Down-regulation of ERK but not MEK phosphorylation in cultured endothelial cells by repeated changes in cyclic stretch

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Abstract

Objective: Effects of cyclic stretch on endothelial cells are studied usually by exposing cells cultured under stretch-free conditions to some levels of cyclic stretch, but in vivo these cells experience both increase and decrease in stretch. Experiments were designed to study how endothelial cells maintained under certain levels of cyclic stretch responded to shifts in stretch frequencies and amplitudes.

Methods: Confluent endothelial cells cultured on flexible silicone membranes with or without pre-stretching for 2–12 h were exposed to various levels of stretch amplitude or frequency and assayed for extracellular signal-regulated kinase 1/2 (ERK) phosphorylation.

Results: When endothelial cells without pre-stretching were cyclically stretched, ERK phosphorylation increased, peaking ∼15 min and slowly decreased. In contrast, when pre-stretched cells were exposed to either higher or lower stretch condition, ERK phosphorylation transiently decreased within 5 min, indicating that some mechanism which down-regulated ERK phosphorylation was activated. Because phosphorylation of ERK kinase (MEK) was not inhibited in these cells, this mechanism targeted ERK directly, not the upstream kinases of the Ras→Raf→MEK→ERK cascade. Furthermore, this ERK down-regulation in pre-stretched cells was not induced by agonists, was inhibited by Na3VO4 but not okadaic acid, and was detected in the cytosolic fraction. Repeated shifts in stretch conditions induced continuous down-regulation of ERK but not MEK phosphorylation.

Conclusions: Endothelial cells are capable of down-regulating ERK phosphorylation in a cyclic stretch- and tyrosine phosphatase-dependent manner. Frequent changes in stretch conditions constitutively activated this ability, which could play some role in regulating ERK activity in endothelial cells in vivo.

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1. Introduction

Due to pumping of the heart, cells in the wall of large arteries are cyclically stretched. Effects of cyclic stretch (CS) on cells are studied using devices that consist of a flexible membrane which is cyclically stretched, thus causing cells on the membrane to be passively stretched (for example, [1,2]).
[7,19,20], and angiotensin II [7,21] receptors and G proteins [7,22,23] are involved in initiating CS-induced mechanosignaling in various cell types. These early events are followed by activation of downstream effectors such as extracellular signal-regulated kinase 1/2 (ERK) [24–26] and other mitogen-activated protein kinases [25,27], which activate transcription factors including AP-1, Elk-1, and Egr-1 [25,28,29], leading to expression of stretch-induced phenotypes. It is reported that chronic or deregulated ERK activation is detrimental to cells [30,31] and that sustained ERK activation weakens interendothelial cell association [32]. Because ECs in vivo are constantly under the influence of CS, which could activate ERK chronically, we wondered if there existed a mechanism that countered this possible ERK activating signal. Here we show that under certain CS conditions, ERK phosphorylation is down-regulated. Instead of using ECs cultured under no-CS condition, we first preconditioned them by CS for 2–12 h and then exposed them to CS whose amplitude or frequency was different from that of the pre-conditioning stretch. We report here that ERK response is induced not only by increasing the CS amplitude and frequency but also by decreasing these parameters, indicating that cells respond to CS changes not to just increases in CS parameters. Kinetics of ERK phosphorylation was different between pre-conditioned and non-pre-conditioned (naïve) ECs even though they were exposed to the same CS stimulus. Instead of rapid increases in ERK phosphorylation seen in naïve cells, pre-conditioned cells exhibited a transient decrease. This down-regulation in ERK phosphorylation occurred without parallel down-regulation of MEK phosphorylation, was abolished by Na3VO4, was detected only in CS pre-conditioned cells, and was induced by both increases and decreases in CS amplitude and frequency. Moreover, the similar ERK down-regulation occurred in cells under repetitive shifts in CS parameters.

2. Methods

2.1. Reagents

VEGF, TNF, N-acetylcysteine (NAC), Na3VO4, and monoclonal anti-actin were from Sigma (Saint Louis, MI),

Fig. 1. CS-induced ERK phosphorylation in naïve and pre-conditioned ECs. Confluent BAECs were exposed to various CS conditions for 2 h (0–120 min.) and then to new CS conditions (arrows) for 30 min (+5 to +30; plus indicates minutes after CS shift). Total (ERK) and phosphorylated (p-ERK) ERK were assayed by immunoblotting. Stretch conditions were changed from 8%/1 Hz to 15%/1 Hz (A), from 15%/1 Hz to 8%/1 Hz (B), from 8%/0.5 Hz to 8%/1 Hz (C), or from 8%/1 Hz to 8%/0.5 Hz (D). One series of immunoblots is illustrated for each condition. Note a transient decrease at 5 min after the shift (asterisks). The immunoblot for each condition is one example out of 2–4 independent experiments, all showing the same p-ERK pattern.
and okadaic acid and PD98059 from Calbiochem (San Diego, CA). Anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-phospho-MEK1/2 (Ser217/221) were from Cell Signaling Technologies (Beverly, MA). Mouse monoclonal anti-ERK1/2 and anti-MEK1 were obtained from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgGs were from Pierce (Rockford, IL). An ECL detection kit was from Amersham Biosciences (Buckinghamshire, England). IRDye 800 conjugated anti-mouse and IRDye 680 conjugated anti-rabbit IgGs were from Rockland (Gilbertsville, PA).

2.2. Cells

Bovine aortic endothelial cells (BAECs) were purchased from Clonetics (San Diego, CA) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES and penicillin (50 IU/ml)/streptomycin (50 μg/ml) as described[33]. Cells were used between passages 6 and 10. All culture medium supplies were from MediaTech (Hemdon, VA). Human arterial endothelial cells (HAECs) were obtained from Cascade Biologics (Portland, OR) and cultured in Medium 200 (Cascade Biologic) supplemented with low serum growth supplement (Cascade Biologic) containing 2% FBS, 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.3. Application of cyclic stretch

To apply uniaxial CS with various amplitudes and frequencies, a cell stretch device (K.K. Scholar Tec, Osaka, Japan; Model NS-500) designed by Naruse et al. [2] was operated in a tissue culture incubator. Silicon stretch chambers (30 × 30 mm²) have a thin (0.1 mm) silicon membrane bottom. This surface was treated overnight with 100 μg/ml rat tail type I collagen (BD Bioscience, Bedford, MA) in 0.02 N acetic acid (1.5–2 ml), rinsed thoroughly with PBS, sterilized by UV for 15 min, and ECs plated and cultured until becoming confluent. Six chambers could be stretched simultaneously. A control box outside the incubator provided various pre-set levels of amplitude (5, 6, 8, 10, 12, 15, 20, and 25%) and frequency (1, 5, 10, 20, 30, 40, 50, and 60 cycles/min) of stretch. Shifts in these stretch parameters can be made without stopping the apparatus. When time

Fig. 2. Quantification of ISE. A: Confluent BAECs with (left) or without (right) pre-conditioning with 5%/1 Hz stretch for 12 h were exposed to 15%/1 Hz CS. Phosphorylated (p-ERK) and total (ERK) ERK were determined by immunoblotting. The phosphorylation level in naïve cells increased without ISE (right) while it transiently decreased in the pre-conditioned cells (Precond) at 2 and 5 min (left). B: ERK phosphorylation levels were quantified. Data are presented as means ± s.e.m. (n = 3). *: p < 0.01 and ***: p < 0.0005. All the changes were statistically significant (at least p < 0.05) when compared to the value at 0 time. #: arbitrary units relative to the phosphorylation level at 0 time (≡1).
course studies were done, it was stopped briefly (<5 s) at the no-stretch position to remove one of the chambers and re-started.

2.4. Cell fractionation

The cytosolic, membrane, cytoskeletal, and nuclear fractions were prepared according to a published method [34] using a subcellular proteome extraction kit (Calbiochem, San Diego, CA). A protease inhibitor cocktail and non-specific nuclease inhibitor, benzonase, were supplemented. Ten or 20% of the total extract volume was loaded onto SDS-PAGE gels and analyzed by immunoblotting for the total and phosphorylated ERK.

2.5. Immunoblotting

SDS lysis buffer (2% SDS, 100 mM Tris–HCl, pH 6.8) was added to the chamber, and cells were scraped off and heated at 100 °C for 5 min. Protein concentrations were determined (MicroBCA assay; Pierce), and equal amounts of proteins were loaded for SDS-PAGE, and gel bands transferred to a PVDF membrane (Millipore, Billerica, MA). After blocking with 5% nonfat milk and 0.1% Tween 20 in PBS, membranes were incubated with primary antibodies for 1 h at room temperature. After thorough washing in Tris-buffered saline, the membrane was incubated with horseradish peroxidase- or IRDye-conjugated secondary antibodies and detected using an ECL or an Odyssey (Li-COR; Bioscience, Lincoln, NE) infrared imaging system, respectively. Densitometric analyses of immunoblots were performed using an NIH image software, and ERK and MEK phosphorylation levels were determined by taking the ratio between phosphorylated and total kinases. Results were normalized by setting the densitometry reading of control experiments to 1.0. Group differences were analyzed using the standard T-test. A value of p<0.05 was considered significant.

Fig. 3. MEK is phosphorylated during ISE. A: Naïve BAECs were treated with 20 μM PD98059 for 1 h and exposed to 20%/1 Hz CS for up to 60 min. ERK phosphorylation (p-ERK) was inhibited by the MEK inhibitor. DMSO had no effect. B: BAECs with or without pre-conditioning (5%/1 Hz) for 12 h were exposed to 15%/1 Hz stretch and phosphorylated (p-MEK) and total (MEK) MEK were monitored by immunoblotting. No ISE-like MEK pattern was detected in pre-conditioned ECs. C: Changes in MEK phosphorylation are quantified. Each bar represents mean±s.e.m. (n=3). *: p<0.05; Precond: Pre-conditioned cells; Naïve: naive cells; °: arbitrary units relative to the phosphorylation level at 0 time (t=1).

Fig. 4. ISE is not activated by VEGF and TNF. A: Confluent BAECs with (pre-conditioned) or without (naïve) pre-conditioning (5%/1 Hz) for 12 h were treated with 20 ng/ml of either VEGF or TNF for 10–15 min. ERK phosphorylation (p-ERK) was assayed by immunoblotting. Both naïve and pre-conditioned cells show the same ERK phosphorylation pattern. No down-regulation of ERK phosphorylation occurred in pre-conditioned ECs. B: The same experiments were repeated using lower doses of VEGF and TNF. No down-regulation of ERK phosphorylation in pre-conditioned cells was observed at the 2 and 5 min time points. C: To test if pre-treatment with an agonist could pre-condition BAECs for ISE, cells were treated with 5 ng/ml TNF for 5 h and then challenged with 15%/1 Hz stretch. ISE was not observed. Actin (A) or ERK (B, C) was used as a loading control. All the immunoblots are examples from 3 independent experiments.
3. Results

3.1. Endothelial cells respond to changes in the amplitude and frequency of cyclic stretch

ERK is phosphorylated when naïve ECs are cyclically stretched uniaxially [24,25]. Indeed, when naïve BAECs or HAECs were exposed to CS having the amplitude (expressed as a percent increase of non-stretched substrate length) of 8% and the frequency (expressed in Hz) of 1 Hz (8%/1 Hz stretch), ERK phosphorylation increased within 5 min, peaked at about 15 min, and then declined reaching the general baseline level of unstimulated cells within 2 h (Fig. 1A and B). Further exposure (up to 24 h tested) of these cells to the same level of CS did not change the ERK phosphorylation level. We then used pre-conditioned cells to do similar CS experiments. Unlike naïve cells that can be challenged only with increases in CS, pre-conditioned cells may be exposed to either an increase or a decrease in CS. Because ERK phosphorylation levels did not change in naïve cells that were cyclically stretched for >2 h, we defined that for the purpose of our ERK analysis, ECs treated with CS for 2 h or longer were pre-conditioned. Pre-conditioning was performed by exposing ECs to all the possible ranges of stretch (5, 6, 8, 10, 12, 15, 20 and 25%) for 2–12 h at 0.5 or 1 Hz. We found that all the pre-conditioned ECs (both BAECs and HAECs) responded in the same manner to the subsequent CS shifts. Although the standard pre-conditioning was done by treating cells with 5%/1 Hz stretch for 12 h, results are illustrated using cells pre-conditioned under different conditions in order to demonstrate that pre-conditioning could be achieved at various CS levels.

When ECs pre-conditioned with 8%/1 Hz stretch were challenged with an upward shift in amplitude to 15%, we

Fig. 5. ISE occurs in the cytosolic fraction. Confluent BAECs with (Precond) or without (naïve) pre-conditioning (5%/1 Hz) for 12 h were challenged with 15%/1 Hz stretch for 0, 5, or 15 min. Harvested cells were fractionated into cytosolic (CF), membranous (MF), nuclear (NF), and cytoskeletal (SF) fractions and ERK phosphorylation levels were determined by immunoblotting. One set of immunoblot is illustrated. Decreased ERK phosphorylation was detected only in the cytosolic fraction of pre-conditioned cells. Graphs show relative ERK phosphorylation levels (mean±s.e.m., n=3). *: p<0.05; **: arbitrary units relative to the phosphorylation level at 0 time (=1).
observed an initial drop in ERK phosphorylation followed by an increase (Fig. 1A). The initial decrease peaked at about 5 min and the subsequent increase peaked at about 15 min. The same pattern of ERK response was observed when CS amplitude was shifted down from 15%/1 Hz to 8%/1 Hz stretch (Fig. 1B). We also examined if an up- or down-shift in frequency also affected ERK phosphorylation. As shown in Fig. 1C and D, CS increased ERK phosphorylation in naïve cells, which then reached the baseline level in 2 h (see 0–120 min span), but a transient decrease (asterisks) occurred in pre-conditioned cells when CS frequency was either increased (from 0.5 to 1 Hz) or decreased (from 1 to 0.5 Hz). We repeated several of these experiments using HAECs and found the same pattern of response, indicating that these effects were not unique to bovine ECs.

The observed changes in ERK phosphorylation followed by an increase (Fig. 1A). The initial decrease peaked at about 5 min and the subsequent increase peaked at about 15 min. The same pattern of ERK response was observed when CS amplitude was shifted down from 15%/1 Hz to 8%/1 Hz stretch (Fig. 1B). We also examined if an up- or down-shift in frequency also affected ERK phosphorylation. As shown in Fig. 1C and D, CS increased ERK phosphorylation in naïve cells, which then reached the baseline level in 2 h (see 0–120 min span), but a transient decrease (asterisks) occurred in pre-conditioned cells when CS frequency was either increased (from 0.5 to 1 Hz) or decreased (from 1 to 0.5 Hz). We repeated several of these experiments using HAECs and found the same pattern of response, indicating that these effects were not unique to bovine ECs.

The observed changes in ERK phosphorylation were small, but small changes are typical for mechanoresponses. Unlike chemical stimuli that can be easily applied at ten or more times higher concentrations than the physiological level and elicit a robust response in cells, mechanical stress such as CS that is ten times the physiological range damage cells irreversibly. Thus, mechanoresponse must be elicited using the physiological level of stimulus, which often exhibits only a low level of biochemical changes. Nonetheless, the response we report here was observed consistently and was statistically significant. Our data show that ERK responses are elicited by changing either amplitude or frequency of CS and that the upward or downward directionality of the change may not be an important factor for ERK responses. In all the experiments with pre-conditioned cells, down-regulation of ERK phosphorylation was detected within 5 min after changing stretch conditions. This suggests that a burst of inhibitory signal for ERK phosphorylation (ISE) is generated in pre-conditioned ECs. Fig. 2 demonstrates ISE in more detail. Here, ECs pre-conditioned with 5%/1 Hz stretch for 12 h were exposed to 15%/1 Hz stretch, and ERK phosphorylation was semi-quantified using Western blot data. ERK phosphorylation decreased to about 70% of the basal level within 5 min (Fig. 2B) but the extent of decrease is more substantial when compared to ERK phosphorylation in naïve counterparts at the same time points.

Fig. 6. Effects of okadaic acid, Na3VO4, and NAC on ISE. Confluent BAECS with (pre-conditioned or preconditioned) or without (naïve) pre-conditioning (5%/1 Hz) for 12 h were treated with 10 nM okadaic acid (OA), 1 mM Na3VO4, or 10 mM NAC for 30 min and challenged with 15%/1 Hz CS. Phosphorylated (p-ERK) and total (ERK) ERK were determined by immunoblotting at 0 and 5 min. DMSO or H2O was used as sham treatment. A: Okadaic acid failed to block ISE in pre-conditioned cells. B: NAC increased the basal level of ERK phosphorylation but did not inhibit ISE. Pre-conditioned ECs treated with Na3VO4 failed to show ISE. C: ERK phosphorylation at 5 min was quantified for cells treated with Na3VO4 (mean±s.e.m., n=5). °: arbitrary units relative to the phosphorylation level at 0 time (=1).

Fig. 7. Sustained ERK down-regulation in ECs exposed to repeated changes in CS. Confluent BAECS were pre-conditioned (6%/1 Hz) for 3 h and then subjected to alternating CS between 6%/1 Hz and 15%/1 Hz every 15 min. ERK and MEK phosphorylation levels were assayed by immunoblotting. All samples except for the 5-minute point (second data point for p-ERK) were collected at the end of the first 15%/1 Hz stretch cycle on the hour. Because a maximum of 6 chambers could be stretched for one series of experiment, the 5-minute data were obtained in separate experiments (dotted line). A: A schematics showing the pattern of alternating CS and sampling points. The break indicates a period of 3–6 h during which up and down 15-minute cycles were repeated. X represents 3, 4, 5, or 6. B: Quantification of ERK and MEK phosphorylation (mean±s.e.m., n=4). While MEK phosphorylation remained increased throughout the experiments, ERK phosphorylation was down-regulated except for the first 15 minute point when it is always higher than the baseline. Note that the level of ERK down-regulation by repeated CS changes is similar to ISE. Significance levels are calculated against the value at time 0. °: p<0.05; °°: p<0.001; °°°: arbitrary unit relative to the level at the end of pre-conditioning (time 0, relative value=1).
3.2. ISE occurs in spite of MEK phosphorylation

MEK (MAPK [mitogen-activated protein kinase]/ERK kinase) is the immediate upstream kinase of ERK [35]. When phosphorylated, MEK specifically phosphorylates ERK. Indeed, when ECs were pretreated with PD98059 (20 μM, 1 h), a MEK inhibitor, CS-induced ERK phosphorylation was abolished, indicating that ERK phosphorylation by CS is MEK activity-dependent (Fig. 3A). Thus, we investigated if ISE was due to MEK inhibition. MEK was phosphorylated in naïve ECs exposed to 15%/1 Hz CS, and its phosphorylation kinetics was similar to that of ERK (Compare Fig. 2A, right with Fig. 3B, right). However, the transient inhibition phase observed for ERK was not detected for MEK when ECs pre-conditioned for 12 h were challenged with the same CS level (Fig. 3B left panel). This result indicates that ISE occurs downstream to MEK. We noted that the increase in MEK phosphorylation levels was always higher in the pre-conditioned than in the naïve cells at all times (Fig. 3C), indicating higher ERK kinase activity in pre-conditioned cells. This observation suggests that ISE is a strong inhibitory signal as it must overcome the increased activity of the ERK kinase.

To see if ISE could be activated by EC agonists, ECs with or without pre-conditioning were treated with 20 ng/ml VEGF or TNF for up to 15 min and ERK phosphorylation was assayed. The same ERK response was observed in both pre-conditioned and naïve cells, showing that ISE was not initiated by these agonists (Fig. 4A). These results could mean that ISE was unable to overcome robust ERK phosphorylation induced by 20 ng/ml of the agonists. We tested various concentrations of VEGF and TNF and found that 10 ng/ml VEGF and 5–10 ng/ml TNF induced ERK phosphorylation comparable to the level induced by CS. When pre-conditioned ECs were treated with 10 ng/ml VEGF or 5 ng/ml TNF, no ISE was observed (Fig. 4B). To see if ISE could be induced in ECs pre-treated with agonists, we treated cells with 5 ng/ml TNF for 5 h and then exposed them to 15%/1 Hz stretch. No ISE was detected (Fig. 4C). To see if ISE was mediated by some factors secreted by pre-conditioned ECs, naïve cells were treated with spent media from pre-conditioned ECs for various lengths of time. The medium change caused a slight increase in ERK phosphorylation within 15 min, but no ISE was induced with or without CS. These results suggest that ISE is activated by changes in CS magnitudes in pre-conditioned ECs.

3.3. ISE occurs in the cytosolic fraction

Recent studies indicate that an important regulatory mechanism for ERK signaling is compartmentalization of this kinase within a cell [36,37]. Indeed, in addition to nuclear targets, ERK has been implicated to regulate the cytosolic and cytoskeletal substrates [38]. To determine where within the cell the ISE activity is present, we fractionated cells into cytosolic, membrane and membrane organelle, nuclear, and cytoskeletal fractions. ECs with or without CS pre-conditioning were exposed to an increased level of stretch for up to 15 min. These cells were then fractionated at various time points, and each fraction analyzed for ERK phosphorylation. Within naïve ECs, relative compartmentalization of ERK protein was 63.4±7.9% (cytosolic), 24.6±3.9% (membrane), 7.2±2.8% (nuclear), and 4.9±1.7% (cytoskeletal), and CS stimulation did not change these values. As expected, PECAM-1 and VE-cadherin were present in the membrane and cytoskeletal fractions while MEK1 and phosphorylated ELK-1 were in the cytosolic and nuclear fractions only, respectively. ERK phosphorylation increased in all four fractions of naïve cells within 5 min (Fig. 5). In pre-conditioned cells, ISE was detected only in the cytosolic fraction. MEK was found almost exclusively in the cytosolic fraction and its phosphorylation increased in both naïve and pre-conditioned cells.

3.4. ISE is inhibited by sodium vanadate

Because MEK down-regulation did not occur during ISE, some phosphatase that dephosphorylated ERK could be involved. PP2A is serine/threonine phosphatase and has been shown to inactivate ERK in several cell systems [39–41]. ECs with or without pre-conditioning were treated with okadaic acid, a PP2A inhibitor or DMSO (dimethyl sulfoxide), the carrier solvent, during the last 30 min of pre-conditioning and exposed to increased levels of CS for 5 min in the presence of the added reagents. The basal level of ERK phosphorylation increased slightly, presumably due to the effect of this phosphatase inhibitor (Fig. 6A). However, ISE was observed in pre-conditioned cells treated with okadaic acid (Fig. 6A). Next, we tested the effect of Na3VO4, a protein tyrosine phosphatase inhibitor. ECs with or without pre-conditioning were treated with 1 mM Na3VO4 in the same manner as described for okadaic acid treatment and exposed to higher levels of CS for 5 min. ISE was inhibited in pre-conditioned cells treated with Na3VO4 (Fig. 6B, C), suggesting involvement of protein tyrosine phosphatase(s) in ISE. Some reports indicate that CS effects are mediated by reactive oxygen species [42,43]. We found that NAC, an anti-oxidant, strongly increased the basal level of ERK phosphorylation but was unable to inhibit ISE (Fig. 6B).

3.5. Repetitive changes in cyclic stretch negatively regulate ERK phosphorylation

Experiments described so far were done by exposing pre-conditioned ECs to a single step change in CS condition. In the body, however, the amplitude and frequency of CS would fluctuate. We wondered if ECs responded differently to fluctuating CS. Cells were first pre-conditioned for 3 h with 6%/1 Hz stretch and then exposed to repeating step changes that alternated between 15%/1 Hz and 6%/1 Hz every 15 min.
for over 6 h (Fig. 7A). ERK and MEK phosphorylation levels were determined at various time points and compared to the levels at the end of pre-conditioning (time 0 in Fig. 7B). During the first 15 min of CS shift, ERK phosphorylation briefly decreased within 5 min (ISE) and increased by 15 min, as we have shown in other experiments (Figs. 1 and 2). However, when we looked at time points beyond 3 h, ERK phosphorylation was consistently below the time 0 level (Fig. 7B). ERK down-regulation was not stable within the first 1–2 h of stimulation, indicating that some time is necessary for cells to establish this reduced ERK phosphorylation state. MEK phosphorylation remained up-regulated throughout the experiment. Thus, we tentatively concluded that the reduced ERK phosphorylation was due to the ISE state, which was stably maintained by repeated changes in CS.

4. Discussion

4.1. Endothelial cells respond to changes in cyclic stretch

EC responses to CS are typically studied by exposing naïve cells to CS in one step. However, in situ, ECs are exposed to both increase and decrease in CS magnitude and frequency and must rarely encounter CS in this single step, all-or-none manner. To study how ECs respond to changes in CS parameters, we first pre-conditioned cells with CS, then exposed them to a different CS condition, and assayed ERK phosphorylation, a well-known EC response to CS. Our first finding was that ECs respond to both increase and decrease in CS, indicating that they respond to changes in CS, not to just some increased values in amplitude or frequency. Our observations are in line with the idea that ECs respond to mechanical stress gradients [45–47]. The level of stretch exerted on vascular wall cells under normal physiological conditions has been reported to be between 5% [5] and 9–12% [44] for large human arteries. Thus, the levels of CS used in our studies are within the range of normal fluctuations.

4.2. ISE may not be a transient phenomenon

While only increasing ERK phosphorylation was noted in naïve cells challenged with CS, pre-conditioned cells exposed to the same CS condition showed a different ERK phosphorylation response. Instead of progressively increasing, pre-conditioned ECs showed a transient decrease in ERK phosphorylation when exposed to a new CS condition. This transient decrease was not triggered by agonists including spent medium from pre-conditioned ECs, suggesting that ISE was triggered by CS. Whether or not it could also be triggered by other forms of mechanical force needs to be investigated. The most intriguing discovery is that a continuous ISE state may be achieved by exposing cells to repeated changes of CS. We chose a 15-minute cycle to show the effect of repeated changes in CS because if ISE were only a short-lived phenomenon, ERK phosphorylation levels would have increased to the near peak level in 15 min after each CS shift. However, we observed continued ERK down-regulation. This down-regulation was maintained in spite of increased levels of MEK phosphorylation, a characteristic of ISE we identified in this study. For this reason, we tentatively conclude that the reduced ERK phosphorylation in these cells is due to ISE although this needs to be established by further studies.

Chronic or deregulated ERK activity appears to have negative consequences to cells [30–32]. Thus, it may be expected that cells, especially those constantly exposed to stimuli that activate ERK such as ECs in situ, have some mechanism to dampen ERK activity. ISE could be one such mechanism. While there are numerous examples for mechanical stress-induced ERK phosphorylation, examples for mechanical stress-induced down-regulation of this kinase are less abundant. Frangos and colleagues reported that ECs exposed to slowly ramped CS [47] or fluid shear stress [46] exhibited decreases in ERK phosphorylation. Attenuated ERK phosphorylation was also observed in ECs pre-conditioned by fluid shear stress and subsequently challenged with increased levels of shear stress [48,49]. Whether or not ISE plays a role in these cases needs to be investigated.

4.3. Mechanism for ISE

The typical ERK signaling cascade is the Ras–Raf–MEK–ERK pathway, and an inhibiting signal could come in at any step along the pathway. The tightest connection in this cascade is the MEK-ERK connection. ERK is the only known MEK substrate and ERK is phosphorylated when MEK is phosphorylated [35]. Thus, if ERK phosphorylation decreased, one would expect a decrease in MEK phosphorylation. However, MEK phosphorylation remained up-regulated during ISE, suggesting that ISE was not due to down-regulation of this canonical pathway and that it targeted ERK directly. We wondered if some ERK phosphatase was involved. PP2A is a serine/threonine phosphatase known to inactivate ERK [39–41]. If PP2A is transiently activated, ERK phosphorylation could be down-regulated. However, okadaic acid was unable to abolish ISE, suggesting that PP2A did not play a role in ISE. Interestingly, Na3VO4 was able to inhibit ISE. This indicates the involvement of tyrosine phosphatase(s), but the molecular identity remains elusive.

Phosphatases in the MAP kinase phosphatase (MKP) family may be possible candidates. It consists of 11 members that are dual phosphatases (tyrosine and serine/threonine) with overlapping substrates [50,51]. There are two mechanisms for regulating substrates [32,51]. One is regulation by phosphorylated ERK. It is thought that MKP binds to phosphorylated ERK and this binding in turn activates its phosphatase activity. This mechanism could be responsible for transient ISE and also the constitutive ISE state in cells.
stimulated by repeated CS changes. The other mechanism is increased expression of MKP by ERK activation \[52,53\]. This mechanism will not account for the transient ISE that happens within 5 min of stimulation but could play a role in the constitutive ISE state. Although our preliminary results failed to confirm increased expression of MKP1 and MKP2 in ECs exposed to several hours of CS (data not shown), it is still possible that the expression of other members of the family is increased. Identification of phosphatase(s) is a key to elucidating the mechanism for ISE.

In summary, we have shown that ECs react to CS changes, both increases and decreases in amplitudes and frequencies of CS. Using pre-conditioned ECs, we revealed the presence of a mechanism, which we call ISE, that down-regulates ERK phosphorylation in a CS- and tyrosine phosphatase-dependent manner, and this occurs in the face of increased MEK phosphorylation. ISE is detectable only transiently if cells are challenged with a single shift in CS conditions, but it may become continuously active if shifts are made repeatedly. This latter observation may have relevance in ERK regulation in ECs in situ.

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References


