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Applications for ROCK kinase inhibition

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Abstract

ROCK kinases, which play central roles in the organization of the actin cytoskeleton, are tantalizing targets for the treatment of human diseases. Deletion of ROCK I in mice revealed a role in the pathophysiological responses to high blood pressure, and validated ROCK inhibition for the treatment of specific types of cardiovascular disease. To date, the only ROCK inhibitor employed clinically in humans is fasudil, which has been used safely in Japan since 1995 for the treatment of cerebral vasospasm. Clinical trials, mostly focussing on the cardiovascular system, have uncovered beneficial effects of fasudil for additional indications. Intriguing recent findings also suggest significant potential for ROCK inhibitors in the production and implantation of stem cells for disease therapies.

Introduction

ROCK I (alternatively called ROK β) and ROCK II (also known as Rho kinase or ROK α) were originally isolated as RhoA-GTP interacting proteins [1]. The two kinases have 64% overall identity in humans, with 89% identity in the catalytic kinase domain (Figure 1). Both kinases contain a coiled-coil region (55% identity) and a pleckstrin homology (PH) domain split by a C1 conserved region (80% identity). Although only a single Rho-binding domain (RBD) within the coiled-coil region was originally identified, subsequent analysis revealed multiple contact points [2]. RhoA, RhoB and RhoC associate with and activate, but other GTP-binding proteins inhibit ROCK, as has been found for RhoE [3], Rad and Gem [4], which bind at sites distinct from the canonical RBD (Figure 2). Association with the PDK1 kinase promotes ROCK I activity not through phosphorylation but by blocking RhoE association [5•]. During apoptosis, proteolytic cleavage by caspases (ROCK I; [6,7]) or granzyme B (ROCK II; [8•]) removes a carboxyl-terminal portion that normally represses activity, resulting in the generation of constitutively-active kinases (Figure 2). Interaction with PIP₃ provides an additional regulatory mechanism by localizing ROCK II to the plasma membrane where it can undertake spatially restricted activities [9•]. Phosphorylation at multiple specific sites by polo-like kinase 1 was found to promote ROCK II activation by RhoA [10•], additional Serine/Threonine and Tyrosine kinases may also regulate ROCK activity given that more phosphorylations have been identified (<http://www.phosphosite.org>).

ROCK promotes actin-myosin mediated contractile force generation through the phosphorylation of numerous downstream target proteins (Figure 3). ROCK phosphorylates LIM kinases-1 and -2 (LIMK1 and LIMK2) at conserved Threonines in their activation loops, increasing LIMK activity and the subsequent phosphorylation of cofilin proteins, which blocks their F-actin-severing activity [11]. ROCK also directly phosphorylates the myosin regulatory

light chain (MLC) and the myosin binding subunit (MYPT1) of the MLC phosphatase to inhibit catalytic activity [1]. Many of these effects are also amplified by ROCK-mediated phosphorylation and activation of the Zipper-interacting protein kinase (ZIPK), which phosphorylates many of the same substrates as ROCK [12•]. Taken together, ROCK activation leads to a concerted series of events that promote force generation and morphological changes. These events contribute directly to a number of actin-myosin mediated processes, such as cell motility, adhesion, smooth muscle contraction, neurite retraction and phagocytosis. In addition, ROCK kinases play roles in proliferation, differentiation, apoptosis and oncogenic transformation, although these responses can be cell type-dependent. Given the wide spectrum of biological processes influenced by ROCK, it is not entirely that ROCK has been implicated in a variety of pathophysiological conditions. One reason for this is the availability of potent small molecule ROCK inhibitors which have made it relatively easy to ask questions about ROCK-dependence. A major challenge is to determine whether ROCK truly represents a genuine and viable target for the treatment of human disease.

Knockout mice: Lessons learned from ROCK I and ROCK II

The development of potent ROCK small molecule inhibitors has led to massive interest in their potential clinical use. Deletion of ROCK I or ROCK II in mice allows for the genetic validation of ROCK as the critical target for these inhibitors, given their possible non-selective effects [13]. The specific disruption of each gene also makes it possible to genetically dissect the roles of ROCK I and ROCK II in development and pathological conditions, which is not possible with inhibitors which block both kinases with equal potency.

Genetic deletion of ROCK I or ROCK II resulted in similar phenotypes that reflect defects in epithelial cell motility. Homozygous ROCK I^{-/-} mice were born at expected Mendellian ratios, but newborns had developmental defects in eyelid and ventral body wall closure, respectively resulting in eyes-open at birth (EOB), and omphalocele in which organs such as the liver and gut protrude from the peritoneal cavity [14••]. EOB and omphalocele were observed in newborn ROCK II^{-/-} mice, but the ratio of homozygous deletions were sub-Mendellian since 90% died *in utero* due to defects in the placental labyrinth layer which resulted in decreased blood flow to the embryo [15,16••]. Double ROCK I^{+/-} ROCK II^{+/-} heterozygous mice also had EOB and omphalocele [16••]. These results indicate that both ROCK I and ROCK II act in driving the movement of epithelial sheets that are necessary for eyelid and ventral body wall closure. Formation of actin cables at the leading edge of eyelid epithelial sheets and the actomyosin-rich umbilical ring were impaired in both the homozygous knockouts [14••, 16••], demonstrating that both kinases promote actin bundling *in vivo*, possibly by identical means or by distinct mechanisms that collectively contribute to the same outcome. The lack of phenotype in other organs suggests that loss of one kinase is adequately compensated for by the other during embryogenesis and development.

ROCK I^{-/-} mice have been used to examine how this kinase contributes to certain pathological conditions. Using a variety of models that mimic chronic high blood pressure, partial or full deletion of ROCK I reduced cardiac fibrosis without affecting cardiomyocyte hypertrophy [17••,18••]. The reduced fibrosis is likely the result of lower extracellular matrix (ECM) protein production by cardiac fibroblasts, which might be due to lower levels of fibrogenic cytokines, such as TGFβ2 and connective tissue growth factor, released by cardiomyocytes. Alternatively, the sensing and/or responses of ROCK I deficient cardiac fibroblasts to the biomechanical stress generated by pressure overload might be impaired, resulting in decreased ECM production and possibly proliferation. In addition, pressure overload was less effective at inducing cardiomyocyte apoptosis in ROCK I^{-/-} mice relative to controls, suggesting a role for ROCK I in myocardial failure [19••]. These results are consistent with the idea that ROCK I

plays a significant role in the pathophysiological responses to high blood pressure, and validates ROCK as a critical target in the prevention and treatment of specific cardiovascular diseases.

In contrast to the observed effects in the heart, ROCK I deletion did not prevent renal fibrosis in a model of ureteral obstruction, despite observed protective effects of ROCK inhibitor Y-27632 [20,21], suggesting that ROCK II was either sufficient or had a dominant role in this tissue [22].

Genetic mouse models should continue to be informative in efforts to characterize how ROCK contributes to disease, although the constitutive and ubiquitous deletion of ROCK I and ROCK II, and the associated developmental defects limit their usefulness. These models will be improved significantly through the generation of conditional knockouts that would allow for tissue-selective deletion of ROCK I and/or ROCK II at defined time points. Alternatively, mice engineered to conditionally express shRNA that target ROCK I or ROCK II, in a ubiquitous or tissue-selective manner, may be useful for *in vivo* proof-of-principal studies to validate ROCK as a drug target. In addition, the production of mice expressing conditionally-active ROCK [23] in a tissue-selective manner will make it possible to examine the contribution of ROCK activation to disease initiation and progression. The refinement of these mouse models will make it possible to address significant questions about the role of ROCK in disease and the desirability of targeting ROCK as a therapeutic strategy.

Clinical evaluation of ROCK inhibitors

As mentioned above, ROCK has attracted significant interest as a potential target for the treatment of a wide-range of pathological conditions including; cancer, neuronal degeneration, kidney failure, asthma, glaucoma, osteoporosis, erectile dysfunction and insulin resistance. One of the biggest areas of interest has been in their potential use for cardiovascular diseases, and data from ROCK I knockout mice summarized above supports this concept. Despite the considerable interest and the development of numerous potent ROCK inhibitors by different groups, there is surprisingly little information in the literature reporting clinical trials with selective ROCK inhibitors.

Although selective ROCK inhibitors may not have been investigated extensively in man, the isoquinoline derivative fasudil (also known as HA-1077) has been in clinical use in Japan since 1995. Although originally created as one of a series of compounds that inhibited PKA and PKC, it was subsequently determined that fasudil was significantly more potent for ROCK, with an IC_{50} at least 10-fold lower than for other kinases [24]. Fasudil was demonstrated in animal models [25] to be effective in reversing blood vessel spasm and constriction that may occur after an episode of bleeding into the subarachnoid space surrounding the brain, a condition termed subarachnoid hemorrhage (SAH). Generally, SAH results from head trauma or spontaneously from the rupture of cerebral aneurysms, and vasospasm is a serious complication of SAH that may result in tissue damage, stroke and even death. The standard treatment in most countries is Nimodipine, which blocks L-type voltage-gated calcium channels. In Japan, fasudil has been used to prevent vasospasm associated with SAH with effectiveness at least comparable to Nimodipine treatment [26]. Importantly, post-marketing surveillance studies on SAH patients have found that fasudil was well tolerated and safe in over 1400 patients examined [27]. These findings have encouraged fasudil clinical trials for additional indications.

Given the safety and effectiveness of fasudil in treating vasospasm after SAH, and extensive pre-clinical data in a large variety of model systems, it is not surprising that clinical trials have focused on indications that are linked with the cardiovascular system. Human trials have been carried out to assess the efficacy of fasudil in: acute ischemic stroke [28], cerebral blood flow [29], stable angina pectoris [30-33], coronary artery spasm [34-36], heart failure-associated

vascular resistance and constriction [37], pulmonary arterial hypertension [38,39] essential hypertension [40], atherosclerosis [41,42] and aortic stiffness [43]. In addition, there are currently clinical trials in the United States underway to determine whether fasudil would be useful in treating atherosclerosis and hypercholesterolemia (ClinicalTrials.gov Identifier: NCT00120718), and Reynaud's phenomenon (ClinicalTrials.gov Identifier: NCT00498615), which is a vasospastic disorder that causes painful, pale and cold extremities.

Although the continued development of fasudil for the treatment of a wide variety of human diseases appears to be quite promising, the broad specificity of this inhibitor raises questions about the identity of the critical target. Although ROCK is more potently inhibited by fasudil than related kinases such as PKA and PKC [24], and many effects of fasudil have been reproduced in model systems by structurally distinct inhibitors such as Y-27632, the clinical effects of fasudil may actually result from the inhibition of other kinases or may result from the combined inhibition of ROCK plus additional kinases. Refined mouse models that allow for the conditional and tissue-selective deletion of ROCK I and ROCK II will permit genetic validation for ROCK inhibitors. In addition, clinical trials with alternative and more selective ROCK inhibitors will provide pharmacological validation. The proven safety of fasudil suggests that ROCK inhibition will not be a limiting factor in the future development of ROCK inhibitors for the treatment of human disease. It is also a possibility that inhibitors for kinases downstream of ROCK (*e.g.* ZIPK, LIMK) might be effective alternatives; continued research into downstream pathways will provide further validation.

ROCK, apoptosis and stem cells

During apoptosis, cells undergo significant morphological changes including contraction, dynamic membrane blebbing, and nuclear disintegration, which are driven by ROCK-mediated actin-myosin contractile force generation [44]. When cell death has been triggered by extrinsic factors such as TNF α , ceramide or Fas-receptor ligation, ROCK I activation is a relatively late event [6,7] and ROCK inhibition does not halt the apoptotic process [6]. However, in some contexts chronic or high intensity ROCK activity may contribute to the initiation of apoptosis. In fact, under stressed conditions where there is a small increase in caspase activity, the caspase-mediated cleavage and consequent activation of ROCK I may "feed forward" to amplify or accelerate the apoptotic process [19,45]. As a result, under these conditions ROCK inhibitors or endogenous antagonists of ROCK such as RhoE [45] may have pro-survival effects.

When cells lose survival signals derived from contact with other cells or extracellular matrix, they may undergo a form of apoptotic cell death called anoikis. ROCK inhibition has been reported to counteract anoikis in the ethanol-induced death of primary rat astrocytes [46] and detachment-induced death of tropomyosin-1 expressing MDA MB 231 human breast cancer cells [47]. Culturing embryonic stem cells (ESC) *in vitro* has proven to be technically challenging, especially so for human ESC and particularly when attempting to isolate individual cell clones following gene transfer or during differentiation procedures that require growth in suspension culture conditions, due to high rates of anoikis-induced cell death [48•]. Recently, it was reported that ROCK inhibition was protective against anoikis in dissociated human ESC [49•] and mouse ESC-derived neural precursors [48•]. These pro-survival effects of ROCK inhibitors may facilitate ESC production to industrial scale that would be required for their clinical use. In addition, the survival of ESC-derived neural precursor cells grafts was increased significantly when the cells were pre-treated with Y-27632 prior to dissociation and injected along with ROCK inhibitor into the striatum of mice [48•]. Previous research also found that ROCK inhibitors could be effective in combination with hypoxia mimetics acting via the HIF-1 transcription factor in promoting mesenchymal stem cell differentiation into neuron-like cells [50•,51•]. These results suggest that ROCK inhibitors

could be very valuable at numerous stages in the production and use (*e.g.* culturing, genetic modification, differentiation, implantation) of stem cells in basic research and eventual cell-based therapies. Further research is required to determine whether ROCK is the critical target for these effects and whether ROCK inhibition would be compatible with the survival of cells post-differentiation, given that ROCK inhibitors may actually induce apoptosis in some specialized cells types (*e.g.* [52-54]). The availability of ROCK I^{-/-} and ROCKII^{-/-} mice will make it possible to examine the contributions of each kinase to ESC differentiation and apoptosis.

Conclusions

Evidence from knockout mice and from the clinical use of fasudil supports the hypothesis that ROCK is a genuine and significant drug target. However, there are a number of questions that remain to be answered. Even relatively selective ROCK inhibitors such as Y-27632 have been shown to inhibit additional kinases, so their effects are not conclusively the result of ROCK inhibition. The use of more refined mouse models, including conditional deletion or activation, will provide genetic validation. Inducible shRNA expression *in vivo* to knockdown ROCK I and ROCK II will provide a drug-like system to test the hypothesis that reduced ROCK activity would be therapeutically beneficial. These proof-of-principal studies will be especially valuable for designing experiments to test the effects of chronic ROCK inhibition in diseases such as metastatic cancer. These refined mouse models will also be valuable in addressing the role of ROCK in embryonic stem cell apoptosis and differentiation. The potential for ROCK inhibitors in the production and use of stem cells in future therapies may prove to be very beneficial.

The clinical value and safety of fasudil in the treatment of cerebral vasospasm suggests that ROCK is a legitimate drug target. However, it remains to be determined whether ROCK is the critical target, or the simultaneous inhibition of several targets is required for the clinical effects. The evaluation of more selective ROCK inhibitors in human clinical trials will aid in the determination of fasudil's mechanism of action. An intriguing alternative possibility is that although ROCK inhibition produces the desired outcome, the truly important targets are ROCK-regulated kinases that lay downstream, for example ZIPK, LIMK1 or LIMK2. We await the development of inhibitors for these kinases in order to directly test this hypothesis.

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Figure 1. ROCK functional domains

Common functional domains in human ROCK I and ROCK II with the positions of starting and ending residues as annotated by NCBI. The percentage identities between matched regions were determined by pairwise BLAST comparisons. RBD = Rho Binding Domain. PH = Pleckstrin Homology domain. C1 = Protein kinase C conserved region 1. The representations are not to scale.

Figure 2. Modulators of ROCK I and ROCK II activity

In addition to associating with active GTP-bound RhoA, RhoB and RhoC, additional associations modulate ROCK activity. In the case of ROCK I, RhoE and Gem have been found to inhibit activity by binding to sites distinct from the Rho Binding Domain, while PDK1 antagonizes RhoE-mediated inhibition. Proteolytic cleavage of ROCK I by caspase 3 generates a constitutively active fragment. In the case of ROCK II, Rad inhibits activity while proteolysis by Granzyme B generates an active fragment.

Figure 3. ROCK pathways leading to increased actin-myosin contractility

Active GTP-bound Rho associates with ROCK and increases specific activity with the consequence of increased phosphorylation of numerous substrate proteins. Phosphorylation of the MYPT1 myosin-binding subunit of the myosin light chain phosphatase affects both substrate binding and catalytic activity, resulting in inhibition of myosin light chain dephosphorylation. The regulatory myosin light chain (MLC) has also been reported to be a direct ROCK substrate. As a consequence, there is increased MLC phosphorylation which promotes binding to filamentous actin. ROCK phosphorylation of LIMK1 and LIMK2 increases their specific activity, resulting in phosphorylation and inactivation of cofilin family proteins. Cofilin phosphorylation inhibits its filamentous actin severing activity. The sum total of these events is stabilization of filamentous actin and increased actin-myosin interactions. Additional phosphorylations catalyzed by ROCK promote myosin ATPase activity and tethering of filamentous actin with membrane-bound structural proteins, which additionally contribute to the generation of actin-myosin contractile force.