Receptor Interacting Protein 3 Suppresses Vascular Smooth Muscle Cell Growth by Inhibition of the Phosphoinositide 3-Kinase-Akt Axis*1

Received for publication, September 29, 2009, and in revised form, December 4, 2009. Published, JBC Papers in Press, December 30, 2009, DOI 10.1074/jbc.M109.071332

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Proliferation of vascular smooth muscle cells (VSMCs) is a primary mechanism underlying cardiovascular proliferative disorders. Phosphoinositide 3-kinase (PI3K)-Akt (or protein kinase B) axis has been assigned at the center of pathways that regulate cell proliferation. Here we demonstrate that enhanced PI3K-Akt signaling by mitogenic stimulation or arterial injury profoundly elevates expression of receptor interacting protein 3 (RIP3) in primary cultured rat VSMCs and in vivo and that the up-regulation of RIP3 leads to VSMC growth arrest and apoptosis via inhibiting the PI3K-Akt signaling pathway, thereby alleviating balloon injury-induced neointimal formation. Specifically, mitogenic stimulation with platelet-derived growth factor-BB or angiotensin II leads to a profound increase in RIP3 expression, which is abolished by inhibition of PI3K or Akt, and increased PI3K-Akt signaling by expression of a constitutively active PI3K mutant also elevates RIP3 expression. Importantly, adenosine overexpression of RIP3 not only triggers apoptosis but also causes cycle arrest at G1/G0 phases that is associated with suppressed Akt activation. In sharp contrast, RIP3 gene silencing enhances serum- and platelet-derived growth factor-induced cell proliferation and Akt activation. In vivo adenosine gene delivery of rat RIP3 (rRIP3) increased apoptosis and reduced VSMC proliferation, thus, effectively alleviating balloon injury-induced neointimal formation. The growth-suppressive and pro-apoptotic effects are independent of rRIP3 Ser/Thr kinase activity, because overexpression of a kinase-inactive mutant of rRIP3, similar to its wild type, is sufficient to induce growth arrest and apoptosis. These findings reveal a novel growth-suppressive action of RIP3, marking RIP3 as an important factor to prevent excessive mitogenic stimulation- or injury-induced vascular smooth muscle cell hyperplasia.

To maintain tissue homeostasis, eukaryotic cells must keep a balance of cell proliferation and cell death in response to various sources of injury or stress stimuli. Cell hyper-proliferation has long been considered as an important etiological factor of cardiovascular diseases and cancer. Vascular smooth muscle cells (VSMCs) are normally maintained in a non-proliferative state in the arterial tunica media. But arterial injury, inflammation, or excessive mitogenic stimulation triggers transmigration of VSMCs from the media into the intima layer of the arterial wall, where the VSMCs proliferate and synthesize extracellular matrix proteins, resulting in expansion of the arterial intima, i.e. neointimal formation (1–3). Proliferation of neointimal VSMCs is the most common causes of severe cardiovascular diseases such as hypertension, ischemic heart disease, and subsequent myocardial infarction, strokes, and congestive heart failure (4).

Multiple factors, including growth factors, neurohormones, inflammatory cytokines, and reactive oxygen species, have been implicated in vascular proliferative disorders. For instance, platelet-derived growth factor (PDGF) plays a pivotal role in restenosis (5–8). In addition, both angiotensin-converting enzyme and angiotensin II (Ang II) type 1 receptor are up-regulated in the balloon-injured arteries and contribute to neointimal formation and resultant restenosis (9–13). Although phosphoinositide 3-kinase (PI3K)-Akt (or protein kinase B) signaling cascade was originally identified as a viral signaling pathway for tumorogenesis (14), the PI3K-Akt axis is the common pathway activated by a wide variety of mitogenic stimuli, including PDGF and Ang II, and plays a pivotal role in cell proliferation and cell survival (15–18). It has been shown that Akt-mediated phosphorylation of proapoptotic members of the Bcl-2 protein family, including Bad and Bax, prevents translocation of those proapoptotic molecules from the cytoplasm to the mitochondria, thereby inhibiting apoptotic cell death (19, 20).

Over the past decade increasing evidence has placed the PI3K-Akt signaling cascade at the center of pathways for a wide

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* This work was supported, in whole or in part, by the National Institutes of Health Intramural Research Program, NIA (to K.-H. C. and R.-P. X.). This work was also supported by Peking University 985 Project and Chinese 973 Program (2007CB512100) (to Q. Li, G. Li, X. L., M. Z., C.-M. C, and R.-P. X).

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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4 The abbreviations used are: VSMC, vascular smooth muscle cell; PDGF, platelet-derived growth factor BB; Ang II, angiotensin II; PI3K, phosphoinositide 3-kinase; RIP, receptor-interacting protein; rRIP3, rat RIP3; m.o.i., multiplicity of infection; PCNA, proliferating cell nuclear antigen; TUNEL, terminal transferase-mediated dUTP nick end-labeling; siRNA, short interfering RNAs; WKY, Wistar Kyoto; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; Adv, adenosine; GFP, green fluorescent protein; FBS, fetal bovine serum.
array of cardiovascular disorders, including injury-associated arterial restenosis, hypertensive vascular proliferation, cardiac hypertrophy and heart failure, angiogenesis, and endothelial dysfunction (21–24). In particular, Akt is highly activated in response to arterial injury and contributes to neointimal formation (25–28). Thus, novel control points in the PI3K-Akt signaling pathway represent potentially important therapeutic targets.

As essential sensors to various cellular stress signals, receptor-interacting protein (RIP) family, a group of Ser/Thr protein kinases, consists of seven members, including the relatively well characterized RIP (or RIP1), RIP2 (RICK/CARDIAK), and RIP3 (RIPK3), that have been implicated in the regulation of cell survival and cell death (29). RIP family members share a highly homologous amino-terminal serine-threonine kinase domain but possess distinctly different carboxyl termini (29). Previous studies have demonstrated that RIP1 is obligated to tumor necrosis factor receptor-1-mediated NF-κB activation and induces apoptosis and necrosis when overexpressed (30, 31), whereas RIP1 deficiency prevents tumor necrosis factor-mediated activation of NF-κB and enhances cell death (32). A large body of evidence suggests that RIP3 is also essentially involved in the tumor necrosis factor receptor-1 signaling pathway (33–36) and that RIP3 binds to RIP1 and then exerts a potent apoptotic effect by interacting with some procaspases or by attenuating RIP1- and tumor necrosis factor receptor-1-mediated NF-κB activation through phosphorylation of RIP1(37). Moreover, in response to death receptor ligands, RIP3 can mediate both caspase-dependent and -independent apoptosis as well as NF-κB activation (38). However, this perception has been challenged by the fact that in RIP3-deficient cells or mice, tumor necrosis factor-induced NF-κB activation and cell apoptosis are intact (39). It is noteworthy that assembly of the RIP1-RIP3 complex can switch cell death from programmed apoptosis to programmed necrosis in certain cell types (40–42). Nevertheless, the biological function and the underlying mechanism of RIP3 remain largely elusive.

Here we demonstrate that mitogenic stimulation markedly elevates RIP3 expression via a PI3K/Akt-dependent mechanism and that overexpression of RIP3 suppresses VSMC proliferation and promotes apoptotic cell death by inhibiting the PI3K-Akt signaling pathway. These findings suggest that RIP3 functions as an activation-dependent negative regulator of the PI3K-Akt axis, contributing to the maintenance of the normal growth rate of VSMCs and, thus, preventing vascular proliferative disorders.

**EXPERIMENTAL PROCEDURES**

**Materials**—PDGF, Ang II, and antibodies reacting with rat RIP3 (rRIP3) (catalogue #R4277) or β-actin were purchased from Sigma. Antibodies reacting with phosphorylated Akt at Ser-473 or total Akt were purchased from Cell Signaling Technology. LY294002 and wortmannin were purchased from Calbiochem and Tocris, respectively. A cell death ELISA kit was purchased from Roche Applied Science. 

**Adenoviral Constructs**—Replication-defective adenoviruses encoding the complete rat RIP3 open reading frame (Adv-rRIP3) or a kinase-inactive mutant that was produced by PCR amplification of amino acids from 291 to 478 (Adv-rRIP3-C) was constructed by homologous recombination as described in our previous studies (43). Adenoviral vector expressing GFP (Adv-GFP) was made as a control virus. Standard viral amplification and CsCl purification methods were utilized to amplify and purify these adenoviruses. The titer for each adenovirus was determined by dilution assay in HEK293 cells.

**Animals**—Adult male Wistar Kyoto (WKY) rats were supplied by the Center for Experimental Animals (an Assessment and Accreditation of Laboratory Animal Care-accredited experimental animal facility) at Peking University, Beijing, China. All procedures involving experimental animals were performed in accordance with protocols approved by the committee for animal research of Peking University, China, or from the animal facility at NIA of the National Institutes of Health and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1985).

**Primary VSMC Culture and Adenoviral Infection**—VSMCs from WKY thoracic aorta specimens were isolated using a standard enzymatic digestion technique, as described previously (43). VSMCs were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS, Invitrogen) (the serum medium). VSMC synchronization was achieved by culturing cells in Dulbecco’s modified Eagle’s medium with 0.2% FBS for 48 h. Adenoviral infection was then implemented by adding an indicated titer of Adv-rRIP3, Adv-rRIP3-C, or Adv-GFP. Infection of cultured VSMCs was highly efficient, as evidenced by ∼100% GFP-positive cells for each adenoviral vector at 50 multiplicity of infection (m.o.i.) for only 24 h.

**Cell Counting**—The growth curves of VSMCs were examined using cell counting. Cells were first underwent mitogen
Akt with a specific inhibitor (10^{-6} versus pretreated with LY294002 (10^{-6}) wortmannin significantly attenuates a PDGF-induced increase in rRIP3 protein level. Quiescent cells were untreated or LY294002-treated groups; *GFP or Adv-CA-PI3K (100 m.o.i. for 48 h) (*, whereas the expression. Quiescent cells were pretreated with LY294002 (10^{-6}) or wortmannin (50 nM) for 30 min, then incubated with PDGF (10 ng/ml) for 6 h (*, p < 0.01 versus control and cells treated with either PI3K inhibitor; †, p < 0.01 versus PDGF; n = 4 for each group). B, inhibition of PI3K with either LY294002 or wortmannin significantly attenuates a PDGF-induced increase in rRIP3 protein level. Quiescent cells were pretreated with LY294002 (10^{-6}) or wortmannin (50 nM) for 30 min, then incubated with PDGF (10 ng/ml) for 6 h (*, p < 0.01 versus control and cells treated with either PI3K inhibitor; †, p < 0.01 versus PDGF; n = 4 for each group). C, adenoviral expression of a constitutively activated PI3K mutant increases the rRIP3 mRNA level (*, p < 0.01 versus Adv-GFP-infected group). D, expression of the constitutively active PI3K mutant elevates rRIP3 protein abundance. The top shows typical Western blots of total (t-) or phosphorylated (p-) Akt and rRIP3, whereas the bottom illustrates the average data of rRIP3 expression. Quiescent cells were infected with Adv-GFP or Adv-CA-PI3K (100 m.o.i. for 48 h) (*, p < 0.01 versus Adv-GFP, n = 3 for each group). E and F, inhibition of Akt with a specific inhibitor (10^{-6}) attenuates PDGF- or Ang II-induced up-regulation of rRIP3. Cells were pretreated with the Akt inhibitor for 30 min, then treated with PDGF (10 ng/ml) for 6 h or Ang II (100 nM) for 12 h (†, p < 0.05 versus control and Akt inhibitor; †, p < 0.05 versus PDGF or Ang II; n = 4 for each group).

**FIGURE 2. Activation of PI3K is both necessary and sufficient for PDGF- and Ang II-mediated up-regulation of rRIP3.** A, inhibition of PI3K with LY294002 (10^{-6}) blocks PDGF- and Ang II-induced increases in rRIP3 expression. Quiescent cells were pretreated with LY294002 (10^{-6}) for 30 min, then incubated with PDGF (10 ng/ml) for 4 h or Ang II (100 nM) for 6 h. rRIP3 mRNA levels were assayed by real time PCR (*, p < 0.01 versus untreated or LY294002-treated groups; n = 3 for each group). B, inhibition of PI3K with either LY294002 or wortmannin significantly attenuates a PDGF-induced increase in rRIP3 protein level. Quiescent cells were pretreated with LY294002 (10^{-6}) or wortmannin (50 nM) for 30 min, then incubated with PDGF (10 ng/ml) for 6 h (*, p < 0.01 versus control and cells treated with either PI3K inhibitor; †, p < 0.01 versus PDGF; n = 4 for each group). C, adenoviral expression of a constitutively activated PI3K mutant increases the rRIP3 mRNA level (*, p < 0.01 versus Adv-GFP-infected group). D, expression of the constitutively active PI3K mutant elevates rRIP3 protein abundance. The top shows typical Western blots of total (t-) or phosphorylated (p-) Akt and rRIP3, whereas the bottom illustrates the average data of rRIP3 expression. Quiescent cells were infected with Adv-GFP or Adv-CA-PI3K (100 m.o.i. for 48 h) (*, p < 0.01 versus Adv-GFP, n = 3 for each group). E and F, inhibition of Akt with a specific inhibitor (10^{-6}) attenuates PDGF- or Ang II-induced up-regulation of rRIP3. Cells were pretreated with the Akt inhibitor for 30 min, then treated with PDGF (10 ng/ml) for 6 h or Ang II (100 nM) for 12 h (†, p < 0.05 versus control and Akt inhibitor; †, p < 0.05 versus PDGF or Ang II; n = 4 for each group).

quiescence by serum starvation and viral infection with Adv-GFP, Adv-rRIP3-C, or Adv-rRIP3 at 100 m.o.i. for 24 h. The cell number under these experimental conditions was used as the base line. To examine the status of VSMC proliferation, the cells were subsequently stimulated with serum (10% FBS) or PDGF (10 ng/ml), and the cell number was counted daily to day 6 after infection (Fig. 3C). Each count was an average of three repeats, whereas each data point was the average of five experiments.

**MTT Assay—**VSMCs seeded in 96-well plates underwent mitogenic quiescence by serum starvation for 24 h. The cells were then infected with adenovirus for 24 h followed by serum (10% FBS) stimulation. Cell proliferation was assayed by the cleavage of the tetrazolium salt MTT. After cells were incubated with MTT for 4 h at 37 °C, the culture medium was removed, and the cells were solubilized in 200 μl of DMSO. The cleavage of the tetrazolium salt MTT was quantified spectrophotometrically at 490 nm with background subtraction at 630 nm, as described previously (43).

**Rat Carotid Artery Injury Model and Adenoviral Gene Transfer—**Balloon dilation of the left common carotid artery of male WKY rats (350–400 g) was performed as previously described (43). Briefly, a 2F embolectomy balloon catheter was inserted into the left common carotid artery via the external carotid artery. Then the inflated balloon was drawn gently toward the external carotid artery. After repeating this procedure three times, the catheter was then removed. The injured artery was washed with phosphate-buffered saline and incubated with 30 μl of adenoviral vectors (5 × 10^{9} plaque-forming units/ml) expressing GFP or rRIP3 for 20 min. Arteries were collected at indicated times and embedded in paraffin to prepare cross-sections. Subsequent morphological analyses were performed in a double-blind manner.

**Immunohistochemical Staining—**Immunostaining of sections from injured rat carotid arteries in the presence of Adv-rRIP3 or Adv-GFP infection was performed with a rabbit anti-rRIP3 antibody (1:250 dilution) following the manufacturer’s instructions at the indicated time points after balloon injury. The same sections were also counterstained with hematoxylin.

**PCNA Assay in Injured Carotid Artery—**To assess the possible effect of rRIP3 gene transfer on VSMC proliferation in vivo, a PCNA immunohistochemical assay was performed 1 week after injury in sections from injured rat carotid arteries infected with Adv-rRIP3 or Adv-GFP using mouse anti-PCNA monoclonal antibody (1:200 dilution) as previously described (43). Sections were also counterstained with hematoxylin. The PCNA-positive cells were determined by cell counts under light microscopy using a computer-based Image-Pro Morphometric System by two independent observers in a double-blind manner.

**Real Time Quantitative PCR—**To quantitate the expression of rRIP3 in response to proliferative stimuli, synchronized VSMCs were stimulated by PDGF (10 ng/ml) or Ang II (10^{-7} M) in 0.2% FBS-containing Dulbecco’s modified Eagle’s medium. Cells were collected at the indicated time points, and RNA isolation and reverse transcription were performed. Real time quantitative PCR was then performed using the DNA Engine Opticon system (MJ Research) in combination with SYBR Green dye (Roche Applied Science) as described previously (43). The primers for rRIP3 were 5’-CTTGAAACCTTTGCC-TACTGC-3’ and 5’-CGAGGACCCTTCA-ACTTGA-3’ as forward and the reverse, respectively. The relative mRNA level of RIP3 was normalized by 18 S levels. Real time PCR data were the average of three independent experiments, whereas each experiment was in triplicate.
Western Blot Analysis—Proteins were prepared from cultured VSMCs or arteries. For Western blot analysis, proteins were electrophoresed on 10% SDS-PAGE and transferred to the polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween 20 (TBS-T) and incubated overnight with an antibody reacting with rRIP3, β-actin, phosphorylated Akt, or total Akt in 4 °C overnight. The membrane was washed and then incubated with secondary antibody conjugated with horseradish peroxidase in 5% nonfat milk in TBS-T buffer for 1–2 h. Detection was carried out using a chemiluminescence detection kit (Cell Signaling Technology).

Cell Death ELISA and DNA Laddering—Cellular DNA fragmentation was quantified by the Cell Death ELISA (Roche Applied Science), which measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation, following the manufacturer’s instruction. 2 × 10⁴ control or infected VSMCs were used for each test. DNA fragmentation was also assayed by DNA laddering as described (44). Briefly, 1.5 μg of DNA was loaded in each lane, size-fractioned on a 1.5% agarose gel in Tris-acetate-EDTA buffer, and then stained with ethidium bromide (Invitrogen).

Propidium Iodide Staining and Fluorescence-activated Cell Sorter Analysis—VSMCs were synchronized and infected with adenoviral vectors for 48 h and subsequently stimulated by serum to proliferate as described above. After adding serum for 18 h, the cells were harvested and fixed overnight with 70% ethanol at −20 °C. The cells were then pelleted, resuspended in 0.5 ml of phosphate-buffered saline containing RNase (100 μg/ml), incubated at 37 °C for 30 min, stained for 10 min at room temperature with propidium iodide (PI) and analyzed by fluorescence-activated cell sorter (BD Biosciences FACScan). G, shown are the average data of the percent of cells in G₀/G₁ phases (†, p < 0.01 versus Adv-GFP; n = 3).

FIGURE 3. Overexpression of rRIP3 suppresses serum- and PDGF-induced VSMC growth and leads to cell cycle arrest at G₀/G₁ phases. A, rRIP3 protein level was assayed by Western blotting using an anti-RIP3 with total cellular proteins from cultured VSMCs infected with Adv-rRIP3 or Adv-GFP (100 m.o.i. for 24 h). B and C, shown is the inhibitory effect of rRIP3 on serum- and PDGF-stimulated VSMC growth. Quiescent VSMCs were infected with Adv-rRIP3 or Adv-GFP (100 m.o.i. for 24 h) and subsequently exposed to the serum medium (10% FBS) or PDGF (10 ng/ml). Cell numbers were determined with a hemocytometer at indicated time points (n = 5 each with triplicates; †, p < 0.01 versus Adv-GFP). D and E, overexpression of rRIP3 inhibits serum- and PDGF-stimulated VSMC proliferation assayed by MTT. Quiescent cells were infected with Adv-GFP or Adv-rRIP3 and then stimulated with 10% FBS for indicated time (†, p < 0.01 versus uninfected or Adv-GFP; n = 3). F, shown are typical examples of cell cycle distribution in VSMCs infected with Adv-GFP or Adv-rRIP3 (100 m.o.i. for 24 h) and stimulated by serum for 18 h. Then cells were stained with propidium iodide (PI) and analyzed by fluorescence-activated cell sorter (BD Biosciences FACScan). G, shown are the average data of the percent of cells in G₀/G₁ phases (†, p < 0.01 versus Adv-GFP; n = 3).
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According to the manufacturer’s protocol (Invitrogen). The efficiency of gene knockdown was detected by Western blotting and functional studies at 72 h after adenoviral siRNA transfection.

Statistic Analysis—All data are expressed as the mean ± S.E. Comparisons among groups were analyzed by 1-way analysis of variance. A Student’s *t* test was used for differences between two groups. Differences were considered statistically significant at a value of *p* < 0.05.

RESULTS

Cloning rRIP3—Using differential display techniques, we cloned a cDNA that was highly expressed in primary cultured rat VSMCs subjected to homocysteine (1.0 mM for 24 h), known as an independent risk factor for atherosclerosis (45), and weakly expressed in untreated VSMCs. Using cDNA library screening and a 5’-rapid amplification of cDNA ends reaction, we cloned the full-length cDNA (GenBank™ no. NM_139342) consisting of 15 bp before a poly(A) tail and encoding 478 amino acids (supplemental Fig. S1A). Sequence analysis revealed that the cloned cDNA shares a high sequence homology with human RIP3 (hRIP3) and mouse RIP3 (mRIP3) (supplemental Fig. S1B). Thus, we named this gene as rat RIP3 (rRIP3).

Mitogenic Stimulation Increases rRIP3 Expression—Mitogenic stimuli, including various growth factors and neurohormones, play a central role in the pathogenesis of vascular proliferative disorders. To investigate the potential function of rRIP3 in vascular biology and pathophysiology, we examined rRIP3 gene expression in response to a prototypical growth factor, PDGF-BB, or a clinically relevant neurohormone, Ang II, in primary cultured WKY rat VSMCs. Both PDGF (10 ng/ml) and Ang II (100 nM) markedly elevated rRIP3 gene expression in response to a prototypical growth factor, PDGF-BB, or a clinically relevant neurohormone, Ang II, in primary cultured WKY rat VSMCs. Both PDGF (10 ng/ml) and Ang II (100 nM) markedly elevated rRIP3 gene expression in response to a prototypical growth factor, PDGF-BB, or a clinically relevant neurohormone, Ang II, in primary cultured WKY rat VSMCs. 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respective receptors activates multiple downstream signaling events, including PI3K- and MAPK-mediated signaling pathways. To determine whether PI3K signaling is required for these mitogenic stimuli-induced up-regulation of rRIP3, we treated cells with mechanistically different PI3K inhibitors, either LY294002 or wortmannin. Pretreatment of cells with LY294002 (10 µM) fully abolished a PDGF- or Ang II-mediated increase in rRIP3 mRNA level (Fig. 2A) and markedly blunted PDGF-induced elevation in rRIP3 protein abundance without altering its basal level (Fig. 2B). Likewise, wortmannin (50 nM) also attenuated a PDGF-induced increase in rRIP3 protein level (Fig. 2B). These results indicate that PI3K activation is essentially involved in both mitogenic stimuli mediated rRIP3 up-regulation in primary cultured VSMCs.

Next, we determined whether activation of the PI3K signaling pathway per se is sufficient to elevate rRIP3 expression in VSMCs. Adenoviral gene transfer of a constitutively active PI3K mutant (Adv-CA-PI3K at 100 m.o.i.) clearly increases phosphorylation of Akt (Fig. 2D), a key downstream protein kinase of PI3K (Fig. 2D), indicating enhanced activation of PI3K-Akt signaling. Importantly, enhanced PI3K-Akt signaling augmented rRIP3 expression at both mRNA (Fig. 2C) and protein levels (Fig. 2D), whereas inhibition of Akt activity with a specific inhibitor (46) significantly attenuated PDGF- and Ang II-induced up-regulation of rRIP3 (Fig. 2, E and F). Although both mitogenic agents increased ERK1/2 activation in VSMCs, inhibition of ERK1/2 with PD98059 did not alter the effects of either PDGF or Ang II on rRIP3 expression (data not shown).

Overexpression of rRIP3 Induces VSMC Growth Arrest and Inhibits Akt Activation—We next examined the potential functional consequence of rRIP3 up-regulation in cultured VSMCs and explored the underlying mechanism. Adenoviral gene transfer of rRIP3 led to a titer-dependent overexpression of rRIP3 in cultured VSMCs (Fig. 3A). For instance, infection of cells with Adv-rRIP3 at a 100 m.o.i. for 24 h increased rRIP3 protein abundance by 6-fold over base line, a level similar to that induced by PDGF (10 ng/ml) treatment (Figs. 1C and 2B). Serum- or PDGF-induced cell growth was markedly suppressed in VSMCs infected with Adv-rRIP3 (100 m.o.i. for 24 h) relative to the control group (Adv-GFP) (Fig. 3, B and C, respectively). The growth suppressive effect of rRIP3 was also confirmed by a MTT assay (Fig. 3, D and E).

In principle, rRIP3-induced growth suppression might be mediated by either blocking cell cycle or increasing cell death or both. To delineate these possibilities, we examined the possible effect of rRIP3 on cell cycle and found that overexpression of rRIP3 markedly blocked the VSMC cell cycle at the G0/G1 phases. In the quiescent VSMCs, 90% of VSMCs were in the G0/G1 phases of the cell cycle. Serum stimulation enabled the majority of the cells to enter the cell cycle, reducing the percentage of G0/G1 cells from 90 to 29% at 18 h after serum stimulation in the control group (Fig. 3, F and G). In contrast, infection VSMCs with Adv-rRIP3 blocked the cell cycle in a titer-dependent manner (Fig. 3, F and G).

To define the mechanism responsible for rRIP3-induced growth arrest, we investigated the possible role of rRIP3 in regulating Akt signaling. Serum (10% FBS) or PDGF (10 ng/ml) induced a robust increase in Akt phosphorylation at Ser-473, which was effectively suppressed by adenoviral overexpression of rRIP3 (Fig. 4 A and B).

rRIP3 Gene Silencing Augments Mitogenic Stimulation-induced Akt Activation and Cell Proliferation—The aforementioned results indicate a negative feedback regulation of the PI3K-Akt axis by rRIP3, implying that knockdown of rRIP3 would, therefore, result in enhanced Akt activation and proliferation in response to mitogenic stimulation. To test this hypothesis, we utilized an siRNA-mediated gene silencing technique to knock down rRIP3. Adenoviral gene transfer of rRIP3 siRNA markedly suppressed endogenous rRIP3 expression and prevented PDGF-mediated up-regulation of rRIP3 (supplemental Fig. S2). Indeed, knockdown of rRIP3 profoundly enhanced PDGF-induced Akt phosphorylation (Fig. 4C) and PDGF- or serum-stimulated cell proliferation (Fig. 4, D and E). Taken together, the present results establish the negative feedback loop; although enhanced PI3K-Akt signaling increases rRIP3 expression, the latter functions as an activation-dependent negative feedback to suppress Akt-mediated cell proliferation, implying that rRIP3 plays an important role in preventing excessive Akt signaling and cell hyperplasia.

Overexpression of rRIP3 Triggers VSMC Apoptosis—Increasing evidence indicates that mitogenic stimulation-dependent cell survival is mediated mainly by activation of the PI3K-Akt signaling cascade. The inhibitory effect of rRIP3 on Akt activation would be expected to block Akt-mediated cell survival.
Indeed, rRIP3 overexpression led to robust VSMC apoptosis, as evidenced by DNA fragmentation assayed by DNA laddering (Fig. 5A) and cell death ELISA (Fig. 5B), consistent with previous studies on other cell types (33–36). Next, we envisioned that normalizing Akt activation by adenoviral gene transfer of the constitutively active PI3K mutant might protect cells from rRIP3-induced cell death. As expected, rRIP3-induced apoptosis was fully abolished by infection of cells with Ad-CA-PI3K (Fig. 5C). In addition, adenoviral gene transfer of Bcl-xL, an anti-apoptotic member of Bcl-2 family, also effectively prevented rRIP3-mediated apoptotic VSMC death (Fig. 5, D), substantiating that overexpression of rRIP3-induced VSMC death is mainly caused by apoptosis.

Ser/Thr Kinase Activity Is Not Essential for rRIP3-mediated Growth Arrest and Apoptosis—All RIP family members, including RIP3, share a highly homologous amino-terminal serine-threonine kinase domain and exhibit protein kinase activity (29). Previous studies have shown that the kinase activity of RIP3 contributes to its necrotic effect (40), whereas other studies suggest that the kinase activity is not necessary for its apoptotic effect (34). To determine whether the kinase activity is involved in rRIP3-induced growth arrest and apoptosis, we made a kinase-inactive mutant of RIP3 (rRIP3-C, which lacks the kinase domain). Adenoviral gene transfer of RIP3-C (Adv-rRIP3-C) and the wild type rRIP3 (Adv-rRIP3) exhibited a similar titer-dependent expression of rRIP3-C and the wild type rRIP3 (Fig. 6A). Expression of rRIP3-C suppressed PDGF-induced VSMC growth, examined by cell counting (Fig. 6B) and MTT assay (Fig. 6C), and triggered profound apoptotic VSMC death detected by cell death ELISA (Fig. 6D). It is noteworthy that the growth arrest and pro-apoptotic effects of rRIP3-C were comparable with those induced by overexpression of the wild type rRIP3 (Fig. 6, B–D). These results indicate that the kinase activity is not essential for rRIP3-mediated cell growth suppression and apoptosis.

Up-regulation of rRIP3 in Balloon-injured Arterial Neointima—Although in using multiple assays we demonstrate that mitogenic stimuli elevate rRIP3 expression via a PI3K-Akt-dependent mechanism, we wish to address whether the same regulation holds true in \textit{in vivo} and, if so, to explore its potential physiological and pathological relevance. We investigated the expression profile of rRIP3 in rat balloon-injured arteries. Real time PCR and Western blotting assays revealed that balloon injury-induced neointimal formation was associated with elevated rRIP3 expression at mRNA and protein levels (Fig. 7, A–C). Interestingly, there was a biphasic temporal profile of rRIP3 gene expression after balloon injury with the first peak occurring 8 h after injury and the second peak detected 1 week after injury (Fig. 7A). Moreover, the up-regulation of rRIP3 protein in balloon-injured arteries also exhibited a biphasic temporal profile with the first and second peaks occurring 1 day and 1 week after the surgery, respectively (Fig. 7B). To determine whether balloon injury-induced up-regulation of rRIP3 gene expression is mediated by a PI3K-dependent mechanism, a subgroup of animals was pretreated with the PI3K inhibitor, wortmannin (30 μg/kg, intravenously twice at 1 h and 5 min, respectively, before balloon injury). Inhibition of PI3K by wortmannin profoundly suppressed balloon-in-
jured mediated up-regulation of rRIP3 gene expression at the time point of 8 h after surgery (Fig. 7C). Thus, atrial injury-induced elevation of rRIP3 expression is mediated by a PI3K-dependent mechanism.

Overexpression of rRIP3 Induces VSMC Apoptosis in Vivo—To further explore the pathological significance of rRIP3 growth suppressive and proapoptotic effects in cultured VSMCs, rat carotid arteries were subjected to balloon injury and simultaneously infected with either Adv-rRIP3 or Adv-GFP, as described previously (43). The efficiency of in vivo adenoviral gene transfer of rRIP3 was tested by immunohistochemical staining with the anti-rRIP3 polyclonal antibody. Four days after the surgery, rRIP3 protein level was significantly increased in arteries infected with Adv-rRIP3 compared with those infected with Adv-GFP (Fig. 8A). On average, rRIP3 protein was increased by 3-fold over baseline (Fig. 8B).

Importantly, the percentage of TUNEL-positive VSMCs was augmented by more than 5-fold in arteries infected with Adv-rRIP3 relative to the control group (n = 10, p < 0.01) (Fig. 8C and D). A PCNA immunohistochemical assay revealed that overexpression of rRIP3 markedly suppressed cell proliferation as manifested by a profound reduction in PCNA-positive cells (Fig. 8, E and F). Thus, rRIP3 overexpression not only increases apoptosis but also suppresses proliferation of VSMCs in culture and in vivo.

Adenovirus-mediated Somatic Gene Transfer of rRIP3 Alleviates Injury-induced Neointimal Formation—Because rRIP3 overexpression suppresses VSMC growth and promotes cell apoptosis, we hypothesized that vascular injury-induced up-regulation of rRIP3 may represent a salutary compensatory mechanism that restricts the neointimal formation of the injured arteries. Indeed, 3 weeks after adenoviral transfection, the ratios of the intima/media and neointimal area in the Adv-rRIP3-infected group were reduced by 40% compared with the control group (n = 6; p < 0.01 versus Adv-GFP; Fig. 8G–I).
and 50%, respectively, relative to that of Adv-GFP-infected group (Fig. 8, G–J), indicating that overexpression of rRIP3 inhibits arterial injury-induced neointimal formation.

**DISCUSSION**

The major finding of the present study is that up-regulation of rRIP3 constitutes a suppressor of VSMC hyperplasia via a negative feedback regulation of the PI3K-Akt signaling axis. This conclusion is based on several lines of evidence. First, mitogenic stimulation- or arterial injury-triggered VSMC proliferation is associated with an elevation of rRIP3 expression in *in vitro* and *in vivo*. Second, adenoviral overexpression of rRIP3 suppresses mitogenic stimulation-mediated Akt phosphorylation and VSMC proliferation and markedly reduces balloon injury-induced neointimal formation. To the contrary, rRIP3 gene silencing augments mitogenic stimuli-induced Akt activation and cell proliferation. These current *in vitro* and *in vivo* data not only reveal a novel growth suppressive action of rRIP3 in addition to its well established role in cell apoptosis but also strongly imply that normal expression and function of rRIP3 is crucially involved in the tight regulation of the PI3K-Akt signaling axis, thus contributing to the maintenance of non-proliferative state of VSMCs under physiological conditions.

**Cellular and Molecular Mechanisms Underlying rRIP3-mediated Suppression of Neointimal Formation—**Proliferative disorders such as cardiovascular disease and tumor might occur if cell proliferation is abnormally enhanced. In this regard, excessive activation of the PI3K-Akt axis has long been implicated as an important cause factor of proliferative disorders, including atherosclerosis, restenosis, and cancer (21, 47). Using cell counting and a MTT assay in conjunction with a PCNA immunohistochemical assay, here we have shown that overexpression of rRIP3 markedly suppresses mitogenic stimuli- and arterial injury-induced VSMC proliferation *in vitro* and *in vivo* via inhibiting the PI3K-Akt signaling pathway. Thus, we have defined rRIP3 as a powerful negative feedback regulator of the PI3K-Akt axis, highlighting an important role of rRIP3 in preventing neointimal formation caused by various etiologies.

Although the exact underlying mechanism is presently unclear, rRIP3 may suppress the PI3K-Akt axis by a couple of potential pathways. These include (a) suppression of the PI3K-Akt signaling by rRIP3-mediated intermolecular interaction and subsequent inhibition of Ras, a small GTPase serving as an important upstream signaling event of the PI3K-Akt axis or (b) counteracting RIP1-dependent PI3K-Akt signaling (48, 49) perhaps via RIP3 physically binding and subsequently phosphorylating RIP1, thereby inhibiting RIP1-mediated activation of PI3K-Akt signaling. It has been shown RIP1 activates PI3K-Akt using dual mechanisms; that is, interruption of the mTOR negative feedback loop and down-regulation of cellular PTEN levels (49). Nonetheless, these potential underlying mechanisms merit future investigation.

In addition to inhibition of cell proliferation, growing evidence has indicated that apoptosis also plays an essential role in the control of neointimal thickening. VSMC apoptosis in both the intima and the media can limit neointimal formation at a defined time point and is inversely correlated with restenosis (50–52). The present study has shown that adenoviral gene transfer of rRIP3 markedly triggers apoptosis of VSMCs in culture and in balloon-injured rat carotid arteries. Thus, the inhibitory effect of RIP3 overexpression on neointimal formation is likely attributable to the increased apoptosis as well as suppressed proliferation of VSMCs. But it is still a matter of debate as to what is the impact of VSMC apoptosis on neointimal formation, as some evidence suggests that early apoptosis of medial VSMCs may increase late neointimal formation *in vivo* (53). Our preliminary data have demonstrated that overexpression of rRIP3 overtly attenuates VSMC migration (data not shown). This newly identified anti-migration effect of rRIP3 may also contribute to its beneficial effect. Altogether, the net outcome of rRIP3 up-regulation is to suppress VSMC hyperplasia and attenuates angioplasty-induced neointima thickening, marking rRIP3 as a potential therapeutic target in treating cardiovascular proliferative disorders.

**Potential Clinical Implications of rRIP3 in the Treatment of Vascular Proliferative Disorders—**The PI3K-Akt axis is one of the major signaling pathways involved in neointimal formation in response to vascular injury, as manifested by enhanced activation of Akt in neointima and alleviation of neointimal formation by inhibiting Akt activity (25–28). In this study we have demonstrated for the first time that expression of rRIP3 is abundantly elevated by balloon injury via a PI3K-dependent mechanism and that overexpression of rRIP3 using somatic adenoviral gene delivery effectively alleviates balloon injury-induced neointimal formation. These findings indicate that an up-regulation of rRIP3 serves as a negative feedback to negate injury-evoked vascular VSMC growth and lesion formation, likely representing a salutary adaptation of arteries in response to injury or exaggerated proliferative signals.

In summary, we have shown for the first time that rRIP3 is up-regulated by mitogenic stimulation and arterial injury and subsequently constitutes a negative feedback regulator of the PI3K-Akt axis in primary cultured rat VSMCs and *in vivo*. Importantly, overexpression of rRIP3 markedly attenuates balloon injury-induced neointimal formation in rat carotid arteries, suggesting that rRIP3 might represent a clinically important therapeutic target in cardiovascular proliferative diseases.

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