Intracellular Transactivation of Epidermal Growth Factor Receptor by $\alpha_{1A}$-Adrenoceptor Is Mediated by Phosphatidylinositol 3-Kinase Independently of Activation of Extracellular Signal Regulated Kinases 1/2 and Serine-Threonine Kinases in Chinese Hamster Ovary Cells

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ABSTRACT

Transactivation of epidermal growth factor receptor (EGFR) by $\alpha_{1}$-adrenoceptor ($\alpha_{1}$-AR) is implicated in contraction and hypertrophy of vascular smooth muscle (VSM). We examine whether all $\alpha_{1}$-AR subtypes transactivate EGFR and explore the mechanism of transactivation. Chinese hamster ovary (CHO) cells stably expressing one subtype of $\alpha_{1}$-AR were transiently transfected with EGFR. The transactivation mechanism was examined both by coexpression of a chimeric erythropoietin (EPO)-EGFR with an extracellular EPO and intracellular EGF domain, and by pharmacologic inhibition of external and internal signaling routes. All three $\alpha_{1}$-AR subtypes transactivated EGFR, which was dependent on the increase in intracellular calcium. The kinase inhibitor AG1478 [4-((3′-chloroanilino)-6,7-dimethoxyquinazoline] abrogated $\alpha_{1A}$-AR and $\alpha_{1D}$-AR induced phosphorylation of EGFR, but both the inhibition of matrix metalloproteinases by GM6001 [(S)-2-(1H-indol-3-yl)-1-methyl[carbamoyl-ethyl]-2-isobutyl-succinamide] or blockade of EGFR by cetuximab did not. Stimulation of $\alpha_{1A}$-AR and $\alpha_{1D}$-AR also induced phosphorylation of EPO-EGFR chimeric receptors. Moreover, $\alpha_{1A}$-AR stimulation enhanced phosphorylation of extracellular signal regulated kinase (ERK) 1/2 and serine-threonine kinases (Akt), which were both unaffected by AG1478, indicating that ERK1/2 and Akt phosphorylation is independent of EGF transactivation. Accordingly, inhibitors of ERK1/2 or Akt did not influence the $\alpha_{1A}$-AR-mediated EGFR transactivation. Inhibition of calcium/calcmodulin-dependent kinase II (CaMKII), phosphatidylinositol 3-kinase (PI3K), and Src, however, did block EGFR transactivation by $\alpha_{1A}$-AR and $\alpha_{1D}$-AR. These findings demonstrate that all $\alpha_{1}$-AR subtypes transactivate EGFR, which is dependent on an intracellular signaling route involving an increase in calcium and activation of CaMKII, PI3K, and Src, but not the phosphatidylinositol 3-kinase/protein kinase B (Akt) pathway.

Introduction

The major importance of cardiovascular (CV) disease has generated interest in the factors that induce proliferation, hypertrophy, and contraction of vascular smooth muscle cells (VSMC) and cardiomyocytes. Prolonged stimulation of $\alpha_{1}$-adrenergic receptors ($\alpha_{1}$ARs) by catecholamines induces hyper trophy of VSMC (Chen et al., 1995) and cardiomyocytes (Barki-Harrington et al., 2004) and constitutes an important

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ABBREVIATIONS: AG1478, 4-((3′-chloroanilino)-6,7-dimethoxyquinazoline; Akt, serine-threonine kinases; A7R5, rat smooth muscle embryonic aorta; $\alpha_{1}$-AR, $\alpha_{1}$-adrenoceptor; CaMKII, calcium/calcmodulin-dependent kinase II; CHO, Chinese hamster ovary; CV, cardiovascular; DMEM, Dulbecco’s modified Eagle’s medium; EGFR, epidermal growth factor receptor; EGFR, epidermal growth factor receptor; EPO, erythropoietin; EPO, erythropoietin receptor; ERK1/2, extracellular signal regulated kinases; GM6001, ([S]-2-(1H-indol-3-yl)-1-methyl[carbamoyl-ethyl]-2-isobutyl-succinamide; GPCR, G-protein–coupled receptor; KN93, N-[2-[[4-(4-chlorobutyl)phenoxy]ethyl]-2-(1H-indol-3-yl)-1-methyl[carbamoyl-ethyl]]-32H]-pyrazolo[1,5-a][1,3]benzimidazole; MMP, matrix metalloproteinase; MK2206, 8-[4-(1-amino-cyclobutyl)[phenyl]-9-phenyl]-2,4,6-triazolo[3,4-f][1]benzimidazol-5-one; pCamKII, phosphorylated CaMKII; pEGFR, phosphorylated EGFR; pERK, phosphorylated ERK1/2; pPI3K, phosphorylated PI3K; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzo[4,5]pyridazin-4-one; PE, phenylephrine; PKB, phosphatidylinositol 3-kinase; PKI-166, 4-[1[(1R)-1-phenyethyl][aminol]-7H-pyrrolo[2,3-d]pyrimidin-6-yl]ethanol; PP2, 4-amino-3-(4-chlorophenyl)-1-[(1-butyl)-(1H-pyrazolo[3,4-d]pyrimidin-4-one; VSMC, vascular smooth muscle cells; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride.
risk factor for CV diseases. In this process, transactivation of epidermal growth factor receptor (EGFR) by α1-ARs has been implicated as a major pathway involved in catecholamine-mediated CV hypertrophy (Asakura et al., 2002; Zhang et al., 2004; Li et al., 2011).

We recently demonstrated that transactivation of EGFR in VSMC also contributes to α1-AR-mediated vascular contraction. In that study, α1-AR stimulation resulted in EGFR phosphorylation in rat aorta, while the EGFR kinase inhibitors AG1478 [4-(4′-chloroanilino)-6,7-dimethoxyquinazoline] and DAPH (4,5-dianilinophthalimide) concentration-dependently attenuated phenylephrine (PE)-induced contractile responses (Ulu et al., 2010). Moreover, in our recent study, EGFR kinase inhibitor PKI-166 [4-[4-(1H)-1-phenylethylamino]-7H-pyrrolo [2,3-d]pyrimidin-6-yl]phenol] attenuated the progression of hypertension and maintained cardiac function in the hyperensive chronic kidney disease rat model (Ulu et al., 2013).

The induction of vascular contraction and cellular growth and proliferation by α1-AR involves the activation of complex signaling pathways including phospholipase C, protein kinase C, calcium/calmodulin-dependent kinase II (CaM-KII), phosphatidylinositol 3-kinase (PI3K), Src, mitogen-activated protein kinases, extracellular signal regulated kinases (ERK1/2), and serine-threonine kinases (Akt) (Wu et al., 1992; Xiao et al., 2001; Ilario et al., 2003; Koshimizu et al., 2003; Cipolletta et al., 2010; Haba et al., 2010). Modulation of all these intracellular signaling molecules may, at least in part, be dependent on transactivation of EGFR by α1-AR.

Previous studies have suggested that G-protein–coupled receptors (GPCRs) induce transactivation of EGFR by an extracellular route involving matrix metalloproteinase (MMP)-dependent shedding of growth-factor-like substances, for example, heparin-binding EGF-like growth factor (HB-EGF) dependent shedding of growth-factor-like substances, for receptors (GPCRs) induce transactivation of EGFR by an involved in the transactivation of EGFR by examining whether an intracellular or extracellular signaling route is involved in the transactivation of EGFR by examining whether an intracellular or extracellular signaling route is involved in the transactivation of EGFR. Therefore, in this study, we address whether an intracellular or extracellular signaling route is involved in the transactivation of EGFR by examining α1A-AR–induced phosphorylation of a chimeric erythropoietin receptor (EPOR/EGFR) in Chinese hamster ovary (CHO) cells. In addition, we used pharmacological tools interfering with extracellular and intracellular sites of EGFR or modulating specific intracellular signaling pathways. To conclusively determine whether all three α1-AR subtypes can transactivate EGFR, we investigated transactivation of EGFR in CHO cells stably expressing a single subtype of α1-AR. As ERK1/2 and Akt activation by GPCRs are often thought to originate from cross-talk with classic receptor tyrosine kinases (such as protein kinase C and EGFR) or effectors (such as arrestins) (Luttrell, 2005; Engelhardt, 2007), we also explored whether α1A-AR–dependent activation of ERK1/2 and Akt requires EGFR transactivation.

**Materials and Methods**

**Cell Culture.** A7R5 (rat smooth muscle embryonic aorta) cells were grown in 75-cm² nontreated cell culture flasks (Corning, Tewksbury, MA) in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO/Invitrogen, Carlsbad, CA) enriched with 10% fetal bovine serum and 1% penicillin/streptomycin. Before the experiments, the cells were trypsinized and replated at 50% confluence on six-well plates. Stimulations were performed on day 3 after 24 hours of serum starvation.

CHO K1 cells lacking intrinsic α1-AR were stably transfected with a plasmid containing one of the human α1-AR subtypes: α1A-AR, α1B-AR, or α1D-AR. They were grown in 75-cm² nontreated cell culture flasks in DMEM/Ham’s F-12 medium (DMEM/F-12) enriched with 10% fetal bovine serum, 1% penicillin/streptomycin, and 200 μg/ml Geneticin (G418; Invitrogen, Carlsbad, CA). For each experiment, 10⁴ cells were plated in each well of a six-well plate and grown for 2 days to reach a confluence of 35–40%. Subsequently, the cells were transiently transfected with the pBabe-puro plasmid (1 μg) containing human EGFR sequence (AddGene, Cambridge, MA). Transfection was performed with Lipofectamine 2000 (Invitrogen) at a concentration of 2.5 μl/well according to the protocol. The next day, the cells were serum-starved for 24 hours before they were used in experiments.

**Intracellular Calcium Measurements.** PE-mediated calcium (Ca²⁺) responses were measured in nontransfected and EGFR-transfected CHO cells stably transfected with one of the α1-AR subtypes. Intracellular Ca²⁺ was measured by fura-2 fluorescence at room temperature (21 ± 2°C). Cells were incubated for 50 minutes with 1 μM fura-2-AM (Sigma-Aldrich, St. Louis, MO) in HEPES buffer (10 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.5 mM KH₂PO₄, 1 mM MgCl₂·6H₂O, 5 mM NaHCO₃, and 1.5 mM CaCl₂·H₂O pH 7.4) for loading and then washed with fresh HEPES buffer. Fluorescence was recorded using a PTI Ratiometer microspectrophotometer and FELIX software (Photon Technology International, Inc., Birmingham, NJ).

**Stimulation Experiments with ATR5 and CHO Cells.** A7R5 cells were pretreated with 10 μM EGFR inhibitor AG1478 or 1 μM prazosin for 30 minutes and then incubated with 10 μM PE (for 5 or 10 minutes) or 1 nM epidermal growth factor (EGF; positive control, for 10 minutes) after 24 hours of serum starvation. CHO cells were pretreated for 30 minutes with one of these inhibitors: 10 μM EGFR kinase inhibitor AG1478 (Sigma-Aldrich), 10 μM PI3K inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride] (Sigma-Aldrich), 10 μM Src inhibitor PP2 [4-amino-3-(4-chlorophenyl)-1-(3-butyl)-1H-pyrrozolo[3,4-d]pyrimidine] (Sigma-Aldrich), 100 μM Ca²⁺/CaM inhibitor W7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride] (Tocris Bioscience, Bristol, United Kingdom), 10 μM CaMKII inhibitor KN93 (N-[2-[4-chlorocinnamyl]-N-methylaminomethylphenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt) (Tocris Bioscience), 10 μM broad spectrum MMP inhibitor GM6001 ([β-NH₂]-hydroxy-N-[((S)-2-(1H-indol-3-yl)-1-methylcarbamoyl-ethyl]-2-isobutyl-succinimide) (Calbiochem, Merck KGaA, Darmstadt, Germany), 10 μM ERK1/2 inhibitor PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one] (Sigma-Aldrich), and 1 μM Akt inhibitor MK2206 [8-[4-(4-1-amino-3-cyclobutylphenyl)-9-phenyl-1,2,4-triazolo[3,4-f][1,6]naphthyridin-3(2H)-one] (Selleckchem, Houston, TX). Pretreatment with the anti-EGFR monoclonal antibody cetuximab (Merck Serono, Geneva, Switzerland) was performed for 2 hours at a concentration of 5 μM. Stimulation with 10 μM PE or 1 nM EGF was terminated after 10 minutes of incubation by removing the media, placing the plates on ice, and adding the homogenization buffer. Each experiment was performed in triplicate and repeated as specified under Results.

**EPOR-EGFR Chimeric Receptor.** On the 3rd day after plating, CHO cells containing the α1A-AR subtype were transfected with the pcDNA3 plasmid containing the EPOR-EGFR chimeric receptor, a generous gift of Dr. Hong-Jian Zhu (Ludwig Institute for Cancer Research, Melbourne, VIC, Australia). The chimera embodied the extracellular domain of the EPOR fused to the transmembrane and intracellular domains of the EGFR (Zhu et al., 2003). On the 5th day, 24-hour serum-starved cells were stimulated with 10 μM PE or 100 IU·ml⁻¹ EPO (positive control) for 10 minutes. Each experiment was performed in triplicate and was repeated as specified under Results.
Protein Isolation and Western Blotting. Cells were immediately put on ice after stimulation, and 100 μL of buffer containing 1% Nonidet P40, 0.02 M sodium vanadate, protease inhibitor (Roche Molecular Diagnostics, Mannheim, Germany) in phosphate-buffered saline was added. After homogenization, cells were incubated for 15 minutes and centrifuged at 5000 g for 5 minutes at 4°C. Supernatant was collected and stored at −80°C.

We performed electrophoresis on newly cast 4–7% SDS-polyacrylamide gels and then transferred them onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with the appropriate primary antibodies overnight, washed three times for 10 minutes with Tris-buffered saline and 0.2% Tween 20 and incubated for 1 hour with the secondary antibody. After subsequent washes, the membranes were soaked in Luminol (Santa Cruz Biotechnology, Santa Cruz, CA) and imaged on Kodak film.

We used the following antibodies (with the concentration for Western blot analysis): anti-EGFR (1:500), anti-Src (1:1000), phosphorylated EGFR 1173 (pEGFR; 1:500), phosphorylated EPOR (pEPOR; 1:500), phosphorylated Erk1/2 (pErk1/2; 1:5000), phosphorylated PI3K (pPI3K; 1:400), phosphorylated CamKII (pCamKII; 1:2000), β-actin (1:5000), goat anti-rabbit (1:10,000), rabbit anti-goat (1:10,000), and donkey anti-mouse (1:10,000) from Santa Cruz Biotechnology, and phosphorylated Akt (pAkt; 1:1000) from Cell Signaling Technology (Danvers, MA). When needed, membranes were stripped with 2% SDS, 0.7% mercaptoethanol in 10 ml of phosphate-buffered saline, and then incubated again with another antibody overnight. Band intensities were measured by Bio-Profil Image analysis system (Vilber Lourmat, France) and were corrected for β-actin expression.

Statistical Analysis. Data are reported as mean ± S.E.M., and n represents the number of independent experiments for each indicated condition. Analyses were performed by SPSS 17.0 for Windows software (SPSS, Inc., Chicago, IL). Semiquantitative comparisons between the groups were calculated by Mann–Whitney U test. *P < 0.05 (two-tailed) was considered statistically significant.

Results

Acute Stimulation of α1-AR Transactivates EGFR in A7R5 Cells. We recently had demonstrated that α1-AR induced transactivation of EGFR contributes to VSMC contraction in rat aorta (i.e., to immediately affect the α1-AR response) (Ulu et al., 2010), so we first explored whether a similar transactivation occurs in serum-starved cultured A7R5 VSMCs, which express all three α1-AR subtypes (data not shown). Stimulation of α1-AR by 10 μM PE and EGFR by 1 nM EGF induced phosphorylation of EGFR in A7R5 VSMCs. To examine the role of Ca²⁺ entry in the transactivation, A7R5 cells were pretreated with prazosin (1 μM) to fully block the increase in cellular Ca²⁺ levels (data not shown). Whereas prazosin fully blocked α1-AR–induced phosphorylation of EGFR, it did not affect EGF-mediated EGFR phosphorylation (Supplemental Fig. 1A). Furthermore, the PE-induced increase in pEGFR was also antagonized by 10 μM AG1478 (Supplemental Fig. 1B).

All Three α1-AR Subtypes Transactivate EGFR in CHO Cells. As acute stimulation of α1-AR in A7R5 VSMCs leads to EGFR transactivation, in the next step we addressed the question whether all three α1-AR subtypes can transactivate EGFR. To this end, CHO cells stably expressing a single subtype of α1-AR and cotransfected with EGFR were stimulated with 10 μM PE or 1 nM EGF in the absence and presence of AG1478 (Fig. 1). The concentration of PE was selected as 10 μM because a more pronounced phosphorylation of EGFR was observed with this concentration than with
1 μM (Supplemental Fig. 2). In α1A-AR–expressing CHO cells transfected with EGFR, both PE and EGF significantly increased the phosphorylation of EGFR (Fig. 1A; the percentage increase in pEGFR was 160.0 ± 18.1 and 231.3 ± 9.6 for PE and EGF, respectively, n = 16–23). Likewise, PE and EGF significantly increased pEGFR in α1B-AR (Fig. 1B; the percentage increase in pEGFR was 138.3 ± 25.0 and 198.4 ± 35.3 for PE and EGF, respectively, n = 4–15; P < 0.05 versus control for both) and α1D-AR (Fig. 1C; the percentage increase in pEGFR was 266.5 ± 77.1 and 748.2 ± 178.8 for PE and EGF, respectively, n = 4–14; P < 0.05 versus control for both) expressing CHO cells. Increases in pEGFR mediated by all three α1-AR subtypes or EGF were antagonized by AG1478 (Fig. 1).

α1-AR–Mediated Transactivation of EGFR Is Intracellular in CHO Cells. In the third step, we intended to identify the mechanism of α1-AR–mediated transactivation of EGFR. To pharmacologically determine whether transactivation of EGFR by α1A-AR–expressing CHO cells was conveyed via an extracellular route, cells were pretreated with cetuximab, a humanized anti-EGFR monoclonal antibody (Piascik and Perez, 2001). Cetuximab did not prevent the α1A-AR–mediated increase in pEGFR (Fig. 2A; the percentage increase in pEGFR was 110.3 ± 18.1 and 231.3 ± 9.6 for PE and EGF, respectively, n = 8 for each condition), whereas the EGF-mediated increase in pEGFR was fully blocked (Fig. 2A; the percentage increase in pEGFR was 133.9 ± 55.3 for EGF + cetuximab, n = 8; P < 0.05 versus EGF). Also, the inhibition of MMPs by GM6001 did not abrogate α1A-AR–induced phosphorylation of EGFR (Fig. 2A; the percentage increase in pEGFR was 286.5 ± 97.5, n = 7). The phosphorylation of EGFR appeared to be enhanced in the presence of GM6001, with a relatively higher S.E.M. In line with our observations, GM6001 was also reported previously as causing a slightly higher EGFR phosphorylation induced by insulin (Roztocil et al., 2008).

These experiments strongly suggest that transactivation of EGFR by α1A-AR depends on an intracellular route. To conclusively demonstrate this, CHO cells were transfected with a chimeric EPOR-EGFR in which the extracellular domain of EGFR had been replaced by EPOR. In these cells, α1A-AR stimulation by PE induced a substantial phosphorylation of the intracellular domain of the transiently expressed EPOR/EGFR chimeric receptor (Fig. 2B; the percentage increase was 258.2 ± 53.7, n = 23; P < 0.05 versus control), as did EPO (Fig. 2B; the percentage increase was 198.8 ± 22.7, n = 23; P < 0.05 versus control). Collectively, these data demonstrate that transactivation of EGFR by α1A-AR depends on an intracellular route.

We additionally examined this mechanism in α1D-ADrenoreceptor–expressing CHO cells, as α1A-AR and α1D-AR are predominantly found in VSMC (Piascik and Perez, 2001; Hein and Michel, 2007). Stimulation of α1D-AR by PE in CHO cells also induced phosphorylation of the intracellular domain of a transiently expressed EPOR/EGFR chimeric receptor (Supplemental Fig. 3A), which was abrogated by the EGFR kinase inhibitor AG1478. Thus, our data demonstrate that both α1A-AR and α1D-AR subtypes employ an intracellular route to transactivate EGFR.

EGFR Transfection of CHO Cells Does Not Alter α1-AR–Mediated Intracellular Ca2+. To substantiate the functionality of α1-AR subtypes and to investigate the contribution of EGFR transfection to α1-AR–mediated intracellular Ca2+ responses, intracellular Ca2+ was measured in CHO cells. All three α1-AR subtypes induced an intracellular Ca2+ response, of which the α1A-AR–induced increase in fura-2 fluorescence was the highest compared with α1B-AR and α1D-AR (Supplemental Fig. 4). Further, EGFR transfection of CHO cells did not alter the intracellular Ca2+ response mediated by all three α1-AR subtypes (Supplemental Fig. 4). Thus, Ca2+ measurements indicate that all three α1-AR subtypes are functional when stably transfected in CHO cells.
α1-AR-Mediated Transactivation of EGFR Involves CaMKII, PI3K, and Src Activation, but Not the ERK1/2 and Akt Pathways. It is known that α1-AR stimulation involves activation of Ca$$^{2+}$$/CaM, CaMKII, Src, PI3K, ERK1/2, and Akt; thus, in the next step, we sought to identify the roles and order of these signaling mediators in α1-AR-mediated transactivation of EGFR. Hereeto, CHO cells were stimulated with α1-AR or EGFR ligands in the absence and presence of AG1478, GM60001, PD98059 (ERK1/2 inhibitor), PP2 (Src inhibitor), or LY294002 (PI3K inhibitor). We found that α1A-AR stimulation by PE increased phosphorylation of CaMKII, which was blocked by Ca$$^{2+}$$/CaM inhibitor W7 (Fig. 3A; the percentage increase was 251.2 ± 48.9 and 79.2 ± 23.7 for PE and PE + W7, respectively, n = 17 for each condition; P < 0.05 for PE versus control). This finding substantiates the involvement of Ca$$^{2+}$$ as one of the major routes activated by α1-AR.

Likewise, PE induced increases in pSrc (Fig. 3B; the percentage increase was 436.2 ± 205.1, n = 12; P < 0.05 for PE versus control) and pPI3K (Fig. 3C, percentage increase was 238.4 ± 75.4, n = 7, P < 0.05 for PE versus control) were blocked by PP2 (Fig. 3B; the percentage increase was 42.6 ± 8.0, n = 12) and LY294002 (Fig. 3C, the percentage increase was 93.7 ± 50.6, n = 7 independent experiments), respectively. Importantly, α1A-AR-mediated transactivation of EGFR (Fig. 4; the percentage increase was 244.0 ± 52.4, n = 14) was completely blocked by preincubation with W7 (Fig. 4; the percentage increase was 117.8 ± 40.6, n = 17), PP2 (Fig. 4; the percentage increase was 156.5 ± 36.3, n = 12), LY294002 (Fig. 4; the percentage increase was 126.2 ± 38.0, n = 7), and KN93 (Fig. 4; the percentage increase was 127.3 ± 41.1, n = 14).

Similarly to the α1A-AR subtype, blockade of PI3K, Ca$$^{2+}$$/CaM, and Src also attenuated the PE-induced phosphorylation of EGFR in α1D-AR-expressing CHO cells (Supplemental Fig. 3B), supporting the hypotheses that both α1-AR subtypes employ a common intracellular pathway of EGFR transactivation.

PE-induced increases in pSrc were blocked both by W7 (percentage increase was 84.5 ± 27.5, n = 15) and KN93 (percentage increase was 124.1 ± 9.9, n = 8), indicating involvement of Ca$$^{2+}$$/CaM and CaMKII upstream of Src (Fig. 5A). In addition, the PE-induced increase in pPI3K was blocked by W7 (percentage increase was 116.1 ± 42.3, n = 4), KN93 (percentage increase was 109.9 ± 48.6, n = 17), and PP2 (percentage increase was 95.3 ± 23.0, n = 6), indicating involvement of Src upstream of PI3K (Fig. 5B).

Stimulation of α1A-AR by PE also strongly increased pERK1/2 (Fig. 6, B and C; the percentage increase was 3693.5 ± 933.1, n = 17; P < 0.05 for PE versus control), which was blocked both by LY294002 (Fig. 6C; percentage increase was 155.7 ± 116.9, n = 4) and PD98059 (Fig. 6C; percentage increase was 317.6 ± 132.9, n = 6), but not by AG1478 (Fig. 6, A and C; percentage increase was 3623.9 ± 962.1, n = 7; P < 0.05 for PE versus control). Moreover, the PE-induced increase in pEGFR was not antagonized by PD98059 (Fig. 6D; percentage increase was 239.3 ± 75.5, n = 7; P < 0.05 for
PE versus control), indicating that \(\alpha_1\)-AR–mediated transactivation of EGFR is independent of ERK1/2 activation. These findings also demonstrate that \(\alpha_1A\)-AR–mediated activation of ERK1/2 is not through EGFR.

Likewise, \(\alpha_{1A}\)-AR stimulation by PE increased pAkt (Fig. 7, A, B, and C; percentage increase was 223.9 ± 19.3, \(n = 14; P < 0.05\) for PE versus control), which was blocked both by LY294002 (Fig. 7, B and C; percentage increase was 35.9 ± 38.5, \(n = 3\)) and the Akt inhibitor MK2206 (Fig. 7, B and C; percentage increase was 65.4 ± 31.3, \(n = 14\), but not by AG1478 (Fig. 7, A and C; percentage increase was 221.4 ± 94.6, \(n = 4; P < 0.05\) for PE versus control) or PD98059 (Fig. 7, A and C; percentage increase was 208.7 ± 50.5, \(n = 3\)). Moreover, the PE-induced increase in pEGFR was not antagonized by MK2206 (Fig. 7D; percentage increase was 254.9 ± 53.0, \(n = 11; P < 0.05\) for PE versus control), indicating that \(\alpha_1\)-AR–mediated transactivation of EGFR is independent of Akt activation. These findings also demonstrate that \(\alpha_{1A}\)-AR–mediated activation of Akt is not through transactivation of EGFR.

**Discussion**

This study shows that all three \(\alpha_1\)-AR subtypes (\(\alpha_{1A}\)-AR, \(\alpha_{1B}\)-AR, and \(\alpha_{1D}\)-AR) can transactivate the EGFR. Transactivation is dependent on an intracellular route rather than an extracellular route. First, EGFR transactivation is unaffected by cetuximab and the pan-MMP blocker GM6001, both acting extracellularly, whereas transactivation is blocked by AG1478, which acts intracellular. Second, \(\alpha_{1A}\)-AR and \(\alpha_{1D}\)-AR stimulation induced phosphorylation of the intracellular domain of a transiently expressed EPOR/EGFR chimeric receptor, which lacks the extracellular binding sites for EGF ligands. Collectively, these findings indicate that the transactivation of EGFR by \(\alpha_{1A}\)-AR and \(\alpha_{1D}\)-AR is mediated by intracellular signaling. Further, \(\alpha_{1A}\)-AR stimulation

![Fig. 4. Phosphorylation of EGFR (pEGFR) was investigated after 10 \(\mu\)M PE (10-minute) stimulation in the absence or presence of calcium/calmodulin (Ca\(^{2+}\)/CaM) inhibitor W7 (100 \(\mu\)M, 30-minute preincubation), Src inhibitor PP2 (10 \(\mu\)M, 30-minute preincubation), PI3K inhibitor LY294002 (10 \(\mu\)M, 30-minute preincubation), or calcium/calmodulin-responsive kinase II inhibitor KN93 (10 \(\mu\)M, 30-minute preincubation), respectively, in EGFR transfected CHO cells stably expressing \(\alpha_{1A}\)-AR.](image1)

Fig. 4. Phosphorylation of EGFR (pEGFR) was investigated after 10 \(\mu\)M PE (10-minute) stimulation in the absence or presence of calcium/calmodulin (Ca\(^{2+}\)/CaM) inhibitor W7 (100 \(\mu\)M, 30-minute preincubation), Src inhibitor PP2 (10 \(\mu\)M, 30-minute preincubation), PI3K inhibitor LY294002 (10 \(\mu\)M, 30-minute preincubation), or calcium/calmodulin-responsive kinase II inhibitor KN93 (10 \(\mu\)M, 30-minute preincubation), respectively, in EGFR transfected CHO cells stably expressing \(\alpha_{1A}\)-AR. Representative Western blot detections of pEGFR and \(\beta\)-actin blots are shown. The pEGFR band intensities were corrected with \(\beta\)-actin and expressed as the percentage of control. Data are expressed as mean ± S.E.M. *\(P < 0.05\) versus control.

![Fig. 5. Phosphorylation of Src (pSrc) was investigated after 10 \(\mu\)M PE (10-minute) stimulation in the absence or presence of calcium/calmodulin (Ca\(^{2+}\)/CaM) inhibitor W7 (100 \(\mu\)M, 30-minute preincubation) or calcium/calmodulin-responsive kinase II inhibitor KN93 (10 \(\mu\)M, 30-minute preincubation) in EGFR transfected CHO cells stably expressing \(\alpha_{1A}\)-AR (A). Phosphorylation of pPI3K was also investigated after 10 \(\mu\)M PE (10-minute) stimulation in the absence or presence of W7, KN93, or Src inhibitor PP2 (10 \(\mu\)M, 30-minute preincubation) in EGFR transfected CHO cells stably expressing \(\alpha_{1A}\)-AR (B). Representative Western blot detections of pSrc, pPI3K, and \(\beta\)-actin blots are shown. The pSrc and pPI3K band intensities were corrected with \(\beta\)-actin and expressed as the percentage of control. Data are expressed as mean ± S.E.M. *\(P < 0.05\) versus control.](image2)
enhanced phosphorylation of ERK1/2 and Akt, and both were unaffected by the EGFR kinase inhibitor AG1478, indicating that ERK1/2 and Akt phosphorylation is independent of EGFR transactivation. Accordingly, inhibitors of ERK1/2 or Akt did not influence the α1A-AR–mediated EGFR transactivation. Importantly, inhibition of CaMKII, Src, and PI3K blocked both α1A-AR– and α1D-AR–mediated EGFR transactivation. Collectively, these results demonstrate an intracellular EGFR transactivation route involving the phosphorylation of CaMKII, Src, and PI3K, but not of ERK1/2 and Akt pathways in CHO cells.

Several studies have been performed to identify the exact mechanism of EGFR transactivation mediated by GPCRs. Proteolytic metalloprotease-dependent shedding of EGFR ligands has been proposed as a key regulator of EGFR signaling for heart development and angiogenesis (Dong et al., 1999; Iwamoto et al., 2003; Kurohara et al., 2004; Zhou et al., 2004). Moreover, it has been postulated that cross-talk between GPCRs (including α1-ARs) and EGFR is mediated via MMP-dependent shedding of EGFR ligands (Prenzel et al., 1999; Asakura et al., 2002; Zhang et al., 2004). In our recent and present studies, we sought to identify the mechanism of α1-AR–dependent EGFR transactivation. In this study, neither EGFR monoclonal antibody (cetuximab) nor broad spectrum MMP inhibitor (GM60001) antagonized PE-mediated EGFR phosphorylation. Furthermore, we took a novel approach to conclusively determine whether an intracellular or extracellular signaling route is involved in the transactivation of EGFR. In these experiments, PE induced phosphorylation of the intracellular part of EPOR-EGFR chimeric receptor, which lacks binding sites for EGFR ligands. These results strongly support that the transactivation of EGFR by α1-AR is mediated by intracellular signaling molecules.

On the one hand, acute stimulation of α1-ARs by catecholamines enhances vascular tone by the activation of direct signaling pathways involving Gq and PI3K and elevation of intracellular Ca2+. On the other hand, the present study and earlier studies indicate that acute and prolonged stimulation of α1-ARs also influences the cellular function by an indirect route consisting of transactivation of EGFR (Asakura et al., 2002; Zhang et al., 2004; Ulu et al., 2010; Li et al., 2011). It is obvious that based on the localization of α1-ARs this signaling has physiologic and pathologic outcomes, particularly in CV diseases. Of the three different α1-AR subtypes, α1A-AR and α1B-AR are mainly localized in the myocardium, and α1A-AR and α1D-AR are predominant in VSMC (Piascik and Perez, 2001; Hein and Michel, 2007). As this study shows, all three

Fig. 6. Phosphorylation of extracellular signal regulated kinases (pERK1/2) was investigated after 10 μM PE (10-minute) or 1 nM epidermal growth factor (EGF, 10-minute) stimulations in the absence or presence of EGFR tyrosine kinase inhibitor AG1478 (30-minute preincubation) (A), phosphatidylinositol 3-kinase inhibitor LY294002 (10 μM, 30-minute preincubation), or ERK1/2 inhibitor PD98059 (10 μM, 30-minute preincubation) (B) in EGFR transfected CHO cells stably expressing α1A-AR. Phosphorylation of pEGFR was also investigated after 10 μM PE (10-minute) stimulation in the absence or presence of PD98059 in EGFR transfected CHO cells stably expressing α1A-AR (D). Representative Western blot detections of pERK1/2, pEGFR, and β-actin blots are shown. The pERK1/2 and pEGFR band intensities were corrected with β-actin and expressed as the percentage of control (C and D). Data are expressed as mean ± S.E.M. *P < 0.05 versus control.
α1-AR subtypes are able to transactivate EGFR, so EGFR signaling seems be an alternative target in CV diseases of different origin (Ulu et al., 2013).

We observed that α1A-AR stimulation activated Akt and ERK1/2 via an EGFR-independent route, as blockade of EGFR phosphorylation by AG1478 did not antagonize α1A-AR–mediated activation of Akt or ERK1/2. Conversely, inhibitors of Akt or ERK1/2 did not influence the phosphorylation of EGFR after PE stimulation, which precludes the involvement of Akt or ERK1/2 in α1A-AR–mediated transactivation. All three α1-AR subtypes couple to Gq and result in activation of phospholipase C with an increase in inositol triphosphate and subsequent mobilization of intracellular Ca2+, which leads to activation of the calcium/calmodulin (Ca2+/CaM) pathway (Wu et al., 1992; Bylund et al., 1994; Della Rocca et al., 1997; Koshimizu et al., 2003). The Ca2+/CaM pathway directly and indirectly influences EGFR and has a regulatory function in EGFR signaling (Murasawa et al., 1998; Aifa et al., 2002; Li and Villalobo, 2002; Sanchez-Gonzalez et al., 2010). Other important signaling molecules activated by α1-AR are the Src (Han et al., 2008) and PI3K, which are also implicated in EGFR transactivation.

In this study, we found that α1A-AR stimulation activated CaMKII, indicating the activation of Ca2+/CaM pathway. Moreover, Ca2+/CaM and CaMKII inhibitors (W7 and KN93, respectively) not only blocked α1A-AR– and α1D-AR–mediated EGFR transactivation but also the phosphorylation of Src and PI3K. Consequently, CaMKII activation seems to represent the most upstream event in transactivation of EGFR. Furthermore, as the PE-induced activation of PI3K was also blocked by PP2, Src activation seems to be upstream of PI3K.

Taken together, the cascade of α1A-AR– and α1D-AR–mediated transactivation of EGFR is triggered by Ca2+ influx and subsequent Ca2+/CaM and CaMKII, which triggers signaling through Src and PI3K, as depicted in Fig. 8. This activation cascade is consistent with previous...
reports (Della Rocca et al., 1997; Ballou et al., 2000; Gentili et al., 2002; Kubo et al., 2005). These results extend our findings for an intracellular route involving a Ca\(^{2+}\)/CaM-CaMKII pathway, PI3K, and Src, but not Akt or ERK1/2 for \(\alpha\)-AR–mediated transactivation of EGFR. In line with our results, it was previously reported that \(\alpha\)-AR–mediated growth of adventitial cells activation of the downstream ERK1/2 pathway does not mediate the transactivation of EGFR (Zhang et al., 2004).

The pathway identified in our present study may be of significant physiologic and pathophysiologic importance, and interruption of EGFR transactivation by targeting one of the dissected key components may provide a novel approach in the treatment of CV pathologies. The main limitation of our study is that we investigated the mechanism of \(\alpha\)-AR–mediated EGFR transactivation in an isolated CHO cell culture model system. Therefore, the mechanism of this transactivation both in VSMC and under in vivo conditions still requires confirmation. Furthermore, our data do not provide conclusive evidence on the role of \(\alpha\)-AR–mediated EGFR transactivation in vascular hypertrophy and VSMC proliferation. Further research using specific assays in cell culture and animal models will allow testing the role of the proposed mechanism in CV hypertrophy and proliferation.

Our study demonstrates that all \(\alpha\)-AR subtypes can mediate EGFR signaling, which is dependent on an intracellular route involving CaMKII, PI3K, and Src activation but is independent from ERK1/2 and Akt in CHO cells. Moreover, we demonstrate that \(\alpha\)-AR–dependent activation of Akt or ERK1/2 is independent of EGFR transactivation. Therefore, our study not only establishes \(\alpha\)-AR– and \(\alpha\)-AD–dependent transactivation of EGFR to be governed by the intracellular route, but also extends the evidence that Akt and ERK1/2 need not necessarily require cross-talk of GPCRs with receptor tyrosine kinases.

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Authorship Contributions

Participated in research design: Ulu, Henning, Guradal. Conducted experiments: Ulu, Guner, Zoto, Duman-Dalkili, Duin, Guradal. Contributed new reagents or analytic tools: Ulu, Henning, Guner, Duman-Dalkili, Guradal. Performed data analysis: Ulu, Henning, Zoto, Duman-Dalkili, Duin, Guradal. Wrote or contributed to the writing of the manuscript: Ulu, Henning, Guradal.

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