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Hyperglycemia upregulates translation of the fibroblast growth factor 2 mRNA in mouse aorta via internal ribosome entry site

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Abbreviations footnote: IRES, internal ribosome entry site; LucF, Firefly luciferase; LucR, *Renilla* luciferase; RT, reverse transcriptase; SMC, smooth muscle cells; STZ, streptozotocin

Abstract

Fibroblast growth factor 2 (FGF-2) is normally synthesized at low levels but is elevated in various pathophysiological conditions including diabetes-associated vascular diseases. FGF-2 expression is regulated translationally through an internal ribosome entry site (IRES) located in its mRNA, which allows a non classical cap-independent translation. We addressed the pathophysiological regulation of the IRES *in vivo* by using a streptozotocin-induced hyperglycemic model known to suppress markedly overall translation. Evaluation of FGF-2 IRES-dependent translation was performed with transgenic mice expressing dual luciferase bicistronic mRNA containing the FGF-2 IRES.

FGF-2 IRES-dependent reporter activity increased 240% of control in the diabetic aorta although where the reporter mRNA levels significantly decreased. Expression of endogenous FGF-2 protein in the aorta closely correlated with the IRES activity, but not with FGF-2 mRNA levels. Moreover, the biosynthesis of endogenous FGF-2 protein was stimulated in an IRES-dependent manner by high glucose that significantly suppressed global protein synthesis in aortic smooth muscle cells from the transgenic mice. These results suggest that IRES-dependent translational regulation could play a pathological role in FGF-2 expression *in vivo*, especially in the cardiovascular consequences of diabetes.

Introduction

FGF-2, also known as basic FGF (bFGF), is a pleiotropic factor that promotes proliferation of most mesoderm- and neuroectoderm-derived cells as well as differentiation of mesenchymal cells (1). It stimulates proliferation of endothelial and smooth muscle cells (2), whose proliferation is involved in pathological processes such as neovascularization and atherosclerosis. *In vivo*, FGF-2 is implicated in the homeostatic process of vascular tone control (3, 4) and in diabetes-induced vascular dysfunction (5).

Five FGF-2 isoforms are expressed by a single FGF-2 mRNA in human, resulting from a process of alternative initiation of translation from five different initiation codons, including four noncanonical CUGs (6, 7). Expression of the human and mouse FGF-2 isoforms undergoes the same tissue-specific control in mice overexpressing a human FGF-2 transgene (8), suggesting conservation of the regulatory mechanisms between species. Expression of FGF-2 is translationally controlled through several cis-acting elements located within the mRNA leader sequence; in particular an internal ribosome entry site (IRES) (9). The IRES allows translation of FGF-2 mRNA via noncanonical cap-independent mechanism (10, 11) and could account for the expression of CUG1, -2, and -3-initiated FGF-2 isoforms (12).

Recently, IRESs have been discovered in numerous cellular mRNAs (see refs. 13, 14 for review and our web site, <http://www.rangueil.inserm.fr/iresdatabase>). In *ex vivo* studies, most cellular IRESs have been shown to function preferentially when cap-dependent translation is impaired in cells exposed to various types of stress, such as γ -irradiation (15), hypoxia (16), heat shock (11, 17) and amino acid starvation (18). We previously demonstrated the *in vivo* behavior of FGF-2 IRES activity under physiological conditions, which have been tested in transgenic mice which express a bicistronic mRNA encoding two different luciferase reporter genes, the second being under the control of the human FGF-2 IRES (19). In that study, a stringent regulation was observed, as the IRES was essentially active in the embryo, whereas it was silenced or highly tissue-specific in adult mice. However, IRES activity in

pathophysiological conditions remained unexplored.

To address this issue, we chose to explore the vascular tissue in an experimental diabetic mouse model induced by streptozotocin (STZ). It has been shown that STZ-induced diabetes decreases markedly the global protein synthesis activity in several tissues (20, 21). Also, tissue-specific variations of FGF-2 mRNA expression have been reported in STZ-treated rats, but little information is available about the *in vivo* modulation of FGF-2 protein expression in these conditions (22, 23). Here, we show a specific regulation of IRES-dependent FGF-2 translation in the aortic tissue of diabetic mice and in vascular smooth muscle cell culture under high glucose conditions.

Materials and Methods

Induction of diabetes in transgenic mice

The transgenic mouse line RFL12, expressing bicistronic *Renilla* luciferase (LucR)-FGF-2 IRES-Firefly luciferase (LucF) mRNA, were generated as described previously (19). Male, 8- to 12-week-old RFL12 mice were treated with STZ (Sigma). STZ was dissolved in sterile citrate buffer (0.05 M sodium citrate, pH 4.5) and injected intraperitoneally into mice (60 mg/kg, ~25 μ l) for 5 consecutive days. Control age-matched mice received the same volume of citrate buffer. Serum glucose was measured from tail vein blood using the glucose oxidase method (Sigma) in non-fasted mice. One, 3 and 7 wk after initial injection, mice were anesthetized with 100 mg/kg pentobarbital and tissues were excised, washed twice with ice-cold phosphate buffered saline (PBS), frozen immediately in liquid nitrogen, and stored at -80°C .

Isolation and culture of aortic smooth muscle cells (SMC)

Thoracic aortas were removed sterilely from 3 adult (12-14 weeks old) male transgenic RFL12 or non-transgenic mice. The vessels were transferred into culture medium (DMEM including 10% FCS, 0.5% gentamicin, and 1% amphotericin). They were stripped carefully of connective tissue, cut into rings (3 to 5 mm long), and placed on to sterile plastic 6-well tissue culture plates. Secondary cultures were established by trypsinization of primary cultures and replating 60 diameter culture dishes. SMC were identified by their typical hill-and-valley morphology in culture and their characteristic immunocytochemical staining for α -smooth muscle actin (monoclonal anti-smooth muscle α -actin antibody, Sigma). Cells at passage 3 to 6 were used for experiments.

Luciferase activity analysis

Two luciferase, LucR and LucF, activities were measured in the cell or tissue extracts as

described previously (19). Each luciferase activities were normalized to the protein content of the cell or tissue extracts, which was measured using the Bio-Rad Dc assay.

Western blot analysis

FGF-2 was partially purified on heparin-Sepharose beads except for SMC sample and separated in a 12.5% acrylamide/SDS gel. FGF-2 protein was immunodetected with rabbit polyclonal anti-FGF-2 antibody (Santa Cruz. Inc.) as previously described (7).

Quantitative Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of mRNAs for the reporter luciferase and FGF-2 was determined by real-time TaqMan technology with a Sequence Detection System model 5700 (Perkin Elmer) according to the instruction manual. Total RNA was isolated according to the TRIZOL protocol (Gibco Life Technology) and possible DNA contamination was removed using DNA-free kit (Ambion Inc.). RT reaction was performed using Reverse Transcription Core Kit (Eurogentec, Belgium), and RT reaction products were treated with RNase H to remove RNA.

For real-time PCR, Specific primers for mouse FGF-2, LucR, and LucF, were designed using TaqMan® Primer & Probe Design of Primer Express® Ver 1.5 (Mac) software (Perkin Elmer/Applied Biosystem). The sequences of primer set for mouse FGF-2, LucR, and LucF were as follows: mouse FGF-2 sense, 5'-CACCAGGCCACTTCAAGGA-3'; mouse FGF-2 antisense, 5'-GATGGATGCGCAGGAAGAA-3'; LucR sense, 5'-AAGGTGAAGTTCGTCGTCCAA-3'; LucR antisense, 5'-GTACAACGTCAGGTTTACCACCTTT-3'; LucF sense, 5'-TTCCATC TTCCAGGGATACGA-3'; LucF antisense, 5'-ATCATCCCCCTCGGGTGTA-3'. The housekeeping gene 18S rRNA served as internal control (Pre-developed TaqMan® Assay Reagent, 18S rRNA, Applied Biosystem).

Metabolic labeling with ^{35}S -methionine, immunoprecipitation of FGF-2, and

^{35}S -methionine incorporation

Cells were incubated for 6 d under normal (5.5 mM) or high (25 mM) glucose conditions, followed by a further 6-h incubation in methionine-free DMEM, but containing 30 $\mu\text{Ci/ml}$ L- ^{35}S methionine/ ^{35}S cysteine (Amersham-Pharmacia). After washing with ice-cold PBS, cells were harvested, collected in a microcentrifuge tube, and pelleted by centrifugation at 300 g for 20 sec. One half of the pelleted cell were resuspended in ice-cold lysis buffer, containing of 10 mM Tris-HCl (pH 7.2), 0.125% (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail (Roche, , Mannheim, Germany) for immunoprecipitation as described previously (24). Immunoprecipitation of FGF-2 was performed with a monoclonal anti-FGF-2 (clone bFM-2; Upstate Group Inc.) and a polyclonal anti-FGF-2 (sc-79, SantaCruz Inc.) antibodies and protein A-sepharose beads. Samples were analyzed by autoradiography after electrophoresis on a 12.5% polyacrylamide/SDS gel. The relative levels of FGF-2 were then determined by densitometric scanning. The other half was used for analysis of ^{35}S -methionine incorporation into cellular proteins. Cells were washed four times with 10% TCA and twice with ethanol, air-dried, dissolved with 0.3 M NaOH, and neutralized with 1.5M HCl. One half of them was subjected to liquid scintillation counting, and the other half was used for measurement of total protein concentration. Data were expressed as c.p.m./ μg protein.

Cell transfection

The bicistronic plasmids have already been described (19). The two luciferase genes, LucR and LucF, are controlled by the cytomegalovirus promoter and separated by an IRES of human FGF2 (pCRFL), human c-myc (pCRMyl), and human PDGF (pCRPL), respectively. Cells were treated with normal (5.5 mM) or high (25 mM) glucose for 72 h, then transfected with 0.5 μg of plasmid by using JetPEI cationic transfection reagent (Qbiogen) in 12-well

tissue culture plates. Twenty hours of post-transfection, cell lysates were prepared for luminescence activity.

Statistical analysis

ANOVA and Scheffé's test were used to determine statistically significant differences. Differences were considered significant if the p value was less than 0.05.

Results

The FGF-2 IRES activity is stimulated in vascular tissue in response to hyperglycemia

We used our transgenic mouse model, expressing the bicistronic mRNA containing two open reading frames (ORFs) encoding two distinct luciferases, LucR and LucF (Fig. 1A). In these conditions, translation of the second ORF (LucF) is driven by the FGF-2 IRES located between LucR and LucF.

The transgenic mice were treated with STZ for induction of diabetes (serum glucose concentration: 1 wk, 440 ± 36 mg/dl; 3 wk, 508 ± 79 mg/dl; 7 wk, 623 ± 81 mg/dl). Serum glucose was 170-200 mg/dl in vehicle-treated control mice during all the experimental period. STZ-treated mice with serum glucose > 350 mg/dl were defined as diabetic. Four different organs were tested for FGF-2 expression, on the one hand, eye, heart and aorta, which are typical target organs of hyperglycemia and diabetes, and on the other hand, brain in which FGF-2 IRES activity is very high (19) and where FGF-2 plays a critical physiological role (3).

As shown in Fig. 1B, STZ-induced hyperglycemia affected LucR reporter activity in the aorta, the heart, and the eye. LucR activities were correlated with levels of the reporter mRNA in all organs tested (Fig. 1D), indicating that LucR activity reflects its mRNA levels.

Furthermore, mRNA quantification using LucR primers was always equal to that using LucF primers, indicating the bicistronic status of the mRNA in all conditions. In contrast to the results of LucR activity, FGF-2 IRES-dependent LucF activity was independent on the level of its mRNA (Fig. 1C and D). In the aorta, the reporter mRNA decreased significantly during hyperglycemia, however LucF activity increased until week 3 (243% of control mice) and then decreased to the basal level (Fig. 1C). In contrast, LucF activity decreased selectively in the brain and was not affected significantly in the eye during hyperglycemia (Fig. 1C). Only the heart showed a close relationship between the levels of reporter mRNA and reporter activities (Fig. 1, B-D). These data indicate that hyperglycemia affects the FGF-2 IRES

activity in a tissue-specific manner.

Hyperglycemia upregulates FGF-2 expression at the level of translation in aorta

To further examine the relationships between hyperglycemia-associated translational activity and endogenous FGF-2 protein expression, we analyzed the levels of FGF-2 mRNA and protein (Fig. 2). In the aorta (Fig. 2A-C), hyperglycemia significantly increased FGF-2 protein levels, with a kinetics similar to that observed for IRES activity in Fig. 1: both endogenous FGF-2 protein expression and IRES activation disappeared at week 7, and this feature was specific to aorta. In contrast, FGF-2 mRNA expression was not significantly altered by hyperglycemia (Fig. 2C), indicating that hyperglycemia-induced FGF-2 expression in the aorta could be mainly regulated by the IRES-dependent translation.

In the heart, FGF-2 protein levels slightly decreased in association with a decrease in its mRNA during hyperglycemia (Fig. 2D-F). In the eye the levels of FGF-2 protein and mRNA both increased during hyperglycemia (Fig. 2G-I). Altogether the results shown here and in Fig. 1 suggest that FGF-2 expression in the heart and the eye might be regulated transcriptionally rather than translationally.

In the brain, FGF-2 protein levels showed no change during all experimental period (Fig. 2J and K), although its mRNA significantly increased during hyperglycemia (Fig. 2L) in agreement with previous reports (22, 23). The absence of correlation between FGF-2 mRNA and protein levels suggests that FGF-2 protein expression might be inhibited at the translational level except at week 1, in concordance with a decrease of the FGF-2 IRES activity observed in Fig. 1C.

In conclusion, translational changes of FGF-2 expression induced by hyperglycemia were observed in the aorta and the brain. Translational activation of endogenous FGF-2 in the aorta fully correlated with the activation of FGF-2 IRES.

Glucose stimulates biosynthesis of FGF-2 protein in an IRES-dependent mechanism

To test the hypothesis that an increase in ambient glucose concentrations alone was sufficient to activate FGF-2 IRES and stimulate biosynthesis of endogenous FGF-2 protein in aortic tissue, we isolated and cultivated aortic smooth muscle cells (SMC) from non-diabetic transgenic mice. This cell model likely provides a more physiological significance of FGF-2 IRES than a transient transfection model, since a transient transfection generates cellular stress that could alter the IRES activity (12).

Cells were incubated in normal glucose (5.5 mM) or high glucose (25 mM) conditions for 3 and 6 days, then luciferase activities and integrity of the reporter mRNA were determined (Fig. 3A). Consistent with *in vivo* data shown in Fig. 1, the FGF-2 IRES-dependent LucF activity was independent on the levels of reporter mRNA (Fig. 3A). LucF activity increased in 180% of control at 3 d post-exposure with high glucose, and it remained elevated at 6 d. However, the reporter mRNA was slightly decreased by high glucose. Again, quantification of LucF mRNA paralleled that of LucR mRNA, confirming the mRNA bicistronic status. In contrast to LucF activity, LucR activity was gradually and significantly reduced during high glucose exposure; by 6 d, the activity decreased to 58% of control cells incubated in normal glucose. LucR activity showed a larger decrease than the reporter transcript level, suggesting that high glucose stress could suppress cap-dependent translational activity in the cells. This translational modulation by high glucose did not result from an increase in osmolarity insofar as the nonmetabolized analog L-glucose (5.5 mM D-glucose plus 19.5 mM L-glucose) had no effect on the IRES activities (Fig. 3A).

Next, we examined whether glucose-stimulated FGF-2 IRES led to upregulation of biosynthesis and protein levels of endogenous FGF-2 in the cells. After cells were metabolically radiolabeled with [³⁵S]methionine/[³⁵S]cysteine, we analyzed an incorporation of radioactivity into total cellular proteins (Fig. 3B) and the FGF-2 biosynthesis (Fig. 3C).

Exposure of the cells with high glucose for 6 d reduced a rate of overall protein synthesis to 57% of control cells (Fig. 3B), which was closely consistent with a decrease in LucR activity (Fig. 3A). On the contrary, biosynthesis of FGF-2 protein increased in 220% of control by high glucose (Fig. 3C), which was correlated with the FGF-2 IRES activity (Fig. 3A). We further confirmed by Western blot analysis that treatment of the cells with high glucose for 6 d augmented total levels of endogenous FGF-2 protein to 280% of control (Fig. 3D). Endogenous FGF-2 transcripts also increased in 220% of control in response to high glucose (Fig. 3E), consistent with a previous report (25). Finally, we confirmed that treatment with STZ compound had no effect on basal and high glucose-induced expression of endogenous FGF-2 protein in the SMC (Fig. 3F) and NIH3T3 cells (data not shown).

Although both FGF-2 mRNA and protein increased after glucose stimulation, these data indicated an activation of the FGF-2 IRES: indeed in low glucose conditions the FGF-2 mRNA can be translated by both cap- and IRES-dependent mechanisms (10), whereas FGF-2 synthesis in high glucose is mainly IRES-dependent (consequently to the decrease of cap-dependent translation measured in Fig. 3B). These results indicated that high glucose-induced FGF-2 protein accumulation was regulated not only transcriptionally but also translationally and that the FGF-2 IRES allowed efficient translation under high glucose conditions that markedly reduced the activity of global protein synthesis in the cells.

Effect of high glucose on cellular IRESs

The function of the FGF-2 IRES could allow efficient translation under high glucose conditions. To investigate whether this property might be a specific feature of the FGF-2 IRES or could be shared by other cellular IRESs, we compared the effect of glucose on the FGF-2 IRES to the effect on the activity of the IRESs from PDGF and c-myc whose gene product was induced by high glucose (26, 35) (Fig. 4). Aortic SMC from non-transgenic mice were transiently transfected with different bicistronic vectors, and IRES activity was assessed by

luciferase assay. The LucF activity from FGF-2 IRES was significantly increased by high glucose, whereas the activity from other IRESs maintained the level seen in normal glucose conditions. In contrast, LucR activity was significantly reduced to around 70% of control. Thus, the maintenance of translational activity may be a general property of cellular IRES contained in glucose-induced genes, whereas the ability to be activated by high glucose seems specific to the FGF-2 IRES.

Discussion

IRESs, which allow non-classical cap-independent translation initiation, have been discovered in several cellular capped mRNAs. The presence of such elements in capped mRNAs has raised the question of their function in the regulation of gene expression. Little is known about a significance of IRES-dependent translational control of gene expression, in particular *in vivo* under disease states. The present study showed for the first time that IRES-dependent translational control is a key step in the changes in regulation of FGF-2 expression under hyperglycemic conditions.

Our data presented here show that induction of FGF-2 by hyperglycemia is regulated at the level of translation in a tissue-specific manner. FGF-2 expression in the aorta and the brain of hyperglycemic mice could depend on FGF-2 IRES-mediated translational activity but not on the level of its mRNA (Fig. 1 and 2). This phenomenon observed in the brain is in agreement with our previous observation that showed a strong, constitutive FGF-2 IRES activity in brain (19). These observations indicate that FGF-2 expression in brain could be mainly regulated by the IRES in both physiological and pathophysiological conditions. The pathological activation of FGF-2 IRES observed in the diabetic aorta, among all organs tested, suggests that FGF-2 IRES activating trans-acting factors (ITAFs) might be induced and/or activated in the aorta but not in other organs, whereas such ITAFs could be negatively regulated in the brain (Fig. 5). Alternatively, we can hypothesize the involvement of inhibitory ITAFs such as p53 previously shown to inactivate the FGF-2 IRES (27, 28), whose activity would be removed in aorta in response to hyperglycemia (Fig. 5). These data also indicate that the levels of FGF-2 protein do not always correlate with those of its transcript abundance in certain tissues, which further support the importance of translational regulation of gene expression (29). In contrast, the level of FGF-2 protein in the eye and the heart was correlated with both its mRNA levels (Fig. 2), suggesting that hyperglycemia-induced change of FGF-2 expression was mainly regulated transcriptionally in these organs.

To further demonstrate the component of the diabetic state responsible for the activation of FGF-2 IRES, we used *ex vivo* model by using aortic SMC from the transgenic mice. In parallel with our *in vivo* findings, exposure to high concentrations of extracellular glucose increases FGF-2 IRES activity in spite of a decrease in the reporter mRNA (Fig. 3A). Furthermore, our *ex vivo* study clearly showed that high glucose stress suppressed overall protein synthesis activity in the cells. Experimental controls with the nonmetabolized enantiomer L-glucose confirms that this translational control required intracellular metabolism of glucose and do not reflect the potential influence of osmolar stimulus. Our data show that induction of FGF-2 protein by glucose results from not only an increase in its transcripts but also translational upregulation in an IRES-dependent manner (Fig. 3).

Diabetes mellitus is associated with a characteristic array of vascular complications including macrovascular disease characterized by accelerated atherosclerosis, and microvascular disease characterized by vascular hypertrophy and neovascularization (30). FGF-2 is a potent growth factor for vascular cell types including endothelial and smooth muscle cells, whose proliferation appears to be involved in pathological processes such as neovascularization and atherosclerosis. In addition, several lines of evidence show an *in vivo* important implication of FGF-2 in the pathogenesis of vascular dysfunction induced by hyperglycemia, including vascular hyperpermeability and hemodynamic change (5), increased aortic smooth muscle growth response to vascular injury (31), and the development of diabetic retinopathy (32). Therefore, translational or transcriptional upregulation of FGF-2 expression by glucose could contribute to the development of diabetes-associated vascular complications in selected tissues such as aorta and eye. The translational upregulation appeared as a transitory phenomenon in aorta despite the persistent hyperglycemia. A pathological state could then be initiated, although an uncontrolled persistence could also constitute a risk factor. Such a transient or permanent activation of the distinct regulation of FGF-2 expression observed in aorta and eye (Fig. 1 and 2) could be involved in the

paradoxical consequences of diabetes, retinal angiogenesis versus limb ischemia.

In the simplified model based on our data (Fig. 5), we propose that IRES-dependent regulation of translation is an important parameter in diabetes-associated vascular complications resulting from FGF-2 overexpression. However, these data related to previous ones enable us to affirm that translational regulation in vessels is more general and a key phenomenon in the link between diabetes and atherosclerosis: a previous report has shown that another gene, the CD36 scavenger receptor, contributing to accelerated atherosclerosis in response to glucose is translationally activated in vascular wall macrophages (33). The glucose sensitive translation is mediated by the CD36 mRNA 5'UTR and involves the translation of small upstream open reading frames (uORF). Although the mechanism used for CD36 translational upregulation seems to be different from that regulating FGF-2 mRNA, we know today that uORF can be coupled with IRES-dependent translation: a recent report has shown that activation of the arginine/lysine transporter cat-1 mRNA IRES in response to amino-acid deficiency is mediated by translation of a uORF responsible for mRNA leader remodeling (34). Several arguments that maintenance of translation efficiency under high glucose conditions might be a common feature of cellular IRESs included, at least, in glucose-induced genes and that glucose upregulates translation of specific gene (CD36) suggest that the mechanism of IRES-mediated translational activation in response to glucose may be able to regulate several genes involved in the pathophysiology of diabetes.

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References

1. Bikfalvi, A., Klein, S., Pintucci, G., and Rifkin, D.B. 1997. Biological roles of fibroblast growth factor-2. *Endocr. Rev* **18**:26-45.
2. Gospodarowicz, D., Ferrara, N., Schweigerer, L., and Neufeld, G. 1987. Structural characterization and biological function of fibroblast growth factor. *Endocrinol. Rev.* **8**:95-114.
3. Dono, R., Texido, G., Dussel, R., Ehmke, H., and Zeller, R. 1998. Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *EMBO J.* **17**:4213-4225.
4. Zhou, M., Sutliff, R.L., Paul, R.J., Lorenz, J.N., Hoying, J.B., Haudenschild, C.C., Yin, M., Coffin, J.D., Kong, L., Kranias, E.G., et al. 1998. Fibroblast growth factor 2 control of vascular tone. *Nat. Med.* **4**:201-207.
5. Stephan, C.C., Chang, K.C., LeJeune, W., Erichsen, D., Bjercke, R.J., Rege, A., Biediger, R.J., Kogan, T.P., Brock, T.A., Williamson, J.R., and Tilton, R.G. 1998. Role for heparin-binding growth factors in glucose-induced vascular dysfunction. *Diabetes* **47**:1771-1778.
6. Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lelias, J.M., Liauzun, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A., and Caput, D. 1989. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. *Proc. Natl. Acad. Sci. USA* **86**:1836-1840.
7. Arnaud, E., Touriol, C., Boutonnet, C., Gensac, M.C., Vagner, S., Prats, H., and Prats, A.C. 1999. A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. *Mol. Cell. Biol.* **19**:505-514.
8. Coffin, J.D., Florkiewicz, R.Z., Neumann, J., Mort-Hopkins, T., Dorn II, G.W., Lightfoot, P., German, R., Howles, P.N., Kier, A., O'Toole, B.A., et al. 1995. Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic

mice. *Mol. Biol. Cell* **6**:1861-1873.

9. Prats, A.C., Vagner, S., Prats, H., and Amalric, F. 1992. Cis-acting elements involved in the alternative translation initiation process of human basic fibroblast growth factor. *Mol. Cell. Biol.* **12**:4796-4805.

10. Vagner, S., Gensac, M.C., Maret, A., Bayard, F., Amalric, F., Prats, H., and Prats, A.C. 1995. Alternative translation of human fibroblast growth factor 2 mRNA occurs by internal entry of ribosomes. *Mol. Cell. Biol.* **15**:35-44

11. Vagner, S., Touriol, C., Galy, B., Audigier, S., Gensac, M.C., Amalric, F., Bayard, F., Prats, H., and Prats, A.C. 1996. Translation of CUG- but not AUG-initiated forms of human fibroblast growth factor 2 is activated in transformed and stressed cells. *J. Cell Biol.* **135**:1391-1402

12. Galy, B., Maret, A., Prats, A.C., and Prats, H. 1999. Cell transformation results in the loss of the density-dependent translational regulation of the expression of fibroblast growth factor 2 isoforms. *Cancer Res* **59**:165-171.

13. Vagner, S., Galy, B., and Pyronnet, S. 2001. Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. *EMBO Rep.* **2**:893-898.

14. Bonnal, S., Boutonnet, C., Prado-Lourenco, L., and Vagner, S. 2003. IRESdb: the Internal Ribosome Entry Site database. *Nucleic Acids Res.* **31**:427-428.

15. Holcik, M., Lefebvre, C., Yeh, C., Chow, T., and Korneluk, R.G. 1999. A new internal-ribosome-entry-site motif potentiates XIAP-mediated cytoprotection. *Nat. Cell Biol.* **1**:190-192.

16. Stein, I., Itin, A., Einat, P., Skaliter, R., Grossman, Z., and Keshet, E. 1998. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. *Mol. Cell. Biol.* **18**:3112-3119.

17. Coldwell, M.J., deSchoolmeester, M.L., Fraser, G.A., Pickering, B.M., Packham, G., and Willis, A.E. 2001. The p36 isoform of BAG-1 is translated by internal ribosome entry

following heat shock. *Oncogene* **20**:4095-4100.

18. Fernandez, J., Yaman, I., Mishra, R., Merrick, W.C., Snider, M.D., Lamers, W.H., and Hatzoglou, M. 2001. Internal ribosome entry site-mediated translation of a mammalian mRNA is regulated by amino acid availability. *J. Biol. Chem.* **276**:12285-12291.

19. Créancier, L., Morello, D., Mercier, P., and Prats, A.C. 2000. Fibroblast growth factor 2 internal ribosome entry site (IRES) activity ex vivo and in transgenic mice reveals a stringent tissue-specific regulation. *J. Cell Biol.* **150**: 275-281.

20. Arnqvist, H.J., and Dahlkvist, H.H. 1979. Effect of experimental diabetes on the incorporation of amino acids into protein in rat aorta. *Horm. Metab. Res.* **11**:384-388.

21. Pain, V.M., Albertse, E.C., and Garlick, P.J. 1983. Protein metabolism in skeletal muscle, diaphragm, and heart of diabetic rats. *Am. J. Physiol.* **245**:E604-E610.

22. Karpen, C.W., Spanheimer, R.G., Randolph, A.L., and Lowe Jr, W.L. 1992. Tissue-specific regulation of basic fibroblast growth factor mRNA levels by diabetes. *Diabetes* **41**:222-226.

23. Lowe Jr, W.L., Florkiewicz, R.Z., Yorek, M.A., Spanheimer, R.G., and Albrecht, B.N. 1995. Regulation of growth factor mRNA levels in the eyes of diabetic rats. *Metabolism* **44**:1038-1045.

24. Rokutan, K., Teshima, S., Miyoshi, M., Nikawa, T., and Kishi, K. 1997. Oxidant-induced activation of nuclear factor-kappa B in cultured guinea pig gastric epithelial cells. *Dig. Dis. Sci.* **42**:1880-1889.

25. McClain, D.A., Paterson, A.J., Roos, M.D., Wei, X., and Kudlow, J.E. 1992. Glucose and glucosamine regulate growth factor gene expression in vascular smooth muscle cells. *Proc Natl Acad Sci U S A.* **89**:8150-8154.

26. Hamet, P., Sugimoto, H., Umeda, F., Lecavalier, L., Franks, D.J., Orth, D.N., and Chiasson, J.L. 1985. Abnormalities of platelet-derived growth factors in insulin-dependent diabetes. *Metabolism.* **34**: 25-31.

27. Galy, B., Créancier, L., Zanibellato, C., Prats, A.C., Prats, H. 2001. Tumour suppressor p53 inhibits human fibroblast growth factor 2 expression by a post-transcriptional mechanism. *Oncogene*. 20:1669-1677.
28. Galy, B., Créancier, L., Prado-Lourenco, L., Prats, A.C., Prats, H. 2001. p53 directs conformational change and translation initiation blockade of human fibroblast growth factor 2 mRNA. *Oncogene*. 20:4613-4620.
29. Gygi, S.P., Rochon, Y., Franza, aB.R., nd Aebersold, R. 1999. Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* **19**:1720-1730.
30. Williamson, J.R., Tilton, R.G., Chang, K., and Kilo, C. 1988. Basement membrane abnormalities in diabetes mellitus: relationship to clinical microangiopathy. *Diabetes Metab. Rev.* **4**:339-370.
31. Lindner, V. and Reidy, M.A. 1991 Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA*. 88: 3739-3743.
32. Sivalingam, A., Kenney, J., Brown, G.C., Benson, W.E., and Donoso, L. 1990. Basic fibroblast growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. *Arch. Ophthalmol.* 108: 869-872.
33. Griffin, E., Re, A., Hamel, N., Fu, C., Bush, H., McCaffrey, T., Asch, A.S. A link between diabetes and atherosclerosis: 2001. Glucose regulates expression of CD36 at the level of translation. *Nat. Med.* 7:840-846.
34. Yaman, I., Fernandez, J., Liu, H., Caprara, M., Komar, A.A., Koromilas, A.E., Zhou, L., Snider, M.D., Scheuner, D., Kaufman, R.J., Hatzoglou, M. 2003. The zipper model of translational control: a small upstream ORF is the switch that controls structural remodeling of an mRNA leader. *Cell* 113:519-531.
35. Jonas, J.C., Laybutt, D.R., Steil, G.M., Trivedi, N., Pertusa, J.G., Van de Castele, M., Weir, G.C., and Henquin, J.C. 2001. High glucose stimulates early response gene c-Myc

expression in rat pancreatic beta cells.

J.Biol.Chem. 276:35375-35381.

Figure legend

Figure 1. Aorta-specific activation of the FGF-2 IRES by hyperglycemia

(A) Schema of the bicistronic transgene expressed by the transgenic mice. The bicistronic cassette is under the control of the CMV promoter. It expresses the renilla luciferase (LucR) in a cap-dependent manner and the firefly luciferase (LucF) in an IRES-dependent manner. The FGF-2 IRES is located between the two cistrons. (B and C) Tissue extracts were prepared from the transgenic mice at the indicated time post-injection of STZ or from control mice treated with vehicle (V), and the luciferase activities were measured as described in Materials and Methods. Each luciferase activity is shown relative to the activity of vehicle-treated control mice, which is expressed as 100%. Results represent mean $\pm$ SE (n=6-10). B) LucR activity; C) LucF activity. [#]*P*<0.01, ^{*}*P*<0.05 vs. vehicle-treated control. (D) Tissue RNA was prepared from the transgenic mice at the indicated time post-injection of STZ or from vehicle-treated control mice, and the level of reporter mRNA was measured by a real-time RT-PCR as described in Materials and Methods. Results are expressed relative to the level of individual tissue for vehicle-treated control mice and represent mean $\pm$ SD (n=4-6). [#]*P*<0.01, ^{*}*P*<0.05 vs. vehicle-treated control.

Figure 2. Tissue-specific increase in endogenous FGF-2 expression by hyperglycemia

Tissue protein or total RNA was prepared from the transgenic mice treated with vehicle (V) or STZ for the indicated time. Equal amounts of tissue protein from each sample were incubated with heparin-sepharose, and heparin-binding fraction was analyzed by immunoblot analysis using an anti-FGF-2 antibody. Representative data are shown in A, D, G, and J. Bands that correspond to the three different isoforms of FGF-2 are indicated by arrows. Results are also expressed relative to the level of individual tissue for vehicle-treated control mice (B, E, H, and K) and represent mean $\pm$ SE (n=4-6). Levels of endogenous FGF-2 mRNA was determined by a real time RT-PCR analysis as described in Materials and Methods (C, F,

I, and L). Results are expressed relative to the level of individual tissue for vehicle-treated control mice and represent mean $\pm$ SE (n=4-6). [#]*P*<0.01, ^{*}*P*<0.05 vs. vehicle-treated control.

Figure 3. Glucose-stimulated biosynthesis of FGF-2 protein in an IRES-dependent

manner (A) Stimulation of FGF-2 IRES activity by glucose *ex vivo*. SMC from transgenic mice were incubated in medium containing 5.5 mM normal (NG), 25 mM high D-glucose (HG), or 5.5 mM D-glucose plus 19.5 mM L-glucose (L-glucose) for the indicated time. Cell extracts were prepared, and luciferase activities were measured. Total RNA was prepared from the cells, and the level of reporter mRNA was measured by a real-time RT-PCR as described in Materials and Methods. Results are presented as described in the legend to Fig. 1. The data represent the mean $\pm$ SE of three independent experiments. [#]*P*<0.01, ^{*}*P*<0.05 vs. NG. (B and C) Decrease of global protein synthesis and increase in biosynthesis of FGF-2 protein by HG. Cells incubated in NG and HG conditions for 6 d were metabolically radiolabeled with [³⁵S]methionine/[³⁵S]cysteine for 6 h. B) Incorporation of the radioactivities into TCA-precipitated proteins was analyzed by a liquid scintillation counter. The data show the mean $\pm$ SE (n=3). [#]*P*<0.01 vs. NG. C) Equal amounts of protein were immunoprecipitated as described in Materials and Methods. Representative data is shown, and bands that correspond to the three different isoforms of FGF-2 are indicated by arrows. Histograms represent relative level of all isoforms of FGF-2 protein determined by densitometric analysis. (D) Increase in total level of endogenous FGF-2 protein by HG. Whole-cell extracts were prepared from the cells cultured in NG and HG conditions for 6 d. FGF-2 protein levels were analyzed by immunoblot analysis. Representative data is shown, and bands corresponding to the three different isoforms of FGF-2 are indicated by arrows. The loading control is given by the level of β -actin. Histograms represent relative level of all isoforms of FGF-2 protein to β -actin determined by densitometric analysis. The data are the mean $\pm$ SE (n=3). [#]*P*<0.01 vs. NG. (E) Increase in endogenous FGF-2 mRNA by HG. Total RNA was prepared from the

cells cultured in NG and HG conditions for 6 d. Levels of endogenous FGF-2 transcripts was determined by a real-time RT-PCR. Results are expressed relative to the level of control cells and represent mean $\pm$ SE (n=5). [#]*P*<0.01 vs. NG. (F) Treatment with STZ did not affect the stability of endogenous FGF-2 protein. After the cells were cultured in NG and HG conditions for 3 d, they were treated with 0.25 mM STZ for the indicated time. Whole-cell extracts were prepared from the cells, and FGF-2 protein levels were analyzed by immunoblot analysis. The loading control is given by the level of β -actin.

Figure 4. Comparison of the activities of FGF-2, PDGF, and c-myc IRESs in response to high glucose

SMC from non-transgenic mice were treated with 5.5 mM or 25 mM glucose for 72 h, then they were transiently transfected with bicistronic plasmid containing the indicated IRES. Twenty hours post-transfection, cell lysates and total RNA were prepared for luminescence activity and reporter mRNA integrity, respectively. (A and B) Each luciferase activity is shown relative to the activity under normal glucose conditions, which is expressed as 100%. The data represent the mean $\pm$ SE of three independent experiments. [#]*P*<0.01 vs. NG. (C) The level of reporter mRNA was measured by a real-time RT-PCR as described in Materials and Methods. Results are expressed relative to the level of NG-treated control cells and represent mean $\pm$ SE (n=6).

Figure 5. Schematic diagram of vascular tissue-specific activation of FGF-2 IRES in response to hyperglycemia

Our data lead us to propose the following model: on the one hand (left), hyperglycemia results in inhibition of global protein synthesis activity, which could lead to reduction of overall gene expression in these tissue. On the other hand (right), hyperglycemia, by regulating expression,

localization, and/or activity of IRES trans-acting factors (ITAFs), leads to activation of FGF-2 IRES, resulting in increased FGF-2 biosynthesis by an IRES-dependent mechanism.

The FGF-2 mRNA is schematized, with the five initiation codons, and the IRES located upstream from the CUG 1 codon (able to control the use of CUG1, 2, 3 and AUG codons). The binding of ITAFs on the IRES is represented. Such proteins are thought to behave as chaperones that will stabilize the IRES structure (in the case of activating factors). In the case of p53 which has an inhibitory effect, we have shown that the FGF-2 IRES is unfolded following the p53 binding (28). According to our results, hyperglycemia prevents the 43S ribosome recruitment at the capped 5' end (binding the initiation factor eIF4F) but increases 43S internal entry mediated by the IRES. IRES-dependent translation stimulated by hyperglycemia may lead to overproduction of FGF-2 in vascular tissues and thus create a risk factor for diabetes-associated vascular complications.

Figure 1

A Bicistronic transgene

B

C

D

Figure 2

Figure 3

Figure 4

A

B

C

Figure 5