Hyperglycemia and insulin resistance accelerate atherosclerosis by an unclear mechanism. The two factors down-regulate insulin receptor substrate-1 (IRS-1), an intermediary of the insulin/IGF-I signaling system. We previously reported that IRS-1 down-regulation leads to vascular smooth muscle cell (VSMC) dedifferentiation and that IRS-1 deletion from VSMCs in normoglycemic mice replicates this response. However, we did not determine IRS-1’s role in mediating differentiation. Here, we sought to define the mechanism by which IRS-1 maintains VSMC differentiation. High glucose or IRS-1 knockdown decreased p53 levels by enhancing MDM2 proto-oncogene (MDM2)-mediated ubiquitination, resulting in decreased binding of p53 to Krüppel-like factor 4 (KLF4). Exposure to nutlin-3, which dissociates MDM2/p53, decreased p53 ubiquitination and enhanced the p53/KLF4 association and differentiation marker protein expression. IRS-1 overexpression in high glucose inhibited the MDM2/p53 association, leading to increased p53 and p53/KLF4 levels, thereby increasing differentiation. Nutlin-3 treatment of diabetic or Irs1−/− mice enhanced p53/KLF4 and the expression of p21, smooth muscle protein 22 (SM22), and myocardin and inhibited aortic VSMC proliferation. Injecting normoglycemic mice with a peptide disrupting the IRS-1/p53 association reduced p53, p53/KLF4, and differentiation. Analyzing atherosclerotic lesions in hypercholesterolemic, diabetic pigs, we found that p53, IRS-1, SM22, myocardin, and KLF4/p53 levels are significantly decreased compared with their expression in nondiabetic pigs. We conclude that IRS-1 is critical for maintaining VSMC differentiation. Hyperglycemia- or insulin resistance–induced IRS-1 down-regulation decreases the p53/KLF4 association and enhances dedifferentiation and proliferation. Our results suggest that enhancing IRS-1–dependent p53 stabilization could attenuate the progression of atherosclerotic lesions in hyperglycemia and insulin-resistance states.

Insulin-like growth factor-I (IGF-I) and insulin coordinately regulate cellular growth and differentiation in response to changes in nutritional status and intermediary metabolism (1). However, the mechanisms by which both hormones signal cells to grow or differentiate are not well defined. Both hormones stimulate these processes through their receptors, which directly phosphorylate insulin receptor substrate-1 (IRS-1) and Src homology 2 domain-containing–transforming protein C (Shc) to transmit their signals to downstream signaling components of the PI3K and mitogen-activated protein kinase pathways (2). During normal physiologic conditions, the PI3K pathway promotes glucose influx; glycogen, lipid, and protein synthesis; and changes in gene expression that help to maintain cellular differentiation (3, 4). However, in response to hyperglycemia, IRS-1 is down-regulated in multiple cell types, and insulin or IGF-I signaling through IRS-1 is impaired (5, 6). In cell types that are capable of undergoing dedifferentiation, such as vascular smooth muscle cells (VSMC), IRS-1 down-regulation is associated with up-regulation of a cell surface–associated scaffolding protein termed SHPS-1 (7). Under these conditions, stimulation of the IGF-I receptor leads to recruitment of the tyrosine kinase CTK to the plasma membrane, and CTK directly phosphorylates SHPS-1 (8). SHPS-1 functions as a scaffold and recruits kinases that activate both the PI3K and mitogen-activated protein kinase pathways (6, 9). This signaling switch occurs in vivo in VSMCs of diabetic mice in response to hyperglycemia. This change is accompanied by VSMC dedifferentiation (10) and an enhanced cellular proliferative response to injury (10). To determine whether loss of IRS-1 expression in response to hyperglycemia was mediating these changes, we

The abbreviations used are: IGF-I, insulin-like growth factor-I; IRS-1, insulin receptor substrate-1; Shc, Src homology 2 domain-containing–transforming protein C; VSMC, vascular smooth muscle cell; mSMC, mouse smooth muscle cell; pSMC, porcine smooth muscle cell; PI3K, phosphatidylinositol 3-kinase; NS, not significant; ID3, inhibitor of differentiation 3; NG, normal glucose; HG, high glucose; NGH, normal–high glucose transient exposure; i.p., intraperitoneally; DAPI, 4′,6-diamidino-2-phenylindole; WCL, whole-cell lysates; SM22, smooth muscle protein 22; MyoC, myocardin; DM, diabetic WT mice; NM, nondiabetic WT mice.
IRS-1 maintains VSMC differentiation

deleted IRS-1 in VSMC in mice. Aortic VSMC in which IRS-1 expression had been deleted underwent dedifferentiation under normoglycemic conditions and had a hyperproliferative response to vascular injury that was similar to the response of diabetic mice. Therefore, the loss of IRS-1 was sufficient to induce these changes.

Previous work shows that VSMC dedifferentiation is associated with enhanced expression of the transcription factor KLF4 (11) and that enhanced KLF4 expression, which occurs in response to cytokine-induced stress, suppresses transcription of the primary determinant of VSMC differentiation, myocardin (11, 12). We demonstrated that KLF4 expression increased in arteries of diabetic or normoglycemic IRS-1−/− mice, and myocardin was suppressed (10). Although the results showed that IRS-1 was required for myocardin expression and differentiation, they did not delineate the mechanism by which maintenance of IRS-1 expression enhances differentiation and inhibits dedifferentiation. Some studies show that increased KLF4 expression in VSMC leads to dedifferentiation, but other studies suggest that KLF4 expression increases expression of p21, which inhibits cell cycle progression and increases myocardin to promote VSMC differentiation. This discrepancy was resolved by Yoshida et al. (13), who showed that the variable that accounts for this difference is the balance between p53 and KLF4. In the presence of adequate p53, there is increased nuclear p53/KLF4 association, which enhances myocardin and p21 expression. In the absence of p53 association, KLF4 inhibits myocardin and p21. Because dedifferentiation is an important component of the atherosclerotic process (14) and hyperglycemia and insulin resistance, which down-regulate IRS-1, are mediated in arteries of diabetic or normoglycemic IRS-1−/− mice, and myocardin was suppressed (10). Although the results showed that IRS-1 was required for myocardin expression and differentiation, they did not delineate the mechanism by which maintenance of IRS-1 expression enhances differentiation and inhibits dedifferentiation. Some studies show that increased KLF4 expression in VSMC leads to dedifferentiation, but other studies suggest that KLF4 expression increases expression of p21, which inhibits cell cycle progression and increases myocardin to promote VSMC differentiation. This discrepancy was resolved by Yoshida et al. (13), who showed that the variable that accounts for this difference is the balance between p53 and KLF4. In the presence of adequate p53, there is increased nuclear p53/KLF4 association, which enhances myocardin and p21 expression. In the absence of p53 association, KLF4 inhibits myocardin and p21. Because dedifferentiation is an important component of the atherosclerotic process (14) and hyperglycemia and insulin resistance, which down-regulate IRS-1, are known to accelerate the development of atherosclerosis (15, 16), we undertook these studies to determine the mechanism by which IRS-1 functions to maintain VSMC differentiation.

Results

To investigate whether changes in p53 could be related to changes in KLF4 function, we determined whether hyperglycemia regulated p53. The levels of p53 decreased 3.4 ± 0.5-fold (mean ± S.D. throughout; p < 0.01) (n = 3), and the concentration of the p53/KLF4 complex was reduced 3.9 ± 0.5-fold (p < 0.01) (n = 3) in high glucose (Fig. 1A). Furthermore, nuclear p53 and p53/KLF4 were significantly decreased (Fig. 1B). Analysis of cells in which IRS-1 was deleted showed markedly reduced total p53 and p53/KLF4 complexes (cell lysate and nuclear fraction) (Fig. 1, C and D), although they were maintained in 5 mM glucose. Because p53 inhibits VSMC proliferation and counteracts the deleterious effects of oxidative stress on VSMC function, we utilized p53 knockdown and overexpression to further delineate the role of p53 in regulating VSMC differentiation. p53 knockdown resulted in no significant change in KLF4 or IRS-1 (Fig. 1E), but there was marked reduction in nuclear p53 and p53/KLF4 (Fig. 1F). Importantly, the differentiation markers myocardin and SM22 were decreased by 4.0 ± 0.6-fold (p < 0.01, n = 3) and 4.4 ± 1.1-fold (p < 0.001, n = 3), respectively, following p53 knockdown (Fig. 1G). Furthermore, p21, which is necessary to arrest cell cycle progression, was markedly inhibited (Fig. 1G). To confirm that these changes were p53-dependent, we overexpressed p53 in high glucose. This resulted in maintenance of higher total cellular p53 levels (Fig. 2A) and increased KLF4/p53 association (Fig. 2B), but there were no changes in IRS-1 and KLF4 (Fig. 2A). Nuclear p53 increased 3.0 ± 0.5-fold (p < 0.01) (n = 3), and p53/KLF4 association in the nuclear fraction was significantly enhanced 3.5 ± 0.5-fold (p < 0.01) (n = 3) despite hyperglycemia (Fig. 2C). This led to a 3.7 ± 0.8-fold (p < 0.001) increase in myocardin, a 4.5 ± 1.5-fold (p < 0.001) increase in p21, and a 3.3 ± 0.9-fold (p < 0.001) increase in SM22 expression (Fig. 2D). Therefore, maintenance of high levels of nuclear p53 promoted differentiation even in the presence of hyperglycemia.

To determine how p53 was down-regulated in VSMC following exposure to high glucose, we investigated the role of the ubiquitin ligase MDM2, which ubiquitinates p53 and targets it for proteasomal degradation. MDM2 increased 3.8 ± 0.8-fold (p < 0.05) (n = 3) during a 6–8 h exposure to high glucose, which also increased p53/MDM2 complexes (Fig. 3A). To determine how the glucose-induced increase in MDM2 mediated the reduction in p53, we utilized nutlin-3, a compound that disrupts p53/MDM2 association (17). Exposure to nutlin-3 inhibited p53/MDM2 (Fig. 3B) and inhibited p53 ubiquitination (Fig. 3C), confirming that MDM2 was functioning to lower p53 levels primarily by this mechanism. Exposure of cells maintained in high glucose to nutlin-3 increased total p53 and p53/KLF4 to levels that were similar to VSMC maintained in 5 mM glucose (Fig. 3D). Importantly, nutlin-3 increased nuclear p53 and p53/KLF4 (7.2 ± 1.4-fold, p < 0.01, n = 3 and 9.1 ± 2.6-fold, p < 0.01, n = 3, respectively) (Fig. 3E). The downstream factors myocardin, SM22, and p21 were increased (Fig. 3F). Total KLF4 was unchanged, suggesting that changes in myocardin and p21 expression were dependent upon increased KLF4/p53 association (Fig. 3D). To confirm these results and assess their importance for regulating differentiation, we utilized a cell-permeable peptide that was designed to disrupt p53/MDM2 association. Exposure of cells maintained in 25 mM glucose to the disrupting peptide was associated with a decrease in p53/MDM2 association and a decrease in p53 ubiquitination (Fig. 3G). Nuclear p53 concentrations as well as p53/KLF4 increased 2.6 ± 0.3-fold (p < 0.05) (n = 3) and 6.6 ± 0.6-fold (p < 0.01) (n = 3), respectively (Fig. 3H). Furthermore, p21, myocardin, and SM22 expression increased significantly even in the presence of high glucose to a concentration similar to that in cells maintained in normal glucose (Fig. 3I). In contrast, the addition of the peptide to VSMC maintained in 5 mM glucose resulted in no change in p21, SM22, or myocardin (Fig. 3J).

Because the differentiation response was p53-dependent and reduced IRS-1 expression is associated with decreased p53 and enhanced dedifferentiation, we wished to determine the role of IRS-1 in regulating p53. Following IRS-1 knockdown in VSMC in 5 mM glucose (Fig. 1C), there was a 2.9 ± 0.7-fold (p < 0.05) (n = 3) increase in p53/MDM2 association and a 14.2 ± 1.9-fold (p < 0.001) increase in ubiquitinated p53 (Fig. 4A). This resulted in a significant reduction in total cellular p53 levels (Figs. 1C and 4B) as well as nuclear p53/KLF4 (Fig. 1D). These changes were associated with significant reductions in p21, SM22, and myocardin (Fig. 4C).
To directly determine the role of IRS-1 in high glucose, we prepared cells overexpressing IRS-1. IRS-1 overexpression resulted in a substantial increase in p53 in whole-cell lysate and the nucleus as well as p53/KLF4 association (Fig. 5A and B). This was accompanied by a 4.2 ± 0.1-fold \((p < 0.01)\) \((n = 3)\) decrease in MDM2/p53 association, a 2.9 ± 0.6-fold \((p < 0.01)\) \((n = 3)\) increase in IRS-1/p53, and a 3.9 ± 0.6-fold \((p < 0.01)\) \((n = 3)\) decrease in p53 ubiquitination (Fig. 5C and D). These changes were accompanied by enhanced expression of myocardin, p21, and SM22 (Fig. 5E–G). The findings suggest that IRS-1 is functioning to alter MDM2-mediated p53 degradation because overexpression of IRS-1 even in the presence of high glucose increases nuclear p53 and p53/KLF4 association, which enhances differentiation. To confirm the importance of p53/KLF4 association, we prepared a peptide that disrupted their association (Fig. 5F). Exposure of cells maintained in 5 mM glu-
IRS-1 maintains VSMC differentiation

cose to the peptide reduced p53/KLF4 and decreased the expression of myocardin, SM22, and p21 significantly (Fig. 5G). The peptide had no effect on the expression of these proteins when added to cells maintained in high glucose (Fig. 6D). To further examine the role of IRS-1, we prepared a synthetic peptide that disrupted p53/IRS-1 association. In the presence of normal glucose, this peptide increased MDM2/p53 association (2.7 ± 0.2-fold, p < 0.01 (n = 3)) and enhanced p53 ubiquitination (5.2 ± 1.1-fold, p < 0.01 (n = 3) (Fig. 6A)). These changes were accompanied by a decrease in nuclear p53 and nuclear p53/KLF4 association (Fig. 6B) and significant reductions in differentiation marker proteins (Fig. 6C). Differentiation marker protein expression was unchanged when the peptide was added to cells maintained in 25 mm glucose (Fig. 6D).

To determine the role of p53 and IRS-1 in vivo, we examined the effect of disrupting p53/KLF4 in diabetic and IRS-1−/− mice. Both groups of mice showed decreased total p53 (e.g. 3.8 ± 0.1-fold (p < 0.001) (n = 3) and 3.5 ± 0.1-fold (p < 0.01) (n = 3) reductions, respectively) compared with non diabetic, WT mice. p53/KLF4 was decreased 4.0 ± 0.1-fold (p < 0.001) and 3.4 ± 0.1-fold (p < 0.01) (n = 3) in diabetic and IRS-1−/− mice, respectively (Fig. 7, A and B). Injection of nutlin-3 for 5 days significantly increased p53 and p53/KLF4 in diabetic mice (3.9 ± 0.2-fold (p < 0.01, n = 3) and 3.6 ± 0.8-fold (p < 0.05, n = 3), respectively) and in IRS-1−/− mice (2.2 ± 0.3-fold (p < 0.05, n = 3) and 2.4 ± 0.2-fold (p < 0.01, n = 3), respectively) (Fig. 7, A and B). In addition, the p53 ubiquitination level was significantly reduced by nutlin-3 treatment in diabetic or IRS-1−/− mice. These changes were accompanied by significant increases in p21, SM22, and myocardin expression in aortic VSMC (Fig. 7A and B). Reduced p53/KLF4 was also detected in the nuclear fraction of the aortic extracts from both groups of mice (Fig. 7, C and D), and this change was normalized by nutlin-3 treatment. The mechanism by which nutlin-3 was functioning was confirmed by showing decreased p53/MDM2 in the diabetic and IRS-1−/− mice (Fig. 7E and F). The importance of IRS-1 for regulating the stability of p53 in diabetic mice was confirmed using an IRS-1/p53-disrupting peptide. The peptide disrupted IRS-1/p53 association in normoglycemic mice, which led to a 4.2 ± 0.3-fold (p < 0.01) (n = 3) reduction in total p53 protein and 3.8 ± 0.4-fold (p < 0.01) (n = 3) reduction in p53/KLF4 association in the nuclear fraction (Fig. 7G). This was associated with a significant decrease in myocardin, SM22, and p21 expression (Fig. 7H). Importantly, nutlin-3 exposure resulted in a significant decrease in VSMC proliferation as determined by IGF-1 stimulated Ki67 labeling in the diabetic mice (1.7 ± 0.4-fold increase, p < 0.01, n = 3 versus 1.0 ± 0.1-fold, p = NS). Similarly, the IRS-1−/− mice had a substantial reduction in their cell proliferation response following nutlin-3 exposure (1.8 ± 0.1-fold increase, p < 0.05 versus 1.1 ± 0.2-fold, p = NS) (Fig. 8, A and B). A similar pattern was detected when aortic thickness was examined (Fig. 8C). Nutlin-3 exposure also attenuated macrophage recruitment to the
IRS-1 maintains VSMC differentiation

A

IP MDM2 IB p53
IB MDM2
IB β-actin

HG (hrs) 0 2 4 6 8 16

B

NHG 8h

IP MDM2 IB p53
Input IB MDM2

NG Ctrl Nutlin-3

C

IP p53 IB Ub
IB p53

NG Ctrl Nutlin-3

D

HG

Ctrl Nutlin-3 NG

IB p53
IB β-actin
IP KLF4 IB p53
IB KLF4

55 KDa
55 KDa
70 KDa

E

HG

IB p53
55 KDa

IP KLF4 IB p53
IB PARP-1

NG Ctrl Nutlin-3

F

HG

Ctrl Nutlin-3 NG

IB MyoC
IB p21
IB SM22
IB β-actin

110 KDa
25 KDa
25 KDa
55 KDa

G

HG

IP MDM2 IB p53
IB MDM2
IP p53 IB Ub

NG Ctrl Nutlin-3

H

Nuclear (HG)

Ctrl P33/MDM2 DP

IP KLF4 IB p53
IB p53
IB PARP-1

55 KDa
55 KDa
100 KDa

I

HG

IB MyoC
IB p21
IB SM22
IB β-actin

100 KDa
25 KDa
25 KDa
55 KDa

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2411
aorta, which was increased in diabetic and IRS-1−/− mice compared with control mice (Fig. 8, D and E).

Although VSMCs from diabetic mice undergo several changes in signaling that are similar to changes that occur in atherosclerotic lesions, they do not develop typical subintimal plaques unless they are also hyperlipidemic. To determine if the changes in p53, IRS-1, and KLF4 noted in our mice were present in an animal model that does develop subintimal lesions, we analyzed femoral arteries obtained from diabetic pigs that had been fed a high fat diet and had been shown to have extensive atherosclerosis (18). The results show that arterial extracts from the diabetic animals had a 2.6 ± 0.1-fold reduction (p < 0.001) (n = 5) in IRS-1 and a 2.8 ± 0.1-fold decrease (p < 0.01) (n = 5) in p53 compared with nondiabetic pigs (Fig. 9A). MyoC and SM22 were also significantly decreased. Most importantly the extracts from the diabetic animals showed a marked reduction in p53/KLF4 association (3.5 ± 0.2-fold (n = 8), p < 0.001) (Fig. 9B). These changes are consistent with the changes that occur in diabetic and IRS-1−/− mouse aorta.

Discussion

VSMCs possess the unusual characteristic that fully differentiated cells can dedifferentiate, leading to both accelerated proliferation and dysfunctional expression of proteins that are nec-

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**Figure 3. Prevention of p53 down-regulation via blocking ubiquitin proteasomal degradation rescues mSMC differentiation during hyperglycemia.**

A, cell lysates from mSMCs exposed to HG for the indicated times were immunoprecipitated (IP) with anti-MDM2 and immunoblotted (IB) with anti-p53. They were also directly immunoblotted with anti-MDM2 or β-actin. B–F, cell lysates or nuclear fraction extracts from mSMCs cultured in NG or HG in the presence or absence of nutlin-3 (10 µM). B, cell lysates (n = 3) were immunoprecipitated with anti-MDM2 and immunoblotted with anti-p53 or directly immunoblotted with anti-MDM2. Scanning densitometry showed that nutlin-3 treatment significantly impairs high-glucose-induced p53/MDM2 association (e.g. 2.4 ± 0.6-fold reduction, n = 4, p < 0.05). C, cell lysates were immunoprecipitated with anti-p53 and immunoblotted with an anti-ubiquitin (Ub) or only immunoblotted with an anti-p53. Scanning densitometry showed that high-glucose exposure increased p53 ubiquitination 3.2 ± 0.6-fold (p < 0.01) (n = 3), and nutlin-3 treatment prevented this increase (1.1 ± 0.3-fold increase, p = NS compared with cells exposed to NG). D, cell lysates were immunoprecipitated with an anti-KLF4 and immunoblotted with an anti-p53 or directly immunoblotted with anti-p53, anti-β-actin, or anti-KLF4. The bar graph shows the ratios ± S.D. (error bars) (n = 4) of scanning densitometry values of p53 divided by KLF4. E, nuclear fraction extracts were immunoprecipitated with anti-KLF4 and immunoblotted with anti-p53 or directly immunoblotted for p53. PARP-1 was the loading control. F, cell lysates were immunoblotted with anti-MyoC, anti-p21, anti-SM22, or anti-β-actin. The bar graph shows the ratios ± S.D. (n = 4) of scanning densitometry values of MyoC or p21 or SM22 divided by β-actin. G–I, whole-cell lysates or nuclear fraction extracts were obtained from mSMCs exposed to NG, NHG, or HG in the presence of a control peptide (Ctrl) or a p53/MMDM2-disrupting peptide (DP). G, cell lysates were immunoprecipitated with anti-MDM2 or p53 and immunoblotted with anti-p53 or ubiquitin. Cell lysates were also directly immunoblotted with anti-MDM2 or p53 as loading controls. Scanning densitometry showed that the p53/MMDM2-disrupting peptide reduced MDM2/p53 association by 3.7 ± 0.1-fold (p < 0.05) (n = 3) and p53 ubiquitination by 3.5 ± 0.1-fold (p = 0.01) (n = 3). H, nuclear fraction extracts were immunoprecipitated with anti-KLF4 and immunoblotted with anti-p53. They were directly immunoblotted for PARP-1 as a loading control. I, cell lysates were immunoblotted with an anti-MyoC, p21, SM22, or β-actin. The bar graph shows the ratios ± S.D. (n = 3) of scanning densitometry values of MyoC, p21, or SM22 divided by the β-actin. p < 0.05 and p < 0.01 indicate the significant differences.

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**Figure 4. Knockdown of IRS-1 enhances MDM2-mediated p53 degradation and impairs cell differentiation, which is rescued by nutlin-3.** Whole-cell lysates were obtained from mSMCs exposed to NG and treated with control siRNA (Ctrl Si) or IRS-1 siRNA (IRS-1 Si) in the presence or absence of nutlin-3 (Nt-3). A, cell lysates were immunoprecipitated (IP) with anti-MDM2 or ubiquitin and immunoblotted (IB) with anti-p53. Cell lysates were also directly immunoblotted with anti-MDM2 or p53 as loading controls. B, cell lysates were immunoprecipitated with anti-p53 and immunoblotted with anti-KLF4. p53 and β-actin were used as loading controls. The bar graph shows the ratios ± S.D. (error bars) (n = 3) of scanning densitometry values of p53 divided by β-actin or KLF-4, respectively. C, cell lysates were immunoblotted with anti-MyoC, p21, SM22, or β-actin. The bar graph shows the ratios ± S.D. (n = 3) of scanning densitometry values of MyoC, p21, or SM22 divided by β-actin. p < 0.05 and p < 0.01 indicate the significant differences.
Figure 5. Overexpression of IRS-1 up-regulates p53 and its association with KLF4, which maintains mSMC differentiation during hyperglycemia. A–E, whole-cell lysates or nuclear fraction extracts were obtained from mSMCs expressing LacZ or IRS-1 exposed to HG or NHG. A, cell lysates (n = 3) were immunoprecipitated (IP) with anti-KLF4 and immunoblotted (IB) with anti-p53. They were also directly immunoblotted with anti-IRS-1, p53, β-actin, and KLF4. Scanning densitometry showed that overexpression of IRS-1 resulted in a 3.0 ± 0.8-fold increase in p53 (p < 0.01) and 3.3 ± 0.8-fold increase of KLF4-associated p53 (p < 0.001) under hyperglycemic conditions. B, nuclear extracts were immunoprecipitated with anti-KLF4 and immunoblotted with anti-p53. PARP-1 was used as a loading control. The bar graph shows the ratios ± S.D. (error bars) (n = 3) of scanning densitometry values of p53 divided by PARP-1. C, cell lysates were immunoprecipitated with anti-MDM2 or IRS-1 and immunoblotted with anti-p53 or IRS-1. They were also immunoblotted with anti-β-actin. D, cell lysates were immunoprecipitated with anti-ubiquitin (Ub) and immunoblotted with anti-p53. E, cell lysates were immunoprecipitated with anti-MyoC, p21, SM22, or β-actin. The bar graph shows the ratios ± S.D. (n = 3) of scanning densitometry values of MyoC, p21, or SM22 divided by β-actin. F and G, nuclear fraction extracts and cell lysates (n = 3) were obtained from mSMCs exposed to NG treated with control peptide (Ctrl P) or p53/KLF4-disrupting peptide (DP). F, nuclear fraction extracts were immunoprecipitated with anti-KLF4 and immunoblotted with anti-p53. They were also directly immunoblotted with anti-KLF4 or PARP-1. G, cell lysates were immunoblotted with an anti-MyoC, anti-p21, anti-SM22, or anti-β-actin. The bar graph shows the ratios ± S.D. (n = 3) of scanning densitometry values of MyoC or p21 or SM22 divided by β-actin. p < 0.05, p < 0.01, and p < 0.001 indicate the significant differences.
Essential for normal contractile function (19). Hyperglycemia and insulin resistance predispose to increased VSMC dedifferentiation and atherosclerotic lesion development (10, 15, 20). Therefore, identification of the factors that regulate the ability of VSMC to maintain the differentiated state as well as those that lead to dedifferentiation will further our understanding of how changes in metabolism lead to arterial dysfunction and atherosclerosis. Both hyperglycemia and increased insulin resistance down-regulate IRS-1 in VSMCs and in blood vessels of diabetic animals (10, 21). This reduction is due to enhanced IRS-1 serine phosphorylation and increased ubiquitination that targets it to a proteasome (22). Recently, we reported that down-regulation of IRS-1 in the arteries of diabetic mice was accompanied by the enhanced expression of KLF4, a transcription factor that enhances VSMC dedifferentiation under certain conditions (10). KLF4 suppressed expression of myocardin, a transcription factor that is required to maintain VSMC in the differentiated state. The importance of IRS-1 in regulating this response was confirmed by deleting IRS-1 expression in normoglycemic mice and then demonstrating increased KLF4, reduced myocardin expression, and enhanced the proliferative response to injury. We concluded that down-regulation of IRS-1 in response to hyperglycemia led to dedifferentiation, but the mechanism by which IRS-1 maintained differentiation was not defined.

Our results show that IRS-1 stabilizes p53 concentrations, which enhances nuclear p53/KLF4 and drives expression of myocardin and other proteins required for VSMC differentiation (Fig. 10). Additionally, expression of p21, a cell cycle inhibitor, increased significantly. Knocking down IRS-1 in VSMC in vitro and in vivo in the presence of normal glucose replicated the effects of hyperglycemia, suggesting that hyperglycemia functions by decreasing IRS-1. The importance of maintaining p53 was confirmed by demonstrating that hyperglycemia down-regulates p53 and overexpression of p53 in VSMC during hyperglycemia restores p53/KLF4 as well as differentiation marker protein expression. High glucose enhances MDM2-mediated ubiquitination of p53, which down-regulates p53, but IRS-1 overexpression inhibits the p53/MDM2 interaction and p53 ubiquitination, thereby stabilizing p53 and promoting p53/KLF4-mediated induction of differentiation during chronic hyperglycemia. Although diabetic mice have signaling abnormalities that are present in atherosclerotic vessels, they do not develop lesions in the absence of hyperlipidemia; therefore, we analyzed arterial tissue obtained from diabetic pigs that develop extensive lesions (18). The results showed that p53 and IRS-1

Figure 6. Disruption of p53/IRS-1 interaction increases p53 ubiquitination and degradation and decreases p53/KLF4 association, leading to attenuated differentiation during normoglycemia. Whole-cell lysates or nuclear fraction extracts were obtained from mSMCs exposed to normal glucose (NG) treated with control peptide (CtrlP) or p53/IRS-1–disrupting peptide (DP). A, cell lysates \( n = 3 \) were immunoprecipitated with anti-p53, ubiquitin (Ub), or MDM2 and immunoblotted with anti-IRS-1 or p53. They were also directly immunoblotted with anti-KLF4 and immunoblotted with anti-p53. PARP-1 was used as a loading control. The bar graph shows the ratios ± S.D. \( n = 3 \) of scanning densitometry values of p53– or KLF4–associated p53 divided by PARP-1. B, nuclear extracts were immunoprecipitated with an anti-KLF4 and immunoblotted with anti-p53. The bar graph shows the ratios ± S.D. \( n = 3 \) of scanning densitometry values of MyoC, p21, or SM22 divided by β-actin. \( p < 0.001 \) and \( p < 0.01 \) indicate the significant differences. C, whole-cell lysates \( n = 3 \) were obtained from mSMCs exposed to NG (5 mM) or HG (25 mM) in the presence of a control peptide (Ctrl) or a p53/MDM2–disrupting peptide (DP), a p53/KLF4–disrupting peptide, or a p53/IRS-1–disrupting peptide. Cell lysates were immunoblotted with an anti-MyoC, anti-p21, anti-SM22, or anti-β-actin antibody.
were down-regulated in porcine lesions as well as the markers of differentiation. Importantly, there was a 3.5-fold reduction in p53/KLF4 association. These results are consistent with our findings in diabetic and IRS-1 knockout mice and suggest that these changes may be an important component of diabetic animal atherosclerotic lesion development.

P53 regulates VSMC proliferation, and its deletion enhances VSMC proliferation in response to hypercholesterolemia (23). Bone marrow transplant studies comparing p53-expressing and non-p53–expressing VSMC showed that endogenous p53 reduces atherosclerosis in APOE knockout mice (24). Similarly, overexpression of the p55γ subunit of PI3K blocked MDM2/p53 interaction, thereby increasing p53 and attenuating the proliferative response to rat carotid artery injury (25). Additionally, a long noncoding RNA, link RNA p21, that inhibited p53 ubiquitination in APOE−/− mice enhanced p53-mediated repression of VSMC proliferation (26). Although p53 knockdown in VSMC in APOE−/− mice did not alter lesion size, it did increase cell number in lesions, and it enhanced invasiveness (27). Because lesion size is regulated by multiple factors, such as blood pressure, hyperlipidemia, and cytokines, it is possible that they increased lesion size in these mice independently of

Figure 7. Down-regulation of p53 and p53/KLF4 association impairs SMC differentiation, which is rescued by nutlin-3 in vivo. Total aortic lysates or nuclear fraction extracts were prepared from nondiabetic WT mice (NM) (n = 9), diabetic WT mice (DM) (n = 15), nondiabetic IRS-1 WT (n = 9), or nondiabetic IRS-1 SM C knockout mice (IRS-1−/−/−) (n = 15) that had been injected with nutlin-3 or PBS (Ctrl) as described under “Experimental procedures.” A and B, aortic lysates were immunoprecipitated (IP) with anti-KLF4 or ubiquitin (Ub) and immunoblotted (IB) with anti-p53. They were also directly immunoblotted with anti-KLF4, p53, SM22, MyoC, or β-actin. Each bar shows the ratios ± S.D. (n = 3) of the value of the scanning units for p21, SM22, or MyoC divided by the scanning units of β-actin. Scanning densitometry values showed that nutlin-3 treatment dramatically decreased p53 ubiquitination in DM (e.g. 5.2 ± 1.9-fold reduction, n = 3, p < 0.001) or in IRS-1−/−/− NM (e.g. 5.9 ± 1.4-fold reduction, n = 3, p < 0.001). C and D, nuclear fraction extracts were immunoprecipitated with anti-KLF4 and immunoblotted with anti-p53. They were also directly immunoblotted with an anti-KLF4. Each bar shows the ratios ± S.D. (n = 3) of the value of the scanning units for KLF4-associated p53 divided by the scanning units of KLF4. E and F, aortic lysates were immunoprecipitated with anti-MDM2 and immunoblotted with anti-p53. They were also directly immunoblotted with anti-β-actin. Scanning densitometry values showed that nutlin-3 treatment decreased MDM2/p53 association in DM 2.9 ± 0.2-fold (p < 0.01) (n = 3) or 3.9 ± 0.4-fold (p < 0.01) (n = 3) in IRS-1−/−/− NM. G, in a separate experiment, aortic nuclear extracts and lysates were prepared from normal mice (n = 6) treated with a peptide that disrupted p53/IRS-1 (DP) or a control peptide (Ctrl P) as described under “Experimental procedures.” Nuclear extracts were immunoprecipitated with anti-p53 or KLF4 and immunoblotted with anti-p53 or KLF4. They were also directly immunoblotted with anti-p53, KLF4, or PARP-1. H, aortic lysates were immunoprecipitated with anti-Myc, p21, SM22, or β-actin antibody. Each bar shows the ratios ± S.D. (n = 3) of the value of the scanning units for MyoC, p21, or SM22 divided by β-actin. p < 0.001, p < 0.01, and p < 0.05 indicate the significant differences, and NS indicates no significant difference.
changes in p53. Diabetes is known to accelerate the rate of lesion progression, but it is not believed to be the cause of lesion initiation. Therefore, glucose-induced changes in p53 would be expected to contribute more to histologic changes that are related to lesion progression.

p53 is down-regulated during hyperglycemia (28); however, induction of AMP kinase stimulated p53 and p21 and down-regulated cyclin D1 in high glucose–exposed VSMC, leading to inhibition of cell proliferation in vitro (29). However, AMPK was equally effective in inhibiting cyclin D1 in normal and high glucose, making it difficult to conclude that it specifically counteracts the effect of high glucose. Our studies show that inhibition of p53/MDM2 with nutlin-3 or a specific peptide that disrupted their interaction reduced ubiquitinated p53, increased

IRS-1 maintains VSMC differentiation

Figure 8. Nutlin-3 treatment inhibits IGF-I–stimulated cell replication in diabetic or smooth muscle–specific IRS-1−/− mice. NM (n = 6) or DM (n = 12) with or without nutlin-3 (5 days of treatment) mice were injected with IGF-I or PBS i.p. twice (24 h and 15 min) before sacrifice. Sections from aorta were stained with anti-Ki67, and the number of proliferating cells was determined as in methods. The results are expressed as the percentage of Ki67-positive nuclei. Representative images are also shown (A). The mean values ± S.D. (error bars) from six mice per treatment group (three sections measured/mouse) are shown graphically (B). C, aortic thickness were measured from each group (n = 5) following the procedure described under “Experimental procedures.” D and E, total aortic extracts were prepared from NM, nondiabetic WT (WT), DM, and nondiabetic IRS-1SMC knockout mice (IRS-1−/−) that had been injected with nutlin-3 or PBS (Ctrl) as described under “Experimental procedures.” The aortic extracts were immunoblotted with an anti-macrophage scavenger receptor (MSR) (1:500; Trans Genic Inc.) or β-actin antibody. Scanning densitometry values showed that MSR expression was significantly increased in DM (e.g., 3.0 ± 0.4-fold, n = 3, p < 0.001) compared with NM or WT mice, respectively. Nutlin-3 treatment prevented this increase in both types of mice (e.g., 2.4 ± 0.7-fold, n = 3, p < 0.001) compared with NM or WT mice, respectively. Inhibition of cell proliferation in vitro (29). However, AMPK was equally effective in inhibiting cyclin D1 in normal and high glucose, making it difficult to conclude that it specifically counteracts the effect of high glucose. Our studies show that inhibition of p53/MDM2 with nutlin-3 or a specific peptide that disrupted their interaction reduced ubiquitinated p53, increased

J. Biol. Chem. (2019) 294(7) 2407–2421
p53 and p53/KLF4 association, and enhanced differentiation in diabetic or IRS-1−/− mice. p53 overexpression gave similar findings. Therefore, we conclude that MDM2 activation is an important mechanism by which hyperglycemia down-regulates p53 in VSMC. Overcoming the hyperglycemia-induced reduction in p53 allows VSMC to retain the ability to differentiate.

Figure 9. Important signaling events are confirmed in diabetic pigs. Diabetes was induced in pigs using streptozotocin, and femoral artery extracts were prepared from normal pigs (NP; n = 4) or diabetic pigs (DP; n = 8) as described under “Experimental procedures.” A, the extracts were immunoblotted (IB) with an anti-IRS-1, anti-p53, anti-MyoC, anti-KLF4, anti-p21, anti-SM22, or anti-β-actin antibody. Each bar shows the average ratio ± S.D. (error bars) of scanning densitometry values of the indicated proteins divided by the β-actin. p < 0.001 and p < 0.01 indicate the significant differences. B, the extracts (n = 4 for normal pigs, n = 8 for diabetic pigs) were immunoprecipitated with an anti-KLF4 antibody and immunoblotted with an anti-p53 antibody. The same amount of extract was immunoblotted with an anti-β-actin antibody. Each bar is the average ratio ± S.D. of scanning densitometry values of KLF4-associated p53 divided by the β-actin. p < 0.001 indicates the significant differences.

Figure 10. Schematic of IRS-1 regulation of mSMC differentiation. Under normoglycemic conditions, IRS-1 prevents MDM2-mediated p53 degradation, leading to enhanced p53/KLF4 association, and maintains VSMC differentiation. During hyperglycemia, IRS-1 is down-regulated, resulting in increased MDM2-mediated p53 ubiquitination and degradation. This decreases p53/KLF4 association, which stimulates VSMC dedifferentiation.
IRS-1 maintains VSMC differentiation

Hyperglycemia-induced p53 down-regulation resulted in decreased p53 nuclear content and decreased p53/KLF4 association. The role of nuclear p53/KLF4 in regulating VSMC differentiation has been analyzed (13). Pidkoka et al. (30) found that KLF4 was a potent transcriptional repressor of VSMC differentiation markers, such as myocardin, in the absence of p53 association. Other studies confirmed that KLF4 is induced during dedifferentiation, and this is consistent with our findings in both hyperglycemic and IRS-1−/− mice (31, 32). KLF4 associates with histone deacetylase 2 or 5 and the cofactors SRF and ELK-1, which results in inhibition of differentiation marker gene expression (31). Yoshida et al. (33) showed that KLF4-stimulated dedifferentiation was induced by stimulation of NF-κB association with KLF4 and that inhibition of NF-κB following neointimal injury inhibited KLF4-induced dedifferentiation. Our prior studies showed that high glucose induced a signaling switch from IRS-1 to SHPS-1 (8). This resulted in activation of p65Rel A by PKCζ on the SHPS-1 scaffold and NF-κB activation (34). Therefore, in high glucose, the increased activated nuclear NF-κB could bind to KLF4, leading to suppression of myocardin expression.

In contrast to its role as a mediator of dedifferentiation, Wassmann et al. (35) demonstrated that KLF4 enhanced expression of the VSMC differentiation marker SM22A. Shi et al. (36) found that all transretinoic acid induced multiple VSMC differentiation marker genes in a KLF4-dependent manner. Several studies confirm that the transactivation function of KLF4 to induce specific genes depends upon post-translational modifications, which mediate cofactor association and determine the response to KLF4 induction (37). These discrepant findings regarding KLF4 function in VSMC have been addressed by Yoshida et al. (13). They demonstrated that conditional deletion of KLF4 delayed down-regulation of VSMC differentiation markers but also accelerated neointimal proliferation (38). Enhanced expression of KLF4 in VSMC was associated with induction of p21 and reduced cellular proliferation. Importantly, they and others (39) documented that increased binding of p53 and KLF4 to the p21 promoter led to increased p21 and inhibited proliferation. Wassmann et al. (35) confirmed these findings by demonstrating that in the presence of p53, KLF4 induced VSMC differentiation. They also reported that inhibitor of differentiation 3 (ID3) binding to KLF4 resulted in p53 repression and enhancement of VSMC proliferation and that in the absence of ID3 overexpression, the predominant function of p53/KLF4 was to inhibit VSMC proliferation (40). Thus, high-glucose suppression of p53 may be a predominant mechanism by which phenotypic switching from the quiescent, differentiated phenotype in the presence of low glucose and high p53 levels is reversed, allowing unrestrained KLF4 suppression of differentiation. Our finding that disruption of p53/KLF4 during normoglycemia resulted in loss of p21 expression and dedifferentiation as characterized by reduced myocardin and SM22 further strengthens the conclusion that p53/KLF4 association is required for maintaining the differentiated phenotype in VSMC.

Our results show that the major mechanism by which IRS-1 functions to retain VSMC in the differentiated phenotype is to protect p53 from degradation. Overexpression of IRS-1 in high glucose resulted in decreased MDM2/p53 association and p53 ubiquitination. This increased nuclear p53 and p53/KLF4 association. Conversely, knockdown of IRS-1 promoted p53 ubiquitination and decreased p53/KLF4 and the downstream differentiation marker proteins. Furthermore, in diabetic mice, nutlin-3, which increased nuclear p53 and p53/KLF4 association, stimulated VSMC differentiation, indicating that reversal of p53 ubiquitination would reverse hyperglycemia-induced phenotypic switching. Because IRS-1 overexpression results in similar findings, we conclude that a basal level of IRS-1 expression is necessary to retain sufficient p53 in the nucleus to induce the differentiation-promoting effects of KLF4. This conclusion was confirmed by disrupting p53/IRS-1 in normoglycemic mice, which led to p53 degradation, ubiquitination, loss of nuclear p53, and a major reduction in myocardin and p21. Taken together, our results show that IRS-1 prevents p53 ubiquitination, which promotes downstream signaling events that maintain the differentiated phenotype. Conversely, loss of this association during exposure to high glucose results in loss of p53, dedifferentiation, and increased VSMC proliferation. Because our prior study showed that loss of IRS-1 led to a hyperproliferative response to injury, we conclude that IRS-1 is an important modulator of VSMC proliferation and that loss of IRS-1 in hyperglycemia could account for the acceleration in VSMC proliferation noted in diabetics with atherosclerosis.

There are minimal published data regarding IRS-1 and maintenance of the normal vascular phenotype or loss of IRS-1 in atherosclerosis. Sobue and co-workers (41) demonstrated that dephosphorylation of IRS-1 Tyr-895 by SHP-2 resulted in blockade of ERK activation in vitro. Therefore, maintenance of high IRS-1 concentrations in the presence of adequate SHP-2 would be expected to help to maintain a low rate of VSMC proliferation (41). Similarly, adiponectin induced VSMC differentiation and stabilized IRS-1 concentrations in vitro (3). Conversely, Thomas et al. reported that overexpression of SHPS-1 in skeletal muscle during normoglycemia resulted in loss of IRS-1 activation by the insulin receptor and inhibition of protein synthesis, suggesting that activation of SHPS-1 inhibited this differentiated function. T cadherin expression down-regulated IRS-1, and this was associated with VSMC dedifferentiation and reversed with rapamycin (42). Similarly, Taniyama et al. (43) showed that induction of reactive oxygen species down-regulated IRS-1 in VSMC in response to angiotensin II, and this was associated with increased proliferation. Because hyperglycemia induces reactive oxygen species, it is likely that both mechanisms are operating in parallel. One study suggested that attenuating IRS-1 function may be relevant to the development of human atherosclerosis. Baroni et al., who demonstrated that the G792R mutation in IRS-1 impairs insulin signaling (44), reported that this mutation was associated with a 2.93-fold increase in the relative risk ratio for the presence of coronary artery disease (45). Therefore, these studies support the conclusion that maintenance of IRS-1 facilitates VSMC differentiation and may reduce the risk of developing atherosclerosis.

In summary, glucose-induced down-regulation of IRS-1 results in loss of P53/KLF4 association, thereby decreasing p53/KLF4 complex induction of myocardin and p21. Reduced myocardin and p21 leads to enhanced VSMC dedifferentiation and
proliferation in diabetic mouse aorta. The results have important implications for understanding the mechanism by which hyperglycemia facilitates atherosclerotic lesion formation.

Experimental procedures

Human IGF-I was a gift from Genentech (South San Francisco, CA). Immobilon-P membranes and antibody against IRS-1 were from EMD-Millipore. Antibodies against phospho-AKT (Ser-473) and phospho-Erk1/2 were from Cell Signaling Technology Inc. Anti-phosphotyrosine (pTyr-99), PARP-1, MDM2, p53, and KLF4 antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-β-actin antibody was from Sigma-Aldrich. Antibodies against Ki67, ubiquitin, and SM22 antigen-Aldrich. Antibodies against Ki67, ubiquitin, and SM22 antigen were from Abcam. An anti-myocardin antibody was purchased from Santa Cruz Biotechnology, Inc. mSMCs were transfected using a method that had been described previously (46).

Cell culture

VSMCs were isolated from mouse (mSMCs) or pig (pSMCs) aortas using a method that had been described previously (46). The mSMCs were maintained in Dulbecco’s modified Eagle’s medium with glucose (1 g/liter normal glucose (NG) or 4.5 g/liter high glucose (HG)) supplemented with 10% fetal bovine serum (Atlanta Biologics, Flowery Branch, GA), streptomycin (100 ng/ml), and penicillin (100 units/ml). Cultures were grown to confluent density prior to the initiation of the experiment. In the case of normal–high glucose transient exposure (NHG), mSMCs were cultured in the NG until confluence before adding 20 mM glucose for the indicated times. For MDM2/p53-disrupting experiments, the MDM2/p53-disrupting peptide (2 μM) was added and incubated for 6 h. Nutlin-3 (10 μM) was added when 20 mM glucose was added and incubated for 8 h. For differentiation marker studies, disrupting peptides (10 μg/ml) or nutlin-3 (10 μM) were added every 24 h, and cultures were incubated for 48 h before cells were harvested. The cells that were used in these experiments were used between passages 5 and 15.

Transient transfection with siRNA targeting IRS-1 and p53

siRNA targeting IRS-1 (sc-29377), siRNA targeting p53 (sc-29436), and a control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. mSMCs were transfected using a concentration of 30 pm for IRS-1 and 90 pm for p53 and using the PepMute Plus reagent (SignaGen Laboratories, MD) following the manufacturer’s instructions. The experiments were initiated 24–48 h after transfection.

Establishment of mSMCs/pSMCs expressing p53, IRS-1, and LacZ

Human p53 cDNA was amplified from pcDNA3 p53 (Addgene, Cambridge, MA) using a 5’ primer sequence (5’-cacc atg gag gag cgc cag tca gat cag-3’) and a 3’ primer sequence (5’-ttt AGCGTAACTGGAACATCGTATGGGTA ggc cct tct gtc ttg aac atg-3’). mSMCs expressing p53 and LacZ were established using procedures described previously (47). The establishment of pSMCs expressing IRS-1 or LacZ was described previously (48).

Immunoprecipitation and immunoblotting

The immunoprecipitation and immunoblotting procedures were performed as described (47). Immunoprecipitation was performed by incubating 0.5 mg of cell lysate protein with 1 μg of anti-KLF4 or MDM2 or p53 or ubiquitin antibodies at 4 °C overnight. Immunoblotting was performed using a dilution of 1:1000 for anti-PARP-1, SM22, and β-actin antibodies and a dilution of 1:500 for anti-KLF4, p53, MDM2, IRS-1, p21, and myocardin antibodies. The proteins were visualized using enhanced chemiluminescence (Thermo Fisher Scientific).

Mice

All mouse experiments were approved by the institutional animal care and use committees of the University of North Carolina (Chapel Hill, NC). The floxed IRS-1–mice were provided by Dr. Morris White (Harvard Medical School). The generation of smooth muscle–specific IRS1−/− mice has been described (10). Mice were maintained at 22 °C with a 12-h light/dark cycle and given free access to regular chow (2018 Teklad global rodent diet containing 18.6% protein, 6.2% fat, and 3.5% crude fiber) and water. All groups of mice maintained normal nutrient intake and growth during the experiment.

Induction of hyperglycemia in mice and preparation of whole aortic lysates for biochemical analysis

Hyperglycemia was induced in WT male mice (C57BL/6) using low-dose streptozotocin (49). All mice had serum glucose concentrations that were >250 mg/dl, and the levels were maintained during the experiments. After acclimation, mice underwent conscious tail-cuff blood pressure measurement (10 times for each mouse), recording systole/diastole averaged over 40 cycles (Code 8, Kent Scientific, Inc., Torrington, CT), which showed no differences between diabetic mice and nondiabetic mice (103 ± 15/141 ± 12 versus 107 ± 9/141 ± 8 mm Hg, p = NS) or WT mice and smooth muscle–specific IRS-1−/− mice (117 ± 12/151 ± 5 versus 108 ± 4/145 ± 11 mm Hg, p = NS). There were 16 mice per group (WT, WT with diabetes, and smooth muscle–specific IRS-1−/− mice) that were used for biochemical analyses and Ki67 staining studies. IGF-I (1 mg/kg) (n = 6) or PBS (n = 6) was administered i.p. 24 h and 15 min before sacrifice for assessment of Ki67 labeling. For nutlin-3 treatment, 5 mg/kg was administered i.p. every day for 5 days. PBS was used as a control. For the aortic thickness experiments, IGF-I was administered i.p. every day for 5 days. For the p53/IRS-1–disrupting experiment, the disrupting peptide (2 mg/kg) or control peptide (2 mg/kg) was injected every day for
IRS-1 maintains VSMC differentiation

5 days in nondiabetic WT mice (n = 6). The whole aortic lysate preparation followed a procedure described previously (10).

Induction of diabetic pigs and preparation of femoral artery extracts

Diabetes was induced in pigs using streptozotocin, and femoral artery extracts were prepared as described previously (18). The animals were diabetic for 6 months prior to analysis.

Nuclear fraction extract preparation

Nuclear fraction extracts from cells or mice aortas were isolated using NE-PER® nuclear extraction reagents (Thermo Scientific) following a procedure provided by the manufacturer. Protein concentrations of extracts were measured using a BCA assay (Thermo Scientific). Equal amounts of protein were used for direct immunoblotting. For protein/protein interaction studies, that a similar amount input of the protein of interest was added was determined based on the results obtained from direct immunoblotting.

Immunohistochemistry

The aortas from mice were fixed with 4% paraformaldehyde overnight, and paraffin-embedded sections were prepared by the University of North Carolina histology core facility. An immunohistochemistry paraffin protocol provided by Abcam was followed, and the procedures were described previously for Ki67 and DAPI staining (50). The Ki67-positive and total nuclei (DAPI-positive) in a whole aortic ring were quantified using ImageJ (version 1.50i, National Institutes of Health) and expressed as the percentage of the total nuclei. To study aortic thickness, eight adjacent 5-μm sections were cut every 500 μm and stained with hematoxylin and eosin. The thickness was measured following the procedures described previously (10).

Statistical analysis

The results that are shown in all experiments are representative of at least three independent experiments and expressed as the mean ± S.D. Student’s t test was used when two data points were compared. Analysis of variance was applied when multiple points were compared. p < 0.05 was considered statistically significant.

Author contributions—G. X. designed and performed many of the experiments. He planned the mouse breeding program as well as supervising technical work that was necessary to complete the manuscript. D. R. C. helped to design experiments and plan the studies. He reviewed the data extensively and prepared the manuscript. M. F. W. prepared and provided the floxed IRS-1 mice. X. S. performed some in vitro experiments. C. W. maintains the mouse breeding program and assisted in preparation of the tissues for biochemical and immunohistochemical analysis.

References

1. Sadagurski, M., and White, M. F. (2013) Integrating metabolism and longevity through insulin and IGF1 signaling. Endocrinol. Metab. Clin. North Am. 42, 127–148 CrossRef Medline
2. Hakuno, F., Fukushima, T., Yoneyama, Y., Kamei, H., Ozoe, A., Yoshihara, H., Yamanaka, D., Shibano, T., Sone-Yonezawa, M., Yu, B. C., Chida, K., and Takahashi, S. (2015) The novel functions of high-molecular-mass complexes containing insulin receptor substrates in mediation and modulation of insulin-like activities: emerging concept of diverse functions by IRS-associated proteins. Front. Endocrinol. (Lausanne) 6, 73 CrossRef Medline
3. Ding, M., Xie, Y., Wagner, R. J., Jin, Y., Carrao, A. C., Liu, L. S., Guzman, A. K., Powell, R. J., Hwa, J., Rzucidlo, E. M., and Martin, K. A. (2011) Adiponectin induces vascular smooth muscle cell differentiation via repression of mammalian target of rapamycin complex 1 and FoxO4. Arterioscler. Thromb. Vasc. Biol. 31, 1403–1410 CrossRef Medline
4. Navé, B. T., Ouwens, M., Withers, D. J., Alesi, D. R., and Shepherd, P. R. (1999) Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem. J. 344, 427–431 CrossRef Medline
5. Saad, M. J., Araki, E., Miralpeix, M., Rothenberg, P. L., White, M. F., and Kahn, C. R. (1992) Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. J. Clin. Invest. 90, 1839–1849 CrossRef Medline
6. Nemoto, S., Matsumoto, T., Taguchi, K., and Kobayashi, T. (2014) Relationships among protein tyrosine phosphatase 1B, angiogenin II, and insulin-mediated aortic responses in type 2 diabetic Goto-Kakizaki rats. Atherosclerosis 233, 64–71 CrossRef Medline
7. Maile, L. A., Capps, B. E., Ling, Y., Xi, G., and Clemmons, D. R. (2007) Hyperglycemia alters the responsiveness of smooth muscle cells to insulin-like growth factor-I. Endocrinology 148, 2435–2443 CrossRef Medline
8. Radhakrishnan, Y., Shen, X., Maile, L. A., Xi, G., and Clemmons, D. R. (2011) IGF-I stimulates cooperative interaction between the IGF-I receptor and CSK homologous kinase that regulates SHPS-1 phosphorylation in vascular smooth muscle cells. Mol. Endocrinol. 25, 1636–1649 CrossRef Medline
9. Radhakrishnan, Y., Maile, L. A., Ling, Y., Graves, L. M., and Clemmons, D. R. (2008) Insulin-like growth factor-I stimulates Shc-dependent phosphatidylinositol 3-kinase activation via Grb2-associated p85 in vascular smooth muscle cells. J. Biol. Chem. 283, 16320–16331 CrossRef Medline
10. Xi, G., Wai, C., White, M. F., and Clemmons, D. R. (2017) Down-regulation of insulin receptor substrate 1 during hyperglycemia induces vascular smooth muscle cell dedifferentiation. J. Biol. Chem. 292, 2009–2020 CrossRef Medline
11. Liu, Y., Sinha, S., McDonald, O. G., Shang, Y., Hoofnagle, M. H., and Owens, G. K. (2005) Kruppel-like factor 4 abrogates myocardin-induced activation of smooth muscle gene expression. J. Biol. Chem. 280, 9719–9727 CrossRef Medline
12. Zheng, X. L. (2014) Myocardin and smooth muscle differentiation. Arch. Biochem. Biophys. 543, 48–56 CrossRef Medline
13. Yoshiida, T., Yamashita, M., Horimai, C., and Hayashi, M. (2014) Kruppel-like factor 4 protein regulates isoproterenol-induced cardiac hypertrophy by modulating myocardin expression and activity. J. Biol. Chem. 289, 26107–26118 CrossRef Medline
14. Chistiakov, D. A., Orekhov, A. N., and Bobryshev, Y. V. (2015) Vascular smooth muscle cell in atherosclerosis. Acta Physiol. (Oxf) 214, 33–50 CrossRef Medline
15. Holman, R. R., Paul, S. K., Bethel, M. A., Matthews, D. R., and Neil, H. A. (2008) 10-year follow-up of intensive glucose control in type 2 diabetes. N. Engl. J. Med. 359, 1577–1589 CrossRef Medline
16. Tsuchiya, K., Nakayama, C., Iwashima, F., Sakai, H., Izumiyama, H., Doi, M., and Hirata, Y. (2007) Advanced endothelial dysfunction in diabetic patients with multiple risk factors: importance of insulin resistance. J. Atheroscler. Thromb. 14, 303–309 CrossRef Medline
17. Lazo, P. A. (2017) Reverting p53 activation after recovery of cellular stress to resume with cell cycle progression. Cell. Signal. 33, 49–58 CrossRef Medline
18. Maile, L. A., Busby, W. H., Xi, G., Gollahan, K. P., Flowers, W., Gabfack, N., Gabfack, S., Stewart, K., Merricks, E. P., Nichols, T. C., Bellinger, D. A., and Clemmons, D. R. (2017) An anti-αVβ3 antibody inhibits coronary artery atherosclerosis in diabetic pigs. Atherosclerosis 258, 40–50 CrossRef Medline
34. Xi, G., Shen, X., Wai, C., Vilas, C. K., and Clemmons, D. R. (2015) Hyperglycemia stimulates p62/PKCζ interaction, which mediates NF-kappaB activation, increased Nox4 expression, and inflammatory cytokine activation in vascular smooth muscle. FASEB J. 29, 4772–4782 CrossRef Medline

35. Wassmann, S., Wassmann, K., Jung, A., Velten, M., Knuefermann, P., Petoumenos, V., Becher, U., Werner, C., Mueller, C., and Nickenig, G. (2007) Induction of p53 by GKLF is essential for inhibition of proliferation of vascular smooth muscle cells. J. Mol. Cell Cardiol. 43, 301–307 CrossRef Medline

36. Shi, J. H., Zheng, B., Chen, S., Ma, G. Y., and Wen, J. K. (2012) Retinoic acid receptor α mediates all-trans-retinoic acid-induced Klf4 gene expression by regulating Klf4 promoter activity in vascular smooth muscle cells. J. Biol. Chem. 287, 10799–10811 CrossRef Medline

37. Zheng, B., Han, M., and Wen, J. K. (2010) Role of Kruppel-like factor 4 in phenotypic switching and proliferation of vascular smooth muscle cells. IUBMB Life 62, 132–139 Medline

38. Yoshida, T., Kaestner, K. H., and Owens, G. K. (2008) Conditional deletion of Kruppel-like factor 4 delays downregulation of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury. Circ. Res. 102, 1548–1557 CrossRef Medline

39. Zhang, W., Geiman, D. E., Shields, J. M., Dang, D. T., Mahanta, C. S., Kaestner, K. H., Biggs, J. R., Kraft, A. S., and Yang, V. W. (2000) The gut-enriched Kruppel-like factor (Kruppel-like factor 4) mediates the transactivating effect of p53 on the p21WAF1/Cip1 promoter. J. Biol. Chem. 275, 18391–18398 CrossRef Medline

40. Wassmann, K., Mueller, C. F., Becher, U. M., Werner, C., Jung, A., Zimmer, S., Steinmetz, M., Nickenig, G. and Wassmann, S. (2010) Interaction of inhibitor of DNA binding 3 (Id3) with Gut-enriched Kruppel-like factor (GKLF) and p53 regulates proliferation of vascular smooth muscle cells. Mol. Cell Biochem. 333, 33–39 CrossRef Medline

41. Hayashi, K., Shibata, K., Morita, T., Iwasaki, K., Watanabe, M., and Sobue, K. (2004) Insulin receptor substrate-1/SHP-2 interaction, a phenotype-dependent switching machinery of insulin-like growth factor-1 signaling in vascular smooth muscle cells. J. Biol. Chem. 279, 40807–40818 CrossRef Medline

42. Frisianti, A., Dasen, B., Pfaff, D., Erne, P., Resink, T. J., and Philippova, M. (2016) T-cadherin promotes vascular smooth muscle cell dedifferentiation via a GSK3β-inactivation dependent mechanism. Cell. Signal. 28, S16–S30 CrossRef Medline

43. Taniyama, Y., Hitomi, H., Shah, A., Alexander, R. W., and Griendling, K. K. (2005) Mechanisms of reactive oxygen species-dependent down-regulation of insulin receptor substrate-1 by angiotensin II. Arterioscler. Thromb. Vasc. Biol. 25, 112–1147 CrossRef Medline

44. Frisianti, A., Dasen, B., Pfaff, D., Erne, P., Resink, T. J., and Philippova, M. (2016) T-cadherin promotes vascular smooth muscle cell dedifferentiation via a GSK3β-inactivation dependent mechanism. Cell. Signal. 28, S16–S30 CrossRef Medline

45. Baroni, M. G., D’Andrea, M. P., Montali, A., Pannitteri, G., Barilla, F., Campagna, F., Mazzei, E., Lovari, S., Seccareccia, F., Campa, P. P., Ricci, G., Pozzilli, P., Urbiniati, G., and Arca, M. (1999) A common mutation of the insulin receptor substrate-1 gene is a risk factor for coronary artery disease. Arterioscler. Thromb. Vasc. Biol. 19, 2975–2980 CrossRef Medline

46. Gockerman, A., and Clemmons, D. R. (1995) Porcine aortic smooth muscle cells secrete a serine protease for insulin-like growth factor binding protein-2. Circ. Res. 76, 514–521 CrossRef Medline

47. Xi, G., Shen, X., and Clemmons, D. R. (2008) p66shc negatively regulates insulin-like growth factor 1 signal transduction via inhibition of p52shc binding to Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 leading to impaired growth factor receptor-bound protein-2 membrane recruitment. Mol. Endocrinol. 22, 2162–2175 CrossRef Medline

48. Radakrishnan, Y., Busby, W. H., Jr, Shen, X., Maile, L. A., and Clemmons, D. R. (2010) Insulin-like growth factor-1-stimulated insulin receptor substrate-1 negatively regulates Src homology 2 domain-containing protein-tyrosine phosphatase substrate-1 function in vascular smooth muscle cells. J. Biol. Chem. 285, 15682–15695 CrossRef Medline

49. Maile, L. A., Capps, B. E., Miller, E. C., Aday, A. W., and Clemmons, D. R. (2010) Induction of p53 by GKF is essential for inhibition of proliferation of vascular smooth muscle cells. J. Mol. Cell Cardiol. 43, 301–307 CrossRef Medline

50. Frisianti, A., Dasen, B., Pfaff, D., Erne, P., Resink, T. J., and Philippova, M. (2016) T-cadherin promotes vascular smooth muscle cell dedifferentiation via a GSK3β-inactivation dependent mechanism. Cell. Signal. 28, S16–S30 CrossRef Medline