Endocrine, *vol. 20, no. 3, 247–252, April 2003* 0969–711X/03/20:247–252/$20.00

Exploring Levels of Hexosamine Biosynthesis Pathway Intermediates and Protein Kinase C Isoforms in Muscle and Fat Tissue of Zucker Diabetic Fatty Rats

Remko R. Bosch,[1,2] Susan W. J. Janssen,[2,4] Paul N. Span,[1] André J. Olthaar,[1] Sjenet E. van Emst-de Vries,[3] Peter H. G. M. Willems,[3] Gerard J.M. Martens,[4] Ad R. M. M. Hermus,[2] and C. G. J. (Fred) Sweep[1]

Departments of [1]Chemical Endocrinology, [2]Endocrinology, and [3]Biochemistry, University Medical Centre Nijmegen, Nijmegen, The Netherlands; and [4]Department of Animal Physiology, Faculty of Science, University of Nijmegen, Nijmegen, The Netherlands

Many studies suggest that insulin resistance develops and/or is maintained by an increased flux of glucose through the hexosamine biosynthesis pathway. This pathway may attenuate insulin-stimulated glucose uptake by activating protein kinase C (PKC). Therefore, we investigated whether the concentrations of the major hexosamine metabolites, uridine diphosphate-*N*-acetyl-glucosamine (UDP-GlcNAc) and uridine diphosphate-*N*-acetyl-galactosamine (UDP-GalNAc), and the expression levels of PKC isoforms were affected in Zucker Diabetic Fatty (ZDF) rats, an animal model widely used to study type 2 diabetes mellitus. At the age of 6 wk, control and ZDF rats were normoglycemic. Whereas control rats remained normoglycemic, the ZDF rats became hyperglycemic. The amount of UDP-GlcNAc and UDP-GalNAc in muscle tissue of ZDF rats was similar at 6, 12, 18, and 24 wk of age. Moreover, the concentration of both hexosamines did not differ among ZDF, phlorizin-treated ZDF, and control rats. Western blot analysis revealed that PKCα, δ, ε, and ζ, but not PKCβ and γ, were expressed in muscle and fat tissues from 6- and 24-wk-old control and ZDF rats. In addition, we did not observe changes in the expression levels of the PKC isoforms following prolonged hyperglycemia. Taken together, these findings indicate that the amounts of several metabolites from the hexosamine biosynthesis pathway and PKC isoforms, both hypothesized to be important in the development and/or maintenance of the insulin-resistant state of muscle and fat tissue, are not different in ZDF compared with nondiabetic rats.

Received September 30, 2002; Revised December 2, 2002; Accepted January 9, 2003.

Author to whom all correspondence and reprint requests should be addressed: R. R. Bosch, Department of Chemical Endocrinology, University Medical Centre Nijmegen, PO Box 9101, NL-6500 HB Nijmegen, The Netherlands. E-mail: R.Bosch@ace.umcn.nl

Key Words: Zucker diabetic fatty rats; uridine diphosphate-*N*-acetyl glucosamine; uridine diphosphate-*N*-acetyl galactosamine; protein kinase C.

Introduction

Hyperglycemia is the clinical hallmark of type 2 diabetes mellitus, and its presence plays a major role in the development of the long-term complications of diabetes (reviewed in refs. *1* and *2*). The increase in blood glucose level results mainly from a diminished glucose uptake in insulin-sensitive tissues, especially skeletal muscle and adipose tissue. At present, it is a challenge to elucidate the molecular mechanisms responsible for the reduction in insulin-stimulated glucose disposal.

Glucose that enters the cell is metabolized into glucose-6-phosphate and fructose-6-phosphate. A minor part (1–3%) of the fructose-6-phosphate enters the hexosamine biosynthesis pathway by conversion into glucosamine-6-phosphate through the action of the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) *(3)*. Several lines of evidence suggest that the hexosamine biosynthesis pathway controls the responsiveness of insulin-sensitive tissues to take up glucose (reviewed in refs. *3–5*). Marshall et al. *(6)* were the first to demonstrate that increased amounts of the cellular hexosamine metabolites induce insulin resistance in adipocytes. Others have confirmed this finding in various insulin-sensitive cells from rat and human origin *(7–12)*. Moreover, cloning of human GFAT *(13)* enabled the construction of transgenic mice that overexpress this enzyme *(14,15)*. These mice suffer from insulin resistance. Taken together, these findings suggest that the hexosamine biosynthesis pathway functions as a glucose sensor able to regulate insulin-stimulated glucose uptake, and it is hypothesized that this pathway plays a role in the pathogenesis of insulin resistance in patients with type 2 diabetes mellitus (reviewed in ref. *4*). At present, the underlying principle via which hexosamine metabolites affect insulin-mediated glucose

uptake is not clear, but impaired insulin-stimulated translocation of the glucose transporter GLUT-4 to the plasma membrane has been observed *(14,16,17)*. Two studies provide insights into the possible mechanism of action of the hexosamine biosynthesis pathway intermediates *(18,19)*. First, vesicles containing the insulin-responsive GLUT-4 transporter in rat skeletal muscle are more glycosylated upon glucosamine infusion *(18)*, suggesting that a marked reduction in insulin action results from altered glycosylation of proteins in the GLUT-4-containing vesicles. Second, glucosamine-induced insulin resistance in rat epididymal fat pads was abrogated following treatment with the protein kinase C (PKC)-inhibitor Ro-31-8220 *(19)*, suggesting that PKC is activated via the hexosamine biosynthesis pathway to inhibit insulin-mediated GLUT-4 translocation to the plasma membrane.

The PKC family consists of 12 isoforms that can be divided into three classes: the classic or conventional PKC isotypes (α, β_I, β_{II}, and γ), the novel isoforms (δ, ε, η, θ, and μ), and the atypical isozymes (ζ, λ, and τ) *(20–22)*. Reports on changes in PKC activity in muscle tissue of obese Zucker rats that develop insulin resistance are conflicting. Cooper et al. *(23)* observed a decrease in immunoreactivity of PKCα, β, and ε. By contrast, others have reported an increase in PKC activity, which is explained either by increased levels of PKCε and θ *(24)* or by increased translocation of PKCα, β, δ, and ε to the plasma membrane *(25)*.

Given the apparent importance of the hexosamine biosynthesis pathway for the generation of insulin resistance, we investigated the activity of this pathway in Zucker Diabetic Fatty (ZDF) rats, a widely used animal model for type 2 diabetes mellitus. The amount of two major end products of the hexosamine biosynthesis pathway, uridine diphosphate-*N*-acetyl-glucosamine (UDPGlcNAc) and UDP-*N*-acetyl-galactosamine (UDP-GalNAc), were determined in control and ZDF rats. In the latter, we also investigated the effect of prolonged treatment with phlorizin, a potent inhibitor of renal tubular glucose transport, which almost totally prevented the development of hyperglycemia in the rats. In addition, the discrepancy in changes in the PKC isoform expression in obese Zucker rats urged us to assess the expression of several PKC isoforms in muscle and adipose tissue from control and ZDF rats, since the latter originate from a colony of obese Zucker rats *(26)*.

Results

Blood Glucose and Insulin Concentrations

The blood glucose concentrations in male ZDF and control rats at the age of 6, 12, 18, and 24 wk were determined shortly before they were sacrificed. Figure 1 shows that at wk 6 both control and ZDF rats were normoglycemic. The control rats remained normoglycemic during the whole study period. As described previously *(27)*, the blood glucose concentration in the ZDF rats was significantly higher than in the control rats at wk 12, 18, and 24. Starting at 6 wk of age, ZDF rats (n = 6) were treated with phlorizin (ZDF/phlorizin), a potent inhibitor of renal tubular glucose transport, until they were 12 (n = 3) or 18 (n = 3) wk old. Compared with ZDF rats injected with vehicle solution only (ZDF/control), the blood glucose concentration of ZDF/phlorizin rats was significantly lowered and in the range of control rats (Fig. 1).

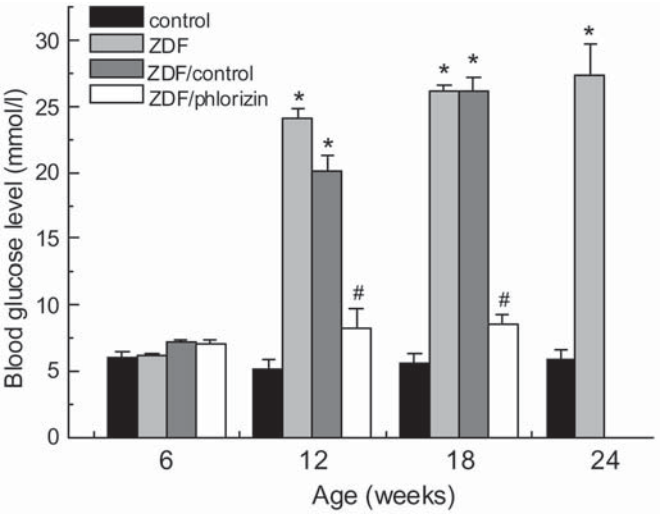

Fig. 1. Blood glucose concentrations in ZDF and control rats. Blood glucose levels from male control, ZDF, ZDF vehicle-injected (ZDF/control), and ZDF phlorizin-treated (ZDF/phlorizin) rats were determined when the rats were 6, 12, 18, and 24 wk of age. Data are presented as means ± SEM from three animals. *Significantly different from control rats of identical age; #significantly different from ZDF/control rats of identical age.

As described previously *(27)*, plasma insulin levels in control rats increased gradually from ~1 ng/ml at 10 wk of age to ~1.5 ng/mL at 24 wk of age. In ZDF rats, plasma insulin levels were 19.7 ± 2.1 ng/mL at 10 wk of age and thereafter decreased to 2.4 ± 0.3 ng/mL at 17 wk of age.

Determination of Nucleotide-Linked Hexosamine Metabolites in Muscle and Fat Tissue

Muscle tissues from control and from the three groups of differently treated ZDF rats were collected and the amounts of UDP-GlcNAc (Fig. 2A) and UDP-GalNAc (Fig. 2B) were determined. No significant differences were observed in the concentrations of both nucleotide-linked hexosamine metabolites among control, ZDF, ZDF/control, and ZDF/phlorizin rats at the different ages. In addition, we observed no significant change in the concentration of either UDP-GlcNAc or UDP-GalNAc within the four different groups when the rats became older (from 6 until 24 wk of age). Finally, no significant correlation was observed when the blood glucose levels and the concentration of UDP-GlcNAc and UDP-GalNAc in muscle tissues from all the different

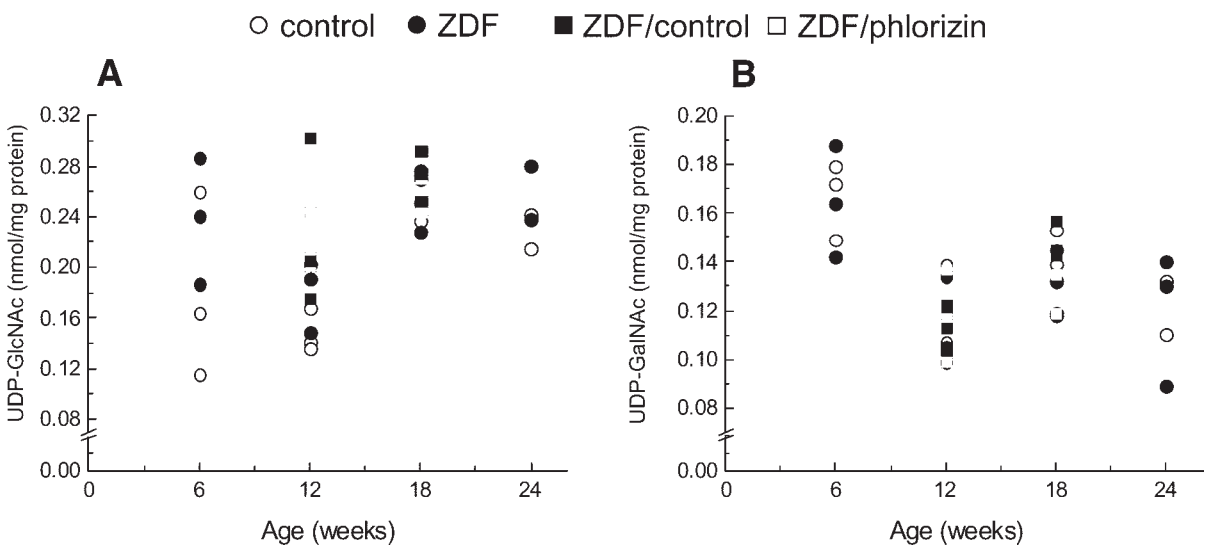

Fig. 2. UDP-linked hexosamine metabolites in muscle tissue of control and ZDF rats. Muscle tissue was isolated from control ($n = 3$) and ZDF ($n = 3$) rats sacrificed when they were 6, 12, 18, or 24 wk of age. In addition, tissues were collected from phlorizin-treated (ZDF/phlorizin; $n = 3$) and vehicle-injected (ZDF/control; $n = 3$) rats. The amount of UDP-GlcNAc (**A**) and UDP-GalNAc (**B**) was determined by high-performance liquid chromatography (HPLC). Each plotted symbol represents the value from one rat.

groups and ages were compared (linear fit, UDP-GlcNAc: $R = 0.25$, $p = 0.14$; UDP-GalNAc: $R = –0.29$, $p = 0.10$).

We analyzed fat tissue of control and ZDF rats for the presence of the two nucleotidelinked hexosamines, but both were consistently below the limit of quantification, which is 30 pmol/mg of protein (data not shown).

Expression of PKC Isoforms in Muscle and Fat Tissue from Control and ZDF Rats

In the muscle of 6- and 24-wk-old control and ZDF rats, we detected PKCα, δ, ε, and ζ (Fig. 3), whereas PKCβ and γ were not detected. In fat tissue from 6- and 24-wk-old control and ZDF rats, PKCα, δ, and ζ were present. We observed also the presence of PKCε in fat tissue of both control and ZDF rats, but the level was very low (data not shown). By contrast, we did not detect PKCβ and γ in the different fat samples. No significant changes in the level of PKC expression were observed in both tissues between ZDF rats and their age-matched controls after densitometric analysis of the Western blots (data not shown). Since we did not observe changes in the expression profile or any major differences in the level of expression of the PKC isoforms, we did not analyze PKC expression in the phlorizin-treated ZDF rats.

Of note, the antibody we used to quantify the expression of PKCζ also recognizes PKCλ (Gibco, personal communications). It may therefore be very well possible that the immunochemical staining represents both atypical PKCs. However, Kanoh et al. *(28)* demonstrated that Goto-Kakizaki rat tissues contain much more PKCζ than PKCλ. Possibly, the same holds true for ZDF rats, indicating that the immunochemical staining predominantly represents PKCζ.

Discussion

We investigated the hexosamine biosynthesis pathway and PKC in ZDF rats, an animal model for type 2 diabetes mellitus. We observed no difference in the concentration of UDP-GlcNAc and UDP-GalNAc among muscle tissues from young (normoglycemic) ZDF, older (hyperglycemic) ZDF, and control (normoglycemic) rats. In addition, lowering of the blood glucose concentration in ZDF rats by phlorizin did not change the muscular concentration of these two nucleotide-linked hexosamines. Furthermore, both the muscle and fat tissue of 6-wk-old (normoglycemic) control and (normoglycemic) ZDF rats contained similar levels of PKCα, δ, ε, and ζ, which was unchanged in 24-wk-old (normoglycemic) control and (hyperglycemic) ZDF rats. Taken together, these findings do not support the hypothesis that either the hexosamine biosynthesis pathway or PKC is involved in the generation or maintenance of insulin resistance in ZDF rats.

Many reports have demonstrated that activation of the hexosamine biosynthesis pathway by administration of glucosamine induces insulin resistance in a number of in vitro and in vivo models *(6–12,19)*. Therefore, it has been hypothesized that in case of human type 2 diabetes mellitus, an increased flux of glucose into the hexosamine biosynthesis pathway induces and/or worsens insulin resistance. Although there is evidence for a role for this pathway in the pathogenesis of the long-term complications associated with type 2 diabetes mellitus *(29,30)*, evidence for its involvement in the pathogenesis of insulin resistance in type 2 diabetes mellitus is very limited. In humans, it has been shown that the enzymatic activity of GFAT is increased in skeletal muscle

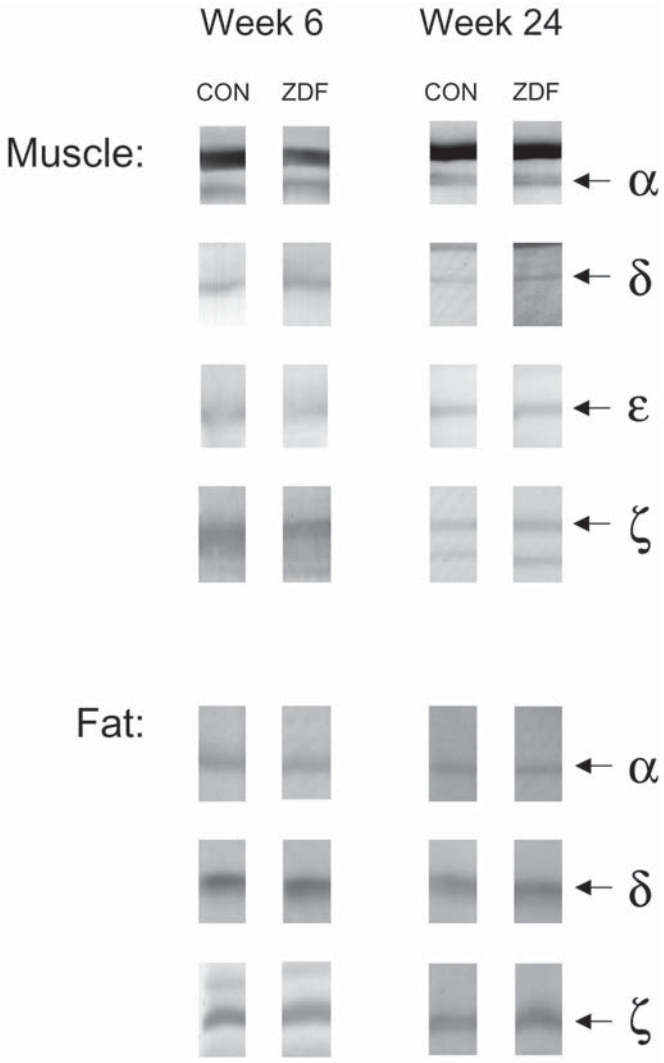

Fig. 3. PKC isoform expression in muscle and fat tissues from control and ZDF rats. Total cell lysates (50 μg) from muscle and fat tissues from 6- or 24-wk-old control (CON) and ZDF rats were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, proteins were transferred to polyvinylidine difluoride membranes by Western blotting and probed with rabbit anti-PKCα, β, γ, δ, ε, or ζ. Muscle tissue from control and ZDF rats (at 6 and 24 wk of age) contains PKCα, δ, ε, and ζ, whereas the fat tissue from these rats contains PKCα, δ, and ζ, and PKCε at very low concentrations (not shown). Each blot is a representative result from three individual rats.

from patients suffering from type 2 diabetes mellitus compared with that in nondiabetic individuals *(31)*. In male Wistar BR rats, streptozotocin-induced diabetes or a 2-h hyperglycemic clamp resulted in an increase in the total UDP-hexosamines content in muscle tissue of 50 and 40%, respectively *(32)*. However, these observations do not prove that the increased activity of the hexosamine biosynthesis pathway is causally related to insulin resistance. A recent publication seriously questions the involvement of the hexosamine biosynthesis pathway in hyperglycemia-associated insulin resistance *(33)*. In 3T3-L1 adipocytes the amount of UDP-hexosamines increased only 30% following excessive glucose loading whereas glucosamine incubation required an increase of 900% to induce insulin resistance. The latter argues against a role for the hexosamine biosynthesis pathway in hyperglycemia-induced insulin resistance. In the present study, we report that the levels of UDP-GlcNAc and UDP-GalNAc in muscle tissue from ZDF rats do not change when these rats develop hyperglycemia and were comparable with the levels observed in muscle tissue from normoglycemic control rats.

Recently it has been shown that with an identical flux of the hexosamine biosynthesis pathway, insulin resistance is induced with an altered glycosylation pattern of cytosolic and nuclear proteins *(34,35)*. UDP-GlcNAc is enzymatically attached to serine and threonine residues by O-linked GlcNAc transferase and, like phosphorylation, modulates the function of the protein. In addition, O-GlcNAcase is a nucleocytoplasmic enzyme that catalyzes the removal of O-linked GlcNac from proteins. It has been shown that altered functioning of O-linked GlcNAc transferase *(34)* and O-GlcNAcase *(35)* induces insulin resistance without changes in the amount of UDP-GlcNAc. Whether different glycan-dependent signaling is the underlying principle for the development of diabetes mellitus type 2 in ZDF rats is unclear.

The enzyme PKC is involved in cell signaling by catalyzing the phosphorylation of specific serine and threonine residues of various cellular proteins, thereby modulating their function. Here we report that muscle tissue from 6- and 24-wk-old control rats contains PKCα, δ, ε, and ζ but not PKCβ and γ. A similar PKC expression profile is observed in muscle tissue of 6-wk-old normoglycemic and 24-wk-old hyperglycemic ZDF rats. In fat tissue of 6- and 24-wk-old control rats, we detected the same expression pattern as in the muscle tissue, albeit that the expression of PKCε was extremely low. Similar to in the muscular cells, this pattern was not different in fat tissue from 6- and 24-wk-old ZDF rats. These results show that chronic hyperglycemia did not change the PKC expression profile, because we did not observe expression of PKCβ or γ nor loss of expression of any of the detected PKC isoforms in muscle and fat tissue of 24-wk-old ZDF rats. Furthermore, densitometric analysis on the Western blot results revealed no significant differences in the expression levels of the four PKC isoforms between ZDF and control rats of identical age.

In contrast to our findings, the levels of several PKC isoforms were found to be affected in muscle tissue from insulin-resistant obese Zucker rats. Cooper et al. *(23)* observed a 70–90% decrease in immunoreactivity of PKCα, β, and ε in soleus muscle of the obese Zucker rat compared with their lean agematched counterpart. Confusingly, others report an increase in the levels of PKCε and θ in soleus muscle from insulin-resistant obese Zucker rats compared with their lean control rats *(24)*. We did not observe either a decrease or an increase in the expression levels of the PKC isoforms in the

muscular cells of the ZDF rats following prolonged hyperglycemia. Of note, the possibility remains that in our (hyperglycemic) ZDF rats more PKC may be translocated to the plasma membranes of both muscle and fat cells, which is considered to result in enhanced PKC activity *(36)*. Such an increased translocation of PKC isoforms to the plasma membrane has been observed in muscle tissue from insulin-resistant obese Zucker rats *(25)*. The total amount of PKC, however, was not affected. Interestingly, Considine et al. *(37)* reported that in the liver of hyperglycemic ZDF rats PKCα, β, ε, and ζ expression was increased compared with that in lean and obese control rats. Prolonged phlorizin treatment, which prevented severe increases in the blood glucose concentration, did not lower the expression of these four PKC isoforms, and, therefore, it was concluded that PKC expression levels in the liver of ZDF rats are more likely to be genetically determined rather than caused by hyperglycemia.

Taken together, our data do not reveal a role for either the hexosamine biosynthesis pathway or PKC in the development of insulin resistance in ZDF rats. Whether these two factors are involved in other models of diabetes mellitus type 2 remains to be determined.

Materials and Methods

Animals

Male ZDF (ZDF/Gmi-fa/fa or ZDF rats) and lean Zucker control rats (ZDF/Gmi- +/? or control rats) were from Genetic Models (Indianapolis, IN). They arrived at our animal facility when they were 5 wk of age. The rats were maintained on a 12-h light/dark cycle (lights on at 7:00 AM, lights off at 7:00 PM) and had ad libitum access to tap water and chow (RMH-B: 22.8% protein, 5.1% fat, 4.2% fiber, and 67.9% other carbohydrates; Hope Farms, Woerden, The Netherlands).

Chemicals

Goat anti–rabbit IgG antibodies conjugated to alkaline phosphatase, leupeptin, soybean trypsin inhibitor, and phlorizin were obtained from Sigma (St. Louis, MO). Phenylmethylsulfonyl fluoride (PMSF) was obtained from SERVA (Heidelberg, Germany) and aprotinin from Fluka (Buchs, Switzerland). I-Block reagent was purchased from Tropix (Bedford, MA). Isotype-specific PKC antibodies and PKC isotype–specific peptides were obtained from Gibco (Paisley, Scotland). All other chemicals were of analytical grade.

Phlorizin Treatment

Male ZDF rats (ZDF/phlorizin) were subcutaneously injected (250 μL) with phlorizin (50 mg/kg of body wt) dissolved in phosphate-buffered saline (PBS) (pH 7.4) and propylene glycol (4:6 [v/v]). Control rats (ZDF/control) were injected with vehicle. The injections were given twice a day at 9:00 AM and 5:00 PM starting when the rats were 6 wk of age. At the ages of 12 and 18 wk, three phlorizin- and three vehicle-treated rats were sacrificed.

Analysis of Blood and Insulin Glucose Concentrations

Approximately 50 μL of venous blood was collected from a tail vein at 9:00 AM when the rats were 6, 10, 12, 17, 18, and 24 wk of age. Blood glucose concentrations were measured by a glucose oxidase method, using the Glucocard Memory device (Menarini Diagnostics, Brussels, Belgium). Plasma insulin levels were assayed using a rat insulin radioimmunoassay kit (RI-13k; Linco) as described previously *(27)*.

Collection of Fat and Muscle Tissue

Rats ($n = 3$) were killed when they were 6, 12, 18, or 24 wk of age, and inguinal adipose tissue and hind-limb muscles (mixture of gastrocnemius, soleus, and plantaris muscle *[38]*) were rapidly removed, snap frozen in liquid N_2, and stored at –80ºC until further use.

Analysis of Nucleotide-Linked Hexosamines

Analyses were performed as described in detail previously *(39)*. In brief, 200 mg wet wt of muscle tissue was homogenized in liquid N_2 with a dismembrator (Braun, Melsungen, Germany). The tissue powder was taken up in 1 mL of extraction buffer containing 0.1 *M* KCl, 1 m*M* EDTA, and 50 m*M* KH_2PO_4 adjusted with KOH to pH 7.5. Subsequently, the homogenate was centrifuged at 60,000*g* for 15 min at 4ºC. The supernatant (300 μL) was deproteinized by adding 1 vol of 1.2 *M* perchloric acid. After 5 min on ice, the mixture was centrifuged for 10 min at 13,500*g* and 4ºC. The supernatant was diluted with 10 vol of 10 m*M* KH_2PO_4, adjusted with H_3PO_4 to pH 2.5, and applied to LC-SAX ion-exchange columns (Amersham, Buckinghamshire, UK). After washing, the samples were eluted with 1.5 mL of KH_2PO_4 (150 m*M*) and adjusted with KOH to pH 7.5. Finally, samples (150 μL) were injected on two LC-18T columns in series (25 cm × 4.6 mm, 5 μ*M*; Supelco, Zwijndrecht, The Netherlands) and separated by HPLC. Detection of UDP-linked glucose and galactose and UDP-linked glucosamine and galactosamine was performed by ultraviolet detection at 262 nm. Values were normalized for cytosolic protein content (bicinchoninic acid; Pierce, Rockford, IL).

Measurement of Different PKC Isoforms by Western Blotting

Western blotting was performed essentially as described previously *(40)*. Approximately 50 mg wet wt of muscle or fat tissue was taken up in 1 mL of homogenization buffer containing 2 m*M* EDTA, 10 m*M* EGTA, 0.25 *M* sucrose, 5 m*M* dithiothreitol, 0.2 mg/mL of trypsin inhibitor, 0.5 μg/mL of aprotinin, 20 μ*M* leupeptin, 1 m*M* PMSF, and 20 m*M* Tris adjusted with HCl to pH 7.5. The tissue was homogenized at 4ºC using a Potter-Elvehjem homogenizer (glass-Teflon) with 10 strokes at 500 rpm, 5 strokes at 1000 rpm, and 5 strokes at 1200 rpm, after which the total protein content was determined (Bio-Rad, Hertfordshire, UK). Subsequently, 500 μg of total protein was precipitated with 5% (v/v) trichloroacetic acid. After 10 min on ice, the homo-

genate was centrifuged for 10 min at 13,000*g*. The pellet was taken up in sample buffer, and 50 µg of total protein was subjected to SDS-PAGE. Proteins were transferred overnight to polyvinylidene difluoride membranes (Immobillon P; Millipore, Bedford, MA) by Western blotting. Membranes were blocked for 1 h with PBS (pH 7.4) containing 0.2% (w/v) I-Block reagent and 0.1% (v/v) Tween-20 and incubated overnight with PKC isotype–specific antibodies diluted 1:500 in PBS containing 0.1% (w/v) I-Block reagent and 0.2% (v/v) Tween-20. To demonstrate the specificity of the reaction, control membranes were incubated in the presence of corresponding PKC isotype–specific peptides (1:1000). Membranes were washed with PBS containing 0.3% (v/v) Tween-20 (washing buffer) and incubated for 1 h with goat anti–rabbit IgG antibodies conjugated to alkaline phosphatase diluted 1:1000 in PBS (pH 7.4) containing 0.1% (w/v) I-Block reagent and 0.2% (v/v) Tween-20. Membranes were washed with washing buffer and PBS before staining with 0.1 *M* diethanolamine containing 0.34 mg/mL of Nitroblue tetrazolium, 0.18 mg/mL of 5-bromo-4-chloro-3-indolylphosphate, and 50 m*M* $MgCl_2$. Membranes were scanned using an Imaging Densitometer (Bio-Rad, Munich, Germany).

Data Analyses

The results presented are the mean ± SEM of three individual rats and statistical significance was determined by analysis of variance. In the case of significance ($p < 0.05$), individual groups were compared according to Tukey-Kramer. When appropriate, Spearman correlation coefficients were determined. A value of $p < 0.05$ was considered significant.

Acknowledgments

We wish to thank Annet Verleg and Henk Arnts (Central Animal Laboratory, University of Nijmegen, The Netherlands) for biotechnical assistance with the animal experiments, and Joop Heuvel (Department of Chemical Endocrinology, University Medical Centre, Nijmegen) for technical assistance with the phlorizin experiments. This study was supported by grant no. 95.116 from The Netherlands Diabetes Foundation (Diabetes Fonds Nederland).

References

1. Gerich, J. E. (1988). *Endocr. Rev.* **19,** 491–503.
2. Lebovitz, H. E. (1999). *Clin. Chem.* **45,** 1339–1345.
3. Hussain, M. A. (1998). *Eur. J. Endocrinol.* **139,** 472–475.
4. McClain, D. A. and Crook, E. D. (1996). *Diabetes* **45,** 1003–1009.
5. Rossetti, L. (2000). *Endocrinology* **141,** 1922–1925.
6. Marshall, S., Bacote, V., and Traxinger, R. R. (1991). *J. Biol. Chem.* **266,** 4706–4712.
7. Ciaraldi, T. P., Carter, L., Nikoulina, S., Mudaliar, S., McClain, D. A., and Henry, R. R. (1999). *Endocrinology* **140,** 3971–3980.
8. Hawkins, M., Barzilai, N., Chen, W., et al. (1996). *Diabetes* **45,** 1734–1743.
9. Hawkins, M., Hu, M., Yu, J., et al. (1999). *J. Biol. Chem.* **274,** 31312–31319.
10. Patti, M.-E., Virkamäki, A., Landaker, E. J., Kahn, R., and Yki-Järvinen, H. (1999). *Diabetes* **48,** 1562–1571.
11. Robinson, K. A., Sens, D. A., and Buse, M. G. (1993). *Diabetes* **42,** 1333–1346.
12. Virkamäki, A., Daniels, M. C., Hämäläinen, S., Utriainen, T., McClain, D., and Yki-Järvinen, H. (1997). *Endocrinology* **138,** 2501–2507.
13. McKnight, G. L., Mudri, S. L., Mathews, S. L., et al. (1992). *J. Biol. Chem.* **267,** 25208–25212.
14. Cooksey, R. C., Hebert, L. F., Zhu, J.-H., Wofford, P., Garvey, W..T., and McClain, D. A. (1998). *Endocrinology* **140,** 1151–1157.
15. Hebert, L. F., Daniels, M. C., Zhou, J., et al. (1996). *J. Clin. Invest.* **98,** 930–936.
16. Baron, A. D., Zhu, J. S., Zhu, J. H., Weldon, H., Maianu, L., and Garvey, W. T. (1995). *J. Clin. Invest.* **96,** 2792–2801.
17. Heart, E., Chol, W. S., and Sung, C. K. (2000). *Am. J. Physiol. Endocrinol. Metab.* **278,** E103–E112.
18. Hawkins, M., Angelov, I., Liu, R., Barzilai, N., and Rossetti, L. (1997). *J. Biol. Chem.* **272,** 4889–4895.
19. Fillipis, A., Clark, S., and Proietto, J. (1997). *Biochem. J.* **324,** 981–985.
20. Hofmann, J. (1997). *FASEB J.* **11,** 649–669.
21. Jaken, S. (1996). *Curr. Opin. Cell Biol.* **8,** 168–173.
22. Nishizuka, Y. (1995). *FASEB J.* **9,** 484–496.
23. Cooper, D. R., Watson, J. E., and Dao, M. L. (1993). *Endocrinology* **133,** 2241–2247.
24. Qu, X., Seale, J. P., and Donnelly, R. (1999). *J. Endocrinol.* **162,** 207–214.
25. Avignon, A., Yamada, K., Zhou, X., et al. (1996). *Diabetes* **45,** 1396–1404.
26. Clark, J. B., Palmer, C. J., and Shaw, W. N. (1983). *Proc. Soc. Exp. Biol. Med.* **173,** 68–75.
27. Janssen, S. W., Martens, G. J., Sweep, C. G., Ross, H. A., and Hermus, A. R. (1999). *Horm. Metab.Res.* **31,** 610–615.
28. Kanoh, Y., Bandyopadhyay, G., Sajan, M. P., Standaert, M. L., and Farese, R. V. (2000). *J. Biol. Chem.* **275,** 16990–16996.
29. Nerlich, G. A., Sauer, U., Kolm-Litty, V., Wagner, E., Koch, M., and Schleicher, E. D. (1998). *Diabetes* **47,** 170–178.
30. Schleicher, E. D. and Weigert, C. (2000). *Kidney* **58,** S13–S18.
31. Yki-Järvinen, H., Daniels, M. C., Virkamäki, A., Mäkimattila, S., DeFronzo, R. A., and McClain, D. A. (1996). *Diabetes* **45,** 302–307.
32. Robinson, K. A., Weinstein, M. L., Lindenmayer, G. E., and Buse, M. G. (1995). *Diabetes* **44,** 1438–1446.
33. Nelson, B. A., Robinson, K. A., and Buse, M. G. (2000). *Diabetes* **49,** 981–991.
34. McClain, D. A., Lubas, W. A., Cooksey, R. C., et al. (2002). *Proc. Natl. Acad. Sci. USA* **99,** 10695–10699.
35. Vosseller, K., Wells, L., Lane, M. D., and Hart, G. W. (2002). *Proc. Natl. Acad. Sci. USA* **99,** 5313–5318.
36. Nishizuka, Y. (1992). *Science* **258,** 607–614.
37. Considine, R. V., Nyce, M. R., Allen, L. E., et al. (1995). *J. Clin. Invest.* **95,** 2938–2944.
38. Armstrong, R.B. and Phelps, R.O. (1994). *Am. J. Anat.* **171,** 259–272.
39. Span, P. N., Pouwels, M. J., Olthaar, A., Bosch, R. R., Hermus, A. R., and Sweep, C. G. (2001). *Clin. Chem.* **47,** 944,945.
40. Smeets, R. L., Garner, K. M., Hendriks, M., et al. (1996). *Cell Calcium* **20,** 1–9.