Chronic limb ischemia, a complication commonly observed in conjunction with cardiovascular disease, is characterized by insufficient neovascularization despite the up-regulation of pro-angiogenic mediators. One hypothesis is that ischemia induces inhibitory signals that circumvent the normal capillary growth response. FoxO transcription factors exert anti-proliferative and pro-apoptotic effects on many cell types. We studied the regulation of FoxO1 protein in ischemic rat skeletal muscle following iliac artery ligation and in cultured endothelial cells. We found that FoxO1 expression was increased in capillaries within ischemic muscles compared with those from rats that underwent a sham operation. This finding correlated with increased expression of p27Kip1 and reduced expression of Cyclin D1. Phosphorylated Akt was reduced concurrently with the increase in FoxO1 protein. In skeletal muscle endothelial cells, nutrient stress as well as lack of shear stress stabilized FoxO1 protein, whereas shear stress induced FoxO1 degradation. Endogenous FoxO1 co-precipitated with the E3 ubiquitin ligase murine double minute-2 (Mdm2) in endothelial cells, and this interaction varied in direct relation to the extent of Akt and Mdm2 phosphorylation. Moreover, ischemic muscles had a decreased level of Mdm2 phosphorylation and a reduced interaction between Mdm2 and FoxO1. Our results provide novel evidence that the Akt-Mdm2 pathway acts to regulate endothelial cell FoxO1 expression and illustrate a potential mechanism underlying the pathophysiological up-regulation of FoxO1 under ischemic conditions. (Am J Pathol 2011, 178:935–944; DOI: 10.1016/j.ajpath.2010.10.042)

Successful reperfusion of the chronically ischemic limb depends both on re-establishment of blood flow via atherosclerotic feeding arteries and restoration of functioning microvessels through angiogenesis. Compensatory tissue neovascularization is therefore an important adaptive response to restore oxygen homeostasis in ischemic tissue. Unfortunately, the physiologic mechanisms that control microvascular growth and function ultimately fail within ischemic limbs. Although efforts have been made to deliver key mediators of hypoxia-induced angiogenesis such as vascular endothelial growth factor-A (VEGF-A) and hypoxia-inducible factor-1α (HIF-1α),1,2 clinical trials with pro-angiogenic agents have revealed little to no practical utility in patients who have chronic limb ischemia.3–6 These findings suggest a lack of responsiveness of endothelial cells to the growth factors or an elevated level of anti-angiogenic signals that might circumvent the actions of angiogenic factors, but underlying mechanisms remain unknown. Thus it is crucial to better understand how anti-angiogenic signaling pathways may be induced by the ischemic environment.

An increasing body of evidence supports essential roles of the forkhead transcription factors (FOX) in vascular development and maturation. The FOX family is distinguished by a conserved 100 amino acid domain called the “forkhead box.” The O subgroup of this family (FoxO) comprises four members: FoxO1, FoxO3a, and FoxO4, which are widely expressed, and FoxO6, which is restricted to the brain.7–9 In response to a variety of metabolic, oxidative, and mechanical stresses, FoxO

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transcription factors promote cell cycle arrest, repair of damaged DNA, gluconeogenesis, apoptosis, and detoxification of reactive oxygen species.10–12 Of the three highly related members of the FoxO family, only the loss of FoxO1 causes embryonic lethality due to severe defects in vascular development.13,14 FoxO transcription factors play substantive roles in vascular homeostasis by modulating several downstream genes important for vessel formation and maturation. Angiopoietin-1, which promotes vascular stability, inhibits the expression of its antagonist angiopoietin-2 through activation of the FoxO pathway.15 FoxO1 and FoxO3a may elicit anti-angiogenic effects in part by transcripional repression of endothelial nitric oxide synthase (eNOS) in endothelial cells.16 Members of the FoxO family are known to induce both cell cycle arrest via the enhanced transcription of cell cycle inhibitors such as p27Kip1 and p21 and apoptosis through synthesis of bim, a pro-apoptotic Bcl-2 family member.17 Conversely, VEGF-induced survival and proliferation of endothelial cells is associated with the repression of FoxO1 transcriptional activity and down-regulation of the FoxO1 target gene p27Kip1.18 The FoxO protein family is regulated primarily by post-translational modifications, including phosphorylation, acetylation, mono-ubiquitination, and polyubiquitination.8,9 These modifications control FoxO protein levels and subcellular localization, as well as the efficacy of DNA-binding and transcriptional activity. FoxO factors are regulated negatively by the serine/threonine protein kinase Akt. Dephosphorylated FoxO1 localizes to the nucleus and exhibits transcriptional activity, whereas Akt-dependent phosphorylation of FoxO1 protein causes its nuclear exportation and promotes its proteasomal degradation.10,11 Earlier work identified Skp2-dependent ubiquitination of FoxO1 subsequent to serine 256 phosphorylation.20 Two recent studies provided evidence that murine double minute-2 (Mdm2), an E3 ubiquitin ligase targets, and regulates FoxO protein levels.22,23 FoxO1 expression is suppressed by Akt-dependent phosphorylation in cells stimulated by growth factors or insulin or in endothelial cells subjected to sustained fluid shear stimulation.10,24–26 Considering that ischemia conversely generates an environment in which cellular activation by these factors is diminished, we hypothesized that chronic ischemia would reduce Akt signaling, thus promoting accumulation of FoxO1 and resulting in expression of factors that may override or reduce the angiogenic response.

Materials and Methods

Ethical Approval

Animal studies were approved by the York University Committee on Animal Care and performed in accordance with Animal Care Procedures at York University and the American Physiological Society’s Guiding Principles in the Care and Use of Animals.

Animal Protocol

Male Sprague-Dawley rats (200 to 250 g, Charles River Laboratories, Saint-Constant, QC, Canada) were used for all experiments. Rats were subject to hind limb ischemia for 4 (n = 4), 7 (n = 4), or 14 days (n = 4). Limitation of blood flow was induced by unilateral ligation of the right common iliac artery performed about 0.5 cm below the aortic bifurcation. Surgical procedures were carried out under anesthesia (intraperitoneal injection of ketamine, 80 mg/kg, and xylazine, 10 mg/kg) as previously described.27 Rats that underwent a sham operation (n = 8) were used as controls. At the end of each experiment, rats were anesthetized and the right extensor digitorum longus (EDL) and the tibialis anterior (TA) muscles were removed, weighed, and frozen either in liquid nitrogen or in cooled isopentane for histochemistry samples. Animals were euthanized by exsanguination.

Muscle Histology and Capillary to Fiber Ratio

Cryosections of TA muscle (10 μm) were fixed in 3.7% formaldehyde and stained with toluidine blue (10 mg/ml toluidine blue in 70% ethanol, diluted 1:10 in 1% NaCl) for 20 minutes. Sections were rinsed with PBS and mounted with AquaPerm media (Fisher Thermoscientific, Whiby, ON, Canada). Capillary to muscle fiber ratios were determined as an indicator of angiogenesis. Cryosections of TA muscle (10 μm) were fixed in cold acetone and stained with isocitrate (fluorescein isothiocyanate–conjugated Griffonia simplicifolia I; Vector Laboratories, Burlington, ON, Canada) diluted in PBS (1:100) for 30 minutes. In each case, sections were viewed with an Olympus microscope (×20 objective) and images were captured using a digital color charge coupled device camera (Hitachi, Tokyo, Japan). Capillary and muscle fiber counts were averaged from five independent fields of view per rat (sampling from equivalent oxidative and glycolytic regions of the muscle in each condition).

Extraction and Western Blotting of Skeletal Muscle Samples

Proteins were extracted from 20 to 40 mg of frozen muscles using a radioimmunoprecipitation assay lysis buffer containing 1 mg/ml phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, 1 mmol/L NaF (Sigma-Aldrich, Montreal, QC, Canada), and 1X protease inhibitor cocktail (Complete mini; Roche Diagnostics, Laval, QC, Canada). Muscle lysis was performed using a Retsch MM400 tissue lyser (Retsch GmbH, Haan, Germany). Proteins were quantified by bicinchoninic acid assay (Pierce, Fisher Thermoscientific). Samples were separated on SDS-polyacrylamide gel electrophoresis under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Immobilon P; Millipore Corporation, Billerica, MA). Forty (40) μg of total protein were loaded, except for FoxO1 and phosphoserine 256 FoxO1 detection, for which 80 μg total protein were required. After blocking with 5% fat-free milk, membranes were probed overnight at 4°C using antibodies from Cell Signaling Technology
RNA Extraction and Real-Time PCR Analysis

A small amount of frozen skeletal muscle (<10 mg) was homogenized in 300 μl RLT buffer (Qiagen Inc. Canada, Mississauga, ON, Canada) using a Retsch MM400 tissue lyser (Retsch GmbH). Total RNA was purified using the RNaseasy Fibrous Tissue Mini Kit (P/N 74704; Qiagen Inc. Canada) following deoxyribonuclease treatment. RNA was reverse transcribed using MMLV reverse transcriptase (Applied Biosystems Canada, Streetsville, ON, Canada) with anti-Mdm2 (clone 2A10) antibody and then with protein A/G plus-agarose beads (Santa Cruz Biotechnology) as previously described to detect phospho-ERK (#9102), p38 (#9211), phospho-p38 (9212), p38 (9211), phospho-Mdm2 (3521), β-actin (4967), αβ-tubulin (#2148), eNOS antibody (#9586), or cleaved caspase3 (#9664). Alternatively, blots were probed with HIF1α (NB100-105, Novus Biologicals, Littleton, CO), VEGF-A (sc152; Santa Cruz Biotechnology, Santa Cruz, CA) or Mdm2 (Clone 2A10; kindly provided by Dr. M.E. Perry, National Cancer Institute, Frederick, MD). Membranes were incubated with donkey anti-rabbit horseradish peroxidase secondary antibody (GE Healthcare Bio-Sciences, Inc., Baie d’Urle, QC, Canada or Dako Canada, Mississauga, ON, Canada) or Mdm2 (Clone 2A10; kindly provided by Dr. M.E. Perry, National Cancer Institute, Frederick, MD). Membranes were incubated with donkey anti-rabbit horseradish peroxidase secondary antibody (GE Healthcare Bio-Sciences, Inc., Baie d’Urle, QC, Canada or Dako Canada, Mississauga, ON, Canada). Bound antibodies were detected using Super West Pico (Pierce, Fisher Scientific, Woodbridge, CT). Bands were quantified using FluorChem software (Alpha Innotech, Cell Biosciences, San Leandro, CA).

Immunoprecipitation

Cell lysates or muscle extracts were incubated overnight with anti-Mdm2 (clone 2A10) antibody and then with protein A/G plus-agarose beads (Santa Cruz Biotechnology) for 2 to 3 hours at 4°C. Supernatants were subjected to Western blot analysis as previously described to detect FoxO1 (Cell Signaling #9454) or Mdm2 (2A10).

Statistical Analysis

Results were expressed as mean ± SEM and analyzed by one-way analysis of variance and Tukey post-tests (Prism4; Graphpad Software Inc., La Jolla, CA). P values of less than 0.05 were considered statistically significant.

Results

Ischemia-Induced HIF1α and VEGF-A Expression Is Not Associated with an Increase in Capillary to Fiber Ratio in Rat Hind Limb Muscle

Rats underwent ligation of the right common iliac artery or a corresponding sham operation and muscles were examined after 4, 7, or 14 days. We observed significant and sustained increases in hypoxia-sensitive HIF1α and VEGF-A protein levels between 4 to 14 days after ligation (Figure 1, A and B), similar to previous reports.27,30 Despite the elevation in these pro-angiogenic factors, capillary to
fiber ratio was not increased over the 14-day period (Figure 1C), consistent with previous studies.\textsuperscript{27,30} Histologic examination of muscle morphology did not reveal overt signs of inflammatory cell infiltration, edema, or necrosis 4 or 14 days after ligation (Figure 1D).

**Ischemia Induces FoxO1 Expression in Hind Limb Muscle**

We next examined FoxO1 expression in ischemic hind limb muscles. Phospho-Ser256-FoxO1 and total FoxO1 protein levels were determined by Western blotting (Figure 2A). In ischemic muscles, FoxO1 protein levels were elevated significantly at the 4-day and 7-day time points (2.3-fold and twofold increase, respectively; $P < 0.05$ versus controls that underwent a sham operation). Although the levels of phospho-Ser256-FoxO1 did not change at any experimental time point in comparison with sham values, the relative levels of phospho-FoxO1 compared with total FoxO1 protein levels were reduced substantially at 4 and 7 days after ligation (both $P < 0.05$ versus sham). No differences in protein or mRNA levels of the examined genes were observed between the 4-day and 14-day control animals; therefore data from these muscle samples were pooled together to form a single sham group. FoxO1 protein was difficult to detect by immunofluorescent staining in EDL cross-sections from control animals (Figure 2B). In contrast, FoxO1 protein was strongly visible 7 days after artery ligation and colocalized with capillary staining (Figure 2C). Real-time PCR analysis revealed a transient small induction of FoxO1 mRNA in ischemic muscles with maximal expression at day 4 of ligation ($0.78 \pm 0.08$ in sham group versus $1.07 \pm 0.07$ after 4 days ligation; $P < 0.05$).

**Ischemia Alters Downstream Targets of FoxO1 Involved in Cell Cycle Regulation**

We examined the expression of downstream targets of FoxO1 involved in cell-cycle progression. The cell cycle inhibitor p27\textsuperscript{Kip1} was augmented in ischemic muscle extracts at day 4 and 7 after ligation (twofold and 0.5-fold increase, respectively, $P < 0.05$ versus sham), returning to sham values after 14 days (Figure 3A). Conversely, levels of cyclin D1 mRNA were attenuated significantly at 7 days after ligation compared with controls that underwent a sham operation (~40% reduction, $P < 0.05$ versus sham; Figure 3B). Levels of cleaved caspase-3 (an indicator of apoptosis) were not altered in the ischemic muscle (Figure 3C). Immunostaining for cleaved caspase-3 also did not reveal significant differences between ischemic and sham conditions (data not shown). We did not observe significant changes in eNOS protein level during the 2-week course of ischemia (Figure 3D). Spry2 mRNA, a downstream target of FoxO1 reported to influence angiogenesis,\textsuperscript{31} significantly decreased 4 days after the induction of ischemia (30% reduction, $P < 0.05$ versus sham; Figure 3E).

**Ischemia Alters the Phosphorylation of ERK and Akt, Two Pathways Involved in the Regulation of FoxO1 Protein Level**

To further investigate the mechanisms responsible for FoxO1 up-regulation, we analyzed ERK, Akt, and p38 pathways, because each of them has been shown to regulate the stability or the activity of the transcription factor.\textsuperscript{22,23,32} Phosphorylation of mitogen-activated protein kinase ERK 1/2 was enhanced transiently (threefold) at 4 days after iliac artery ligation ($P < 0.05$ versus sham; Figure 4A). In contrast, no significant change in p38 phosphorylation was observed (Figure 4B). Significant decreases in phospho-Akt levels (in relation to total Akt) were detected at day 4 and day 7 (60% reduction for both time points; $P < 0.05$ versus sham; Figure 4C). Phospho-Akt levels returned to levels seen in nonischemic muscle by 14 days after ligation.
Nutrient Deprivation, but not Hypoxic or Oxidative Stress, Enhances FoxO1 Expression in Cultured Endothelial Cells

In the animal model of hind limb ischemia, restriction of blood flow generates numerous disturbances, including an inadequate oxygen supply, shortage of glucose and serum factors, and an accumulation of reactive oxygen species. To further investigate the stimuli underlying FoxO1 up-regulation in response to ischemia, we evaluated FoxO1 expression in cultured microvascular endothelial cells that were exposed to ischemia-mimetic conditions. Low oxygen pressure (5% O₂ for 24 hours), which enhanced VEGF-A expression (~twofold increase versus control, \( P < 0.05 \)), did not affect FoxO1 protein expression in endothelial cells (Figure 5A). Treatment of cells with \( H_2O_2 \) induced a concentration-dependent increase in free radicals as estimated by the oxidation of the dichlorodihydrofluorescein into the fluorescent dichlorofluorescein [1.2 ± 0.01-fold increase (200 \( \mu \)mol/L) to 3.0 ± 0.09-fold increase (800 \( \mu \)mol/L) vs untreated cells, \( P < 0.05 \)] and stimulated intense phosphorylation of oxidative stress-sensitive kinase ERK 1/2 but did not modulate FoxO1 protein levels. Conversely, prolonged nutrient stress for 24 hours significantly elevated FoxO1 protein expression by twofold versus control (Figure 5C).

In the Absence of Shear Stimulation, Cultured Endothelial Cells Are Characterized by Low Levels of Akt Phosphorylation and Elevated FoxO1 Protein

Ischemia also induces a reduction in capillary blood flow and shear stress, which may affect endothelial cell gene expression. Thus we compared Akt phosphorylation and FoxO1 expression in endothelial cells maintained under no-flow or shear-stimulated conditions. In agreement with previous reports, we observed that cells kept under no-flow conditions had 65% less Akt phosphorylation on residue serine 473 com-
pared with cells subjected to 2 hours of shear stimulation (P < 0.05 versus shear-stimulated cells; Figure 6A). Conversely, FoxO1 protein expression was increased significantly in microvascular endothelial cells maintained under no-flow conditions (1.8-fold increase, P < 0.05 versus shear-stimulated cells; Figure 6B). FoxO1 mRNA expression was not different between shear-stimulated or no-flow endothelial cells (1.0 versus 0.81 ± 0.14 (30 minutes shear) or 1.0 versus 3.0 ± 0.87 (2 hours shear); n = 4, P > 0.05 shear-stimulated versus time-matched no-flow cells). Akt activation is associated with enhanced proteasomal degradation via ubiquitin ligase pathways.24 The E3 ubiquitin ligase Mdm2 is a substrate for Akt and was reported recently to mediate FoxO ubiquitination.22,23 We detected phospho-Ser166 (active) Mdm2 in shear-stimulated endothelial cells; however, this phosphorylation substantially decreased in

Figure 3. Responsiveness of putative FoxO target genes to ischemia. Protein or RNA was extracted from rat TA and EDL, respectively, at 4, 7, or 14 days following induction of hind limb ischemia or a corresponding sham (Sh) operation. A: p27Kip1 protein level was analyzed by Western blot. Blots were stripped and reprobed for α/β-tubulin as a loading control. p27 levels were normalized to α/β-tubulin and expressed relative to sham (n = 4; *P < 0.05 versus sham). B: Cyclin D1 mRNA expression was analyzed by real-time PCR, with values normalized to housekeeping genes GAPDH and HPRT (n = 4; *P < 0.05 versus sham). C: Levels of cleaved caspase-3 were analyzed by Western blot. Blots were stripped and reprobed for α/β-tubulin as a loading control (n = 4, P > 0.05). D: eNOS protein expression was analyzed by Western blot and normalized to α/β-tubulin as a loading control (n = 4; *P < 0.05). E: Spry-2 mRNA expression was analyzed by real-time PCR, with values normalized to housekeeping genes glyceraldehyde-3-phosphate dehydrogenase and hypoxanthine phosphoribosyltransferase (n = 4; *P < 0.05 versus sham).

Figure 4. ERK 1/2, p38 MAPK, and Akt phosphorylation in response to ischemia. Protein was extracted from rat tibialis anterior at 4, 7, or 14 days following induction of hind limb ischemia or a corresponding sham (Sh) operation. A: Phospho-ERK 1/2 and total ERK 1/2 protein levels were analyzed by Western blot. Phospho-ERK 1/2 was expressed relative to total ERK 1/2 (n = 4; *P < 0.05 versus sham). B: Phospho-p38 and total p38 protein levels were analyzed by Western blot. Phospho-p38 was expressed relative to total p38 (n = 4; not significant). C: Phospho-(Ser473)-Akt levels were normalized to total Akt in each sample and expressed as a value relative to the 4-day sham (n = 3; *P < 0.05 versus 4-day sham). β-actin levels are shown to indicate loading.
cells maintained under no-flow conditions (25-fold decrease, "P" < 0.05 versus shear-stimulated cells; Figure 6C).

**FoxO1 Interaction with Mdm2 Is Reduced in Ischemic Muscle**

Based on our observation of reduced Akt phosphorylation under ischemic conditions, we postulated that Mdm2 phosphorylation also would be reduced under these conditions. We observed that phospho-(Ser166)-Mdm2 was reduced substantially in response to nutrient stress (~50% reduction, "P" < 0.05 versus time-matched control; Figure 7A). Conversely, serum stimulation of starved cells resulted in increased phospho-(Ser166)-Mdm2 levels and reduced FoxO1 protein expression, correlating also with the appearance of a higher molecular weight band (marked by asterisk on Figure 7B). Treatment of serum-stimulated cells with the proteasomal inhibitor MG132 augmented FoxO1 protein level, particularly that of the higher molecular weight band (Figure 7C). Given the inverse relationship observed between FoxO1 protein and Mdm2 phosphorylation under various experimental conditions, we looked for direct evidence of Mdm2-FoxO1 interaction. FoxO1 protein was detectable following immunoprecipitation of Mdm2 from serum-starved cell lysates, and serum stimulation of these cells enhanced this interaction (Figure 7D). We also found that the amount of phospho-(Ser166)-Mdm2 was reduced significantly at day 4 of ischemia (65% reduction, "P" < 0.05 versus controls that underwent a sham operation; Figure 7E). This decline also was noticeable but not statistically significant at day 7 (48% reduction; "P" = 0.065). Consistent with our observations in cultured endothelial cells that the extent of Mdm2-FoxO1 interaction correlates directly with Mdm2 phosphorylation, immunoprecipitation of Mdm2 from ischemic muscles pulled down a reduced amount of FoxO1 compared with sham muscles (Figure 7F).

**Discussion**

Our study provides novel evidence of the enhanced endogenous expression of the transcription factor FoxO1 within the microcirculation of hind limb skeletal muscles in response to an ischemic insult. The increased level of FoxO1 correlates with the modulation of cell cycle regulators and thus may contribute to the inhibition of recovering angiogenesis. Using cultured endothelial cells, we provide evidence that nutrient deprivation or lack of shear stress is
a sufficient stimulus to promote enhanced levels of FoxO1. Furthermore, we provide novel evidence of endogenous Mdm2-FoxO1 interaction within cultured endothelial cells as well as in vivo in muscle tissue. Our data suggest that reduced phosphorylation of Akt/Mdm2 may contribute to the increased levels of FoxO1 in response to ischemia.

**FoxO1 is a Factor Associated with Ischemia-Mediated Alterations in Muscle Microcirculation**

While a potential role for the FoxO family during ischemia has been proposed previously, the mechanisms by which these proteins regulate the process of neovascularization in ischemic tissue remains largely unknown. Potente et al assessed posts ischemia revascularization in skeletal muscles of transgenic FoxO3a-/- mice. The authors observed a significant enhancement of postischemia limb perfusion relative to wild-type animals, and this enhancement was associated with enhanced capillary density and improved blood flow recovery in a murine model of hind limb ischemia.

Our results showed an increased expression of p27CIP1 (which is an established transcriptional target of FoxO1) at days 4 and 7 after ligation, correlating closely with the time course of increase in FoxO1 protein. Our findings are congruent with a previous report showing induction of FoxO transcriptional activation in mouse hearts subjected to ischemia-reperfusion. The same study reported that transgenic mice over-expressing cardiac FoxO1 were characterized by decreased cardiomyocyte cell proliferation through up-regulation of p27CIP1 and p27Kip1 genes.

Conversely, levels of cleaved caspase-3 did not change, indicating no elevation in apoptotic signaling. We did not detect an increase in Spry2 mRNA (a negative regulator of the MEK pathway that may be controlled by FoxO transcription factors) in ischemic muscles. Although unexpected, this finding might be explained by the fact that Spry2 gene expression also is regulated by Ets-1. We measured a transient decrease in Ets-1 mRNA in response to ischemia (data not shown), and Spry2 mRNA appears to follow the same expression pattern. We did not observe any change in eNOS levels in ischemic muscle in contrast to in vitro studies showing in cultured cells that over-expression of either FoxO1 or FoxO3a repressed eNOS expression. However, our results are consistent with a previous study using the rat iliac artery ligation model of ischemia that reported no change in eNOS protein level despite elevated levels of VEGF-A.
Together, these results point to the complexities associated with understanding FoxO1 function within a multicellular environment that is responding to a pleiotropic stimulus. The role of FoxO in regulating the expression of these and other genes in response to ischemia requires further elucidation, because the pattern of gene regulation likely is dependent on the extent of FoxO up-regulation and the levels of transcriptional co-regulators.

Role of Nutrient Stress and Shear Stress in the Regulation of FoxO1

We examined upstream regulation of FoxO1 to further our understanding of the mechanisms underlying the ischemia-dependent increase in FoxO1 protein. While numerous kinases are known to regulate FoxO1 protein stabilization and functional activity by phosphorylation, we found that the decline in phosphorylation of Akt, but not that of ERK 1/2 or p38 MAPK, fits closely with the time-course increase in FoxO1 protein. This finding is consistent with the established mechanism of Akt stimulating FoxO1 cytoplasmic sequestration and subsequent proteasomal degradation. Our findings in cultured endothelial cells further supported this relationship, because nutrient stress or lack of a shear stress stimulus promoted a reduced state of Akt phosphorylation and enhanced FoxO1 protein levels. Our data suggest that nutrient stress and reduction of a shear stress stimulus may both contribute to the elevation in FoxO1 protein observed in ischemic muscles.

Interestingly, the pattern of Mdm2 phosphorylation correlated positively with that of Akt phosphorylation, increasing in response to shear stimulation and decreasing during nutrient or ischemic stress. Brenkman and colleagues previously reported that Mdm2 induced polyubiquitination of FoxO4 and that Akt phosphorylation of FoxO was required for Mdm2-FoxO interaction and the subsequent ubiquitination/degradation of FoxO4. Consistent with their observations, we found that the extent of Mdm2-FoxO1 interaction correlated positively with Mdm2 phosphorylation both in cultured endothelial cells and in ischemic muscles. While Mdm2-FoxO1 interactions have been reported in a cell-free system, our data provide novel in vivo measures of FoxO1-Mdm2 interaction and suggest that Mdm2 may act as a critical regulator of FoxO1 protein level in endothelial cells.

Conclusion

This study has demonstrated for the first time that the development of ischemia in skeletal muscle is associated with up-regulation of FoxO1. Enhanced expression of FoxO1 during ischemia correlates with reduced Akt phosphorylation and may be triggered by nutrient stress and reduction of shear stress. Our study provides the first evidence of endogenous Mdm2-FoxO1 interactions within microvascular endothelial cells and in ischemic muscle. Because Mdm2 ubiquitin ligase activity itself is regulated via Akt-dependent phosphorylation, Akt appears to be a critical regulator of FoxO1 function at multiple levels. In the ischemic environment, FoxO1 escapes these repressive signals as illustrated by the decreased interaction between Mdm2 and FoxO1 observed in ischemic muscle postligation, which correlated with increased total levels of FoxO1 and enhanced expression of cell cycle inhibitor p27Kip1 (Figure 8). In light of the established effects of FoxO1 on cell cycle regulation, it is compelling to consider this transcription factor as a modulator able to override the pro-angiogenic signaling pathways. Further clarification of FoxO protein regulation and function during ischemic episodes may lay the foundation for the successful application of clinical therapies for ischemia.

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