Mechanical stretch stimulates growth of vascular smooth muscle cells via epidermal growth factor receptor

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†Division of Endocrinology and Metabolism, Second Department of Internal Medicine, Tokyo Medical and Dental University, Tokyo 113-8519; and ‡Molecular Cardiology Unit, Research Institute of Angiocardiology and Cardiovascular Clinic, Kyushu University School of Medicine, Fukuoka 812-8582, Japan

Iwasaki, Hiroaki, Satoru Eguchi, Hikaru Ueno, Fumiaki Marumo, and Yukio Hirata. Mechanical stretch stimulates growth of vascular smooth muscle cells via epidermal growth factor receptor. Am. J. Physiol. Heart Circ. Physiol. 278: H521–H529, 2000.—We have studied whether activation of epidermal growth factor receptor (EGFR) is involved in stretch-induced extracellular signal-regulated kinase 1/2 (ERK1/2) activation and protein synthesis in cultured rat vascular smooth muscle cells (VSMC). Cyclic stretch (1 Hz) induced a rapid (within 5 min) phosphorylation of ERK1/2, an effect that was time and strength dependent and inhibited by an EGFR kinase inhibitor (AG-1478) but not by a platelet-derived growth factor receptor kinase inhibitor (AG-1296). The stretch rapidly (within 2 min) induced tyrosine phosphorylation of several proteins, among which 180-kDa protein was shown to be EGFR as revealed by blockade with AG-1478 as well as immunoprecipitation with anti-EGFR antibody coupled with immunoblotting with anti-phosphotyrosine antibody. The stretch rapidly (within 2 min) induced association of tyrosine-phosphorylated EGFR with adaptor proteins (Shc/Grb2) as revealed by coprecipitation with glutathione-S-transferase-Grb2 fusion protein coupled with immunoblotting with anti-phosphotyrosine, anti-EGFR, and anti-Shc antibodies. Transfection of a dominant-negative mutant of H-Ras also inhibited stretch-induced ERK1/2 activation. Treatment with a stretch-activated channel blocker (Gd³⁺) and an intracellular Ca²⁺ antagonist (TMB-8) inhibited stretch-induced phosphorylation of EGFR and ERK1/2. Treatment with AG-1478 and a mitogen-activated protein kinase kinase inhibitor (PD-98059), but not AG-1296, blocked [³H]leucine uptake stimulated by a high level of stretch. These data suggest that ERK1/2 activation by mechanical stretch requires Ca²⁺-sensitive EGFR activation mainly via stretch-activated ion channels, thereby leading to VSMC growth.

stretch-activated ion channel; adaptor protein; p21ras; extracellular signal-regulated kinases 1 and 2; protein synthesis

VASCULAR SMOOTH MUSCLE CELLS (VSMC) are constantly exposed to static mechanical strains from superimposed pulsatile and mean pressure loads by the cardiac contractile cycle in vivo. These strains are thought to be altered under pathological conditions such as hypertension, which may contribute to the process of vascular remodeling (19, 25, 40). The mechanical strain modulates cellular orientation, synthesis of extracellular matrix, myosin isoform expression, and cellular proliferation.

Although the cellular mechanism by which mechanical strain stimulates VSMC growth remains obscure, recent studies have focused on the potential involvement of extracellular signal-regulated kinase 1/2 (ERK1/2) and/or p42/p44 mitogen-activated protein kinase (MAPK) in the long-term responses including cell proliferation and differentiation. In cultured VSMC, mechanical strain has been shown to activate ERK1/2 in vitro (29). Moreover, activation of ERK1/2 in arterial walls has been shown to occur after acute hypertension in rats (42) and in balloon-overstretched porcine coronary and carotid arteries in vivo (27). However, the initial signaling event(s) leading to ERK1/2 activation by mechanical stress remains elusive.

It is well recognized that activated receptor tyrosine kinases (RTK), such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), provide docking sites for the adaptor proteins (Shc, Grb2) and recruit Sos (GDP/GTP exchange factor) to the plasma membrane, thereby leading to p21ras-dependent ERK1/2 activation (26). Recent studies revealed that a variety of stimuli, such as G protein-coupled receptor (GPCR) agonists, cytokines, irradiation, and osmotic stress, induce ERK1/2 activation through activation of several RTKs in various cell types including VSMC (39). Mechanical stress induces protein tyrosine phosphorylation in cardiac myocytes (32, 33), endothelial cells (24), and fetal lung cells (21), suggesting the possible involvement of RTK in the mechanism of stretch-induced ERK1/2 activation in cardiovascular tissues.

Submitting given cells to mechanical strain spurs a transient increase in Ca²⁺ and divalent cations via mechanosensitive or stretch-activated (SA) ion channels. There exist at least two classes of SA ion channels in VSMC (9, 18): a nonselective cation channel that is permeable to Na⁺ and Ca²⁺, and a Ca²⁺-activated K⁺ channel. Because an increase in intracellular Ca²⁺ by ANG II constitutes a pivotal signal for activation of ERK1/2 and its growth-promoting effect in VSMC (10),...
SA ion channels may also contribute to the early signaling event by mechanical stress.

In the present study, we examined whether mechanical stretch stimulates ERK1/2 activation and cell growth via Ca$^{2+}$-dependent RTK in rat VSMC. We report herein that Ca$^{2+}$-dependent activation of EGFR mainly via SA ion channels is required for the stretch-induced ERK1/2 activation and hypertrophy of VSMC.

MATERIALS AND METHODS

Materials. AG-1478, AG-1296, and 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) were purchased from Calbiochem-Novabiochem (La Jolla, CA); PD-98059 from New England Biolabs (Beverly, MA); polyclonal anti-phosphorylated ERK1/2 antibody from Promega (Madison, WI); agarose-conjugated glutathione-S-transferase (GST)-Grb2(1–217) fusion protein, protein A/G-agarose, polyclonal anti-ERK2, polyclonal anti-EGFR, monoclonal anti-H-Ras and anti-rat horseradish peroxidase (HRP)-conjugated second antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); recombinant human PDGF-BB, monoclonal anti-phosphotyrosine, polyclonal anti-human EGFR, and polyclonal anti-Shc antibodies from Upstate Biotechnology (Lake Placid, NY); anti-mouse and anti-rabbit HRP-conjugated second antibodies from Amersham (Amerham, UK); and gadolinium chloride from Wako Chemicals (Osaka, Japan). Nicardipine was a generous gift from Yamanouchi Pharmaceutical (Tokyo, Japan).

Cell culture. VSMC were prepared from the thoracic aorta of 12-wk-old male Sprague-Dawley rats by the explant method previously (17). Quiescent VSMC (passages 5–15) were subjected to mechanical stretch for the indicated times, with the conditioned media after stretch being collected and added to the cells. To determine whether cyclic stretch induces ERK1/2 activation in a time- and strength-dependent manner in cultured rat VSMC, ERK1/2 activation was assessed by immunoblotting with the polyclonal antibody that selectively recognizes the dually phosphorylated active form of ERK1/2.

RESULTS

Cyclic stretch activates ERK1/2 in a time- and strength-dependent fashion in rat VSMC. To determine whether cyclic stretch induces ERK1/2 activation in a time- and strength-dependent manner in cultured rat VSMC, ERK1/2 activation was assessed by immunoblotting with the polyclonal antibody that selectively recognizes the dually phosphorylated active form of ERK1/2. Mechanical stretch (25% elongation) caused phosphorylation of ERK1/2 as early as 3 min, which peaked at 5 min and then decreased by 20 min (Fig. 1A). The phosphorylation of ERK1/2 by mechanical stretch was strength dependent; it increased by as low as ~3% elongation, and a maximal response was induced by 25% elongation (Fig. 1B). Therefore, subsequent cyclic stretch experiments were performed by 25% elongation for 5 min unless otherwise stated.

Mechanical stress has been reported to release ANG II (33, 34) and endothelin-1 (44) from cardiac myocytes and PDGF (41), fibroblast growth factor (5, 6), and ATP (14) from VSMC, which may activate ERK1/2 in an autocrine and/or paracrine manner. To exclude the possibility that stretch-induced ERK1/2 phosphorylation may be mediated by such a diffusible factor(s), we examined the effect of conditioned media collected from VSMC after stretching (25% elongation for 5 min) on ERK1/2 phosphorylation. Treatment of unstretched, fresh VSMC with the conditioned media after stretching did not affect ERK1/2 phosphorylation (data not shown), although the possible autocrine mechanism by
diffusible factor(s) that may be accumulated after longer incubation or rapidly degraded or otherwise nontransferable factor(s) could not be excluded.

Cyclic stretch activates ERK1/2 via AG-1478-sensitive protein tyrosine kinase. To determine whether RTKs are involved in the stretch-induced ERK1/2 activation, we examined the effects of two selective inhibitors (20), one for EGFR (AG-1478) and the other for PDGFR (AG-1296). The stretch-induced phosphorylation of ERK1/2 was completely blocked by pretreatment with AG-1478 (250 nM) (Fig. 2A). In contrast, AG-1296 (25 µM), although completely blocking PDGF-
induced ERK1/2 activation, had no effect on the stretch-induced phosphorylation of ERK1/2 (Fig. 2B).

To ascertain whether an AG-1478-sensitive protein tyrosine kinase is indeed activated in response to stretch, the effect of AG-1478 on protein tyrosine phosphorylation induced by mechanical stretch was studied by immunoblotting with anti-phosphotyrosine antibody (Fig. 3). Mechanical stretch rapidly and transiently (within 2 min) induced tyrosine phosphorylation of several proteins with different molecular weights, among which phosphorylation of ~180-, ~120-, ~60-, ~52-, and ~46-kDa proteins was decreased by pretreatment with AG-1478 (250 nM) (Fig. 3A, top blot). The tyrosine-phosphorylated ~180-kDa protein was assumed to be EGFR, because this protein was comigrated with the band recognized by anti-EGFR antibody (Fig. 3A, middle blot).

To determine whether mechanical stretch activates EGFR, the phosphotyrosine content of EGFR after stretching was examined by immunoprecipitation with anti-EGFR antibody coupled with immunoblotting with anti-phosphotyrosine and two different anti-EGFR antibodies. Mechanical stretch rapidly (within 2 min) increased the amount of tyrosine-phosphorylated EGFR (Fig. 3B), suggesting that the stretch-induced tyrosine phosphorylation is partly mediated via EGFR activation.

Mechanical stretch stimulates association of tyrosine-phosphorylated EGFR and Shc with Grb2. Because the association of adaptor proteins (Shc/Grb2) with tyrosine-phosphorylated RTK plays a critical role to recruit Sos, a GDP/GTP exchange factor of p21ras (26), we further examined whether mechanical stretch stimulates the association of EGFR with these adaptor proteins by coprecipitation with GST-Grb2 fusion protein followed by immunoblotting with anti-phosphotyrosine, anti-EGFR, and anti-Shc antibodies (Fig. 4). The stretch rapidly (within 2 min) increased association of the tyrosine-phosphorylated ~180-kDa protein with GST-Grb2 fusion protein (Fig. 4A, top blot); the phosphorylated 180-kDa protein associated with the fusion protein was identified as EGFR by immunoblotting with anti-EGFR antibody (Fig. 4A, bottom blot). Three tyrosine-phosphorylated Shc isoforms (p66, p52, and p46) were concomitantly associated with the fusion protein after stretching (Fig. 4B). These data suggest that the tyrosine-phosphorylated EGFR by mechanical stretch provides binding sites for the adaptor proteins Shc and Grb2, thereby possibly leading to p21ras-dependent ERK1/2 activation in VSMC.

p21ras is essential for ERK1/2 activation by mechanical stretch. p21ras has been shown to play a key role in signal transduction for ERK1/2 activation by RTK and GPCR agonists (12, 26, 38). To elucidate whether p21ras...
also contributes to ERK1/2 activation by mechanical stretch in VSMC, we examined the effect of adenovirus-mediated transfection of a dominant-negative mutant of H-Ras (Fig. 5). The transfection of RasY57, but not control LacZ, blocked the stretch-induced ERK1/2 phosphorylation (Fig. 5, top blot). The expression of ERK2 protein was comparable after either transfection (Fig. 5, middle blot); the expression of transfected RasY57 was confirmed by immunoblotting with anti-H-Ras antibody (Fig. 5, bottom blot). These data suggest that ERK1/2 activation by mechanical stretch requires p21ras activation in VSMC.

Fig. 4. Association of tyrosine-phosphorylated EGFR and Shc with GST-Grb2 fusion protein by mechanical stretch. Cells were stimulated with mechanical stretch (25%) for 2 min. Cell lysates were coprecipitated with GST-Grb2 fusion protein and then immunoblotted with anti-pTyr antibody (top blots) and anti-EGFR antibody (A, bottom blot) or anti-Shc antibody (B, bottom blot). Arrowheads indicate tyrosine-phosphorylated EGFR (A) or 3 Shc isoforms (B). Summary data graphs are shown (bottom). Data are means ± SE for 3 independent experiments. *P < 0.05 vs. basal.

Fig. 5. Effect of dominant-negative mutant of H-Ras (AdRasY57) on stretch-induced ERK1/2 activation. After transfection with AdRasY57 (100 multiplicity of infection [MOI]) or AdLacZ (100 MOI), cells were stretched (25%) for times indicated. Cell lysates were immunoblotted with anti-phosphorylated ERK1/2 antibody (top blot), anti-ERK2 antibody (middle blot), and anti-H-Ras antibody (bottom blot). Arrowheads indicate phosphorylated ERK1/2 and H-Ras. Right: summary data graph. Data are means ± SE for 3 independent experiments. LacZ, control; DNRas, dominant-negative mutant. **P < 0.01 vs. basal.
Ca\textsuperscript{2+} influx via SA ion channels is required for EGFR and ERK1/2 activation. Because stretch increases Ca\textsuperscript{2+} influx through SA ion channels in fibroblasts (3) and an increase in intracellular Ca\textsuperscript{2+} is a pivotal signal for EGFR activation in VSMC (11), we examined the effects of Gd\textsuperscript{3+}, an SA ion channel inhibitor (45), and TMB-8, an intracellular Ca\textsuperscript{2+} antagonist that blocks the release of Ca\textsuperscript{2+} from intracellular stores (7, 23), on the stretch-induced activation of ERK1/2 and EGFR. Pretreatment with both Gd\textsuperscript{3+} (50 µM) and TMB-8 (50 µM) inhibited the stretch-induced phosphorylation of EGFR (Fig. 6) as well as ERK1/2 activation (Fig. 7); neither Gd\textsuperscript{3+} nor TMB-8 alone affected basal levels of EGFR or ERK1/2. However, nicardipine (1 µM), a voltage-dependent Ca\textsuperscript{2+} channel inhibitor, had no effect on the stretch-induced phosphorylation of EGFR and ERK1/2 (data not shown). These results suggest that accumulation of intracellular Ca\textsuperscript{2+} mainly via SA ion channels is required for EGFR and subsequent ERK activation.

Stretch-induced protein synthesis is mediated by EGFR and ERK1/2 activation. To determine whether the stretch-induced EGFR and ERK1/2 pathway contributes to VSMC growth, the effects of two RTK inhibitors (AG-1478 and AG-1296) and a selective inhibitor of MAPK kinase (PD-98059) (2) were tested (Fig. 8). Mechanical stretch (20% elongation) after 20 h caused an approximately twofold greater increase in [\textsuperscript{3}H]leucine incorporation than the unstretched condition, and this effect was completely inhibited by pretreatment with AG-1478 (250 nM) and PD-98059 (25 µM), but not with AG-1296 (25 µM) (Fig. 8A); either compound alone was without effect. To examine the question of whether increases or decreases in stretch produce changes in protein synthesis linked to the EGFR pathway, we compared the effects of a low (3.125% elongation) and high (20% elongation) level of stretch on protein synthesis. Both AG-1478 and PD-98059 again completely blocked protein synthesis stimulated by a high level of stretch, whereas a low level of stretch did not significantly stimulate protein synthesis and either compound alone was without effect (Fig. 8B). Thus the stretch-induced EGFR and subsequent ERK1/2 activation is essential for VSMC growth by a high level of stretch.

**DISCUSSION**

The present study has demonstrated that mechanical stretch induces p21ras-dependent ERK1/2 activation via Ca\textsuperscript{2+}-dependent activation of EGFR, thereby leading to stimulation of protein synthesis in rat VSMC. Recent in vivo studies revealed that ERK1/2 in arterial wall is transiently activated by acute hypertension (42) and balloon-overstretched injury (27). On the other hand, recent accumulating lines of evidence have shown that a variety of agonists, such as ANG II (11), endothelin-1, thrombin, and lysophosphatidic acid (LPA) (8), stimulate ERK1/2 activation via EGFR activation and that oxidized low-density lipoprotein (35) and H\textsubscript{2}O\textsubscript{2} (28) also activate EGFR in VSMC. The present results...
clearly reveal that cyclic strain also shares EGFR activation as an early signaling event with other growth-promoting factors.

The activated autophosphorylated EGFR provides binding sites for the Src homology-2 domain of adaptor proteins, such as Shc and Grb2, to bring a p21ras activator, Sos, to the plasma membrane (26). In fact, the present study demonstrated that both phosphorylated EGFR and Shc were coprecipitated with GST-Grb2 fusion protein in response to stretch. We also demonstrated that the selective inhibition of EGFR by AG-1478 or p21ras by transfection of a dominant-negative mutant (RasY57) effectively blocked the stretch-induced ERK1/2 activation. Thus EGFR activation appears to be an early signaling required for the ERK1/2 activation by mechanical stretch in VSMC.

Our present study showed that mechanical strain induced a rapid (within 2 min) and transient tyrosine Fig. 7. Effects of Gd3+ and TMB-8 on stretch-induced phosphorylation of ERK1/2. Cells pretreated with or without Gd3+ (50 µM) for 60 min or TMB-8 (50 µM) for 30 min were stimulated with mechanical stretch (25%) for 5 min. Left: cell lysates were subjected to immunoblotting with anti-phosphorylated ERK1/2 antibody (top blots) and anti-ERK2 antibody (bottom blots). Arrowheads indicate phosphorylated ERK1/2. Right: summary data graphs. Data are means ± SE for 3 independent experiments. **P < 0.01 vs. basal.

Fig. 8. Effects of EGFR kinase inhibitor (AG-1478), PDGFR kinase inhibitor (AG-1296), and MAPK kinase inhibitor (PD-98059) on stretch-stimulated protein synthesis. After pretreatment with or without AG-1478 (250 nM), AG-1296 (25 µM), or PD-98059 (25 µM) for 30 min, cells were stimulated with or without stretch (20%) for 20 h (A) or with 20% or 3.125% stretch for 20 h (B). [3H]leucine incorporated during 4 h was measured. Each column represents mean ± SE for 3 independent experiments. **P < 0.01 vs. basal.
phosphorylation of several proteins with different molecular weights as revealed by immunoblotting with anti-phosphotyrosine antibody. Among these proteins, the tyrosine-phosphorylated 180-kDa protein is assumed to be EGFR as evidenced by its inhibition by AG-1478 as well as immunoprecipitation with anti-EGFR antibody. Because AG-1478 also inhibited tyrosine phosphorylation of several proteins with different molecular weights (~120, ~60, ~52, and ~46 kDa) other than that of EGFR, these proteins may represent downstream substrates for EGFR, including Shc isoforms. Indeed, our study revealed that tyrosine-phosphorylated Shc isoforms (p66, p52, and p46) after stretching formed a rapid (within 2 min) complex with Grb2 as evaluated by coprecipitation with GST-Grb2 fusion protein coupled with immunoblotting with anti-phosphotyrosine and anti-Shc antibodies. These data suggest that three Shc isoforms were rapidly phosphorylated and recruited by the stretch-induced activated EGFR.

It has previously been reported that mechanical stimuli induced an increase in intracellular Ca\(^{2+}\) levels in fibroblasts (3) and VSMC (31). In the present study, both an SA ion channels inhibitor (Gd\(^{3+}\)) and an intracellular Ca\(^{2+}\) antagonist (TMB-8) used at the appropriate dose (50 µM) on the basis of previous reports (7, 22), but not a voltage-dependent Ca\(^{2+}\) channel inhibitor (nicardipine), inhibited the stretch-induced EGFR and ERK1/2 activation in rat VSMC. These data are consistent with the notion that Ca\(^{2+}\) influx mainly via SA ion channels plays a critical role for the stretch-induced EGFR and subsequent ERK1/2 activation. Furthermore, this is in good agreement with a recent study (24) showing that tyrosine phosphorylation by stretching is mediated by an increase in intracellular Ca\(^{2+}\) levels via Gd\(^{3+}\)-sensitive SA ion channels in endothelial cells. However, our data are in contrast to those of previous studies showing that Ca\(^{2+}\) influx via voltage-sensitive Ca\(^{2+}\) channels is a sufficient signal to induce tyrosine phosphorylation of EGFR in PC12 cells (30) and that stretch-induced ERK1/2 activation is mediated by Na\(^+\)/H\(^+\) exchanger, but not by Gd\(^{3+}\)-sensitive SA ion channels, in cardiac myocytes (43). Collectively, the stretch-induced EGFR and ERK1/2 activation may utilize different ion channels in a cell- and tissue-specific manner.

It has been shown that mechanical strain causes cell proliferation by modulating transcription factors via ERK1/2 pathway (40). In the present study, we have confirmed that a high level of stretch caused an approximately twofold greater increase in protein synthesis than unstretched conditions or a low level of stretch. Furthermore, protein synthesis stimulated by a high level of stretch was completely inhibited by AG-1478 and PD-98059, but not by AG-1296. Although the pharmacological inhibitors used at a single dose may cause nonselective effects, we cautiously tested various doses of these compounds against ERK activation (11) and chose the appropriate doses of AG-1478 (250 nM), AG-1296 (25 µM), and PD-98059 (50 µM). These doses appear to be almost comparable to those that selectively inhibit the effects as reported by other laboratories (2, 8, 36). Thus our data strongly suggest that increased protein synthesis by mechanical stretch is dependent on EGFR and ERK activation. The present in vitro results also appear to be important from the standpoint of vascular growth and remodeling because VSMC in vivo are normally and constantly exposed to pulsatile stretch almost comparable to the low level of cyclic stretch as applied in this study.

Recently, it has been reported that mechanical stress directly induced tyrosine phosphorylation of PDGFR and activation of ERK1/2 in rat VSMC (15). The present in vitro study, however, demonstrated that AG-1296 did not affect the stretch-induced ERK1/2 activation and protein synthesis. This is consistent with a recent report demonstrating that the LPA-induced ERK1/2 activation via PDGFR activation is driven only when the targeted cell lacks EGFR (13). However, our study does not exclude the importance of PDGFR in vascular remodeling after mechanical strain. In fact, it has been shown that both PDGFR-α and -β are induced in balloon-stretched arteries (1) and that a PDGFR kinase inhibitor, AG-1295, markedly inhibited neointimal formation after balloon injury (4). Thus preferential activation of EGFR and/or PDGFR may determine the long-term process of vascular remodeling depending on the mechanical strain applied and experimental models used.

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