CELL BIOMECHANICS

ROCK in a Stiff Place

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Understanding the molecular and cellular mechanisms involved in the progression of atherosclerosis has revealed a vital role for the Rho kinase (ROCK) pathway associated with stiffening of the underlying extracellular matrix (Huynh *et al.*, this issue).

BLOOD VESSELS STIFFEN UP

Atherosclerosis, which leads to heart attack and stroke, is still the most prevalent cause of death in the Western world. Despite the effectiveness of cholesterol-lowering statin drugs in reducing mortality among patients with cardiovascular disease, additional approaches to slow disease progression remain an important therapeutic need. Because atherosclerosis is increasingly thought of as an inflammation-based pathology, inflammation-related cellular and molecular processes are becoming the focus for the ongoing search for new drug targets (*1*). In this issue of *Science Translational Medicine*, Huynh, Reinhart-King, and colleagues shed light on how the Rho kinase (ROCK) cell signaling pathway may serve as a promising anti-atherosclerosis target, owing to its role in several inflammatory processes, as well as its key role in arterial stiffness and aging (*2*).

Atherosclerosis develops through a complex series of events in which order may be highly convoluted. In certain regions of the vasculature, increased permeability of the blood vessel endothelium permits enhanced accumulation of cholesterol-laden low-density lipoprotein (LDL), along with transmigration of neutrophil leukocytes from the bloodstream into the vessel wall intimal layer (Fig. 1). Recruitment of monocytes and their subsequent differentiation into macrophages builds a hallmark atherosclerotic plaque that compromises blood flow and eventually leads to clot formation. Although several factors can aggravate the pathology—and are accordingly considered to be potential avenues for therapeutic treatment—the increased endothelial permeability is an especially attractive target because of its synergistic role in facilitating intimal accumulation of both LDL and leukocytes.

Thus, a critical question is the origin of increased permeability. The study by Huynh *et al.* derives its central hypothesis from knowledge that blood vessel wall–stiffening has been observed to correlate with age, as well as with atherosclerosis. Previous work has shown that endothelial cells exhibit less cell-cell contact on extracellular matrix substrata of higher stiffness (*3*), which suggests that endothelial cell layers may correspondingly possess looser cell-cell junctions, and thus allow less hindered passage of molecules and cells in stiffer vessels. The Reinhart-King group has now tested fundamental aspects of this theory in multiple ways: in vitro with endothelial cell culture, ex vivo with mouse aortas, and in vivo in mice (*2*).

ENDOTHELIAL CELLS ROCK OUT

Huynh *et al.* first seeded endothelial cells in vitro on synthetic hydrogel matrices possessing a range of physiologically relevant mechanical stiffnesses and then measured their molecular permeabilities, cell-cell junction separation distances, and leukocyte transmigration. Fluorescent imaging of vascular endothelial–cadherin (marking cell membrane locations in adhesive interactions)

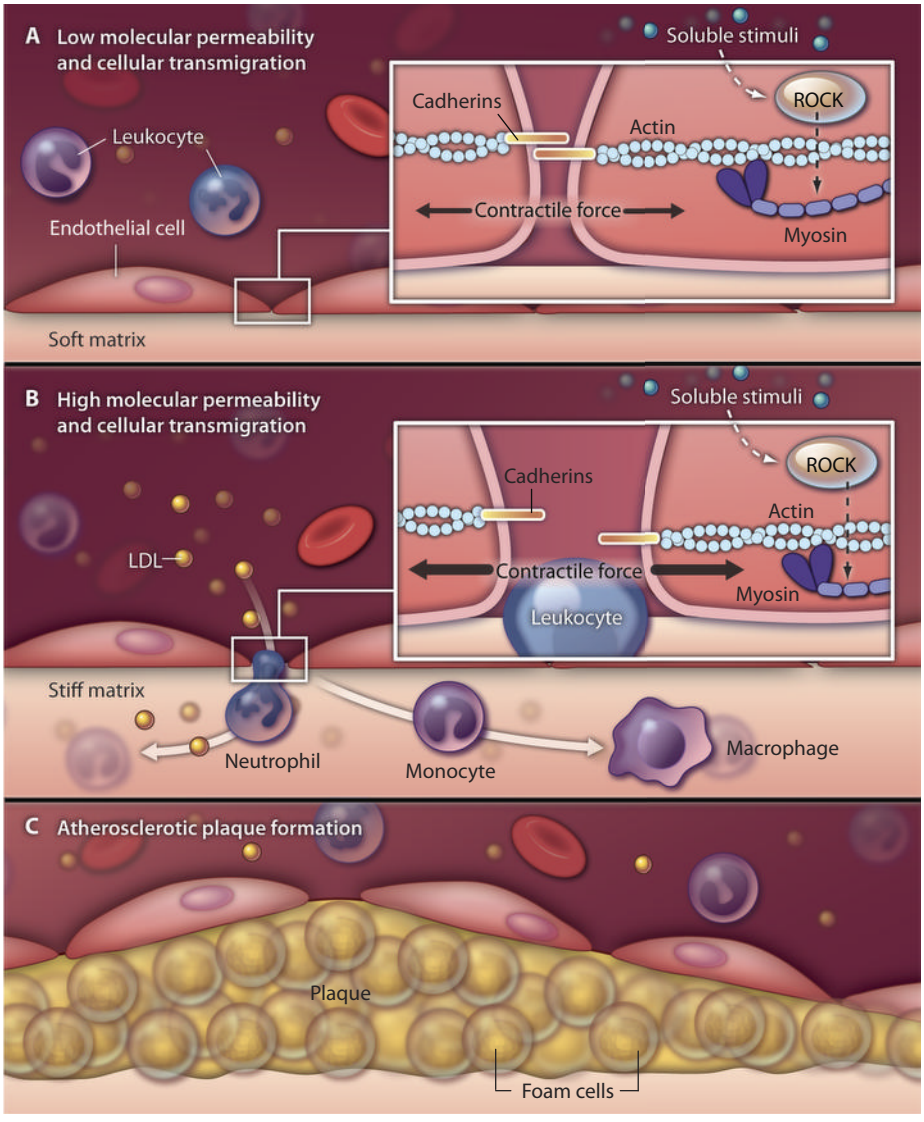

Fig. 1. Contractile forces at play. ROCK-mediated enhancement of endothelial cell contractile forces permits easier passage of molecules, such as LDL, and white blood cells (neutrophils, monocytes) across the blood vessel wall, thus facilitating plaque formation underlying atherosclerosis.

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showed an increase in intercellular separation distance from about 3.5 to nearly 5 μm when endothelial layers were cultured on 2.5- versus 10-kPa matrices, respectively. Fluorescently labeled dextran (an albumin mimic) was determined to diffuse across the endothelial cell barrier more than twice as rapidly on the stiffer substrata (10 kPa). It must be noted that because albumin is much smaller than the LDL molecules of principal relevance here, the corresponding quantitative enhancement for LDL (the more physiological entity) is not known. When the seeded endothelial cells were treated with tumor necrosis factor α, an inflammatory cytokine that stimulates an up-regulation of adhesion ligands, neutrophil transmigration—a hallmark of atherogenesis—was increased twofold for the stiffer constructs.

Moving in vivo into mice, the authors investigated the relationship between aging and stiffening by measuring vascular wall stiffness, endothelial intercellular separation distance, and endothelial permeability for blood vessels taken from young (10 to 11 weeks) and old (21 to 25 months) mice. Stiffness was found to be roughly threefold greater for the aged mice, concordant with a junction separation increase of about 20% and enhanced albumin permeability (followed via Evans blue dye) of about 50%. Leukocyte transmigration was not assessed in vivo.

From here, two major avenues of follow-up experimentation could be envisioned in order to identify approaches for preventing or slowing this dangerous permeability enhancement: either understanding the cause of the matrix stiffening underlying permeability enhancement, or, alternatively, the cause of the widening of endothelial cell junctions induced by vessel stiffening. Reasoning that vascular wall stiffening seems to be a natural feature of aging—which is itself a risk factor for atherosclerosis (*4*)—Huynh *et al.* focused on the regulatory mechanisms governing the cellular response to stiffening.

A key role for active endothelial cell contractile force, induced by growth factor stimuli in concert with cell-to-matrix adhesion, has been established (*5*), with the ROCK signaling pathway known to provide particular vitality in activating force generation (*6*). For this reason, ROCK is considered to be a promising therapeutic target for a broad range of cardiovascular pathologies (*7*). ROCK has been shown to modulate endothelial barrier operation (*8*) (Fig. 1), although how its signaling hyperactivity is elicited is yet unclear. The study by Huynh *et al.* offers enticing evidence that stiffening of the intimal matrix underlying the vascular endothelial cell layer may be largely responsible. Direct measurement of endothelial cell traction force in the in vitro assays here showed that it is indeed greater on the stiffer matrices. Across the 2.5- to 10-kPa matrix stiffness range, traction force nearly tripled, while ROCK activity increased by about 50%. The authors found that Y-27632, a small molecular pharmacological inhibitor of ROCK, prevented or reversed all of these observations both in vitro and in vivo. A chief contribution by ROCK was further validated by finding that the in vitro effects of matrix stiffening were abrogated by siRNA-mediated knockdown of *ROCK1*.

INHIBITING ATHEROSCLEROSIS

Taken together, the aggregate findings link together a complex set of fundamental molecular and cellular processes known individually to be associated with aging and atherosclerosis: matrix stiffening, ROCK activation, endothelial cell contractile force generation, cell-cell junction loosening, enhanced molecular permeability, and leukocyte transmigration (Fig. 1). These findings therefore make an attractive argument for focusing on endothelial cell contractility responses to age-related—and perhaps inflammation-related—matrix stiffness changes toward improved therapeutic approaches to slowing the progression of atherosclerosis.

Interestingly, it is now appreciated that at least a substantial contribution to the effectiveness of statins derives from pleiotropic, LDL-independent effects likely residing in modulation of inflammation-related processes, including, prominently, amelioration of ROCK activity (*9*). This suppressive effect arises naturally as a by-product of the statin inhibition of 3-hydroxyl-3-methylglutaryl CoA reductase, by diminishing the capability of the small GTPase Rho to be activated by membrane association upstream of ROCK; hence, ROCK inhibition may already be presumed to be an aspect of current treatments for atherosclerosis. The ROCK inhibitor fasudil is approved for clinical use in certain cardiovascular indications and is being considered for additional indications in current trials. Moreover, a variety of pharmaceutical industry efforts are aimed in the direction of interfering with the Rho-signaling pathway (*7*).

An important issue, however, will be whether ROCK itself is the best target rather than alternative downstream effectors. This is especially germane because of the many other cell types that will be affected by ROCK inhibition. In some cases, suppression of ROCK-regulated functions in other cell types, including leukocytes and smooth muscle cells, could be beneficial. But a more detailed study of the ROCK-regulated effectors may present opportunities for greater therapeutic specificity. One central downstream effector that is directly involved in contractile force generation is myosin light chain phosphorylation, via a kinetic trade-off between myosin light-chain kinase and myosin light-chain phosphatase, both of which are regulated by ROCK (*6*, *7*). Nevertheless, the full spectrum of ROCK effectors has yet to be enumerated, and doing so using a systems biology approach—especially one that is analogous to a recent study that identifed several previously unknown effectors for extracellular-regulated kinase (*10*)—ought to bring both scientific and clinical reward for improved treatment of age-related endothelial dysfunction in atherosclerosis.

REFERENCES AND NOTES

1. I. F. Charo, R. Taub, Anti-inflammatory therapeutics for the treatment of atherosclerosis. *Nat. Rev. Drug Discov.* **10**, 365–376 (2011).
2. J. Huynh, N. Nishimura, K. Rana, J. M. Peloquin, J. P. Califano, C. R. Montague, C. B. Schaffer, M. R. King, C. A. Reinhart-King, Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration. *Sci. Transl. Med.* **3**, 112ra122 (2011).
3. C. A. Reinhart-King, M. Dembo, D. A. Hammer, Cell-cell mechanical communication through compliant substrates. *Biophys. J.* **95**, 6044–6051 (2008).
4. F. U. Mattace-Raso, T. J. van der Cammen, A. Hofman, N. M. van Popele, M. L. Bos, M. A. Schalekamp, R. Asmar, R. S. Reneman, A. P. Hoeks, M. M. Breteler, J. C. Witteman, Arterial stiffness and risk of coronary heart disease and stroke: The Rotterdam Study. *Circulation* **113**, 657–663 (2006).
5. M. T. Yang, D. H. Reich, C. S. Chen, Measurement and analysis of traction force dynamics in response to vasoactive agonists. *Integr. Biol. (Camb)* **3**, 663–674 (2011).
6. M. Schwartz, Rho signalling at a glance. *J. Cell Sci.* **117**, 5457–5458 (2004).
7. Q. Zhou, C. Gensch, J. K. Liao, Rho-associated coiled-coil-forming kinases (ROCKs): Potential targets for the treatment of atherosclerosis and vascular disease. *Trends Pharmacol. Sci.* **32**, 167–173 (2011).
8. G. Loirand, P. Guérin, P. Pacaud, Rho kinases in cardiovascular physiology and pathophysiology. *Circ. Res.* **98**, 322–334 (2006).
9. P. Y. Liu, Y. W. Liu, L. J. Lin, J. H. Chen, J. K. Liao, Evidence for statin pleiotropy in humans: Differential effects of statins and ezetimibe on rho-associated coiled-coil containing protein kinase activity, endothelial function, and inflammation. *Circulation* **119**, 131–138 (2009).
10. S. M. Carlson, C. R. Chouinard, A. Labadorf, C. J. Lam, K. Schmelzle, E. Fraenkel, F. M. White, Large-scale discovery of ERK2 substrates identifies ERK-mediated transcriptional regulation by ETV3. *Sci. Signal.* **4**, rs11 (2011).

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