Glycated Human Serum Albumin Induces Interleukin 8 mRNA Expression through Reactive Oxygen Species and NADPH Oxidase-Dependent Pathway in Monocyte-Derived U937 Cells

Koji HIGAI,* Risa SANO, Misaki SATAKE, Yutaro AZUMA, and Kojiro MATSUMOTO

Department of Clinical Chemistry, School of Pharmaceutical Sciences, Toho University; 2–2–1 Miyama, Funabashi, Chiba 274–8510, Japan. Received May 7, 2007; accepted July 24, 2007; published online July 24, 2007

Glycated human serum albumin (Glc-HSA) has previously been reported (Higai K., *et al.*, 2006) to induce E-selectin expression on human umbilical vein endothelial cells through activation of NADPH oxidase; however, Glc-HSA signaling in monocytes remains obscure. To clarify the influence on human monocyte-derived U937 cells, U937 cells were stimulated with Glc-HSA and glycoaldehyde-dimer-modified HSA (GA-HSA) for 2 h in the absence and presence of the protein kinase C (PKC) inhibitor calphostin and the reactive oxygen species (ROS) scavenger *N*-acetyl-L-cysteine (NAC) and NADPH oxidase inhibitor apocynin; interleukin-8 (IL-8) mRNA expression was determined by RT-PCR. As a result, IL-8 mRNA expression in U-937 cells was time- and dose-dependently enhanced by stimulation with Glc-HSA and GA-HSA. Furthermore, promoter activity of the IL-8 reporter gene was enhanced approximately 2-fold by stimulation with Glc-HSA and GA-HSA. Nuclear factor κ B (NF κ B) and activator protein-1 (AP-1) reporter genes were also enhanced although CCAAT/enhancer binding protein (C/EBP) was not affected. IL-8 mRNA expression was suppressed by NAC and apocynin but not calphostin in cells stimulated with Glc-HSA; however, its expression in cells stimulated with GA-HSA was suppressed by calphostin but not NAC. These results indicated that IL-8 mRNA expression was upregulated by NF κ B and AP-1 in U937 cells stimulated with Glc-HSA and GA-HSA, but the signaling pathways were different.

Key words glycated albumin; interleukin 8; protein kinase C; reactive oxygen species; NADPH oxidase

Accelerated atherosclerosis and microvascular dysfunction are major vascular complications of diabetes mellitus. Non-enzymatic glycation of proteins is a significant factor in the development of these complications.¹⁾ Glucose reacts with amino groups on proteins to produce Schiff base intermediates and Amadori rearrangement products, early glycated products, which are converted to various advanced glycation end-products (AGEs) by further chemical reactions.

AGEs transduce multiple signaling pathways and activate nuclear factor κ B (NF κ B) and activator protein-1 (AP-1)²⁾ different AGE ligands induce different signaling cascades in different cell types. *N*^ε-(carboxymethyl)-lysine (CML)-modified albumin, a representative ligand for receptor for AGE (RAGE), stimulates human monocyte-derived THP-1 cells to express interleukin-1 β (IL-1 β),³⁾ vascular endothelial growth factor (VEGF), tumor necrosis factor α (TNF α) and IL-8.⁴⁾ Glycated albumin or fructosyllysine-modified albumin, an early glycation product, is also linked to the pathogenesis of diabetic nephropathy and other complications, and activates NF κ B and AP-1 through similar signaling cascades to AGEs,⁵⁾ although the receptors for the glycated albumin, and the signaling cascades, remain to be clarified.

Chemokines are crucial immune mediators in many physiological and pathophysiological conditions. IL-8 levels are significantly elevated in patients with type 2 diabetes.⁶⁾ Levels of IL-8 and monocyte chemoattractant protein-1 (MCP-1) are high in the vitreous fluid of patients with proliferative diabetic retinopathy (PDR) and correlate with PDR activities.⁷⁾ IL-8 and MCP-1 are major mediators of acute neutrophil and chronic macrophage-mediated inflammation, respectively.⁸⁾

We reported previously that glycated human serum albumin (Glc-HSA), an Amadori rearrangement product, induces E-selectin expression on human umbilical vein endothelial

cells (HUVEC) through activation of NADPH oxidase and reactive oxygen species (ROS); this expression is not suppressed by the protein kinase C (PKC) inhibitor calphostin.⁹⁾ However, glycoaldehyde-dimer-modified HSA (GA-HSA), a CML-rich AGE, induces E-selectin expression through PKC but not ROS. These results indicated that both Glc-HSA and GA-HSA could regulate E-selectin mRNA expression. In this study, we have compared chemokine IL-8 mRNA expression induced with Glc-HSA or GA-HSA in human monocyte-derived U937 cells.

MATERIALS AND METHODS

Human Cell Lines and Cell Culture Human monoblastoid leukemia U937 cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (JRC Biosciences, Lenexa, KS, U.S.A.), 0.6 mg/ml L-glutamate (Wako Pure Chemical Co., Osaka, Japan), 100 U/ml penicillin (Banyu Pharmaceutical Co., Tokyo, Japan) and 100 μ g/ml streptomycin (Meiji Seika Co., Tokyo, Japan) under a humidified atmosphere containing 5% CO₂ at 37 °C.

Preparation of Glc-HSA and GA-HSA Glc-HSA and GA-HSA were prepared according to our previous report.⁹⁾ Briefly, 42.5 mg of HSA (Wako) and 180 mg of glucose (Wako) in 10 ml of phosphate-buffered saline (PBS; pH 7.4) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin were filtered (pore size 0.20 μ m) and incubated at 37 °C for 7 d. HSA (20 mg) was also reacted with 33 mmol/l glycoaldehyde dimer (GA) at 37 °C for 7 d. The reaction mixtures were dialyzed against PBS and concentrated using

* To whom correspondence should be addressed. e-mail: koji@phar.toho-u.ac.jp

an Amicon Ultra device (Millipore Co., Billerica, MA, U.S.A.) then used without further purification. The modified HSA preparations were assumed to be Amadori-modified HSA (Glc-HSA) and AGE-HSA (GA-HSA), respectively. The Glc-HSA content in the preparations was estimated to be approximately 25% using TSKgel Boronate-5PW affinity chromatography (150×4.6 mm i.d., Tosoh Co., Tokyo, Japan) according to the manufacturer's instructions. Protein contents were determined using the Advanced Protein Assay kit (Cytoskeleton, Denver, CO, U.S.A.) calibrated with HSA.

Stimulation of U937 Cells with Glc-HSA and GA-HSA U937 cells (1.0×10^6 cells/ml) were incubated with 0.5 mg/ml Glc-HSA and GA-HSA for 0 to 3 h at 37°C or with 0 to 1.0 mg/ml Glc-HSA and GA-HSA for 2 h at 37°C. U937 cells were also incubated at 37°C for 1 h in the presence of the PKC inhibitor calphostin (2.4 nmol/l) (Wako), the ROS scavenger *N*-acetyl-L-cysteine (NAC) (2.0 mmol/l) (Sigma Aldrich, St. Louis, MO, U.S.A.), an NADPH oxidase inhibitor apocynin (5 mmol/l) (Calbiochem, La Jolla, CA, U.S.A.) and then stimulated with 1.0 mg/ml Glc-HSA and GA-HSA for 2 h.

RT-PCR of IL-8 mRNA Total cellular RNA was extracted from U937 cells stimulated with Glc-HSA and GA-HSA for 1 h at 37°C using TRIzol reagent (Invitrogen Co., Carlsbad, CA, U.S.A.). The first-strand cDNA (20 µl) was prepared from total RNA (5 µg) using ReverTra Ace reverse transcriptase (Toyobo, Tokyo, Japan) and oligo (dT)₂₀ primers followed by DNase treatment according to the manufacturer's instructions.

cDNA (5 µl) was amplified by PCR on a PC-812 Thermal cycler (Astec Co., Fukuoka, Japan) using KOD Dash (Toyobo) and the specific primer sets 5'-ATGACTTCCAAGC-TGCCCGTGGCT-3' (sense) and 5'-TCTCAGCCCTCTTC-ATCAAAAACCTTCTC-3' (antisense) for IL-8 (GenBank accession no. NM-000584), and 5'-CCTGGCCAAGGTC-ATCCATG-3' (sense) and 5'-GGAAGGCCATGCCAGTG-AGC-3' (antisense) for GAPDH (M-33197), respectively. PCR conditions for IL-8 were 95°C for 2 min, followed by 25 cycles of 98°C for 20 s, 65°C for 20 s, 72°C for 30 s and 72°C for 5 min; conditions for GAPDH were 95°C for 2 min, followed by 20 cycles of 98°C for 20 s, 63°C for 20 s, 72°C for 30 s and 72°C for 5 min. PCR products were separated by 1.5% agarose gel electrophoresis and stained with 0.05% ethidium bromide.

Dual Luciferase Assay for IL-8, NFκB, AP-1 and C/EBP Reporter Genes Genomic DNA from U937 cells was extracted with the Wizard SV Genomic DNA preparation system (Promega Co., Madison, WI, U.S.A.). The IL-8 reporter gene was cloned from the nt-415 5'-flanking region to the transcription initiation site by PCR from genomic DNA using the KOD plus system (Toyobo) and the specific primers 5'-TGGCTTATCTTCACCAT-3' (sense) and 5'-TGCTCCGGTGGCTTTTATATC-3' (antisense), respectively. After purification of the products with Wizard SV Gel and PCR Cleanup system (Promega) followed by 5'-phosphorylation with T4-polynucleotide kinase and ligation with Ligation High (Toyobo) according to the manufacturer's instructions, the reporter gene was transformed into competent cell DH5α (Invitrogen) by heat shock at 42°C for 30 s. After selection on an LB plate containing 100 µg/ml ampicillin (Invitrogen), the IL-8 reporter plasmid was purified using the Pure Yield plasmid Midi prep System (Promega). NFκB, AP-1 and CCAAT/enhancer binding protein (C/EBP) reporter vectors (Stratagene Co., La Jolla, CA, U.S.A.) were transformed into competent cell DH5α and these reporter plasmids were prepared as described above.

The IL-8 reporter plasmid, NFκB reporter plasmid, AP-1 reporter plasmid, C/EBP reporter plasmid (each 1.5 µg) or pGL4.11 vector (0.15 µg) was transfected into U937 cells (1×10^6 cells) together with pRL-CMV (0.1 µg) using Lipofectamine 2000 (Invitrogen). After 16 h of transfection, the cells were stimulated with 1.0 mg/ml Glc-HSA and GA-HSA for 2 h and luciferase activities were determined by 20/20n luminometer (Promega) using the Dual Luciferase Assay Kit (Promega) and normalized to those of the control cells transfected with the pGL4.11 vector.

RESULTS

IL-8 mRNA Expression in U937 Cells Stimulated with Glc-HSA and GA-HSA We determined IL-8 mRNA expression in monocyte-derived U937 cells that had been stimulated with Glc-HSA or GA-HSA (0.5 mg/ml) for 0 to 3 h at 37°C or for 2 h at 37°C with 0 to 1.0 mg/ml Glc-HSA or GA-HSA (Fig. 1). RT-PCR revealed that the enhancement of IL-8 mRNA expression levels was time-dependent following stimulation with both Glc-HSA (Fig. 1A) and GA-HSA (Fig. 1B). Both Glc-HSA (Fig. 1C) and GA-HSA (Fig. 1D) dose-

Fig. 1. IL-8 mRNA Expression in U937 Cells Stimulated with Glc-HSA or GA-HSA

U937 cells were stimulated with 0.5 mg/ml Glc-HSA (A) or GA-HSA (B) for 0 to 3 h at 37°C. U937 cells were also stimulated with 0 to 1 mg/ml Glc-HSA (C) or GA-HSA (D) for 2 h at 37°C. IL-8 mRNA expression in the cells was determined by RT-PCR.

dependently enhanced IL-8 mRNA expression, with maximum levels reached at 1.0 mg/ml.

Promoter Activities of IL-8, NF κ B, AP-1 and C/EBP Reporter Genes We cloned the IL-8 reporter gene from the nt-414 5'-flanking region to the transcription initiation site: this covers the transcription initiation site of IL-8 (determined by the oligocapping method) and contains the binding sites for transcriptional factors including AP-1 (−127 to −119 nt), C/EBP (−94 to −81 nt) and NF κ B (−80 to −70 nt). After 16 h of transfection, the cells were stimulated with 1.0 mg/ml Glc-HSA or GA-HSA for 2 h and the luciferase activities determined using the dual luciferase assay (Fig. 2). The luciferase activity of the IL-8 reporter gene in U937 cells stimulated with Glc-HSA and GA-HSA was approximately 2-fold higher than that in control cells with pGL4.11.

To clarify the contribution of the NF κ B, AP-1 and C/EBP binding sites in this region, we transfected NF κ B, AP-1 and C/EBP reporter genes into U937 cells in the presence and absence of 2.4 nmol calphostin and 2.0 mmol NAC. After 16 h of transfection and 2 h stimulation with 1.0 mg/ml Glc-HSA and GA-HSA, luciferase activities of these reporter genes were determined (Fig. 3) and found to be approximately 1.5- to 2-fold higher for NF κ B and AP-1 in cells treated with Glc-HSA (Figs. 3A, B) and GA-HSA (Figs. 3C, D) compared with those of control pGL4.11, while activity of the C/EBP reporter gene was not affected by stimulation with Glc-HSA and GA-HSA (data not shown). The AP-1 and NF κ B promoter activities enhanced with Glc-HSA stimulation were significantly suppressed by NAC but not by calphostin and those with GA-HSA stimulation *vice versa* although the suppression was insignificant.

Signaling Pathways for IL-8 mRNA Expression To clarify the PKC activation and ROS production in Glc-HSA-

and GA-HSA-induced signaling, the cells were treated with 2.4 nmol/l calphostin, 2.0 mmol/l NAC and 5 mmol/l apocynin for 1 h then stimulated with 1.0 mg/ml Glc-HSA or GA-HSA for 2 h; IL-8 mRNA expression levels were determined by RT-PCR (Fig. 4). Calphostin did not suppress IL-8 mRNA expression in U937 cells stimulated with Glc-HSA, although it almost completely suppressed IL-8 mRNA expression in cells stimulated with GA-HSA. On the other hand, NAC and apocynin suppressed Glc-HSA-induced IL-8 mRNA expression but not GA-HSA-induced IL-8 mRNA expression. These results indicate that Glc-HSA and GA-HSA enhance

Fig. 2. Promoter Activities of the IL-8 Reporter Gene in U937 Cells Stimulated with Glc-HSA or GA-HSA

IL-8 reporter gene from nt-415 to transcription initiation site and pGL4.11 vector were transfected into U937 cells together with pRL-CMV using Lipofectamine 2000. After 16 h of transfection, the cells were stimulated with Glc-HSA (A) or GA-HSA (B) for 2 h. Luciferase activities were determined using dual luciferase assay and normalized to those of control pGL4.11. The results were obtained by three independent experiments and represented by mean $\pm$ S.D. Asterisks indicate significant differences ($p < 0.05$).

Fig. 3. Promoter Activities of NF κ B, AP-1 and C/EBP Reporter Genes in U937 Cells Stimulated with Glc-HSA or GA-HSA

NF κ B (A, C), AP-1 (B, D), reporter genes and pGL4.11 vector were transfected into U937 cells together with pRL-CMV using Lipofectamine 2000. After 16 h of transfection, the cells were stimulated with Glc-HSA (A, B) or GA-HSA (C, D) for 2 h in the presence and absence of 2.4 nmol/l calphostin and 2.0 mmol/l NAC. Luciferase activities were determined using dual luciferase assay and normalized to those of control pGL4.11. The results were obtained by three independent experiments and represented by mean $\pm$ S.D. Asterisks indicate significant differences ($p < 0.05$).

A) Glc-HSA

B) GA-HSA

Fig. 4. Effects of Signaling Inhibitors on IL-8 mRNA Expression in U937 Cells Stimulated with Glc-HSA or GA-HSA

After 1 h treatment in the absence or presence of calphostin (2.4 nmol/l), NAC (2.0 mmol/l) and apocynin (5 mmol/l), U937 cells were stimulated with Glc-HSA (A) or GA-HSA (B) for 2 h and IL-8 mRNA expression levels were determined by RT-PCR.

IL-8 mRNA expression through different signaling pathways.

DISCUSSION

We have shown that both Glc-HSA and GA-HSA can enhance IL-8 mRNA expression in human monocyte-derived U937 cells by transcription factors NF κ B and AP-1, through different cellular signalings, ROS and NADPH oxidase-dependent in the case of Glc-HSA, and ROS and NADPH oxidase-independent in the case of GA-HSA, respectively.

Bian *et al.* have reported that glycated albumin stimulates human retinal pigment epithelial cells and corneal keratocytes to induce IL-8 and monocyte chemotactic protein-1 (MCP-1).^{10–13} IL-8 expression is generated by a combination of three different mechanisms, including depression of the gene promoter, transcriptional activation of the gene by NF κ B and JNK pathways, and stabilization of the mRNA by the p38 mitogen-activated protein kinase (MAPK) pathway.¹⁴ IL-8 gene transcription is regulated at the –1 to –133 nt of 5'-flanking region, in which NF κ B is essential and AP-1 and C/EBP are required for maximum gene expression.^{15,16} The p38 MAPK pathway induces IL-8 gene expression by marked stabilization of mRNA.¹⁷ In this study, we demonstrated that Glc-HSA and GA-HSA enhanced the promoter activities of the IL-8 reporter gene that contained AP-1, C/EBP and NF κ B binding sites and also enhanced the promoter activities of AP-1 and NF κ B but not C/EBP reporter genes in human monoblastoid leukemia U937 cells.

We also previously reported that calphostin suppressed E-selectin expression on HUVEC stimulated with GA-HSA but not with Glc-HSA.⁹ In the present study, NAC and apocynin suppressed IL-8 mRNA expression induced with Glc-HSA but not with GA-HSA in U937 cells and calphostin suppressed IL-8 mRNA expression induced with GA-HSA but not with Glc-HSA. Signaling involved in stimulation with glycated albumin is reported to be mediated by PKCs and is suppressed significantly by the PKC inhibitor RO318220 and partially by calphostin in RPE cells,¹² GF109203X (general)

and rottlerin (δ -specific) in vascular smooth muscle cells,¹⁸ GF109203X and LY379196 (β -specific) in mesangial cells,¹⁹ GF109203X in mesangial cells,²⁰ GO6976 in monocytes²¹ and GF109203X in endothelial cells.²² The PKC family of serine/threonine kinases is classified into three major types of isoenzymes: classical isoforms (α , β 1, β 2, γ), novel isoforms (δ , ϵ , θ , μ) and atypical isoforms (ζ , τ / λ). Different PKC isoforms catalyze the phosphorylation of different proteins *via* different signaling pathways so are inhibited by different inhibitors.

We previously reported that the NADPH oxidase inhibitor diphenylene iodide (DPI) and the ROS scavenger NAC suppressed E-selectin expression on HUVEC stimulated with Glc-HSA but not with GA-HSA.⁹ In the present study, NAC and apocynin suppressed IL-8 mRNA expression induced with Glc-HSA but not with GA-HSA in U937 cells. MCP-1 expression induced with glycated albumin is also suppressed by NAC in vascular smooth muscle cells.^{23,24} Glycated albumin generates ROS to activate PKC in human mesangial cells, which is inhibited by the NADPH oxidase inhibitor DPI and apocynin.²⁰ Furthermore, signaling from RAGE to NF κ B in monocytic THP-1 cells is reported to involve ROS generation.²⁵ These results indicate that not only AGE but also glycated albumin can activate NADPH oxidase and the generated ROS can modulate various signaling molecules to activate NF κ B and AP-1.

We have revealed here that Glc-HSA and GA-HSA induce ROS generation and PKC activation, respectively, to up-regulate IL-8 mRNA expression by activation of the transcription factors NF κ B and AP-1 in monocyte U937 cells. Receptors for multiple ligands of Glc-HSA and GA-HSA have not yet been elucidated and their expression and intracellular signaling molecules will be different in different cell and tissue types. Further studies are necessary to clarify the mechanism for diabetic complications induced by non-enzymatic glycation of proteins.

Acknowledgements A part of this work was supported by the "Open Research Center" Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

REFERENCES

- 1) Wautier J. L., Schmidt A. M., *Circ. Res.*, **95**, 233–238 (2004).
- 2) Bierhaus A., Humpert P. M., Morcos M., Wendt T., Chavakis T., Arnold B., Stern D. M., Nawroth P. P., *J. Mol. Med.*, **83**, 876–886 (2005).
- 3) Westwood M. E., Thornalley P. J., *Immunol. Lett.*, **50**, 17–21 (1996).
- 4) Pertynska-Marczewska M., Kiriakidis S., Wait R., Beech J., Feldmann M., Paleolog E. M., *Cytokine*, **28**, 35–47 (2004).
- 5) Cohen M. P., *Arch. Biochem. Biophys.*, **419**, 25–30 (2003).
- 6) Herder C., Haastert B., Muller-Scholz S., Koenig W., Thorand B., Holle R., Wichmann H. E., Scherbaum W. A., Martin S., Kolb H., *Diabetes*, **54** (Suppl. 2), S11–S17 (2005).
- 7) Hernandez C., Segura R. M., Fonollosa A., Carrasco E., Francisco G., Simo R., *Diabet. Med.*, **22**, 719–722 (2005).
- 8) Mukaida N., Harada A., Matsushima K., *Cytokine Growth Factor Rev.*, **9**, 9–23 (1998).
- 9) Higai K., Shimamura A., Matsumoto K., *Clin. Chim. Acta*, **367**, 137–143 (2006).
- 10) Bian Z. M., Elner S. G., Strieter R. M., Glass M. B., Lukacs N. W., Kunkel S. L., Elner V. M., *J. Leukoc. Biol.*, **60**, 405–414 (1996).
- 11) Bian Z. M., Elner V. M., Lukacs N. W., Strieter R. M., Kunkel S. L.,

- Elnér S. G., *Curr. Eye. Res.*, **17**, 65—72 (1998).
- 12) Bian Z. M., Elnér V. M., Yoshida A., Kunkel S. L., Elnér S. G., Bian Z. M., Elnér V. M., Yoshida A., Kunkel S. L., Elnér S. G., *Invest. Ophthalmol. Vis. Sci.*, **42**, 1660—1668 (2001).
- 13) Bian Z. M., Elnér S. G., Yoshida A., Elnér V. M., *Invest. Ophthalmol. Vis. Sci.*, **45**, 1887—1896 (2004).
- 14) Hoffmann E., Dittrich-Breiholz O., Holtmann H., Kracht M., *J. Leukoc. Biol.*, **72**, 847—855 (2002).
- 15) Matsusaka T., Fujikawa K., Nishio Y., Mukaida N., Matsushima K., Kishimoto T., Akira S., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 10193—10197 (1993).
- 16) Mukaida N., Okamoto S., Ishikawa Y., Matsushima K., *J. Leukoc. Biol.*, **56**, 554—558 (1994).
- 17) Holtmann H., Winzen R., Holland P., Eickemeier S., Hoffmann E., Wallach D., Malinin N. L., Cooper J. A., Resch K., Kracht M., *Mol. Cell. Biol.*, **19**, 6742—6753 (1999).
- 18) Hattori Y., Kakishita H., Akimoto K., Matsumura M., Kasai K., *Biochem. Biophys. Res. Commun.*, **281**, 891—896 (2001).
- 19) Cohen M. P., Ziyadeh F. N., Lautenslager G. T., Cohen J. A., Shearman C. W., *Am. J. Physiol.*, **276**, 684—690 (1999).
- 20) Yoo C. W., Song C. Y., Kim B. C., Hong H. K., Lee H. S., *Cell. Physiol. Biochem.*, **14**, 361—368 (2004).
- 21) Salazar R., Brandt R., Krantz S., *Glycoconj. J.*, **18**, 769—777 (2001).
- 22) Chen S., Cohen M. P., Lautenslager G. T., Shearman C. W., Ziyadeh F. N., *Kidney Int.*, **59**, 673—681 (2001).
- 23) Ichiki T., Funakoshi Y., Ito K., Takeshita A., *Biochem. Biophys. Res. Commun.*, **269**, 666—670 (2000).
- 24) Hattori Y., Suzuki M., Hattori S., Kasai K., *Hypertension*, **39**, 22—28 (2002).
- 25) Yeh C. H., Sturgis L., Haidacher J., Zhang X. N., Sherwood S. J., Bjerkke R. J., Juhasz O., Crow M. T., Tilton R. G., Denner L., *Diabetes*, **50**, 1495—1504 (2001).