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Molecular mechanisms of the angiogenic effects of low-energy shock wave therapy: roles of mechanotransduction

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Hatanaka K, Ito K, Shindo T, Kagaya Y, Ogata T, Eguchi K, Kurosawa R, Shimokawa H. Molecular mechanisms of the angiogenic effects of low-energy shock wave therapy: roles of mechanotransduction. *Am J Physiol Cell Physiol* 311: C378–C385, 2016. First published July 13, 2016; doi:10.1152/ajpcell.00152.2016.—We have previously demonstrated that low-energy extracorporeal cardiac shock wave (SW) therapy improves myocardial ischemia through enhanced myocardial angiogenesis in a porcine model of chronic myocardial ischemia and in patients with refractory angina pectoris. However, the detailed molecular mechanisms for the SW-induced angiogenesis remain unclear. In this study, we thus examined the effects of SW irradiation on intracellular signaling pathways in vitro. Cultured human umbilical vein endothelial cells (HUVECs) were treated with 800 shots of low-energy SW (1 Hz at an energy level of 0.03 mJ/mm^2). The SW therapy significantly upregulated mRNA expression and protein levels of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS). The SW therapy also enhanced phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2) and Akt. Furthermore, the SW therapy enhanced phosphorylation of caveolin-1 and the expression of HUTS-4 that represents β_1-integrin activity. These results suggest that caveolin-1 and β_1-integrin are involved in the SW-induced activation of angiogenic signaling pathways. To further examine the signaling pathways involved in the SW-induced angiogenesis, HUVECs were transfected with siRNA of either β_1-integrin or caveolin-1. Knockdown of either caveolin-1 or β_1-integrin suppressed the SW-induced phosphorylation of Erk1/2 and Akt and upregulation of VEGF and eNOS. Knockdown of either caveolin-1 or β_1-integrin also suppressed SW-induced enhancement of HUVEC migration in scratch assay. These results suggest that activation of mechanosensors on cell membranes, such as caveolin-1 and β_1-integrin, and subsequent phosphorylation of Erk and Akt may play pivotal roles in the SW-induced angiogenesis.

shock wave; mechanotransduction; angiogenesis; caveolin-1; β_1-integrin

SHOCK WAVES (SW) have been clinically introduced for lithotripsy since the 1980s; urinary stones are broken up by high-energy SW (7). The waveform of an SW is similar to that of a blast wave, which is composed of discontinuous compression of leading shock propagating with supersonic speed, subsequent rarefaction, and negative pressure (27). Over the past 20 years, low-energy SW therapy has also been put into clinical application (3, 17). We have previously demonstrated that low-energy SW (about 10% of the energy used for urolithotripsy treatment) significantly upregulated vascular endothelial growth factor (VEGF) in human umbilical vein endothelial cells (HUVECs) (25). Low-energy SW therapy has also been reported to enhance nitric oxide (NO) production via activation of endothelial NO synthase (eNOS) in vitro (23, 24). We have demonstrated that extracorporeal low-energy cardiac SW therapy enhances angiogenesis and contractile function in a porcine model of chronic myocardial ischemia and in patients with refractory angina pectoris without any adverse effects (12, 19, 25). Moreover, low-energy SW therapy has been widely used for treatment of orthopedic diseases, such as bone nonunions, tendinosis calcarea, epicondylitis, and calcaneal spur through anti-inflammatory effects (3, 26, 37).

Although we have demonstrated that extracorporeal cardiac SW therapy is an effective and noninvasive therapy for severe ischemic heart disease, the detailed molecular mechanisms of the SW-induced angiogenesis remain unclear. Vascular endothelial cells, which cover the inner surface of blood vessels, are exposed to fluid shear stress caused by blood flow and transmit extracellular mechanical stimuli to intracellular signaling pathways, leading to angiogenesis, cell proliferation, vasodilatation, and antithrombotic effects (6, 14, 21, 36). This process is called mechanotransduction. Many molecules have been reported to play important roles in mechanotransduction, including ATP-gated P2X4 purinoceptor, transient receptor potential channels, mechanosensitive channels, and cytoskeletons (4, 8, 41). Caveolins, integrins, and their complex have also been proposed as mechanotransducers that could convert physical stresses into biochemical signals and have been reported to be involved in angiogenesis and proliferation cascades (9, 28, 29). Therefore, we paid attention to vascular endothelial cells among various types of cells in the present study and examined whether caveolin-1 and β_1-integrin play important roles in sensing mechanical stress induced by low-energy SW, and, if so, we aimed to elucidate the downstream signaling pathways.

METHODS

Cell culture and SW therapy. Single-donor HUVECs were used as in our previous study (25). Cells were purchased from Lonza (Basel, Switzerland) and were cultured in a complete endothelial cell growth medium (EGM-2 BulletKit; Lonza). The same batch of cells were used in all experiments at *passages 3* to *5* and were maintained in EGM-2. The cells at the same passage were used for each set of experiments. Twenty-four hours before the SW therapy, the cells (5×10^4) were resuspended in about 1.8-cm^2 cell culture plates. We treated

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the cells with 800 shots of SW with 1 Hz at an energy level of 0.03 mJ/mm^2 using an SW system (Duolith SD1; Storz Medical, Tägerwilen, Switzerland) based on our preliminary studies (Fig. 1*A*). In the preliminary studies, low-energy levels of SW (~0.03–0.10 mJ/mm^2; <10% of the energy density used for urolithiasis) enhanced VEGF expression in HUVECs. The energy level of 0.03 mJ/mm^2 was the highest energy level that allowed us stable measurements without cell detachment from the surface of the culture plates.

Lactate dehydrogenase assay. To evaluate potential cell damage induced by SW, the activity of lactate dehydrogenase (LDH) in the supernatants of treated cells was quantified using the LDH cytotoxicity detection kit (Takara Bio, Kusatsu, Japan). The cell-free supernatants were collected and incubated with the reaction mixture from the kit, and the LDH activity was determined by an enzymatic reaction, which generates formazan and dyes the supernatant a red color, the intensity of which directly correlates with the LDH activity. FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) was used for measuring the absorbance of the samples at 490 nm. Triton X-100 (1%; Sigma-Aldrich, St. Louis, MO) solution was used to induce maximal cell damage in the cultured cells. Twenty-four and 48 h after SW irradiation, cell damage was evaluated with LDH activity.

RNA isolation, real-time PCR. Total RNA was isolated from cultured HUVECs using a total RNA extraction kit (RNeasy Plus Mini Kit; QIAGEN, Venlo, Netherlands). cDNA was synthesized with the PrimeScript RT Master Mix (Takara Bio). The following oligonucleotide primers were used in the present study: human VEGF-A (GenBank Acc. NM_001025366.2) (forward) 5′-TCACAGGTACAGGGATGAGGACAC-3′ and (reverse) 5′-CAAAGCACAGCAATGTCCTGAAG-3′; eNOS (GenBank Acc. NM_001160109.1) (forward) 5′-AAAGACAAGGCAGCAGTGGAAAT-3′ and (reverse) 5′-TCCACGATGGTCACTTTGGCTA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank Acc. NM_002046.5) (forward) 5′-GCACCGTCAAGGCTGAGAAC-3′ and (reverse) 5′-TGGTGAAGACGCCAGTGGA-3′. After reverse transcription, real-time PCR was performed with SYBR Premix Ex Taq II (Takara Bio) and a CFX96 Real-Time system C1000 Thermal Cycler (Bio-Rad, Hercules, CA). The PCR conditions were 40 cycles of 2 s at 98°C and 5 s at 55°C. mRNA expression levels were compared between the control and SW groups. Results are reported as the quotients of the copy number of the gene of interest, relative to that of *GAPDH*, as a housekeeping gene.

Western blot analysis. To quantify the expression levels of VEGF, eNOS, phosphorylated eNOS (phospho-eNOS), Erk1/2, phospho-Erk1/2, Akt, phospho-Akt, Fyn, phospho-Fyn, FAK, phospho-FAK, caveolin-1, phospho-caveolin-1, β_1-integrin, HUTS-4, and α-tubulin, protein samples were loaded on SDS-PAGE gel and transferred to PVDF membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were immunoblotted with anti-VEGF (sc-507; Santa Cruz Biotechnology, Dallas, TX), anti-eNOS (ADI-905-386; Enzo, Farmingdale, NY), anti-phospho-eNOS (9571; Cell Signaling Technology, Danvers, MA), anti-Erk1/2 (sc-94; Santa Cruz Biotechnology), anti-phospho-Erk1/2 (sc-7383; Santa Cruz Biotechnology), anti-Akt (9272; Cell Signaling Technology), anti-phospho-Akt (4060;

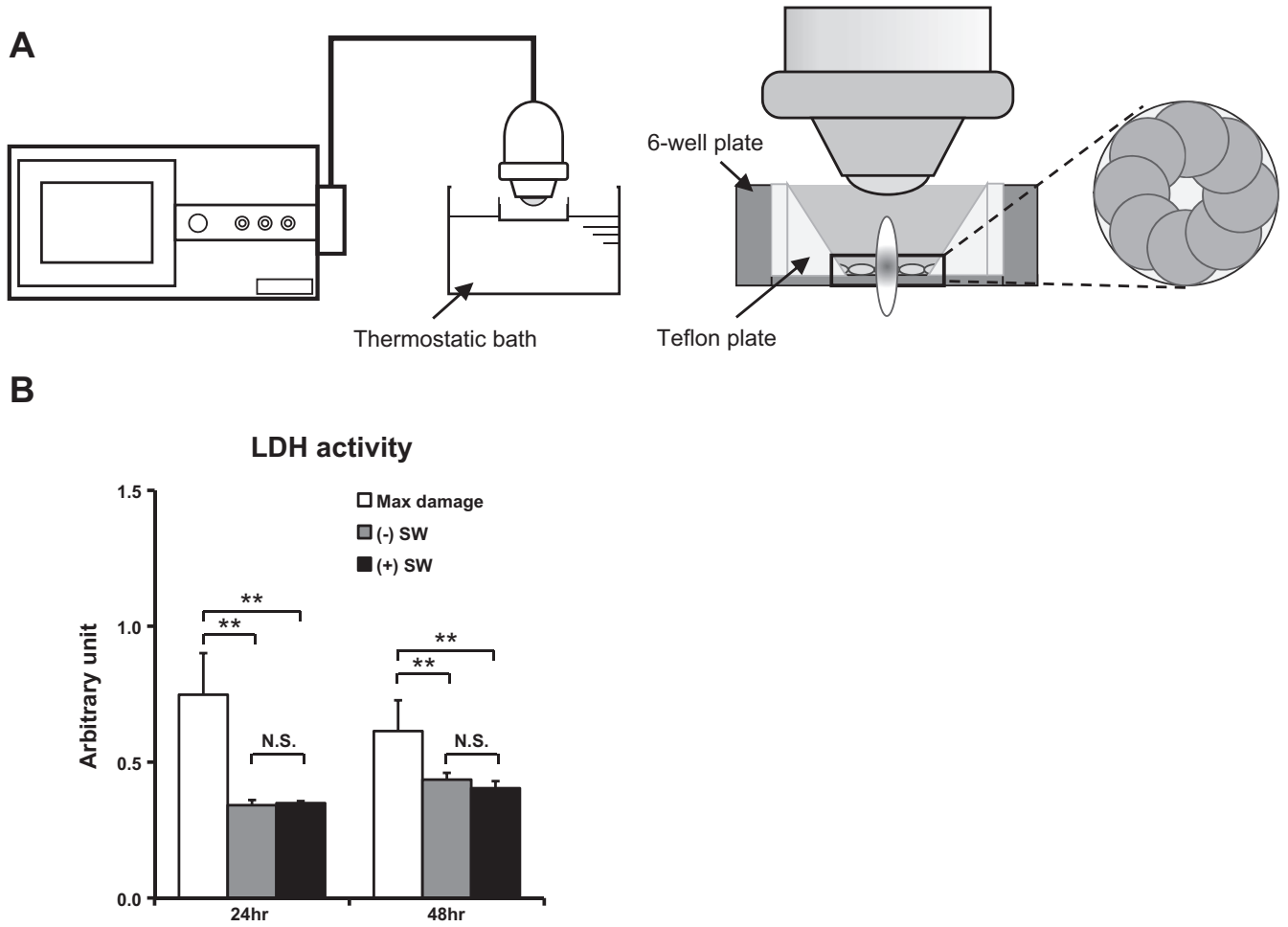

Fig. 1. Experimental setup and lactate dehydrogenase (LDH) assay. *A*: illustrations of experimental setup. *B*: low-energy shock wave (SW) therapy did not cause cell injury ($n = 6$ each). Results are expressed as means ± SD. $^{**}P < 0.01$ in each comparison.

Cell Signaling Technology), anti-Fyn (4023; Cell Signaling Technology), anti-phospho-Fyn (6943; Cell Signaling Technology), anti-FAK (3285; Cell Signaling Technology), anti-phospho-FAK (8556; Cell Signaling Technology), anti-caveolin-1 (610407; BD Biosciences, Franklin Lakes, NJ), anti-phospho-caveolin-1 (3251; Cell Signaling Technology), anti-β_1-integrin (ab52971; Abcam, Cambridge, UK), and anti-HUTS-4 (LS-B2861; LifeSpan BioSciences, Seattle, WA). The regions containing proteins were visualized by an electrochemiluminescence Western blotting luminal reagent (RPN2232, GE Healthcare).

Small-interference RNA and its transfection. Small-interference RNA (siRNA) duplex targeting caveolin-1, β_1-integrin, Fyn, and FAK was purchased from QIAGEN. A functional nontargeting siRNA that was bioinformatically designed by QIAGEN (AllStars Negative Control siRNA) was used as a control. HUVECs were transfected with HiPerFect Transfection Reagent (QIAGEN) with 10 nmol/l siRNA specific for each protein. Seventy-two hours after siRNA transfection, HUVECs were treated with SW and mRNA, or protein expressions were evaluated. Efficacy of knockdown was assessed 72 h after transfection by real-time PCR and Western blot analysis.

Scratch assay. As one of the indices of angiogenesis, scratch assay was performed in HUVECs (22). To further elucidate the roles of mechanotransduction on the SW-induced angiogenesis, scratch assay was performed with siRNA targeting either caveolin-1 or β_1-integrin and scramble siRNA. Cells were seeded on six-well plates and grown to confluence. The cell monolayers were scratched with a cell scraper and photographed at 0, 24, and 48 h after SW irradiation (33). Distances between one side of the scratch and the other were measured with image analysis software ImageJ.

Statistical analysis. All results are expressed as means ± SD. Statistical comparisons between two groups were performed by Student's *t*-test. Multiple groups were analyzed by one-way ANOVA followed by Tukey's or Games-Howell multiple-comparison test as appropriate to determine statistical significance. Probability values <0.05 were considered statistically significant.

RESULTS

LDH assay. To evaluate cell damage induced by the SW, we performed LDH assay in HUVECs. There was no difference in the degree of LDH release between the SW group and the control group (Fig. 1*B*), suggesting that the low-energy SW used in the present study did not cause cell injury.

Effects of the SW therapy on the expression of angiogenic factors. To study the molecular mechanisms for the SW-induced angiogenic effects, mRNA expression of VEGF and eNOS was evaluated. The SW therapy significantly upregulated the expression of VEGF and eNOS (Fig. 2*A*). Protein levels of VEGF and eNOS were also enhanced by the SW therapy (Fig. 2, *B* and *C*). The phosphorylation of eNOS at Ser1177 was enhanced by the SW therapy (Figure 2*D*).

Effects of the SW therapy on protein phosphorylation. To elucidate the signaling pathways responsible for the SW-induced upregulation of VEGF and eNOS, we examined the phosphorylation state of several proteins. Immediately after the SW therapy, phosphorylation of Erk1/2 (Thr202-Tyr204) and

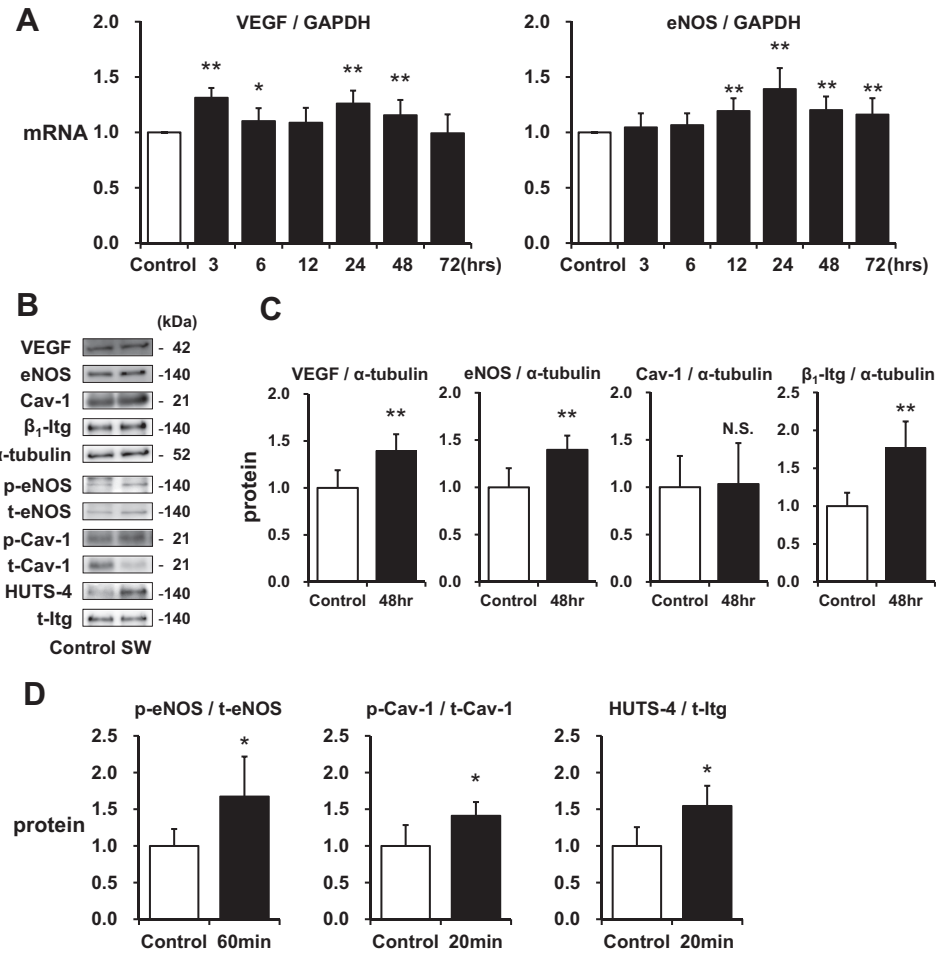

Fig. 2. Upregulation of angiogenic factors and mechanoreceptors on cell membranes by SW therapy. *A*: mRNA expression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) ($n = 12$ each). *B*: representative images of Western blot analysis. *C*: quantitative data of protein levels of VEGF, eNOS, caveolin-1 (Cav-1), and β_1-integrin (β_1-Itg) ($n = 6$ each). *D*: quantitative data of Western blot analysis for phosphorylation state of eNOS at Ser1177 and caveolin-1 at Tyr14 and the expression of HUTS-4 ($n = 6$ each). Results are expressed as means ± SD. $*P < 0.05$, $**P < 0.01$ compared with the control group (without SW therapy).

Akt (Ser473) was significantly enhanced (Fig. 3). Because FAK is known to be involved in mechanotransduction by interacting with integrins and the Src family and in regulating downstream MAP kinases (20, 44), we also examined the phosphorylation state of FAK and Fyn. Phosphorylation of FAK (Tyr397) was enhanced by the SW therapy, whereas that of Fyn (pSrc) was not affected (Fig. 3).

Effects of the SW therapy on potential mechanosensors. Because both caveolin-1 and β_1-integrin on cell membranes are known to play key roles in mechanotransduction (31, 34, 38, 43), we examined the protein levels of caveolin-1 and β_1-integrin. We also examined the phosphorylation state of caveolin-1 and the expression of HUTS-4 that represents the β_1-integrin activity (10). The SW therapy significantly enhanced the protein levels of β_1-integrin but not those of caveolin-1 (Fig. 2, *B* and *C*). On the other hand, the SW therapy significantly enhanced both the phosphorylation of caveolin-1 and the β_1-integrin activity (Fig. 2*D*).

Studies with siRNA. To further elucidate the signaling pathways involved in the SW-induced angiogenesis, HUVECs were transfected with siRNA of either caveolin-1 or β_1-integrin. Knockdown of either caveolin-1 or β_1-integrin suppressed the SW-induced upregulation of VEGF and eNOS (Fig. 4, *A* and *B*). Knockdown of either caveolin-1 or β_1-integrin also suppressed the SW-induced phosphorylation of Erk1/2 and Akt (Fig. 4, *C* and *D*). Furthermore, knockdown of either Fyn or FAK with siRNA also inhibited the SW-induced upregulation of VEGF and phosphorylation of Erk1/2. Knockdown efficiency of each molecule was ~72–95% in the mRNA levels (Fig. 4*E*) and ~85–98% in the protein levels (Fig. 4*F*). These results suggest that both caveolin-1 and β_1-integrin play pivotal roles in the SW-induced angiogenesis and that Fyn, FAK, Erk1/2, and Akt are also involved in the SW-induced angiogenic effects.

Effects of the SW therapy on cell migration. To confirm the effects of the SW therapy on cell migration and to elucidate the roles of caveolin-1 and β_1-integrin in the process, we performed scratch assay. The SW therapy significantly enhanced cell migration (Fig. 5*A*), which was blunted by knockdown of either caveolin-1 or β1-integrin with siRNA (Fig. 5, *B* and *C*).

DISCUSSION

In the present study, we demonstrated that the mechanotransduction system, including caveolin-1 and β_1-integrin and its downstream pathways, plays pivotal roles in the upregulation of angiogenic factors induced by low-energy SW. To the best of our knowledge, this is the first study that demonstrates the importance of membrane proteins caveolin-1 and β_1-integrin in the SW-induced angiogenic responses.

Potential intracellular signaling pathways for angiogenic effects of SW therapy. We have previously demonstrated that SW therapy upregulates mRNA expression of VEGF in HUVECs in vitro and ameliorates myocardial ischemia in a porcine model of chronic myocardial ischemia in vivo (25) and in patients with refractory angina pectoris (12, 19). However, the detailed molecular mechanisms of the angiogenic effects of SW therapy remain to be elucidated. Living cells recognize their surrounding environment by sensing deformation and mechanical forces and transmitting extracellular mechanical stimuli into biochemical signals (14). Mechanosensitive feedback modulates cellular functions, such as proliferation, differentiation, migration, and apoptosis, and is also crucial to maintain cytoskeletal structure and homeostasis (14). In the present study, we confirmed that SW therapy affects the mechanotransduction pathways. It has been reported that caveolae are disassembled in response to physical stress but rapidly restore their structure in the resting condition (35). Because caveolin-1 and β_1-integrin, both of which are important components of caveolae membranes, are known to play key roles in mechanotransduction (9, 16, 28, 29), we focused on caveolin-1 and β_1-integrin and their downstream pathways. In the present study, we demonstrated that SW therapy upregulated the expression of VEGF and eNOS and also enhanced phosphorylation of Erk1/2 and Akt, which are known to be involved in cell proliferation (45). Furthermore, experiments with siRNA revealed that knockdown of either caveolin-1 or β_1-integrin blunted the SW-induced upregulation of VEGF and eNOS and phosphorylation of Erk1/2 and Akt. These results suggest that both caveolin-1 and β_1-integrin are indispensable for SW-induced angiogenesis. We also elucidated that SW therapy

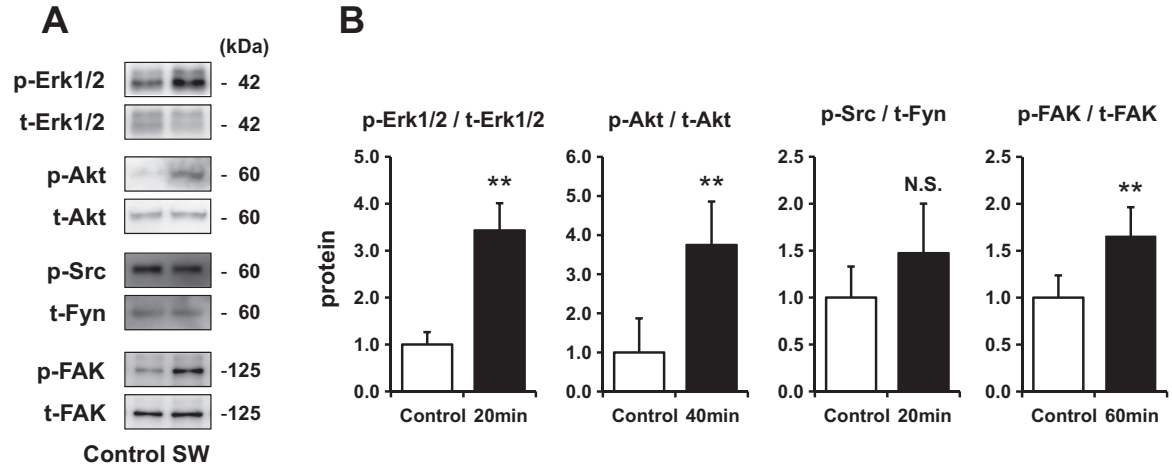

Fig. 3. Enhanced phosphorylation of intracellular proteins by SW therapy. *A*: representative images of Western blot analysis. *B*: quantitative data of Western blot analysis for phosphorylation state of Erk1/2 at Thr202-Tyr204, Akt at Ser473, Src at Tyr416, and FAK at Tyr397 ($n = 6$ each). Results are expressed as means ± SD. $^{**}P < 0.01$ compared with the control group (without SW therapy).

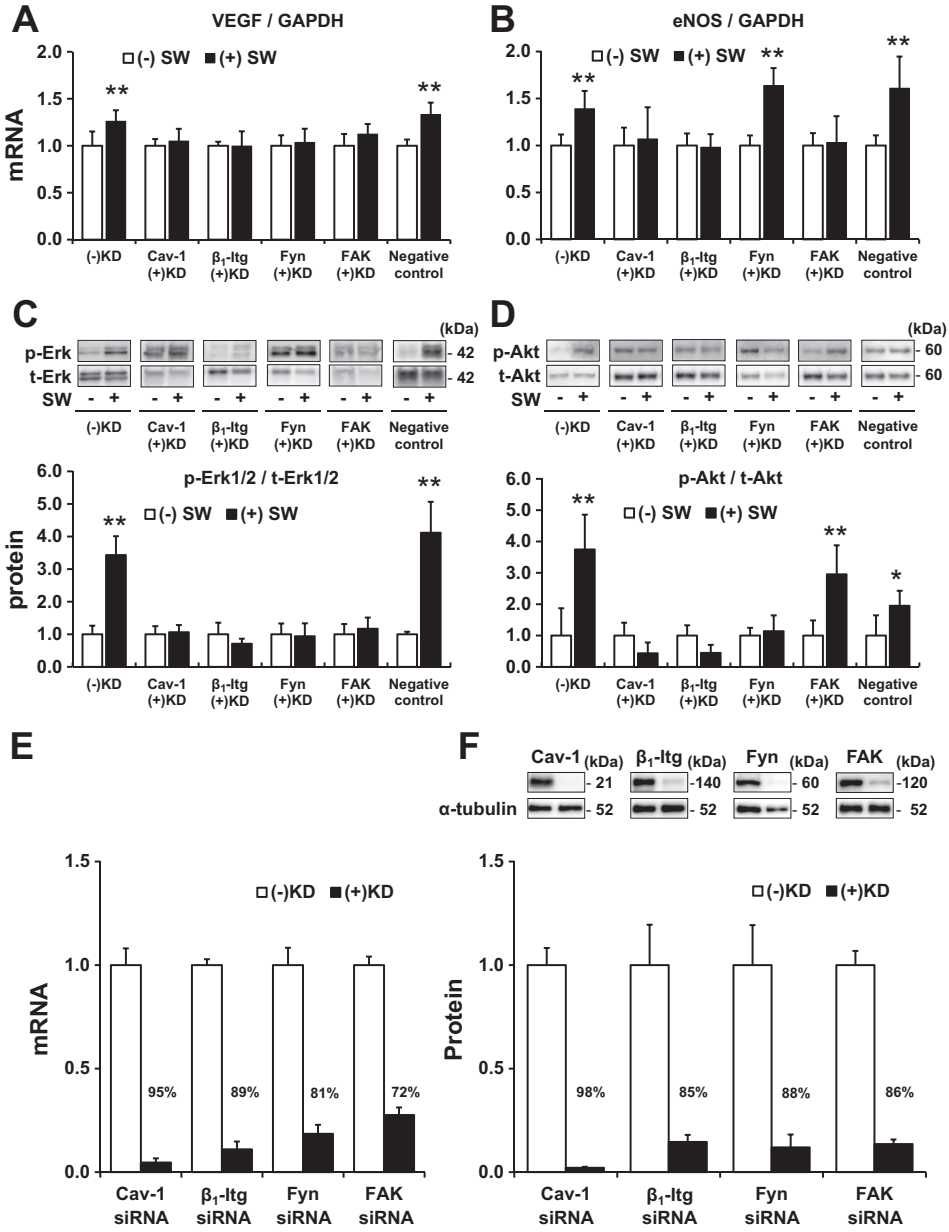

Fig. 4. Crucial role of focal adhesion pathway in the angiogenic effects of SW therapy. *A*: effects of knockdown of caveolin-1, β_1-integrin, Fyn, and FAK with siRNA on VEGF expression ($n = 6$ each). *B*: effects of knockdown (KD) of caveolin-1, β_1-integrin, Fyn, and FAK with siRNA on eNOS expression ($n = 6$ each). *C*: effects of knockdown of caveolin-1, β_1-integrin, Fyn, and FAK with siRNA on phosphorylation level of Erk1/2 at Thr202-Tyr204 ($n = 6$ each). *D*: effects of knockdown of caveolin-1, β_1-integrin, Fyn, and FAK with siRNA on phosphorylation level of Akt at Ser473 ($n = 6$ each). Results are expressed as means ± SD. $^*P < 0.05$, $^{**}P < 0.01$ compared with the corresponding control group (without SW therapy). *E*: knockdown rates of each molecule with siRNA by real-time PCR ($n = 6$ each). *F*: knockdown rates of each molecule with siRNA by Western blot analysis ($n = 6$ each). Results are expressed as means ± SD.

activates the focal adhesion pathway. FAK is a kinase that plays a critical role in integrin-mediated signal transductions and also participates in signaling pathways derived from other cell surface receptors (46). Mechanical stimuli, such as shear stress, phosphorylate FAK at Tyr397, transmit extracellular signals to downstream pathways, and activate Akt and MAP kinase (20). In the present study, we demonstrated that SW therapy enhances phosphorylation of FAK and that knockdown of FAK suppresses the SW-induced phosphorylation of Erk1/2. Knockdown of Fyn suppressed the SW-induced phosphorylation of Erk1/2 and Akt. Fyn and FAK are reported to be downstream molecules of caveolin-1 and β1-integrin, respectively (38). These results suggest that SW therapy enhances angiogenic signaling pathways by stimulating caveolae on endothelial membranes with subsequent activation of the focal adhesion pathway (Fig. 6). Although negative control siRNA had no effects on the levels of VEGF, eNOS, or p-Erk/t-Erk, the p-Akt/t-Akt levels with the negative control siRNA were rather lower than those without it. This might be due to off-target effects of siRNA (18).

Regulation of the expression of angiogenic factors. In the present study, although both mRNA and protein levels of VEGF and eNOS increased in response to SW irradiation, mRNA and protein did not change in parallel. Also, the time course of VEGF mRNA expression after SW therapy showed sustained or bimodal upregulation. These results suggest that multiple pathways are involved in upregulation of VEGF and eNOS and that the expression of VEGF and eNOS is posttranscriptionally regulated. Knockdown of Fyn reduced the SW-induced upregulation of VEGF but not that of eNOS, suggesting that the upregulation of VEGF and eNOS was mediated by different pathways.

Roles of caveolin-1 in cell migration. In the scratch assay, the SW-enhanced cell migration was blunted by knockdown of

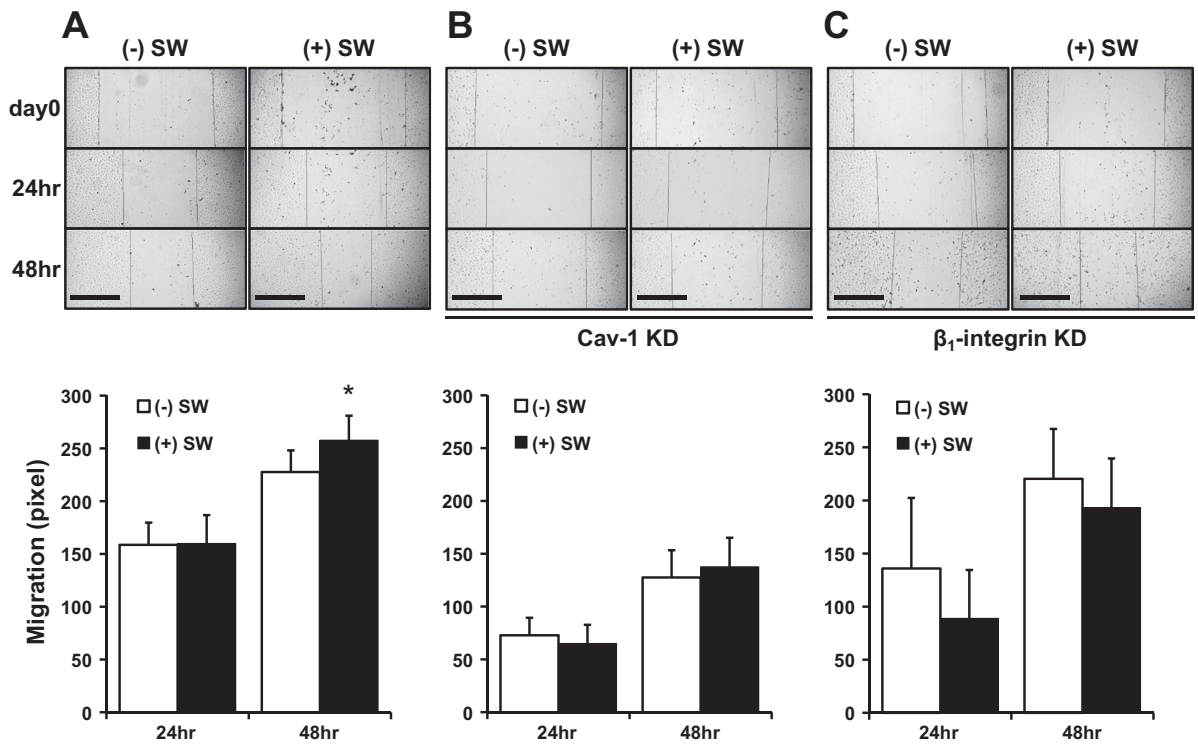

Fig. 5. Enhanced endothelial cell migration by SW. Human umbilical vein endothelial cells were treated with SW therapy, and cell migration was examined by the scratch assay. The representative images and quantitative data are shown under control condition (*A*), after transfection of caveolin-1 siRNA (*B*), and after transfection of β_1-integrin siRNA (*C*) ($n = 6$ each). Results are expressed as means ± SD. *$P < 0.05$ vs. control group without SW therapy. Scale bar = 500 μm.

either caveolin-1 or β1-integrin (Fig. 5). Furthermore, cell migration was reduced by knockdown of caveolin-1 at 24 and 48 h after SW therapy. These results suggest that caveolin-1 plays a crucial role in migration even under basal conditions without SW irradiation.

Roles of caveolae in the effects of SW. Caveolae are invaginated organelles that are found in the plasma membrane ubiquitously. Caveolin-1, a constitutive protein of caveolae, has been implicated in the regulation of cell growth, lipid trafficking, endocytosis, and cell migration (31). Phosphorylation of caveolin-1 at Tyr14 is involved in the integrin-regulated caveolae trafficking and also in signaling at focal adhesions in migrating cells (31). β_1-Integrin is also an important component of caveolae membranes (28, 16, 40). In the present study, SW therapy enhanced protein levels of β_1-integrin, but not those of caveolin-1, and enhanced the phosphorylation state of caveolin-1 and β_1-integrin activity. Activation of β_1-integrin and phosphorylation of caveolin-1 have been reported to mediate the shear stress-induced intracellular signaling (30). In addition, β_1-integrin-mediated activation of Erk1/2 and Akt is mediated by caveolin-1 (11). These findings suggest the importance of close interactions between caveolin-1 and β_1-integrin. It has been reported that SW therapy enhances cell proliferation through activating Erk1/2 (39), a consistent finding with the present result of the scratch assay. Furthermore, we demonstrated that SW-induced enhancement of cell migration was blunted by the knockdown of caveolin-1 or β_1-integrin with siRNA. These results indicated that both caveolin-1 and β_1-integrin in caveolae are required in SW-induced angiogenic responses. However, it is still not clear whether SW therapy drives mechanotransduction by directly impinging on endothelial cell membranes or by flow-induced shear stress. Further studies are needed.

Study limitations. Several limitations should be mentioned for the present study. First, in the present study, we mainly examined intracellular signaling pathways that are related to angiogenesis. We have previously reported that low-energy SW therapy suppresses post-MI left ventricular remodeling in

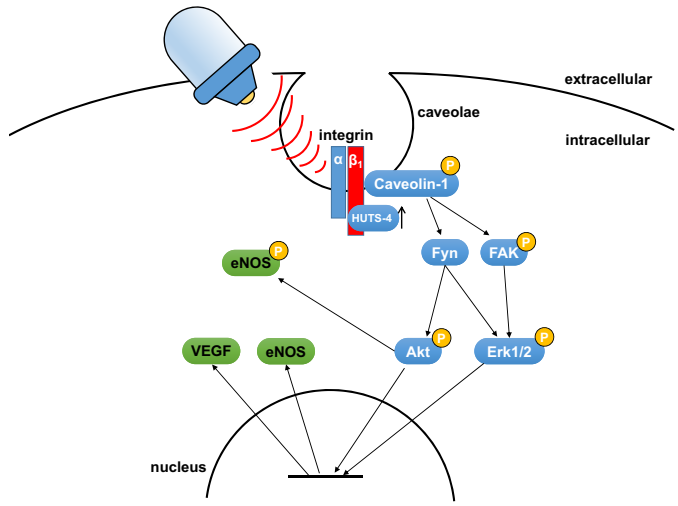

Fig. 6. Possible molecular mechanisms for the angiogenic effects of SW. Mechanoreceptors in caveolae and its downstream pathways may play pivotal roles in the SW-induced angiogenesis.

rats through its anti-inflammatory effects in addition to its angiogenic effects (1). Thus other intracellular signaling pathways may also be involved in the beneficial effects of SW. Second, in the present study, we only examined the molecular mechanisms of the angiogenic effects of SW therapy in vitro. To confirm the potential intracellular signaling pathways, in vivo studies with genetically modified animals (e.g., caveolin-1 knockout mice) may be useful. Third, it was previously reported that vascular endothelial cadherin, platelet endothelial cell adhesion molecule-1, and Toll-like receptor 3 may also be involved in the effects of SW therapy (13, 15). Thus it is possible that these molecules interact with caveolin-1 and β_1-integrin. Fourth, in the present study, we only examined the effects of low-energy SW therapy in HUVECs. However, other cell types, such as vascular smooth muscle cells, fibroblasts, cardiac myocytes, and inflammatory cells, and complex interactions among various cell types may also play important roles in the ischemic myocardium. All these points remain to be examined in future studies.

Clinical implications. Low-energy SW therapy has been reported to promote migration and differentiation of bone marrow-derived mononuclear cells (32, 42), and the effects of the combination therapies of cell transplantation and SW have also been reported (2, 5). Understanding of the detailed mechanisms of SW-induced angiogenesis may enable us to develop new therapeutic strategies (e.g., combination of pharmacotherapy and SW therapy).

Conclusions. In the present study, we were able to demonstrate that SW therapy may enhance angiogenic signaling pathways through mechanotransduction proteins (caveolin-1 and β_1-integrin) in caveolae and its downstream pathways (e.g., FAK, Erk1/2, Akt, and eNOS).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.H., K.I., T.S., Y.K., T.O., K.E., R.K., and H.S. conception and design of research; K.H. and T.S. performed experiments; K.H., T.S., and H.S. analyzed data; K.H., K.I., T.S., Y.K., T.O., K.E., R.K., and H.S. interpreted results of experiments; K.H. and T.S. prepared figures; K.H., K.I., T.S., and H.S. drafted manuscript; K.H., K.I., T.S., and H.S. edited and revised manuscript; K.H., K.I., T.S., Y.K., T.O., K.E., R.K., and H.S. approved final version of manuscript.

REFERENCES

1. **Abe Y, Ito K, Hao K, Shindo T, Ogata T, Kagaya Y, Kurosawa R, Nshimiya K, Stoh K, Miyata S, Kawakami K, Shimokawa H.** Extracorporeal low-energy shock-wave therapy exerts anti-inflammatory effects in a rat model of acute myocardial infarction. *Circ J* 78: 2915–2925, 2014.
2. **Aicher A, Heeschen C, Sasaki K, Urbich C, Zeiher AM, Dimmeler S.** Low-energy shock wave for enhancing recruitment of endothelial progenitor cells: a new modality to increase efficacy of cell therapy in chronic hind limb ischemia. *Circulation* 114: 2823–2830, 2006.
3. **Al-Abbad H, Simon JV.** The effectiveness of extracorporeal shock wave therapy on chronic Achilles tendinopathy: A systematic review. *Foot Ankle Int* 34: 33–41, 2013.
4. **Alenghat FJ, Ingber DE.** Mechanotransduction: All signals point to cytoskeleton, matrix, and integrins. *Sci Signal* 119: pe6, 2002.
5. **Assmus B, Walter DH, Seeger FH, Leistner DM, Steiner J, Ziegler I, Lutz A, Khaled W, Klotsche J, Tonn T, Dimmeler S, Zeiher AM.** Effect of shock wave-facilitated intracoronary cell therapy on LVEF in patients with chronic heart failure: The CELLWAVE randomized clinical trial. *JAMA* 309: 1622–1631, 2013.
6. **Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH.** Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling. *J Am Coll Cardiol* 49: 2379–2393, 2007.
7. **Chaussy CG, Fuchs GJ.** Current state and future developments of noninvasive treatment of human urinary stones with extracorporeal shock wave lithotripsy. *J Urol* 141: 782–789, 1989.
8. **Davies PF.** Flow-mediated endothelial mechanotransduction. *Physiol Rev* 75: 519–560, 1995.
9. **del Pozo MA, Balasubramanian N, Alderson NB, Kiosses WB, Grande-Garcia A, Anderson RG, Schwartz MA.** Phospho-caveolin-1 mediates integrin-regulated membrane domain internalization. *Nat Cell Biol* 7: 901–908, 2005.
10. **Du J, Chen X, Liang X, Zhang G, Xu J, He L, Zhan Q, Feng XQ, Chien S, Yang C.** Integrin activation and internalization of soft ECM as a mechanism of induction of stem cell differentiation by ECM elasticity. *Proc Natl Acad Sci USA* 108: 9466–9471, 2011.
11. **Echarri A, Del Pozo MA.** Caveolae internalization regulates integrin-dependent signaling pathways. *Cell Cycle* 5: 2179–2182, 2006.
12. **Fukumoto Y, Ito A, Uwatoku T, Matoba T, Kishi T, Tanaka H, Takeshita A, Sunagawa K, Shimokawa H.** Extracorporeal cardiac shock wave therapy ameliorates myocardial ischemia in patients with severe coronary artery disease. *Coron Artery Dis* 17: 63–70, 2006.
13. **Ha CH, Kim S, Chung J, An SH, Kwon K.** Extracorporeal shock wave stimulates expression of the angiogenic genes via mechanosensory complex in endothelial cells: Mimetic effect of fluid shear stress in endothelial cells. *Int J Cardiol* 168: 4168–4177, 2013.
14. **Hahn C, Schwartz MA.** Mechanotransduction in vascular physiology and atherogenesis. *Nat Rev Mol Cell Biol* 10: 53–62, 2009.
15. **Holfeld J, Tepeköylü C, Reissig C, Lobenwein D, Scheller B, Kirchmair E, Kozaryn R, Albrecht-Schgoer K, Krapf C, Zins K, Urbschat A, Zacharowski K, Grimm M, Kirchmair R, Paulus P.** Toll-like receptor 3 signalling mediates angiogenic response upon shock wave treatment of ischaemic muscle. *Cardiovasc Res* 109: 331–343, 2016.
16. **Israeli-Rosenberg S, Chen C, Li R, Deussen DN, Niesman IR, Okada Patel HHH, Roth DM, Ross RS.** Caveolin modulates integrin function and mechanical activation in the cardiomyocyte. *FASEB J* 29: 374–384, 2015.
17. **Ito K, Fukumoto Y, Shimokawa H.** Extracorporeal shock wave therapy for ischemic cardiovascular disorders. *Am J Cardiovasc Drugs* 11: 295–302, 2011.
18. **Jackson AL, Linsley PS.** Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov* 9: 57–67, 2010.
19. **Kikuchi Y, Ito K, Ito Y, Shiroto T, Tsuburaya R, Aizawa K, Hao K, Fukumoto Y, Takahashi J, Takeda M, Nakayama M, Yasuda S, Kuriyama S, Tsuji I, Shimokawa H.** Double-blind and placebo-controlled study of the effectiveness and safety of extracorporeal cardiac shock wave therapy for severe angina pectoris. *Circ J* 74: 589–591, 2010.
20. **Li S, Kim M, Hu YL, Jalali S, Schlaepfer DD, Hunter T, Chien S, Shyy JY.** Fluid shear stress activation of focal adhesion kinase: Linking to mitogen-activated protein kinases. *J Biol Chem* 272: 30455–30462, 1997.
21. **Li YS, Haga JH, Chien S.** Molecular basis of the effects of shear stress on vascular endothelial cells. *J Biomech* 38: 1949–1971, 2005.
22. **Liang CC, Park AY, Guan JL.** In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2: 329–333, 2007.
23. **Mariotto S, Cavalieri E, Amelio E, Ciampa AR, Prati AC, Marlinghaus E, Russo S, Suzuki H.** Extracorporeal shock waves: From lithotripsy to anti-inflammatory action by NO production. *Nitric Oxide* 12: 89–96, 2005.
24. **Mariotto S, Prati AC, Cavalieri E, Amelio E, Marlinghaus E, Suzuki H.** Extracorporeal shock wave therapy in inflammatory diseases: Molec-

ular mechanism that triggers anti-inflammatory action. *Curr Med Chem* 16: 2366–2372, 2009.
25. **Nishida T, Shimokawa H, Oi K, Tatewaki H, Uwatoku T, Abe K, Matsumoto Y, Kajihara N, Eto M, Matsuda T, Yasui H, Takeshita A, Sunagawa K.** Extracorporeal cardiac shock wave therapy markedly ameliorates ischemia-induced myocardial dysfunction in pigs in vivo. *Circulation* 110: 3055–3061, 2004.
26. **Ogden JA, Alvarez RG, Levitt R, Marlow M.** Shock wave therapy (Orthotripsy) in musculoskeletal disorders. *Clin Orthop Relat Res* 387: 22–40, 2001.
27. **Ogden JA, Tóth-Kischkat A, Schultheiss R.** Principles of shock wave therapy. *Clin Orthop Relat Res* 387: 8–17, 2001.
28. **Radel C, Carlile-Klusacek M, Rizzo V.** Participation of caveolae in β_1 integrin-mediated mechanotransduction. *Biochem Biophys Res Commun* 358: 626–631, 2007.
29. **Rizzo V, Radel C.** Integrin mechanotransduction stimulates caveolin-1 phosphorylation and recruitment of Csk to mediate actin reorganization. *Am J Physiol Heart Circ Physiol* 288: H936–H945, 2005.
30. **Rizzo V, Sung A, Oh P, Schnitzer JE.** Rapid mechanotransduction in situ at the luminal cell surface of vascular endothelium and its caveolae. *J Biol Chem* 273: 26323- 26329, 1998.
31. **Salanueva IJ, Cerezo A, Guadamillas MC, del Pozo MA.** Integrin regulation of caveolin function. *J Cell Mol Med* 11: 969–980, 2007.
32. **Sheu JJ, Sun CK, Chang LT, Fang HY, Chung SY, Chua S, Fu M, Lee FY, Kao YH, Ko SF, Wang CJ, Yen CH, Leu S, Yip HK.** Shock wave-pretreated bone marrow cells further improve left ventricular function after myocardial infarction in rabbits. *Ann Vasc Surg* 24: 809–821, 2010.
33. **Shimizu T, Fukumoto Y, Tanaka S, Satoh K, Ikeda S, Shimokawa H.** Crucial role of ROCK2 in vascular smooth muscle cells for hypoxia-induced pulmonary hypertension in mice. *Arterioscler Thromb Vasc Biol* 33: 2780–2791, 2013.
34. **Shyy JY, Chien S.** Role of integrins in endothelial mechanosensing of shear stress. *Circ Res* 91: 769–775, 2002.
35. **Sinha B, Köster D, Ruez R, Gonnord P, Bastiani M, Abankwa D, Stan RV, Butler-Browne G, Vedie B, Johannes L, Morone N, Parton RG, Raposo G, Sens P, Lamaze C, Nassoy P.** Cells respond to mechanical stress by rapid disassembly of caveolae. *Cell* 144: 402–413, 2011.
36. **Traub O, Berk BC.** Laminar shear stress mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Bol* 18: 677–685, 1998.
37. **Wang CJ.** Extracorporeal shockwave therapy in musculoskeletal disorders. *J Orthop Surg Res* 7: 11, 2012.
38. **Wary KK, Mariotti A, Zurzolo C, Giancotti FG.** A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell* 94: 625–634, 1998.
39. **Weihs AM, Fuchs C, Teuschl AH, Hartinger J, Slezak P, Mittermayr R, Redl H, Junger WG, Sitte HH, Rünzler D.** Shock wave treatment enhances cell proliferation and improves wound healing by ATP release-coupled extracellular signal-regulated kinase (ERK) activation. *J Biol Chem* 289: 27090–27104, 2014.
40. **Yang B, Rizzo V.** Shear stress activates eNOS at the endothelial apical surface through β1 containing integrins and caveolae. *Cell Mol Bioeng* 6: 346–354, 2013.
41. **Yin J, Kuebler WM.** Mechanotransduction by TRP channels: General concepts and specific role in the vasculature. *Cell Biochem Biophys* 56: 1–18, 2009.
42. **Yip HK, Chang LT, Sun CK, Youssef AA, Sheu JJ, Wang CJ.** Shock wave therapy applied to rat bone marrow-derived mononuclear cells enhances formation of cells stained positive for CD31 and vascular endothelial growth factor. *Circ J* 72: 150–156, 2008.
43. **Yu J, Bergaya S, Murata T, Alp IF, Bauer MP, Lin MI, Drab M, Kurzchalia TV, Stan RV, Sessa WC.** Direct evidence for the role of caveolin-1 and caveolae in mechanotransduction and remodeling of blood vessels. *J Clin Invest* 116: 1284- 1291, 2006.
44. **Zebda N, Dubrovskyi O, Birukov KG.** Focal adhesion kinase regulation of mechanotransduction and its impact on endothelial cell functions. *Microvasc Res* 83: 71–81, 2012.
45. **Zhang W, Liu HT.** MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* 12: 9–18, 2002.
46. **Zhao X, Guan JL.** Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv Drug Deliv Rev* 63: 610–615, 2011.