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Transactivation of epidermal growth factor receptor in vascular and renal systems in rats with experimental hyperleptinemia: Role in leptin-induced hypertension

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ABSTRACT

We examined the role of epidermal growth factor (EGF) receptor in the pathogenesis of leptin-induced hypertension in the rat. Leptin, administered in increasing doses (0.1–0.5 mg/kg/day) for 10 days, increased phosphorylation levels of non-receptor tyrosine kinase, c-Src, EGF receptor and extracellular signal-regulated kinases (ERK) in aorta and kidney, which was accompanied by the increase in plasma concentration and urinary excretion of isoprostanes and H_2O_2. Blood pressure and renal Na^+,K^+-ATPase activity were higher, whereas urinary sodium excretion was lower in animals receiving leptin. The effects of leptin on renal Na^+,K^+-ATPase, natriuresis and blood pressure were abolished by NADPH oxidase inhibitor, apocynin, Src kinase inhibitor, PP2, EGF receptor inhibitor, AG1478, protein farnesyltransferase inhibitor, manumycin A, and ERK inhibitor, PD98059. In contrast, inhibitors of insulin-like growth factor-1 and platelet-derived growth factor receptors, AG1024 and AG1295, respectively, only slightly reduced ERK phosphorylation and had no effect on blood pressure in rats receiving leptin. These data indicate that: (1) experimental hyperleptinemia is associated with oxidative stress and c-Src-dependent transactivation of the EGF receptor, which stimulates ERK in vascular wall and the kidney, (2) overactivity of EGF receptor-ERK pathway contributes to leptin-induced hypertension by stimulating renal Na^+,K^+-ATPase and reducing sodium excretion, (3) inhibitors of c-Src, EGF receptor and ERK may be considered as a novel therapy for hypertension associated with hyperleptinemia, e.g. in patients with obesity and metabolic syndrome.

1. Introduction

Obesity is a leading cause of arterial hypertension, however, the pathogenesis of obesity-associated hypertension is incompletely elucidated. Studies performed during the last decade indicate that adipose tissue hormone, leptin, plays an important role in this context. Plasma leptin concentration is increased in obese subjects [1], and some clinical studies indicate that leptin level is higher in patients with essential hypertension than in normotensive individuals independently of adiposity scores [2]. Chronic leptin administration or transgenic overexpression increases blood pressure in experimental animals [3,4], and some studies indicate that hypertension develops in obese animals only if they are hyperleptinemic [5]. Leptin increases blood pressure through several mechanisms including: (1) stimulation of sympathetic

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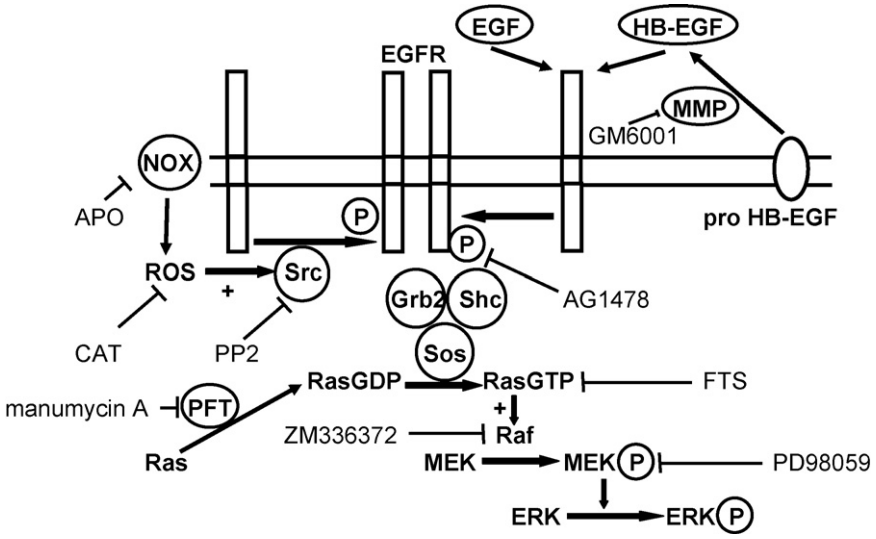

Fig. 1 – Mechanisms of activation of EGF receptor (EGFR) and sites of action of inhibitors used in this study. EGF, epidermal growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; MMP, matrix metalloproteases; NOX, NADPH oxidase; APO, apocynin; P, inorganic phosphate; ROS, reactive oxygen species; CAT, catalase; RasGDP, inactive (GDP-bound) Ras protein; RasGTP, active (GTP-bound) Ras protein; Sos, "Son of sevenless" guanine nucleotide exchange factor; FTS, farnesylthiosalicylic acid; PFT, protein farnesyltransferase; MEK, an upstream kinase of ERK; ERK, extracellular signal-regulated kinase.

nervous system [3], (2) impairment of pressure natriuresis [6] and/or induction of sodium retention [7,8], (3) increased expression of vasoconstrictor endothelin-1 [9], (4) induction of oxidative stress and nitric oxide (NO) deficiency, which results in vasoconstriction and enhanced renal Na^+ reabsorption [8,10,11].

Epidermal growth factor receptor (EGFR) is a member of plasma membrane receptor tyrosine kinases. Upon ligand binding, intracellular EGFR domain undergoes autophosphorylation and recruits several adaptor proteins such as Grb2, Shc and Sos, which elicit downstream signaling events. EGF receptor triggers three major signaling pathways: (1) phosphoinositide 3-kinase (PI3K)-protein kinase B/Akt, (2) Ras protein-Raf kinase-mitogen activated protein kinase kinase (MEK)-extracellular signal regulated kinase (ERK) cascade (Fig. 1), and (3) phospholipase Cγ [12]. EGF receptor is widely expressed in tissues, including cardiovascular system and the kidney. Apart from its cognate ligand, EGF receptor may be activated by other related ligands such as heparin-binding epidermal growth factor-like growth factor (HB-EGF). EGF receptor is also activated by many mediators relevant for the regulation of cardiovascular function such as angiotensin II, endothelin-1 and α-adrenergic agonists; the phenomenon referred to as "transactivation". There are two major mechanisms of EGFR transactivation, (1) shedding of plasma membrane-bound proHB-EGF by metalloproteases (MMP) or members of "a disintegrin and metalloprotease (ADAM)" family, to yield mature HB-EGF, (2) stimulation of reactive oxygen species (ROS) formation and subsequent activation of non-receptor tyrosine kinase, c-Src, which phosphorylates intracellular domain of EGF receptor and activates it in a ligand-independent manner [12]. Transactivation of the EGF receptor by both these mechanisms has been demonstrated in vascular smooth muscle cells [13] and renal tubular cells [14], and may contribute to the development of hypertension by inducing vasoconstriction and/or altering renal tubular Na^+ transport [12].

Renal Na^+,K^+-ATPase plays a pivotal role in active tubular sodium reabsorption and is regulated by most mediators involved in maintaining isovolemia [15]. Abnormalities of sodium pump regulation are observed in various forms of hypertension including obesity-associated hypertension [16]. Previously, we have demonstrated that leptin infused locally to the renal artery stimulates ERK through the mechanism involving H_2O_2, c-Src and EGF receptor, leading ultimately to the increase in renal Na^+,K^+-ATPase activity [17]. However, it is unclear if EGF receptor is activated when leptin is administered systemically for a prolonged time period.

The aim of this study was to examine if EGF receptor and ERK are activated in vascular and renal systems in experimental hyperleptinemia. In addition, we examined if inhibition EGFR-ERK pathway can modulate blood pressure and renal Na^+ handling in this model of hypertension.

2. Materials and methods

2.1. *Animals*

The study was performed on adult male Wistar rats weighing 248 ± 6 g. All animals were kept at a temperature of 20 ± 2 °C, on a 12 h light/dark cycle (lights on at 7.00 am) and had free access to food (standard rodent chow, Agropol, Motycz, Poland) and tap water. The study protocol was approved by The Bioethical Committee of the Medical University in Lublin.

2.2. Experimental protocol – systemic 10-day leptin administration

After two weeks of acclimation, the animals were randomized into the following groups ($n = 8$ each): (1) control group, fed standard rat chow ad libitum, (2) group with experimental hyperleptinemia induced by administration of exogenous hormone (see below), (3) group receiving leptin and NADPH oxidase (NOX) inhibitor, apocynin (2 mM in the drinking water), (4) group receiving leptin and EGFR inhibitor, AG1478 (20 mg/kg/day i.p.), (5) group receiving leptin and a structurally related but EGFR-inactive compound, AG43 (A63, 20 mg/kg/day i.p.), (6) group receiving leptin and platelet-derived growth factor receptor (PDGFR) inhibitor, AG1295 (20 mg/kg/day i.p.), (7) group receiving leptin and insulin-like growth factor-1 receptor (IGF-1R) inhibitor, AG1024 (20 mg/kg/day i.p.), (8) group receiving leptin and a specific inhibitor of non-receptor Src tyrosine kinase family, PP2 (10 mg/kg/day i.p.), (9) group receiving leptin and an inactive analogue of PP2, PP3 (10 mg/kg/day i.p), (10) group receiving leptin and ERK inhibitor, PD98059 (5 mg/kg/day i.p.), (11) group receiving leptin and farnesyltransferase inhibitor, manumycin A (5 mg/kg s.c. every other day), (12) group receiving leptin and a broad-spectrum MMP inhibitor, doxycycline (20 mg/kg/day i.p.), (13) group receiving leptin and another MMP inhibitor, galardin (GM6001, 100 mg/kg/day i.p.). Separate groups of animals (14–19) received apocynin, AG1478, PP2, PD98059, manumycin A or doxycycline, but not leptin.

In our previous studies using the model of experimental hyperleptinemia [8], we administered leptin at a dose of 0.25 mg/kg twice daily s.c. for 7 days, which resulted in about 4-fold elevation of plasma leptin concentration. Herein, we modified this protocol to better reflect progressive increase in plasma leptin as it occurs in patients with obesity or type 2 diabetes, i.e. we gradually increased leptin dose from 0.05 mg/kg twice daily to 0.25 mg/kg twice daily in 0.05 mg-steps every two days. Consequently, the full dose (0.25 mg/kg twice daily) was achieved on the 9th day and the same dose was also administered on the 10th day. In this protocol, the cumulative dose of leptin administered to each animal during 10-day experiment was 3 mg/kg vs. 3.5 mg/kg for 7 days in our previous study [8].

The dose of apocynin used by us was previously demonstrated to abolish leptin-induced blood pressure elevation and oxidative stress [18]. Doses of AG1478, PD98059, manumycin A, doxycycline and GM6001 used by us were previously shown to effectively block the respective signaling pathways in rodents in vivo by Johns et al. [19], Muthalif et al. [20], Sugita et al. [21], Hao et al. [22], and Lund et al. [23]. Farnesyltransferase inhibitor, manumycin A, was used to block the activity of Ras proteins which must be farnesylated to become active. Dose of PP2 was empirically established by us in preliminary experiments by measuring its effect on c-Src phosphorylation. Doses of inactive analogues, PP3 and AG43, were identical as doses of their active counterparts. Doses of AG1024 and AG1295 were identical as dose of AG1478, since in our previous study these three compounds administered locally at equimolar amounts effectively and specifically inhibited the effects of IGF-1, PDGF and EGF, respectively [17].

Animals not receiving leptin were injected with bovine serum albumin (BSA) at a respective dose, and those not receiving any inhibitor or receiving apocynin in the drinking water were injected with equivolume amount of dimethyl sulfoxide (DMSO). Injections of leptin or BSA were made every day between 7.00 and 8.00 a.m. and between 7.00 and 8.00 p.m., and injections of inhibitors or their vehicle between 7.00 and 8.00 a.m. The first dose of leptin was injected in the evening and the last dose between 7.00 and 8.00 a.m. in the morning; total number of leptin doses was 20 in each animal.

2.3. Urine collection and blood pressure measurement

At the end of experiment, animals were placed in individual metabolic cages (Tecniplast, Buggugiate, Italy) and daily urine collection was done in all experimental groups. Urine collection was started on day 9 between 1.00 and 2.00 p.m., i.e. before the last two leptin/BSA injections, and was continued until 1.00–2.00 p.m. on the 10th day, i.e. until about 6 h after the last leptin/BSA dose. Urine was collected into sterile containers containing penicillin G (2000 IU) to prevent microbial overgrowth, and 10 μl of 0.05% butylated hydroxytoluene (BHT) to prevent lipid peroxidation in vitro. Urine was centrifuged, separated from sediment, and stored at −80 °C until analysis.

Systolic blood pressure (SBP) was measured with a tail–cuff method in conscious restrained animals prewarmed with a light lamp for 5 min at 37 °C. Three consecutive measurements were taken and the average value was recorded. Daily SBP measurements were initiated 3 days before starting leptin administration to train animals to the procedure, and were performed every day between 1.00 and 2.00 p.m. until the end of experiment. The last measurement taken before starting leptin injections was used for statistical comparisons as baseline value (day 0).

After completing urine collection and the last blood pressure measurement, animals were anesthetized with pentobarbital (50 mg/kg i.p.) and blood was withdrawn from the abdominal aorta into EDTA-containing tubes and immediately centrifuged to separate plasma. The kidneys and aorta (thoracic + abdominal) were removed and immediately frozen in liquid nitrogen. Plasma, urine and tissue samples were stored at −80 °C until analysis. For the assay of isoprostanes, plasma samples were stored in the presence of 0.05% BHT.

2.4. Experimental protocol – acute studies

In separate animals, acute effect of leptin and/or selected inhibitors on c-Src, ERK and EGFR phosphorylation in the aortic wall was investigated. For this purpose, we modified our model previously used to study the role of EGF receptor in the regulation of renal Na^+,K^+-ATPase [17]. Animals were anaesthetized with pentobarbital (50 mg/kg i.p.) and a thin catheter was inserted through the femoral artery into the abdominal aorta about 2 cm proximally from the renal arteries. Saline was infused for 30 min at a rate of 4 ml/h (stabilization period), and then leptin and/or various inhibitors were infused (see Section 3 for details). When infusion was performed for >1 h, anesthesia was supplemented at hourly intervals by i.p. injections of 2.5 mg pentobarbital/kg. After the end of

infusion, abdominal cavity was opened, and two parts of aorta, proximal and distal to the end of the catheter, were excised and immediately frozen in liquid nitrogen.

2.5. EGFR phosphorylation assay

For the measurement of EGFR activity, we assayed the levels of EGF receptor phosphorylated at two residues, Tyr^{1068} and Tyr^{845}. Upon activation, EGF receptor is phosphorylated at several tyrosine residues within its intracellular domain. Whereas some of them including Tyr^{1068} are phosphorylated in response to ligand binding, phosphorylation at Tyr^{845} is specifically carried out by c-Src [24]. We measured the levels of total EGFR (regardless of its phosphorylation state), EGFR phosphorylated at Tyr^{1068}, and EGFR phosphorylated at Tyr^{845} by Total EGF Receptor, Phospho-EGF (Tyr1068) Receptor and Phospho-EGF (Tyr845) Receptor PathScan Sandwich ELISA Kits (Cell Signaling Technology, Inc., Danvers, MA, USA), respectively.

In brief, thawed tissue samples were homogenized in 10 volumes of cell lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged (15,000 × *g* at 4 °C for 10 min). Supernatant (100 μl) was mixed with 100 volumes of sample diluent provided in the kit to fit within the linearity range (protein concentration in the diluted sample <0.1 mg/ml), and vortexed for a few seconds. Then, 100 μl of this mixture was added to the microplate wells coated with anti-EGFR antibody recognizing both phosphorylated and non-phosphorylated EGFR. The plate was incubated overnight at 4 °C to capture EGFR contained in the sample. Then, samples were removed and wells were washed 4 times with 200 μl of wash buffer. After washing, 100 μl of rabbit detection antibody specific for EGFR regardless of its phosphorylation state, antibodies specific for EGFR phosphorylated at Tyr^{1068} or antibodies specific for EGFR phosphorylated at Tyr^{845} was added, and the plate was incubated for 1 h at 37 °C. After the next washing, 100 μl of anti-rabbit IgG linked to horseradish peroxidase was added to the wells. After 30-min incubation at 37 °C and washing, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) + H_2O_2 substrate was added and the plate was incubated for 30 min at a room temperature, Then, 100 μl of stop solution was added and absorbance was read at 450 nm. Each sample was assayed in duplicate and the mean was used for further calculations. The ratio between optical density (OD) measured with anti-phosphoEGFR(Tyr1068) and OD measured with anti-EGFR, and the ratio between OD measured with anti-phosphoEGFR(Tyr845) and OD measured with anti-EGFR were calculated for each sample, and the results are presented as percent values taking the mean ratio in the control group as 100%. The relationship between protein concentration in diluted sample and OD was linear when protein concentration was <0.1 mg/ml. The intraassay/interassay CVs for OD measured for total EGFR, Tyr^{1068}-phosphorylated EGFR and Tyr^{845}-phosphorylated EGFR were 4%/7%, 8%/11% and 7%/12%, respectively. Total concentration of EGFR in the sample was expressed as OD_{450} × (mg protein/ml)$^{-1}$.

Although anti-EGFR antibodies used in these kits are primarily designed for human sequence, we confirmed their usefulness in the rat by several approaches. First, we found no absorbance if homogenate was processed without protease inhibitors. Second, EGFR was not detected in rat plasma. Third, EGFR was detected in microsomal/membrane fraction but not in cytosolic fraction separated by 100,000 × *g* centrifugation. Fourth, we observed linear relationship between total protein concentration and EGFR in serially diluted samples. Finally, increase in EGFR phosphorylation after administration of EGF but not other growth factors, and its abolishment by AG1478 but not by other tyrphostins (see Section 3) support the specificity of antibodies toward EGFR.

2.6. ERK phosphorylation assay

The activity of ERK in aortic and renal tissues was measured as the ratio between phosphorylated ERK and total (phosphorylated + nonphosphorylated) ERK. Phospho-ERK and total ERK concentrations were measured by enzyme immunoassay using kits obtained from Assay Designs, Inc. (Ann Arbor, MI, USA) as previously described in detail [17].

2.7. c-Src phosphorylation assay

The levels of active c-Src kinase (the most ubiquitously expressed member of Src kinase family) phosphorylated at Tyr^{418} and total c-Src were quantified by Sigma Phospho-Src($pTyr^{418}$) and Sigma Src ELISA kits, respectively, as previously described [17]. The sensitivity of phospho-c-Src assay was <1 U/ml; 1 U is defined as the amount of phospho-c-Src derived from the autophosphorylation of c-Src contained in 0.17 μg of total protein isolated from blood platelets. The sensitivity of the total c-Src assay is <1 ng/ml. The ratio between phospho-c-Src and total c-Src was calculated for each sample and the results are expressed in U/ng.

2.8. Renal Na^+,K^+-ATPase assay

Na^+,K^+-ATPase activity was assayed in microsomal fraction isolated from the renal cortex and medulla by measuring ouabain-inhibitable ATP hydrolysis to inorganic phosphate (P_i) at 100 mM NaCl (i.e. under V_{max} conditions), and was expressed in μmol of P_i liberated by 1 mg of microsomal protein during 1 h (μmol/mg protein/h) [17]. P_i was assayed by the method of Hurst [25].

2.9. Indices of oxidative stress

Plasma and urinary 8-isoprostanes, which are produced during ROS-mediated peroxidation of arachidonic acid, were measured by enzyme immunoassay using 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI, USA), after extraction on C-18 SPE cartridges (Waters Corporation, Milford, MA, USA). The procedures of extraction and assay were described in detail previously [8]. The detection limit of the assay was 5 pg/ml, whereas the intra- and interassay CVs were 5% and 8%, respectively.

Hydrogen peroxide in urine was measured by the FOX method based on the catalase-sensitive oxidation of Fe^{2+} to Fe^{3+}, which subsequently forms a blue-violet chromogen with xylenol orange [17,26]. Plasma H_2O_2 was measured by the same method using BIOXYTECH H_2O_2-560 assay kit (Oxis International, Portland, OR, USA).

2.10. Other assays

Plasma leptin was measured using Leptin Enzyme Immunoassay Kit (Cayman Chemical). Antibodies used in this assay are designed to detect human leptin, but we demonstrated 80% cross-reactivity with recombinant rat leptin (R&D Systems, Abingdon, Oxon, UK). Na^+ concentration in plasma and urine was measured by flame photometry. Creatinine was assayed by standard picric acid colorimetric method. Protein concentration in tissue samples was measured by the method of Lowry et al. [27].

2.11. Chemicals

Recombinant human leptin was purchased from Calbiochem (San Diego, CA, USA). Leptin (5 mg) was dissolved in 2.5 ml of 15 mM HCl and then 1.5 ml of 7.5 mM NaOH was added to bring pH to 5.2. This solution was diluted with the 15 mM HCl/7.5 mM NaOH mixture (5:3, v/v) to yield the appropriate concentration, frozen, stored at −80 °C and thawed immediately before use. The same HCl/NaOH mixture was used as a vehicle for BSA in animals not receiving leptin. The specific inhibitor of H^+,K^+-ATPases, Sch 28080, was provided by the Schering-Plough Research Institute (Kenilworth, NJ, USA). HB-EGF neutralizing antibody was obtained from R&D Systems. The following reagents were obtained from Calbiochem: PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), PP3 (4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine), GM6001 (*N*-[(2R)-2-(hydroxamido-carbonylmethyl)-4-methyl-pentanoyl]-L-tryptophan methylamide), inactive analogue of GM6001, *N*-*t*-butoxycarbonyl-L-leucyl-L-tryptophan methylamide, and ZM336372 (*N*-[5-(3-dimethylaminobenzamido)-2-methylphenyl]-4-hydroxybenzamide). Catalase (from bovine liver, specific activity 2000–5000 units/mg protein), tyrphostin AG1478 (*N*-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine), tyrphostin AG1024 (6,7-dimethyl-2-phenylquinoxaline), tyrphostin AG1295 (6,7-dimethyl-2-phenylquinoxaline), PD98059 (2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one), manumycin A, doxycycline, human EGF and HB-EGF, CRM197, IGF-1, recombinant rat PDGF-BB, angiotensin II and all other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

For chronic experiments, daily doses of AG1478, AG1024, AG1295, AG43, PP2 and PP3 for each animal were dissolved in 0.2 ml of DMSO. PD98059 was suspended in 0.5 ml of 20% DMSO in 0.9% NaCl. Manumycin A was dissolved in 0.5 ml of 0.4% DMSO in phosphate-buffered saline (PBS) [21]. GM6001 was prepared as 20 mg/ml slurry in 4% carboxymethylcellulose in PBS [23].

For acute experiments, stock solutions of tyrphostins (100 mM) were prepared in DMSO, stored at −20 °C, and diluted with 0.9% NaCl immediately before infusion. Doses of inhibitors used for infusion were chosen according to our recent study [17], in which these compounds have been demonstrated to effectively block renal Src-EGFR-Ras-Raf-MEK-ERK pathway. The final concentration of DMSO in infused solution was <1% and at this concentration DMSO had no effect on aortic or renal c-Src, EGFR or ERK if administered alone. To block Ras proteins, farnesylthiosalicylic acid (FTS) instead of manumycin A was used in acute experiments. FTS inactivates Ras by dislodging it from its membrane anchorage domains. Although manumycin A may be more effective in blocking Ras [21], it is unsuitable for acute experiments since due to its indirect mechanism of action (inhibition of Ras farnesylation, Fig. 1), it requires more time to become effective.

2.12. Calculations and statistical analysis

Glomerular filtration rate (GFR) was estimated by calculating endogenous creatinine clearance (urine output × urinary creatinine concentration/plasma creatinine concentration). Fractional excretion of Na^+ was counted as the ratio between urinary excretion and filtered load (GFR × plasma concentration).

Results are presented as mean ± S.E.M. from eight animals in each group. Blood pressure in a given group at various time points was compared by repeated-measures ANOVA followed by Newman–Keul's test. Between-group comparisons of other variables were done by single-measures ANOVA and Newman–Keul's post-hoc test. Data with nonGausian distribution or with variances differing between groups (plasma leptin, EGFR, c-Src and ERK phosphorylation) were logarithmically transformed before analysis. $p < 0.05$ was considered significant.

3. Results

3.1. Acute effect of leptin on c-Src, ERK and EGFR phosphorylation in aortic wall

First, we checked if acutely administered leptin stimulates c-Src and ERK phosphorylation in aortic wall, similar to that what was previously demonstrated in the kidney [17]. For this purpose, leptin was infused intraaortally under general anesthesia at a dose of 1 μg/kg/min. Initially, we infused leptin for 3 h because this period was required to observe stimulation of renal ERK [17]. However, in preliminary experiments we observed that similar stimulation of ERK in aortic wall was observed after 2 h of leptin infusion. Therefore, this time of infusion was used in subsequent experiments.

Administration of leptin at a dose of 1 μg/kg/min for 2 h had no effect on hormone concentration in peripheral blood, as demonstrated by measuring plasma leptin in samples withdrawn from the jugular vein after 2 h of infusion (control group: 3.12 ± 0.45 ng/ml, leptin-infused group: 3.21 ± 0.61 ng/ml). These data demonstrate that exogenous leptin was rapidly metabolized. Leptin increased ERK phosphorylation level in the part of aorta distal to the site of infusion, but not in its proximal segment (Fig. 2A). The effect of leptin on ERK phosphorylation was abolished by NADPH oxidase (NOX) inhibitor, apocynin, H_2O_2 scavenger, catalase, Src inhibitor, PP2, EGFR inhibitor, AG1478, Ras inhibitor, FTS, Raf inhibitor, ZM336372, and ERK inhibitor, PD98059, but not by PI3K inhibitor, wortmannin (Fig. 2A). PDGFR and IGF-1R inhibitors, AG1295 and AG1024, respectively, partially attenuated leptin-induced ERK phosphorylation (Fig. 2A). Superoxide scavenger, tempol, as well as PP3 and AG43 had no effect on leptin-induced ERK phosphorylation (not shown). AG1478 slightly

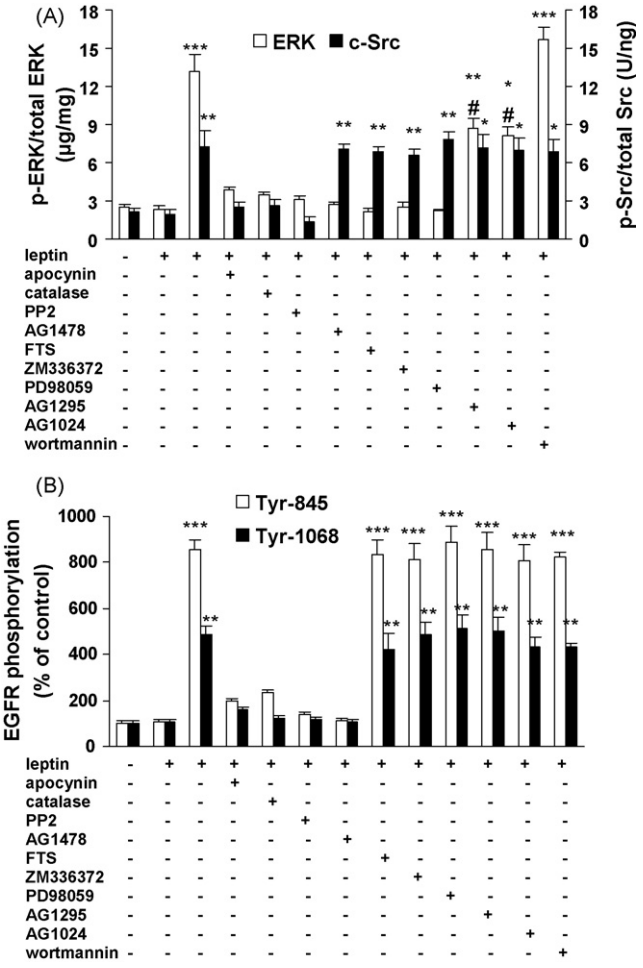

Fig. 2 – Acute effect of leptin on c-Src-EGFR-ERK pathway in aortic wall. (A) ERK phosphorylation (the ratio between phosphorylated and total ERK in μg/mg, left scale) and c-Src phosphorylation (the ratio between c-Src phosphorylated at Tyr^{418} and total c-Src, right scale). (B) Effect of leptin on EGFR phosphorylation at Tyr^{845} and Tyr^{1068}. The second pair of bars counting from the left represents a portion of aorta proximal to the site of infusion whereas the remaining bars – a part of aorta distal to the site of infusion. Leptin was infused at 1 μg/kg/min for 2 h and inhibitors expected to block its effect for the last 60 min of leptin administration. Inhibitors were administered at 1 μmol/kg/min (apocynin), 1 mg/kg/min (catalase), 10 nmol/kg/min (PP2 and wortmannin) or 0.1 μmol/kg/min (other compounds). Control group received 0.9% NaCl for 2 h. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group, $^{\#}p < 0.05$ vs. group receiving leptin alone.

reduced ERK phosphorylation also in animals not receiving leptin, but other inhibitors had no effect (not shown).

Apart from ERK, leptin increased c-Src phosphorylation and this effect was abolished only by apocynin, catalase and PP2, but not by other inhibitors (Fig. 2A). These results suggest that leptin activates ERK in aortic tissue through the mechanism involving ROS (presumably H_2O_2), c-Src, EGF receptor and partially PDGF and IGF-1 receptors.

Next, we examined the effect of leptin on EGF receptor phosphorylation (Fig. 2B). Leptin increased EGFR phosphorylation at Tyr^{845} more than 8-fold and at Tyr^{1068} more than 4-fold. These effects were abolished by apocynin, catalase, PP2 and AG1478, but not by other inhibitors. AG1478 reduced EGFR phosphorylation at both sites by about 50% in animals not receiving leptin (not shown). AG1295 and AG1024 had no effect on EGFR phosphorylation at either Tyr^{845} or Tyr^{1068} (Fig. 2B).

3.2. *Acute effect of H_2O_2 and growth factors on c-Src, ERK and EGFR phosphorylation in aortic tissue*

Similar to leptin, infusion of H_2O_2 resulted in the increase in c-Src and ERK phosphorylation (Fig. 3A). In addition, H_2O_2 increased EGFR phosphorylation at Tyr^{845} and, more modestly, at Tyr^{1068} (Fig. 3B). H_2O_2-induced increase in c-Src phosphorylation was abolished only by PP2 but not by AG1478, PD98059 or manumycin A (Fig. 3A). Stimulation of EGFR phosphorylation by H_2O_2 was abolished by PP2 and AG1478, and stimulation of ERK by both these inhibitors as well as by PD98059 but not by wortmannin. These data suggest that H_2O_2 stimulates ERK in c-Src and EGFR-dependent but PI3K-independent manner.

Exogenous EGF increased EGFR phosphorylation more markedly at Tyr^{1068} than at Tyr^{845}, and its effect was sensitive to AG1478 but not to PP2. In addition, EGF increased ERK phosphorylation, which was abolished by AG1478, FTS and PD98059. Interestingly, although wortmannin had no effect on leptin- or H_2O_2-induced ERK phosphorylation, it partially attenuated EGF-induced ERK phosphorylation (Fig. 3C), suggesting that PI3K is involved in ERK activation by EGF. The similar results were observed following the infusion of HB-EGF, however, the equimolar dose of this growth factor had less marked effects on EGF receptor and ERK than EGF (not shown).

Exogenous PDGF as well as IGF-1 induced a slight increase in ERK phosphorylation, however, this effect was much less marked than the effect of leptin or EGF (Fig. 3D). The effects of these growth factors were abolished by AG1295 and AG1024, respectively, but not vice versa. Moreover, AG1478 had no effect on PDGF or IGF-1-induced ERK phosphorylation (Fig. 2D), confirming that PDGF and IGF-1 receptors are active in the aortic wall, and that three tyrphostins used, AG1478, AG1295 and AG1024, specifically inhibit the activity of their target receptors.

3.3. *Effect of experimental hyperleptinemia on c-Src, EGFR, and ERK phosphorylation in aorta and kidney*

Having established that leptin stimulates EGFR-ERK signaling in the vascular wall in the short run, we examined the effect of long-lasting hyperleptinemia. For this purpose, we injected animals with gradually increasing doses of leptin for 10 days (see Section 2). Neither leptin nor any of the coadministered inhibitors (see below) had any effect on body weight. Leptin tended to decrease food intake by about 15% on days 8–10, but this effect did not reach the level of significance. Therefore, separate ''pair-fed'' groups with food intake restricted to match the level observed in group receiving leptin were not included in this study. Apocynin, PP2, PP3, AG1478, AG43, AG1024, AG1295, manumycin A and PD98059 had no effect on

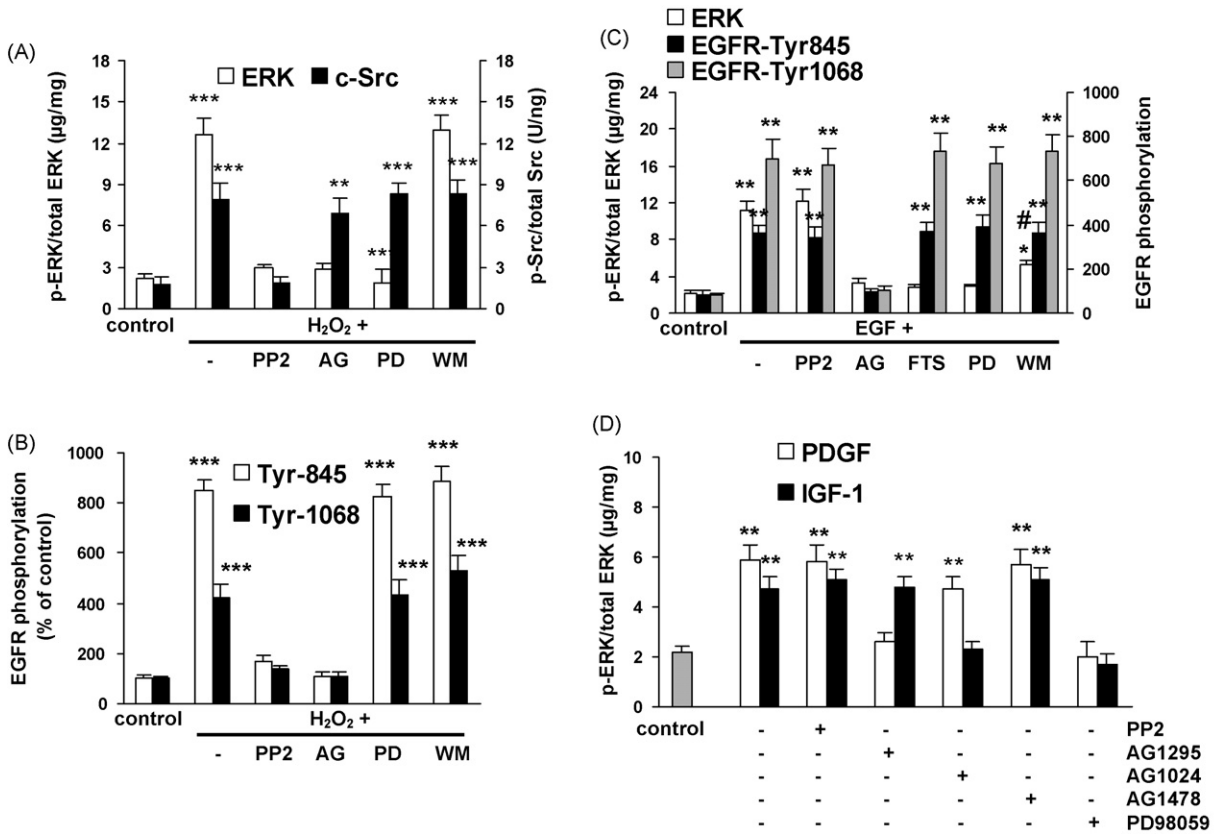

Fig. 3 – Acute effect of H_2O_2 (left panels) and growth factors (right panels) on c-Src-EGFR-ERK pathway in the rat aorta. H_2O_2 was infused at 200 nmol/kg/min for 30 min (Ref. [17]) and c-Src and ERK phosphorylation (A) as well as EGFR phosphorylation at Tyr^{845} and Tyr^{1068} (B) were measured. Separate groups received PP2 (10 nmol/kg/min), AG1478 (AG, 0.1 μmol/kg/min), PD98059 (PD, 0.1 μmol/kg/min) or wortmannin (WM, 10 nmol/kg/min) for 30 min before and during H_2O_2 infusion. (C) EGF was infused at 1 nmol/kg/min for 30 min, and inhibitors: PP2 (10 nmol/kg/min), AG1478 (AG, 0.1 μmol/kg/min), farnesylthiosalicylic acid (FTS, 0.1 μmol/kg/min), PD98059 (PD, 0.1 μmol/kg/min) or wortmannin (WM, 10 nmol/kg/min) were administered for 30 min before (i.e. during the stabilization period) and during EGF infusion. The ratio between phospho-ERK and total ERK (in μg/mg, left scale) and EGFR phosphorylation at Tyr^{845} and Tyr^{1068} (% of control, right scale) were measured in aortic wall. (D) PDGF or IGF-1 was infused at 1 nmol/kg/min for 30 min. Separate groups received PP2 (10 nmol/kg/min), PD98059 (0.1 μmol/kg/min) or tyrphostins AG1295, AG1024 or AG1478 (all at 0.1 μmol/kg/min) for 60 min (30 min before and during growth factor infusion). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control, $^{\#}p < 0.05$ vs. group receiving EGF alone.

food intake (not shown). Plasma leptin was higher in animals receiving leptin than in control rats (14.2 ± 1.8 vs. 3.7 ± 0.4 ng/ml, $p < 0.001$), but was unaffected by any of the co-administered inhibitors.

Leptin administered for 10 days increased c-Src, ERK and EGFR phosphorylation in aorta (Fig. 4A) and renal cortex (Fig. 4B). Because in acute experiments leptin had a similar effects on ERK, c-Src and Na^+,K^+-ATPase in the renal cortex and medulla [17] and because the amount of medullary tissue was insufficient to make all assays (c-Src, ERK, EGFR and Na^+,K^+-ATPase) in the single sample without pooling, herein we did not measure c-Src, ERK and EGFR phosphorylation in the renal medulla. Importantly, leptin had no effect on absolute amount of total c-Src, EGF receptor and ERK (not shown). Leptin-induced increase in c-Src phosphorylation in the aortic wall and renal cortex was abolished by apocynin and PP2 (Fig. 4). Increase in EGFR phosphorylation in animals receiving leptin was abolished by coadministration of apocynin, PP2 and AG1478, whereas increase in ERK phosphorylation was abolished by apocynin, PP2, AG1478, manumycin A and PD98059 in both aortic wall and renal cortex (Fig. 4). AG1024 and AG1295 slightly reduced ERK phosphorylation in aorta but not in the kidney of rats injected with leptin for 10 days, but had no effect on EGFR phosphorylation. Neither PP3 nor AG43 had any effect on c-Src, EGFR and ERK phosphorylation (not shown). These data indicate that 10-day systemic administration of leptin stimulates c-Src-EGFR-Ras-MEK-ERK pathway.

In animals not receiving leptin, AG1478 reduced EGFR phosphorylation at Tyr^{845} and Tyr^{1068} respectively by 23% and 29% in aortic wall, and by 16% and 19% in the renal cortex, but had no effect on either c-Src or ERK phosphorylation. PD98059 administered alone tended to reduce ERK phosphorylation, but this effect was not significant. PP2 had no effect on

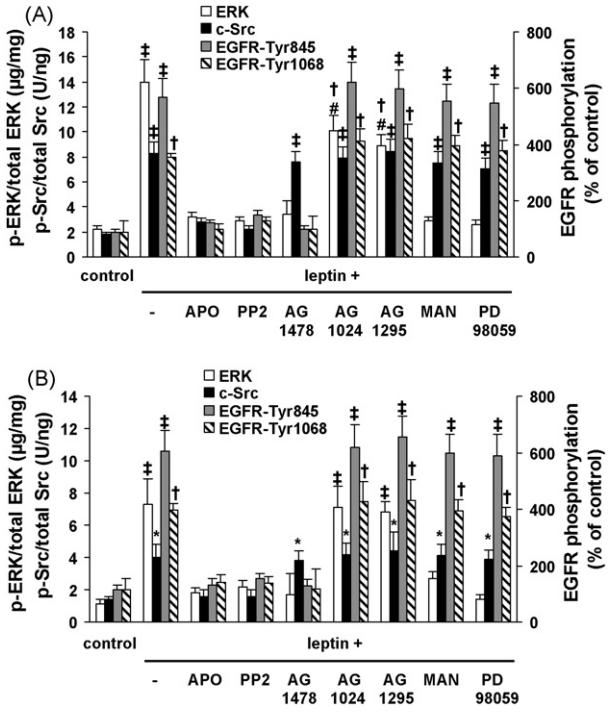

Fig. 4 – Effect of experimental hyperleptinemia on ERK, c-Src and EGFR phosphorylation in aortic wall (A) and renal cortex (B). Leptin was administered for 10 days in increasing doses and separate groups of animals received apocynin (APO), PP2, AG1478, AG1024, AG1295, manumycin A (MAN) or PD98059 together with leptin (see Section 2 for details). ERK phosphorylation (white bars) and c-Src phosphorylation (black bars) are presented on the left axis, and EGFR phosphorylation at Tyr^{845} (grey bars) and Tyr^{1068} (batched bars) are presented as % of control on the right axis. $^{*}p < 0.05$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$ vs. control group, $^{\#}p < 0.05$ vs. group receiving leptin alone.

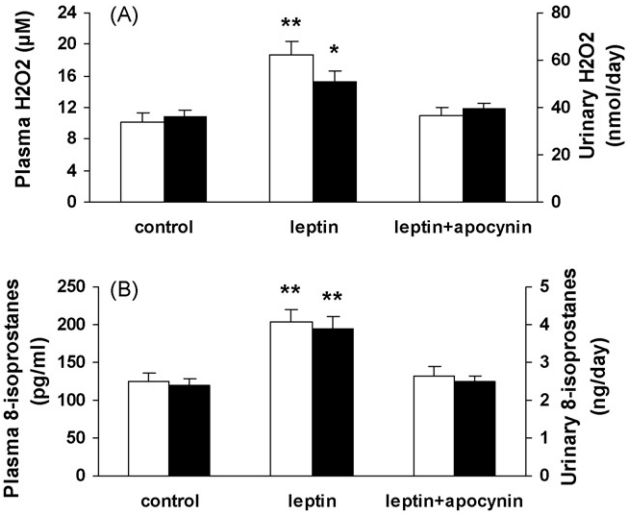

Fig. 5 – Plasma concentration (white bars, left scale) and urinary excretion (black bars, right scale) of H_2O_2 (A) and 8-isoprostanes (B) in selected experimental groups. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group.

phosphorylation levels of c-Src, EGFR and ERK in rats not receiving leptin (not shown).

3.4. *Effect of experimental hyperleptinemia on oxidative stress*

Plasma concentration and urinary excretion of H_2O_2 and 8-isoprostanes were higher in animals receiving leptin than in control group. Apocynin restored these markers of oxidative stress in animals receiving leptin to the levels observed in control group (Fig. 5). In contrast, PP2, AG1478 and PD98059 had no effect on H_2O_2 and isoprostanes. Apocynin had no effect on H_2O_2 and isoprostanes in animals not receiving leptin (not shown).

3.5. *Effect of experimental hyperleptinemia on systolic blood pressure*

There was no significant difference in SBP between groups before treatment, and blood pressure did not change in the control group throughout the experiment. Blood pressure increased significantly following leptin administration since the 5th day, and remained elevated until the 10th day (Fig. 6). Coadministration of apocynin, PP2, AG1478, manumycin A or PD98059 reduced blood pressure in rats receiving leptin to values observed in the control group (Fig. 6). Neither of these compounds had any effect on blood pressure in rats not receiving leptin. In addition, inactive analogues of PP2 and AG1478, PP3 and AG43, respectively, as well as AG1024 and AG1295 had no effect on blood pressure (not shown). These data indicate that NOX-derived reactive oxygen species, Src tyrosine kinase(s), EGF receptor, Ras proteins and ERK are involved in leptin-induced blood pressure elevation.

3.6. *Effect of experimental hyperleptinemia on renal Na^+ handling and renal Na^+,K^+-ATPase*

Plasma Na^+, plasma creatinine, and creatinine clearance did not differ between groups (not shown). Both absolute and fractional sodium excretion were lower in group receiving leptin than in control rats (Fig. 7A), which was accompanied by the stimulation of renal cortical and medullary Na^+,K^+-ATPase (Fig. 7B). The effects of leptin on absolute Na^+ excretion and Na^+,K^+-ATPase were abolished, and the effect on fractional Na^+ excretion was markedly attenuated, by apocynin, PP2, AG1478, manumycin A or PD98059 (Fig. 7), but not by PP3, AG43, AG1024 or AG1295 (not shown). These data suggest that ROS-Src-EGFR-Ras-ERK pathway is involved in antinatriuretic effect of leptin.

3.7. *Acute effect of inhibitors of c-Src-EGFR-ERK pathway in rats with experimental hyperleptinemia*

Stimulation of c-Src-EGFR-ERK pathway induced by 10-day leptin administration could be secondary to hypertension, structural changes in the kidney/vasculature and/or altered

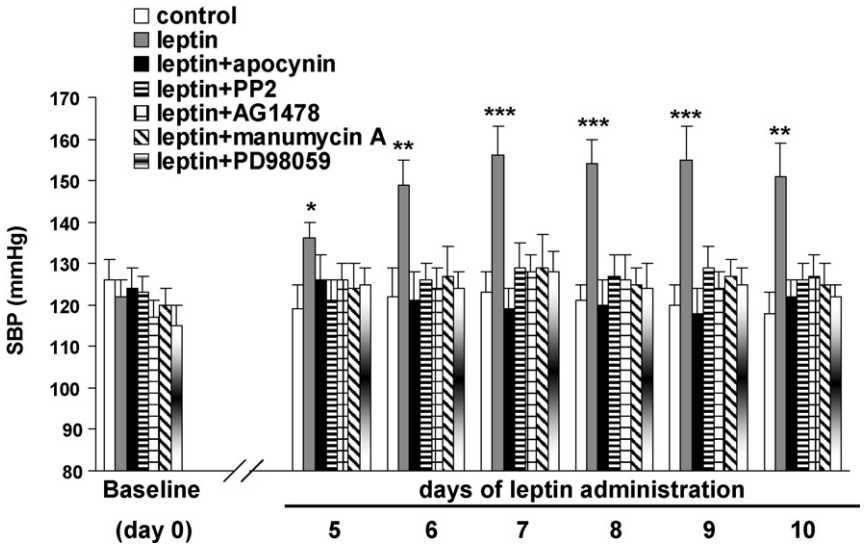

Fig. 6 – Systolic blood pressure (SBP) in various experimental groups. For clarity, data recorded on days 1–4 of leptin administration, when SBP did not differ between groups, are not presented. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. baseline in the respective group by repeated-measures ANOVA.

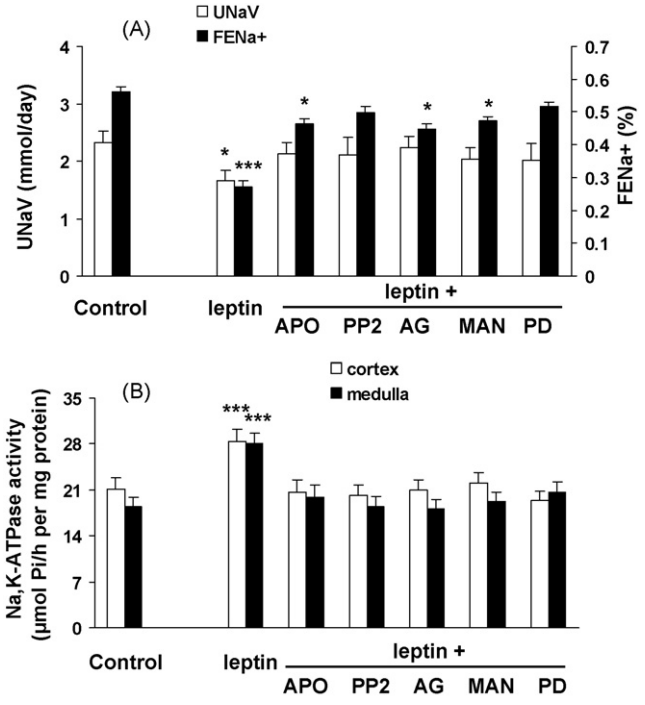

Fig. 7 – Renal sodium handling (A) and renal Na^+,K^+-ATPase activity (B). (A) absolute (white bars, left scale) and fractional (black bars, right scale) sodium excretion on the 10th day of experiment (24-h urine collection bracketing the last two leptin doses and finished ca. 6 h after the last leptin injection) in various experimental groups. (B) Na^+,K^+-ATPase activity in the renal cortex (white bars) and medulla (black bars) after 10 days of administration of leptin and/or various inhibitors. APO, apocynin; AG, AG1478; MAN, manumycin A; PD, PD98059. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control group.

gene expression of these kinases. To examine if this pathway may be modulated in hyperleptinemic rats in the short run by the respective inhibitors, separate animals previously receiving leptin for 10 days were anesthetized, and apocynin, PP2, AG1478 or PD98059 were infused into the abdominal aorta for 60 min. Because leptin increased EGFR phosphorylation predominantly at Tyr^{845}, only phosphorylation at this site was measured in these studies. In rats receiving leptin, the level of c-Src phosphorylation was normalized by apocynin or PP2, EGFR phosphorylation was normalized by apocynin, PP2 or AG1478, whereas ERK phosphorylation and Na^+,K^+-ATPase activity were restored to control values by either of these inhibitors as well as by PD98059 (Fig. 8). These results suggest that alterations of c-Src-EGFR-ERK pathway induced by 10-day leptin administration are at least partially independent of hypertension and/or vascular and renal hypertrophy, but are induced by leptin itself.

3.8. *Role of MMP/ADAM-dependent HB-EGF shedding in leptin-induced EGFR transactivation*

Cleavage of HB-EGF from its membrane-bound precursor, proHB-EGF, by MMPs or ADAMs is a common mechanism of EGFR activation by various vasoactive substances. We used several approaches to examine if this mechanism is involved in the effect of leptin. First, we investigated the effect of angiotensin II, which is well known to transactivate the EGF receptor through the HB-EGF-dependent mechanism. For this purpose, we infused angiotensin II into the abdominal aorta of anesthetized animals for 2 h and then measured EGFR and ERK phosphorylation in aortic wall and renal cortex (Fig. 9A and B). Angiotensin II markedly stimulated EGFR phosphorylation both at Tyr^{845} and at Tyr^{1068} as well as ERK phosphorylation, and these effects were partially attenuated by a broad-range MMP and ADAM inhibitor, GM6001, but not by its inactive

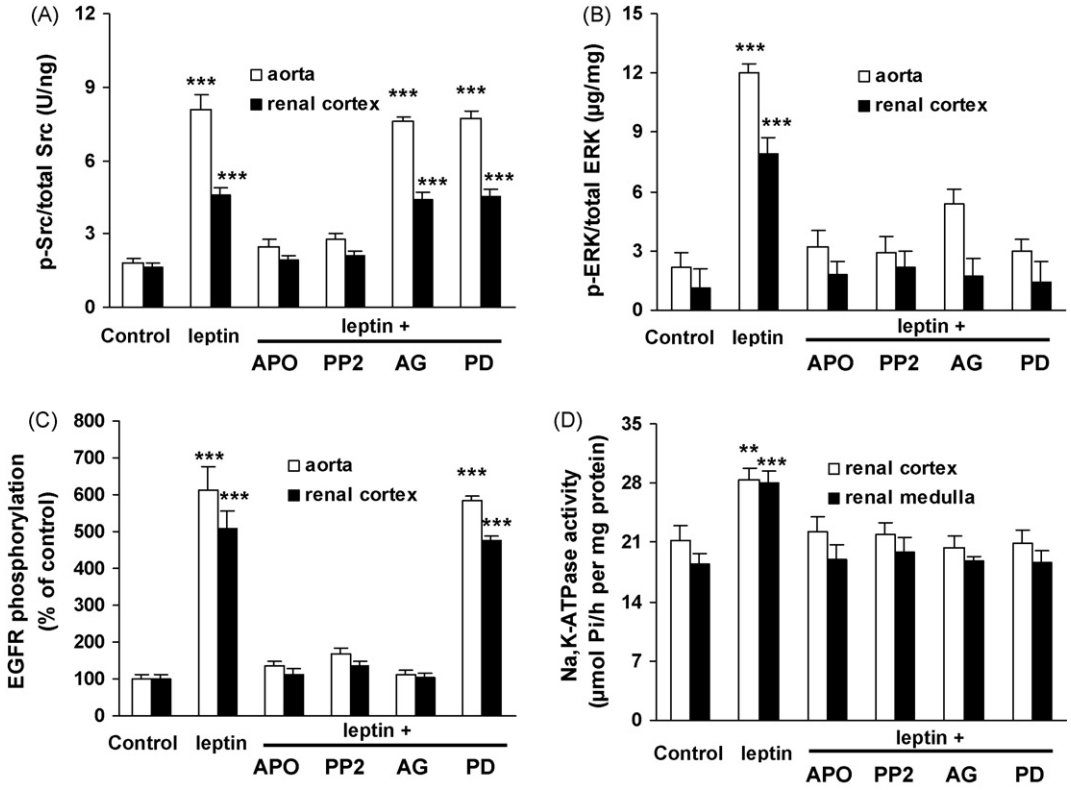

Fig. 8 – Acute effect of apocynin (APO), PP2, AG1478 (AG) and PD98059 (PD) on c-Src phosphorylation (A), EGFR phosphorylation at Tyr^{845} (B) and ERK phosphorylation (C) in aortic wall and renal cortex, and on renal Na^+,K^+-ATPase activity (D). Leptin was administered for 10 days, animals were anesthetized 6 h after the last leptin injection, and apocynin (1 μmol/kg/min), PP2 (10 nmol/kg/min), AG1478 (0.1 μmol/kg/min) or PD98059 (0.1 μmol/kg/min) were infused into the abdominal aorta for 60 min. Control and hyperleptinemic rats not receiving any inhibitor were infused with saline for 60-min before harvesting tissue samples. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

analogue. Similarly, a high affinity HB-EGF ligand, CRM197, as well as HB-EGF neutralizing antibody, but not nonimmune IgG, also attenuated the effects of angiotensin II on EGFR and ERK phosphorylation. These data confirm that GM6001, CRM197 and anti HB-EGF antibody effectively block the MMP/ADAM-HB-EGF pathway. However, GM6001, CRM197 and anti HB-EGF antibody failed to attenuate the effect of acutely infused leptin (Fig. 9)

Next, we examined if these inhibitors administered acutely affect the EGFR-ERK pathway in rats previously injected with leptin for 10 days (Fig. 9C and D). In these animals, infusion of GM6001, CRM197 or anti HB-EGF antibody partially attenuated EGFR phosphorylation at Tyr^{1068} but not at Tyr^{845} in aortic wall, and had no effect on EGFR phosphorylation at either site in the kidney. ERK phosphorylation was unaltered in both tissues. These data indicate that although HB-EGF may be, to some extent, involved in EGFR transactivation in aorta of rats with experimental hyperleptinemia, this mechanism plays only a minor role in ERK stimulation.

Finally, we tested the effect of GM6001 as well as of another broad-spectrum MMP inhibitor, doxycycline, administered systemically together with leptin for 10 days (Fig. 10). GM6001 as well as doxycycline slightly reduced EGFR (Tyr^{1068}) and ERK phosphorylation in aorta but not in the renal cortex. However, GM6001 and doxycycline had no effect on blood pressure or renal Na^+,K^+-ATPase.

4. Discussion

4.1. *Role of ERK and EGFR in leptin-induced hypertension*

Extracellular signal-regulated kinases-1 and -2, belonging to a large family of mitogen-activated protein kinases, constitute one of the most important signaling pathways controlling cell growth, proliferation and survival, but are also involved in the regulation of vascular tone. ERKs are activated in response to various vasoconstrictors such as α-adrenergic agonists, endothelin-1 and angiotensin II, and increased ERK phosphorylation and/or activity has been demonstrated in several animal models of hypertension [28,29], as well as in hypertensive humans [30]. Moreover, ERK inhibitors such as PD98059 improve endothelial function, dilate blood vessels and/or reduce blood pressure in hypertensive but not normotensive animals [20,31–34]. ERK may also activate renal Na^+,K^+-ATPase [35,36]. In addition, several lines of evidence suggest that EGF and its receptor are involved in the pathogenesis of hypertension. EGF induces vasoconstriction

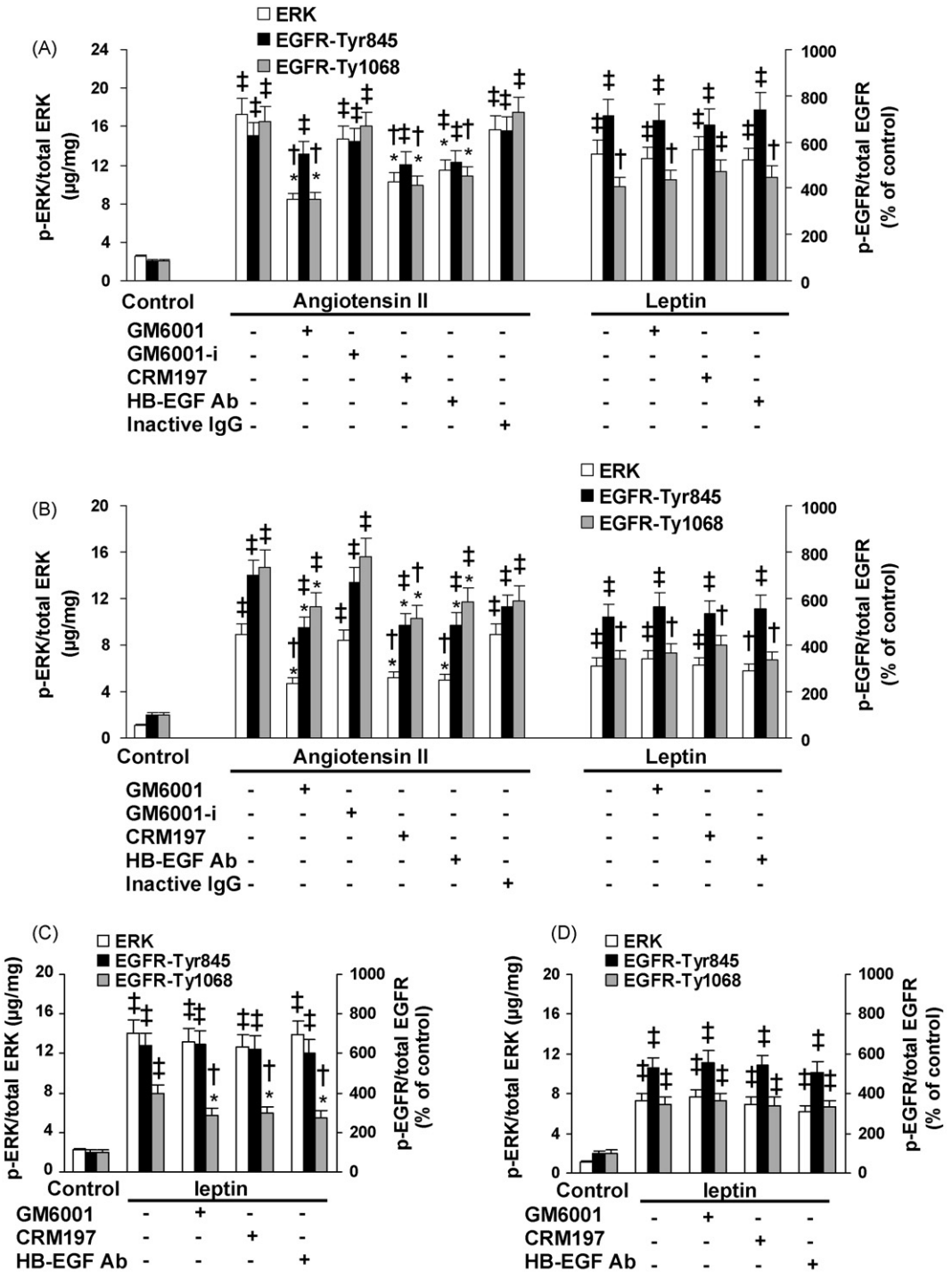

Fig. 9 – Effect of inhibitors of MMP/ADAM – HB-EGF pathway on ERK phosphorylation (left scale) and EGFR phosphorylation (right scale) induced by angiotensin II or leptin. (A and B) Anaesthetized rats were acutely infused with angiotensin II (100 ng/kg/min) or leptin (1 μg/kg/min). Angiotensin II or leptin were infused into the abdominal aorta for 2 h, and separate groups received simultaneously GM6001, its inactive analogue, GM6001-i (both at 100 nmol/kg/min), CRM197 (1 nmol/min/rat), HB-EGF neutralizing antibody ((HB-EGF Ab, 10 pg/min/rat) or control inactive IgG (10 pg/min/rat). ERK and EGFR phosphorylation were subsequently measured in aortic wall (A) and renal cortex (B). (C and D) leptin was administered for 10 days, and then GM6001, CRM197 or HB-EGF neutralizing antibody were infused into the abdominal aorta under general anesthesia at doses specified above for 2 h. ERK and EGFR phosphorylation were subsequently measured in aortic wall (C) and renal cortex (D). $^{\dagger}p < 0.01$ $^{\ddagger}p < 0.001$ vs. control group, $^{*}p < 0.05$ vs. group receiving angiotensin II or leptin alone.

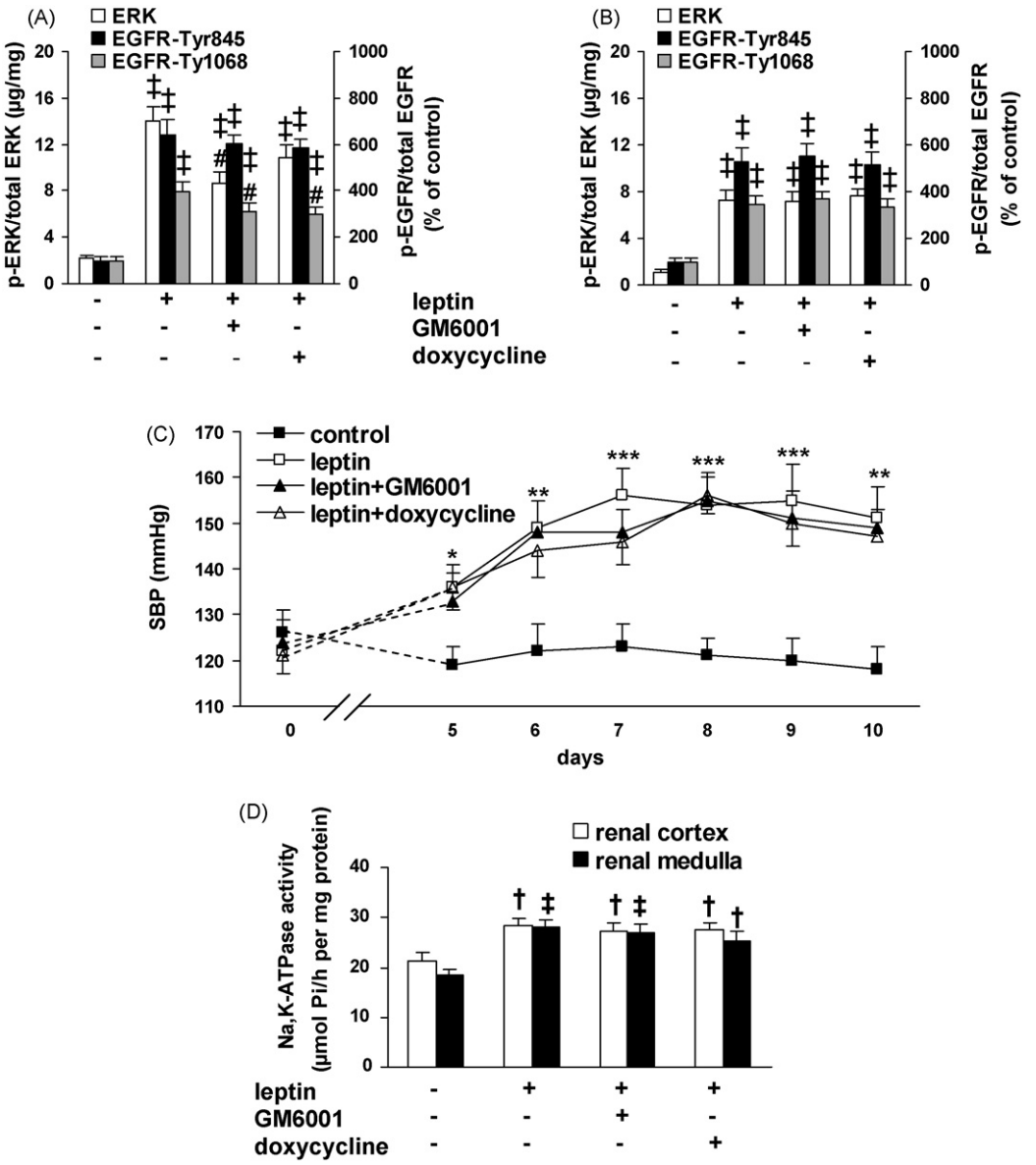

Fig. 10 – Effect of MMP and ADAM inhibitors, GM6001 and doxycycline, on ERK and EGFR phosphorylation in the aortic wall (A) ERK and EGFR phosphorylation in the renal cortex (B), blood pressure (C) and renal Na^+,K^+-ATPase activity (D). Leptin was administered in increasing doses (see Section 2) for 10 days. Separate groups received also doxycycline (20 mg/kg/day i.p.) or GM6001 (100 mg/kg/day i.p.). $^{\dagger}p < 0.01$ $^{\ddagger}p < 0.001$ vs. control group, $^{\#}p < 0.05$ vs. group receiving leptin alone (by single-measures ANOVA), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. day 0 (before starting leptin administration) by repeated-measures ANOVA.

[37,38] and elicits a pressor response in vivo [39]. Moreover, although its effect on renal tubular transport is controversial, EGF has been demonstrated to stimulate renal Na^+,K^+-ATPase [40] and thus may promote sodium retention. However, the greatest interest in role of EGFR in hypertension is associated with its ligand-independent transactivation. The expression and/or activity of EGFR is increased in various experimental models of hypertension [41–45]. EGFR inhibitors attenuate vasoconstriction induced by angiotensin II or α-adrenergic agonists [22,45], and EGFR antisense oligonucleotides reduce blood pressure in rats with angiotensin II-induced hypertension [46].

Recently, leptin has been demonstrated to activate ERK in a catalase-sensitive manner in human coronary artery endothelial cells [47], but the involvement of EGFR was not studied. Nevertheless, leptin transactivates EGF receptor in cultured gastric cancer [48], esophageal cancer [49] and vascular smooth muscle cells [9]. Moreover, EGFR inhibitor attenuated the beneficial effect of leptin on gastric ulcer healing [50]. Herein, we demonstrate that EGF receptor and ERK are activated in vascular wall and kidney of rats with experimental hyperleptinemia and are involved in the pathogenesis of leptin-induced hypertension. In addition, to our knowledge we demonstrate for the first time the blood pressure-lowering potential of

pharmacological EGFR inhibition. Previous studies addressing the hypotensive effect of EGFR blockade gave conflicting results. Whereas Kagiyama et al. [46] have demonstrated that EGFR antisense oligonucleotides reduce blood pressure in rats with angiotensin II-induced hypertension, pharmacological blockade of EGFR with gefitinib had no effect on blood pressure in rats with hypertension induced by chronic nitric oxide synthase blockade with L-NAME [51]. The reasons of these discrepancies are unclear at present. However, in the latter study (51) L-NAME was administered for 4 weeks, which resulted in more marked blood pressure elevation (to almost 180 mmHg) than in our study. In addition, L-NAME model is characterized by severe deterioration of renal structure and function evidenced by proteinuria and almost 2-fold elevation of plasma creatinine [51]. It is likely that EGFR inhibitors are effective hypotensive agents only in relatively mild and short-lasting models of hypertension such as the model used by us. Nevertheless, EGFR is potentially a very attractive target for antihypertensive therapy. First, EGFR-ERK pathway is a common final pathway for many vasoconstricting and Na^+-retaining mediators and therefore, inhibiting its activity may be superior to therapies targeting specific mediators. Second, EGFR blockade may exert not only functional effects on vascular tone and/or renal function, but also may inhibit end-organ damage associated with long-lasting hypertension. If states associated with hyperleptinemia are considered, it should be noted that leptin may contribute to renal damage [52], vascular remodeling [53] and left ventricular hypertrophy [54] independently of blood pressure elevation. Finally, EGFR inhibitors were first introduced to the market as anticancer drugs, and leptin may promote tumor progression [55]. Simultaneous beneficial effects on cardiovascular system and the risk of cancer may be a unique feature of EGFR inhibitors.

4.2. *Mechanism of leptin-induced EGFR activation*

There are two principal mechanisms of EGFR transactivation: (1) c-Src-dependent phosphorylation, (2) MMP-dependent shedding of HB-EGF from its membrane precursor. Both these mechanisms may be elicited by oxidative stress, and the latter one has been demonstrated in response to various vasoconstrictors including sympathetic system and endothelin-1 [12], which are stimulated by leptin [3,9]. Leptin stimulates MMPs in endothelial and vascular smooth muscle cells [56], and MMPs have been implicated in leptin-induced EGFR activation in gastric cancer, esophageal cancer and vascular smooth muscle cells [9,48,49], although the evidence of this was confined to inhibition of the effect by GM6001. However, the results of our study strongly suggest that MMP/HB-EGF dependent mechanism plays only a minor role in rats with experimental hyperleptinemia. First, MMP and HB-EGF inhibitors had no effect on leptin-induced EGFR and ERK stimulation in acute experiments, although at the same doses markedly attenuated angiotensin II-induced effects (Fig. 9A and B). Second, leptin increased EGFR phosphorylation more markedly at Tyr^{845}, a c-Src phosphorylation site [24] than at Tyr^{1068}, which is one of typical autophosphorylation (ligand-induced phosphorylation) sites. Indeed, both EGF and HB-EGF increased EGFR phosphorylation more markedly at Tyr^{1068} than at Tyr^{845}. Third, leptin-induced phosphorylation of EGFR and ERK as well as blood pressure elevation were abolished by PP2, a specific inhibitor of Src kinase family. Although c-Src may also be activated by EGFR, in our hands PP2 abolished leptin-induced EGFR phosphorylation but AG1478 did not attenuate leptin-induced c-Src phosphorylation, suggesting that this was not the case. Finally, PI3K inhibitor, wortmannin, partially attenuated EGF- or HB-EGF-stimulated ERK phosphorylation, but had no effect on leptin- or H_2O_2-induced ERK phosphorylation, suggesting that although both ligand-dependent and c-Src-dependent EGFR activation results in the stimulation of ERK, the precise intracellular mechanisms linking this receptor with ERK may differ, presumably due to various profiles of tyrosine residues phosphorylated in both cases.

In contrast to acute leptin infusion, GM6001, CRM197 or anti HB-EGF antibody infused intraaortally partially attenuated EGFR phosphorylation at Tyr^{1068} in vascular wall in rats receiving leptin for 10 days. Similarly, either GM6001 or doxycycline administered systematically reduced EGFR and ERK phosphorylation in aortic wall of rats receiving leptin. These results suggest that MMP/HB-EGF pathway is stimulated in aortic wall of animals receiving leptin for 10 days, however, this pathway plays only a minor role in hemodynamic and renal effects of leptin, since GM6001 or doxycycline did not alter blood pressure or renal Na^+,K^+-ATPase activity. It should be noted that doxycycline administered at a dose used by us reduced blood pressure in spontaneously hypertensive rats after 5 weeks of treatment [22]. We cannot exclude that more prolonged treatment with MMP inhibitors would have resulted in reduction of blood pressure in leptin-induced hypertension.

4.3. *Possible role of other growth factor receptors*

Apart from EGFR, other growth factor receptor such as PDGFR and IGF-1R are expressed in the vascular wall and may be transactivated by oxidative stress-dependent mechanisms [57,58]. Although AG1295 and AG1024, administered at doses which abolished ERK stimulation by PDGF and IGF-1, respectively (Fig. 3D), slightly reduced ERK phosphorylation in aorta of rats receiving leptin either acutely (Fig. 2) or for 10 days (Fig. 4), these tyrphostins had no effect on blood pressure. These data suggest that although PDGFR and IGF-1R are involved in leptin-induced ERK stimulation, neither of these receptors contributes to the development of hypertension. Consistently with our findings, PDGFR inhibitor, imatinib, and IGF-1R antisense oligonucleotides had no effect on blood pressure in other models of hypertension [57,59,60]. However, imatinib attenuated myocardial and arterial hypertrophy in angiotensin II-dependent hypertension [57,59] which, together with our finding that AG1295 reduced ERK phosphorylation in aorta of rats receiving leptin, suggests that PDGFR inhibitors might ameliorate hypertrophic response of the cardiovascular system in hyperleptinemic states.

4.4. *Study limitations*

There are several important limitations of the present study. First, we measured only systolic blood pressure and did it by an indirect tail–cuff method. Although the limitations of this method are well-known, this procedure allowed as repeating the measurements in all animals throughout the entire

experimental period. In addition, direct measurement in anesthetized animals would require more extensive and longer instrumentation, which might affect signaling pathways in subsequently harvested tissue samples. Second, although the effect of leptin was mimicked by H_2O_2 and abolished by catalase, we cannot identify the specific ROS involved (H_2O_2, superoxide, peroxynitrite, etc.) apart from saying that its primary source is NOX, as evidenced by effectiveness of apocynin. Third, we measured EGFR and ERK phosphorylation in aorta which plays a minor role in the regulation of vascular resistance. Further studies are required to examine if the same signaling pathway is triggered by leptin in small resistance arteries. Fourth, both aortic wall and the kidney are composed of many cell types. Because we measured EGFR, ERK and c-Src phosphorylation in whole-tissue homogenate, we cannot conclude in which cells the observed effects occurred. Finally, we did not measure the effect of leptin on vascular tone and thus cannot conclude if EGFR-ERK pathway contributes to blood pressure elevation by increasing vascular resistance in addition to its effect on renal Na^+ handling. However, the role of EGFR-ERK pathway in vasoconstriction is well established in other models [12]. Moreover, leptin has multiple effects on vascular tone including regulation of NO production [61], attenuation of angiotensin II-induced vasoconstriction [62], and possibly stimulation of NO-independent vasorelaxation [63]. The net effect of leptin on vascular tone is thus a result of these counteracting mechanisms, and dissecting the precise role of EGFR-ERK pathway might be difficult in vitro. Despite these limitations, the results strongly suggest the role of EGFR in leptin-induced hypertension, although the precise contribution of changes of vascular tone and renal Na^+ handling remains to be established.

4.5. *Conclusions*

The results of this study indicate that hyperleptinemia is associated with ROS and c-Src-dependent activation of the EGF receptor in the vascular wall and the kidney, and that this receptor contributes to blood pressure elevation by inducing Na^+,K^+-ATPase-dependent sodium retention and, possibly, increasing vascular tone. Prohypertensive effect of EGF receptor involves Ras proteins and extracellular signal-regulated kinases. These data suggest that inhibitors of c-Src-EGFR-Ras-ERK pathway may be helpful in the treatment of hypertension associated with hyperleptinemic states, such as obesity and type 2 diabetes.

Acknowledgments

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