

# Integrin-Associated Protein Association With Src Homology 2 Domain Containing Tyrosine Phosphatase Substrate 1 Regulates IGF-I Signaling In Vivo

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**OBJECTIVE**—Smooth muscle cell (SMC) maintained in medium containing normal levels of glucose do not proliferate in response to IGF-I, whereas cells maintained in medium containing 25 mmol/l glucose can respond. The aim of this study was to determine whether signaling events that have been shown to be required for stimulation of SMC growth were regulated by glucose concentrations in vivo.

**RESEARCH DESIGN AND METHODS**—We compared IGF-I-stimulated signaling events and growth in the aortic smooth muscle cells from normal and hyperglycemic mice.

**RESULTS**—We determined that, in mice, hyperglycemia was associated with an increase in formation of the integrin-associated protein (IAP)/Src homology 2 domain containing tyrosine phosphatase substrate 1 (SHPS-1) complex. There was a corresponding increase in Shc recruitment to SHPS-1 and Shc phosphorylation in response to IGF-I. There was also an increase in mitogen-activated protein kinase activation and SMC proliferation. The increase in IAP association with SHPS-1 in hyperglycemia appeared to be due to the protection of IAP from cleavage that occurred during exposure to normal glucose. In addition, we demonstrated that the protease responsible for IAP cleavage was matrix metalloproteinase-2. An anti-IAP antibody that disrupted the IAP-SHPS-1 association resulted in complete inhibition of IGF-I-stimulated proliferation.

**CONCLUSIONS**—Taken together, our results support a model in which hyperglycemia is associated with a reduction in IAP cleavage, thus allowing the formation of the IAP-SHPS-1 signaling complex that is required for IGF-I-stimulated proliferation of SMC. *Diabetes* 57:2637–2643, 2008

**D** diabetes is an independent risk factor for atherosclerotic heart disease (1). Studies have shown a correlation between glucose levels and risk of developing atherosclerosis (2–4). Atherosclerosis is characterized by an increase in smooth muscle cell (SMC) migration and proliferation from the vessel wall into the lumen (5). Both in vitro and in vivo studies have demonstrated that IGF-I is a stimulator of SMC migration and proliferation (6–8). When SMCs grown in 5 mmol/l glucose are exposed to IGF-I, there is no increase in migration or proliferation (9). However, when glucose is

increased to 25 mmol/l, IGF-I stimulates significant increases in migration and proliferation (10). Phosphorylation of Shc and subsequent activation of the mitogen-activated protein kinase (MAPK) pathway is absolutely required for the migration and proliferation of SMCs in response to IGF-I (11). When SMCs are grown in 25 mmol/l glucose and then exposed to IGF-I, Shc and MAPK phosphorylation are significantly increased. In contrast, when SMCs are grown in 5 mmol/l glucose, there is no increase in Shc or MAPK phosphorylation (10).

We have determined that in contrast to SMCs grown in 5 mmol/l glucose, when SMCs are exposed to 25 mmol/l glucose, Src homology 2 domain containing tyrosine phosphatase substrate 1 (SHPS-1) binds to the extracellular domain of IAP, via its extracellular domain. This interaction is required for IGF-I to stimulate SHPS-1 phosphorylation, which is required for recruitment and phosphorylation of Shc (12). The aims of this study were to determine whether IAP/SHPS-1 interacted in vivo, whether this interaction was regulated by changes in blood glucose levels, and whether this interaction regulated cellular responsiveness to IGF-I stimulation.

## RESEARCH DESIGN AND METHODS

Human (endotoxin-free) IGF-I was a gift from Genentech (South San Francisco, CA). Polyvinyl difluoride membranes (Immobilon P) were from Millipore Corporation (Billerica, MA). Autoradiographic film was from Pierce (Rockford, IL). Fetal bovine serum, Dulbecco's modified medium, penicillin, and streptomycin were purchased from Life Technologies (Grand Island, NY). The monoclonal anti-phosphotyrosine (PY99) and the polyclonal anti-IGF-I receptor (IGF-IR) antibodies were from Santa Cruz (Santa Cruz, CA). The phospho/total extracellular signal-related kinase (ERK)1/2 and anti-Shc antibodies were purchased from BD Transduction Laboratories (Lexington, KY). The anti-SHPS-1 antibody was purchased from Upstate Cell Signaling Solutions (Charlottesville, VA). The  $\beta$ -actin was purchased from Chemicon (Temecula, CA). The matrix metalloproteinase-2 (MMP-2) inhibitor IV (category number 444274) was purchased from EMD Biosciences (San Diego, CA). All other reagents were from Sigma (St. Louis, MO) unless stated.

**Anti-IAP antibodies.** The anti-IAP monoclonal antibody, B6H12, was purified from a cell line derived from a B-cell hybridoma (13). The anti-IAP antibody (referred to as R569), which recognizes amino acids 41 and 61 in the extracellular domain of IAP, has been described previously (14).

**Induction of hyperglycemia in mice.** Hyperglycemia was induced in C57/B6 mice (Taconic Hudson NY) using the low-dose streptozotocin (STZ) protocol (15). After a 4-h fast, mice were injected intraperitoneally with either STZ (50 mg/kg) in citrate buffer (pH 4.5) or citrate buffer alone daily for 5 days. The protocols used were derived from those published by the Animal Models of Diabetic Complications Consortium, which were developed to allow direct comparison of the studies from a large consortium of multiple investigators (15).

**Blood glucose measurements.** Blood glucose levels were measured using a Freestyle Glucose monitor (Abbott Laboratories, Alameda CA). Blood was obtained from the cheek pouch using GoldenRod animal lancets (Medipoint International, Mineola, NY). Glucose levels were measured before the administration of IGF-I and after 15 min, 30 min, and 30 h.

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**Measurement of total cholesterol.** Total cholesterol levels were measured in whole blood using a Cardiocheck meter with cholesterol testing strips (Polymer Technology Systems, Indianapolis, IN).

**Measurement of IGF-I levels.** An enzyme-linked immunoassay was used to measure IGF-I levels in the serum from mice obtained at necropsy by direct heart puncture (Diagnostic Systems Laboratories, Webster, TX). Samples were treated as described by the manufacturer. Absorbance was measured by a plate reader (measuring wavelength: 450 nm, background wavelength: 530 nm) Appropriate standards ranging in concentration from 0 to 4,000 ng/ml and internal controls (provided by the manufacturer) were used.

**Treatment with IGF-I and assessment of SMC proliferation.** Fasted hyperglycemic and control mice were injected intraperitoneally with IGF-I (1 mg/kg), PBS alone, B6H12 (1 mg/kg), control IgG, or B6H12 plus IGF-I 30 h before death. Aortas were removed and fixed before embedding in paraffin. Serial sections (5  $\mu$ m thick) were cut from the same region of aorta, and every 10th section (four sections/aorta) was incubated with an anti-Ki67 antibody (Abcam, Cambridge, MA) followed by a biotin-conjugated goat anti-rabbit secondary antibody. Staining was visualized using the ABC Elite kit (Vector Laboratories, Burlingame, MA). The number of SMCs staining positive for Ki67 was determined as a percentage of the total number of SMCs in each section. There were a total of six mice in each treatment group. The data shown are the mean percentages of Ki67<sup>+</sup> cells from all four sections from each of the mice.

**Treatment of mice with IGF-I for assessment of signaling events.** Fasted, hyperglycemic, and control mice were injected intraperitoneally with IGF-I (1 mg/kg) or PBS for 15 and 30 min before death. Additional mice were injected with B6H12 (1 mg/kg) or IgG 30 h before death. Aortas were removed and immediately snap-frozen.

**Homogenization of aorta samples for protein analysis.** The aortas were homogenized in ice-cold buffer, 20 mmol/l Tris, 150 mmol/l sodium chloride (pH 7.4), 2 mmol/l EDTA, and 0.05% Triton X100, using a glass tissue grinder. Protein levels were measured (bicinchoninic acid; Pierce), and equal amounts were either examined directly by Western immunoblotting after SDS-PAGE or after immunoprecipitation.

Proteins were separated by SDS-PAGE (under nonreducing conditions to visualize IAP; otherwise, reducing conditions were used) and then visualized by Western immunoblotting with appropriate antibodies (at concentrations between 1:500 and 1:1,000).

**Analysis of MMP-2 gelatinase activity by zymography.** MMP-2 gelatinase activity was assessed in aorta homogenates and serum-free conditioned medium from murine aortic SMCs (mSMCs) by gelatin zymography (14).

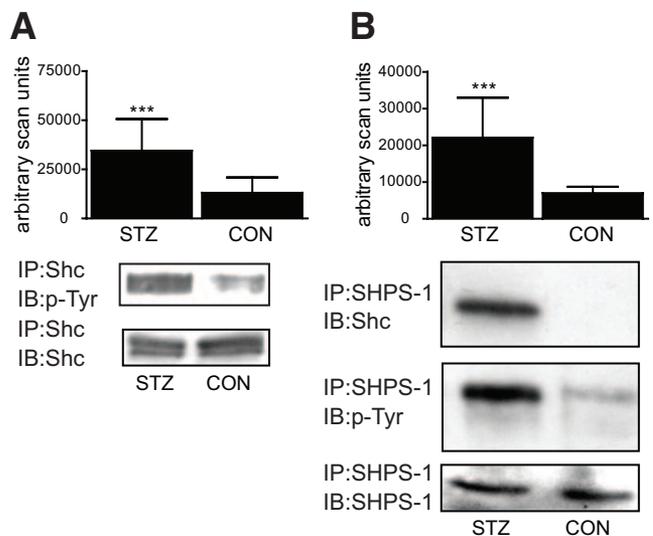
**Isolation of mSMCs.** mSMCs were isolated from the aorta of wild-type C57/B6 male mice and maintained in medium containing either 5 or 25 mmol/l glucose (16). Medium containing 5 mmol/l glucose was supplemented to 25 mmol/l with mannitol (the addition of mannitol does not affect IAP cleavage when SMCs are grown in 5 mmol/l glucose [14]). Both types of medium contained 1.0 mmol/l pyruvate.

**Analysis of IAP from lysates of mSMCs.** mSMCs were plated in medium containing either 25 or 5 mmol/l glucose. The medium was changed after 3 days. After an additional 3 days, the monolayers were rinsed three times with SFM (5 mmol/l glucose supplemented to 25 mmol/l with mannitol) and incubated 16–17 h. The MMP-2 inhibitor (3  $\mu$ g/ml) or an equivalent amount of vehicle (dimethylsulphoxide) was added for 4 h. Cells were lysed in modified radioimmunoassay buffer. After centrifugation, equal amounts of cellular protein were mixed with nonreducing gel-loading buffer, heated to 70°C for 10 min and separated by SDS-PAGE (8%). Proteins were visualized by immunoblotting (13).

**Data quantification and statistical analysis.** For the biochemical analysis of aortic extracts, a representative Western immunoblot for each experiment is shown. Chemiluminescent images obtained from analysis of at least six mice from each treatment group (from at least three independent sets of mice analyzed over a period of 12 months) were scanned using a DuoScan T1200 (AGFA, Brussels, Belgium), and band intensities were analyzed using NIH Image, version 1.61. An unpaired *t* test and ANOVA were used to compare the differences in band intensities between the different groups.

## RESULTS

**Characterization of hyperglycemic mice compared with control mice.** The mean fasting glucose concentration of mice treated with STZ was 285  $\pm$  47 mg/dl compared with 118  $\pm$  17 mg/dl in control mice (mean  $\pm$  SD, *n* = 25). Treatment with IGF-I for 30 h had no significant effect on the glucose levels in either the STZ or control mice. After 15 min, there was a transient 66  $\pm$  7% decrease that returned to pretreatment levels by 20 min.



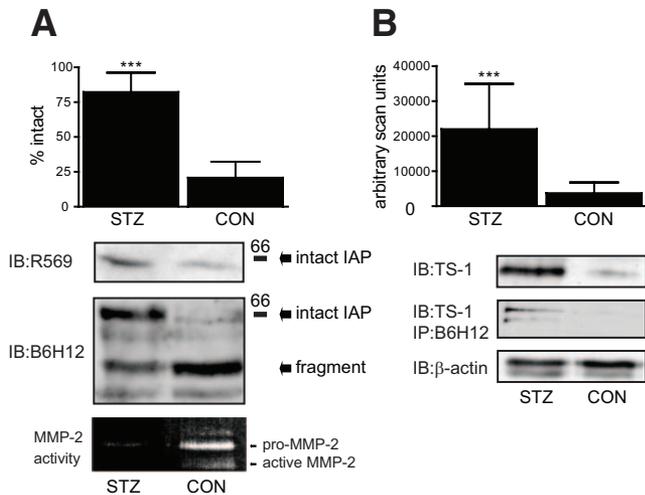
**FIG. 1. A:** Shc phosphorylation and Shc recruitment to SHPS-1 and SHPS-1 phosphorylation in aorta homogenates from hyperglycemic compared with control mice. **A:** Aortas from hyperglycemic (STZ) and control (CON) mice were homogenized. Shc phosphorylation was determined following immunoprecipitation (IP) with an anti-Shc antibody and immunoblotting (IB) with an anti-phosphotyrosine antibody (*p*-Tyr). Total Shc levels were determined by immunoblotting with an anti-Shc antibody after Shc immunoprecipitation. The graph shows the difference in Shc phosphorylation when the aortas from the STZ mice are compared with the CON mice expressed as arbitrary scanning units (mean  $\pm$  SD, *n* = 12, \*\*\**P* < 0.005). **B:** Aortas from hyperglycemic (STZ) and control (CON) mice were homogenized. Shc recruitment to SHPS-1 was determined after immunoprecipitation with an anti-SHPS-1 antibody and immunoblotting with an anti-Shc antibody (*Shc*) (*top panel*). SHPS-1 phosphorylation was determined from using the same homogenate by immunoprecipitation with the anti-SHPS-1 antibody and immunoblotting with an antiphosphotyrosine (*p*-Tyr) antibody (*middle panel*). Total SHPS-1 levels were determined after immunoprecipitation and immunoblotting with the anti-SHPS-1 antibody. The graph shows the difference in Shc recruitment to SHPS-1 between the aortas from the STZ compared with the CON mice expressed as arbitrary scanning units (mean  $\pm$  SD, *n* = 12, \*\*\**P* < 0.005).

There was no significant difference in the body weight between the mice treated with STZ (31  $\pm$  2 g) and controls (29  $\pm$  2 g). Cholesterol levels in the control and STZ-treated mice showed no significant difference (e.g., STZ-treated, 104  $\pm$  3 mg/dl; control, 104  $\pm$  6 mg/dl; NS). There was no significant difference in the levels of IGF-I (STZ-treated, 130  $\pm$  7 ng/ml; control, 130  $\pm$  5 ng/ml; NS). Administration of IGF-I resulted in a 5  $\pm$  0.1-fold increase in serum IGF-I at 15 min; however, after 30 h, it had returned to baseline.

**Shc phosphorylation, Shc recruitment to SHPS-1, and SHPS-1 phosphorylation are increased in aorta from hyperglycemic mice.** The proliferative response of cultured SMCs to IGF-I is determined by the extent of Shc phosphorylation (11). The aortic homogenates from the hyperglycemic mice showed a significant 2.8-fold increase in phosphorylated p52 Shc compared with the control mice (scanning units 34,570  $\pm$  16,048 [hyperglycemic], 12,160  $\pm$  2,459 [control], mean  $\pm$  SD [*n* = 12, *P* < 0.005]) (Fig. 1A).

There was a fourfold increase in the amount of Shc associated with SHPS-1 in the hyperglycemic mice compared with the control mice (Fig. 1B, *top panel*). The scanning units for the hyperglycemic mice were 29,779  $\pm$  14,414 compared with control (7,619  $\pm$  5,848 [mean  $\pm$  SD, *n* = 12, *P* < 0.005]).

Shc recruitment to SHPS-1 requires SHPS-1 phosphorylation (11). When SHPS-1 phosphorylation in aorta homog-



**FIG. 2. Hyperglycemia regulates cleavage of IAP and TS-1 association with IAP.** *A:* Homogenates from hyperglycemic (STZ) and control (CON) mice were separated by SDS-PAGE and IAP protein levels visualized by Western immunoblotting (IB) with the anti-IAP antibody that selectively recognizes intact IAP (R569) and the anti-IAP monoclonal antibody B6H12 that detects intact and fragmented IAP. MMP-2 activity in homogenates from both groups of mice was assessed by gelatin zymography. The graph shows the difference in the amount of intact IAP between the aortas from the STZ compared with the CON mice expressed as percent (mean  $\pm$  SD,  $n = 10$ ,  $***P < 0.005$ ). *B:* Homogenates from hyperglycemic (STZ) and control (CON) mice were either immunoblotted (IB) with an anti-TS-1 antibody (*top panel*), immunoprecipitated (IP) with the anti-IAP antibody B6H12 before immunoblotting with the anti-TS-1 antibody (*middle panel*), or immunoblotted with the anti- $\beta$ -actin antibody. The graph shows the difference in the amount of TS-1 between the aortas from the STZ compared with the CON mice expressed as arbitrary scanning units (mean  $\pm$  SD,  $n = 8$ ,  $***P < 0.005$ ).

enates from hyperglycemic and control mice were compared, there was a sevenfold increase in SHPS-1 phosphorylation (Fig. 1*B*, *middle panel*). The scanning units for the hyperglycemic mice were  $27,466 \pm 14,652$  compared with control ( $4,146 \pm 3,305$  [mean  $\pm$  SD,  $n = 8$ ,  $P < 0.005$ ]).

**Hyperglycemia regulates IAP cleavage.** In order for SHPS-1 to be phosphorylated, it must be associated with IAP (12). Our recent *in vitro* studies have determined that in SMCs cultured in 5 mmol/l glucose, SHPS-1 phosphorylation and formation of the SHPS-1-SHP-2-c-Src-Shc signaling complex (which is required for cellular replication) is impaired because of constitutive cleavage of IAP, which disrupts SHPS-1 binding (14). When the status of IAP in the aortic homogenates was determined, there was a significant (fivefold) increase in intact IAP detected in the homogenates from the hyperglycemic mice compared with controls. The samples were immunoblotted using an anti-IAP antibody (R569) that is specific for intact IAP (Fig. 2*A*, *upper panel*). The homogenates were also immunoblotted with an anti-IAP antibody that can detect both intact and fragmented IAP (Fig. 2*A*, *lower panel*). In the aortas obtained from the hyperglycemic mice,  $82 \pm 13.9\%$  (mean  $\pm$  SD,  $n = 10$ ) of the total IAP was intact. This was significantly greater than the  $20 \pm 11.52\%$  (mean  $\pm$  SD,  $n = 10$ ,  $P < 0.005$ ) of the total IAP that was intact in aortas from the control mice. Our previous studies determined that in SMCs cultured in normal glucose, IAP cleavage is mediated by an increase in MMP-2 protease activity (14). Analysis of the aorta homogenates from the STZ mice using gelatin zymography showed that there was a  $68.5 \pm 0.05\%$  decrease in the amount of protease activity associated with the pro-form of MMP-2 and a similar  $59.3 \pm 3\%$

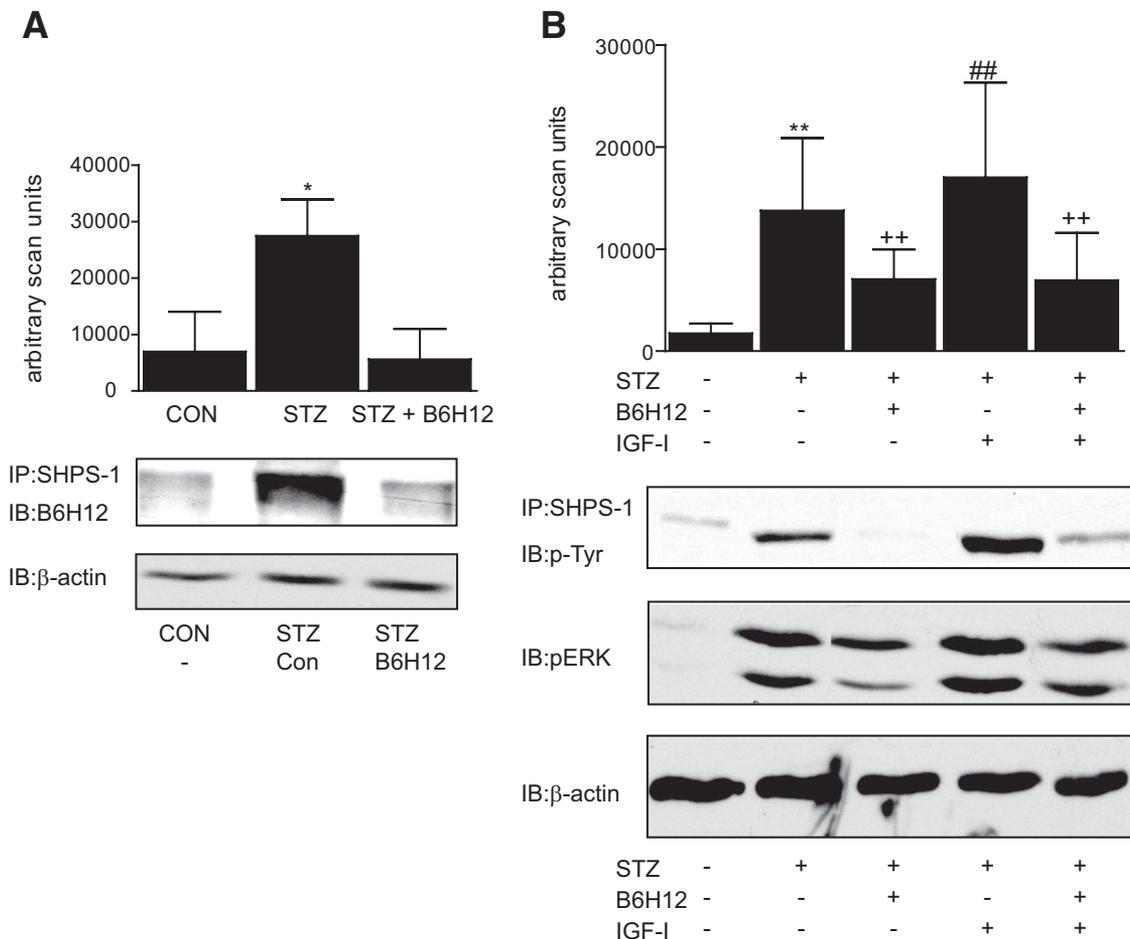
decrease in the amount of protease activity associated with the active form of MMP-2 compared with the control mice (Fig. 2, *bottom panel*; mean  $\pm$  SD,  $n = 3$ ,  $P < 0.05$ ). **Hyperglycemia is associated with an increase in thrombospondin-1 and enhanced IAP-thrombospondin-1 binding.** The IAP ligand thrombospondin (TS)-1 enhances the response of SMC to IGF-I, in part through its binding to IAP (13). There is significantly more TS-1 associated with IAP in SMCs maintained in 25 mmol/l glucose compared with 5 mmol/l glucose (9). Therefore, we determined if the increase in intact IAP in hyperglycemic mice was associated with an increase in TS-1 binding to IAP. There was a sixfold increase in the amount of TS-1 in the aortas of the hyperglycemic mice. The scanning units were  $21,968 \pm 12,948$  (hyperglycemic) and  $3,715 \pm 3,059$  (control) (mean  $\pm$  SD,  $n = 8$ ,  $P < 0.005$ ). Furthermore, there was a significant 3.8  $\pm$  1.3-fold (mean  $\pm$  SD,  $n = 8$ ,  $P < 0.005$ ) increase in the amount of TS-1 that could be coprecipitated with IAP (Fig. 2*B*).

**IAP-SHPS-1 association regulates IGF-I-stimulated signaling events in hyperglycemic mice.** Consistent with the increase in intact IAP in the hyperglycemic mice, we detected a fourfold increase in the amount of IAP that could be coprecipitated with SHPS-1 in the aorta homogenates from hyperglycemic mice compared with controls (Fig. 3*A*). The scanning units were  $27,463 \pm 6,453$  (hyperglycemic) and  $960 \pm 7,046$  (control) (mean  $\pm$  SD,  $n = 6$ ,  $P < 0.05$ ).

The anti-IAP antibody B6H12 disrupts the association between the two proteins and consequently it inhibits IGF-I signaling (12). To determine whether IAP-SHPS-1 association was required for IGF-I signaling *in vivo*, we treated the hyperglycemic mice with IGF-I with or without the systemic administration of B6H12. Treatment with B6H12 induced an eightfold decrease between IAP and SHPS-1 association (Fig. 3*A*) (scanning units  $5,517 \pm 550$  [B6H12] and  $27,463 \pm 6,453$  [hyperglycemic control] [mean  $\pm$  SD,  $n = 6$ ,  $P < 0.05$ ]). Basal SHPS-1 phosphorylation was higher in the hyperglycemic mice compared with controls. After IGF-I, there was a 10-fold increase in SHPS-1 phosphorylation (Fig. 3*B*, *top panel*). Administration of B6H12 significantly decreased SHPS-1 phosphorylation in both the hyperglycemic (a twofold decrease) and the hyperglycemic mice treated with IGF-I (a fourfold decrease) (Fig. 3*B*, *top panel*). The scanning units were as follows: control mice:  $1,725 \pm 980$ , hyperglycemic mice:  $13,751 \pm 7,117$ , the hyperglycemic mice treated with IGF-I:  $17,162 \pm 6,704$ , hyperglycemic mice treated with B6H12:  $7,041 \pm 2,931$ , and hyperglycemic mice treated with IGF-I and B6H12:  $6,539 \pm 4,653$  (mean  $\pm$  SD,  $n = 6$ ,  $P < 0.05$ ).

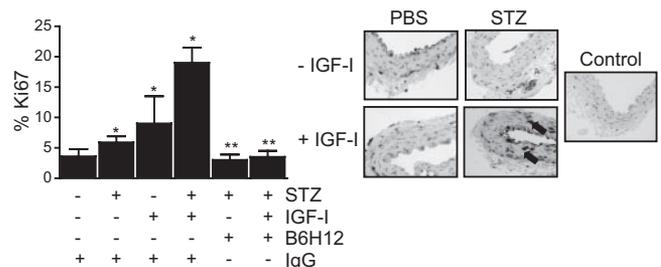
Our previous studies have shown that activation of MAPK and stimulation of SMC proliferation in response to IGF-I requires formation of an IAP-SHPS-1-Shc signaling complex and Shc phosphorylation. Immunoblotting showed an eightfold increase in ERK1/2 phosphorylation in hyperglycemic mice compared with control mice (Fig. 3*B*, *middle panel*). Treatment of the hyperglycemic mice with IGF-I resulted in a 9.8-fold increase in ERK1/2 phosphorylation (compared with no IGF-I treatment). Treatment with B6H12 significantly inhibited the increase in ERK1/2 phosphorylation both in the presence or absence of IGF-I in the hyperglycemic mice (Fig. 3*B*, *middle panel*).

**Increased cell proliferation in response to IGF-I in hyperglycemic mice.** To demonstrate if these differences in signaling observed in the aortas from hyperglycemic

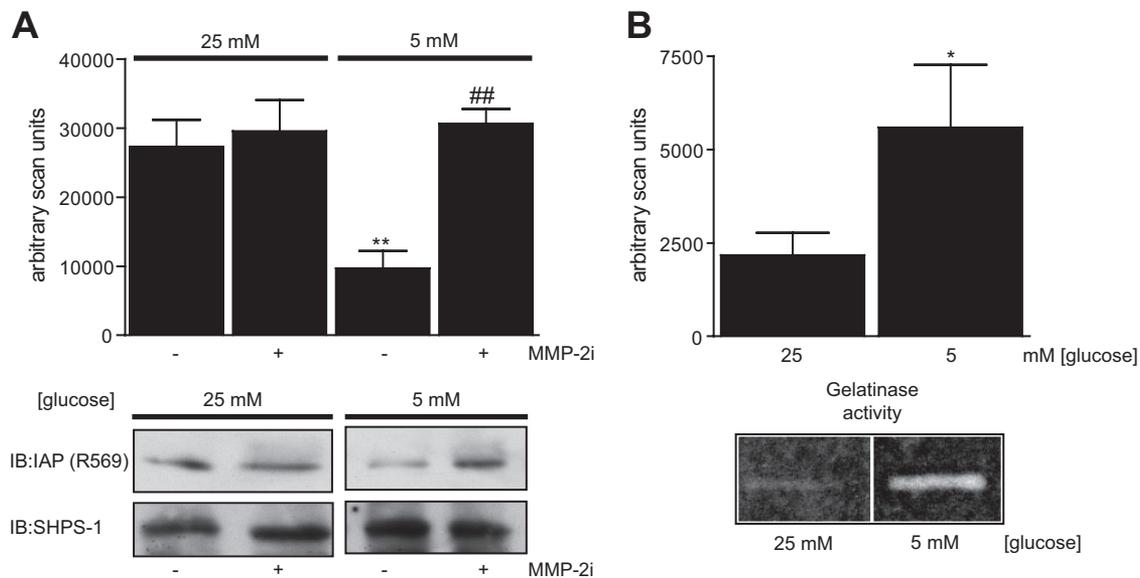


**FIG. 3. Disrupting the association between IAP and SHPS-1 inhibits SHPS-1 phosphorylation and downstream signaling in response to IGF-I.** *A:* Homogenates from control (CON), hyperglycemic (STZ), and hyperglycemic mice that had been treated with the anti-IAP antibody (B6H12) in vivo were immunoprecipitated (IP) with the anti-SHPS-1 antibody, and IAP association with SHPS-1 was determined by immunoblotting for IAP (B6H12) (*top panel*). Equal quantities of homogenate from each sample were also immunoblotted with an anti- $\beta$ -actin antibody (*bottom panel*). The graph shows the difference in IAP association with SHPS-1 between the aortas from the STZ (with or without injection with B6H12) compared with the CON mice, expressed as arbitrary scanning units (mean  $\pm$  SD,  $n = 6$ , \* $P < 0.05$ ). *B:* Homogenates from control, hyperglycemic (STZ), and hyperglycemic mice treated with the anti-IAP antibody B6H12 and/or IGF-I were immunoprecipitated with the anti-SHPS-1 antibody and immunoblotted with the anti-phosphotyrosine antibody (*p-Tyr*) (*top panel*). Homogenates were also immunoblotted directly with the anti-phospho ERK1/2 antibody. To demonstrate that there was no difference in the amount of protein in each sample, equal quantities of aortic homogenates were also immunoblotted with the anti- $\beta$ -actin antibody (*bottom panel*). The graph shows the difference in SHPS-1 phosphorylation between the aortas from the different treatment groups expressed as arbitrary scanning units (mean  $\pm$  SD,  $n = 6$ , \*\* $P < 0.01$  when STZ-treated mice are compared with control, ## $P < 0.01$  when IGF-I treatment of STZ mice is compared with STZ-treated mice alone, and + $P < 0.05$  when treatment with B6H12 is compared with STZ alone).

mice resulted in a change in SMC proliferation, hyperglycemic and control mice were injected with IGF-I, and percentage of SMCs that were Ki67<sup>+</sup> was quantified. Treatment of the control mice with IGF-I increased the percentage of SMCs positive for Ki67 from 3.6  $\pm$  1.2% (PBS alone) to 9  $\pm$  4.5% (mean  $\pm$  SD,  $n = 6$ ). Hyperglycemia alone increased the number to 5.9  $\pm$  1% (mean  $\pm$  SD,  $n = 6$ ;  $P < 0.05$ ). Treatment of hyperglycemic mice with IGF-I resulted in a significant increase in the percentage of Ki67<sup>+</sup> cells to 19  $\pm$  2.5% (mean  $\pm$  SD,  $n = 6$ ;  $P < 0.05$  when compared with both control and hyperglycemic mice) (Fig. 4). Analysis of IGF-I receptor protein levels showed no difference between the control mice and STZ-treated mice. (STZ, 103  $\pm$  3% of the levels in the control mice [mean  $\pm$  SD,  $n = 12$ ]). To determine whether the hyperglycemia-induced increase in IAP association with SHPS-1 was related to enhanced SMC proliferation, hyperglycemic mice were injected with the anti-IAP antibody (B6H12) to disrupt IAP and SHPS-1 association (Fig. 3). After B6H12, the increase in Ki67<sup>+</sup> cells induced by either hyperglycemia



**FIG. 4. Hyperglycemia enhances the proliferative response of SMCs to IGF-I in vivo.** Control (PBS) and hyperglycemic mice (STZ) were treated with IGF-I for 30 h (in the presence of the anti-IAP antibody B6H12 or control IgG). The aortas were removed and paraffin sections prepared. After staining with an anti-Ki67 antibody, the number of proliferating cells in the layer was counted and expressed as the percentage of Ki67 cells. The mean data from six mice per treatment group (with four sections counted/mouse) is shown graphically, and representative images are also shown. \* $P < 0.05$  when the number of Ki67 cells are compared with control mice. \*\* $P < 0.01$  when Ki67 staining in the presence of B6H12 is compared with STZ or STZ plus IGF-I.



**FIG. 5. Hyperglycemia-regulated activation of MMP-2 regulates IAP cleavage in murine SMCs.** **A:** Murine SMCs were grown in medium containing either 25 or 5 mmol/l glucose before overnight incubation in serum-free medium. mSMCs grown in both 25 and 5 mmol/l glucose were then exposed to either an MMP-2 inhibitor (MMP-2i) or vehicle alone before lysis. Intact IAP was visualized after immunoblotting with the anti-IAP antibody that recognizes intact IAP (R569). Blots were then stripped and reprobed with the anti-SHPS-1 antibody. The graph shows the amount of IAP expressed as arbitrary scanning units (mean  $\pm$  SD,  $n = 3$ ,  $**P < 0.01$  when IAP levels in lysate from SMCs grown in 5 mmol/l glucose are compared with lysate from SMCs grown in 25 mmol/l glucose and  $##P < 0.01$  when IAP levels in SMCs grown in 5 mmol/l glucose are compared with lysates from SMCs grown in 5 mmol/l glucose and treated with the MMP-2 inhibitor). The scanning units were as follows: 25 mmol/l glucose  $27,321 \pm 3,862$ , 25 mmol/l glucose plus MMP-2 inhibitor  $28,052 \pm 3,641$ , 5 mmol/l glucose  $9,697 \pm 2,516$ , 5 mmol/l glucose plus MMP-2 inhibitor  $30,637 \pm 2,117$  (mean  $\pm$  SD,  $n = 3$ ,  $P < 0.01$ ). **B:** Conditioned medium collected from mSMCs grown in 25 and 5 mmol/l glucose and incubated overnight in serum-free medium was analyzed by gelatin zymography (top panel). The graph shows the difference in MMP-2 gelatinase activity expressed as arbitrary scanning units (mean  $\pm$  SD,  $n = 3$ ,  $*P < 0.05$ ). The scanning units for medium collected from mSMCs in 25 mmol/l glucose were  $2,173 \pm 576$  and for mSMCs in 5 mmol/l glucose they were  $5,593 \pm 1,671$  (mean  $\pm$  SD,  $n = 3$ ,  $P < 0.05$ ).

mia alone or hyperglycemia plus IGF-I was completely inhibited ( $3 \pm 0.9$  and  $3.5 \pm 1\%$  of cells, respectively, were  $Ki67^+$ ; mean  $\pm$  SD,  $P < 0.01$ ).

**MMP-2 cleaves IAP in murine SMCs.** We determined previously, using both RNA interference and metalloprotease inhibitors, that the protease in porcine SMCs responsible for the cleavage of IAP is MMP-2 (14). Therefore, we analyzed the ability of the MMP-2 inhibitor (14) to protect IAP from cleavage in mSMCs grown in 5 mmol/l glucose. Basally, there was a significant ( $65 \pm 5\%$ ) decrease in the amount of intact IAP compared with mSMCs grown in 25 mmol/l glucose. When SMCs were incubated with the MMP-2 inhibitor, the level of intact IAP in the SMCs grown in 5 mmol/l glucose increased to  $113 \pm 7\%$  of the level of intact IAP in SMCs grown in 25 mmol/l glucose (Fig. 5A). To confirm that MMP-2 activation is regulated by changes in glucose, we compared the amount of active MMP-2 in the conditioned medium from murine SMCs grown in 5 and 25 mmol/l glucose. There was a significant (2.6-fold) increase in active MMP-2, as detected by gelatin zymography, in the conditioned medium from mSMCs in 5 mmol/l glucose compared with the conditioned medium from mSMCs in 25 mmol/l glucose (Fig. 5B).

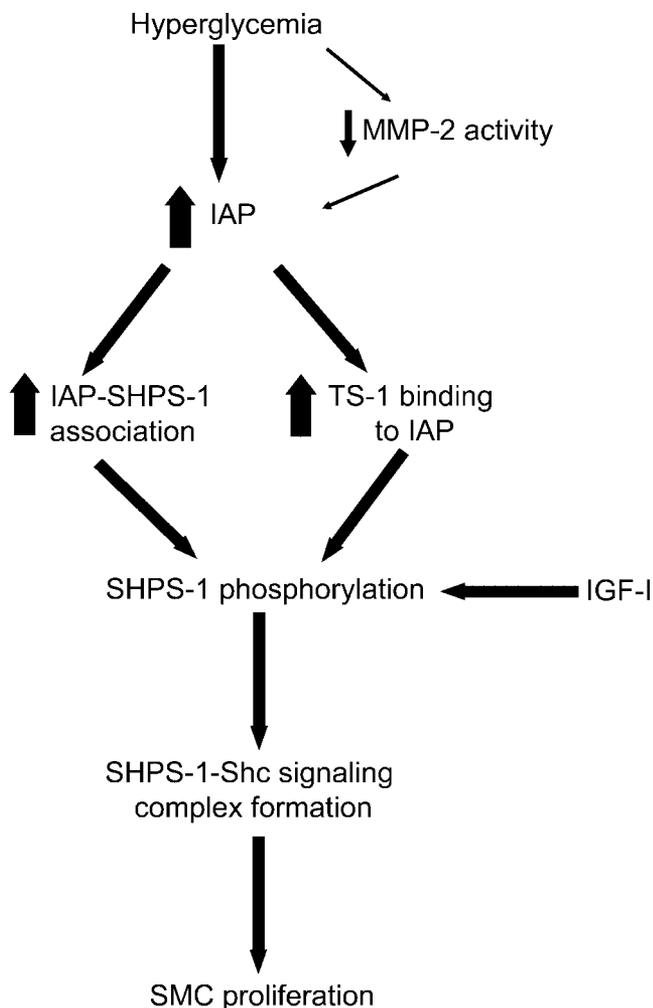
## DISCUSSION

SMCs grown in medium containing 5 mmol/l glucose do not proliferate or migrate in response to IGF-I (9). In contrast, SMCs grown in 25 mmol/l glucose show significant increases after IGF-I stimulation (9). This difference in the responsiveness of SMCs in vitro is due to impaired IAP association with SHPS-1, preventing the recruitment and phosphorylation of Shc (9). The results of this study show that IAP is cleaved constitutively in aortic extracts from normoglycemic animals and does not bind to SHPS-1.

In contrast, the induction of hyperglycemia in mice is associated with inhibition of IAP cleavage. This allows SHPS-1 and Shc phosphorylation and stimulation of DNA synthesis in response to IGF-I. Although we cannot rule out the possibility that other changes induced by STZ also contribute to changes in IAP cleavage, our data strongly support our conclusion that hyperglycemia is a critical regulator of IAP cleavage and thus allows IGF-I signaling leading to increased SMC replication (Fig. 6).

Hyperglycemia has been associated with an increase in SMC proliferation in response to several growth factors, including IGF-I in vitro (9,17–19). While studies in experimental animal models show that manipulation of IGF-I levels in vascular tissue is related to changes in neointimal formation and SMC proliferation, those studies have not assessed the effect of hyperglycemia on vascular cell proliferation (7,8,20,21). Our results clearly demonstrate that the presence of hyperglycemia results in enhanced responsiveness of vascular SMC to IGF-I in mice. Importantly, they also demonstrate that the signaling mechanism that accounts for enhanced SMC responsiveness to IGF-I in vitro (14) is activated in this animal model, thus leading to the conclusion that it accounts for the increase in cell replication.

Shc phosphorylation was significantly increased in the aorta from hyperglycemic mice, clearly demonstrating that the effect of glucose concentration on Shc phosphorylation is not an artifact of culture conditions or an in vitro phenomenon. For Shc to be phosphorylated in response to IGF-I, it must be recruited to phosphorylated tyrosine residues within the cytoplasmic domain of SHPS-1 (11). In normoglycemic mice, there is minimal SHPS-1 phosphorylation, but in hyperglycemic animals, there is a marked increase in basal and IGF-I-stimulated SHPS-1 phosphor-



**FIG. 6.** Proposed model by which hyperglycemia enhances IGF-I signaling in SMCs in vivo. Hyperglycemia protects IAP from cleavage by downregulating MMP-2 protease activity that enhances IAP binding to both TS-1 and SHPS-1. This in turn enhances SHPS-1 phosphorylation in response to IGF-I, generating a high-affinity binding site to recruit the SHP-2-c-Src-Shc. Phosphorylation of Shc then leads to activation of downstream signaling pathways including MAPK, which in turn lead to SMC proliferation.

ylation. The elevated basal levels presumably reflect enhanced tissue responsiveness to endogenous IGF-I. The increase in SHPS-1 phosphorylation results in enhanced Shc recruitment to SHPS-1 and Shc phosphorylation (11). That this enhanced IGF-I responsiveness in the hyperglycemic animals has important consequences was confirmed in the experiments that showed increased MAPK activation and SMC proliferation.

IAP cleavage results in loss of SHPS-1 association, which disrupts the ability of IGF-I to stimulate MAPK pathway activation and SMC proliferation. Our results show that, in normoglycemic mice, the extracellular domain of IAP is cleaved, resulting in impaired IAP-SHPS-1 association and SHPS-1 phosphorylation. The importance of this association for IGF-I actions is underscored by the result of the experiment in which we disrupted IAP/SHPS-1 using the anti-IAP antibody B6H12. This resulted in attenuation of SHPS-1 phosphorylation and downstream signaling basally and in response to IGF-I. More importantly, this disruption of IAP-SHPS-1 resulted in complete inhibition of the IGF-I-stimulated increase in cell proliferation. This strongly supports the conclusion that failure of

IAP-SHPS-1 to associate in normoglycemic conditions leads to attenuation of IGF-I signaling events.

Sajid et al. (22) demonstrated that after balloon injury in baboons, there is a significant increase in IAP in the neointima and media, whereas in uninjured vessels, IAP is only detected in endothelium. A significant increase in TS-1 was detected in the same areas after injury (22). Consistent with our findings TS-1 binding to IAP was associated with enhanced SMC proliferation in vitro. While the mechanism that resulted in the increase in detection of intact IAP in the injured vessel was not determined in that study, it seems reasonable to propose that increased cellular levels of IAP and TS-1 are common responses of vascular SMCs to stress (e.g., mechanical injury or hyperglycemia). The increase in IAP and its association with TS-1 leads to enhanced responsiveness of SMCs to IGF-I.

Our finding of increased TS-1 in aorta from hyperglycemic mice is consistent with prior studies that detected increased levels of TS-1 in blood vessels of diabetic Zucker rats (23). Other models of diabetes have also demonstrated increases in TS-1 in cardiac fibroblasts (24), myocytes (25,26), and SMCs (27). TS-1 binding to IAP enhances IGF-I signaling by its ability to modulate the association between IAP and SHPS-1 (13). Because the increase in TS-1 that was detected in the hyperglycemic mice was associated with an increase in the amount of TS-1 associated with the IAP, it seems likely that this change is related to the increase in IAP/SHPS-1 association. Thus, the increased expression of TS-1 in response to hyperglycemia and the associated enhancement of IGF-I signaling is likely to be an important contributor to increased cell proliferation.

In a prior study using MMP-2 RNAi, we determined that the protease responsible for IAP cleavage in porcine SMCs was MMP-2 (14). Our studies using SMCs isolated from murine aorta and an MMP-2 inhibitor strongly support our conclusion that MMP-2 is the protease responsible for IAP cleavage (14). In this study, we demonstrate that there is more MMP-2 activity associated with the aorta from hyperglycemic mice than from control mice. Taken together, these findings suggest that hyperglycemia is functioning to regulate the amount of active MMP-2 in the extracellular environment, thereby regulating IAP cleavage (Fig. 6).

Understanding the role of MMPs in atherosclerosis is complicated by evidence suggesting they play a dual role by regulating SMC migration, matrix deposition, and instability caused by matrix destruction (28). The role of MMPs is further complicated by the use of inhibitors that are not specific for just one MMP and by the possibility of compensatory function of another MMP when one is inhibited. In MMP-2/Apo E<sup>-/-</sup> mice fed a high-fat diet, lesion size was reduced compared with control mice, apparently as a result of decreased SMC migration (29). In another study, after balloon injury to rats, inhibition of MMP-2 and -9 resulted in decreased SMC migration and early lesion development, but the intimal lesions eventually increased to control levels because of an increased SMC replication rate in the rats treated with the MMP inhibitor (30,31). These findings are consistent with our results that suggest that inhibition of MMP-2 cleavage of IAP enhances SMC proliferation in response to IGF-I. It is interesting to note that Webb et al. (31) observed that, unlike the other MMPs, MMP-2 was produced constitutively in normal rat vessels, but that there was a decrease in levels immediately after injury, rebounding again over time. Other studies have demonstrated either a decrease in MMP-2 (32,33) or an

increase in its inhibitor TIMP-1 (34) in cells or tissues from diabetic animals. Thus, our studies support the conclusion that disruption of the association between IAP and SHPS-1 by modulating MMP-2 activity may provide opportunities for delaying the progression of lesion formation that occurs in response to hyperglycemia.

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