Mechanical strain has been implicated in phenotypic changes, including alteration of gene expression in vascular smooth muscle cells; however, the molecular basis for mechanotransduction leading to nuclear gene expression is largely unknown. We demonstrate in the present study that cyclic stretching of vascular smooth muscle cells dramatically activates Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) through an autocrine mechanism. Stretch causes time- and strength-dependent rise of the ATP concentration in media. The stretch-induced activation JNK/SAPK is attenuated by the addition of hexokinase or apyrase that scavenge ATP in media. Both the P2 receptor antagonist and the A1 subtype-selective P1 receptor antagonist partially inhibit stretch-induced activation of JNK/SAPK. The conditioned medium from stretched cells contains an activity to stimulate JNK/SAPK. The JNK-stimulating activity in the conditioned medium from stretched cells is attenuated by the addition of apyrase or P1 and P2 receptor antagonists. The addition of exogenous ATP or adenosine induces dose-dependent activation of JNK/SAPK. These results indicate that stretch activates JNK/SAPK in vascular smooth muscle cells through mechanisms involving autocrine stimulation of purinoceptors by ATP and its hydrolyzed product adenosine.

The vascular wall is constantly exposed to mechanical forces of hemodynamic origin. The tensile stress resulting from transmural pressure activates a contractile mechanism, which confers a mechanistic basis for autoregulation of blood flow (1, 2). Recent studies also reveal that mechanical stretch exerts regulatory influences on gene expression and thereby influences vascular tone and remodeling. We recently demonstrated that cyclic stretching of vascular smooth muscle dramatically increases the expression of the gene for the vasorelaxant parathyroid hormone-related peptide (3–5). Others have reported that stretch induces an increase in the gene expression of platelet-derived growth factor-A chain in vascular smooth muscle cells (6). However, little is known about the mechanisms by which mechanical force is converted into intracellular signals coupled to nuclear gene expression in vascular smooth muscle cells (7, 8).

Epilogue: The role of Stretch in Vascular Smooth Muscle Cells

Mechanical stretch is a major determinant of vascular responses. The vascular wall is constantly exposed to mechanical forces of hemodynamic origin. The tensile stress resulting from transmural pressure activates a contractile mechanism, which confers a mechanistic basis for autoregulation of blood flow (1, 2). Recent studies also reveal that mechanical stretch exerts regulatory influences on gene expression and thereby influences vascular tone and remodeling. We recently demonstrated that cyclic stretching of vascular smooth muscle dramatically increases the expression of the gene for the vasorelaxant parathyroid hormone-related peptide (3–5). Others have reported that stretch induces an increase in the gene expression of platelet-derived growth factor-A chain in vascular smooth muscle cells (6). However, little is known about the mechanisms by which mechanical force is converted into intracellular signals coupled to nuclear gene expression in vascular smooth muscle cells (7, 8).

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confluent cells were serum-deprived by incubation in serum-free Dulbecco's modified Eagle's medium containing 0.2% bovine serum albumin (Fraction V, Sigma) for 24 h. RASM cells were subjected to repetitive cycles of mechanical stretch and relaxation with a Flexercell strain unit (FX-2000, Flexcell Corp., McKeesport, PA) as described previously (3). The negative pressure to the flexible bottoms was set to provide a maximum elongation of 3, 7, 15, or 20% of the silicone rubber and cells. All experiments were carried out using alternation cycles of 0.5-s stretch and 0.5-s relaxation at a rate of 60 cycles/min at 37 °C in 5% CO2 in a humidified air.

**Northern Blot Analysis**—Total RNA, isolated from RASM cells by the acid-phenol/chloroform/phenol/chloroform method, was separated by formaldehyde, 1% agarose gel electrophoresis, transferred onto a nylon membrane (Hybond N, Amersham Life Science) and hybridized by cDNA probes with [α-32P]dCTP (NEN Life Science Products) by the random priming method as described (3, 28). The radioactivity of corresponding bands was quantitated by Fuji BAS 2000 bioimage analyzer (Fuji Film Co. Ltd., Tokyo, Japan). The rat c-jun, human junB, and mouse junD cDNAs were obtained from RIKEN Gene Bank (Tsukuba, Japan). The human c-fos and c-myc cDNAs were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

**JNK and ERK Assay**—The solid phase JNK assay was performed as described in Ref. 13. JNK was recovered by incubation of cell lysate with 10 μg of glutathione S-transferase (GST)-c-Jun-(5–89) fusion protein bound to glutathione-Sepharose beads. The pelleted beads were incubated with [γ-32P]ATP at 30 °C for 20 min, and the reaction was terminated by adding 10 μl of 4 × Laemmli's SDS sample buffer and boiling for 5 min. The samples were analyzed as described (30). The bacterial expression plasmid of N-terminal amino acids 5–89 of c-Jun fused to GST, pGEX-2T-c-Jun-(5–89) (29), was provided by Dr. A. S. Kraft (University of Alabama School of Medicine, Birmingham, AL). The immune complex JNK activity was determined as described (14, 30). JNK was immunoprecipitated, using rabbit polyclonal anti-JNK1 C-terminal antibody (Santa Cruz, C-17). The immunoprecipitate was incubated with 30 μl of the JNK assay buffer containing 3 μg of GST-c-Jun-(5–89) at 30 °C for 30 min. The reaction was terminated and analyzed as described for the solid phase JNK assay.

The immune complex ERK assay was carried out as described (31). The MAPKs ERK1 and ERK2 were immunoprecipitated using a mouse monoclonal anti-MAPK antibody that recognizes both ERK1 and ERK2 (Zymed Laboratories Inc., 03-6600).

**Measurement of ATP Concentrations**—The ATP concentration in culture media was measured with the Enlighten ATP assay system using luciferase and luciferin (Promega). Briefly, culture media were collected, mixed with trichloroacetic acid (a final concentration of 0.3%), and kept at 4 °C for 30 min. The media were then neutralized by adding eight volumes of 250 mM Tris acetate (pH 7.75), and mixed with the ATP assay reagents. Luminescence was measured with a Lumat LB9501 luminometer (Berthold, Bad Wildbad, Germany). The standard curve was generated by using known concentrations of ATP.

**RESULTS**

We first determined whether cyclic stretching (60 cycle/min) of vascular smooth muscle cells causes any change in the expression of immediate-early genes. Northern blot analysis revealed that all of the five immediate-early genes examined (c-jun, jun B, jun D, c-fos, and c-myc) showed increased expression in response to mechanical stretch (a maximal elongation of 15%) (Fig. 1A). Among them, the induction of c-jun mRNA was most prominent with a maximal 6-fold increase by 30 min. The induction of c-jun mRNA increased with increasing strength of stretch and became maximal at 15% stretch (Fig. 1B). In contrast, stretch hardly changed the glyceraldehyde-3-phosphate dehydrogenase mRNA level.

We next examined whether stretch activated JNK/SAPK in vascular smooth muscle cells. Stretch caused a rapid and sustained activation of JNK. The JNK activity, measured with a solid phase kinase assay (13), was increased significantly over the basal value by 5 min, reached a maximal value of 6-fold at 30 min, and then declined to a lower level of 3-fold at 60 min (Fig. 2A). Measurement with an immune complex kinase assay using anti-JNK C-terminal antibody gave similar results (data not shown). The JNK activation was dependent on the magnitude of stretch applied to cells (Fig. 2B). The relationship between the extent of the stretch and the changes in JNK activity was quite similar to that observed for c-jun mRNA expression (Fig. 1B). The vasoconstrictor AII, at a maximal concentration in terms of its Ca2+ mobilizing activity, also activated JNK, but provided a much weaker stimulus than stretch (Fig. 2A). We also examined the effect of stretch on the activity of ERK, another MAPK family member. As shown in Fig. 3, the application of stretch stimulated the ERK activity with a maximal stimulation of approximately 2-fold over the basal activity at 30 min. AII induced activation of ERK to a comparable extent as stretch (Fig. 3).

We examined how vascular smooth muscle cells convert mechanical stimuli into signals for activating JNK. Knowing that stretch causes activation of stretch-activated (SA) cation chan-
nels in a variety of cell types (32, 33), we sought to determine if SA cation channel activation is involved in stretch-induced JNK/SAPK activation, by examining the effect of Gd3⁺, a specific inhibitor of SA cation channel function (34, 35). As shown in Fig. 4, Gd3⁺ failed to suppress stretch-induced activation of JNK/SAPK (Fig. 4). Removal of extracellular Ca²⁺ was also without any effect on stretch-induced activation of JNK/SAPK (Fig. 4). Thus, SA cation channels or any other Ca²⁺ channels are not likely to be involved in stretch-induced JNK activation.

The possible involvement of autocrine factors in stretch-induced JNK activation was next examined. Conditioned media from cells subjected to stretch for varied time periods were collected and applied to non-stretched cells. The conditioned media from stretched cells did cause the activation of JNK/SAPK (Fig. 5). In this assay, the JNK/SAPK activating “factor” in conditioned media was detectable by 2 min, and accumu-
lacked with time to reach a plateau value by 10 min.

Previous studies have shown that imposing flow on vascular endothelial cells (36) and mechanical deformation of the cell membrane in mast cells (37) and in mammary epithelial cells (38) induces release of ATP. It is also known that vascular smooth muscle cells express cell surface receptors for ATP and its hydrolyzed product, adenosine (39). Therefore, we examined the possibility that ATP might be the autocrine factor involved in stretch-induced JNK activation. The application of stretch induced a rapid and sustained rise in the ATP concentration in the extracellular medium (Fig. 6A). The ATP concentration in the medium reached a maximal level of 50-fold increase over the basal value by 5 min, and remained elevated over the basal value for at least 30 min. When the magnitude of stretch was increased, the ATP concentration in the medium also increased (Fig. 6B), like the activity of JNK and c-jun mRNA expression (Figs. 1B and 2B). To determine whether or not released ATP is indeed involved in stretch-induced activation of JNK, we examined the effects of agents that reduce the ATP concentration in media. Apyrase is a well known ATP- and ADP-hydrolyzing enzyme. Hexokinase catalyzes phosphorylation of glucose to generate glucose 6-phosphate at the expense of ATP (51). The enzyme. Hexokinase catalyzes phosphorylation of glucose to

conditioned medium harvested from stretched cells, inhibited its JNK-stimulating activity (Fig. 8). These observations support the notion that ATP acts as a mediator of stretch-induced JNK activation.

ATP and ADP act as agonists for P2 receptors, while the hydrolyzed products of ATP, adenosine and AMP, act as agonists for P1 receptors (40, 41). We examined which of P1 and P2 receptors mediated the JNK activation in response to stretch. The P2 receptor antagonist reactive blue-2 (RB-2) (42, 43) partially inhibited stretch-induced JNK activation in a dose-dependent manner (Fig. 9A). Suramin, another P2 receptor antagonist with a different subtype selectivity (44, 45), was without any inhibitory effect. The A1 subtype-selective P1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (46, 47), but not the A2 subtype-selective P2 receptor antagonist 8-(3-chlorostyryl)caffeine (47), also inhibited JNK activation (Fig. 9A). These results suggest that both RB-2-sensitive P2 and DPCPX-sensitive P1 receptors are involved in stretch-induced JNK activation. By contrast, these antagonists were without any effect on AII-induced JNK activation (data not shown). Moreover, RB-2 and DPCPX both inhibited the JNK-stimulating effect of the conditioned medium harvested from stretched cells (Fig. 9B). We examined the ability of exogenous purinoceptor agonists to activate JNK in vascular smooth muscle cells. ATP, ADP, adenosine, and UTP induced a time-de-
dependent activation of JNK (Fig. 9C). ATP, ADP, and adenosine each activated JNK with similar dose-response relationships (Fig. 9D). UTP was the most potent in activating JNK. Thus, ATP, adenosine, and presumably ADP appear to mediate stretch-induced JNK activation.

**DISCUSSION**

The present study demonstrates for the first time that mechanical stress causes potent activation of JNK/SAPK in vascular smooth muscle cells (Fig. 2). Stretch also induces modest activation of MAPK/ERK (Fig. 3). Although stretch and the receptor agonist AII are similar in that either stimulus can activate both JNK/SAPK and MAPK/ERK in vascular smooth muscle cells, stretch activates JNK/SAPK much more strongly than AII (Figs. 2 and 3). Stretch-induced JNK activation is transient in the continued presence of stretch stimuli. This transient nature might be explained by possible up-regulation by stretch of JNK-inactivating phosphatase such as MKP-1, possible inactivation of JNK kinase by phosphorylation, or other mechanisms. The JNK/SAPK pathway is a recently identified downstream mediator linking extracellular stimuli to nuclear events (27, 48). c-Jun, ATF-2, and Elk1 are well-characterized substrates for JNK/SAPK, and JNK/SAPK-mediated phosphorylation activates these transcription factors (13, 14, 16, 19, 20). These transcription factors appear to be essential for transcriptionally activating a variety of genes in response to external stimuli. Thus, JNK/SAPK likely acts as a mediator for stretch-induced regulation of gene expression.

The activation of JNK/SAPK is induced by a variety of bioactive substances including growth factors, cytokines, and G protein-coupled receptor agonists. Accordingly, we pursued the possibility that a factor released from vascular smooth muscle cells upon stretching might act in an autocrine/paracrine manner to activate JNK/SAPK. The present study demonstrates that the conditioned medium harvested from stretched cells contains a factor that activates JNK/SAPK (Fig. 5) and that ATP is released in large quantities from cells upon stretching (Fig. 6). The results shown in the present study imply that ATP is an autocrine/paracrine factor to activate JNK/SAPK. First, when released ATP was scavenged by the addition of the ATP-trapping agents, apyrase and hexokinase, stretch-induced JNK/SAPK activation was considerably suppressed (Fig. 7, A and B). Second, the P2 receptor antagonist RB-2 inhibited stretch-induced JNK/SAPK activation (Fig. 9A). The partial inhibition of stretch-induced JNK activation by RB-2 might be explained by its weak antagonistic potency and/or the partial contribution of P2 receptor (see below). Third, the JNK-stimulating effect of the conditioned medium from stretched cells was suppressed by apyrase treatment or the addition of RB-2 (Figs. 8 and 9B). Fourth, the addition of exogenous ATP caused activation of JNK/SAPK in vascular smooth muscle cells (Fig. 9C). However, the magnitude of JNK/SAPK activation by exposure to the conditioned medium from stretched cells was considerably smaller that induced by stretch (compare Figs. 2 and 5). These differences might imply that stretch activates JNK/SAPK through both autocrine-dependent and autocrine-independent mechanisms. This notion is supported by the findings showing that nearly complete inhibition of stretch-induced increase in extracellular ATP by apyrase or hexokinase could...
not totally abolish stretch-induced JNK activation (Fig. 7, A and B). However, the incomplete inhibition of stretch-induced JNK activation by hexokinase and apyrase could also be because ADP, AMP, and adenosine in the presence of hexokinase, and AMP and adenosine in the presence of apyrase still could activate purinoceptors in vascular smooth muscle cells (see below) (Fig. 9, C and D).

Exogenous ATP at the concentration slightly higher than the maximal medium concentration of ATP in stretched cells gives approximately 2–3-fold increase in the JNK activity (Fig. 9D), while stretch usually causes more than 5-fold increase in the JNK activity. The difference may suggest the contribution of the hydrolyzed products of ATP, i.e., ADP, AMP, and adenosine, besides ATP, as mentioned above. Second, it is known that extracellular nucleotides are subjected to active hydrolysis by ectonucleotidases existing on the plasma membrane (49, 50). Therefore, the concentration of ATP in the medium might have been rapidly reduced by avid hydrolysis, although much attention was paid for allowing for quick collection and transfer of conditioned media. It is also possible that the ATP concentration in the bulk media may be lower than that in the vicinity of ATP transporters on the plasma membrane where purinoceptors also exist. In airway epithelial cells, it was suggested that an ATP transporter and a P2 receptor were present in close proximity on the plasma membrane (51). It has been shown previously that medium concentrations of autocrine peptides endothelin-1 and angiotensin II in stretched cardiomyocyte cultures are lower than those of exogenous peptides required for activation of MAPK (11, 12).

The A1 subtype-selective P1 receptor antagonist DPCPX inhibited stretch-induced activation of JNK/SAPK (Fig. 9A). Further, the addition of exogenous adenosine caused activation of JNK/SAPK. Cells were stimulated with various doses of ATP, ADP, adenosine, or UTP for 20 min (for ATP and ADP) or 30 min (for adenosine and UTP). The JNK/SAPK activity was measured with the solid phase kinase assay method. The value represents fold stimulation over the JNK/SAPK activity in non-stimulated cells. The asterisk denotes a statistical significance (p < 0.05) as compared with stretch (A) or stretch conditioned medium (B).

**Fig. 9. Effects of purinoceptor antagonists on stretch-induced activation of JNK/SAPK and effects of exogenous nucleotides and nucleoside on JNK/SAPK activity.** A, inhibition of stretch-induced JNK/SAPK activation by purinoceptor antagonists. Cells were subjected to stretch (a maximal elongation of 15%) for 30 min in the presence of the P2 receptor antagonists suramin (3 × 10⁻¹⁴ to 3 × 10⁻⁴ M), the A1 receptor antagonist DPCPX (10⁻⁷ to 10⁻⁶ M), or the A2 receptor antagonist 8-(3-chlorostyryl)caffeine (10⁻⁶ M). The autoradiogram and quantitation of the results are shown. The JNK/SAPK activities were measured with the solid phase kinase assay and are expressed relative to the activity in non-stretched cells, which was given an arbitrary value of 1. The asterisk denotes statistically significant difference (p < 0.05) as compared with stretched cells. B, inhibition by RB-2 and DPCPX of the JNK-stimulating activity in the conditioned medium (CM) harvested from stretched cells. The conditioned medium was rapidly harvested from cells subjected to stretch for 10 min or non-stretched cells and were immediately applied with or without a receptor antagonist to non-stretched cells. The incubation was carried our for 30 min. C, time-dependent activation of JNK/SAPK. Cells were stimulated with 10⁻⁶ M amounts of ATP, ADP, adenosine, or UTP for the indicated time periods. D, activation of the JNK/SAPK activity by exogenous nucleotides. Cells were stimulated with various doses of ATP, ADP, adenosine, or UTP for 20 min (for ATP and ADP) or 30 min (for adenosine and UTP). The JNK/SAPK activity was measured with the solid phase kinase assay method. The value represents fold stimulation over the JNK/SAPK activity in non-stimulated cells. The asterisk denotes a statistical significance (p < 0.05) as compared with stretch (A) or stretch conditioned medium (B).
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The results of the antagonist sensitivity (Fig. 9A) and the relative nucleotide potency (Fig. 9C) favor the view that P2Y6 largely mediates stretch-induced JNK activation.

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(38, 54) demonstrated that mechanical stimuli induced release of UTP as well as ATP from non-smooth muscle cells. It is an interesting possibility that uracil nucleotides also contribute to stretch-induced JNK activation. Cloned rat P2Y2 receptor, but not rat P2Y6, is sensitive to the P2 receptor antagonist RB-2, whereas both receptor subtypes are substantially resistant to suramin (5).2 The results of the antagonist sensitivity (Fig. 9A) and the relative nucleotide potency (Fig. 9C) favor the view that P2Y6 largely mediates stretch-induced JNK activation.

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