

Rho kinase in the regulation of cell death and survival

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Abstract

Rho kinase (ROCK) belongs to a family of serine/threonine kinases that are activated via interaction with Rho GTPases. ROCK is involved in a wide range of fundamental cellular functions, such as contraction, adhesion, migration, and proliferation. Recent studies have shown that ROCK plays an important role in the regulation of apoptosis in various cell types and animal disease models. Two ROCK isoforms, ROCK1 and ROCK2, are assumed to be function redundant, this based largely on kinase construct overexpression and chemical inhibitors (Y27632 and fasudil) which inhibit both ROCK1 and ROCK2. Gene targeting and RNA interference approaches allow further dissection of distinct cellular, physiological, and patho-physiological functions of the two ROCK isoforms. This review, based on recent molecular, cellular, and animal studies, focuses on the current understanding of ROCK signaling in the regulation of apoptosis and highlights new findings from recently generated ROCK-deficient mice.

Key words: ROCK1, ROCK2, apoptosis, survival, gene targeting.

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INTRODUCTION

Rho GTPase family proteins, which include Rho, Rac1, and Cdc42, control a wide variety of cellular processes, such as cell adhesion, motility, proliferation, differentiation, and apoptosis [27, 44, 143]. One of the best-characterized effectors of Rho is Rho-associated coiled-coil-containing protein kinase (hereafter referred to as ROCK), which was discovered in 1996 [54, 73, 82, 95]. During the last decade, the Rho/ROCK signaling pathway has attracted much attention in various research fields, and more than 2,000 articles have been published; many of which focus on ROCK function in the cardiovascular system, central nervous system, cancers, and embryonic development. Several excellent recent reviews have covered large aspects [11, 33, 51, 78, 94, 97, 113, 123]. The progress to date in translational research has demonstrated that ROCK is an important therapeutic target for the treatment of various cardiovascular diseases and neurological disorders, cancers, etc. In these studies, two relatively selective ROCK inhibitors, Y27632 [141] and fasudil [5], were extensively used to dissect the roles of ROCK in cellular signaling and in animal disease models, and these studies sug-

gest that inhibition of ROCK has great therapeutic potential.

During the last decade, we have also witnessed dramatic progress in the understanding of the physiological and patho-physiological roles as well as molecular mechanisms of apoptosis. The involvement of ROCK in apoptosis has recently been extensively examined. In this review, we summarize the current understanding of ROCK signaling in the regulation of apoptosis and highlight new findings from recently generated ROCK-deficient mice.

APOPTOSIS

Three types of cell death are known: apoptosis, necrosis, and autophagy. Apoptosis is a controlled, energy-dependent, active form of cell death characterized by morphological changes such as shrinkage of the cell, condensation of chromatin, and disintegration of the cell into small fragments that can be removed by phagocytosis [19]. Necrosis is an uncontrolled, energy-independent process characterized by cellular edema and disruption of the plasma membrane, leading to

release of the cellular components and inflammatory tissue response [57]. Autophagy is characterized by the sequestration of bulk cytoplasm and organelles in double or multi-membrane autophagic vesicles and their delivery to and subsequent degradation by the cell's own lysosomal system [124]. A role for ROCK in cell autophagy has not been reported.

Apoptosis occurs during development and throughout life and plays an important role in normal tissue homeostasis [19]. The deregulation of apoptosis has been associated with cancer, neurodegeneration, autoimmunity, and cardiovascular diseases. Apoptosis results from the activation of caspases, which are a family of cysteine proteases that cleave target proteins at specific aspartate residues. Apoptosis can be initiated by two evolutionarily conserved pathways (Fig. 1). The extrinsic pathway, which utilizes cell surface death receptors, is responsive to a highly specialized subset of death signals (e.g. inflammatory signals). In contrast, the intrinsic pathway relies on signaling through the mitochondria and the endoplasmic reticulum [19, 31]. Both types of triggering pathways converge in the activation of downstream executioner caspases, including caspase 3, 6, and 7.

In the extrinsic pathway, death ligands, such as FasL and tumor necrosis factor (TNF) α , bind to their respective receptors and stimulate recruitment of the adapter proteins Fas-associated via death domain (FADD) [6] and TNFR-associated death domain (TRADD) [20], respectively. FADD and TRADD recruit procaspase 8 into a complex named death-inducing signaling complex, where it undergoes dimerization and concomitant activation. Activated caspase 8 then activates procaspase 3 directly, which then cleaves various subcellular cytoplasmic proteins and fragments nuclear DNA. Caspase 8 can also signal indirectly through the mitochondria via activation of Bid, which translocates to the mitochondria and activates Bax and Bak to trigger the release of cytochrome *c* [42, 75, 79].

In many cell types, cytochrome *c*-dependent apoptosome formation, and the resulting activation of caspase 9, is the principal mechanism of caspase 3 activation via the intrinsic apoptosis pathway. Mitochondria play an important role in transmitting and amplifying death signals in the intrinsic pathway. The precise connections between apoptotic stimuli and the activation of the intrinsic pathway are not clear in many instances. However, it is clear that many stress stimuli activate caspases through the activation of BH3-only proteins of the Bcl-2 family, such as Bid, Bad, Bim, Nix, etc. The activated BH3-only proteins initiate mitochondrial outer membrane permeabilization by activating proapoptotic Bcl-2 family proteins, Bax and Bak, in mitochondria, leading to the release of cytochrome *c* as well as the release of Smac/DIABLO and Omi/HtrA2. This process is antagonized by anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-X_L. After release from the mitochondria, cytochrome *c* forms a complex with the adapter protein Apaf-1, dATP, and procaspase 9, result-

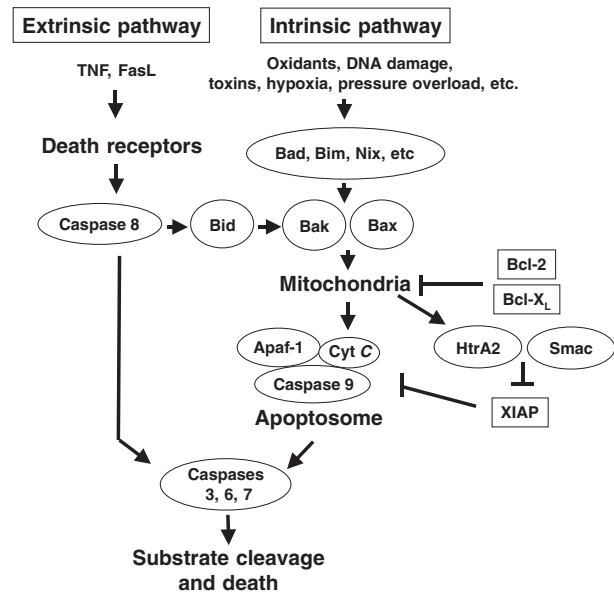


Fig. 1. The extrinsic and intrinsic pathways of caspase activation. In the extrinsic pathway, death ligands, such as FasL and TNF α , bind to their respective receptors, leading to caspase 8 activation. Activated caspase 8 directly activates procaspase 3, which then starts the death cascade. Caspase 8 can also signal indirectly through the mitochondria via activation of Bid, which translocates to the mitochondria and activates Bax and Bak to trigger the release of cytochrome *c* (Cyt *C*). In the intrinsic pathway, many stress stimuli activate caspases through the activation of BH3-only proteins of the Bcl-2 family, such as Bid, Bad, Bim, Nix, etc. The activated BH3-only proteins then activate Bax and Bak, leading to the release of cytochrome *c* as well as the release of Smac and HtrA2 from the mitochondria. This process is antagonized by Bcl-2 and Bcl-X_L. Cytochrome *c* forms a complex with the adaptor protein Apaf-1 and procaspase 9, resulting in the formation of an apoptosome, which leads to the proteolytic cleavage and concomitant activation of caspase 9. Active caspase 9 directly cleaves and activates procaspase 3. This process is antagonized by XIAP, which can be inhibited by Smac and HtrA2 following their release from the mitochondria.

ing in the formation of a structure known as the apoptosome, which leads to the proteolytic cleavage and concomitant activation of caspase 9. Active caspase 9 directly cleaves and activates procaspase 3. When a critical amount of activated caspase 3 is present within a cell, apoptosis is triggered. This process is antagonized by X-linked inhibitor of apoptosis (XIAP), which can be inhibited by Smac/DIABLO and Omi/HtrA2 following their release from the mitochondria.

Mitochondrial cytochrome *c* release can also follow mitochondrial permeability transition (mPT), an event triggered by changes in the permeability of the inner mitochondrial membrane. mPT can be induced by increased cytosolic Ca²⁺ levels as well as moderate oxidative stress (resulting from, for example, exposure to toxins). mPT results in a loss of mitochondrial membrane potential ($\Delta\Psi_m$), mitochondrial swelling, and outer membrane rupture, which in turn results in release of cytochrome *c* [7]. mPT has also been impli-

cated in necrosis [7, 96]. Caspase 3 activation resulting from cytochrome *c* release can stimulate mPT [69, 111], which can subsequently act as a feed-forward mechanism for further cytochrome *c* release.

Most knowledge about apoptotic cell death has come from the study of dividing or undifferentiated cells. The mechanisms of cell death in terminally differentiated, non-dividing cells such as cardiomyocytes and neurons are less well defined. Recent studies have shown that Apaf-1 levels in post-mitotic cells such as cardiomyocytes and sympathetic neurons are markedly reduced (compared with mitotic cells) [108, 109, 116], resulting in a significant decrease in apoptosome activity. The reduced activity of E2F1 [30], which is a transcriptional regulator of Apaf-1 [93] in terminally differentiated cardiomyocytes, is likely to contribute to the marked reduction of Apaf-1 in these cells. Given the reduced apoptosome activity (resulting from low levels of Apaf-1), endogenous XIAP has a greater inhibitory impact on apoptosis in post-mitotic cells. These results suggest that the inhibition of apoptosome activity by endogenous XIAP may be a rate-limiting step for cardiomyocyte and neuron apoptosis induced by pathological stimuli.

STRUCTURE AND EXPRESSION OF ROCK ISOFORMS

ROCK was initially identified as a Rho-binding protein with serine/threonine protein kinase activity and with a molecular mass of about 160 kDa [54, 73, 82, 95]. The protein contains a catalytic kinase domain at the amino terminus, followed by a central coiled-coil domain including a Rho-binding domain (RBD), and a carboxyl-terminal pleckstrin-homology (PH) domain with an internal cysteine-rich domain (Fig. 2). The ROCK family contains two members: ROCK1 (also called ROK β or p160ROCK) and ROCK2 (also known as ROK α), which share 65% overall identity and 92% identity in the kinase domain [54, 82, 95]. ROCK shares 45–50% homology with myotonic dystrophy kinase (DMPK), DMPK-related cdc42-binding kinase (MRCK), and citron kinase [113].

ROCK has auto-inhibitory activity [3]. In the inactive form, the carboxyl terminal PH domain and RBD of ROCK interact with the kinase domain, which forms an auto-inhibitory loop. The kinase domain is about 300 amino acids in length and contains the conserved motifs associated with serine/threonine protein kinases [46].

ROCK inhibitors such as Y27632 and fasudil bind to the kinase domain and inhibit ROCK1 and ROCK2 with similar potency [10, 56, 141]. The coiled-coil domain is thought to interact with other α -helical proteins. The RBD localized in the coiled-coil domain is about 80 amino acids in length and interacts only with activated Rho GTPases, including RhoA, RhoB, and RhoC [36]. The PH domain is believed to interact with lipid mediators such as arachidonic acid (AA) [29, 35] and sphingosylphosphorylcholine (SPC) [29, 35, 126], and might also participate in protein localization [14, 60, 151]. The amino-terminal regions, upstream of the kinase domain of ROCK, can be involved in the interaction with the substrates (e.g. RhoE) [112] and can also be important for the kinase activity [73].

Both ROCK1 and ROCK2 are ubiquitously expressed across human, rat, and mouse tissues [54, 73, 95, 147]. ROCK2 is more prominent in brain and skeletal muscle, whereas ROCK1 is more pronounced in liver, testes, and kidney. Both ROCK1 and ROCK2 are expressed in vascular smooth muscle and heart. In early mouse embryos, both ROCK1 and ROCK2 are expressed, with ROCK1 highly enriched in the developing heart and ROCK2 ubiquitously expressed [146].

ROCK1 and ROCK2 are essentially cytosolic in the resting state, but are translocated to the membrane upon Rho activation [74, 82]. In addition, ROCK2 has been found to be located at cleavage furrows during cytokinesis [65], at stress fiber [14], and the vimentin intermediate filament network [128], whereas ROCK1 has been shown to be co-localized with the centrosomes [15]. Two recent reports, using the siRNA approach, have shown that ROCK1 and ROCK2 have distinct distributions in primary rat embryonic fibroblasts [151] and are involved with different myosin compartments [151, 152]. In these fibroblasts, ROCK1 is mainly diffuse and perinuclear, and is required for stress fiber and focal adhesion formation, whereas ROCK2 exhibits both cell membrane and intense perinuclear distributions and is required for phagocytosis [151].

SUBSTRATES OF ROCK

Both ROCK1 and ROCK2 phosphorylate a variety of protein substrates at serine or threonine residues. The consensus sequence of ROCK substrates is R/K-X-S/T or R/K-X-X-S/T (R, arginine; K, lysine; S, serine; T, threonine) [4, 37, 41, 61, 83]. More than 20 ROCK

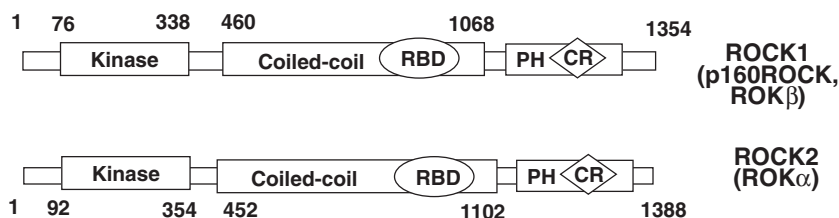


Fig. 2. Molecular structure of ROCK1 and ROCK2. ROCK sequences comprise a serine/threonine kinase domain located at the amino terminus of the protein, followed by a central coiled-coil domain containing the Rho-binding domain (RBD) and a carboxyl terminal pleckstrin homology (PH) domain with an internal cysteine-rich (CR) domain.

substrates have been identified (reviewed in refs. [51, 78, 113]).

The first characterized targets of ROCK are myosin light chain (MLC) [4, 66] and the myosin binding subunit of MLC phosphatase (MYPT1) [4, 61, 62]. ROCK can increase MLC phosphorylation through direct effect on MLC or indirectly by inactivating MLC phosphatase. The increased MLC phosphorylation results in stimulation of actomyosin contractility [2, 55, 73]. Inactivation of MLC phosphatase by ROCK plays important physiological roles, such as agonist-induced Ca^{2+} sensitization in smooth muscle contraction [28, 29, 141]. Most of the ROCK substrates are cellular proteins associated with the regulation of the actin cytoskeleton. Besides MLC and MYPT1, this important subset of ROCK substrates includes CPI-17 [63], calponin [58], LIM kinases [80, 99, 132], ezrin/radixin/moesin (ERM) [83], adducin [37], sodium-hydrogen exchanger (NHE1) [137], and ZIP kinase [43].

Several ROCK substrates are involved in the regulation of cell death and survival (Fig. 3). Increased MLC phosphorylation and actomyosin contraction in apoptotic cells regulate plasma membrane blebbing [88]. Caspase 3-mediated ROCK1 activation is responsible for the increased MLC phosphorylation in a variety of cell types [16, 119]. Phosphatase and tensin homologue

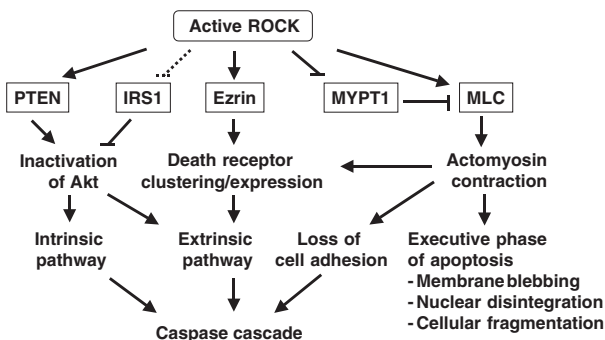


Fig. 3. Potential ROCK targets in apoptotic signaling. Several ROCK substrates are involved in the regulation of apoptosis. ROCK can increase MLC phosphorylation through direct effect on MLC or indirectly by inactivating MLC phosphatase (MYPT1). The increased MLC phosphorylation results in stimulation of actomyosin contractility, which regulates morphological apoptotic events during the execution phase of apoptosis, including plasma membrane blebbing, nuclear disintegration, and fragmentation of apoptotic cells. Under some conditions, the actin cytoskeleton rearrangement induced by ROCK activation can also be involved in the intracellular signaling involved in the initiating stages of apoptosis through regulation of assembly of death receptor complex or loss of cell adhesion. In addition, ROCK has been shown to stimulate phosphatase and tensin homologue (PTEN) and inhibit insulin receptor substrate 1 (IRS1) signaling (broken line indicates that both positive and negative regulations of IRS1 signaling by ROCK have been reported), resulting in inactivation of Akt. Akt has important roles in promoting cell survival, possibly through inhibition of both the extrinsic and the intrinsic pathways. Finally, ROCK activation was found to promote apoptosis through increasing ezrin phosphorylation, which in turn led to Fas clustering and membrane expression.

(PTEN) is a newly identified ROCK substrate [77]. The phosphorylation of PTEN by ROCK stimulates its phosphatase activity. PTEN dephosphorylates both proteins and phosphoinositides and is a negative regulator of the phosphatidylinositol (PI)3-kinase/Akt pathway, which has important roles in a diverse range of biological processes, including cell survival [22]. PTEN mediates the reduction of Akt phosphorylation induced by ROCK activation in HEK cells [13, 77]. Reduced PTEN activity may contribute to the protective effect of ROCK inhibition in endothelial cells [148] and to the protective effects of suppression of ROCK1 in cardiomyocytes [13]. In addition, ROCK has been shown to interact with and negatively regulate insulin receptor substrate 1 (IRS1) signaling and PI3-kinase activation in vascular smooth muscle cells [9] and in fibroblasts derived from p190B RhoGAP null mice [131]. In contrast, a recent study has shown that ROCK interacts with and phosphorylates IRS1 at Ser632/635 sites, thereby enhancing PI3-kinase activation in adipocytes and muscle cell lines and in isolated soleus muscle *ex vivo* [39]. ROCK appears to be involved in both positive and negative regulation of PI3-kinase/Akt signaling and the outcome may be cell-type and stimulus dependent. Finally, ROCK2 activation was found to promote apoptosis through increasing ezrin phosphorylation, which then leads to Fas clustering and membrane expression in Raf-1 deficient embryonic fibroblasts [107]. Fas activation stimulates the formation of Raf-1-ROCK2 complexes, thereby down-regulating ROCK2 activity. This mechanism may contribute to the phenotype of Raf-1-deficient mice, such as fetal liver apoptosis, embryonic lethality, and selective hypersensitivity to Fas-induced cell death [52, 87].

The majority of ROCK substrates have been identified from cell culture experiments. In most cases, only one ROCK isoform (more generally ROCK2) has been tested. Because ROCK1 and ROCK2 share 92% identity in the kinase domain, it has been assumed that they share the same substrates. However, ROCK1 and ROCK2 may have different targets, as only ROCK1, but not ROCK2, binds to and phosphorylates RhoE [114].

REGULATION OF ROCK ACTIVITY

ROCK activity can be regulated by several distinct mechanisms (Table 1). The kinase activity is increased after Rho binding [54, 73, 82]. The interaction between RBD and the active GTP-bound form of Rho disrupts the interaction between the catalytic domain and the inhibitory carboxyl-terminal region of ROCK. The Rho/ROCK pathway is activated by numerous extracellular stimuli. The consequence of Rho-dependent ROCK activation is highly cell-type dependent, ranging from changes in contractility, cell permeability, migration, and proliferation, to apoptosis.

Lipid mediators such as AA [29, 35] and SPC [126] can interact with the ROCK carboxyl-terminal inhibito-

Table 1. Known mechanisms of ROCK activation and inactivation

Regulators	Site of interaction on ROCK	Effects	References
Rho	interacts with RBD, ROCK1 and ROCK2	activation	54, 73, 82
AA	interacts with the PH domain, ROCK2	activation	29, 35
SPC	interacts with the PH domain, ROCK2	activation	126
PI3,4,5P ₃ , PI4,5P ₂	interact with the PH domain, ROCK2 only	activation	151
Gem, Rad	interact with the coiled-coil domain, ROCK1 and ROCK2	inactivation	145
Caspase 3	cleaves at DETD1113, ROCK1 only	activation	16, 119
RhoE	interacts between amino acids 1-420, ROCK1 only	inactivation	101, 112
STK15	phosphorylates at serine residues between amino acids 726 -1,034, ROCK1	inactivation	25
Granzyme B	cleaves at IGLD1131, ROCK2 only	activation	118
Caspase 2	cleavage site is not known, ROCK2 only	activation	117
Raf-1	site of interaction is not known, ROCK2 only	inactivation	107
p21 ^{Cip1/WAF1}	site of interaction is not known, ROCK2	inactivation	81

ry domain and stimulate ROCK activity independently of Rho. At the cellular level, the activation of the AA or SPC/ROCK pathways was mainly observed in smooth muscle cells to increase calcium sensitivity [92, 126]. In addition, inositol phospholipids such as PI3,4,5P₃ and PI4,5P₂ can interact with the PH domain of ROCK2, but not ROCK1, suggesting that ROCK2 may be regulated by PI-3 kinase activity [151]. Dimerization and/or trans-phosphorylation may also play a role in regulating ROCK activity by regulating its affinity for ATP [24].

ROCK can be activated constitutively by proteolytic cleavage of the inhibitory carboxyl-terminal domain. ROCK1 is cleaved by caspase 3 at the cleavage site DETD1113 during apoptosis [16, 119]. This consensus sequence for caspase 3 cleavage is conserved in human, rat, and mouse, but is not present in ROCK2. Caspase 3 is believed to be responsible for activating ROCK1 in apoptotic cells, as evidenced by the absence of ROCK1 cleavage in caspase 3-deficient MCF-7 breast carcinoma cells and the presence of ROCK1 cleavage after restoring procaspase 3 expression [119]. In addition, ROCK1 cleavage by caspase 3 can be inhibited by caspase inhibitors in a variety of apoptotic cells [16, 23, 70, 86, 104, 119, 127, 139, 140, 155]. However, caspase 3-independent cleavage of ROCK1 was observed in extracellular ATP-induced and P2X7 ATP receptor-mediated apoptosis [91] and in cancer cells subjected to combined BGC9331 (a thymidylate synthase inhibitor) and SN-38 (a topoisomerase I inhibitor) treatment [17]. The nature of the proteases involved and the cleavage site for caspase 3-independent cleavage of ROCK1 remain to be identified [17, 91]. On the other hand, during cytotoxic lymphocyte granule-induced cell death, human ROCK2 can be cleaved by the proapoptotic protease granzyme B at the IGLD1131 site, and this site is not present in ROCK1 [118]. Human ROCK2, but not ROCK1, can also be activated by caspase 2-dependent cleavage in endothelial cells in response to thrombin, but the cleavage site remains to be identified [117].

Besides these positive regulators, negative control of ROCK activity has also been described (Table 1). The small GTP-binding protein RhoE interacts with the N-terminal region of ROCK1 (amino acids 1-420) and

prevents Rho binding to RBD [101, 112]. Other negative regulators have been found to bind to and inhibit ROCK activity, such as the small GTP-binding proteins Gem and Rad [145], Raf-1 [107], p21(Cip1/WAF1) [81], and Aurora-A/serine/threonine kinase 15 (STK15) [25]. However, their mechanisms of action are not defined.

As discussed below, the caspase 3/ROCK pathway is believed to play a major role in regulating membrane blebbing [16, 119], a characteristic feature of apoptotic cells, in a variety of cell types, independent of apoptotic stimuli. An apoptotic role for other ROCK activation pathways, such as Rho/ROCK [59, 89], granzyme B/ROCK2 [118], or caspase 2/ROCK2 [117], may be highly cell-type dependent and/or apoptotic stimulus dependent. Importantly, ROCK1 and ROCK2 can be distinctly activated or inhibited by a number of positive or negative regulators, and in turn can have distinct cellular or physiological functions.

ROCK1- AND ROCK2-KNOCKOUT MICE

Recently ROCK1-knockout (ROCK1^{-/-}) and ROCK2-knockout (ROCK2^{-/-}) mice have been generated [115, 122, 135, 153] and their phenotypes are different. ROCK1^{-/-} mice under a C57BL/6N genetic background exhibit eyelids open at birth (EOB) and omphalocele phenotype due to disorganization of actin filament in the epithelial cells of the eyelid and of the umbilical ring [122]. ROCK2^{-/-} mice in a mixed genetic background between 129/SvJ and C57BL/6N are embryonic lethal because of placental dysfunction and have intrauterine growth retardation caused by thrombus formation in the labyrinth layer of the placenta, indicating that there is no compensation for the loss of ROCK2 by ROCK1 [135]. Interestingly, ROCK2^{-/-} mice under a C57BL/6N genetic background exhibit not only the placental phenotype, but also EOB and omphalocele phenotype [136], indicating that genetic background affects the EOB and omphalocele phenotype in ROCK2^{-/-} mice. In addition the shared EOB and omphalocele phenotype in ROCK1^{-/-} and ROCK2^{-/-} mice under the C57BL/6N genetic background indicates

that they act together to regulate the assembly of actin bundles essential for closure of the eyelid and ventricular body wall in mouse embryos.

The genetic background may also affect the EOB and omphalocele phenotype in ROCK1^{-/-} mice, as this phenotype was not observed in ROCK1^{-/-} mice under the mixed C57BL/6N-129/SvJ or FVB background [153], although differences in targeting vectors may also contribute to the different phenotypes of ROCK1^{-/-} mice generated by different laboratories [115, 122, 153]. In addition, the ROCK1^{-/-} mice under the FVB background have no detected anatomical abnormalities from embryonic day 9.5 to adulthood, although they are underrepresented, possibly due to lethality at very early developmental stages [153]. Further studies with ROCK1 deletion in various strain backgrounds and at early developmental stages (prior to E9.5) should provide additional information on the developmental role of ROCK1. A common characteristic of ROCK1^{-/-} and ROCK2^{-/-} mice is that after surviving their intrauterine and perinatal problems, these mice develop normally and are apparently healthy and fertile [115, 122, 135, 153]. In addition, there is no compensatory up-regulation of the ROCK1 expression in ROCK2^{-/-} mice and vice versa.

Systemic and conditional ROCK1- and ROCK2-knockout mice offer a unique opportunity to analyze *in vivo* physiological and pathological functions of ROCK1 and ROCK2, as ROCK inhibitors do not distinguish these two isoforms. A recent study has reported that ROCK1^{+/-} mice exhibit cardiac hypertrophy but decreased cardiac fibrosis in response to angiotensin II infusion [115]. We have observed that ROCK1^{-/-} mice exhibit cardiac hypertrophy but reduced cardiac fibrosis in response to pressure overload [153]. These observations suggest an important role for ROCK1 in the development of cardiac fibrosis, but not hypertrophy. The molecular and cellular mechanisms underlying the cardiac fibrotic role of ROCK1 remain to be defined. On the other hand, renal fibrosis induced by unilateral ureteral obstruction is not reduced by ROCK1 deletion, suggesting that the presence of ROCK2 may compensate for the loss of ROCK1 in this renal fibrosis model [34]. Interestingly, *in vivo* study with ROCK1^{-/-} mice also revealed an important role for ROCK1 in cardiomyocyte apoptosis [13], which is a critical contributing factor of heart failure progression [31]. Deletion of ROCK1 indeed prevents or delays the development of dilated cardiomyopathy in several pathological models (Shi et al., unpublished observations). These studies strongly suggest that ROCK1 and ROCK2 have some non-overlapping *in vivo* functions.

THE ROLE OF ROCK IN REGULATING MORPHOLOGICAL APOPTOTIC EVENTS

ROCK is recognized as a major regulator of the morphological events that occur during the execution phase of apoptosis, including cell contraction, dynamic mem-

brane blebbing, nuclear disintegration, and fragmentation of apoptotic cells into apoptotic bodies (Fig. 3).

Membrane blebbing

The execution phase of apoptosis is characterized by a number of morphological changes, which consist of three stages: release, membrane blebbing, and condensation. During the release stage, a dying cell loses contact with the extracellular matrix and surrounding cells and begins to round up. This retraction stage is followed by plasma membrane blebbing. After a period of membrane blebbing, which is normally transient, cells move into the condensation stage; where cells either condense into a single apoptotic body or fragment into multiple apoptotic bodies. Plasma membrane blebbing is regulated by MLC phosphorylation and actomyosin contraction [88]. A role for caspase 3-mediated cleavage and activation of ROCK1 in the regulation of membrane blebbing of apoptotic cells was first demonstrated in anti-Fas antibody-treated Jurkat cells and TNF α -treated NIH3T3 [16, 119]. This caspase 3-mediated ROCK1 activation is both necessary and sufficient for the formation of membrane blebs through increasing MLC phosphorylation and actomyosin contraction [16, 119]. The importance of this pathway in bleb formation was then confirmed by other studies in a variety of cell types, independent of apoptotic stimuli [23, 70, 86, 104, 127, 155]. In addition, ROCK1-mediated blebbing facilitates cellular fragmentation and/or apoptotic cell clearance, as discussed below.

Even though caspase 3-mediated ROCK1 activation appears to be a major pathway inducing membrane blebbing in apoptotic cells, membrane blebbing could occur via Rho-dependent and caspase-independent activation of ROCK in some apoptotic conditions [59, 89]. Both ROCK1 and ROCK2 could be involved in this process, as both can be activated by Rho to regulate MLC phosphorylation and actomyosin contraction. In agreement with this, over-expression of wild-type ROCK2 or a constitutively active mutant of ROCK2 is able to induce membrane blebbing through activation of actin-myosin contractility [130]. Finally, ROCK2, but not ROCK1, can be activated by granzyme B-mediated cleavage (at Asp 1131) in a context of cytotoxic lymphocyte granule-induced apoptosis. This cleaved (activated) ROCK2 is able to induce membrane blebbing independent of caspase 3 activation [118].

Fragmentation of apoptotic cells

During the execution phase of apoptosis, a cell packages itself into apoptotic bodies; therefore its contents are not released. The resulting apoptotic bodies are removed from the surrounding tissue through phagocytosis [71]. Apoptotic cells undergo a number of changes to prepare for phagocytosis. These include nuclear fragmentation, relocation of fragmented DNA into membrane blebs, and shrinkage and/or fragmentation into apoptotic bodies, and they express phagocytic markers

on their surface. ROCK activation and subsequent MLC phosphorylation are required for apoptotic nuclear disintegration [18] as well as relocation of fragmented DNA into blebs and apoptotic bodies [16] in NIH3T3 cells. ROCK activity and actomyosin contraction also contribute to the formation of endoplasmic reticulum and chromatin-containing blebs in the late apoptotic cells which are likely the progenitors of apoptotic bodies [70]. Moreover, inhibition of ROCK with Y27632 prevents fragmentation of apoptotic cells and blocks Golgi fragmentation in apoptotic COS-7 and PC12 cells, respectively, and these ROCK-dependent execution events require actomyosin contraction [102, 103]. Interestingly, ROCK controls surface expression phagocytic markers independently of actomyosin contraction [102]. Importantly, cells dying in the presence of Y27632 are less efficiently phagocytized than those dying without the inhibitor; this suggests that ROCK plays an important role in the preparation of dead cells for phagocytosis [102]. Additionally, siRNA knock-down of ROCK1 but not ROCK2 inhibits fragmentation of dying cells, consistent with ROCK1, but not ROCK2, being cleaved and activated by caspases [103]. However, Jurkat cells dying in the presence of Y27632 are phagocytosed by macrophages as efficiently as those dying without the inhibitor [127], suggesting that the importance of ROCK in the preparation of dead cells for phagocytosis may depend on cell type and/or apoptotic stimulus.

Phagocytosis of apoptotic cells

Deletion of apoptotic cells from tissues involves their phagocytosis by macrophages, dendritic cells, and tissue cells. The rapid and efficient phagocytosis of apoptotic cells plays a critical role in preventing secondary necrosis and inflammation as well as in tissue remodeling and regulating immune responses. Phagocytic clearance of apoptotic cells consists of four distinct steps: accumulation of phagocytes at the site where apoptotic cells are located in response to specific attraction signals released by cells undergoing apoptosis; recognition by phagocytes through a number of bridge molecules and receptors; engulfment of apoptotic cells; and degradation of the engulfed cells within phagocytes. Recent studies have shown that Rho and ROCK are involved in the clearance of apoptotic cells through the regulation of the actin cytoskeleton [26, 138].

A decrease in Rho activity was observed during the engulfment of apoptotic cells [138]. In addition, Rho seems to negatively affect basal phagocytosis, such that inhibition of Rho-mediated signaling enhances the uptake of apoptotic cells. ROCK seems to be primarily responsible for this inhibitory effect on engulfment [138]. The negative effect of Rho/ROCK during engulfment of apoptotic cells differs from other types of phagocytosis mediated via the complement receptor and Fc receptor, respectively. Rho and ROCK have been shown to be critical for complement receptor-mediated phagocytosis,

and but not for Fc receptor-mediated phagocytosis [12, 100]. Recently, ROCK2 activity, but not ROCK1 activity, has been shown to facilitate phagocytic uptake of fibronectin-coated beads [151].

During the degradation of apoptotic cells within macrophages or fibroblasts, the Rho/ROCK/ERM signaling pathway contributes to the rapid maturation of phagosomes, as blockage of Rho/ROCK/ERM signaling delays maturation rates of phagosomes containing apoptotic cells [26]. The maturation of phagosomes containing cells taken up through Fc receptors is slower and is independent of Rho and ROCK.

THE ROLE OF ROCK IN MEDIATING APOPTOTIC SIGNALS

Although a role for ROCK in regulating morphological apoptotic events during the execution phase of apoptosis is well recognized, the importance of ROCK in regulating apoptotic caspase cascades is highly cell-type and/or apoptotic-stimulus dependent. Under some conditions, ROCK activation or inhibition is not important for mediating apoptotic signals. For example, inhibition of ROCK does not affect caspase 3 activation and progression of apoptosis in anti-Fas antibody-treated Jurkat cells and TNF α -treated NIH3T3 [16, 119]. Inhibition of ROCK disrupts actin stress fibers, but does not induce apoptosis in 3T3 cells [90]. Expression of the cleaved (activated) ROCK1 is sufficient to induce membrane blebbing, but does not result in chromatin condensation in NIH3T3 cells [16, 18] and Jurkat cells [119]. ROCK activation is required for membrane blebbing, but not for other apoptotic events such as the loss of mitochondrial respiration and cytochrome *c* release induced by eosinophil peroxidase in lung epithelial cells [86]. However, there is increasing recognition that the actin cytoskeleton rearrangement induced by ROCK activation may be involved not only in the end-stage execution of apoptosis, but in the intracellular signaling involved in the initiating stages, as well (Fig. 3). ROCK-mediated apoptotic signaling may involve a cell death domain receptor-dependent extrinsic pathway (e.g. regulation of assembly of death receptor complex) and/or a mitochondrial-dependent intrinsic pathway (e.g. regulation of expression of apoptotic or anti-apoptotic molecules).

In vitro evidence

Several studies have shown that Rho/ROCK activation is required for endothelial cell death caused by cytokines or drugs which induce MLC phosphorylation and actin cytoskeletal alterations. ROCK inhibition by Y27632 or Rho inhibition by the overexpression of dominant negative Rho significantly attenuates TNF α -induced apoptosis and caspase 8 activity through reduction of MLC phosphorylation and actomyosin contraction, which may be critical for the assembly of the TNF α death receptor complex in bovine pulmonary artery

endothelial cells [105, 106]. Rho and ROCK activation by combretastatin A-4-phosphate, a tumor vascular-targeting agent responsible for microtubule depolymerization, leads to increased MLC phosphorylation and actomyosin contractility, which then induce membrane blebbing and loss of cell adherence in human umbilical vein endothelial cells [59]. Consistent with this observation, up-regulation of ROCK activity via inhibition of ERK-MAPK signaling during tumor vascularization promotes endothelial cell retraction and death [85].

Several studies also point to a pro-apoptotic role of ROCK in other cell types. ROCK-mediated MLC phosphorylation acts as an upstream event required for membrane contraction and the subsequent caspase 8, 10, and 3 activation during phorbol-12-myristate-13-acetate (PMA) stimulation in erythroblastic TF-1 cells [67]. In addition to MLC phosphorylation, ROCK activation mediates other molecular events in PMA-treated apoptotic cells, such as prevention of nuclear distribution of phospho-ERK, inhibition of induction of the cell cycle inhibitor p21(Cip1/Waf1) [68], and cytosolic translocation of heterogeneous nuclear ribonucleoprotein C1 and C2, which are two nuclear restricted pre-mRNA binding proteins [72]. In primary thymocytes, apoptosis induced by activation of the receptor for thromboxane A₂, which is coupled to G α (12/13) subunits, is inhibited by deletion of Lsc RhoGEF (an activator of GTPases of the Rho family), associated with inactivation of Rho/ROCK and increased Akt phosphorylation [47]. Ethanol is an important teratogen that induces marked central nervous system dysfunctions and ethanol-induced ROCK activation in astrocytes promotes MLC phosphorylation and actomyosin contraction, thereby leading to apoptosis induced by loss of anchorage [89].

As we already know, the majority of the studies have been performed with ROCK inhibitors or dominant negative mutants of ROCK, which inhibit both ROCK1 and ROCK2. However, ROCK1 and ROCK2 may not share identical apoptotic functions. For example, ROCK2 activation has been found to promote apoptosis through increasing ezrin phosphorylation, leading to Fas clustering and membrane expression in Raf-1-deficient embryonic fibroblasts [107]. Reduced ROCK2 expression by siRNA reduced Fas-induced apoptosis of Raf-1-deficient embryonic fibroblasts [107]. On the other hand, ROCK1 physically associates with STK15, which is implicated in the regulation of the centrosome replication cycle. Suppression of ROCK1 expression by siRNA bypasses G(2)/M cell cycle arrest after STK15 depletion and alleviates apoptosis in HeLa cells [25].

ROCK1 has been demonstrated to be a key mediator which links pro-apoptotic stimuli to apoptosis in cultured neonatal cardiomyocytes [13]. Multiple pro-apoptotic stimuli result in the caspase 3-dependent proteolytic cleavage and concomitant activation of ROCK1 in cardiomyocytes. Additionally, reduction of ROCK1 activity via siRNA treatment blocks ceramide-induced caspase 3 activation and apoptosis. Moreover, expression of an activated ROCK1 mutant is sufficient to induce caspase 3

activation in cultured neonatal rat cardiomyocytes, suggesting a positive feed-forward regulatory loop. The mechanism underlying the apoptotic role of ROCK1 in amplification of caspase 3 remains to be defined, and may be mediated by PTEN activation. In contrast, a previous study has shown that treatment of cultured rat neonatal cardiomyocytes with Y27632 promoted cardiomyocyte apoptosis [98], and inhibition of both ROCK1 and ROCK2 by this chemical inhibitor may contribute to the different observations compared with our study.

In vivo evidence

In a variety of animal disease models, inhibition of ROCK produces protective effects, including reduced apoptosis, which is accompanied in most cases by reduced inflammatory responses. Inhibition of ROCK significantly reduces medial smooth muscle cell apoptosis, inflammatory cell infiltration, and cytokine production and attenuates abdominal aortic aneurysm formation induced by angiotensin II [144]. ROCK mediates the production of reactive oxygen species and inflammatory cytokines which are substantially involved in the pathogenesis of hepatocellular necrosis and apoptosis induced by cold ischemia-reperfusion injury after liver transplantation in rats [125]. ROCK mediates the infiltration of leukocytes as well as the production of TNF α and CXC chemokines and hepatocellular apoptosis in endotoxemic liver injury (challenged with lipopolysaccharide and D-galactosamine) [134]. The inhibition of ROCK by Y27632 during acute ischemia/reperfusion injury significantly reduces cardiomyocyte apoptosis, prevents the down-regulation of Bcl-2 protein, and attenuates inflammatory responses [8]. ROCK inhibition by fasudil reduces endothelin-1-induced myocardial injury, such as multifocal myocardial necrosis with infiltration of neutrophils and macrophages in the rabbit [149]. The underlying mechanisms for the apoptotic role of ROCK in these studies are not defined, and the effects of ROCK inhibitors in other cellular events, such as inflammatory cell infiltration and cytokine production, may be related to the anti-apoptotic effect of ROCK inhibitors. Consistent with the studies mentioned above, ROCK is involved in the regulation of inflammatory responses, such as the production of inflammatory cytokines, inflammatory cell infiltration, and vehicle secretions, in a number of experimental models [48, 50, 117, 133], and these inflammatory responses may subsequently promote apoptosis. Finally, ROCK also regulates oxidative stress [49, 125, 150], which in turn can induce apoptosis.

THE ROLE OF ROCK IN MEDIATING CELL SURVIVAL SIGNALS

There is increasing evidence that ROCK-mediated focal adhesion and integrin activation are involved in cell survival [32, 45, 142]. Perturbations of the actin

cytoskeleton integrity via ROCK inhibition can initiate events that commit a cell to apoptosis.

In vitro evidence

A ROCK-mediated survival effect has been documented in epithelial, cancer, and endothelial cells as well as in other cell types. In airway epithelial cells, inhibition with either Y27632 or HA1077 induces membrane ruffling, loss of actin stress fibers, and apoptosis that can be blocked by inhibiting caspase function or by inhibiting protein synthesis [90]. Rho/ROCK activation also plays a pro-survival role during oxidative stress (H_2O_2)-induced intestinal epithelial cell injury via activation of PKC δ and PKD [129]. In anaplastic thyroid cancer cells, inhibition of Rho by lovastatin or inhibition of ROCK by Y27632 induces cytochrome *c* release, activation of caspases 3 and 9, and apoptosis which required *de novo* protein synthesis [154]. Inhibition of Rho or ROCK results in the activation of caspases 3, 8, and 9 and glioma cell apoptosis [110]. Treatment of human umbilical vein endothelial cells with ROCK inhibitors Y27632 and HA1077 causes dose-dependent cell death which is dependent on *de novo* protein synthesis and is associated with an increase in p53 protein level [76].

ROCK may play a pro-survival role in hepatic stellate cells (HSCs), which play a central role in the development of hepatic fibrosis. Inhibition of ROCK by Y27632 causes apoptosis with increased DNA fragmentation, condensation of nuclear chromatin, and caspase 3 activity in rat cultured HSCs, possibly through decreasing cell spreading and attachment [53]. However, another study using the ROCK inhibitor fasudil has shown that inhibition of ROCK does not induce apoptosis of rat cultured HSCs, but inhibits cell spreading, suppresses collagen production, and enhances collagenase activity [38].

ROCK has been shown to regulate cell survival of the central nervous system. Activation of G-protein-coupled neurotransmitter receptors, including cholinergic muscarinic receptors, can provide neuroprotection from a wide variety of potentially lethal toxic insults. In human neuroblastoma SH-SY5Y cells, ROCK activation by muscarinic receptor stimulation is critical for the protective effect of muscarinic receptor activation against H_2O_2 (oxidative stress)- and camptothecin (DNA damage)-induced apoptosis [21].

In vivo evidence

A pro-survival role of ROCK in vascular smooth muscle cells was first observed in neointima formation after balloon injury in the rat carotid artery [120]. Y27632 treatment significantly increases the neointimal TUNEL-positive smooth muscle cells at 7 and 14 day after vascular injury [120], and this increased apoptosis is associated with significant up-regulation of the proapoptotic Bax, suggesting that ROCK inhibition induces neointimal smooth muscle cell apoptosis

through Bax up-regulation [121]. Consistent with this finding, the inhibition of ROCK with fasudil enhances smooth muscle cell apoptosis in the neointima after stent implantation in the porcine coronary artery, and this increased apoptosis is associated with down-regulation of the anti-apoptotic protein Bcl-2 [84]. Moreover, inhibition of ROCK with fasudil significantly enhances smooth muscle cell apoptosis in autologous vein grafts in rabbits [40] and in pulmonary hypertension in rats [1], further supporting a pro-survival role of ROCK in smooth muscle cells. Increased smooth muscle cell apoptosis along with inhibition of smooth muscle cell proliferation, inflammatory cell infiltration, and/or extracellular matrix protein production contribute to the inhibition of neointima formation via ROCK inhibition [40, 84, 120]. Therefore, these studies suggest that ROCK plays a pro-survival role in neointimal smooth muscle cells. The underlying mechanisms are not defined, and the effects of ROCK inhibitors in other cellular events such as smooth cell proliferation, inflammatory cell infiltration, and cytokine production may be related to their effect on smooth muscle cell apoptosis.

ROCK also plays an essential role in the survival of the spinal motor neurons during mammalian embryogenesis, as conditional expression of a dominant-negative form of ROCK (inhibits both ROCK1 and ROCK2) in spinal motor neurons in transgenic mice increases apoptosis [64]. Moreover, the Rho/ROCK pathway is involved negatively in the regulation of the glioma cell death pathway, as inhibition of Rho or ROCK induces apoptosis of C6 glioma cell tumor cells and results in a significantly smaller tumor mass *in vivo* [110].

CONCLUSIONS AND FUTURE DIRECTIONS

ROCK plays an important role in the regulation of morphological changes of apoptotic cells by regulating the cytoskeleton and actomyosin contractility. ROCK, either as a pro-apoptotic or anti-apoptotic regulator, has also been reported in a variety of cell types. However, how the basic components of the apoptotic machinery are regulated by ROCK is not completely understood in many instances, and is likely different depending on the cell type and the apoptotic stimulus. Clearly, more studies are needed in order to understand the molecular and cellular mechanisms through which ROCK regulates cell death and survival as well as in association with its roles in physiology and patho-physiology.

ROCK1 and ROCK2 not only function in a redundant manner, each being able to compensate for the loss of the other in some systems, but also may have their own distinct roles in some tissues during embryonic development (e.g. placenta) or under certain patho-physiological conditions in adulthood (e.g. cardiac fibrosis, cardiomyocyte apoptosis). There are a number of factors that could contribute to their distinct functions, including the differences in their tissue distribution, subcellular localization, activation by upstream sig-

nals (e.g. caspases), and interaction with downstream molecules. The difficulty in studying specific cellular functions of the two ROCK isoforms is due to the high degree of their sequence homology in the kinase domain and the lack of isoform-specific inhibitors. Genetic targeting approaches using systemic and/or conditional ROCK1 and ROCK2 knockout mice coupled with molecular analysis using RNA interference and gene transfer strategies will become a focus of future efforts to understand the distinct cellular, physiological, and patho-physiological functions of the two ROCK isoforms. A greater understanding of the physiological and patho-physiological roles of each ROCK isoform is much desired for better evaluating the beneficial effects and side effects of ROCK inhibitors in animal and clinical studies.

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