Cell Type-specific Post-transcriptional Regulation of Production of the Potent Antiangiogenic and Proatherogenic Protein Thrombospondin-1 by High Glucose

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Hyperglycemia is an independent risk factor for development of vascular diabetic complications. Vascular dysfunction in diabetes manifests in a tissue-specific manner; macrovasculature is affected by atherosclerotic lesions, and microvascular complications are described as “aberrant angiogenesis”; in the same patient angiogenesis is increased in some tissues (e.g. retinal neovascularization) and decreased in others (e.g. in skin). Molecular cell- and tissue-specific mechanisms regulating the response of vasculature to hyperglycemia remain unclear. Thrombospondin-1 (TSP-1), a potent antiangiogenic and proatherogenic protein, has been implicated in the development of several vascular diabetic complications (atherosclerosis, nephropathy, and cardiomyopathy). This study examines cell type-specific regulation of production of thrombospondin-1 by high glucose. We previously reported the increased expression of TSP-1 in the large arteries of diabetic animals. mRNA and protein levels were up-regulated in response to high glucose. Unlike in macrovascular cells, TSP-1 protein levels are dramatically decreased in response to high glucose in microvascular endothelial cells and retinal pigment epithelial cells (RPE). This down-regulation is post-transcriptional; mRNA levels are increased. In situ mRNA hybridization and immunohistochemistry revealed that the level of mRNA is up-regulated in RPE of diabetic rats, whereas the protein level is decreased. This cell type-specific post-transcriptional suppression of TSP-1 production in response to high glucose in microvascular endothelial cells and RPE is controlled by untranslated regions of TSP-1 mRNA that regulate coupling of TSP-1 mRNA to polysomes and its translation. The cell-specific regulation of TSP-1 suggests a potential mechanism for the aberrant angiogenesis in diabetics and TSP-1 involvement in development of various vascular diabetic complications.

Despite the significant advances in the therapeutic methods to control blood glucose and insulin levels in diabetic patients, the precise regulation of these levels remains a problem. Vascular diabetic complications remain most prevalent and dangerous and account for the greatest numbers of deaths and hospitalizations in diabetic patients. The molecular basis for the vascular complications of diabetes is not well understood. Recent reports indicate that both microvascular and macrovascular complications of diabetes correlate directly with glucose levels in both patients and animal models (1–5). Some of these reports revealed the pathogenic role of impaired glucose tolerance and post-prandial hyperglycemia even in the absence of diabetes, e.g. (6). In vascular cells, glucose regulates expression of many genes that have been linked to the development of atherosclerosis or abnormal angiogenesis (reviewed in Ref. 7). One of them is thrombospondin-1 (TSP-1), a cell matrix protein implicated in both atherogenesis (8–12) and angiogenesis (13–19). Several lines of evidence indicate that TSP-1 may represent a link between diabetes and vascular complications. Patients with diabetes have increased TSP-1 in their plasma and kidneys (20). The expression of TSP-1 in mesangial cells was increased by high levels of glucose (21). TSP-1 promotes the events associated with diabetic nephropathy: mesangial cell proliferation and increased matrix production by mesangial cells (21–24). A peptide blocking the activation of transforming growth factor-β activation by TSP-1 prevented progression of cardiac fibrosis and improved cardiac function in a rat model, implicating TSP-1 in development of diabetic cardiomyopathy (25). Recently, we reported increased levels of TSP-1 in macrovessels of diabetic Zucker rats (26). Moreover, the major cell types present in large vessels responded to stimulation with high glucose concentrations by increasing their expression of TSP-1. TSP-1 is produced by microvascular cells and retinal cells (27–29), and, in contrast to large vessels and kidney, TSP-1 levels in the diabetic eye are significantly decreased (28). Because TSP-1 is a potent antiangiogenic agent that in the normal eye may prevent uncontrolled growth of microvessels, such down-regulation of TSP-1 in the diabetic eye suggests its involvement in diabetic retinopathy. A direct effect of TSP-1 deficiency on retinal vascularity has been demonstrated recently in TSP-1-deficient mice (30).
Regulation of Production of TSP-1

Elevated glucose levels are associated with the development of diabetic vascular complications, and regulation of TSP-1 production may control both macro- and microvascular changes. The aberrant angiogenesis in diabetics (increased angiogenesis in some tissues and decreased in others in the same patient), which does not have any mechanistic explanation to date, may be dependent on a cell type- and tissue-specific production of regulators of angiogenesis. TSP-1, one of the most potent antiangiogenic proteins, is a good candidate for such a regulator.

We now report a finding of a cell type- and cell origin-specific post-transcriptional mechanism of down-regulation of TSP-1 expression by glucose. This mechanism is not present in endothelial cells (EC) from large blood vessels, vascular smooth muscle cells (SMC), and fibroblasts, where the transcriptional increase in TSP-1 mRNA upon acute glucose stimulation of cells leads to the increased expression of TSP-1 protein (26). However, in microvascular endothelial cells (MVEC) and in retinal pigment epithelial (RPE) cells, the expression of TSP-1 protein is dramatically down-regulated despite increased TSP-1 mRNA levels. The mechanism controlling TSP-1 production is activated by high glucose, operates at the level of mRNA translation and is controlled by untranslated regions of mRNA. The finding of such a cell type- and cell origin-specific mechanism of suppression of the protein production may provide a molecular basis for aberrant angiogenesis in diabetic individuals. Thus, cell- and tissue-specific regulation of TSP-1 production may represent an important link between diabetes, hyperglycemia, and a variety of vascular complications of diabetes.

MATERIALS AND METHODS

Cell Culture, Stimulation with Glucose, TSP-1 Protein, and mRNA Detection—Primary vascular cells were purchased from Cambrex or kindly provided by the investigators listed in the Acknowledgments. The cell lines were from ATCC unless otherwise stated. CDC.EU/HMEC1 were obtained from Dr. Candal (Centers for Disease Control, Atlanta, GA). The stimulation with 30 mM glucose was performed as described (26). TSP-1 protein and mRNA were detected by Western or Northern blotting as described before (26).

Labeling of Protein with [35S]Methionine and Immunoprecipitation of Labeled TSP-1—Labeling of proteins began by the addition of 10 μCi/ml of [35S]methionine for 2 h either 1) prior to stimulation with glucose in the case of experiments on protein degradation/secretion or 2) 22 h after the start of stimulation with glucose in the case of detection of de novo synthesized TSP-1 protein. In protein degradation/secretion experiments, synthesis of labeled protein was stopped by an excess of cold methionine 1 h before the experimental time point zero. TSP-1 was precipitated from cell lysates overnight at 4 °C using anti-TSP-1 antibody (Lab Vision), followed by a 2-h incubation with anti-mouse IgG antibody (Bio-Rad) and protein A/G magnetic beads (Dynal). Precipitated proteins were resolved in 8% SDS-PAGE. The gels were dried and exposed to x-ray film (Amersham Biosciences) to detect TSP-1.

Short Pulse Labeling—200 μCi/ml of [35S]methionine was added for 5 min to RF/6A cells treated with 30 mM glucose for 0, 1, 2, 3, and 6 h. Prior to glucose stimulation, the cells were incubated overnight in low glucose, 0.2% fetal bovine serum medium without methionine. Labeling was stopped by the addition of excess of cold methionine and 10 μg/ml cyclohexamide, and the cells were immediately lysed. Immunoprecipitation of TSP-1 was performed as described above.

Reporter Construct Design—5'- and 3'-untranslated regions (UTR) of TSP-1 mRNA were amplified by PCR from clones IMAGE 6718543 (MRC Geneservice, Cambridge, UK) and IMAGE 6028666 (ATCC), respectively. We used primers 5'-TCTAGAGTCATCAAAATTGTGATTGAAAG-3' and 5'-GGCCCAGCCGTTGTTCTTGTACATAAGAA-3' for 5'-UTR and 5'-AAGCCTAGCCGCTGCGCCGAGCTG-GCC-3' and 5'-CCATGTTGAGCTGTGGTTGCCCAGCAGG-3' for 3'-UTR. 5' was cloned into HindIII and NcoI restriction sites of the pGL3 Control vector (Promega), and 3' was cloned in FseI and XbaI sites of the same vector. Thus, the final constructs included the full 5'-UTR inserted immediately before the luciferase cDNA, the full 3'-UTR inserted immediately after the luciferase cDNA, or both full 5'-UTR and full 3'-UTR.

Transient Transfections and Luciferase Assay—Lipofectin reagent (Invitrogen) was used for transient transfections according to the manufacturer’s instructions. After a 24-h recovery, full growth media were changed to a low glucose medium (5 mM or 1 mg/ml glucose, 5% fetal bovine serum) for 2 h prior to a 24-h stimulation with 30 mM glucose. Luciferase activity was measured in 100 μg of total protein using reagents from Promega according to the manufacturer’s instructions.

Fractionation of RNA on Sucrose Gradient—RF/6A cells transfected with TSP1–5', 3'-UTR/luciferase cDNA plasmid were grown in 150-mm cell culture dishes to 100% confluency in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium, 10% fetal bovine serum (Atlanta Biologicals), 0.1% G418 (Mediatech, Inc.). Medium was changed to a low glucose (5 mM) medium (0.2% fetal bovine serum) 24 h before the experiment. Cyclohexamide (CHX; 50 μg/ml) was added to each plate 5 min before lysing. The cells were washed with 5 ml of ice-cold phosphate-buffered saline containing CHX 50 μg/ml, and then the cells were scraped in phosphate-buffered saline/CHX and transferred to a tube, collected at 600 × g, washed twice with phosphate-buffered saline/CHX, resuspended in 500 μl of ice-cold polysome lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 300 mM KCl in DEPC (diethyl pyrocarbonate) water; 10 mM DTT, 100 units/ml ribonuclease inhibitor (Promega), 200 μg/ml CHX, protease inhibitor mixture (Roche Diagnostics), passed through a 27-gauge needle, incubated on ice for 5 min, and spun down at 1000 × g for 5 min. 1 mg of protein (0.5 ml) was layered on 4.5 ml of 30% sucrose prepared in polysome buffer and spun down at 150,000 × g at 4 °C for 2 h.

After centrifugation, the supernatant (nonpolysomal fraction) was collected into a new tube and precipitated with 1/10 volume of 3 mol/liter NaOAc, 2.5 volumes of ethanol overnight at −80 °C. RNA was purified from nonpolysomal fraction using TRIzol reagent. The pellet (polysomal fraction) was resuspended in 100 μl of polysome lysis buffer, and RNA was extracted with 750 μl of TRIzol reagent.
Real Time RT-PCR—First strand cDNA was synthesized using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen) from 1 μg of RNA isolated from polysomal or nonpolysomal fractions. IQ™ SYBR Green Supermix (Bio-Rad) was used for PCR. The primers used were: luciferase, 5’-gacaattgcactgctgaactctt and 5’-cagccacattgctgaatctcc; TSP-1, 5’-ccaaagacgggtttcattagag and 5’-ttcaggtcagagaaatcct; TSP-1, 5’-H11032/H11032-ccaaagacgggtttcattagag and 5’-H11032/H11032-ttcaggtcagagaaatcct; TSP-1, 5’-H11032/H11032-gacaattgcactgctgaactctt and 5’-H11032/H11032-cagccacattgctgaatctcc. Ct values for TSP-1 and luciferase were normalized to the Ct value for β-actin (dCt). dCt values for each time point of glucose treatment were normalized to the control value (dCt), and the value for fold change represents the exponential of ddCt. β-Actin Ct remained constant in response to glucose treatment in all of the cell types in both polysomal and nonpolysomal fractions.

In Situ mRNA Hybridization and Immunohistochemistry—12-Week-old control and diabetic Zucker rats (Genetic Models, Inc.) were sacrificed by CO2 inhalation. Rat eyes were enucleated and placed into ice-cold 4% paraformaldehyde in 50 mM phosphate buffer, pH 7.4, following a penetrating incision made through the middle of the cornea. After overnight fixation at 4 °C, anterior segments and the lens were removed under preparative microscope, and the posterior eyecups were dehydrated, embedded in paraffin, and cut into 10-μm-thick sections. In situ mRNA hybridization was performed as described previously (26). Immunohistochemistry was performed as described previously (26) with minor modifications; biotinylated anti-TSP-1 antibody (Lab Vision) and Texas Red-labeled streptavidin were used.

Statistical Analysis—All of the described experiments were performed at least three times, and the data were presented as the mean values ± S.E. The p values were determined from the t test using Microsoft Excel. Differences with p values ≤ 0.05 were considered statistically significant. The analyses of images obtained using in situ mRNA hybridization and immunohistochemistry were performed using ImagePro 4.5.1 in eye sections obtained from four eyes for each experimental condition (30 microscopic fields from each eye).

RESULTS
Increased TSP-1 Production in Macrovascular EC—We recently reported that the expression of TSP-1 is increased in the aorta and carotid artery of diabetic Zucker rats at the protein and mRNA levels (26). This increase in production of the potent antiangiogenic protein TSP-1 in macrovessels was associated with the decreased number of vasa vasorum, or small blood vessels, growing through the vascular wall and feeding the inner layers of vascular cells. TSP-1 was up-regulated by glucose stimulation in all major vascular wall cell types from large vessels (EC, smooth muscle cells, and fibroblasts) in vitro (26). Both the protein and mRNA for TSP-1 were rapidly up-regulated after stimulation of cells with 10–30 mM D-glucose but not biologically inactive L-glucose or sorbitol (26, 31). In addition to our published data, similar experiments have been performed in human pulmonary artery EC, several isolates of human aortic EC, and human coronary artery EC with similar results (Fig. 1). In all the isolates of primary EC from large blood vessels, TSP-1 protein production was increased in response to high glucose.

Decreased TSP-1 Production in MVEC and RPE in Response to High Glucose—In contrast to the increased TSP-1 protein production in cultured cell types from macrovessels (26), levels of TSP-1 were dramatically reduced in lysates of primary MVEC and RPE (human dermal MVEC, rat lung MVEC, and human RPE) in response to high glucose (Fig. 2). The decreased levels of TSP-1 in primary MVEC in response to high glucose were described earlier by Sheibani et al. (28). Interestingly, cell lines derived from MVEC and RPE (MVEC, CDC.EU/HMEC1 and RF/6A; RPE, ARPE19 and D407) retained this ability to suppress TSP-1 production upon incubation with high glucose (Fig. 2), suggesting that the differential regulation of TSP-1 in MVEC and RPE versus macrovascular EC and other cells from large blood vessels is an inherited property that is preserved in cell culture and in transformed cell lines. All of the MVEC and RPE produced high levels of TSP-1 in basal conditions.

The dissimilarity in the regulation of TSP-1 protein expression by high glucose between macrovascular cell types versus MVEC and RPE is not due to a difference in transcriptional regulation or to a decreased stability of TSP-1 mRNA, because mRNA levels were increased in response to high glucose in all cell types (Fig. 3), similar to macrovascular cells (26). These disparities between mRNA levels and protein levels identified the mechanism of TSP-1 regulation in MVEC and RPE as post-transcriptional.

Decreased TSP-1 Protein Synthesis in Glucose-stimulated Cells—Decreased levels of TSP-1 protein in cell lysates may result from an increased rate of TSP-1 secretion, increased rate of protein degradation, or a post-transcriptional regulation of protein synthesis. To address these possibilities, we: 1) examined the levels of TSP-1 in cell culture supernatants; similar to the levels of TSP-1 in cell lysates, the TSP-1 levels in supernatants were decreased (Fig. 4A), suggesting that the secretion of TSP-1 is not increased in response to high glucose and cannot account for the decrease in intracellular TSP-1; and 2) monitored the change in the levels of prelabeled intracellular TSP-1 in control and high glucose-stimulated cells (Fig. 4B). In ARPE19 and RF/6A, proteins were labeled by the addition of 10 μCi of [35S]methionine for 2 h, and then an excess of cold methionine was added for 1 h to stop the synthesis of newly labeled protein, and the cells were stimulated with high glucose for 1 h. Starting at this time (time point zero), the cells were
incubated for 15 min to 20 h and lysed. Labeled TSP-1 synthesized before stimulation with glucose was immunoprecipitated using two anti-TSP-1 antibodies (Labvision; Ab4 that recognizes the middle part of TSP-1 and Ab3 that recognizes the N terminus of TSP-1) and resolved in SDS-PAGE. As shown in Fig. 4B, the amount of labeled TSP-1 in cell lysates declined with time, presumably because of the secretion of the protein. However, there was no difference in labeled TSP-1 levels between samples from control cells and high glucose-stimulated cells at any given time point. This observation suggested that the decrease in TSP-1 levels was not due to the increased secretion or degradation of the protein but rather was due to decreased synthesis of the protein.

To confirm that the synthesis of TSP-1 protein is inhibited in response to high glucose, we have performed a short pulse labeling (200 μCi/ml of [35S]methionine) of newly synthesized proteins 1, 2, 3, and 6 h after stimulation of RF/6A with high glucose. Labeled TSP-1 was immunoprecipitated from the lysates and detected in SDS-PAGE (Fig. 4C). The difference in the amount of TSP-1 synthesized during the pulse time (5 min) was detected as early as 2 h after the glucose treatment, and the newly synthesized TSP-1 was barely detected after 6 h of treatment.

Additionally, ARPE19 and HASMC were stimulated with 30 mM glucose, and the newly synthesized proteins were labeled with [35S]methionine for 2 h prior to the cell harvesting at the 24-h time point. ARPE19 and HASMC, two cell types with different mechanisms of TSP-1 protein regulation in response to glucose, were used in these experiments. TSP-1 was immunoprecipitated from the control of each cell type, and the cells were stimulated with 30 mM glucose for 24 h. At 24 h of stimulation, up-regulation of TSP-1 reached maximal levels in HASMC (26), whereas maximum down-regulation of TSP-1 in ARPE19 is detected at the same time point. Although in HASMC the synthesis of TSP-1 was dramatically increased in cells stimulated with high glucose as compared with control cells, in ARPE the synthesis of new TSP-1 was notably decreased (Fig. 4D). No labeled protein was detected at this time point in the medium, suggesting that 2 h (labeling time) are not sufficient for the secretion of detectable amount of TSP-1, and the decrease of newly synthesized TSP-1 in lysates in cells stimulated with high glucose is not a result of increased secretion. These series of experiments confirmed that the decrease in intracellular TSP-1 is due to the decreased synthesis of the protein rather than its increased secretion or degradation.

Control of the Reporter Gene Expression by Untranslated Region of TSP-1 mRNA—UTR of mRNA frequently control protein production by inhibiting translation of a protein or sequestering mRNA (reviewed in Ref. 32, 33). We examined the expression of luciferase in cells transfected with plasmids containing the complete 5′-UTR of TSP-1 cloned in front of the luciferase cDNA, the complete 3′-UTR cloned immediately at the 3′ of the luciferase cDNA, or both 5′ and 3′-UTR. HASMC, bovine aortic endothelial cells (BAEC), ARPE19, and retinal microvascular endothelial cell line RF/6A were transiently transfected with these constructs and stimulated with 30 mM glucose for 15 min to 20 h and lysed. Labeled TSP-1 synthesized before stimulation with glucose was immunoprecipitated using two anti-TSP-1 antibodies (Labvision; Ab4 that recognizes the middle part of TSP-1 and Ab3 that recognizes the N terminus of TSP-1) and resolved in SDS-PAGE. As shown in Fig. 4B, the amount of labeled TSP-1 in cell lysates declined with time, presumably because of the secretion of the protein. However, there was no difference in labeled TSP-1 levels between samples from control cells and high glucose-stimulated cells at any given time point. This observation suggested that the decrease in TSP-1 levels was not due to the increased secretion or degradation of the protein but rather was due to decreased synthesis of the protein.

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glucose for 24 h. In response to high glucose, the activity of 5'-TSP-1-luciferase was increased up to 4-fold (Fig. 5) in all of the cell lines tested. Luciferase expression was inhibited by TSP-1 3'-UTR in all four cell types; the basal expression was inhibited by several orders of magnitude (not shown), and there was no increase in luciferase activity in response to glucose (Fig. 5). However, when the luciferase construct contained both 5'- and 3'-UTR of TSP-1, a significant difference in reporter activity was observed in MVEC and RPE versus macrovascular cell types. The activity of luciferase and the response to high glucose were restored in macrovascular cell types (HASMC and BAEC) but not in ARPE19 or RF/6A. These data suggest that differential cell type-specific regulation of TSP-1 synthesis is controlled by a cell type-specific post-transcriptional mechanism, which depends on the UTR of TSP-1 mRNA. As expected, based on the experiments that determined the increased levels of endogenous TSP-1 mRNA in all glucose-stimulated cells (Fig. 3), the levels of luciferase mRNA were not reduced by glucose treatment (Fig. 5E). These data suggest that differential cell type-specific regulation of TSP-1 synthesis is controlled by a cell type-specific post-transcriptional mechanism, which depends on the UTR of TSP-1 mRNA.

Change in Osmolarity Regulates TSP-1 Production—The transcriptional increase of TSP-1 mRNA that we observed in all cell types is dependent on the intracellular glucose metabolism; cell-impermeable sorbitol and L-glucose used as osmolarity control failed to up-regulate TSP-1 mRNA (26, 31). In contrast, the regulation of protein production by UTR of TSP-1 mRNA in response to high glucose is regulated by a change in osmolarity; biologically inactive cell-impermeable L-glucose had a similar effect on luciferase production regulated by TSP-1 UTR and on the endogenous TSP-1 levels (Fig. 5, A–D and F, respectively).

High Glucose Regulates Coupling of TSP-1 and TSP-1–5'-3'-UTR/Luciferase mRNA with Polysomes—Actively translated mRNA can be found in association with polysomes. Uncoupling from polysomes is a common mechanism of inhibition of mRNA translation. In response to glucose, polysomes were observed in RPE and MVEC (Fig. 5, A–D and F). The coupling of TSP-1 and TSP-1–5'-3'-UTR with polysomes was disrupted in ARPE19 and RF/6A by glucose treatment (Fig. 5, A–D and F).
Regulation of Production of TSP-1

FIGURE 5. UTR of TSP-1 mRNA regulates a reporter gene expression in response to high glucose. A–D, macrovascular cells (HASMC and BAEC), MVEC, and RPE were transiently transfected with reporter plasmids containing TSP-1 5′-UTR, 3′-UTR, or both 5′- and 3′-UTR and stimulated with 30 mM d-glucose or l-glucose for 24 h. Luciferase activity was measured in lysates of transfected cells. E, total RNA from glucose-stimulated RF/6A stably expressing 5′,3′-UTR-TSP/luciferase was analyzed by Northern Blotting using luciferase probe. F, TSP-1 protein levels were analyzed by Western blotting in RF/6A stimulated with 30 mM l- or d-glucose.

transcription. To detect the effect of high glucose on coupling of TSP-1 mRNA with polysomes, we have performed fractionation of RNA from glucose-stimulated RF/6A, HUVEC, and HASMC followed by detection of TSP-1 mRNA in polysomal and nonpolysomal fractions by real time RT-PCR. The cells were preincubated in low glucose medium for 24 h and stimulated with 30 mM d-glucose or l-glucose for 1, 2, 3, 6, and 24 h. As shown in Fig. 6B, there was no detectable shifting of 5′TSP/luciferase mRNA between polysomal and nonpolysomal fractions. However, both 3′TSP/luciferase and 5′,3′TSP/luciferase translocated to the nonpolysomal fraction, suggesting that UTR of TSP-1 mRNA controls the translocation of the heterologous luciferase mRNA.

Decreased Level of TSP-1 in RPE and Retina of Diabetic Zucker Rats—To find out whether the described mechanism is operative in vivo, we examined RPE and retina of diabetic Zucker rats. TSP-1 mRNA was detected by in situ mRNA hybridization. Comparison of control nondiabetic RPE to RPE of diabetic animals revealed that mRNA levels are increased in diabetic rats (Fig. 7). In contrast, TSP-1 protein detected in the extracellular matrix of choroid layers adjacent to RPE and in the extracellular matrix of retina was visibly down-regulated in diabetic animals (Fig. 8). The changes on the amounts of mRNA and protein were estimated by measuring the intensity of fluorescence sections from four diabetic and control rat eyes (Figs. 7B and 8B).

DISCUSSION

TSP-1 expression by MVEC regulates cell survival, and the correlation between TSP-1 and TSP-2 expression and abnormal angiogenesis has been well documented in animal models and in human pathologies (34, 35). High expression of TSP-1 in RPE in normoglycemic conditions is consistent with the anatomical position of RPE in the eye, where TSP-1, a potent antiangiogenic protein, may participate in the maintenance of the normal density of microvessels in the retina and choroid. The expression of TSP-1 in the normal eye and its decreased expression in the diabetic eye were reported, as well as increased vascularity in the retina of TSP-1-deficient animals (28, 30).

At the mRNA level, both macrovascular cell types and MVEC and RPE respond similarly to high glucose stimulation by dramatically increasing TSP-1 mRNA levels. Surprisingly, in response to glucose there is a striking difference between TSP-1 protein regulation in macrovascular cell types and MVEC and RPE; although the protein level is dramatically increased in macrovascular cell types (26), these levels are significantly

HF/6A and 2.7 ± 0.14-fold increase in HASMC at 24 h, \( p = 0.04 \) and 0.0001, respectively). These data indicated that the newly transcribed mRNA is actively translated, and this observation is consistent with increased production of the protein in both HUVEC and HASMC in response to high glucose. However, in RF/6A, the TSP-1 mRNA amount was rapidly decreased in actively translated polysomal fraction, and all the newly transcribed TSP-1 mRNA was accumulated in nonpolysomal fraction (4.1 ± 0.41-fold increase at 24 h, \( p = 4.9E-08 \)). These data clearly indicate that the uncoupling of TSP-1 mRNA from polysomes is the cause of decreased production of the protein in response to high glucose.

To confirm that UTR of TSP-1 mRNA is responsible for the translocation to the untranslated fraction of RNA and decreased production of protein, we used RF/6A stably transfected with a fusion constructs expressing heterologous coding region of luciferase surrounded with 5′-UTR, 3′-UTR, or 5′,3′-UTR of TSP-1. The cells were preincubated in low glucose medium for 24 h and stimulated with 30 mM glucose for 1, 2, 3, 6, and 24 h. As shown in Fig. 6B, there was no detectable shifting of 5′TSP/luciferase mRNA between polysomal and nonpolysomal fractions. However, both 3′TSP/luciferase and 5′,3′TSP/luciferase translocated to the nonpolysomal fraction, suggesting that UTR of TSP-1 mRNA controls the translocation of the heterologous luciferase mRNA.
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decreased in MVEC and RPE. This observation suggests a cell type-specific post-transcriptional mechanism of TSP-1 regulation (e.g. this may be a specific mechanism suppressing the production of TSP-1 in MVEC and RPE or a specific mechanism relieving the block of protein production in macrovascular cells).

Decreased levels of TSP-1 protein in cell culture supernatants of glucose-stimulated cells and the identical time course of loss of labeled intracellular TSP-1 from control and glucose-stimulated cells suggest that increased secretion of TSP-1 cannot account for the difference. Indeed, TSP-1 levels decrease dramatically within the time insufficient for complete protein processing and secretion; the decrease in protein levels can be detected as early as 1 h, whereas 2 h from the start of labeling were insufficient for the detection of labeled TSP-1 in cell culture supernatants. Immunoprecipitation of labeled newly synthesized TSP-1 from lysates of microvascular EC RF/6A and ARPE19 confirmed the decrease in TSP-1 protein synthesis in response to high glucose.

The regulation of TSP-1 production in RPE and MVEC in response to glucose occurs at the level of protein synthesis; transcriptional inhibition is excluded because of the increased levels of TSP-1 mRNA, and protein degradation and secretion are not affected, whereas the synthesis of TSP-1 protein is decreased upon glucose stimulation. UTR of mRNA frequently control the protein expression at the post-transcriptional levels. Such regulation may involve a direct inhibition of mRNA translation or sequestering of mRNA. To find out whether TSP-1 UTR controls the expression of TSP-1 protein in MVEC and RPE in response to high glucose, we decided to investigate the effect of 5'- and 3'-UTR of TSP-1 mRNA on the expression of a heterologous reporter gene in macrovascular cells.

Figure 6 confirms the decrease in TSP-1 protein, and glucose-stimulated cells suggest that increased secretion of TSP-1 cannot account for the difference. Indeed, TSP-1 levels decrease dramatically within the time insufficient for complete protein processing and secretion; the decrease in protein levels can be detected as early as 1 h, whereas 2 h from the start of labeling were insufficient for the detection of labeled TSP-1 in cell culture supernatants. Immunoprecipitation of labeled newly synthesized TSP-1 from lysates of microvascular EC RF/6A and ARPE19 confirmed the decrease in TSP-1 protein synthesis in response to high glucose.

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TSP-1 upon glucose stimulation. Indeed, in response to high glucose, both endogenous TSP-1 mRNA and chimeric 5′,3′-UTR-TSP-1-luciferase mRNA were translocated from actively translated polysome-associated pool of mRNA to nonpolysomal fraction in microvascular EC, but not in macrovascular EC or HASMC. It appears that 3′-UTR alone is sufficient for the translocation to the nontranslated RNA fraction and that both 5′- and 3′-UTR are required for the cell-specific regulation. Further analysis of the requirements for the inhibition of the protein production in MVEC and RPE will be needed to attempt the identification of the RNA-binding protein(s) responsible for this effect. The use of deletion/mutant UTR and identification of protein(s) binding to the UTR fragments that control the cell-specific effect will be necessary to describe the precise mechanism of the observed cell type-specific regulation.

The difference in distribution of the endogenous TSP-1 mRNA in microvascular EC and macrovascular cells was striking; as the levels of TSP-1 mRNA in all the cell types increased, TSP-1 mRNA accumulated in nonpolysomal fraction in RF/6A, but not in HUVEC or HASMC, where it was found with actively translated polysome-associated mRNA. The cell-specific regulation of TSP-1 mRNA translocation to the nonpolysomal fraction and inhibition of translation is clearly not a general effect of high glucose on protein synthesis; β-actin mRNA did not translocate to the nonpolysomal fraction (not shown), and there was no decrease in production of β-actin protein.

The regulation of TSP-1 protein production by UTR does not depend on the uptake and intracellular metabolism of glucose; biologically inactive and cell-impermeable l-glucose had an effect similar to the effect of d-glucose, unlike in case of the up-regulation of levels of TSP-1 mRNA that was dependent on the glucose uptake/intracellular metabolism and was not reproduced with sorbitol or l-glucose.

We have visualized the TSP-1 mRNA and protein expression in RPE of diabetic and control Zucker rats, and the results of the

in situ mRNA hybridization and protein detection by immunohistochemistry revealed that TSP-1 expression is controlled in a tissue-specific manner at the post-transcriptional level; although the TSP-1 mRNA level was increased in RPE of diabetic Zucker rats, the protein level was decreased, suggesting that the cell type-specific mechanism of regulation found in cell culture is operative in vivo.

Our data suggest that TSP-1 expression is regulated by high glucose in a cell type- and tissue-specific manner by a post-transcriptional mechanism present in selected cell types. Such cell type-specific regulation would increase TSP-1 expression in response to high glucose in some tissues (wall of large vessels, skin fibroblasts, etc.) while decreasing the production of TSP-1 expression in others (retina). Cell type-specific regulation of expression of this potent antiangiogenic protein may explain the aberrant angiogenesis in diabetic patients, when at the same time the increased vascularization can be observed in selected tissues, whereas the decreased angiogenesis can cause diabetic complications in other tissues. Thus, tissue-specific regulation of TSP-1 expression in response to hyperglycemia may contribute to a variety of vascular diabetic complications.

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