Sugar-Induced Modification of Fibroblast Growth Factor 2 Reduces Its Angiogenic Activity *in Vivo*

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Both clinical and animal studies have shown that angiogenesis is impaired in diabetes mellitus; however, the mechanisms responsible for this effect are poorly characterized. The major aims of the present study were to evaluate the effect of hyperglycemia on fibroblast growth factor 2 (FGF2)-induced angiogenesis *in vivo* and to determine whether FGF2 non-enzymatic glycation occurs in hyperglycemic mice. New blood vessel formation was examined in reconstituted basement membrane protein (Matrigel) plugs containing FGF2 in control normoglycemic CD1 and in hyperglycemic nonobese diabetic (NOD) mice. FGF2-induced angiogenesis in NOD mice was inhibited by 75% *versus* control mice ($P < 0.001$). When recombinant FGF2 was mixed with Matrigel and injected in mice, it was found that recombinant FGF2 glycation was significantly enhanced in plugs from NOD *versus* control mice ($P < 0.01$). In the Boyden chamber assay, the chemotactic effect of glycated FGF2 toward endothelial cells was lower than that of unmodified FGF2 ($P < 0.01$). Further, FGF2 glycated *in vitro* and co-injected with Matrigel in CD1 mice was a weaker angiogenic stimulus than unglycated FGF2 ($P < 0.005$). These results indicate that FGF2-induced angiogenesis is inhibited in diabetic mice, FGF2 glycation is enhanced in hyperglycemic mice, and glycation markedly reduces FGF2 chemotactic effect *in vitro* and its angiogenic properties *in vivo*. Thus, FGF2 glycation may represent a mechanism responsible for the impairment of angiogenesis in diabetes mellitus. (Am J Pathol 2002, 161:531–541)

It has been previously shown that angiogenesis¹ and microvascular function are impaired in animal models of diabetes mellitus² and that collateral vessel development is inhibited in diabetic patients.^{3–5} The molecular mech-

anisms responsible for these effects are poorly characterized. As a consequence of increased glucose availability, free amino groups of proteins undergo a non-enzymatic reaction with reducing sugars, leading to the formation of unstable Schiff bases and, through the Amadori rearrangement, advanced glycation end-products (AGEs) are produced.^{6,7} Several proteins, including hemoglobin, serum albumin, RNase, crystallins, β -lactoglobulin, extracellular matrix proteins, and many others undergo such non-enzymatic modification leading to precipitation of insoluble proteic material.^{8–11} This posttranslational modification of proteins is responsible for the thickening of the capillary basement membrane, a hallmark of diabetic microangiopathy¹² and AGEs have been shown to be involved in the etiology of both micro- and macrovascular complications in diabetes mellitus.^{13–20} Further, it was reported that glycation reduces the mitogenic activity of basic fibroblast growth factor (FGF2)²¹ *in vitro* and its ability to activate *c-myc*.²² However, neither a cause-effect relationship between FGF2 glycation and diabetes nor the occurrence of FGF2 glycation *in vivo* have been shown. Therefore, the aims of the present study were: 1) to evaluate FGF2-induced angiogenesis in hyperglycemic mice; 2) to determine whether glycation modifies FGF2 function *in vitro*; 3) to evaluate the occurrence of FGF2 glycation *in vivo* in a murine diabetic model; and 4) to investigate the effect of such modification on FGF2-induced angiogenesis *in vivo*. Our results show that FGF2 glycation occurs in diabetic mice *in vivo* and that glycated FGF2 exhibits a weaker chemotactic effect toward endothelial cells *in vitro* and weaker angiogenic properties *in vivo*, as compared to unglycated FGF2. These data suggest that angiogenic growth factor(s) glycation may represent a mechanism for the impairment of angiogenesis in diabetes mellitus.

Materials and Methods

Isolation of Bovine Aortic Endothelial Cells

Primary bovine aortic endothelial cells (BAEC) were prepared and cultured as described.²³ Endothelial cell culture purity was consistently >98% as determined by the

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A

B

C

D

cobblestone configuration of the endothelial cell monolayer and by the 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate acetylated low density lipoprotein (Dil-Ac-LDL) uptake (Biomedical Technologies Inc., Stoughton, MA, USA). All experiments were performed with cells at passages 2–6.

Cell Culture

Cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS) (Euroclone Inc., Milan, Italy), 2 mmol/L L-glutamine, and 100 IU/ml penicillin/streptomycin (Gibco BRL, Paisley, UK) in humidified 5% CO₂ atmosphere at 37°C. The culture medium was changed every 3 or 4 days and cells were grown near confluence; cell monolayers were then harvested by exposure to 0.1% trypsin-EDTA (GIBCO BRL) as described.²³

Migration Assay

Migration assays were carried out in modified Boyden chambers (Costar Scientific Corporation, Cambridge, MA, USA) as described.²⁴ BAEC (2×10^5) were placed in the upper chamber of the Boyden apparatus mounted with 12- μ m pores polycarbonate filters (Costar Scientific Corporation) coated with 5 mg/L gelatin solution (Sigma, St. Louis, MO, USA). Human recombinant FGF2 (Gibco BRL) was used as chemoattractant; it was dissolved at 10 ng/ml concentration in DMEM-0.1% BSA and placed in the lower chamber of the Boyden apparatus. For migration assays using unmodified or glycated FGF2 as chemoattractant, the growth factor at the end of glycation time was diluted with DMEM-0.1% BSA. Migration assays were carried out at 37°C in 5% CO₂, for 5 hours, and then filters were removed, fixed with absolute ethanol, and stained with toluidine blue. Cells migrated were counted blindly at $\times 400$ magnification in 15 fields for each filter and the average number of cells/field was reported. All experiments were performed at least three times in triplicate.

Sugar-Dependent Modification of FGF2

Human recombinant FGF2 was dissolved in phosphate-buffered saline (PBS), pH 7.4, at 10 μ g/ml final concentration and immediately frozen at -80°C . Then aliquots of FGF2 were incubated at 37°C for different times (up to 48 hours) with different concentrations (up to 250 mmol/L) of glucose, fructose, or mannitol, as described.²¹ These samples were tested in migration assays. FGF2 incubated with sugars was also subjected to Western blot

and competitive ELISA analysis using a polyclonal antibody raised against glycated RNase or subjected to dot blot analysis with an anti-AGE monoclonal antibody (Dojindo). For radiolabeling studies, FGF2 (1 μ g) was glycated in the presence of tracing amount of D-[1-¹⁴C]glucose or D-[U-¹⁴C]-fructose (2 μ Ci for each sugar) (Amersham Biosciences, Uppsala, Sweden; specific activity 57.0 mCi/mmol and 216 mCi/mmol for glucose and fructose, respectively), at 37°C, and electrophoresed by 8–15% gradient SDS-PAGE. Radiolabeled bands were detected after 5 days of exposure at -80°C on Kodak Optimax (Rochester, NY, USA) film.

Densitometry was carried out on a GS710 calibrated imaging densitometer (Bio-Rad, Hercules, CA, USA). To determine the number of AGE products/FGF2 molecules, the exact amount of FGF2 and labeled sugar within the bands were calculated by comparison with two calibration curves obtained by immunoblot and autoradiography of increasing amounts of FGF2 and labeled fructose, respectively.

Antibodies

Rabbit AGE-RNase polyclonal antibody was obtained against RNase (Sigma) incubated for 20 days with 25 mmol/L methyl glyoxal (MG) (Fluka, Buchs, Switzerland) according to published procedures.²⁵ After incubation, the RNase-MG was dialyzed through a 12-KDa cut-off tube and used as antigen for rabbit immunization. Two 5-month-old New Zealand rabbits were immunized by injecting 0.5 mg of RNase-MG emulsified with complete Freund's adjuvant (1:4 v:v). Rabbits received a booster injection 4 weeks after the first injection. Blood was collected 20 days after the last injection by ear puncture. The serum was separated and stored at -20°C until use.

Competitive ELISA

The antiserum was characterized by competitive ELISA as described.²⁶ AGE-RNase antibody was assayed against different glycated proteins, ie, bovine serum albumin, ovalbumin, and casein, obtained by incubation with 250 mmol/L fructose for 7 days, or with β -lactoglobulin incubated with 250 mmol/L methyl glyoxal for 7 days. For determination of FGF2 glycation, 96-well flexible ELISA plates were coated using a solution of 10 ng/ml glycated BSA (ie, preincubated with methyl glyoxal as described above) and incubated at 4°C overnight. After blocking with 10% horse serum in PBS, 0.1-ml aliquots of rabbit antiserum diluted 1:4000, in the presence of different amounts of glycated FGF2, were added at 37°C for 1

Figure 1. Effect of hyperglycemia on FGF2-induced angiogenesis. **A:** Histological sections of Matrigel plugs removed from CD1 and NOD mice (Trichrome-Masson staining, magnification, $\times 100$; scale bar, 70 μ m). Red blood cells are present in the neovessels (arrowheads). **B:** Histological sections of Matrigel plugs removed from CD1 and NOD mice (anti-PECAM-1 immunostaining, magnification, $\times 100$; scale bar, 70 μ m). Arrowheads indicate the neovessels. **C:** Quantification of the experiments reported in **A**. Two groups of mice, CD1 ($n = 10$) and hyperglycemic NOD mice ($n = 6$) were injected with 600 μ l of Matrigel containing FGF2 (150 ng/ml). After 8 days, plugs were removed and histologically processed, sections were stained with Trichrome-Masson procedure, and neovessel formation was measured. These experiments indicated that FGF2-induced angiogenesis is impaired in diabetic mice. Data represent average $\pm$ SD. **D:** Angiogenesis in Matrigel plugs measured as function of blood glucose levels in NOD mice. For this experiment, 16 NOD mice were used, with blood sugar levels ranging between 50 and 550 mg/100 ml and injected with 600 μ l of Matrigel containing 150 ng/ml FGF2. A significant inverse relation between blood sugar level and blood vessel number was found ($r = 0.81$; $P < 0.001$). These experiments indicated that impairment of FGF2-dependent angiogenesis was related to the level of hyperglycemia.

hour. Peroxidase-conjugated anti-rabbit IgG secondary antibodies and tetramethylbenzidine (Bio-Rad) were used for a colorimetric assay measuring OD at 450 nm.

Quantification of FGF2 Glycation

A competitive ELISA calibration curve was prepared using as reference a glycated β -lactoglobulin, which bound an average of 4 mol of carbohydrates (AGE) per mole of protein. The relationship between percentage of inhibition and picomoles of AGE was given by the equation $y = 17.4x + 7.06$ ($r^2 = 0.9939$).

The amount of AGE/ μ g of FGF2 under our conditions was then calculated by extrapolating the level of glycation from the calibration curve. The amount of AGE in β -lactoglobulin was determined by electrospray mass spectrometry as described.¹¹

Animals

Swiss (CD1) and non-obese diabetic NOD/LtJ mice were purchased from Charles River (Wilmington, MA). NOD mice have previously been shown to develop diabetes with clinical features similar to those of the human insulin-dependent diabetes mellitus, type I.²⁷ Female mice, 18 to 19 weeks of age, were used for all experiments. Glucose blood levels were measured every 4 days after 6 hours of food starvation. Blood glucose levels were also measured immediately before starting angiogenesis assay and only NOD mice showing sugar levels higher than 200 mg/100 ml were considered hyperglycemic. Mice were housed under controlled temperature (23°C) and lighting with free access to water and standard mouse chow. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with international laws and policies (Guide for the Care and Use of Laboratory Animals. United States National Research Council, 1996).

Evaluation of in Vivo FGF2-Induced Angiogenesis

Angiogenesis in control and NOD mice was evaluated through the Matrigel assay.²⁸ Briefly, a mixture of reconstituted basement membrane proteins (Matrigel, Beckton Dickinson, Franklin Lakes, NJ, 600 μ l) was injected subcutaneously in NOD and CD1 mice ($n = 6$ for each group). Other groups were injected with the same mixture containing unmodified or glycated FGF2 (150 ng/ml). For these experiments, before mixing with Matrigel, FGF2 was incubated with 150 mmol/L mannitol, glucose, or fructose for 24 hours. To correlate glycemic levels with FGF2-induced angiogenesis, glucose blood level was measured in mice before angiogenesis assay was started. After 8 days from Matrigel injection, plugs were removed and processed for histology analysis. Histological sections (7 μ m) were stained with Trichrome-Masson procedure (Bio-Optica, Milan, Italy). The vessels within the plugs were recognized by both morphology and

Figure 2. Characterization of anti-AGE antibodies by competitive ELISA. Wells were coated with AGE-BSA and then various proteins as competitor and anti-AGE antiserum were added (100 μ l per well). $\blacktriangle$, BSA-AGE; $\blacksquare$, ovalbumin-AGE; $\bullet$, casein-AGE; $\blacklozenge$, dimer of methyl-glyoxal-lysine (MOLD); $\circ$, fructosyl-lysine; $\times$, unglycated-BSA; $\parallel$, methyl glyoxal- β -lactoglobulin. Early products of glycation, fructosyl lysine and unglycated BSA, showed no competitive activity. These data indicate that anti-AGE serum recognized glycated proteins and the developed competitive ELISA detected glycated proteins with sensitivity partially depending on the proteic substrate of glycation. Data represent means $\pm$ SD of three experiments performed in duplicate. **Inset:** Competitive ELISA calibration curve obtained by using glycated β -lactoglobulin as reference. Within this molecule, an average of 4 mol of sugar (AGE) per mole of protein is present. This curve was then used to calculate the pmol of AGE per pmol of FGF2 under our conditions. The exact amount of AGE in β -lactoglobulin was determined by electrospray mass spectrometry as described.¹¹

presence of red blood cells. Angiogenesis was evaluated blindly by two operators, by considering at least four different sections per Matrigel plug; each section was 100 μ m from the next. The total number of neovessels over the whole area of Matrigel was measured with a Quantimet 970 instrument (Cambridge Instruments, Cambridge, UK) and was expressed as number of vessels/ mm^2 as described.²⁸ When two segments of vessels were cut longitudinally, as indicated by a long axis greater than threefold the short axis, and were close to each other, they were counted as a single vessel. In additional groups of mice injected with Matrigel containing unmodified or glycated FGF2, blood vessels in histological sections were identified by PECAM staining with an anti-PECAM-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), according to manufacturer's instructions.

FGF2 Glycation in Vivo

Recombinant FGF2 (2 μ g per plug) was mixed with Matrigel (600 μ l/mouse) and subcutaneously injected in control CD1 ($n = 6$) and NOD mice ($n = 6$). Control mice ($n = 5$) were injected with Matrigel alone. After 8 days, Matrigel plugs were removed, proteins within the plugs were extracted by homogenization with 10 strikes in a teflon/teflon potter system, in PBS containing the following inhibitors of proteases: 10 μ mol/L aprotinin, 10 μ mol/L leupeptin, 20 μ mol/L pepstatin, 1 mmol/L benzamide, 10 μ mol/L PMSF (Sigma). After 30 minutes of centrifugation at 12,000 rpm at 4°C, supernatant was removed and immediately stored at -80°C . Proteins were analyzed by ELISA and Western blot. For ELISA analysis, 300 μ g of protein extracted from Matrigel were loaded

Table 1. Competitive ELISA Using Anti-Glycated-RNase Antibodies

FGF2 incubated with sugar	Sugar concentration	24 hours' glycation; inhibition (%)	AGE (pmol)	48 hours' glycation; inhibition (%)	AGE (pmol)
FGF2 (0.9 μ g, 50 pmol)	Mannitol, 250 mM	2.0 $\pm$ 0.2	ND	3.2 $\pm$ 0.3	ND
FGF2 (0.9 μ g, 50 pmol)	Glucose, 150 mM	7.4 $\pm$ 1.2	3	13.2 $\pm$ 1.0	53
FGF2 (0.9 μ g, 50 pmol)	Fructose, 50 mM	ND	ND	17.7 $\pm$ 1.2	91
FGF2 (0.9 μ g, 50 pmol)	Fructose, 150 mM	12.4 $\pm$ 1.7	46	22 $\pm$ 2.1	128
FGF2 (0.9 μ g, 50 pmol)	Fructose, 250 mM	16 $\pm$ 1.4	77	33.1 $\pm$ 2.4	226

In this assay the level of protein glycation is directly related to the inhibition measured. FGF2 (0.9 μ g) was incubated in PBS at 37°C for 24 or 48 hours in the presence of sugars. Incubation with mannitol did not significantly inhibit the binding of anti-AGE-RNase antibody to the immobilized glycated BSA (not detectable, ND). For direct quantification of FGF2 glycation, the pmol of AGE were calculated as reported in Materials and Methods. These results indicate that FGF2 glycation was time- and sugar concentration-dependent and that fructose was more reactive than glucose. Data reported represent average $\pm$ standard deviation of three experiments performed in triplicate.

onto a plastic multiwell plate coated with a FGF2 monoclonal antibody; FGF2 glycation was detected with a monoclonal anti-AGE antibody (Dojindo Laboratories, Kumamoto, Japan) followed by a HRP-labeled secondary antibody and colorimetric assay. For Western blot analysis, total proteins extracted from Matrigel were subjected to immunoprecipitation with Sepharose-protein A beads (Pharmacia) activated with a polyclonal serum anti-FGF2 (Amersham) according to standard protocols and the manufacturer's instructions. Equal amounts of FGF2 (85 ng as assessed by using a commercial ELISA assay for FGF2, R&D Systems, Minneapolis, MN, USA) were electrophoresed by SDS-PAGE and then electroblotted onto nitrocellulose membrane. Glycated proteins were detected by means of a polyclonal antibody raised against glycated RNase followed by ECL detection (Amersham).

Molecular Size Fractionation of FGF2 Glycation in Matrigel Plugs

To elucidate whether *in vivo* glycation involves the extracellular matrix-bound pool of FGF2 or the unbound pool, a fractionation study was carried out. Two groups of 5 hyperglycemic NOD mice each with blood glucose levels higher than 400 mg/100 ml were injected subcutaneously with Matrigel (600 μ l) containing recombinant FGF2 (15 μ g/plug). After 48 hours, plugs were removed and homogenized either in the presence of PBS (group A) or in the presence of 2 mol/L NaCl (group B). High ionic strength was used to detach FGF2 from ECM components. The homogenates were then subjected to 5 minutes of 1500-rpm microfuge centrifugation to remove large insoluble particles and the cleared supernatant was considered total homogenate. Both total homogenates from groups A and B were then subjected to 30 minutes at 14,000 rpm centrifugation. The supernatants were removed and subsequently ultrafiltrated through a 30 kd cut-off Millipore filter and proteins with molecular size higher or lower than 30 KDa were collected. The pellets from the centrifugation steps were not assayed due to the insolubility of samples. Each sample at different steps of fractionation was then immunoprecipitated by Sepharose-protein A-conjugated beads preactivated with an anti-FGF2 antibody (Santa Cruz) then assayed as reported above to quantify both FGF2 and glycation levels. The ultrafiltration allowed to identify two pools of FGF2:

the first one with molecular size higher than 30 kd, which represents the high molecular weight (HMW) complexes, likely due to the binding of FGF2 to heparan sulfates and other ECM components. The second one, with molecular size lower than 30 kd, represents the low molecular weight (LMW) unbound pool of FGF2. The recovery of the injected FGF2 was not complete, probably due to diffusion of FGF2 out of the plugs, formation of stable insoluble complexes resistant to the high ionic strength treatment, stickiness of FGF2, and its attachment to the Millipore filters.

Statistical Analysis

Data were expressed as means $\pm$ SD. Student's two-tailed *t*-test was performed and a $P \leq 0.05$ was considered statistically significant. Linear regression fitting of data were carried out with statistical software (SigmaStat for Windows, Jandel Scientific, Chicago, IL, USA).

Results

FGF2-Induced Angiogenesis in Normoglycemic and Hyperglycemic Mice

To evaluate the effect of hyperglycemia on FGF2-induced angiogenesis, FGF2 was assayed in normoglycemic and hyperglycemic mice. CD1 control mice ($n = 10$) with blood sugar concentrations <100 mg/100 ml and NOD mice ($n = 6$) with blood sugar concentrations between 200 and 550 mg/100 ml were injected subcutaneously with 600 μ l of Matrigel mixed with FGF2 (150 ng/ml). After 8 days, the angiogenic response within Matrigel plugs was determined in histological sections either by Trichrome-Masson staining (Figure 1A) or by PECAM-1 immunostaining (Figure 1B). Results were similar regardless of which of these techniques was used to identify neoformed blood vessels. The angiogenic response to FGF2 was markedly reduced in diabetic mice as compared to controls (Figure 1C, $P < 0.001$). Under similar experimental conditions, it was determined whether the impaired angiogenesis was directly related to the blood sugar level; the angiogenic response was assayed in NOD mice ($n = 16$) with blood glucose levels ranging between 50 and 550 mg/100 ml. After 8 days, mice were

sacrificed and FGF2-induced angiogenesis was quantified and reported as a function of blood glucose level (Figure 1D). These experiments indicate that angiogenesis impairment in NOD mice was directly related to the blood sugar level ($r = 0.81$, $P < 0.005$). Since glucose is known to induce non-enzymatic glycation of proteins, we hypothesized that FGF2 glycation may occur in diabetic mice. To test this hypothesis, a polyclonal antibody able

Figure 4. Effect of FGF2 glycation on BAEC migration. FGF2 was incubated for 24 hours in the presence of mannitol, glucose, or fructose (50 or 150 mmol/L), then immediately used as chemoattractant for migration assay (see Materials and Methods). Absolute number of migrated cells in FGF2-mannitol treated cells was 86 ± 7 cells/field. These experiments indicated that glycation reduced FGF2 chemotactic activity: $P < 0.05$ for glucose- (*) and $P < 0.01$ for fructose- (**) FGF2 glycated versus mannitol-treated FGF2. Data represent average $\pm$ SD of three experiments performed in triplicate.

to recognize glycated proteins was developed and an ELISA assay to measure glycated FGF2 was set up.

Characterization of Anti-AGE Antiserum

A competitive ELISA was set up with a polyclonal anti-AGE antibody developed against methyl glyoxal-modified RNase. In this assay, the amount of glycated products is proportional to the competition measured. The antibody recognizes different glycated proteins and the competi-

Figure 3. Evaluation of FGF2 sugar-dependent modification. **A:** Immunoblot of glycated FGF2. FGF2 (1 μ g, about 55 pmol) treated for 24 hours with PBS, mannitol, glucose, or fructose (each sugar at 250 mmol/L) was blotted onto nitrocellulose and detected with an anti-AGE monoclonal antibody followed by HRP-conjugated secondary antibody and ECL detection. Immunolabeled dots were then quantified by densitometric analysis, and the intensities were compared to a calibration curve (not shown) obtained by blotting increasing amounts of glycated BSA whose number of AGE/molecule was previously calculated. The results of the blotting experiments are shown as pmoles of AGE. These data show that FGF2 is potentially modified by glucose and fructose, whereas mannitol was not effective. Data represent average $\pm$ SD of one representative experiment performed in triplicate. Three experiments were performed with similar results. **B:** Western blot of glycated FGF2 (200 ng/lane) with a polyclonal antibody raised against AGE-RNase. Samples were separated under denaturing conditions by SDS-polyacrylamide gel electrophoresis. Positive control is represented by 10 μ g of *in vitro* glycated BSA (gBSA, incubated with glucose for 2 weeks). These data demonstrate that, under our experimental conditions, FGF2 is covalently modified by both glucose and fructose and that such glycated products are recognized by the antibody raised against AGE-RNase. This panel shows one representative experiment. Three experiments were performed with similar results. **C:** Autoradiography of FGF2 exposed to 14 C-labeled glucose or fructose. FGF2 (1 μ g) was incubated for 24 (lanes B and C) or 48 hours (lanes D and E) with glucose (50 mmol/L, lanes B and D) or fructose (50 mmol/L, lanes C and E) and tracing amount of 14 C-labeled glucose or 14 C-labeled fructose (2 μ Ci), respectively, at 37°C. As positive controls, 10 μ g of BSA (lane F) or human serum albumin (lane G) were treated with 14 C-labeled glucose for 2 weeks. Electrophoretic migration of unmodified FGF2 (200 ng) is shown in lane A and detected by Western blot with a polyclonal anti-FGF2 antibody. Autoradiographic evaluation of electrophoresed samples showed the presence of labeled FGF2 with 18 kd molecular mass, ie, in the monomeric form. These experiments confirmed that FGF2 glycation was time-dependent and more evident with fructose than with glucose. Densitometric analysis of labeled bands (run in triplicate) from FGF2 treated with labeled fructose for 24 hours indicated that, under these experimental conditions, 1 pmol of FGF2 covalently bound approximately 1.1 to 1.8 pmol of total sugar. This panel shows one representative experiment. Three experiments were performed with similar results.

tive ELISA was 50% inhibited by 10 ng of AGE-BSA, 100 ng of AGE-caseine, 50 ng of AGE-ovalbumin, or 7 ng of β -lactoglobulin (Figure 2). Further, anti-AGE antibody did not recognize an early glycation product such as fructosyl lysine and poor competition was observed with the dimer of methyl glyoxal lysine (MOLD). Similar results were also obtained using this anti-AGE antibody in Western blotting assay (data not shown). Glycated β -lactoglobulin, whose exact AGE number per molecule has been previously determined by electrospray mass spectrometry, was used as reference to draw a calibration curve. The linear fitting of such curve ($r^2 = 0.9939$) (Figure 2, inset) was used to calculate the picomoles of AGE/pmol of FGF2 by competitive ELISA (Table 1).

FGF2 Modification

In the following experiments, the ability of sugars to glycate FGF2 was investigated *in vitro*. To evaluate the extent of FGF2 glycation, a competitive ELISA using the anti-AGE polyclonal antibody was performed. In this assay, the level of protein glycation is directly related the inhibition measured. As shown in Table 1, the amount of recombinant FGF2 glycation increased with time and with sugar concentration and was detectable after 48 hours exposure to 50 mmol/L fructose. In other experiments, recombinant FGF2 was incubated in the absence or in the presence of mannitol, glucose, or fructose (250 mmol/L for 24 hours). Samples were then subjected to immunoblot, followed by densitometric analysis (Figure 3A) or SDS-PAGE under denaturing conditions, followed by Western blot analysis with anti-AGE polyclonal antibody (Figure 3B). Both analyses confirmed the formation of glycated-FGF2 after incubation with glucose or fructose. The glycation occurring in our experimental conditions did not modify significantly the antigenic properties of FGF2, as assessed by ELISA for FGF2 and in immunoprecipitation assays (not shown).

To assess the number of residues within FGF2 molecule modified by sugars, FGF2 glycation was also evaluated by incubation in the presence of ^{14}C -labeled sugars. After 24 and 48 hours, samples were electrophoresed and labeled FGF2 was detected by autoradiography (Figure 3C). It was found that FGF2 was covalently modified by glucose and, more markedly, by fructose. Radiolabeled bands of FGF2 treated with labeled fructose (Figure 3C) were then evaluated by densitometric quantification, which showed that after 48 hours incubation with fructose, about 1.1–1.8 glycation product(s) per FGF2 molecule had been formed. These data indicate that, under our experimental conditions, FGF2 can be glycated *in vitro*.

Effect of Glycation on FGF2 Chemoattractant Properties

To evaluate the effect of glycation on FGF2 activity, BAEC migration assays were performed using either modified or unmodified FGF2 (10 ng/ml) as chemoattractant (Figure 4). These experiments showed that incubating FGF2 with glucose or fructose (50 or 150 mmol/L) for 24 hours

Figure 5. FGF2 glycation *in vivo*. **A:** ELISA evaluation of *in vivo* glycated FGF2 extracted from Matrigel. Matrigel containing recombinant FGF2 was injected in CD1 mice ($n = 6$) and hyperglycemic NOD mice ($n = 6$). Control mice ($n = 5$) were injected with Matrigel which had not been supplemented with FGF2. After 8 days, plugs were removed, homogenized, and proteic extracts (300 μg of proteins) were loaded onto plastic wells previously coated with anti-FGF2 monoclonal antibody (see Materials and Methods). Immobilized FGF2 was then treated with anti-AGE monoclonal antibody and detected with a secondary HRP antibody followed by colorimetric detection indicating a fourfold higher FGF2 glycation in NOD than in CD1 mice ($P < 0.001$). Data represent average $\pm$ SD of each group. **B: Upper panel:** Western blot analysis of glycated FGF2 extracted from Matrigel plugs kept for 8 days in normoglycemic CD1 or hyperglycemic NOD mice, as described in **A**. FGF2 was immunoprecipitated from proteic extracts and 85 ng of FGF2 were loaded per lane. Thereafter, glycated FGF2 was immunodetected with anti-AGE-RNase polyclonal antibody. As molecular mass control, recombinant FGF2 (200 ng) was used. Samples from two representative mice are shown. Densitometric analysis of both animal groups indicated an average of 9.4-fold increase of glycation in hyperglycemic NOD ($n = 6$) versus control normoglycemic CD1 mice ($n = 6$; $P < 0.001$). **Lower panel:** Western blot analysis of the total amount of FGF2 for samples shown in the upper panel. Equal amounts (85 ng) of FGF2 from CD1 and NOD mice were loaded in each lane. FGF2 was undetectable, both by ELISA (not shown) and by Western blot analysis, in control Matrigel plugs, injected as control in CD1 and NOD mice, without exogenously added recombinant FGF2 (200 μg of total proteins/lane). Results shown in **A** and **B**, obtained by two different experimental approaches, indicate that FGF2 is glycated *in vivo* in hyperglycemic mice.

led to a significant reduction of its chemoattractant properties, as compared to mannitol-treated FGF2 ($P < 0.05$ for glucose- and <0.01 for fructose-modified FGF2 versus control FGF2). These results further support the hypothesis that glycation may modify FGF2 angiogenic effects *in vivo*.

Table 2. Size Fractionation Study of *in Vivo* FGF2 Glycation

	FGF2 per Matrigel plug (pmol)	AGE per Matrigel plug (pmol)
Group A (total homogenate) PBS	1.62 μ g $\pm$ 0.19 (90 pmol)	N/A
A—supernatant HMW-FGF2 pool	89 ng $\pm$ 9 (4.9 pmol)	4.1
A—supernatant LMW-FGF2 pool	24 ng $\pm$ 1.5 (1.3 pmol)	3.7
Group B (total homogenate) NaCl	5.2 μ g $\pm$ 0.5 (288 pmol)	N/A
B—supernatant HMW-FGF2 pool	520 ng $\pm$ 51 (28.9 pmol)	28.2
B—supernatant LMW-FGF2 pool	247 ng $\pm$ 21 (13.7 pmol)	53.2

Amounts of total FGF2 and glycosylated FGF2 at different steps of size fractionation were determined as reported in Materials and Methods. Hyperglycemic NOD mice were injected subcutaneously with Matrigel (600 μ l) containing recombinant FGF2 (15 μ g/plug). After 48 hours, plugs were removed and homogenized in the presence of either PBS (group A) or 2 mol/L NaCl (group B). FGF2 incubated with Matrigel for 48 hours at 37°C in PBS without sugar, and not injected in mice, exhibited no significant glycation (not shown). These results indicate that *in vivo* FGF2 glycation involves both LMW FGF2, likely not bound to heparan sulfates or ECM components, and HMW FGF2, likely bound to heparan sulfates or other ECM components. In both groups, the LMW pool of FGF2 shows a number of picomoles of AGE/pmol of FGF2 higher than the HMW pool ($P < 0.05$).

FGF2 Glycation *in Vivo*

The *in vivo* occurrence of FGF2 glycation was evaluated in hyperglycemic NOD mice using both monoclonal (Dojindo) and polyclonal anti-AGE antibodies. FGF2 (2 μ g per plug) was mixed with Matrigel and injected subcutaneously in normoglycemic CD1 ($n = 6$) and hyperglycemic NOD ($n = 6$) mice. The blood sugar levels of CD1 and NOD mice were lower than 81 mg/100 ml (mean 64 ± 11 mg/100 ml) and higher than 305 mg/100 ml (mean 510 ± 92 mg/100 ml), respectively. After 8 days, Matrigel plugs were removed and the presence of glycosylated-FGF2 in both groups was evaluated by direct ELISA using a monoclonal anti-AGE antibody. To calculate the amount of AGE, both glycosylated β -lactoglobulin and glycosylated BSA were used as standard, with similar results (not shown). FGF2 glycation was enhanced fourfold in hyperglycemic versus normoglycemic mice ($P < 0.001$) (Figure 5A). Similar results were obtained in this ELISA assay using the polyclonal anti-AGE-RNase serum (not shown). FGF2 glycation was confirmed by Western blot analysis using the polyclonal anti-AGE-RNase antibody (Figure 5B, upper panel); in fact, a faint immunolabeling with the anti-AGE serum was detected with FGF2 immunoprecipitated from the plugs kept in normoglycemic mice, while a strong immunolabeling was detected with FGF2 immunoprecipitated from NOD mice. Densitometric analysis of the bands indicated a 9.4 fold increase of FGF2 glycation in NOD versus control CD1 mice ($P < 0.001$). Similar amounts of FGF2 were loaded in each lane (Figure 5B, lower panel). Endogenous murine FGF2 was not detected in control Matrigel, ie, not containing recombinant FGF2, either by ELISA (not shown) or by Western blot (Figure 5B, lower panel). This was likely due to the low amount of endogenous FGF2 in Matrigel plugs and to the inability of antibodies used in ELISA and in Western blot assays to recognize murine FGF2. These experiments demonstrate that enhanced FGF2 glycation occurred in hyperglycemic mice. Since FGF2, similarly to other heparin-binding growth factors, can bind heparan sulfates proteoglycans in the extracellular matrix (ECM), in additional experiments it was determined whether FGF2 glycation *in vivo* involved either the pool of FGF bound to the ECM components, or the pool of unbound FGF2, or both. To address this issue, hyperglycemic

NOD mice were injected with Matrigel containing recombinant FGF2. After 48 hours, the Matrigel plugs were removed and a size fractionation study was carried out to evaluate the amount of glycosylated FGF2 present in the high molecular weight (HMW), ie, likely ECM-bound, and in the low molecular weight (LMW) complexes, ie, the unbound pool of FGF2. The results of the above experiments are summarized in Table 2. These experiments indicate that *in vivo* glycation of FGF2 involves both pools of FGF2 and that the LMW unbound form shows a three- to fourfold higher number of AGE/FGF2 molecule than the HMW form ($P < 0.05$). To test whether FGF2 glycation may be related to the observed impairment of angiogenesis in diabetes, we evaluated the *in vivo* angiogenic properties of glycosylated FGF2.

In Vivo Evaluation of Angiogenesis Induced by Glycosylated FGF2

The angiogenic effect of glycosylated FGF2 versus unglycosylated FGF2 was tested in CD1 mice. The animals were injected with Matrigel mixed with FGF2 previously incubated for 24 hours at 37°C with 150 mmol/L glucose, fructose, or mannitol. Control mice were injected with Matrigel alone or with Matrigel containing FGF2 preincubated with 150 mmol/L mannitol or FGF2 supplemented with 150 mmol/L glucose or fructose immediately before the injection. After 8 days, Matrigel plugs were removed and the formation of new vessels was measured (Figure 6A). Under these conditions, FGF2 glycation *in vitro* inhibited the angiogenic effect of this growth factor *in vivo*. Microphotographs of histological sections from mice treated with unmodified and glycosylated FGF2 are shown in Figure 6B (Trichrome Masson staining) and 6C (PECAM-1 immunostaining). It is noteworthy that angiogenesis induced by unmodified FGF2 was more marked in the experiments shown in Figure 1B versus Figure 6A. This discrepancy is likely related to temperature- and time-dependent loss of angiogenic activity due to the 24 hours incubation at 37°C of FGF2 with sugars. This result strongly supports the hypothesis that the impairment of angiogenesis observed in NOD mice may be due, at least in part, to FGF2 glycation.

Discussion

The present study shows that hyperglycemia is directly related to a significant impairment of FGF2-induced angiogenesis *in vivo*. Further, it shows that hyperglycemia induces FGF2 glycation *in vivo* and that glycated FGF2 is

a weaker endothelial cell chemoattractant *in vitro* and a weaker angiogenic factor *in vivo* as compared to unmodified FGF2.

Glycation is a non-enzymatic reaction involving mainly the arginine and lysine residues in proteins.⁷ Protein glycation has been extensively studied in diabetes mellitus, and it leads to the formation of early, intermediate and advanced glycation products (AGEs).^{6,7} The role of AGEs in diabetic vasculopathy has been studied, and these products have been shown to promote a number of biochemical, cellular and pathophysiologic abnormalities, characteristic of diabetic vasculopathy.²⁹ Presently it is still unclear whether AGEs are the cause of the vascular complications observed in patients suffering from diabetes mellitus.³⁰ The effects of most AGEs are mediated through a receptor-dependent pathway requiring the interaction with a specific receptor for AGE (RAGE) leading to a cascade of intracellular effects.³¹ On the other hand, due to the high chemical reactivity of lysine and arginine residues, these amino acids play a key role in several posttranslational modifications, like methylation, transglutamination, myristoylation, proteolytic cleavage and protein-protein and protein-DNA interactions.³² Therefore, the chemical modification of such residue(s) due to glycation may lead to serious functional defects. Depending on the proteic target of glycation, hyperglycemia may induce at least two different effects: 1) chemical modifications of structural proteins like albumin, collagen, crystallins, and others, leading to a change of chemical-physical properties and therefore a change in solubility and aggregate formation (this process is related to the development of microangiopathy¹² and to cataract formation⁸ in diabetes mellitus) and 2) chemical modifications of regulatory proteins possibly leading to functional defects (for instance, it has been shown an increase of AGE-modified low density lipoprotein (LDL) in plasma of diabetic patients, leading to a significant impairment of LDL-receptor-mediated clearance).³³ FGF2 also may undergo such posttranslational modification: in fact, *in vitro* glycation of FGF2 has been shown to reduce its *in vitro* mitogenic activity.²¹ That study was mainly focused on intracellular FGF2 glycation, while a more recent report was focused on the glycation of extracellular FGF2.²² These *in vitro* studies suggested

Figure 6. *In vivo* angiogenesis induced by sugar-modified or unmodified FGF2. **A:** CD1 mice were injected with Matrigel plugs containing 150 ng/ml FGF2 treated with mannitol ($n = 5$), glucose ($n = 5$), or fructose ($n = 6$), for 24 hours. After 8 days, Matrigel plugs were removed and new vessels formation was measured (see Materials and Methods). Control mice were injected with Matrigel alone ($n = 4$), or with FGF2 supplemented with glucose ($n = 5$) or fructose ($n = 5$) immediately before the injection. Number of vessels in Matrigel plugs containing FGF2 treated with mannitol was $132 \pm 9/\text{mm}^2$. The results show a significant loss of angiogenic activity for fructose- ($P < 0.005$) and for glucose-modified FGF2 ($P < 0.05$) versus unmodified FGF2. Data represent average $\pm$ SD of each group. **B:** Histological sections of Matrigel plugs from CD1 mice (Trichrome-Masson staining, magnification, $\times 100$; scale bar, $70 \mu\text{m}$). **a:** Matrigel alone; **b:** Matrigel containing FGF2 treated with mannitol; **c:** Matrigel containing FGF2 glycated with glucose; **d:** Matrigel containing FGF2 glycated with fructose. **Arrowheads** indicate blood vessels. **C:** Histological sections of Matrigel plugs from CD1 mice. Sections were immunostained with an anti-PECAM-1 antibody reactive against the murine isoform. **a:** Matrigel alone; **b:** Matrigel containing FGF2 treated with mannitol; **c:** Matrigel containing FGF2 glycated with glucose; **d:** Matrigel containing FGF2 glycated with fructose. **Arrowheads** indicate blood vessels (magnification, $\times 100$; scale bar, $70 \mu\text{m}$).

that FGF2 glycation may interfere with FGF2 binding to its receptor. On the other hand, inhibiting the methylation-dependent modification of FGF2 results in a significant decrease in FGF2 nuclear accumulation, suggesting that methylation as well as a correct structural conformation are relevant to the intracellular distribution of this growth factor.³⁴ Although non-enzymatic glycation is generally considered a rather aspecific reaction, potentially involving any protein, it is noteworthy that only a few lysine or arginine residues in proteins are actually modified. In fact, the number of residues modified by fructose per FGF2 molecule was shown to range between 1.6 and 2.4 in a previous report²¹ and 1.1–1.8 in the present study, according to densitometric quantification of labeled FGF2 incubated with ¹⁴C-labeled sugars or between 0.8 and 3.9 pmol of AGE/pmol of FGF2 according to the results of the *in vivo* glycation assay reported in Table 2. Human FGF2 contains 14 lysine and 11 arginine residues per molecule, therefore, according to these findings, only a few residues per FGF2 molecule are glycated, while the remaining lysine and arginine residues are unaffected. This suggests that some residues within FGF2 are more susceptible to glycation than others. Studies currently ongoing in our laboratory are aimed at the biochemical/functional characterization of this specificity.

In the present study endogenous FGF2 glycation could not be measured for several reasons: 1) very low serum levels of FGF2; 2) lack of good antibodies or ELISA kits specific for murine FGF2; and 3) high background from other glycated proteins. Therefore, in the present study, human recombinant FGF2 mixed with Matrigel was used to evaluate the amount of glycated FGF2. Similarly, since FGF2 serum levels in humans are in the picogram/ml range and the analytical methods developed in the present study are effective in the range of 10 to 100 ng of purified proteins/ml, it will be necessary to develop more sensitive assays to determine whether glycated FGF2 is present in human diabetic patients. It is also noteworthy that the competitive ELISA carried out in the present study and the antibodies used in Western blot experiments recognizes only advanced glycation products and may not reveal earlier glycation of FGF2 which is also likely to occur. In conclusion, our results suggest that *in vivo* FGF2 glycation may underlie the reduced angiogenic response to ischemia, observed in diabetic patients^{3,35} and in animal models¹ of diabetes mellitus.

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References

- Rivard A, Silver M, Chen D, Kearney M, Magner M, Annex B, Peters K, Isner JM: Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. *Am J Pathol* 1999, 154:355–363
- Tooke JE: Microvascular function in human diabetes: a physiological perspective. *Diabetes* 1995, 44:721–726
- Towne JB: Management of foot lesions in the diabetic patient. *Vascular Surgery*. Edited by RB Rutherford, Philadelphia, WB Saunders, 1989, p 895
- Aronson D, Rayfield EJ: Diabetes and obesity. *Atherosclerosis and Coronary Artery Disease*. Edited by V Fuster, R Ross, EJ Topol. Philadelphia, Lippincott-Raven, 1996, pp 327–362
- Abaci A, Oguzhan A, Kahraman S, Eryol NK, Unal S, Arinc H, Ergin A: Effect of diabetes mellitus on formation of coronary collateral vessels. *Circulation* 1999, 99:2239–2242
- Ahmed MU, Thorpe SR, Baynes JW: Identification of N-epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 1986, 261:4889–4894
- Fu MX, Knecht KJ, Thorpe SR, Baynes JW: Role of oxygen in cross-linking and chemical modification of collagen by glucose. *Diabetes* 1992, 2:42–48
- Lyons TJ, Silvestri G, Dunn JA, Dyer DG, Baynes JW: Role of glycation in modification of lens crystallins in diabetic and nondiabetic senile cataracts. *Diabetes* 1991, 40:1010–1015
- Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR, Baynes JW: Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J Clin Invest* 1993, 91:2463–2469
- Facchiano F, Libondi T, Stiuso P, Esposito C, Ragone R, Colonna G: Effect of galactose on alpha-crystallin. *Ophthalmic Res* 1996, 28:97–100
- Fogliano V, Monti SM, Visconti A, Randazzo G, Facchiano AM, Colonna G, Ritieni A: Identification of a beta-lactoglobulin lactosylation site. *Biochim Biophys Acta* 1998, 1388:295–304
- Stehbens WE, Lie JT: *Vascular Pathology*. Edited by Stehbens WE, Lie JT. London, Chapman & Hall Medical, 1995
- Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 1988, 318:1315–1321
- Makita Z, Vlassara H, Cerami A, Bucala R: Immunochemical detection of advanced glycosylation end products in vivo. *J Biol Chem* 1992, 267:5133–5138
- Ruderman NB, Williamson JR, Brownlee M: Glucose and diabetic vascular disease. *EMBO J* 1992, 6:2905–2914
- Vlassara H, Bucala R, Striker L: Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Lab Invest* 1994, 70:138–151
- Schleicher ED, Wagner E, Nerlich AG: Increased accumulation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest* 1997, 99:457–468
- Soulis T, Thallas V, Youssef S, Gilbert RE, McWilliam BG, Murray-McIntosh RP, Cooper ME: Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. *Diabetologia* 1997, 40:619–628
- Berg TJ, Dahl-Jorgensen K, Torjesen PA, Hanssen KF: Increased serum levels of advanced glycation end products (AGEs) in children and adolescents with IDDM. *Diabetes Care* 1997, 20:1006–1008
- Berg TJ, Clausen JT, Torjesen PA, Dahl-Jorgensen K, Bangstad HJ, Hanssen KF: The advanced glycation end product N(epsilon)-(carboxymethyl)lysine is increased in serum from children and adolescents with type 1 diabetes. *Diabetes Care* 1998, 21:1997–2002
- Giardino I, Edelstein D, Brownlee M: Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters basic fibroblast growth factor activity: a model for intracellular glycosylation in diabetes *J Clin Invest* 1994, 94:110–117
- Nissen NN, Shankar R, Gamelli RL, Singh A, Di Pietro LA: Heparin and heparan sulphate protect basic fibroblast growth factor from non-enzymic glycosylation. *Biochem J* 1999, 338:637–642
- D'Arcangelo D, Facchiano F, Barlucchi LM, Melillo G, Illi B, Testolin L, Gaetano C, Capogrossi MC: Acidosis inhibits endothelial cell apoptosis and function and induces basic fibroblast growth factor and vascular endothelial growth factor expression. *Circ Res* 2000, 18:86:312–318
- Facchiano A, De Marchis F, Turchetti E, Facchiano F, Guglielmi M, Denaro A, Palumbo R, Scocciati M, Capogrossi MC: The chemotactic and mitogenic effects of platelet-derived growth factor-BB on rat aorta smooth muscle cells are inhibited by basic fibroblast growth factor. *J Cell Sci* 2000, 113:2855–2863

25. Shamsi FA, Partal A, Sady C, Glomb MA, Nagaraj R: Immunological evidence for methylglyoxal-derived modifications in vivo. *J Biol Chem* 1998, 273:6928–6936
26. Harlow E, Lane D: *Antibodies: A Laboratory Manual*. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press, 1988
27. Fujishima Y, Koide Y, Kaidoh T, Nishimura M, Yoshida TO: Restriction fragment length polymorphism analysis of major histocompatibility complex genes in the non-obese diabetic mouse strain and its non-diabetic sister strains. *Diabetologia* 1989, 32:118–125
28. Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS, Martin GR: A simple, quantitative method for assessing angiogenesis and anti-angiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* 1992, 67:519–528
29. Vlassara H, Fuh H, Makita Z, Krungkrai S, Cerami A, Bucala R: Exogenous advanced glycosylation end products induce complex vascular dysfunction in normal animals: a model for diabetic and aging complications. *Proc Natl Acad Sci USA* 1992, 89:12043–12047
30. Singh R, Barden A, Mori T, Beilin L: Advanced glycation end-products: a review. *Diabetologia* 2001, 44:129–146
31. Bierhaus A, Hofmann MA, Ziegler R, Nawroth PP: AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus: I. the AGE concept. *Cardiovasc Res* 1998, 37:586–600
32. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD: *Molecular Biology of the Cell*. New York, Garland Publishing, 1995
33. Bucala R, Makita Z, Vega G, Grundy S, Koschinsky T, Cerami A, Vlassara H: Modification of low density lipoprotein by advanced glycation end products contributes to the dyslipidemia of diabetes and renal insufficiency. *Proc Natl Acad Sci USA* 1994, 91:9441–9445
34. Pintucci G, Quarto N, Rifkin DB: Methylation of high molecular weight fibroblast growth factor-2 determines post-translational increases in molecular weight and affects its intracellular distribution. *Mol Biol Cell* 1996, 7:1249–1258
35. Aronson D, Rayfield EJ: *Diabetes. Textbook of Cardiovascular Medicine*. Edited by EJ Topol. Philadelphia, Lippincott-Raven, 1998, pp 171–194