Cellular Mechanism of Nutritionally Induced Insulin Resistance in *Psammomys Obesus*

Overexpression of Protein Kinase Cε in Skeletal Muscle Precedes the Onset of Hyperinsulinemia and Hyperglycemia

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The sand rat (*Psammomys obesus*) is an animal model of nutritionally induced diabetes. We report here that several protein kinase C (PKC) isoforms (α, ε, and ζ) representing all three subclasses of PKC are overexpressed in the skeletal muscle of diabetic animals of this species. This is most prominent for the ε isotype of PKC. Interestingly, increased expression of PKCε could already be detected in normoinsulinemic, normoglycemic (prediabetic) animals of the diabetes-prone (DP) line when compared with a diabetes-resistant (DR) line. In addition, plasma membrane (PM)-associated fractions of PKCε and PKCζ were significantly increased in skeletal muscle of diabetic animals, suggesting chronic activation of these PKC isoforms in the diabetic state. The increased PM association of these PKC isoforms revealed a significant correlation with the diacylglycerol content in the muscle samples. Altered expression/activity of PKCε, in particular, may thus contribute to the development of diabetes in these animals; along with other PKC isoforms, it may be involved in the progression of the disease. This may possibly occur through inhibition of insulin receptor (IR) tyrosine kinase activity mediated by serine/threonine phosphorylation of the IR or insulin receptor substrate 1 (IRS-1). However, overexpression of PKCε also mediated downregulation of IR numbers in a cell culture model (HEK293), resulting in attenuation of insulin downstream signaling (reduced protein kinase B [PKB] activity). In accordance with this, we detected decreased 125I-labeled insulin binding, probably reflecting a downregulation of IR numbers in skeletal muscle of *Psammomys* animals from the DP line. The number of IRS was inversely correlated to both the expression and PM-associated levels of PKCε. These data suggest that overexpression of PKCε may be causally related to the development of insulin resistance in these animals, possibly by increasing the degradation of IRS.

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DAG, diacylglycerol; DP, diabetes-prone; DPA, diabetes-prone stage A; DP/C, diabetes-prone stage C; DR, diabetes-resistant; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; HE, high energy; IRI, insulin receptor substrate 1; LE, low energy; PKB, protein kinase B; PKC, protein kinase C; PM, plasma membrane; TPA, tetradecanoylphorbol acetate.

Psammomys obesus, often nicknamed “sand rat,” is a herbivorous desert gerbil living in the eastern Mediterranean and North Africa. The Jerusalem colony was established by domesticating the animals collected from the shores of the Dead Sea (1,2). In its native habitat, the *Psammomys* feeds on the halophilic plant *Atriplex halimus* and has never been found to be hyperglycemic or hyperinsulinemic. In captivity, it remains nondiabetic when fed a low energy (LE) diet containing 2.4 kcal/g. However, when transferred to a relatively high energy (HE) diet of 3.0 kcal/g, similar to the regular rodent diet, it gradually develops hyperinsulinemia and hyperglycemia. Four generally consecutive stages of progression to diabetes have been described (3). The basal stage A is followed by hyperinsulinemia without hyperglycemia (stage B), which precedes hyperinsulinemia with hyperglycemia (stage C), and lastly, stage D, marked by pancreatic insulin secretion collapse with apoptosis (4) and dependence on external insulin supply for survival.

Two *Psammomys* lines have been separated by selective breeding: the diabetes-resistant (DR) line, which remains normoglycemic and normoinsulinemic even on an HE diet, and the diabetes-prone (DP) line, which is susceptible to diabetes when exposed to the HE diet (4). The main difference between the two lines seems to be the cost of weight gain: the DP line uses 6.0 kcal/g for growth during 2 weeks after weaning, whereas the DR line requires 9.3 kcal/g (4). The DP animals exhibit insulin resistance even in the state of normoglycemia, as evidenced by the failure to induce hypoglycemia after external insulin administration and a minimal reduction of hepatic gluconeogenesis during the euglycemic-hyperinsulinemic clamp (5). Therefore, the insulin resistance in the *Psammomys* may be considered an innate characteristic of a desert animal according to the thrifty gene hypothesis (6).

Alterations in the expression level and/or activity of several protein kinase C (PKC) isoforms were found to be associated with insulin resistance in type 2 diabetic patients, animal models of diabetes, and different cell models (7–10). The serine/threonine kinase PKC consists of several isoforms, which according to structure and cofactor requirement for activation can be grouped into classic (α, β1, β2, γ), novel (δ, ε, η, μ), and atypical (λ, ζ, η) isoforms.
(11–13). Evidence from in vitro studies indicated that several PKC isoforms can modulate insulin signaling at different levels of the signaling cascade. Several studies have suggested that PKC can inhibit insulin receptor kinase (IRK) activity through serine/threonine phosphorylation of the insulin receptor (IR) β-subunit (14–17). Furthermore, classic PKC isoforms have been implicated in degradation of receptor tyrosine kinases, including the IR (18).

The aim of this study was to determine whether increased expression or chronic activation of PKC isoforms in insulin-responsive tissues (skeletal muscle and liver) are associated with the development of insulin resistance in an animal model of nutritionally induced diabetes, *P. obesus*. This animal system is extremely well characterized with respect to the development of diabetes and thus provides readily defined cohorts at various stages to determine whether alterations in PKC expression/activation are causally related to the development of the disease or a consequence of the diabetic milieu. Because in vitro studies have suggested that the IR could be a target for PKC-mediated downregulation, we used 125I-labeled insulin binding as an indirect measure of the number of IRs in skeletal muscle of these animals. Our data suggest that overexpression and chronic activation of PKCβ might be causally related to the development and progression of diabetes in these animals and negatively regulate insulin signaling through degradation of the IR.

**RESEARCH DESIGN AND METHODS***

**Materials.** Rabbit antipeptide antibodies against PKC isoforms α, β1, β2, δ, ε, θ, η, ι, and z and glutathione S-transferase (GST) were from Santa Cruz Biotechnology (Santa Cruz, CA). PKC isoforms were from Transduction Laboratories (Lexington, KY). Phospho-Akt (Ser473) antibody and Akt antibody were from New England Biolabs (Beverly, MA). Polyclonal rabbit antisera against the IR were purchased from Amersham (Braunschweig, Germany). Recombinant human insulin and [125I]Tyrotide-labeled human insulin were obtained from Becton Dickinson (San Jose, CA).

The cDNAs for the type A IR (19), PKCε (Wang et al., unpublished data, Genbank accession number AF028009), and GST (kindly provided by R.A. Prendergast) were cloned into a cytomegalovirus promotor-enhancer (kindly provided by R.A. Prendergast) and GST (kindly provided by R.A. Prendergast) and GST (kindly provided by R.A. Prendergast) were transfected into HEK293 cells transiently transfected using CaCl2, as previously described by Chen and Okayama (21) and Gorman et al. (22).

**Preparation of lysates.** Muscles and livers were extracted in ice-cold solubilization buffer using a motor-driven Potter homogenizer (final concentrations: 20 mmol/l HEPES, 8 mmol/l EDTA, 0.2 mmol/l Na3VO4, 10 mmol/l NaF, 2.5 mmol/l phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 2.5 mg/ml benzamidine, 2.5 μg/ml pepstatin, 2.5 μg/ml leupeptin, 100 mmol/l NaF, 2 mmol/l phenylarsenic acid, 1 μg/ml Triton X-100, pH 7.4). After 20 min at 4°C, the samples were centrifuged at 20,000g for 60 min to remove insoluble material and the clarified extracts were stored at −80°C.

**Subcellular fractionation.** In 2 ml of KH buffer (0.15 mol/l KCl, 20 mmol/l HEPES, pH 7.5, 1 mmol/l ATP), 0.2 g of powdered muscle was homogenized using a Polytron. Samples were maintained on ice during subsequent steps, and all centrifugations were performed at 4°C. KC1 was added to a final concentration of 0.65 mol/l, followed by centrifugation at 700g for 10 min. The supernatant S1 was saved and the pellet was reextracted in 2 ml of KH buffer and centrifuged, yielding supernatant S2. S1 and S2 were combined in a total of 5 ml of KH buffer and centrifuged at 183,000g for 60 min. The supernatant was designated as the cytosolic fraction. The pellet was resuspended in 0.2 mol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, 1 mmol/l EGTA, 255 mmol/l sucrose, layered on a 58.3% sucrose gradient and centrifuged in a swing-out rotor at 60,000g for 40 min. The interphase was collected and from supernatant 183,000g for 90 min. The pellet was resuspended in 5 ml of lysis buffer containing 20 mmol/l Tris-acetate, pH 7.0, 0.27 mol/l sucrose, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l 4-(2-aminethyl)benzenesulfonyl fluoride, 1 mmol/l Na3VO4, 100 mmol/l NaF, 1% Triton X-100, 10 mmol/l Na3P2O7, 10 mmol/l MgCl2, 100 mmol/l Na3P2O7, 1% Triton X-100, 10 mmol/l NaF, 5 μg/ml leupeptin, and 10 mmol/l benzamidine. After removal of cellular debris by centrifugation at 15,000g for 10 min at 4°C, the protein content in each sample was measured using Bio-Rad Protein Assay according to the manufacturer’s instructions (Bio-Rad, Richmond, CA).

**Cell transfection.** HEK293 cells were transiently transfected using CaCl2, as previously described by Chen and Okayama (21) and Gorman et al. (22).

**Western blotting, enhanced chemiluminescence, and quantitation of bands.** Equal amounts of cell lysate were dissolved in 2× Laemmli buffer and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, BA85). Immunoreactive proteins were made visible using horseradish-peroxidase–coupled secondary antibodies and enhanced chemiluminescence reagents according to the manufacturer’s instructions (Amersham). The specific bands were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For the Akt experiments, we used Fujifilm LAS1000 Luminescent Image Analyzer for detection of bands and Fujifilm Image Gauge V2.53 for quantification.

**Determination of diacylglycerol (DAG) levels.** Powdered muscle tissue (60 mg) was extracted with 3 ml of chloroform/methanol (1:2, vol/vol) before addition of 0.8 ml of 1 mol/l NaCl. After monophase mixing, 1 ml of chloriform and 1 ml of NaCl were added to break the phases. After centrifugation at 5,000g for 2 min, the chloriform phase was analyzed for DAG using the DAG kinase method (sn-1,2-DAG assay reagents system, Amersham). [3H]Phosphatidic acid was separated by thin-layer chromatography, and the respective bands were scanned on a PhosphorImager and quantitated using ImageQuant software (Molecular Dynamics).

**Insulin binding.** Insulin-binding activity was measured by the method described by Klein et al. (23), with a slight modification. Microwells coated with an anti-IgG antibody were provided by Dr. I.H. Klein (Medical University of Lübeck, Lübeck, Germany). Lysate (60 μl) was added to each well and incubated for 16 h at 4°C. The lysates were then removed, the wells were washed five times with wash buffer (0.05% Triton X-100, 100 mmol/l NaCl, 2.5 mmol/l KCl, 1 mmol/l CaCl2, 0.1 mmol/l Na3VO4, 20 mmol/l HEPES, 10% glycerol, 0.05% NaN3, 0.5% BSA, pH 7.4), and 125I-insulin and unlabeled insulin (6.4 ng/ml and 121.6 ng/ml, respectively) were added. After 16 h at 4°C, the wells were washed again and bound 125I-insulin was subsequently collected by twice adding a solution of 2% SDS at room temperature for 30 min. The radioactivity in the collected SDS solution was measured by β-counting.

**Statistical analysis.** All data are presented as the mean ± SE. Group comparisons were made by unpaired Student’s *t* test. Correlations were assessed by Pearson’s least-squares method. *P* values less than 0.05 were considered significant.

**RESULTS***

**Animal characteristics.** The general data on animals used in this study are summarized in Table 1. There was no difference in body weight between the diabetic and the control animals, but the hyperglycemic diabetic rats (37.2 ± 0.5 mmol/l) had significantly lower body weight (119.2 ± 3.2 g) compared with the control rats (210.2 ± 4.3 g). The diabetic rats showed a marked reduction in food intake (6.4 ± 0.3 g/day) compared with the control rats (11.0 ± 0.6 g/day). The body weight of the diabetic rats was significantly lower than that of the control rats (119.2 ± 3.2 g vs. 210.2 ± 4.3 g, *P < 0.05) at 8 weeks of age. The diabetic rats showed a marked reduction in food intake (6.4 ± 0.3 g/day) compared with the control rats (11.0 ± 0.6 g/day). The body weight of the diabetic rats was significantly lower than that of the control rats (119.2 ± 3.2 g vs. 210.2 ± 4.3 g, *P < 0.05).
significant difference in glucose and insulin values of animals in stage A, whether from the DR line maintained on an HE diet or DP animals maintained on an LE diet. As expected, the transfer of DP animals to the HE diet induced hyperinsulinemia and hyperglycemia. Well defined animal groups (n = 11–14 per group) were taken for PKC assays.

PKC isoforms α, ε, and ζ overexpressed in gastrocnemius muscles of Psammomys of the DP line. We first aimed to determine the expression level of several PKC isoforms in skeletal muscle (gastrocnemius) tissues. Western blots of muscle lysates were probed with isozyme-specific antibodies for the classic PKC isoform α, βI, and βII, the novel PKC isoforms δ, ε, θ, and η, and the atypical PKC isoform ζ, ι, and λ. An example of such a Western blot is shown for a subset of the samples in Fig. 1. The quantitation and statistical analysis of the specific bands from all samples analyzed is summarized in Table 2. Expression levels of PKC isoforms α, ε, and ζ were significantly increased in the diabetic animals (DP/C) as compared with the DR line. Interestingly, there was also a significant increase in PKCe expression level in the normoinsulinemic, normoglycemic animals (which may therefore be termed the “prediabetic stage”) of the DP line as compared with the DR line. The values were calculated from 10–11 animals per group and are expressed as means ± SE. PKC isoforms βI, βII, θ, and η were not changed in the DP line, neither in the prediabetic nor in the diabetic state. The expression level of PKC isoforms δ, ι, and λ were undetectable by Western blotting or too low to allow quantitation of the data.

Increased membrane association of PKCε in diabetic animals. The Western blots had shown a significant increase in the expression level of several PKC isoforms, both in the prediabetic (PKCe) and the diabetic animals (PKCα, ε, ζ). To determine whether this was associated with increased activity of these isoforms, we performed subcellular fractionation of the muscle tissue. Translocation of PKCs from the cytosolic to the particulate fraction is a prerequisite of activation. Because of the lack of sensitive isoform-specific PKC activity assays, plasma membrane (PM) association of PKC is often used as a measurement of its activity. PM and cytosolic fractions of the muscle samples were run on Western blots and probed with PKC isoform-specific antibodies. The autoradiograms were scanned and quantitated using ImageQuant (Molecular Dynamics). The percentages of PM-associated PKCα, βI, βII, ε, η, and ζ in the three groups are shown in Table 3. Interestingly, the samples from diabetic animals revealed significant increases in the PKCα, βI, and ε content of the PM fraction, indicating a chronic activation of these PKC isotypes. Smaller increases in PKCα and ε PM association in the “prediabetic” animals were not significant within the study. PKCζ revealed the highest degree of association with the PM compared with other PKC isoforms; surprisingly, however, the percentage of PM-associated PKCζ decreased in diabetic animals. Despite significant increases in the expression level of PKCζ, we did not observe increased PM association of this isotype in the diabetic animals.

Increased PM association of PKC isoforms correlated with increased DAG levels. Both classic and novel PKC isoforms require DAG for activation (12). To investigate whether the increased PM association of PKCα, ε, and ζ could be caused by changes in DAG levels, we determined the DAG content in the muscle samples. Figure 2 shows linear regression analysis of PM association of PKC isoforms versus the DAG content in the individual samples. A significant correlation between these parameters was observed for α and ε isoforms of PKC. In contrast, we could not detect such a correlation for PKCζ. This was in agreement with the finding of unaltered PM content of PKCζ in the three different groups of animals (see Table 3). It was also expected because the activation of atypical PKC isoforms is independent of DAG (13).

Overexpression of PKCε preceding the onset of insulin resistance. Interestingly, PKCε was already overexpressed in the prediabetic stage. To evaluate whether the increased PKCε expression level correlated with any of the

<table>
<thead>
<tr>
<th>Animal/stage</th>
<th>Weight (g)</th>
<th>Maintenance diet</th>
<th>Serum glucose (mg/dl)</th>
<th>Insulin (mU/l)</th>
<th>Triglyceride (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR (13)</td>
<td>193 ± 9‡</td>
<td>HE</td>
<td>86 ± 5</td>
<td>104 ± 19</td>
<td>201 ± 42</td>
</tr>
<tr>
<td>DP/A (11)</td>
<td>153 ± 6</td>
<td>LE</td>
<td>75 ± 4</td>
<td>75 ± 10</td>
<td>134 ± 14</td>
</tr>
<tr>
<td>DP/C (14)</td>
<td>192 ± 5‡</td>
<td>HE</td>
<td>260 ± 13‡</td>
<td>421 ± 40‡</td>
<td>225 ± 35†</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.01 vs. DR line, †P < 0.05, ‡P < 0.01 vs. DP/A.

FIG. 1. Expression of PKCα, ε, θ, and ζ in gastrocnemius muscle. Muscle lysates were subjected to SDS-PAGE followed by Western blotting and then probed with PKC isotype-specific antibodies. A representative panel for each isotype is shown.
parameters characteristic for insulin resistance/diabetes, we performed linear regression analysis of PKCε versus the serum levels of glucose, insulin, and triglycerides, respectively, in the individual samples. As demonstrated in Fig. 3, there was no significant correlation with any of these parameters.

**PKCε expression in liver of diabetic Psammomys.** To determine whether the changes in PKCε expression levels were specific for skeletal muscle, we prepared liver extracts from the same animals to look at another insulin target tissue. No increase in PKCε expression was detectable in the group of prediabetic animals. The group of diabetic animals (DP/C) showed increased PKCε expression; however, this did not reach significance (DR, 7.5 ± 0.9; DP/A, 7.5 ± 0.9; DP/C, 10.1 ± 1.1, **P** = 0.08 vs. DR).

**PKCε can mediate degradation of the IR and attenuates IR signaling.** A previous report suggested that PKCα was capable of degrading the IR (18). We were therefore interested to see whether a similar effect could be observed with PKCε. HEK293 fibroblasts were transfected either with the IR alone or with the IR and PKCε. As a control, GST was coexpressed in all samples. Cells were stimulated for 1 or 6 h with 1 μmol/l tetradecanoylphorbol acetate (TPA) to activate PKC and were subsequently lysed. Figure 4A shows a representative Western blot of lysates probed with an antibody specifically recognizing the IR β-subunit (upper panel), PKCε (middle panel), or GST (lower panel). Clearly, the endogenous background of PKCs has no effect on the expression level of the IR. However, when PKCε is coexpressed with the IR, there is a marked downregulation of IR expression, which is further enhanced after 6 h of TPA stimulation. PKCε itself is also partly downregulated after 6 h of TPA stimulation, in agreement with previous reports. No effect on the expression level of the GST could be detected, indicating that the changes in the expression level of the IR and PKCε are specific. A quantitation and statistical analysis of six experiments shown in Fig. 4B underlines the significance of

![Figure 2](image-url) Correlation of plasma membrane–associated PKC to DAG levels in gastrocnemius muscle. DAG levels and PM-associated PKC levels were determined as described in RESEARCH DESIGN AND METHODS and the correlations in the individual Psammomys samples (n = 16) are plotted. Correlation coefficients (r) and **P** values are indicated.

**TABLE 2**

<table>
<thead>
<tr>
<th>Total PKC Expression (arbitrary units)</th>
<th>DR</th>
<th>DP/A</th>
<th>DP/C</th>
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<tbody>
<tr>
<td>PKCα</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>PKCβ</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>PKCε</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>PKCδ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PKCε</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>PKCθ</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>PKCη</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>PKCζ</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>PKCε</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
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</table>

Data are means ± SEM. **P** values were determined using Student’s *t* test. *P* < 0.05, †*P* < 0.01, ‡*P* < 0.001. ND, not detectable.

![Figure 3](image-url) Subcellular distribution of PKC isotypes

**FIG. 3** Correlation of plasma membrane–associated PKC to DAG levels in gastrocnemius muscle. DAG levels and PM-associated PKC levels were determined as described in RESEARCH DESIGN AND METHODS and the correlations in the individual Psammomys samples (n = 16) are plotted. Correlation coefficients (r) and **P** values are indicated.

**TABLE 3**

<table>
<thead>
<tr>
<th>Subcellular distribution of PKC isotypes</th>
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<tbody>
<tr>
<td>Membrane/(Cytosolic + Membrane) (%)</td>
</tr>
<tr>
<td>PKCα</td>
</tr>
<tr>
<td>PKCβ</td>
</tr>
<tr>
<td>PKCε</td>
</tr>
<tr>
<td>PKCθ</td>
</tr>
<tr>
<td>PKCη</td>
</tr>
<tr>
<td>PKCζ</td>
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<tr>
<td>PKCε</td>
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</tbody>
</table>

Data are means ± SE. **P** values were determined using Student’s *t* test. *P* < 0.05, †*P* < 0.01, ‡*P* < 0.001 vs. DR; §*P* < 0.01 vs. DP/A.
PKCε OVEREXPRESSION PRECEDES DIABETES

FIG. 3. Correlation of PKCε expression level to serum glucose, insulin, and triglyceride levels. ●, nondiabetic animals (DR + DP/A); ○, diabetic animals (DP/C). There was no significant correlation with any of the parameters in nondiabetic animals (●) nor in all the animals (● + ○).

this finding ($P < 0.001$). Similar effects could be detected for other classic and novel PKC isoforms, but not for atypical PKC isoforms (24).

Because PKCε overexpression and activation led to decreased IR expression, we would obviously expect an effect on IR downstream signaling. To test this hypothesis, we determined the activity of PKB/Akt, which upon insulin stimulation is activated through the IR via the phosphatidylinositol 3-kinase pathway. HEK293 cells were transfected with either the IR alone or a combination of IR and PKCε and stimulated with insulin or TPA plus insulin, respectively. Cells were then lysed; proteins were separated on SDS-PAGE and subjected to Western blotting. The respective bands of the IR and Akt were detected with specific antibodies and quantified. For Akt, we determined the activity of PKB/Akt, which upon insulin stimulation is activated through the IR via the phosphatidylinositol 3-kinase pathway. In this study, we provide evidence that several PKC isoforms ($\alpha, \epsilon, \zeta$) are overexpressed in the skeletal muscle of diabetic $P. obesus$, taking the individuals from the DR line as a basal reference. This may indicate that this overexpression could be the primary reason for the insulin resistance in these animals. It is not a general phenomenon of all PKC isoenzymes, because, for example, PKC $\beta_1$, $\beta_II$, $\theta$, and $\eta$ expression levels are unchanged. Most notable is the expression of PKCε in the skeletal muscle of these animals and may be an early event or mark an inherent susceptibility to the development of the disease. Overexpression of PKCε alone seems to have no consequences for the animals as long as they are restricted in their diet. Only after the introduction of a high-calorie diet is the increased expression of PKCε also reflected in higher PM association and thus presumably increased activity of the enzyme. The finding of increased plasma membrane association and therefore probable chronic activation of PKCε in diabetic DP/C, but not prediabetic DP/A animals, suggests that factors contributed by the diabetic milieu, i.e., hyperlipidemia, hyperglycemia, and/or hyperinsulinemia, are required to activate PKCε. Therefore, in light of a multifactorial origin for diabetes, the overexpression of PKCε might explain the genetic susceptibility of the DP line of $P. obesus$ to the development of insulin resistance, which, together with environmental influences, may lead to development of the disease.

DISCUSSION

PKC overexpression is particularly distinctive in the skeletal muscle of these animals but may also play a role in other insulin target tissues such as liver. In fact, when we determined the expression level of PKCε in the liver samples of the animals, we detected a trend of increased PKCε expression in the diabetic group; however, a
FIG. 4. Effect of PKCε overexpression on IR expression level and Akt activation. A: HEK293 cells were transfected with IR expression plasmid alone or in combination with PKCε expression plasmid as indicated and starved overnight in medium containing 0.5% fetal calf serum. As a control, we cotransfected a GST construct in all samples. The cells were treated with 1 μmol/l TPA for the times indicated. Cell lysates were subject to SDS-PAGE, transferred to nitrocellulose, and incubated with anti-IR-specific antibodies (upper panel). Proteins were made visible using horseradish-peroxidase–coupled secondary antibodies and the enhanced chemiluminescence detection method. The middle panel shows PKCε expression levels, and the lower panel shows GST expression levels, as a control.

B: Quantitation and statistical analysis of IR expression levels. Data are expressed as percentage of control (IR alone, no stimulation) and represent means ± SE of n = 6 independent experiments. P values were determined using Student’s t test. *P < 0.05 vs. IR alone, **P < 0.001 vs. both IR alone and IR + PKCε unstimulated samples.

C: HEK293 cells were transfected with IR expression plasmid alone or in combination with PKCε expression plasmid as indicated and starved overnight in medium containing 0.5% fetal calf serum. The cells were left untreated or stimulated with 10−7 mol/l insulin for 5 min or treated with 1 μmol/l TPA for 6 h before 10−7 mol/l insulin stimulation for 5 min, as indicated. Cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with specific antibodies against the IR β-subunit, Akt, or phospho-Akt (Ser 473). Proteins were made visible using horseradish-peroxidase–coupled secondary antibodies and the enhanced chemiluminescence detection method on Fujifilm LAS1000 Luminescent Image Analyzer and were quantified using the program Fujifilm Image Gauge. The diagram shows the means ± SD of three independent experiments, performed in duplicate. The data are normalized to the expression levels in cells transfected with IR alone and stimulated with insulin.
A larger number of samples would be needed to determine whether this was significant. Overexpression of PKC has previously been demonstrated in the liver of humans and rats with type 2 diabetes (8). In skeletal muscle of high-fat–fed rats (9), chronic activation of PKC isoforms ε and δ was observed. This was connected with decreased expression levels of PKCδ and no changes in PKCε expression level. In this study, the rats were fed a diet containing >30% fat, representing ~60% of their caloric intake. They were insulin-resistant, as determined during an euglyce-

FIG. 5. A: Correlation between PKC expression levels and 125I-insulin binding in muscle homogenates. PKC expression levels and 125I-insulin binding in the individual Psammomys samples (n = 22) are plotted. B: Linear regression analysis of PKCε associated to plasma membrane fraction versus 125I-insulin binding in muscle homogenates. PKCε associated to PM fraction and 125I-insulin binding in the individual Psammomys samples (n = 22) are plotted. Correlation coefficients (r) and P values are indicated.
mic-hyperinsulinemic clamp, but only mildly hyperinsulinemic or hyperglycemic. It was speculated that elevated DAG levels originating from lipolysis of two- to threefold increased triglyceride content in the muscles was responsible for increased PKC activation. This is different from the Psammomys that were fed a regular diet containing only ~4% of fat, whose insulin resistance stemmed from a caloric load inappropriate to their metabolic capacity. Interestingly, however, we detected increased DAG levels in the DP line of P. obesus. The elevated DAG levels, possibly arising from increased synthesis and breakdown of glycerolipids, correlated well with membrane-associated fractions of PKCa and ε, suggesting that they may be causally related to the activation of these PKC isoforms. Increased DAG levels in aorta, heart, and retina of hypergalactosemic and diabetic dogs and rats have been suggested to be responsible for the activation of PKCb (25,26). PKC enzyme activity and levels of immunoreactive PKCa, β, ε, and δ were shown to be increased in the membrane fractions of Goto-Kakizaki rats (10). Furthermore, an increased activity of PKC was linked to reduced IR phosphorylation in the liver of starved rats (27). These data, however, are in contrast to another study that found a decreased expression of PKCa, β, and ε in the soleus muscle of Zucker obese (fa/fa) rats (28). In most of these studies, the elevated expression/activation of PKC seemed to be a consequence of the diabetic milieu and therefore a latent in the development of the disease, correlating with muscle triglyceride levels (9) or hyperinsulinemia (10). The Psammomys have the advantage of being extremely well characterized with respect to the development of diabetes, and relatively homogeneous groups of animals representing the different stages (A, B, C, D) of the disease (4) can be studied. We can show that there is no correlation between PKC overexpression and hyperinsulinemia or serum triglyceride levels. In this animal model, overexpression of PKCe is clearly primary to the development of a diabetic milieu. Of course, it is possible that changes in the diabetic milieu add to the overexpression/activation of PKC in DP/C. Although it has been suggested that PKC activation arises from increased glucose incorporation into DAG (25,26), neither our own nor other studies (9,10) in diabetic animals show conclusive evidence that hyperglycemia could cause the observed changes. Hyperglycemia-induced changes of PKC activity (14) may, however, be an important factor in later stages of diabetes, in which PKCb has been shown to be responsible for late complications of the disease (29).

Several studies in different cell lines as well as in vitro studies have suggested that PKC directly interferes with insulin signaling through serine/threonine phosphorylation of either the IR itself or one of its major substrates, the insulin receptor substrate 1 (IRS-1) (14–17,30). There seems to be agreement that the ε isoform of PKC, in particular, is subject to regulation in diabetic individuals (7–10). Interestingly, an in vitro study provided evidence that PKCe may play an important role in mediating the inhibitory effect of tumor necrosis factor-α on insulin signaling (31,32), the major cause of obesity-linked insulin resistance. It is believed that serine/threonine phosphorylation of IRS-1, via a feedback loop, is able to inhibit the tyrosine kinase activity of the IR (33). In P. obesus, insulin resistance becomes considerably accentuated at stages B and C on the HE diet. This was shown to be associated with impaired IR function, which seems to be related to a decrease in the amount of phosphorylated tyrosine residues on the IR β-subunit (34). The extent of insulin-induced activation of the IRK was markedly reduced in stages B and C, using stage A as a reference. Hyperinsulinemia by itself was shown to be a detrimental factor for IR activation (34), because development of hyperinsulinemia, even without hyperglycemia, was associated with a decrease in insulin-induced IRK activation in liver and muscle. In the skeletal muscle, hyperinsulinemia was accompanied by a reduction in basal IRK activity. Diet restriction leading to the return of insulin level to normal restored the IRK activity (4,34). Therefore, the overexpression of PKC, particularly PKCe, may be an additional factor inducing the IR malfunction. In the present study, we provide evidence that, in addition to serine/threonine phosphorylation of the IR or IRS-1, a downregulation of IR numbers could be a possible mechanism for induction of insulin resistance. Skeletal muscle of diabetic Psammomys DP/C showed a reduction in 125I-insulin binding, most probably representing a decreased number of IRs, although we cannot completely rule out changes in the affinity for insulin. In fact, there was a clear correlation between PKCe expression levels or activity and 125I-insulin binding. This is in good agreement with previous reports showing that PKCe is capable of downregulating tyrosine kinase receptors (18) and that several PKC isoforms mediate degradation of the IR (24). The significant inverse correlation between insulin binding and PKCe expression further suggests that the PKC-induced downregulation of IR number is a causal factor in insulin resistance characterizing P. obesus. Insulin resistance is generally believed to have a major impact on skeletal muscle, liver, and adipose tissue. Surprisingly, mice with a targeted disruption of the IR gene in skeletal muscle displayed elevated levels of fat mass, serum triglycerides, and free fatty acids but never developed diabetes, suggesting that insulin sensitivity in muscle may not be the primary cause for the development of diabetes (35). However, the knockout mice reflect an isolated effect of IR expression in skeletal muscle, whereas in Psammomys, we study the progression of the disease in a much more complex situation of nutritionally induced diabetes. We describe only one aspect of several factors that may lead to insulin resistance in these animals. Other factors, such as the inhibition of IR tyrosine phosphorylation (34) and effects from other tissues, are contributing to the pathogenesis of diabetes. This is also a likely scenario in the context of a growing number of reports that suggest that type 2 diabetes is a multifactorial disease (36). In particular, this has been very elegantly demonstrated in heterozygotes combining several knockouts of genes of the insulin signaling pathway. It is also worthwhile to note that Psammomys has a very small number of IRs compared with the Albino rat (34). Therefore, even a minor change in IR number could have an influence on insulin signaling. In addition, the impact of decreased IR number could become more overt if, at the same time, IR tyrosine phosphorylation is inhibited (34).

Smaller changes in PKCe expression level and insulin binding (data not shown) were found in the liver of Psam-
momys. The hepatic insulin resistance is manifest mainly by nonsuppression of gluconeogenesis by insulin. This aspect of insulin resistance may not be exclusively related to the overexpression of PKCε.

In summary, we have demonstrated changes in the expression level and activity of PKC in gastrocnemius muscle of P. obesus. Our data suggest that, in particular, PKCε could be causally related to the development of insulin resistance in these animals. However, our correlative studies do not provide evidence of causality between PKCε activation and in vivo insulin resistance, although the proposed mechanism of its inhibitory action on insulin signaling through downregulation of IR numbers is consistent with such a model. Further work is required to establish a causative link between the observed changes in PKC expression/activity and the development of insulin resistance.

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