two studies but only partially reduced blood pressure [114, 115]. However, antisense oligonucleotides only partially reduce EGFR expression, and thus could not completely block its activity. EGFR kinase inhibitors were tested in two studies; AG1478 normalized blood pressure in leptin-induced hypertension [92], whereas gefitinib had no effect in hypertension induced by nitric oxide synthase inhibitor, L-NAME [116]. It should be noted that blood pressure is much higher in the L-NAME model (mean BP about 170 mmHg) than in our study (systolic BP about 150 mmHg), and L-NAME was administered for 4 weeks [116] *vs*. 1-week leptin treatment in our study. It is possible that EGFR blockade is more effective in reducing BP in moderate than in severe hypertension. Among MMP/ADAM inhibitors, doxycycline reduced BP in SHR [34], but two more selective inhibitors had no effect in angiotensin II-induced or phenylephrine-induced hypertension [100, 117]. It should be noted that doxycycline is a broad-range MMP/ADAM inhibitor, whereas compounds used in the remaining studies were selective toward specific ADAMs and therefore could be less effective.

Despite these inconsistencies, EGFR is potentially a very attractive target for antihypertensive therapy. First, EGFR is a "final common pathway" for various vasoconstrictors and thus its inhibition may be more effective than specific therapies aimed to block only one of these mediators. Second, inhibiting EGFR may reduce target organ damage such as glomerulopathy or left ventricular hypertrophy even independently of lowering BP. Third, some vessel-dilating hypotensive drugs such as Ca^{2+} antagonists or α_1-adrenergic receptor antagonists may impair renal perfusion and induce fluid retention. If EGFR mediates enhanced tubular Na^+ reabsorption as suggested by some studies, inhibiting it may simultaneously normalize vascular tone and renal Na^+ handling. Finally, hyperleptinemia, which accompanies the metabolic syndrome, may contribute to the development of malignancies [118], possibly by transactivating the EGFR [119,120]. Thus, inhibiting EGFR could simultaneously reduce the risk of cancer in hyperleptinemic states.

EGFR signaling is also affected by less specific antihypertensive therapies such as RAAS inhibitors, ET-1 receptor antagonists and antioxidants [121-124] (Table **3**), some of them are well established antihypertensive drugs or are known to reduce blood pressure in experimental models. The effect of these treatments on EGFR was, however, addressed only in few studies. Angiotensin-converting enzyme inhibitor, imidapril, AT_1 receptor antagonist, losartan, and aldosterone antagonist, spironolactone, have been shown to reduce EGFR phosphorylation level in some, but not all, studies (Table **2**). However, each of these drugs has also EGFR-independent effects, so the causal relationship between inhibiting EGFR and lowering BP remains unclear. ERK in-

Table 3. Effect of Currently Used Antihypertensive Drugs on EGFR

Experimental Model	Species	Treatment	Effect of Treatment on BP	Other Outcomes	Ref.
3/4 nephrectomy	mice	Losartan (AT_1 antagonist)	↓	Reduction of renal EGFR phosphorylation, TACE activity and TGFα level	[100]
Aldosterone + 1% NaCl in drinking water	rat	Imidapril (ACE inhibitor)	No change	↓ EGFR phosphorylation in the left ventricle	[121]
SHR	rat	enalapril +atenolol	↓	↓ HB-EGF in the left ventricle, no effect on total EGFR*	[122]
Aldosterone + 1% NaCl in drinking water	rat	spironolactone	No change	↓ EGFR phosphorylation in the left ventricle	[121]
SHRSP	rat	spironolactone	↓	↓ EGFR mRNA in aorta	[123]
T2DM without obesity	rat	ABT-627 (ET_A receptor antagonist)	↓	↓EGFR phosphorylation in the kidney	[124]
Aldosterone + 1% NaCl in drinking water	rat	apocynin (NOX inhibitor)	No change	No change in EGFR phosphorylation in the left ventricle	[121]
Leptin-induced hypertension	rat	apocynin (NOX inhibitor)	↓	↓ EGFR phosphorylation in aorta and kidney	[92]
AII-induced	rat	PD98059	↓	↓ ERK phosphorylation in vasculature	[125]
DOCA-salt induced	rat	PD98059	↓	↓ ERK phosphorylation in vasculature	[126]
Leptin-induced hypertension	rat	PD98059	↓	↓ EGFR phosphorylation in aorta and kidney	[92]

*No change in absolute level of EGFR mRNA was observed; EGFR phosphorylation was not studied.

hibitor, PD98059, has been consistently demonstrated to reduce BP in several models of hypertension [92,125,126], but ERKs are activated by factors other than EGFR, so ERK inhibitors may be more effective than EGFR inhibitors. Finally, NADPH oxidase inhibitor and antioxidant, apocynin, reduces blood pressure in many experimental models, but its effect on EGFR phosphorylation *in vivo* is controversial [92,121].

CONCLUSIONS

Data presented above strongly suggest that EGFR is involved in the pathogenesis of arterial hypertension, at least in some animal models. EGFR is abundantly expressed in the vascular wall and renal tubules, and this receptor – either activated by its peptide ligand(s) or transactivated by factors such as ROS, angiotensin II or leptin – may elicit vasoconstriction and renal Na^+ retention. Enhanced EGFR signaling has been observed in many animal models of hypertension, and inactivation of this receptor by genetic or pharmacological approaches reduces blood pressure in some of these models.

Nevertheless, many aspects remain to be clarified. It is unknown if EGFR plays any role in hypertension in humans. It is also unclear if other ErbB receptors are transactivated by mechanisms similar to EGFR and, if so, whether these receptors are involved in the regulation of vascular tone and renal Na^+ transport. Currently, it is much too early to recommend EGFR inhibitors for the treatment of hypertension. The effect of these drugs on BP in humans must first be examined, possibly initially in a subset of patients with hypertension and cancers for which these drugs are currently indicated. However, even if positive response is observed, the concern of possible side effects will remain, especially if it is realized that EGFR kinase inhibitors may also target other ErbB receptors, particularly *in vivo*, when compound concentration in all compartments cannot be strictly controlled.

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ABBREVIATIONS

AII = Angiotensin II

ADAM = A disintegrin and metalloprotease

AR = Amphiregulin

BTC = Betacellulin

DAG = Diacylglycerol

DOCA = Deoxycorticosterone acetate

DSRR = Dahl salt-resistant rats

DSSR = Dahl salt-sensitive rats

EGF = Epidermal growth factor

EGFR = Epidermal growth factor receptor

EPR = Epiregulin

ERK = Extracellular signal-regulated kinase

ET-1 = Endothelin-1

GAP = GTPase-activating protein

GDP = Guanosine diphosphate

GEF = Guanine nucleotide exchange factor

GPCR = G protein-coupled receptor

Grb2 = Growth hormone receptor-binding protein-2

GTP = Guanosine triphosphate

HB-EGF = Heparin-binding epidermal growth factor

IP_3 = Inositol triphosphate

Jak = Janus kinase

JNK = c-Jun N-terminal kinase

L-NAME = N_ω-Nitro-L-arginine methyl ester

MAPK = Mitogen-activated protein kinase

MAPKK = Mitogen-activated protein kinase kinase

MMP = Matrix metalloprotease

MR = Mineralocorticoid receptor

mTOR = Mammalian target of rapamycin

Na^+,K^+-ATPase = Sodium, potassium-activated adenosine triphosphatase

NHE = Sodium-proton exchanger

NRG = Neuregulin

PDK = Phosphoinositide-dependent kinase

PI3-K = Phosphoinositide 3-kinase

PKB = Protein kinase B

PKC = Protein kinase C

PLC = Phospholipase C

Pyk = Praline-rich tyrosine kinase

RAAS = Renin-angiotensin-aldosterone system

ROS = Reactive oxygen species

Shc = Src homology-containing protein

SHR = Spontaneously hypertensive rats

SMC = Smooth muscle cells

Sos = Son of sevenless

TACE = Tumor necrosis factor-converting enzyme

TGF-α = Transforming growth factor alpha

TK = Tyrosine kinase

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