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Fibroin Enhances Insulin Sensitivity and Reverses Insulin Resistance in 3T3-L1 Adipocytes

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ABSTRACT

Type 2 diabetes is characterized by hyperglycemia and hyperinsulinemia, features of insulin resistance. *In vivo* treatment of *ob/ob* mice with hydrolyzed fibroin reverses these pathological attributes (6). To explore the mechanism underlying this effect, we have used the 3T3-L1 adipocytes as a cell type which would represent the periphery, *in vivo*. Exposure of 3T3-L1 adipocytes to chronic insulin leads to the a 50% loss of insulin-stimulated glucose uptake. Chronic exposure to fibroin blocked, in part, the response to chronic insulin but also increased the sensitivity of control cells to the acute action of insulin. The later effect was most robust at physiological concentrations of insulin. Fibroin did not prevent the insulin-induced down-regulation of the insulin receptor or the tyrosine kinase activity associated with the receptor. Further, fibroin had no affect on the loss in activity of the insulin-sensitive down-stream kinase, Akt. Interestingly, fibroin accelerated glucose metabolism and glycogen turnover independent of insulin action. In addition, fibroin up-regulated GLUT1 which increased its expression at the cell surface and caused the redistribution of GLUT4 to the plasma membrane. Together, these later effects would lead to an improvement in hyperglycemia *in vivo* which would in turn reduce the need for insulin.

INTRODUCTION

Glycemic control is considered the key to preventing and/or delaying the long term complications of type 2 diabetes which include damage to blood vessels and peripheral nerves, along with increased risk to heart attack, stroke, blindness, and kidney failure. To regulate blood glucose, four classes of hypoglycemic drugs are currently in use: sulfonylureas, α-glucosidase inhibitors, metformin, and thiazolinediones (1). The sulfonylureas are the most widely used drugs and function primarily by stimulating insulin secretion. The so-called second generation drugs (glipizide, glyburide, and glimepiride) have longer half-lives which translates to less frequent administration. The drawback to these drugs is the substantially increased risk of hypoglycemia. Acarbose, an α-glucosidase inhibitor, changes the pattern of glucose absorption in the small intestines which lowers the amount of glucose which crosses the epithelial layer. The side effects are relatively minor. Metformin, a biguanide, has been available in the US since 1995. Much of its hypoglycemic effect is due to the suppression of hepatic glucose production, although there is recent evidence to suggest that glucose metabolism in peripheral tissue is accelerated. Although rare, one of the side effects of metformin is lactic acidosis which has a fatality rate of about 50% when it occurs. The thiazolinediones (TZDs) specifically target muscle and fat to enhance insulin action. Effects include increased insulin receptor phosphorylation, IRS-1 phosphorylation, and Akt (PKB) phosphorylation, along with increased the expression of GLUT4. First generation TZDs, like troglitazone (Rezulin), have been removed from the market because of reports of severe liver injury. Newer TZDs (rosiglitazone and pioglitazone) have a much lower incidence

of liver damage and are thus a more viable alternative in the treatment of diabetes. A number of natural products have been shown to exert anti-diabetic activity (for review, see Broadhurst et al. (2)). For example, in 1990, Khan et al. (3) demonstrated that an ammonium hydroxide extract of cinnamon significantly potentiated the effect of insulin on glucose metabolism in isolated rat adipocytes. The stimulatory activity could be removed from the extract by polyvinylpyrrolidone indicating that the compound contains aromatic hydroxyl groups. Indeed, Anderson and colleagues have demonstrated that the flavonoid, methylhydroxychalcone polymer, is the most active compound in the extract (4). Impari-Radosevich et al. (5) have shown that the site of action of the extract in potentiating insulin action is upstream from PI3-kinase and may involve enhanced insulin receptor autophosphorylation and inhibition of tyrosine phosphatase-1B. In 1995, Nahm and Oh (6) demonstrated that fibroin, the major protein in silk, decreased both blood glucose and insulin in *ob/ob* mice. The mechanism underlying this anti-diabetic activity was not explored. In the present study, we show that fibroin enhances insulin-dependent glucose uptake and blocks the development of insulin resistance in 3T3-L1 adipocytes. The fibroin enhancing activity was sensitive to wortmannin, an inhibitor of PI3-kinase, but did not influence insulin-mediated receptor down-regulation or loss of insulin-dependent autophosphorylation. Further, the loss of the downstream kinase, Akt, was not affected by fibroin. Independent of insulin sensitivity, fibroin increased glucose metabolism. Fibroin increased the expression of GLUT1 which may underlie this phenomenon. Together these data suggest that the *in vivo* actions of fibroin are mediated, at least in part, through an increase in glucose uptake and metabolism in peripheral tissues.

MATERIALS AND METHODS

Fibroin preparations

Fibroin from cocoons of the silkworm, *B. mori*, was supplied by Aminogen Co., Ltd. (Seoul, Korea). Sericin, the other major silk protein, was removed as previously described (7). Briefly, 50 g of cocoons were cut into small pieces and boiled in 2.5 L of 5% (w/v) Na_2CO_3 for 1h and then passed through filter paper. The residue which is mainly fibroin, was washed three times with hot dH_2O to remove any remaining sericin. The fibroin was then solubilized by heating 35 g residue in 346 mL distilled water and 280 mL ethanol containing 226.4 g $CaCl_2$ at 90°C for 1h. The solution was dialyzed against dH_2O for 2 days and then lyophilized. This preparation constituted fibroin protein. To prepare the hydrolysate, fibroin was dissolved in an 80-fold volume of 2 N HCl and boiled for 4h. The brown-colored hydrolysate was neutralized to pH 7.4 with 2 N NaOH and dialyzed against distilled water for 1 day and lyophilized. The efficiency of hydrolysis was about 75%. The unhydrolyzed fibroin was removed by centrifugation through a centrifugal filter membrane with a MWCO of 5,000. This preparation is referred to as the fibroin hydrolysate. To separate bioactive fractions from the hydrolysate, approximately 300 mg dissolved in water was applied to a Sephadex G-25 column (2×110 cm) and eluted with water at a flow rate of 0.6 mL/min. Three fractions were noted: Fraction I (mw～5,000); Fraction II (mw～3,000); and Fraction Ⅲ (mw～1,000). Fraction Ⅲ contained about 30% of the total peptide eluted from the column and is referred to as peptide fraction Ⅲ. Further analysis of this fraction by Sephadex G-15 chromatography indicated that it was composed of at least four peptide fragments. These fragments were not individually collected or further analyzed for bioactivity because of limited quantities. The amino acid compositions of fibroin and peptide fraction III have been reported (7). Of note is the high concentration of glycine and alanine (73% and 80%, respectively, for fibroin and peptide fraction Ⅲ).

Cell culture and induction of insulin resistance

3T3-L1 fibroblasts were grown and differentiated as previously described (8). 3T3-L1 adipocytes were treated or not with fibroin protein, fibroin hydrolysate, or peptide fraction Ⅲ at specific concentrations (0.1～10 mg/mL)

for the times as indicated in the figure legends. To induce insulin resistance, cells were exposed to chronic insulin (10 nM) for 12 h in the presence or absence of fibroin, as indicated in legends. Insulin resistance was also induced with TNF α (500 pM) for 96 h in the presence or absence of fibroin. Prior to the glucose transport assay or in subcellular fractionation studies, cells were washed with three times with 3.0 mL Krebs Ringer phosphate buffer containing 5 mM glucose and 0.1% BSA (Sigma, #7050) at 40 min intervals over a 2 h period.

Glucose transport assay

Insulin-sensitive glucose transport was assayed as previously described (9). In some experiments, the concentration of acute insulin was varied from 0.01 to 1000 nM. Where indicated, wortmannin (100 nM) was added 10 min prior to acute insulin.

Insulin receptor immunoprecipitation and detection

Total membranes were collected and extracted in RIPA buffer containing protease inhibitor cocktail (Sigma), 100 mM NaF, and 1 mM $NaVO_3$. The extracted was clarified by centrifugation. The clarified extract was exposed to Protein A Sepharose beads for 1 h. After centrifugation to remove the beads, the precleared extract was exposed to anti-insulin receptor antibodies, which have been previously described (10). After overnight incubation at 4°C, the immune complexes were collected with Protein A Sepharose. The beads were washed three times with RIPA buffer and three times with RIPA containing 1 M NaCl. Protein was eluted with SDS sample dilution buffer. Proteins were separated by 7.5% SDS-PAGE followed by transfer to nitrocellulose. Blots were screened for total and tyrosine phosphorylated receptor.

Analysis of Akt

To test for the involvement of Akt, cells were extracted in buffer containing NaCl (140 mM), Tris-base (20 mM), EDTA (1 mM), NaF (100 mM), Na_2PO_4 (10 mM), Na_3VO_4 (1 mM), PMSF (1 mM), protease inhibitor cocktail (Sigma), NP-40 (1%), pH 7.5. The extract was mixed at 4°C for 30min and clarified by centrifugation as 16,000×g at 4°C for 10 min. Proteins in the clarified supernatant were separated by reducing SDS-PAGE and transferred to nitrocellulose. Samples were not heated as this caused aggregation in our hands. The concentration of total Akt (anti-Akt, Sigma) was compared against the phosphorylated pool [serine^{473} (Santa Cruz) and threonine^{308} (Cell Signaling Technology)] using phospho-specific antibodies in a western blot analysis.

Membrane isolation

Total membranes were prepared as described by Kitzman et al. (11) and subcellular membranes were isolated from cells as previously described (12). Proteins were separated by SDS-PAGE and transferred to nitrocellulose for western blot analysis. The nitrocellulose membranes were probed for GLUT1 and GLUT4 using polyclonal antibodies which recognize the C-terminus.

Western blotting

For GLUT1, GLUT4, and the insulin receptor, the nitrocellulose containing subcellular membrane fractions was blocked in 5% Carnation Instant Dry Fat Milk in Tris-base (20 mM), NaCl (137 mM), 0.1% Tween pH, 7.4 (TBS-T) for 1.5 h at room temperature. The membrane was washed three×1 min and then two×5 min in TBS-T. Primary antibody was added to TBS-T containing 5% milk and incubated with the membrane for 1.5 h at room temperature. The membrane was again washed with TBST. The secondary antibody with covalently attached horseradish peroxidase was diluted 1:80,000 in TBS-T containing 5% milk for 1 h at room temperature. The membranewas ashed four×1 m in and three×5 min in TBS-T. Detection reagents (Amersham) were added for

1 min and then the nitrocellulose exposed to film. For the detection of pAkt, the procedure varied from the above in that the primary antibody was diluted in TBS-T containing 5% BSA and incubation was carried out overnight as 4EC. The secondary antibody was also diluted in TBS-T with BSA. Analysis of bands was performed by video densitometry (Visage, Millipore).

Northern blot analysis

MRNA was analyzed as previously described (13).

RESULTS

Effect of fibroin on glucose uptake and metabolism

The glucose transport system in 3T3-L1 adipocytes is exquisitely sensitive to the acute addition of pharmacological insulin (1 μM) which is illustrated in Fig. 1. A 10- to 15-fold increase in glucose uptake is routinely observed. As we have shown previously (9), chronic exposure to low concentrations of insulin (10 nM) reduces insulin-sensitive glucose uptake by more than 50% which is also confirmed in Fig. 1. In our hands, this is related to a loss of GLUT4 in the intracellular storage pool which translates to fewer transporter on the cell surface in response to insulin.

With this model system, we have tested the effect of fibroin protein (Panel A), the hydrolysate (Panel B), and peptide fraction III (Panel C) on insulin-stimulated glucose transport in control and insulin-resistant cells. Chronic fibroin protein (FP) treatment enhanced acutely stimulated glucose transport by about 20% and almost completely blocked the loss of insulin sensitivity in response to chronic insulin exposure. Fibroin hydrolysate (FH) was about equally effective in sensitizing cells to acute insulin compared to fibroin protein. However, FH was not as effective as FP in blocking the development of insulin resistance. Fibroin peptide fraction III (FIII) was more effective than either FH or FP in sensitizing cells to acute insulin, increasing insulin-stimulated glucose uptake by about 30~40% relative to controls. FIII was more like FH than FP in its ability to block the development of insulin resistance. While these effects were small in the glucose transport assay (performed at high concentrations

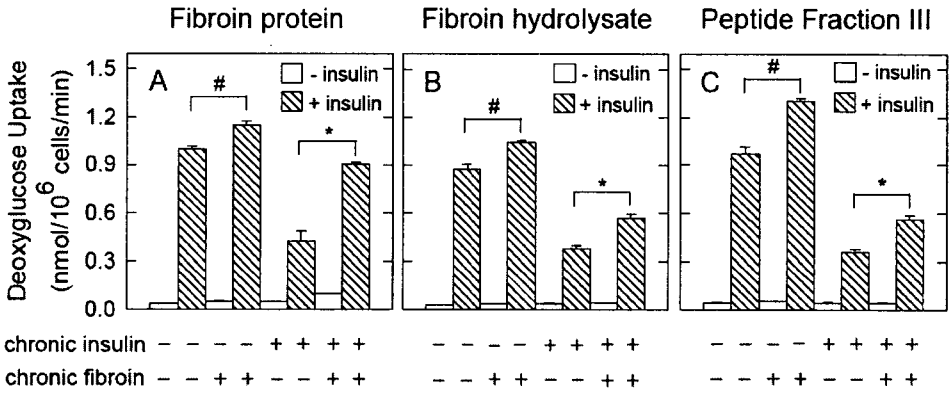

Fig. 1. Fibroin enhances insulin-dependent glucose uptake and blocks insulin-resistance in 3T3-L1 adipocytes.
3T3-L1 adipocytes were exposed to fibroin protein, hydrolysate, or peptide fraction III (5 mg/mL) for 12 h in complete DMEM containing 10% FBS. The medium was changed and insulin (10 nM) was added or not along with fresh fibroin for another 12 h. At the end of 24 h, cells were washed as described in the Methods section and incubated or not with insulin for 10 min. [^{3}H]2-Deoxyglucose (0.2 mM, 0.2 μCi) was added for an additional 10 min. Transport activity was terminated with 3 washes of ice-cold PBS and cells were lysed in 0.1% SDS. An aliquot was for radioactivity. Data represent duplicate experiments of cytochalasin B-inhibitable uptake. $^{\#}p<.001$ relative to control acute, insulin-stimulated value; $^{*}p<.001$ relative to acute, insulin-stimulated value in cells exposed to chronic insulin.

of insulin), they are specific to fibroin as neither albumin nor gamma globulin at the same concentration had any effect on insulin sensitivity or the development of insulin resistance (data not shown). To mimic a more physiological setting, we examined the effect of fibroin on insulin-sensitive glucose transport stimulated at lower concentrations of insulin. In this experiment (Fig. 2), we chose FIII because it was the most efficient insulin-sensitizing agent. Again, we see that FIII increased the maximal insulin-dependent transport activity (Panel A), but importantly, the sensitivity of the cells toward insulin as well (Panel B). Because physiological concentrations of insulin range from 50 to 500 pM, this enhanced sensitivity is striking. Thus, the difference in insulin-stimulated glucose uptake within this range increased by 2~3 fold when cells were exposed to fibroin. These data strongly support the enhancement of insulin-dependent glucose uptake as a major target of fibroin action.

In examining the time-dependency of fibroin action, we noted that basal glucose uptake activity increased at both the 36 and 48 h time points (Fig. 3A). This phenomenon was reminiscent of the increase in glucose transport activity that results from glucose deprivation (11). Because the increase in transport activity occurred within the normal feeding cycle of these cells (every 48 h), it suggested to us that glucose metabolism is accelerated in response to fibroin, which depletes the extracellular concentration of glucose to the point at which the cells become glucose starved. Thus, we tested whether refeeding at the 24 h time point would suppress the observed activation. Indeed, this was the case (Fig. 3B). Thus, fibroin appears to increase glucose metabolism independent of insulin action.

To further examine this phenomenon, we measured the concentration of glucose remaining in the medium from cells exposed or not to fibroin. As expected, glucose concentration fell over time in medium overlaying control cells. By 48 h, glucose was essentially depleted. This illustrates the critical need for refeeding 3T3-L1 adipocytes every 48 hours to avoid glucose deprivation. Note that at each time point during the 48-hour time course, glucose in medium from cells exposed to FIII was further reduced relative to controls (Fig. 4A). By 36 h, there was nearly a 50% difference in the glucose concentration remaining in the medium (see inset). Twelve hours after refeeding, the concentration of glucose in the medium was the same as that after the initial 12 hours of feeding, yet the difference between control and FIII-treated cells remained. Because previous experiments revealed that glucose deprivation increases glycogen turnover (14), we also measured cellular glycogen as an

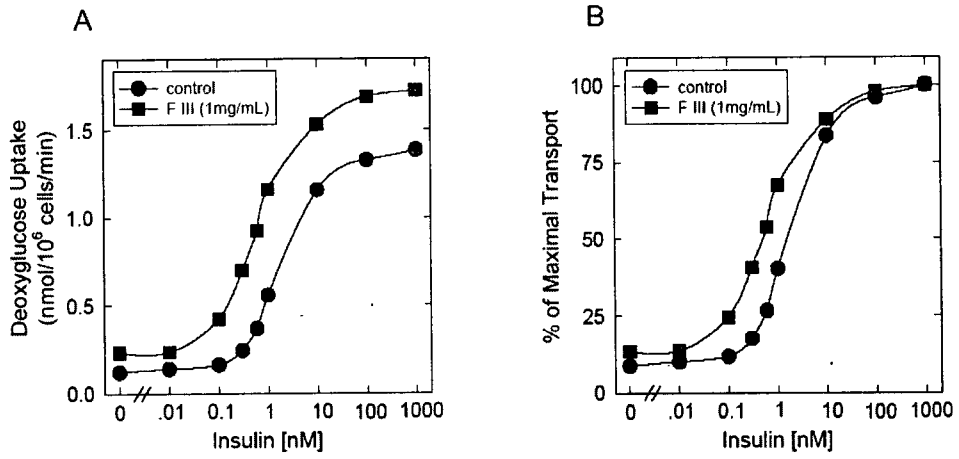

Fig. 2. Peptide fraction III increases sensitivity to acute insulin exposure.
Cells were treated with peptide fraction III (FIII) for 24 h. Cells were washed three times with KRP and allowed to sit for 10 min at 37°C. Insulin, at the indicated concentrations, was added for 10 min and transport activity assessed. The data represent duplicate experiments.

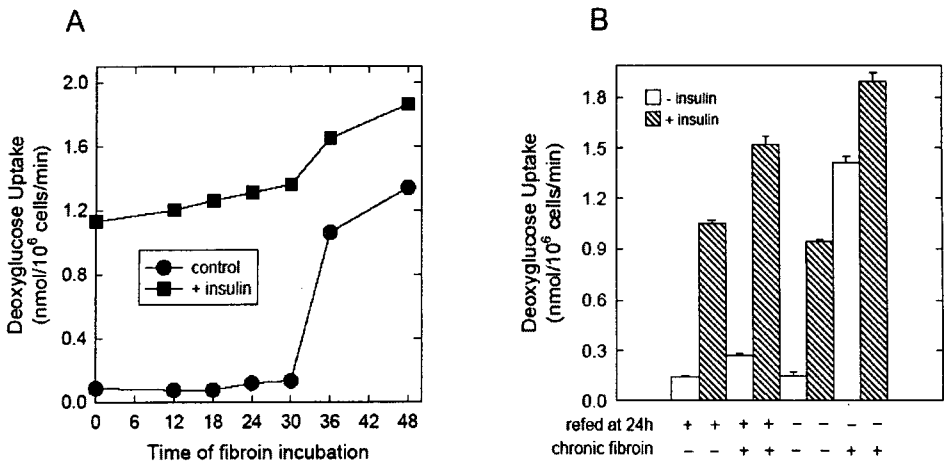

Fig. 3. Fibroin increases basal glucose uptake with extended exposure.
Panel A. Cells were treated with peptide fraction III (2.5 mg/mL) for the indicated times. Glucose uptake was measured at those times in the absence or presence of insulin (1 μM). *Panel B.* Cells were treated with peptide fraction III as in Panel A. Some cells were refed at 24 h with fresh medium and fibroin. At 48 h, glucose uptake was measured in the absence or presence of insulin. Both sets of data are representative of duplicate experiments.

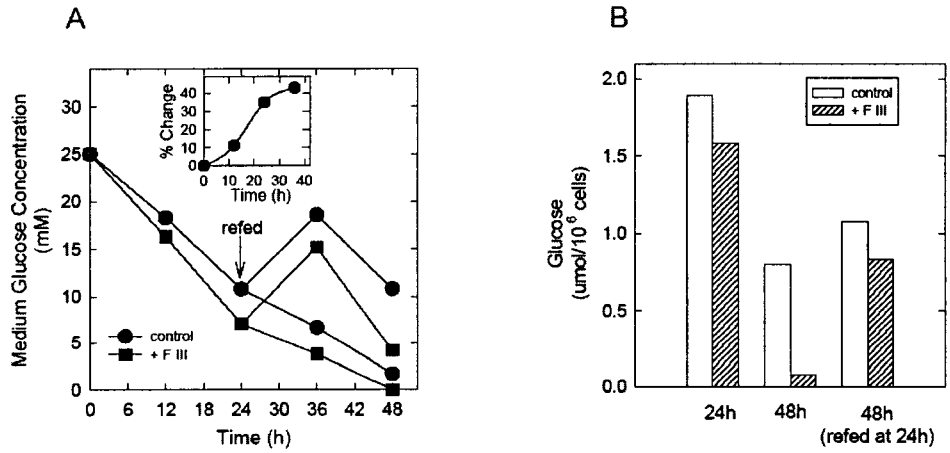

Fig. 4. Fibroin increases glucose and glycogen utilization.
Panel A. Cells were exposed or not to FIII (2.5 mg/mL) for the times indicated. At 24 h, some cells were refed with fresh medium and FIII. Glucose concentration in the medium was assayed using an hexokinase kit from Sigma. These data are representative of two independent experiments. *Inset:* The data in Panel A was used to calculate the percent difference in medium glucose between the control and fibroin-treated cells at each time point. *Panel B.* Cells were treated with or without peptide fraction III for 24 or 48 h: some cells were refed at 24 h. Total glycogen was measured as previously described (14) and reported as total glucose/10^6 cells. These data are representative of duplicate experiments.

indicator glucose utilization (Fig. 4B). At 24 h of exposure to FIII, the concentration of glycogen in the cells was reduced by only 15% compared to controls. However, at 48 h of exposure, the fibroin-treated cells contained only 10% of the control value although this could be prevented by refeeding. Together with Fig. 3, these data support the hypothesis that fibroin increases glucose metabolism. While the mechanism for the increase in glucose metabolism is not known, this infers that adipocytes which are more metabolically active are also more insulin sensitive as has been previously hypothesized (15).

Because the sequence of FIII peptides have not been elucidated, we have synthesized a one of the repeating hexapeptides which comprises fibroin (Gly-Ser-Gly-Ala-Gly-Ala) to determine its affect on glucose uptake. This

synthetic peptide was 95% pure based on HPLC analysis (Protein Chemistry Core, University of Florida). Fig. 5 shows that the synthetic peptide is effective in sensitizing the adipocytes to the acute action of insulin in both control and insulin-resistant cells. As expected from the work of Traxinger and Marshall (16), free amino acids (glycine, alanine, or serine) alone or in combination did not affect on transport activity (data not shown).

In addition to chronic insulin, TNF α has been implicated in the development of insulin resistance (17-21). This phenomenon appears to be related to general cachexia which leads to the de-differentiation of adipocytes and thus loss of GLUT4 expression. To determine if fibroin interferes with this process, we examined the effect of fibroin protein on insulin-stimulated glucose uptake in TNF α-treated 3T3-L1 adipocytes. In our experiments, we found that several days of TNF α treatment were required to demonstrate insulin-resistant glucose transport. Thus we incubated both fibroin protein and TNF α, together, for four days with medium changes each day. The results are shown in Fig. 6. TNF α alone reduced acute, insulin-sensitive transport by nearly 60%. As before, fibroin clearly increased insulin-sensitive transport but was unable to reverse the insulin-resistance induced by TNF α. This observation demonstrates basic differences between the mechanisms which underlie insulin resistance induced by chronic insulin versus TNF α.

Effect of fibroin on insulin signaling elements

To determine if the PI3-kinase pathway is involved in fibroin-induced sensitization, we utilized the PI3-kinase inihibitor, wortmannin. As shown by others, insulin-sensitive glucose uptake is completely blocked by wortmannin (Fig. 7). In fibroin-treated cells, wortmannin was clearly inhibitory, but the inhibition was not as complete as in

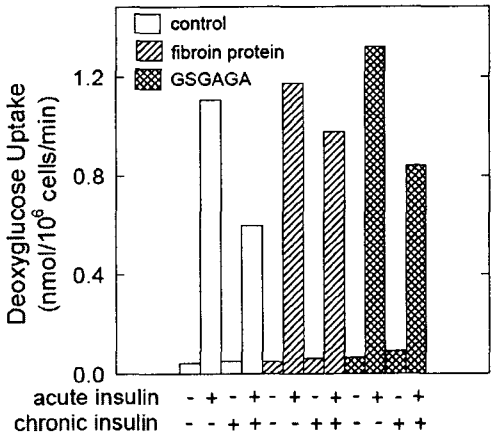

Fig. 5. The fibroin hexapeptide, GSGAGA, increases insulin sensitivity in 3T3-L1 adipocytes.
Cells were treated for 24 h in the presence or absence of fibroin protein (1 mg/mL) or GSGAGA (1 mg/mL: 2.4 mM). Twelve hours into the incubation, cells were exposed to insulin (10 nM) which was added in fresh medium with additional fibroin protein or GSGAGA, as appropriate. Cells were then extensively washed and transport activity measured in the presence or absence of 1 M insulin. Data represent the average of duplicate points in a single experiment. Variation in the data was less than 5%.

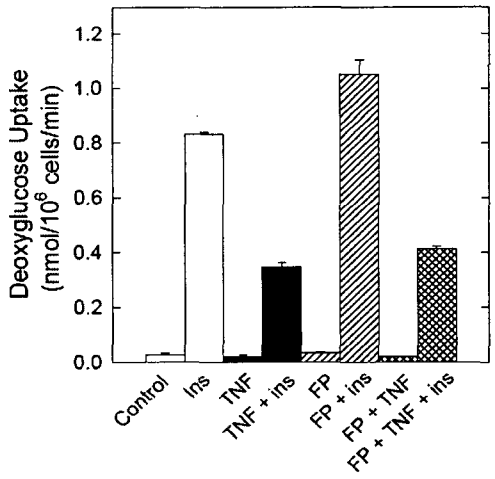

Fig. 6. Fibroin does not block TNF α-mediated insulin resistance.
Cells were incubated for 96 h in the presence or absence of fibroin protein (2.5 mg/mL) and/or TNF α (500 pM). Medium was changed every 24 h. Cells were washed and transport measured as in Fig. 1. Data represent the average SD of duplicate experiments of cytochalasin B-inhibitable uptake. Ins=acute insulin (1 M); FP=fibroin protein; TNF =TNF α. Open bars, controls (no TNF α); closed bars, chronic exposure to TNF α; striated bars, chronic treatment with FP; hatched bars, chronic treatment with TNF α and FP.

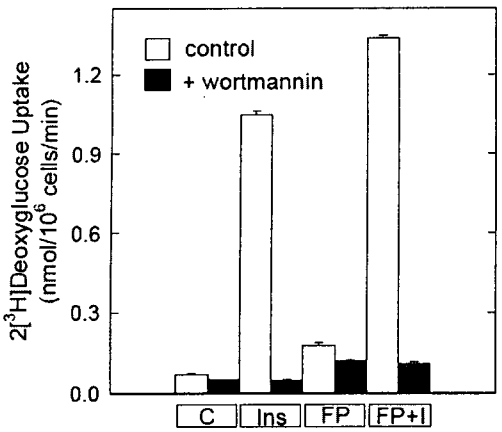

Fig. 7. Wortmannin blocks the effect of fibroin on acute insulin-stimulated glucose uptake.
Cells were exposed to fibroin hydrolysate (5.0 mg/mL) for 24 h. The cells were rinsed in KRP and incubated with or without 20 μM cytochalasin B for 10 min at 37°C. Wortmannin (100 nM) was added or not for 10 min. Insulin (1 M) was then added for an additional 10 min before deoxyglucose uptake was assessed. The data represent cytochalasin B-inhibitable uptake in a single experiment with duplicate points. C=control; Ins=acute insulin; FP =chronic treatment with fibroin hydrolysate; FP+I=chronic fibroin+ acute insulin.

the control cells. This suggests that there may be both PI3-kinase-dependent and -independent paths by which fibroin alters insulin-sensitivity.

Previously, we have shown that chronic insulin reduces insulin binding by about 20% but unaffected by the presence of glucose (9). Based on the argument that only 10% of the receptor pool needs to be occupied for 100% of the insulin-dependent glucose uptake (22), we concluded at that time that the decrease in insulin-dependent glucose uptake after chronic exposure to insulin was not due to loss of insulin binding. The same was observed by Garvey et al. (23) in cultured adipocytes. It is reasonable to conclude that insulin resistance must be due to defects at a postbinding or postreceptor site. Thus, to further explore the effect of fibroin on insulin resistance, we next looked at an event upstream of PI3-kinase activation, i.e., insulin-induced, insulin receptor phosphorylation. As shown in Fig. 8B, chronic insulin even at the relatively low concentration of 10 nM resulted in a 50% loss in the total pool of receptors (25.4±1.8 vs 11.1±1.3 for control and chronic insulin-treated cells, respectively). Even more striking was the 80% reduction in insulin-dependent autopho sphorylation (22.7±2.8 vs

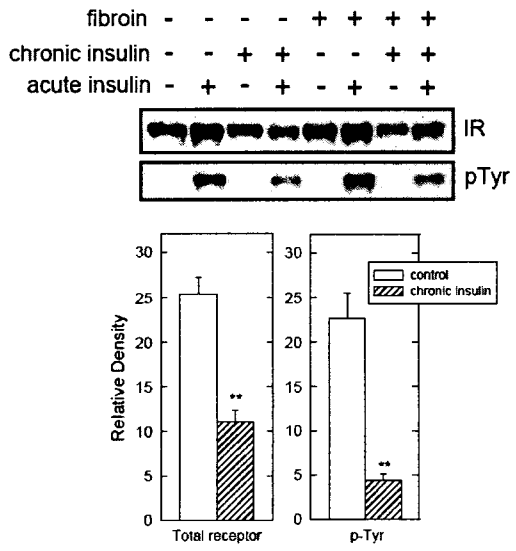

Fig. 8. Fibroin does not affect the insulin-induced down-regulation of receptor number or catalytic activity.
Cells were treated with fibroin hydrolysate (5.0 mg/mL) when indicated for a total of 24 h. Insulin (10 nM) was added 12 h into the incubation. Cells were extensively washed and acute insulin (1 M) added in KRP for 10 min. After washing, cells were scraped in a buffer (Tris, 20 mM; EDTA, 1 mM; NaCl, 140 mM; NaF, 100 mM; Na_sPO_4, 10 mM and Na_3VO_4, 1 mM; pH 7.5) containing protease inhibitors and a total membrane fraction was isolated. Equal protein (3.5 mg) was extracted in RIPA buffer and incubated with Protein A Sepharose beads for 1 h to remove non-specific interactions and then overnight with anti-insulin receptor antibodies (prepared against the C-terminal 12 residues in the β-subunit). Immune complexes were collected with Protein A Sepharose and washed extensively. The complexes were released with SDB, separated by SDS-PAGE, and analyzed by western blotting for total and phosphorylated insulin receptor. Densitometric analysis of three independent experiments. There were no statistical differences between the density of total receptor bands for control vs fibroin-treated cells and chronic insulin- vs chronic insulin plus fibroin-treated cells. Thus, the data from each of these two groups have been compiled for simplicity. $p<.001$ vs control, n=3.

4.4±0.7 for control and chronic insulin-treated cells, respectively). While we might have expected an increase in the receptor pool or an increase in insulin-dependent autophosphorylation in response to chronic fibroin, we observed no changes (Fig. 8).

We next focused on an event down-stream of PI3-kinase activation, i.e., Akt activation by phosphorylation. Others have shown that there are two Akt residues sensitive to insulin action: Ser^{473} and Thr^{308} (24). As expected, acute insulin exposure over a range of concentrations stimulated Akt phosphorylation at both Ser^{473} and Thr^{308} without affecting the total pool (Fig. 9A). Chronic fibroin treatment had no effect on the extent of phosphorylation. Next, we examined the effect of chronic fibroin and insulin on acute, insulin-stimulated Akt phosphorylation. In these experiments, we examined only phosphorylation at Thr^{308} because of the high non-specific background with anti-$pSer^{473}$. These data show that chronic insulin decreased the ability of insulin to stimulate Akt phosphorylation (by about 50%) confirming the earlier observations of Nelson et al. (24). Fibroin had no effect on Akt phosphorylation in either control or insulin resistant cells.

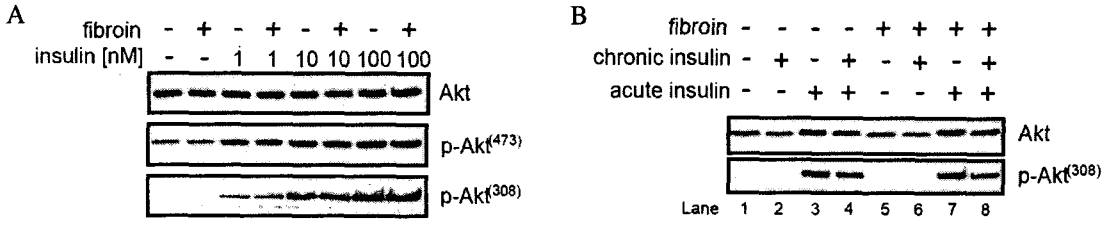

Fig. 9. Fibroin does not affect on insulin-independent Akt phosphorylation.
Panel A. Cells were treated with fibroin hydrolysate (5 mg/mL) for 24 h. Cells were rinsed in KRP and exposed to specific concentrations of insulin as indicated for 10 min. Cells were washed and extracted. The cells were then extracted in buffer containing 1% NP-40, 100 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor cocktail from Sigma (1 μL/mL). The lysed cells were rotated for 30 min at 4°C. An aliquot was used for SDS/PAGE-western blot analysis. Antibodies for total, Akt Ser^{473} and Akt Thr^{308} were purchased from Sigma, Santa Cruz, and Cell Signaling, respectively. *Panel B.* Cells were treated with fibroin, alone, for 12 h. Medium was refreshed and included both fibron and 10 nM insulin. After an additional 12 h, cells were washed and exposed to insulin (1 μM) for 10 min. Cells were extracted as in Panel A and e evaluated for Akt expression and phoshorlation. Each set of data represents a single experiment.

Effect of fibroin on GLUT expression distribution

To determine the effect of fibroin on GLUT1 and GLUT4 expression and distribution, we analyzed their content in specific membrane fractions by western blotting. As observed by us and others, acute insulin alters the distribution of both GLUT1 and GLUT4. Thus, more of each transporter was observed in the PM fraction in response to insulin with a consequent loss from the LDM fraction (Fig. 10). Chronic treatment with fibroin fraction FIII showed two surprising results. First, fibroin treatment alone increased the relative amount of GLUT1 in both the PM and HDM fractions with no affect on the GLUT1 pool in the LDM fraction. Because the HDM represents the biosynthetic compartments (ER and Golgi), this suggests that the increase in GLUT1 arises from new synthesis. The second surprise was that fibroin enhanced the redistribution of GLUT4 from the LDM fraction to the PM, independent of the presence of acute insulin addition although the response to fibroin was significantly less than the response to insulin. Little change in the expression of GLUT4 was noted, and were less reproducible that those of GLUT1.

To determine if the enhanced expression of GLUT1 was due to an increase in the availability of mRNA, we analyzed the content of GLUT1 mRNA by northern blot analysis. Exposure of cells to chronic insulin at high

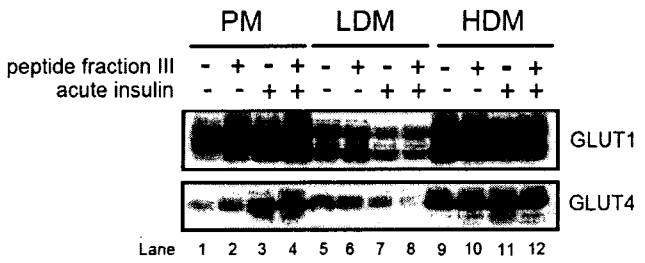

Fig. 10. Fibroin increased the expression of GLUT1 and redistribution of GLUT4.
Cells were treated with peptide fraction III (2.5 mg/mL) for 24 h. Cells were rinsed in KRP for 10 min and then insulin (1 μM) was added for an additional 10 min. Membrane fractions were collected and analyzed by SDS/PAGE and western blotting. These data are representative of duplicate experiments.

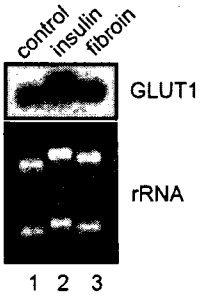

Fig. 11. Fibroin increases GLUT1 mRNA.
Cells were exposed to insulin (100 nM) for 12 h or fibroin hydrolysate (2.5 mg/mL) for 24 h. Total RNA was collected and northern blot analysis was performed using a GLUT1 cDNA probe prepared by PCR from total RNA. This blot represents duplicate experiments.

concentration was used as a positive control, as it is well known that insulin stimulates the transcription of GLUT1 (25,26). Indeed, we were able to confirm these earlier studies as shown in Fig. 11 (lane 2 vs 1). The effect of fibroin on GLUT1 expression was more subtle yet still boosted the level of GLUT1 mRNA by about 2-fold relative to controls (lane 3 vs 1). These data suggest that fibroin controls the expression of GLUT1 protein by influencing the level of mRNA.

DISCUSSION

In these studies, we have pursued the mechanism by which fibroin, the major protein in silk, exerts its hypoglycemic effects *in vivo*. In 3T3-L1 adipocytes, we have shown that fibroin significantly increases insulin-sensitive glucose uptake in response to physiological concentrations of insulin. In addition, it blocks the development of insulin resistance in response to chronic insulin exposure but not that in response to TNF α. Fibroin also increases glucose metabolism, as reflected in the rapid loss of glucose from the medium and the more rapid turnover of glycogen in fibroin-treated cells compared to controls. Accelerated glucose metabolism may be facilitated by the increase in GLUT1 expression at the cell surface, which appears to arise from new synthesis, in addition to the subtle translocation of GLUT4 from its intracellular storage site. Together, these data suggest that fibroin can function to enhance the peripheral utilization of glucose *in vivo*.

To further explore the mechanism which underlies the effects of fibroin on insulin sensitivity and glucose metabolism, we examined several steps in the insulin signaling cascade. First, we show that chronic insulin exposure had a dramatic effect on receptor expression. While this has been observed at high concentrations of insulin, this result was surprising based on the limited reduction in insulin binding noted after exposure to only 10 nM insulin in previous studies (9,23). Subsequent insulin-stimulated autophosphoryation was diminished further yet suggesting that the catalytic activity of the receptor was affected by chronic insulin, independent of the decreased expression. Fibroin did not reverse the effect of chronic insulin at either the level of expression, or at the level of catalytic activity.

It has been suggested that insulin-induced translocation of GLUT4 occurs by both PI3-kinase-dependent and independent (27) paths. In assessing the specific involvement of the PI3-kinase pathway, we treated cells with the inhibitor, wortmannin. The increase in glucose uptake in response to acute insulin was completely blocked by the

addition of wortmannin, as expected. However, wortmannin did not fully inhibit glucose uptake in cells which were pretreated with fibroin. Activation of PI3-kinase leads to the activation of 3-phosphoinositide-dependent protein kinase (PDK1) (28). Among the enzymes that have been reported to be activated downstream of PDK1 are Akt (PKB) (29) and the atypical protein kinase C (aPKC) (30). In our hands, acute addition of insulin stimulated Akt phosphorylation on both Ser^{473} and Thr^{308}. In agreement with the recent study by Buse and colleagues (24), chronic insulin reduced the ability of subsequent insulin challenge to stimulate Akt phosphorylation with little change in its expression. Fibroin did not reverse the effect of chronic insulin. While the involvement of Akt in insulin-induced GLUT4 translocation is still controversial (28,31-33), evidence has been reported which implicates Akt in the insulin-induced increase in GLUT1 expression (34). These investigators showed that dominant negative mutants of PI3-kinase and Akt blocked the insulin-induced increase in GLUT1 expression in 3T3-L1 adipocytes. Further, they showed that rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), also blocked GLUT1 translation. While in our studies, insulin did up-regulate GLUT1, so did fibroin although the effect was more subtle. On the one hand, overexpression of GLUT1 is associated with prolonged exposure to insulin (35) and insulin resistance while on the other hand fibroin, which increases GLUT1 expression to a lesser degree is associated with the reversal of this condition. We have not yet evaluated the Akt/mTOR path for its involvement in fibroin action but it seems unlikely based on the fact that fibroin, alone, does not affect the expression or activation of Akt .

At this point, we are left with a puzzle. Fibroin reduces hyperglycemia *in vivo* and increases glucose uptake and metabolism *in vitro* but apparently not through the insulin receptor or the PI3-kinase arm of the signaling cascade. Yet, fibroin increases the expression of GLUT1 and the redistribution of GLUT4 which are features of the path. This raises the question as to whether fibroin interacts with a unique receptor at the cell surface to independently activate another arm of the insulin signaling cascade or a totally different path. Alternatively, fibroin may be transported (or internalized) to affect cellular metabolism. We are currently pursuing these ideas.

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