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Role of HIF-1 α in maternal hyperglycemia-induced embryonic vasculopathy

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Abstract

OBJECTIVE—Maternal diabetes adversely impacts embryonic vasculogenesis, which results in embryonic vasculopathy. The purpose of our study is to determine whether hypoxia inducible factor (HIF)-1 α plays a role in diabetic embryonic vasculopathy.

STUDY DESIGN—Levels of HIF-1 α were determined in mouse conceptuses. Conceptuses on day 7 of pregnancy were cultured under euglycemic (150 mg/dL glucose) and hyperglycemic (300 mg/dL) conditions with or without AdCA5, or in the presence or absence of 2.0 μ g/mL human recombinant thioredoxin, an endogenous antioxidant protein. AdCA5 is an adenovirus encoding a constitutively active form of HIF-1 α .

RESULTS—Maternal diabetes significantly reduced HIF-1 α protein expression. The administration of 1 μ L (1×10^7 infectious units/mL) per 1 mL culture medium AdCA5 completely reversed hyperglycemia-reduced vasculature morphological scores and vascular endothelial growth factor expression. Thioredoxin treatment reversed hyperglycemia-reduced HIF-1 α levels.

CONCLUSION—We conclude that reduced HIF-1 α plays a critical role in the induction of diabetic embryonic vasculopathy, and that oxidative stress is implicated in hyperglycemia-induced HIF-1 α reduction.

Keywords

constitutively active hypoxia inducible factor-1 α ; diabetic embryonic vasculopathy; hypoxia inducible factor-1 α ; maternal diabetes; vascular endothelial growth factor

Congenital malformations occur in up to 10% of babies born to diabetic women, and the recent rise in diabetes¹ makes this pregnancy complication an extraordinarily important issue.² The embryonic vasculature is the first system to be developed and is the most vulnerable to the in utero environmental conditions. Hyperglycemia has been shown to be associated with embryonic vasculopathy, which leads to embryonic lethality or malformations.^{3,4} The mechanism underlying maternal diabetes-induced embryonic vasculopathy is elusive. Most studies have focused on the mechanisms of maternal diabetes-induced malformations and have determined that congenital malformations during maternal hyperglycemia are the result of a disruption in the balance between intracellular reactive oxygen species and endogenous antioxidant capacities.^{5–8} This oxidative-stress hypothesis is also applicable to maternal diabetes-induced embryonic vasculopathy because our recent study has found that a natural antioxidant purified from green tea, epigallocatechin-3-gallate,

is effective in amelioration of hyperglycemia-induced embryonic vasculopathy in vitro.⁹ Although oxidative stress mediates the negative impact on embryonic vasculogenesis, it is not clear how maternal diabetes and consequent oxidative stress adversely regulate vascular factors leading to embryonic vasculopathy.

Hypoxia inducible factor (HIF)-1 is a key transcriptional factor for hypoxic regulation of embryonic vascular development. HIF-1 α is the oxygen-sensitive subunit of HIF-1.¹⁰ Regulation of HIF-1 activity is critically dependent on the degradation of the HIF-1 α subunit in normoxia.¹¹ HIF-1 acts as a master regulator of angiogenesis by controlling the expression of multiple angiogenic growth factors, including vascular endothelial growth factor (VEGF).¹² Mice lacking HIF-1 activity due to HIF-1 α null mutation develop extensive vascular defects similar to those observed in diabetic embryonic vasculopathy, including inadequate vessel formation and aberrant vascular remodeling.^{13,14} Increased¹⁵ or reduced^{16–18} HIF-1 α protein levels contribute to the pathogenesis of several diabetic complications. Because the stability of HIF-1 α is pivotal to its functions in embryonic vasculogenesis, we chose to assess both HIF-1 α gene expression and protein levels in diabetic embryonic vasculopathy.

Embryonic vasculogenesis begins in the yolk sac (extraembryonic membrane) prior to vasculogenesis in the embryo. In addition, development of the embryonic cardiovascular system and yolk sac vasculature are regulated by the same group of angiogenic and survival factors via common mechanisms.^{3,19,20} Thus, previous studies in hyperglycemia-induced embryonic vasculopathy have specifically focused on the yolk sac vasculature because it has provided a highly reliable experimental system.^{4,19,21,22} The yolk sac is an extraembryonic membrane derived from the same progenitor cells that produce the embryo,²³ and it plays an important role in supporting development of embryos.^{23,24} Adverse effects of hyperglycemia have been documented in the yolk sac of maternal diabetic animal models and in vitro cultured rodent embryos.^{3,19,22,24} Under hyperglycemic conditions, vasculogenesis of the blood vessels in the yolk sac is disrupted, and the cellular structures in the vessels are altered.^{4,19,21,22}

We have morphologically characterized the various adverse effects of hyperglycemia on yolk sac vasculature development by arbitrarily assigning morphological scores to individual vasculatures.⁴ We have successfully used this established morphological score system in our studies to quantify the adverse effect of hyperglycemia on yolk sac vasculature development.^{4,9}

In the present study, we used this yolk sac morphological system to test the hypothesis that hyperglycemia reduces HIF-1 α expression, and blockade of HIF-1 α reduction ameliorates diabetic embryonic vasculopathy. By using in vivo and in vitro models with a constitutively active form of HIF-1 α , AdCA5, we have demonstrated that hyperglycemia reduces HIF-1 α protein expression, but not messenger RNA (mRNA) expression, and reversal of HIF-1 α reduction by AdCA5 reduces diabetic embryonic vasculopathy.

Materials and Methods

Animals and reagents

C57BL/6J mice (median body weight 22 g) were purchased from Jackson Laboratory (Bar Harbor, ME). Streptozotocin from Sigma (St Louis, MO) was dissolved in sterile 0.1 mol/L citrate buffer (pH 4.5). Sustained-release insulin pellets were purchased from LinShen Canada Inc. (Toronto, Canada). Adenoviruses expressing the LacZ (AdLacZ) and the constitutive active form of HIF-1 α (AdCA5) were provided by Dr Gregg L. Semenza at the

Johns Hopkins University School of Medicine, Baltimore, MD. Human recombinant thioredoxin (Trx) was purchased from EMD Chemicals (San Diego, CA).

Mouse models of diabetic embryopathy

The procedures for animal use were approved by the Institutional Animal Care and Use Committee of University of Maryland School of Medicine. Eight-week-old C57BL/6J mice were intravenously injected daily with 75 mg/kg streptozotocin over 3 days to induce diabetes. Once a level of hyperglycemia indicative of diabetes (≥ 250 mg/dL) was achieved, insulin pellets were subcutaneously implanted in these diabetic mice to restore euglycemia prior to mating. The mice were then mated with male mice of the same respective genotype. On day 5 of pregnancy (E5), insulin pellets were removed to permit frank hyperglycemia (>250 mg/dL glucose level), so the developing conceptuses would be exposed to a hyperglycemic environment during organogenesis (E7–11). Nondiabetic female mice with vehicle injections and sham operation of insulin pellet implants served as nondiabetic controls. On E7 and E8, mice were euthanized, and conceptuses were dissected out of the uteri for analysis.

Whole-conceptus culture

C57BL/6J mice were paired overnight. The next morning was designated E0 if a vaginal plug was present. Mouse conceptuses at E7 were dissected out of the uteri in phosphate-buffered saline (Invitrogen, La Jolla, CA). The parietal yolk sac was removed using a pair of fine forceps and the visceral yolk sac was left intact. Conceptuses (4/bottle) were cultured in 4 mL rat serum at 38°C in 30 rpm rotation in the roller bottle system. The culture bottles were gassed with 5% oxygen/5% carbon dioxide/90% nitrogen. Conceptuses were cultured under euglycemic (150 mg/dL glucose, a value close to the blood glucose level of nondiabetic mice) and hyperglycemic (300 mg/dL glucose) conditions in the presence or absence of 0.5 μ L or 1 μ L (1×10^7 infectious units/mL) adenoviral AdCA5 per 1 mL culture medium, or in the presence or absence of 2.0 μ g/mL human recombinant Trx.

Morphologic assessment of the yolk sac vasculature

Conceptuses were examined under a stereomicroscope (MZ16F; Leica Microsystems Inc, Bannockburn, IL) to assess yolk sac vasculature defects. Images of conceptuses were captured by a DFC420 5 MPix digital camera with software (Leica, Wetzlar, Germany) and processed with Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA).

Yolk sac vasculatures were morphologically scored based on visible maldevelopment as previously described.^{4,9} Briefly, a morphological score of 4 indicated a full development of the E11-like yolk sac vasculature with an arborizing interconnecting vascular network composed of arteries, veins, and capillaries exhibiting blood flow. A score of 3 represented only a minor defect of the yolk sac vasculature with fewer blood vessels than that of the yolk sac vasculature with a score of 4. A score of 2 indicated an arrest of the yolk sac vasculature development at the primary capillary plexus stage resulting in few yolk sac vessels. A score of 1 indicated a major defect of the yolk sac vasculature displaying an ecstatic vascular plexus with no signs of arborization and large, nonfused blood islands toward the ectoplacental cone. A score of 0 represented a yolk sac completely devoid of blood vessels with no visible or scattered blood islands.

Embryonic malformations were not examined because at early embryonic stages (E7–E9), structural malformations were not manifested. Our previous studies have extensively described the malformations in embryos of diabetic mice or cultured embryos exposed to hyperglycemia.^{9,25} Especially, at E11, about 25% of embryos from diabetic mice exhibited neural tube defects.²⁵ Our ongoing studies are testing the hypothesis that the early molecular

changes involving embryonic vasculopathy play causative roles in the induction of embryonic malformations in late development stages.

Real-time polymerase chain reaction

Total RNA was isolated from E7 and E8 conceptuses retrieved from nondiabetic or diabetic mice using an RNeasy Mini Kit (Qiagen, Valencia, CA). Real-time polymerase chain reaction (PCR) for HIF-1 α and β -actin were performed using ABI TaqMan Gene Expression Assays (assay ID: Mm00468875_m1 and Mm00607939_s1, respectively; Applied Biosystems, Foster City, CA). Briefly, RNA was reverse transcribed by using the high-capacity cDNA archive kit (Applied Biosystems). Real-time PCR and subsequent calculations were performed by a 7700 ABI PRISM sequence detector system (Applied Biosystems), which detected the signal emitted from fluorogenic probes during PCR.

Western blotting

Western blotting was performed as described by Yang et al.²⁶ Briefly, embryonic samples were sonicated in 80 μ L ice-cold lysis buffer (20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L NaF, 2 mmol/L Na orthovanadate, 1 mmol/L PMSF, and 1% Triton 100) containing a protease inhibitor cocktail (Sigma). Equal amounts of protein (50 μ g) were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Membranes were incubated for 18 hours at 4°C with the following primary antibodies at 1:1000–1:2000 dilutions in 5% non-fat milk: rabbit anti-HIF-1 α (Sigma) or rabbit anti-VEGF (catalog no. ab9953; Abcam, Cambridge, MA). Membranes were exposed to goat antirabbit, anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies. To ensure that equivalent amounts of protein were loaded among samples, membranes were stripped and probed with a mouse antibody against β -actin (Abcam). Signals were detected using an Amersham ECL Advance Detection Kit (GE Healthcare, Piscataway, NJ) and chemiluminescence emitted from the bands was directly captured using a UVP Bioimage EC3 system (UVP, Upland, CA). Densitometric analysis of chemiluminescence signals were performed by VisionWorks LS software (UVP). Images of representative immunoblots were arranged using Adobe Photoshop and Microsoft PowerPoint software (Richmond, CA). All experiments were repeated 3 times with the use of independently prepared tissue lysates.

Statistics

Data are presented as means $\pm$ SE. One-way analysis of variance were performed using SigmaStat 3.5 software (Systat software, San Jose, CA). In analysis of variance, Tukey test was used to estimate the significance of the results. Statistical significance was accepted at $P < .05$.

Results

Hyperglycemia reduces HIF-1 α protein expression with no effects on its mRNA levels

To determine if maternal diabetes affects HIF-1 α expression, E7 and E8 conceptuses from nondiabetic and diabetic mice were used for detection of HIF-1 α protein and mRNA. Maternal diabetes significantly reduced HIF-1 α protein in both E7 and E8 conceptuses (Figure 1, A). HIF-1 α protein levels in E7 nondiabetic conceptuses were higher than those E8 nondiabetic conceptuses, consistence with the notion that the degree of embryonic hypoxia is gradually reduced with the advance of pregnancy. To determine if alternation of HIF-1 α protein is due to the change of its mRNA, HIF-1 α mRNA levels were determined in E7 and E8 conceptuses. Maternal diabetes did not alter HIF-1 α mRNA levels (Figure 1, B).

The levels of HIF-1 α (mRNA and protein) reflect the levels in the whole conceptuses including the embryo and the yolk sac.

Adenoviral gene delivery is effective in cultured conceptuses with no teratogenic effects, and AdCA5 ameliorates hyperglycemia-induced vasculopathy

To establish the novel approach, adenoviral gene delivery to cultured conceptuses, E7 conceptuses were cultured in the presence or absence of 1 μ L (1×10^7 infectious units/mL) adenoviral vector encoding GFP (AD-CMV-GFP, adenovirus expresses enhanced green fluorescent protein under the control of a CMV promoter.) (Vector BioLabs, Philadelphia, PA) per 1 mL culture medium. After 48-hour culture, whole-mount yolk sacs were examined for green fluorescence-labeled cells under a fluorescence microscope. Virtually, all yolk sac cells exposed to AD-CMV-GFP expressed GFP (Figure 2, A). To determine if adenovirus infections have any teratogenic effects, embryonic malformations were examined in conceptuses exposed to AD-CMV-GFP. No malformations were observed in embryos exposed to adenoviruses and embryonic development in conceptuses exposed to AD-CMV-GFP was comparable to that in unexposed conceptuses (Figure 2, B and C). We also did experiments with 3 or 4 μ L (1×10^7 IFU/mL) AD-CMV-GFP and did not observe any teratogenic effects on embryonic development.

AdCA5 is an adenovirus encoding a constitutively active form of HIF-1 α . Cultured conceptuses were treated with AdCA5 to determine if reduced HIF-1 α plays a role in the induction of diabetic embryonic vasculopathy. Morphological scores in yolk sac vasculatures of conceptuses exposed to hyperglycemia were significantly lower than those in yolk sac vasculatures of conceptuses cultured in euglycemia (Figure 3, A). A total of 0.5 μ L AdCA5 increased hyperglycemia-reduced vasculature morphological scores (Figure 3, A). Morphological scores in yolk sac vasculatures of conceptuses cultured in hyperglycemia plus 1 μ L AdCA5 were comparable to those in yolk sac vasculatures of conceptuses cultured in euglycemia (Figure 3, A). Thus, 1 μ L AdCA5 completely reversed hyperglycemia-reduced vasculature morphological scores (Figure 3, A).

AdCA5 blocks hyperglycemia-reduced VEGF protein expression

VEGF is critical for normal embryonic vasculogenesis. Previous studies showed a reduction of VEGF protein expression in diabetic embryonic vasculopathy.²² To determine if reduced HIF-1 α is responsible for the reduction of VEGF protein expression, conceptuses were cultured under euglycemic, hyperglycemic, and hyperglycemia plus 1 μ L AdCA5 for 24 hours were used for detection of VEGF protein levels. AdCA5 reversed hyperglycemia-reduced VEGF protein expression (Figure 3, B).

Trx treatment reverses hyperglycemia-induced HIF-1 α reduction

Hyperglycemia-induced oxidative stress leads to the adverse impacts on embryonic development, and oxidative stress-induced apoptosis resulted in neural tube defects by maternal diabetes.^{25,27} Trx is an antioxidant and antiapoptotic endogenous protein. To test the hypothesis that oxidative stress causes diabetic embryonic vasculopathy, cultured conceptuses were treated by Trx. HIF-1 α levels were determined in E7 conceptuses cultured for 24 hours under euglycemic (150 mg/dL glucose) and hyperglycemic (300 mg/dL) conditions in the presence or absence of 2.0 μ g/mL Trx. Consistent with the observation in vivo (Figure 1, B), hyperglycemia reduced HIF-1 α levels, whereas Trx treatment reversed hyperglycemia-reduced HIF-1 α levels (Figure 4).

Comment

The adverse effects of maternal diabetes on embryonic vasculogenesis have been long recognized. However, the research in diabetic embryonic vasculopathy remains in its infancy. Earlier works have only implicated the roles of a few angiogenic and vasoactive factors in this disease process.^{19,21,22} The direct cellular targets of maternal diabetes and immediate early cellular events upon hyperglycemic exposures remain elusive. Importantly, how diabetes leads to the changes of angiogenic and vasoactive factors resulting in embryonic vasculopathy is completely unknown. In the present study, we for the first time established the causative role of HIF-1 α , a master transcriptional regulator for vascular factors, in maternal diabetes-induced embryonic vasculopathy and VEGF reduction.

The hypoxic environment of the early (E7-E8) conceptuses plays an important role in normal embryonic vasculogenesis. Hypoxia, a naturally occurring phenomenon during normal embryogenesis, is a condition defined by low oxygen levels. Prior to the formation of a definitive embryonic vasculature and organ system, diffusion from extraembryonic sites provides the oxygen necessary for development. During organogenesis, the local hypoxic environment serves as a signal to initiate vasculogenesis.²⁸ Hypoxic response is mediated by HIF-1 α , an oxygen-sensitive subunit of HIF-1, which is essential for initiation and progression of embryonic vasculogenesis.^{13,14} Protein degradation is a main mechanism underlying the regulation of HIF-1 α expression.^{11,29-31} We demonstrated that maternal hyperglycemia reduces HIF-1 α protein expression, but does not effect its transcription. Our results are in agreement with previous observation of reduced HIF-1 α protein expression, without changes of HIF-1 α mRNA in diabetic wound healing and adulthood diabetic vasculopathy.¹⁶⁻¹⁸ In contrast, HIF-1 α protein expression is increased in diabetic retinopathy, which is manifested in blood vessel overgrowth.¹⁵ In diabetic embryopathy, a recent study reports a reduction of HIF-1 α mRNA at E10.5 and decreased levels of HIF-1 α mRNA at E8.5 and E9.5.³² This study's different finding compared to those in the present study may result from different time points being examined. Because HIF-1 α is mainly regulated through protein stability in hypoxic environment, it is important to examine HIF-1 α protein levels. The study mentioned above³² does not measure protein levels of HIF-1 α . Our study demonstrates that maternal hyperglycemia reduces HIF-1 α protein levels at E7 and E8.

We used a valid and widely accepted approach, AdCA5, to reverse maternal diabetes-reduced HIF-1 α protein expression. The molecular basis of HIF-1 α protein degradation is the oxygen-dependent hydroxylation of at least 1 of the 2 proline residues in its oxygen-dependent degradation domain by specific prolylhydroxylases (PHD1, PHD2, and PHD3).^{11,29-31} Hydrolyzed HIF-1 α binds to the von Hippel-Lindau tumor suppressor protein, which acts as an E3 ubiquitin ligase and targets HIF-1 α for proteasomal degradation.^{33,34} Subsequently, HIF-1 α translocates to the nucleus, engages HIF-1 α , and forms the HIF-1 complex that initiates transcription.³⁵ AdCA5, a constitutively active form of human HIF-1 α , contains a deletion (residues 392-520) resulting in constitutive expression and 2 missense mutations (Pro567Thr and Pro658Gln).^{12,36} The mutations of the 2 proline residues in AdCA5 make it resistant to proline hydroxylation-mediated degradation. These modifications of AdCA5 constitutively induce high levels of active HIF-1 α . Consistent with the features of AdCA5, AdCA5 can block hyperglycemia-induced embryonic vasculopathy and VEGF reduction.

VEGF is a crucial vascular factor during embryonic development and loss of even a single VEGF allele results in embryonic lethality due to aberrant vasculogenesis.³⁷ Early studies in diabetic embryonic vasculopathy demonstrated a reduction of VEGF and VEGF receptor activation.²² Similarly, the present study demonstrates that hyperglycemia reduces VEGF

protein expression. The cause of VEGF reduction in diabetic embryonic vasculopathy is not known. VEGF is one of the prominent responsive genes of HIF-1 α .¹² Reversal of HIF-1 α reduction by AdCA5 blocks hyperglycemia-reduced VEGF protein expression accompanying amelioration of vasculopathy, suggesting a causative role of the HIF-1 α - VEGF pathway in diabetic embryonic vasculopathy.

Because we have demonstrated that oxidative stress causes diabetic embryonic vasculopathy, we used an endogenous antioxidant protein, Trx, to determine if suppressing oxidative stress blocks hyperglycemia-reduced HIF-1 α reduction. Trx, a 12-kDa protein, is a potent antioxidant and reduces reactive oxygen species through interactions with its redox-active center.³⁸ Trx protects cells from stress-induced damage through antioxidative, antiapoptotic, and antiinflammatory effects. Trx treatments effectively blocks hyperglycemia-reduced HIF-1 α , suggesting that in diabetic embryonic vasculopathy, oxidative stress is the main mechanism underlying hyperglycemia-reduced HIF-1 α , which is consistent with the findings in diabetic wound healing.^{16,17}

In summary, we provide evidence that reduced HIF-1 α protein expression plays a causative role in the induction of diabetic embryonic vasculopathy, and oxidative stress is responsible for HIF-1 α reduction. Congenital cardiovascular malformations form the most prevalent group of birth defects, affecting around 6–8 per 1000 live birth,³⁹ and is a leading cause of infant morbidity and mortality. Maternal diabetes is an independent risk factor for congenital malformations with a reported risk of malformation in published studies of 1.7–5.0%.^{40–45} Maternal diabetes induces a broad spectrum of cardiovascular malformations including transposition of the great arteries, tricuspid atresia, common atrial trunk, atrial septal defect, mitral atresia, and pulmonary atresia.⁴⁰ Thus, diabetes-induced cardiovascular defects are of urgent clinical significance. Alteration of vascular factors including HIF-1 α and VEGF is linked to cardiovascular defects. The results of the present study reveal the importance of HIF-1 α and VEGF in diabetes-induced vasculopathy and thus will aid future intervention in targeting HIF-1 α and VEGF in diabetes-induced birth defects.

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FIGURE 1. Maternal diabetes reduces hypoxia inducible factor (HIF)-1 α protein expression but does not affect its messenger RNA (mRNA) levels

A, Representative images of HIF-1 α . Western blotting (*top*); graphic data of densitometric analysis (*bottom*). **B**, HIF-1 α mRNA levels were determined in embryonic day (E)7 and E8 conceptuses from nondiabetic and diabetic mice by real-time polymerase chain reaction. Data expressed as mean + SE (n = 3).

*Significant difference ($P < .05$) when compared to nondiabetic groups when analyzed by t test.

FIGURE 2. Adenoviral gene delivery is effective in cultured conceptuses with no teratogenic effects

A, Representative green fluorescent image of whole-mount yolk sac from 48-hour– cultured embryonic day 7 conceptus. During course of culture, conceptuses were incubated with 1 μ L (1×10^7 infectious units/mL) AD-CMV-GFP (adenovirus type 5 expresses enhanced green fluorescent protein under the control of a CMV promoter) per 1 mL medium. *Red arrows* point to blood vessels. Representative images of unexposed **B**, embryos and **C**, adenovirus-exposed embryos. Bars represent 1 mmol/L.

FIGURE 3. AdCA5 blocks hyperglycemia (Hy)-induced embryonic vasculopathy and VEGF reduction

A, Yolk sac vasculature morphological scores in cultured conceptuses were expressed as mean + SE (n = 16 conceptuses each group). *Significant difference ($P < .001$) when compared to Hy group in 1-way analysis of variance (ANOVA) and Tukey test. **B**, Representative images of VEGF Western blotting and graph of densitometric analysis data. *Significant difference ($P < .05$) when compared to groups in 1-way ANOVA and Tukey test. Data were generated from 3 independent experiments.

Eu, euglycemia; *VEGF*, vascular endothelial growth factor.

FIGURE 4. Thioresdoxin (Trx) reverses hyperglycemia (Hy)-reduced hypoxia inducible factor (HIF)-1α protein levels

Top, HIF-1α Western blotting. *Bottom*, Densitometric data. *Significant difference ($P < .05$) when compared to groups in 1-way analysis of variance and Tukey test. Data were generated from 3 independent experiments.

Eu, euglycemia.