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Adiponectin Induces Vascular Smooth Muscle Cell Differentiation via repression of mTORC1 and FoxO4

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Abstract

Objective—The adipocyte-secreted hormone adiponectin exerts important cardioprotective and anti-diabetic effects. Little is known about its effect on vascular smooth muscle cells (VSMC), key cells in restenosis, hypertension, and atherosclerosis.

Methods and Results—Using human coronary artery VSMC, we report that recombinant adiponectin in the HMW or trimeric, but not globular forms induces VSMC differentiation through a mechanism similar to the classic feedback signaling employed by rapamycin, a drug known to effectively inhibit restenosis on drug-eluting stents (DES). Using a combination of pharmacologic agents, siRNA, and overexpression approaches, we demonstrate that adiponectin activates 5′ AMP-activated protein kinase (AMPKα2), leading to inhibition of mammalian target of rapamycin complex 1 (mTORC1) and S6K1. This in turn stabilizes IRS-1, driving Akt2 - mediated inhibition of FoxO4 and subsequent contractile protein induction. While adiponectin and rapamycin have similarly beneficial effects on VSMC phenotype in both cell and organ culture, a direct comparison of the effects of rapamycin versus adiponectin on endothelial cells (EC) revealed distinct differences: rapamycin inhibited, while adiponectin maintained, Akt phosphorylation. Importantly, Akt activity preserves endothelial function.

Conclusions—Adiponectin promotes VSMC differentiation and preserves EC Akt signaling, suggesting that targeting the adiponectin pathway may have advantages over rapamycin in developing new DES therapeutics.

Keywords

Adiponectin; VSMC; differentiation; mTOR; rapamycin; AMPK; Akt2; FoxO4

Introduction

Restenosis is a frequent complication of vascular interventions including bypass grafts, angioplasty, and stenting. Endothelial injury and VSMC phenotypic modulation contribute to this intimal hyperplastic response[1]. Rapamycin-eluting stents dramatically reduce the incidence of coronary restenosis, but have recently been associated with late-stent

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thrombosis, a potentially fatal complication[2,3]. While the underlying mechanisms are still emerging, rapamycin inhibition of re-endothelialization may contribute to late-stent thrombosis[4].

Recent studies revealed that low adiponectin levels can predict late in-stent restenosis[5] and increase cardiovascular disease risk[6]. Adiponectin is a 30 kDa hormone produced by white adipose tissue in inverse proportion to fat mass[7]. Downstream signaling through its known receptors, AdipoR1, AdipoR2[8] and T-Cadherin[9], remains poorly understood. Adiponectin mediates multiple cardioprotective effects, as adiponectin knockout mice exhibit increased neointimal formation and thrombus formation post-injury[10,11], higher blood pressure[12], and impaired recovery from hind-limb ischemia[13]. They also develop increased myocardial infarct (MI) size[14], increased cardiac hypertrophy with pressure overload[15], and exacerbated left ventricular dilation and dysfunction following MI[16]. Adiponectin also exerts anti-inflammatory and anti-diabetic effects[17]. While several studies have documented beneficial adiponectin signaling in endothelial cells[13,18–20], few have assessed the effects of the hormone on VSMC.

VSMC in mature vessels retain remarkable phenotypic plasticity. VSMC dedifferentiation contributes to intimal hyperplasia, atherosclerosis and hypertension[21]. Normal mature VSMC are differentiated, quiescent and contractile, while injured VSMC exhibit a proliferative, dedifferentiated, synthetic phenotype. Differentiation markers include contractile proteins smooth muscle myosin heavy chain (SM-MHC), SM α-actin, calponin, and h-caldesmon. Dedifferentiated VSMC lose these markers and upregulate extracellular matrix synthesis[21].

We previously showed that rapamycin promotes VSMC differentiation via inhibition of mTORC1 and its effector S6 kinase1(S6K1)[22,23]. Herein, we test the hypothesis that adiponectin promotes VSMC differentiation through AMPK-mediated inhibition of mTORC1. As late stent thrombosis may be associated with mTORC1 inhibition[2,3], we also conduct a direct comparison of the effects of adiponectin versus rapamycin in human endothelial cells.

Materials and Methods

All cell culture experiments employ human coronary artery smooth muscle cells purchased from Cascade Biologics (Portland, OR). Transfections and western blot analysis were performed using standard methods as previously published[22–24]. Please see detailed Materials and Methods in supplemental data.

Results

Adiponectin induces VSMC differentiation

To determine the effects of recombinant human adiponectin on VSMC phenotypic modulation, we employed a human coronary artery VSMC culture model. These cells display a synthetic, proliferative phenotype similar to VSMC in intimal hyperplastic lesions. We have previously reported that rapamycin or prostacyclin analogs can induce VSMC differentiation in this system[22–24]. Adiponectin exists in multiple oligomeric forms, including trimeric, hexameric, high molecular weight (HMW), and truncated globular forms in vivo[25]. We examined the effects of different oligomeric preparations on VSMC differentiation. Treatment with a preparation of full length adiponectin enriched in HMW oligomers (12–18mer, ~360–540 kDa) or with trimeric adiponectin (~90 kDa) induced expression of contractile protein markers of VSMC differentiation, including SM-MHC (SM2 isoform), h-caldesmon, calponin, and SM α-actin (Figure 1A). Trimeric adiponectin

was slightly less potent than the HMW preparation. Conversely, the truncated globular form did not efficiently induce contractile protein expression (Figure 1A). We next determined that the HMW-enriched adiponectin promotes VSMC differentiation at concentrations (1–10 μg/ml) (Supplemental Figure IA) within the physiologic range of serum adiponectin levels in healthy people (2 to 17 μg/ml)[7]. We therefore employed 5μg/ml HMW-enriched adiponectin in all subsequent experiments.

Treating VSMC with adiponectin induced VSMC-specific differentiation marker expression in a time-dependent manner (Figure 1B, Supplemental Figure IB). Adiponectin also activated AMPK over time, as measured by increased AMPK-α subunit phosphorylation (Thr172) and confirmed bythe increased phosphorylation of the AMPK substrate acetyl-CoA carboxylase (ACC) (Ser79) (Figure 1B). Adiponectin concomitantly inhibited mTORC1 signaling as evidenced by the reduced phosphorylation of the mTORC1 substrate S6K1 (Thr389), as well as reduced phosphorylation of the S6K1 substrate ribosomal S6 (Ser240/244) (Figure 1B). We have previously shown that rapamycin inhibition of mTORC1 induces VSMC differentiation through relief of the S6K1-mediated feedback inhibition of IRS-1, leading to Akt activation[23]. S6K1-mediated serine phosphorylation of IRS-1 promotes its ubiquitination and degradation, attenuating signal transduction to PI3K and Akt[23]. Like rapamycin, adiponectin also stabilized IRS-1 expression and induced Akt phosphorylation (Ser473) (Figure 1B).

AMPK is necessary for adiponectin-induced differentiation

AMPK has been implicated as a key effector of adiponectin signaling in multiple other cell types[18,25,26]. To determine whether adiponectin induces VSMC differentiation via AMPK, we pretreated VSMC with compound C, a specific inhibitor of AMPK, prior to adiponectin treatment. Compound C inhibited adiponectin-induced AMPK and ACC phosphorylation, as well as contractile protein expression (Figure 2A, Supplemental Figure II). We confirmed this result using siRNA to knockdown the AMPK catalytic subunit isoforms α1 or α2. Notably, only AMPKα2 appears to be required for adiponectin-induced differentiation (Figure 2B). An increase in contractile proteins was noted upon AMPKα1 knockdown, suggesting it may potentially inhibit contractile protein expression. However, treating VSMC with AICAR (5-Aminoimidazole-4-carboxamide-1-b-riboside), a pharmacologic activator of AMPK (which is not known to exert an AMPKα isoform-specific effect), inhibited mTORC1, activated Akt and induced contractile protein expression in a time-dependent manner (Figure 2C). These data indicate that AMPKα2 activation is necessary for adiponectin-induced VSMC differentiation and that AMPK activation is sufficient to induce differentiation.

Adiponectin induces differentiation via mTORC1/S6K1 inhibition

AMPK regulates cellular energy homeostasis by repressing energy-consuming processes, including protein synthesis, while simultaneously enhancing energy-producing processes[27]. Through phosphorylation of the TSC1/2 complex, AMPK inhibits mTORC1 activity and protein synthesis in skeletal muscle[27]. Since we previously reported that mTORC1 inhibition with rapamycin induces VSMC differentiation[22,23], we next determined whether mTORC1 pathway inhibition is required for adiponectin-induced differentiation. We infected VSMC with an adenovirus encoding GFP alone (control) or GFP and an HA-tagged rapamycin-resistant constitutively active S6K1 mutant (S6K1-ED_3E). This mutant is sufficient to block rapamycin-induced VSMC differentiation, IRS-1 stabilization, and Akt activation[22]. This mutant S6K1 also inhibited adiponectin-induced VSMC differentiation (Figure 3), suggesting that adiponectin inhibition of S6K1 is required for this response. Replicate experiments using S6K1 plasmid verified these findings (data not shown).

Akt-2 inhibition of FoxO4 is required for adiponectin-induced differentiation

Increasing evidence reveals that Akt1 and Akt2 differentially regulate cell migration and metabolism[28]. We reported that rapamycin specifically activates Akt2, and that Akt2, but not Akt1, is required for rapamycin-induced VSMC differentiation[23]. Similarly, siRNA knockdown of Akt2, but not Akt1, inhibited adiponectin-induced contractile protein expression (Figure 4). Interestingly, adiponectin increased Akt phosphorylation (Ser473) whether Akt1 or Akt2 was knocked down, suggesting that, unlike rapamycin, adiponectin may activate both Akt isoforms (Figure 4). In other experiments, we were able to detect a modest but selective activation of Akt2 using an isoforms-specific immunoprecipitation approach (Supplemental Figure IIIA). We previously found that only Akt2 activity is sufficient to induce contractile protein expression[23]. Adiponectin may activate both Akt1 and Akt2, but it appears that the actions ofAkt2 predominate given the net adiponectin effect on differentiation. Our siRNA data suggest that Akt1 may inhibit contractile protein expression, as Akt1 knockdown increases baseline MHC expression (Figure 4). We report that overexpression of Akt1 does not inhibit basal levels of contractile proteins, but does inhibit their induction by adiponectin or rapamycin (Supplemental Figure IIIB). Finally, we note a compensatory increase in Akt2 expression and phosphorylated Akt2 when Akt1 is knocked down, suggesting another potential mechanism for opposing actions of these Akt isoforms (See Figures 4, 5B, and Supplemental Figure IIIC).

FoxO proteins are known substrates of Akt. FoxO4, in particular, has been shown to associate with and inhibit the activity of myocardin, a critical transcriptional coactivator in VSMC differentiation[29,30]. Akt phosphorylation of FoxO4 inactivates this transcription factor by promoting its nuclear exclusion[31]. We report that FoxO4 is phosphorylated (Fig 5A) and translocated to the cytosol (Supplemental Fig IV) after adiponectin treatment in a time-dependent manner. Adiponectin-induced FoxO4 phosphorylation was also Akt2-dependent (Figure 5B). Notably, overexpression of FoxO4 prevented adiponectin-induced VSMC differentiation (Fig 5C).

Adiponectin and rapamycin similarly promote VSMC differentiation, but have distinct effects on endothelial cells

As both adiponectin and rapamycin promote VSMC differentiation via mTORC1 inhibition, we conducted experiments to compare their effects on VSMC and endothelial cells (EC) in parallel. Adiponectin and rapamycin induced SM2-MHC expression to a similar extent and with similar (although non-identical) kinetics in VSMC (Supplemental Figure VA). Notably, both adiponectin and rapamycin also prevented culture-induced contractile protein downregulation in intact vessel segments in an organ culture model (Figure 6A). We find that rapamycin also induces FoxO4 phosphorylation and translocation in a time- and dose-dependent manner (Supplemental Figures VB-C).

Importantly, the effects of adiponectin and rapamycin differed when directly compared in human EC. Rapamycin significantly inhibited Akt phosphorylation and completely inhibited mTORC1 in human umbilical artery or vein endothelial cells (HUAEC or HUVEC). Notably, adiponectin did not inhibit Akt in HUAEC and slightly activated Akt in HUVEC. In both EC types, adiponectin only modestly inhibited mTORC1 (Figure 6B). Since Akt/mTOR signaling is cytoprotective and promotes EC survival[32,33], the divergent effect of rapamycin and adiponectin in endothelial cells suggest that the adiponectin pathway may be a preferable route to promote VSMC differentiation while preventing EC injury.

Discussion

We report the novel findings that 1) HMW or trimeric adiponectin induces VSMC differentiation via mTORC1 inhibition; 2) Adiponectin transduces this signal via AMPKα2, and Akt2 suppression of FoxO4; 3) While adiponectin and rapamycin similarly promoteVSMC differentiation, only adiponectin promotes protective signaling in EC. Our study suggests that maintenance of VSMC phenotype may be an additional cardioprotective effect of endogenous adiponectin. In uncovering these roles of adiponectin, we have identified signaling mechanisms that underlie its actions.

Adiponectin induces VSMC differentiation via AMPK/mTORC1

We report that adiponectin promotes differentiation in human VSMC via AMPK-mediated mTORC1 inhibition (see model in Supplemental Figure VI). AMPK has emerged as a critical mediator of adiponectin's beneficial effects on metabolism and insulin sensitivity in other tissues[25], and on the stimulation of angiogenesis[18]. We found that AMPK activation with AICAR was sufficient to inhibit mTORC1 and induce VSMC differentiation. Notably, we believe we are the first to specifically implicate the AMPKα2 isoform as a key adiponectineffector. In contrast to other studies of adiponectin signaling in VSMC, ours is the first using multiple methods to manipulate endogenous levels of AMPK, as opposed to adenoviral overexpression[34]. Our siRNA data interestingly suggest that AMPKα1 may oppose contractile protein expression, as contractile proteins basally increase after AMPKα1 knockdown. AMPKα2 may be the predominant effector of adiponectin induced VSMC differentiation, and maytherefore be a potential therapeutic target for future stent drug development.

Of the two mTOR cellular complexes, only mTORC1 is inhibited by AMPK. mTORC1 and its substrate S6K1 participate in a well-documented feedback loop[35] in which growth factor and nutrient signaling limits the activity of the PI3K/Akt pathway: S6K1 phosphorylates IRS-1 on serine residues that promote its ubiquitination and degradation. This pathway modulates cell growth and insulin sensitivity, and its dysregulation is linked to cancer, diabetes, and obesity[27]. We now report that adiponectin- and rapamycin[23]-induced VSMC differentiation similarly require S6K1 inhibition. Interestingly, adiponectin inhibits mTORC1 to a lesser degree than rapamycin in both VSMC and EC, perhaps because adiponectin inhibits mTORC1 indirectly via AMPK: AMPK phosphorylates TSC2, which, in turn, inhibits Rheb, a requisite mTORC1 activator. In contrast, rapamycin/FKBP12 directly binds and inhibits mTORC1[36]. Our work suggests that adiponectin may provide an endogenous hormonal signal that limits mTORC1 activity in helping to maintain a healthy VSMC phenotype.

We find that adiponectin, like rapamycin, requires Akt2 activation to promote VSMC differentiation. We now report that FoxO4 is an Akt2 substrate and a critical effector of adiponectin in VSMC, as FoxO4 overexpression inhibits adiponectin-induced differentiation. As FoxO4 is known to interact with and inhibit the activity of myocardin[29], and inhibits intimal hyperplasia[30], this provides a likely mechanism for transcriptional regulation of contractile protein expression. Unlike rapamycin, our data suggest that adiponectin may also activate Akt1, but Akt1 does not appear to be required for regulation of FoxO4 or differentiation in this model. Our data suggest that Akt1 opposes contractile protein expression, and we will pursue the mechanism in future studies. It will be interesting to determine whether adiponectin might inhibit Akt3, as this isoform has been suggested to promote VSMC proliferation[37]. We also note potential feedback regulation between Akt isoforms, as Akt1 knockdown increases both total and phospho-Akt2. However, as this Akt2 upregulation did not significantly increase FoxO4 phosphorylation, we hypothesize that

there may be Akt2 substrates in addition to FoxO4 that also contribute to contractile protein regulation.

Although adiponectin was previously shown to inhibit VSMC proliferation and migration by directly binding platelet-derived growth factor-BB and inhibiting growth factor-stimulated ERK signaling[38], our observations in low serum conditions support a role for intracellular signaling mechanisms in adiponectin-induced differentiation. We found that adiponectin also efficientlyinduces VSMC differentiation in 10% serum conditions in cell (data not shown) and organ culture. Adiponectin is also known to inhibit VSMC and EC apoptosis via AMPK[19,39]. In vivo, it is likely that adiponectin exerts pleiotropic effects on VSMC as well as EC that contribute to its anti-restenotic effects[10,11,34].

Divergent effects of adiponectin in VSMC and EC

Despite the success of rapamycin in combating coronary artery restenosis, recent concerns have been raised regarding sudden death from late stent thrombosis[1,3,40]. The cause of the late stent thrombosis is not yet clearly understood, but is likely mediated by incomplete re-endothelialization[40], and diabetes confers a greater risk[2]. Rapamycin and other stent drugs have been shown to inhibit re-endothelialization[1,2,4], which may contribute to this pathogenesis. A fundamental understanding of rapamycin's actions on both VSMC and endothelial cells will be required to address this problem.

An ideal stent agent would inhibit VSMC phenotypic modulation while promoting re-endothelialization. We report that the natural hormone adiponectin may fulfill this dual role. Our data also demonstrate in a careful direct comparison that rapamycin inhibits Akt/mTORC1 in human EC, while adiponectin preserves Akt/mTORC1 activity. Adiponectin-induced Akt signaling has been shown to be pro-angiogenic in EC[18]. Akt promotes eNOS phosphorylation and activity, which is essential for EC migration, and has anti-apoptotic effects in EC[13,18]. Adiponectin additionallybenefits endothelial function bypromoting EC migration[18], inhibiting EC apoptosis[19] and inflammatory activation[41], inducing endothelial nitric oxide (NO) production[20] and decreasing reactive oxygen species production[42], suggesting a global EC protective function. Moreover, adiponectin inhibits thrombus formation[11], while rapamycin promotes platelet aggregation[43]. We found that, in contrast to rapamycin, adiponectin only modestly inhibited mTORC1 activity in EC. Notably, mTORC1 inhibition is anti-angiogenic and inhibits EC migration[44]. The ability of adiponectin to mimic the beneficial effects of rapamycin in VSMC while sparing cytoprotective Akt/mTORC1 signaling in EC suggests that the adiponectin pathway may be a desirable alternative for future DES therapeutic development. As a whole, the adiponectin pathway presents exceptional potential for future anti-restenotic therapy through its unique combination of anti-inflammatory effects, local anti-diabetic properties, and overall promotion of arterial health.

The reasons for the opposing effects of rapamycin on Akt in VSMC versus EC are not yet known, but indirect inhibition of mTORC2 suggests a potential mechanism (see Supplemental Figure VIB). When the mTOR protein resides in mTORcomplex1 with raptor and other accessory proteins, this complexis directly sensitive to rapamycin and regulates processes including protein synthesis. A more recently identified complex, mTORC2, containing mTOR, rictor and other proteins, is required for the phosphorylation of Akt at Ser473, as well as of other kinases including SGK and PKCα[36]. The kinase activity of mTORC2 is itself insensitive to rapamycin, but mTORC2 assembly can be inhibited due to sequestration of newly synthesized mTOR protein in mTORC1/rapamycin-inhibited complexes. This "chronic" rapamycin inhibition of mTORC2 shows dramatic cell type-specificity and has been observed in EC[45]. Given the long half-life of rapamycin, this effect can be substantial in some cell types, and could potentially underlie rapamycin inhibition of

Akt in EC. Adiponectin does not inhibit mTORC1 activity to the complete extent that rapamycin does in VSMC or EC. Furthermore, rapamycin inhibits mTORC1 by directlybinding the complex, while adiponectin inhibition mediated by AMPK occurs through a distcint, TSC-mediated mechanism. It is therefore likely that adiponectin inhibition of mTORC1 does not result in inhibition of mTORC2. Furthermore, we propose that adiponectin may act through as yet undefined signals to activate Akt in EC. Finally, while active in VSMC, the IGF/Insulin-dependent feedback activation of Akt by rapamycin does not occur in all cell types, perhaps due to cell-type specific patterns of phosphorylation and expression of IRS family proteins.

Remaining challenges

The physical properties of adiponectin, including its short half-life, rapid turnover, and multiple oligomeric forms[25], limit its current utility as a DES agent. Understanding the adiponectin-activated pathways in human cells that drive stent pathology is critical for identification of novel targets for development of improved therapeutics. Our data provide important signaling insights as well as identifying the HMW and trimeric, but not globular forms of adiponectin as potent inducers of VSMC differentiation. This is in contrast to skeletal muscle, where globular adiponectin enhanced insulin sensitivity[26]. Interestingly, Kobayashi et al noted that only the HMW form activated AMPK and inhibited apoptosis in EC, and that it was primarily this HMW form that increased upon weight loss in obese patients[19]. Whether there are specific receptors and functions for each oligomeric form of adiponectin remains a subject of intense investigation. A small molecule adiponectin receptor agonist may prove to be an ideal agent for DES.

In summary, we report that adiponectin, through AMPK inhibition of mTORC1 and feedback activation of Akt2 and FoxO4 inhibition, promotes VSMC differentiation, in a manner similar to the stent drug rapamycin. In contrast to rapamycin, adiponectin preserves endothelial cell Akt/mTORC1 activity. This work suggests that adiponectin pathway-based therapeutics may have the potential to avoid pro-thrombotic adverse effects associated with rapamycin while maintaining anti-restenotic efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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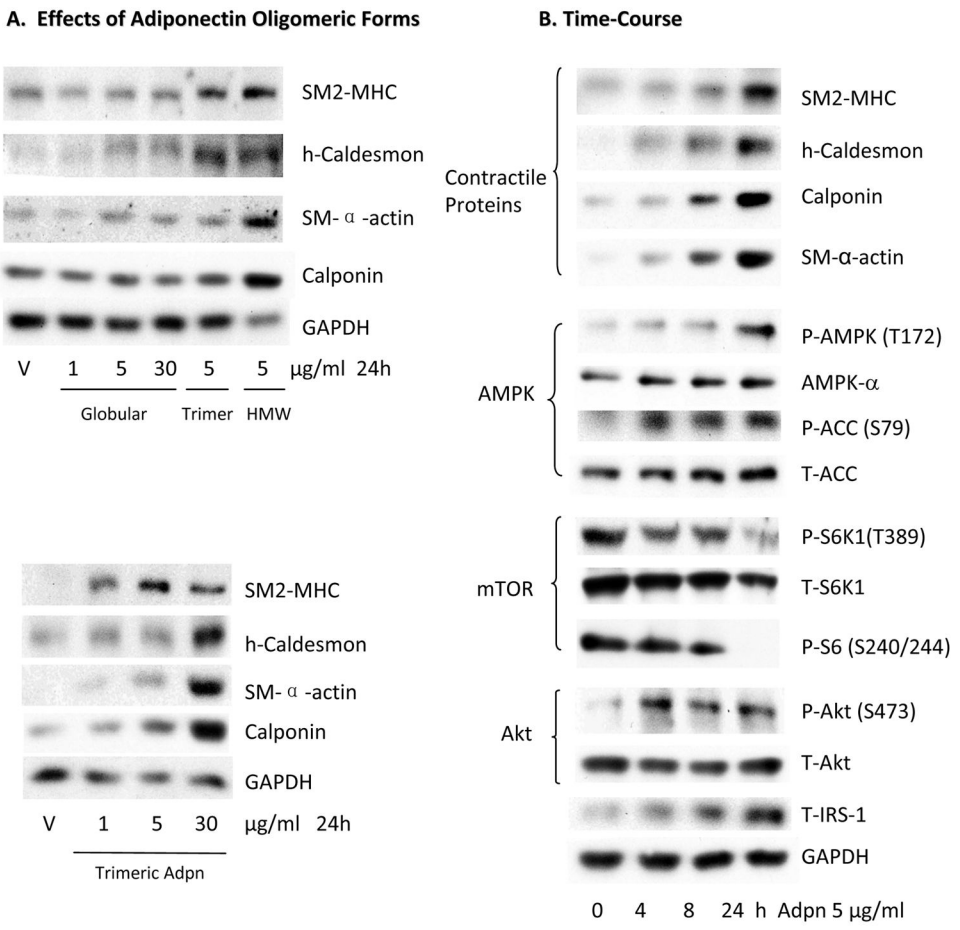

Figure 1.

Adiponectin induces VSMC differentiation marker expression and modulates the mTORC1 signaling pathway. A, Human coronary artery VSMC were treated with recombinant human globular, trimeric or HMW-enriched adiponectin preparations as indicated and tested for 24 h prior to western blotting analysis with the primary antibodies indicated. B, VSMC were treated with 5 μg/ml HMW-enriched adiponectin for the indicated time points and harvested for western analysis as indicated.

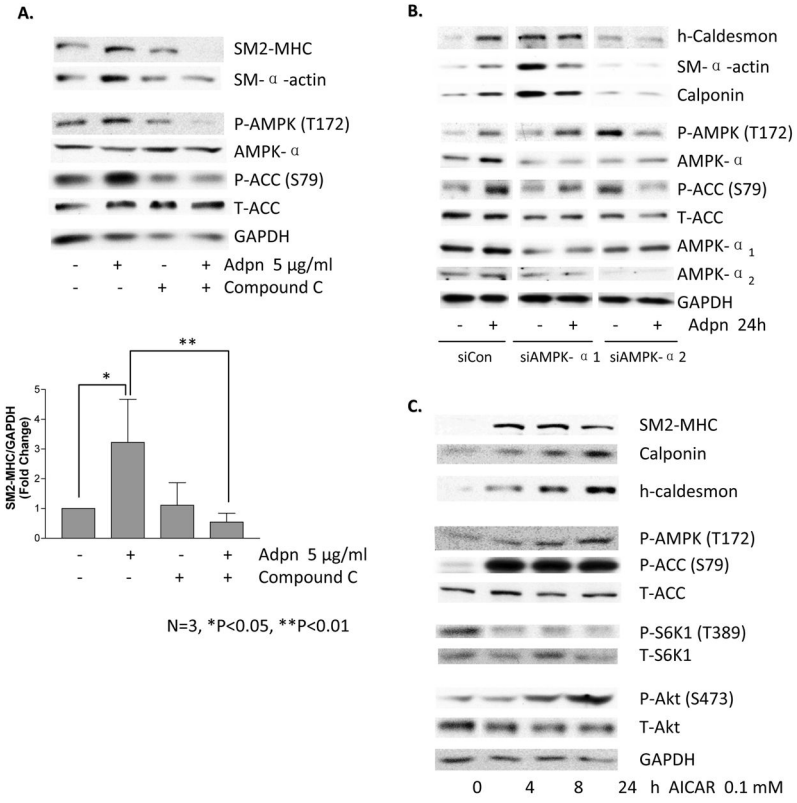

Figure 2.

Adiponectin-induced VSMC differentiation requires AMPK. A, VSMC were pretreated with compound C (20 μM) for 30 min, followed by treatment with 5 μg/ml adiponectin for 24h and western blotting with the indicated antibodies. Bar graphs represent densitometric quantification (mean fold induction plus standard error of the mean) of 3 separate experiments. p-values (Newman-Keuls multiple comparison post hoc tests) are indicated above the bars. B, VSMC were transfected with siControl, siAMPKα1 or siAMPKα2 for 24h, then treated with vehicle or 5 μg/ml adiponectin for 24h and analyzed by western blotting. Data are representative of two experiments. C. VSMC were treated with 0.1 mM AICAR for the indicated time points and harvested for western blotting analysis as above. Data are representative of 3 separate experiments.

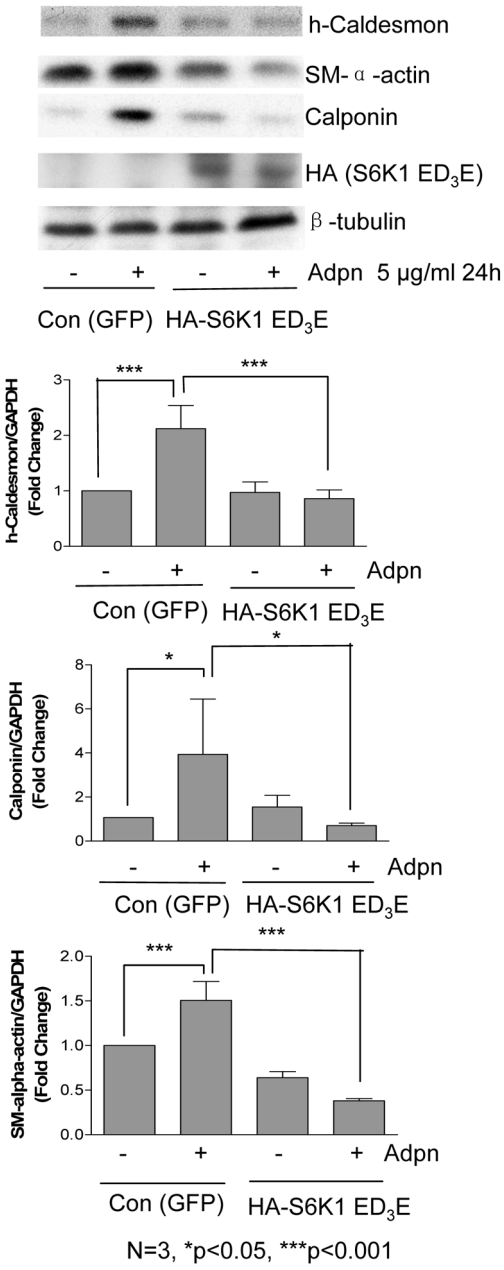

Figure 3.

Adiponectin induces VSMC differentiation via S6K1 inhibition. VSMC were infected with control (GFP) or rapamycin-resistant mutant S6K1 adenovirus overnight, and then treated with vehicle or 5 μg/ml adiponectin for 24h prior to western analysis. Bar graphs were prepared from 3 separate experiments as in Fig. 2A. p-values (Newman-Keuls multiple comparison post hoc tests) are indicated above the bars.

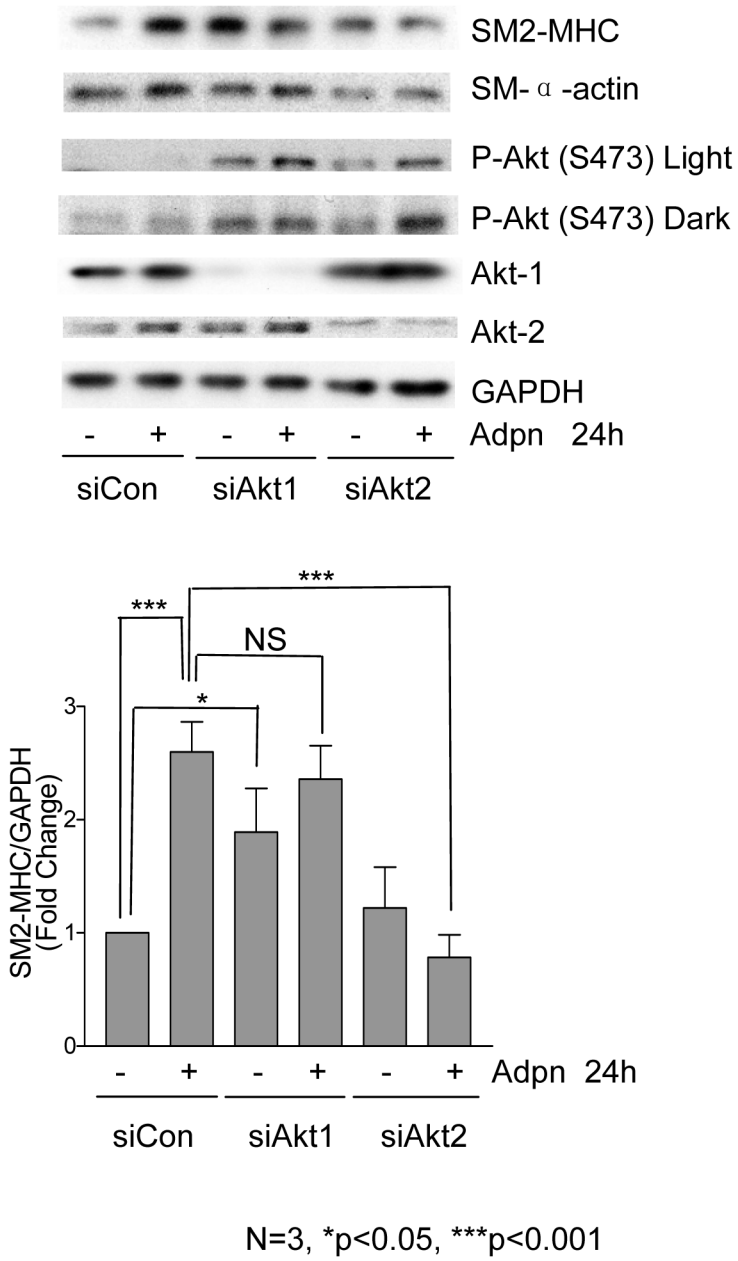

Figure 4.

Adiponectin induces differentiation via Akt2. VSMC were transfected with siControl, siAkt-1 or siAkt-2 for 24 h, then treated with vehicle or 5 μg/ml adiponectin for 24h prior to western analysis. Arepresentative experiment is shown. Bar graphs were prepared from 3 separate experiments as in Fig. 2A.

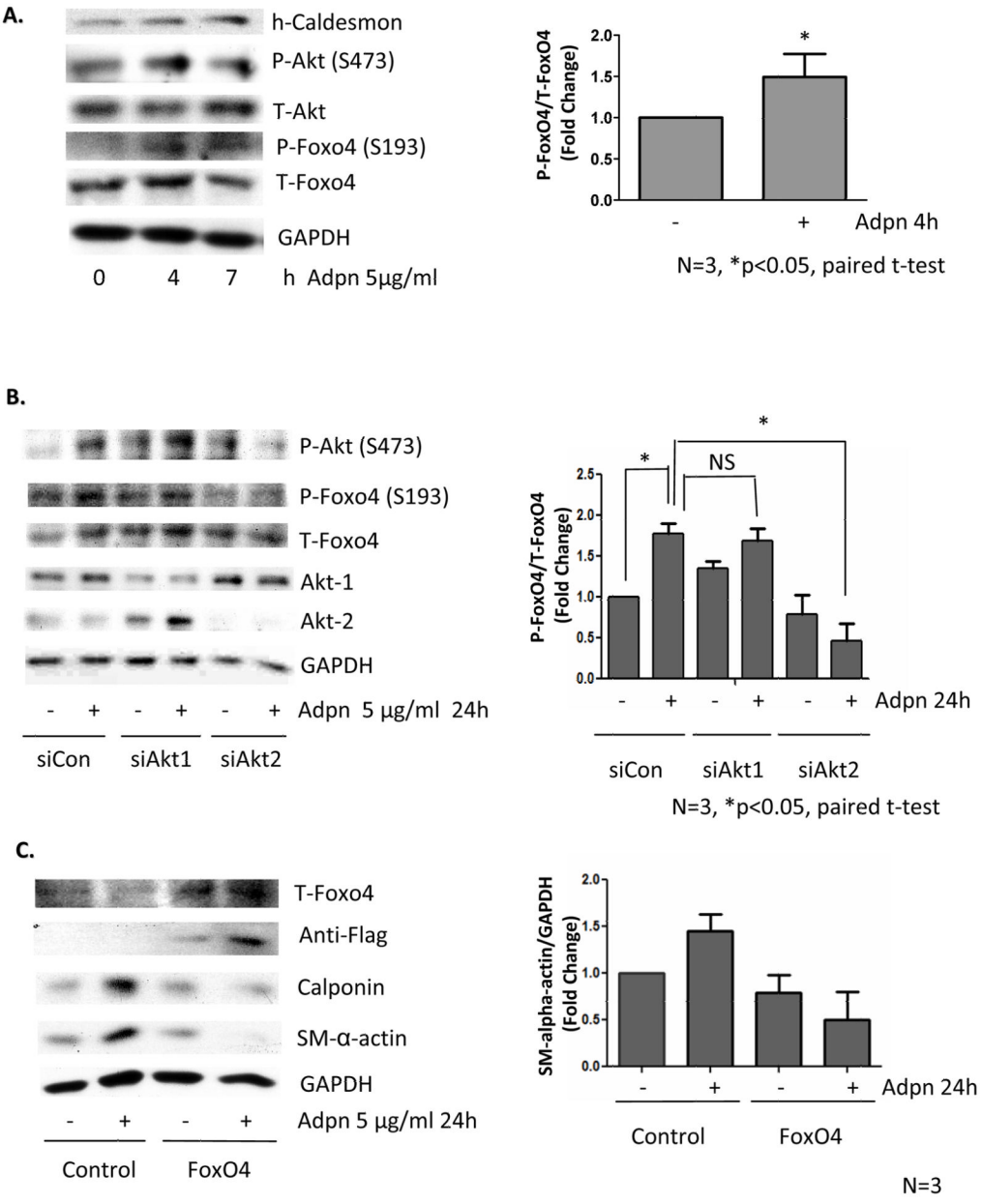

Figure 5.

Adiponectin induces FoxO4 phosphorylation via Akt2. A, VSMC were treated with 5 µg/ml adiponectin for the indicated time points and harvested for western analysis. B, VSMC were transfected, treated, and analyzed as in Fig. 4. C, VSMC were transfected with plasmid encoding GFP or Flag-Foxo4 for 24h, then treated with vehicle or 5 µg/ml adiponectin for 24h and harvested for western analysis. For all panels, bar graphs were prepared from 3 independent experiments.

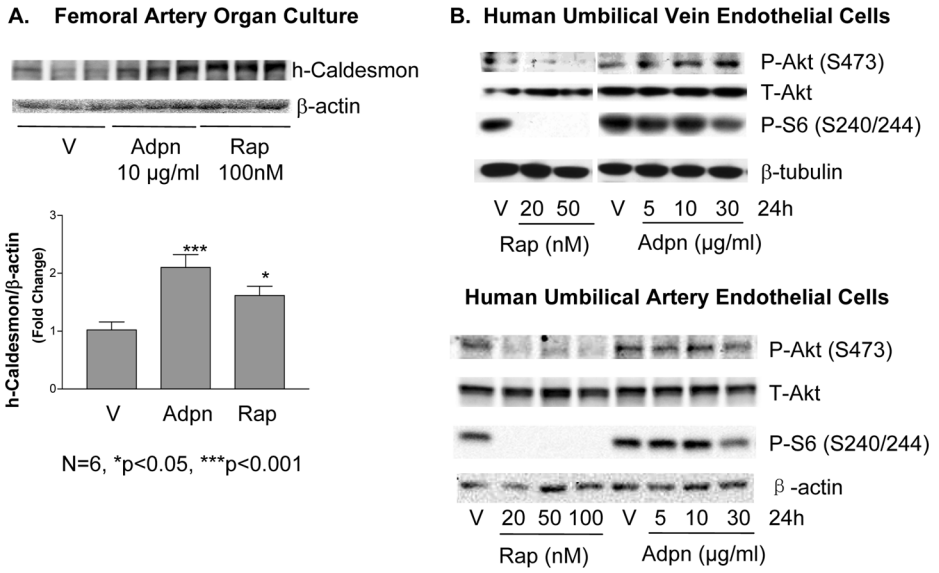

Figure 6.

Adiponectin and rapamycin have similar beneficial effects on vessel phenotype, but differ in adverse effects on endothelial cells. A, Freshly isolated pig femoral arteries were placed in organ culture for 3 days then treated with 100nM rapamycin or 10 μg/ml adiponectin for 4 days before harvesting for western analysis. Data from N=6 were quantitated with statistical analysis as in Fig. 2A. B, HUVEC (top panels) or HUAEC (bottom panels) were treated with the indicated concentrations of rapamycin and adiponectin for 24h and harvested for western analysis. HUAEC blot is representative of N=3.