Protein kinase C isoforms ε, η, δ and ζ in murine adipocytes: expression, subcellular localization and tissue-specific regulation in insulin-resistant states

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The Ca^{2+}-insensitive protein kinase C (PKC) isoforms ϵ, η, δ and ζ are possible direct downstream targets of phosphatidylinositol 3-kinase (PI3-K), and might therefore be involved in insulin signalling. Although isoform-specific changes in PKC expression have been reported for skeletal muscle and liver in insulin-resistant states, little is known about these isoforms in adipocytes. Therefore we studied (1) expression and subcellular localization of these isoforms in murine adipocytes, (2) translocation of specific isoforms to membranes in response to treatment with insulin and phorbol 12-myristate 13-acetate (PMA) and (3) regulation of expression in insulin-resistant states. The PKC isoforms ϵ, η, δ and ζ are expressed in adipocytes. Immunoreactivity for all isoforms is higher in the membranes than in the cytosol, but subcellular fractionation by differential centrifugation shows an isoform-specific distribution within the membrane fractions. PMA treatment of adipocytes induces translocation of PKC-ϵ and -δ from the cytosol to the membrane fractions. Insulin treatment does not alter the subcellular distribution of any of the isoforms. 3T3-L1 adipocytes express PKC-ϵ and -ζ, and PKC-ϵ expression increases with differentiation from preadipocytes to adipocytes. PKC-ϵ expression decreases in an adipose-specific and age/obesity-dependent manner in two insulin-resistant models, the brown-adipose-tissue-deficient mouse and db/db mouse compared with control mice. We conclude that, although none of the isoforms investigated seems to be activated by insulin, the decrease in PKC-ϵ expression might contribute to metabolic alterations in adipocytes associated with insulin resistance and obesity.

INTRODUCTION

The involvement of protein kinase C (PKC) in insulin action and insulin resistance remains controversial. Whereas some groups have shown that insulin induces increased PKC activity or immunoreactivity in membranes of insulin-responsive tissues such as adipose tissue, skeletal muscle and liver, other groups have not been able to show this (reviewed in [1]). Phorbol esters, which activate PKC, have insulin-agonistic effects such as stimulation of glucose transport and GLUT4 translocation, and PKC inhibitors like staurosporine block insulin effects [2]. However, prolonged treatment with phorbol esters and thus down-regulation of PKC often fails to impair insulin action (reviewed in [3]) and this raises doubt as to whether PKCs are necessary for insulin action or are part of the normal insulin signalling pathways. However, several lines of evidence support the involvement of PKCs in the development of insulin resistance [1].

The discovery of several isoforms and subgroups of PKCs complicates interpretation of previous results: activation requirements and substrate specificity varies among different isoforms, the response to prolonged phorbol ester treatment is not uniform (reviewed in [4]) and the subcellular localization, mediated at least in part by specific binding proteins, seems to play an important role in the physiological action of PKCs [5]. Consequently results obtained before the knowledge of the PKC heterogeneity need to be critically re-evaluated and the question of PKC involvement in insulin action or insulin resistance needs to be addressed by testing specific isoforms in specific tissues.

Glucose transport is mediated by a family of facilitated diffusion glucose transporters which are encoded by distinct genes [6]. The glucose transporter GLUT4 is unique in that it is expressed almost exclusively in muscle and fat [7], and in the absence of insulin it is sequestered in an intracellular pool [8]. In response to insulin stimulation GLUT4 is translocated to the cell surface.

Several recent studies suggest that activation of phosphatidylinositol 3-kinase (PI3-K) is involved in GLUT4 translocation and thus in insulin-stimulated glucose uptake in muscle and adipocytes [9,10]. However, the signalling downstream of PI3-K is unclear. Studies in cell-free systems suggest that the products of PI3-K, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, can stimulate the PKC isoforms δ, ϵ, η and ζ [11,12]. Thus, these Ca^{2+}-independent PKC isoforms are candidates for the mediators of pathway(s) from PI3-K to GLUT4 translocation. Further support for a possible role for these isoforms in the exocytotic process of GLUT4 translocation comes from studies showing involvement of PKCs in vesicle movement [13] and PKC-ϵ specifically in secretion [14]. The potential involvement of these atypical/novel isoforms in insulin action and glucose homoeostasis is further suggested by alterations in PKC-ϵ and -ζ expression in insulin-resistant states in muscle and liver [15,16] and finally translocation of PKC-ζ under high-glucose conditions [17] and after insulin stimulation [18].

Whereas none of these Ca^{2+}-independent isoforms seems to be expressed at high levels in skeletal muscle where PKC-θ is dominant [19], expression of PKC-ϵ, -δ and -ζ has been shown in adipocytes [18] where PKC-η has not been studied. The aim of

Abbreviations used: BAT, brown adipose tissue; DMEM, Dulbecco's modified Eagle's medium; HDM, high-density microsomes; HES, Hepes/EDTA/sucrose; KRBH, Krebs–Ringer/bicarbonate/Hepes; LDM, low-density microsomes; Nu/Mit, nuclear-mitochondrial; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PM, plasma membranes; PMA, phorbol 12-myristate 13-acetate; TBS, Tris-buffered saline; TES, Tris/EDTA/sucrose

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this study was to gain further information about the potential role of the Ca^{2+}-independent PKC isoforms in insulin action and insulin resistance. We have determined the relative level of expression of the PKC isoforms δ, ϵ, η and ζ in rodent adipose tissue compared with other tissues, the subcellular distribution of these isoforms in adipocytes, and the impact of insulin treatment compared with treatment with phorbol 12-myristate 13-acetate on this subcellular distribution. Furthermore we have demonstrated tissue-specific and isoform-specific alterations in PKC expression in rodent models of insulin resistance.

MATERIALS AND METHODS

Experimental animals

Mice (FVB) and rats (Sprague–Dawley CD, both from Charles River Laboratory, Wilmington, MA, U.S.A.) were housed in accordance with institutional guidelines on a 12 h light/12 h dark cycle under constant temperature and humidity. Water and food (Purina Chow no. 5008 or Teklad Adjusted Calories Western-Type diet no. 88137) were available *ad libitum*. Mice and rats were killed by CO_2 inhalation. Brown adipose tissue (BAT)-deficient mice (UCP-DTA) were generated by expression of the diphtheria toxin A chain (DTA) specifically in BAT driven by the uncoupling protein (UCP) promoter as previously described [20]. C57BL + db/ + db mice and m + / + db controls were purchased from Jackson Laboratory, Bar Harbor, ME, U.S.A.

Preparation of isolated adipocytes

Isolated adipocytes were prepared by collagenase (1 mg/ml) digestion of perigonadal fat-pads as previously described [21,22]. Cells were incubated in a 20 % (v/v) suspension in Krebs–Ringer/bicarbonate/Hepes buffer (KRBH)/200 nM adenosine/2.5 % BSA for mouse adipocytes or 1 % BSA for rat adipocytes with or without 80 nM insulin (crystalline; porcine; gift from R. Chance, Eli Lilly, Indianapolis, In, U.S.A.) or 1 μM PMA (Sigma, St. Louis, MO, U.S.A.; stock in DMSO) as indicated in the Figure legends. DMSO was added as appropriate to give identical conditions in all incubations. Cell number was evaluated by fixing cells in osmic acid and counting in a Coulter counter [23]; total lipid was determined by the method of Dole [24]. Cell size was calculated by dividing total lipid per aliquot by cell number per aliquot [23].

Preparation of membrane fractions

Total membranes and cytosol from adipocytes were prepared by washing the 20 % cell suspension once in KRBH with adenosine but without BSA. Using a Potter homogenizer, the floating cells were then homogenized in 2 vol. of HES buffer (10 mM Hepes, 5 mM EDTA, 250 mM sucrose, 2 μg/ml each pepstatin, leupeptin and aprotinin, pH 7.4; 18 °C). The homogenate was centrifuged at 1200 ***g*** at −4 °C for 15 min, the fat cake was discarded and the pellet was resuspended in the infranatant. This second homogenate was centrifuged at 220000 ***g*** for 90 min at 4 °C to pellet total membranes. The pellet was resuspended in HES; the supernatant was the cytosol. The protein content of these fractions was determined using the bicinchoninic acid/copper(II) sulphate method with BSA as standard. Total membranes and cytosol from tissues except heart and skeletal muscle were prepared in a similar way using a Polytron (setting 5; 30 s) instead of the Potter homogenizer. The relative volumes of tissue to HES buffer were 1:3 for fat and 1:10 for brain, liver, kidney, spleen and lung.

Membranes from hind limb muscle and heart were prepared by mincing the muscle with scissors into small pieces in SSS buffer (1 mM $NaHCO_3$, 250 mM sucrose, 5 mM NaN_3, pH 7.4) and then homogenizing with a Polytron at setting 5 for 30 s. The tissue/buffer ratio was 1:10. The homogenate was centrifuged at 1200 ***g*** for 10 min at 4 °C and the first pellet was resuspended in SSS buffer. The supernatants of two rounds of centrifugation were combined and subjected to a third centrifugation at 220000 ***g*** for 90 min at 4 °C. The membrane fraction (pellet) was resuspended in SSS buffer.

Subcellular fractionation of isolated rat adipocytes was performed by differential centrifugation as described previously [25]. Briefly, after treatment of the 20 % cell suspension with insulin or PMA, cells were allowed to float at 37 °C, the infranatant was discarded and cells were washed once into 20 mM Tris/HCl 1 mM EDTA/250 mM sucrose buffer (TES, pH 7.4) at 18 °C. The floating cells were homogenized by 10 strokes in a Potter homogenizer and the homogenate was subjected to centrifugation at 16000 ***g*** at −4 °C for 15 min. The fat cake was discarded, the pellet was resuspended in TES buffer and again centrifuged at 16000 ***g*** at 4 °C for 20 min. The resulting pellet was resuspended in TES, layered on top of a 1.12 M sucrose cushion and centrifuged at 48000 ***g*** at 4 °C for 30 min. The resulting interphase [plasma membrane (PM)] and pellet [nuclear/mitochondrial fraction (Nu/Mit)] were resuspended separately in TES and the proteins were recovered by centrifugation at 48000 ***g*** at 4 °C for 30 min (PM) or 16000 ***g*** at 4 °C for 15 min (Nu/Mit). The supernatant of the first 16000 ***g*** centrifugation was centrifuged at 48000 ***g*** at 4 °C for 30 min, and the pellet was resuspended in TES and centrifuged again under the same conditions to give the high-density microsome (HDM) fraction. The supernatant from the 48000 ***g*** spin was centrifuged at 212000 ***g*** at 4 °C for 90 min to pellet the low-density microsome (LDM) fraction.

Cultured 3T3-L1 adipocytes

3T3-L1 fibroblasts were kept in Dulbecco's modified Eagle's medium (DMEM, with 2 mM glutamine, 25 mM glucose, 50 units/ml penicillin and 50 μg/ml streptomycin) supplemented with 10 % calf serum. When cells were confluent, the medium was changed to DMEM with (10 %, v/v) fetal calf serum and differentiation was induced with 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μM dexamethasone and 1 μg/ml insulin for 3 days followed by incubation with DMEM and fetal calf serum for an additional 7 days. Before harvest, cells were starved of serum overnight and washed in PBS. The cells were scraped into cold HES buffer and total membranes were prepared as described above for adipocytes.

Western-blot analysis

SDS/PAGE and electrophoretic transfer of proteins were performed as previously described [26,27] using 1.5 mm-thick 8 or 10 % minigels (Novex, San Diego, CA, U.S.A.), nitrocellulose membranes (0.45 μm; Schleicher & Schuell, Keene, NH, U.S.A.) and the Mini Trans-blot Transfer cell (Bio-Rad, Hercules, CA, U.S.A.) with Towbin buffer (20 % methanol). The quality of transfer and loading of lanes was checked by Ponceau S staining of the nitrocellulose and Coomassie Brilliant Blue staining of the gel after transfer. Prestained molecular-mass markers (Bio-Rad) were phosphorylase *b* (112 kDa), BSA (84 kDa), ovalbumin (53.2 kDa) and carbonic anhydrase (34.9 kDa). Baculovirus-expressed PKC isoforms (described in [11]) were used as standards and were generously provided by A. Toker, Department of Medicine, Beth Israel Hospital, Boston. Isoform-specific polyclonal PKC antibodies were purchased from Gibco (PKC-ϵ, -δ, -ζ) and Santa Cruz Biotechnology (PKC-η), polyclonal GLUT4

antiserum was provided by H. Haspel, Henry Ford Health Center, Detroit, MI, U.S.A. and monoclonal H-ras antibody was purchased from Oncogene Science, Uniondale, NY, U.S.A. Using Tris-buffered saline (TBS) with 0.05 % Tween (PKC-ϵ, -η and -ζ), 0.1 % Tween (PKC-δ) or 0.2 % Tween (GLUT4), the nitrocellulose was blocked with 5 % milk and incubated with primary antibody overnight. Bands were visualized using the ECL system (Amersham, Arlington Heights, IL, U.S.A.), and quantification was performed by scanning and using the Image Quant software (Molecular Dynamics, Benton, NJ, U.S.A.). For preparation of Figures, Western blots were scanned using an Agfa Studioscan II with transparency module.

RESULTS

Tissue distribution and subcellular localization of PKC isoforms

To assess expression of the PKC isoforms ϵ, η, δ and ζ in adipocytes, immunoreactivity was determined in preparations of mouse adipocyte total membranes with isoform-specific antibodies as shown in Figure 1. PKC isoforms expressed in the baculovirus system were run as standards next to the molecular-mass markers and resulted in isolated immunoreactive bands at approx. 90 kDa (ϵ), 80 kDa (η) and 75 kDa (δ and ζ). In adipocyte membranes, corresponding bands at the same molecular mass as each standard were seen for each isoform. The first panel shows a single 90 kDa band for PKC-ϵ in mouse adipocyte membranes. The first lane contains 40 μg of membranes from adipocytes from obese diabetic +db/+db mice which explains the decreased intensity of the band compared with the adjacent lane with protein from m+/+db lean controls. In all other panels both lanes are loaded with adipocyte membrane protein from FVB mice. Whereas the PKC-ϵ antiserum showed no additional bands, a doublet of higher molecular mass appeared with the PKC-η antiserum (second panel) which in every case was regulated in parallel with the specific band which was the same size as the PKC-η standard. For PKC-δ and -ζ several additional immunoreactive bands were detected but these showed no parallel regulation with the 75 kDa band and were determined to be non-specific. Each of the isoform-specific antisera did not cross-react with the other three baculovirus-expressed PKC isoforms in Western blotting (results not shown).

To compare the relative level of expression in adipose tissue with that in other tissues, total membranes and cytosol were prepared from mouse adipose tissue, skeletal muscle, heart, liver, brain, kidney, spleen and lung. Equal amounts of protein were used for Western blotting with isoform-specific antisera, as shown in Figure 2. PKC-ϵ is expressed at highest levels in brain, but can clearly be detected in all other tissues. The cytosolic form (c) appears to be of slightly higher molecular mass in all tissues, and the relative amounts of the cytosolic compared with the membrane-bound form (m) differs in each tissue. Whereas heart, brain and spleen show approximately the same immunoreactivity in both cytosol and membranes, the other tissues show more immunoreactivity in the membrane fraction. However, it should be stressed that, because of the large amount of protein in the cytosolic compartment, a weak signal in the cytosolic fraction may represent a proportionally larger amount of the isoform per cell than a stronger signal in the membrane compartment. PKC-η and -δ can also be detected in all tissues (except PKC-δ in liver) and are present almost entirely in the membrane fraction. PKC-ζ is also most abundant in membranes in all tissues, but this isoform is also present in substantial amounts in the cytosolic fraction of all tissues.

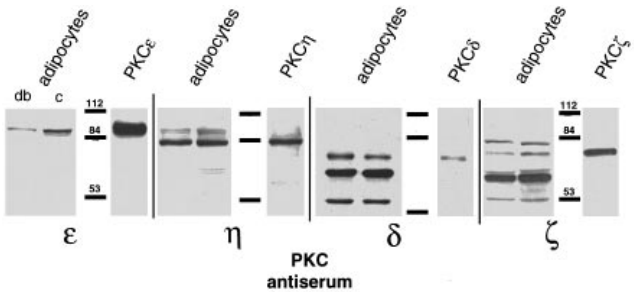

Figure 1 Specificity of PKC antisera

Western blots of total membranes prepared from isolated mouse adipocytes as described in the Materials and methods section were incubated with antisera specific for PKC-ϵ, -η, -δ and -ζ as indicated. In each panel the first two lanes contain 40 μg of total membranes from adipocytes. The third lane shows molecular-mass markers and the fourth lane shows standards consisting of the indicated PKC isoform expressed in the baculovirus system. Note that the first lane in the first panel (PKC-ϵ) is loaded with membranes from +db/+db mouse adipocytes (db), which explains why the immunoreactivity is weaker than in the adjacent lane with membranes from m+/+db lean littermates (c).

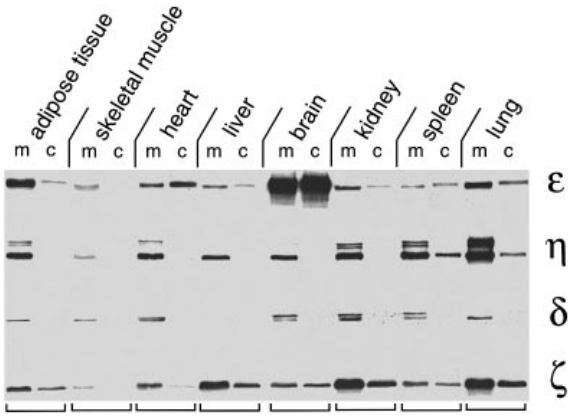

Figure 2 Tissue and subcellular distribution of PKC isoforms

Western blots of membranes (m) and cytosol (c) from different mouse tissues prepared as described in the Materials and methods section. Each lane was loaded with 40 μg of protein. The PKC-isoform-specific antiserum used is specified on the right-hand side of each blot. This is representative of three Western blots from two independent experiments.

Effects of adipocyte differentiation on expression of PKC isoforms

To determine whether the expression of these isoforms changed with adipocyte differentiation, we assessed the amount of the isoforms in total membranes of 3T3-L1 fibroblasts and 3T3-L1 adipocytes 10 days after initiation of differentiation as shown in Figure 3(a). Whereas PKC-η and -δ could not be detected even with considerable overexposure of the Western blots (not shown), PKC-ϵ and -ζ are expressed in 3T3-L1 fibroblasts. With differentiation to adipocytes, PKC-ϵ expression increases twofold (assessed by densitometry), whereas PKC-ζ showed only minor changes. An additional band of slightly higher molecular mass appeared above PKC-ζ upon differentiation, which might well be a modified form of this isoform. Figure 3(b) shows total membranes (TM) and cytosol (Cyto) of untreated (B) and PMA-treated differentiated 3T3-L1 adipocytes. Compared with the distribution in adipose tissue and isolated primary rodent adipocytes, the amount of immunoreactivity in the cytosolic fraction relative to the membranes is increased for both PKC-ϵ and -ζ.

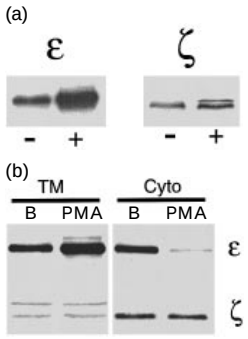

Figure 3 Effect of adipocyte differentiation on expression of PKC isoforms (a) and effect of PMA on the subcellular distribution of PKC-ε and -ζ in 3T3-L1 adipocytes (b)

(**a**) Western blots of total membranes prepared from undifferentiated 3T3-L1 fibroblasts (−) and 3T3-L1 adipocytes 10 days after initiation of differentiation (+) as described in the Materials and methods section. Each lane contained 25 μg of membrane protein. The PKC-isoform-specific antiserum used is specified above the bands. (**b**) Western blots of membranes from differentiated 3T3-L1 adipocytes in the basal state (B) or after 10 min of 1 μM PMA treatment. Each lane was loaded with 20 μg of protein for total membranes (TM) and cytosol (Cyto) prepared as described in the Materials and methods section. This is representative of three Western blots from two independent experiments.

PMA treatment (10 min; 1 μM) leads to a marked translocation of PKC-ϵ but not -ζ.

Effects of insulin and PMA treatment on subcellular localization of PKC isoforms in primary adipocytes

Translocation of PKCs in response to a stimulus is generally regarded as a sign of activation [4]. To identify a possible translocation in response to insulin and to obtain additional information about the subcellular localization of the different isoforms, we performed subcellular fractionation of isolated rat adipocytes before and after 80 nM insulin stimulation for 10 min.

Figure 4 shows the result of Western blots of PM, LDM, HDM and Nu/Mit fractions. Identical amounts of protein were loaded for each fraction. The cytosolic fraction in these preparations was too dilute to give clear results and is not shown. PKC-ϵ shows highest immunoreactivity in the PM fraction and this does not change on treatment with insulin. PKC-ϵ is also present in the HDM and Nu/Mit fractions but is virtually absent from the LDM fraction. PKC-η is most abundant in the Nu/Mit and PM fractions and gives weak signals in the HDM and LDM fractions. Its abundance is also not affected by insulin. PKC-δ is present in the PM, HDM and Nu/Mit fractions, and gives only a very weak signal in the LDM fraction. PKC-ζ is present in every fraction, shows the weakest signal in the Nu/Mit fraction and is the only isoform present in a relatively large proportion in the LDM fraction.

None of the isoforms shows translocation to any fraction with insulin treatment. As a control for insulin action, GLUT4 immunoreactivity in the PM and LDM fractions is shown. As expected, GLUT4 is translocated out of the LDM and increases in PM with insulin treatment. To monitor potential contamination of internal membranes with PM, immunoreactivity for p21^{ras}, which can be used as a PM marker, was determined in the membrane fractions. As can be seen in Figure 4, p21^{ras} is enriched in the PM fraction and is virtually absent from the

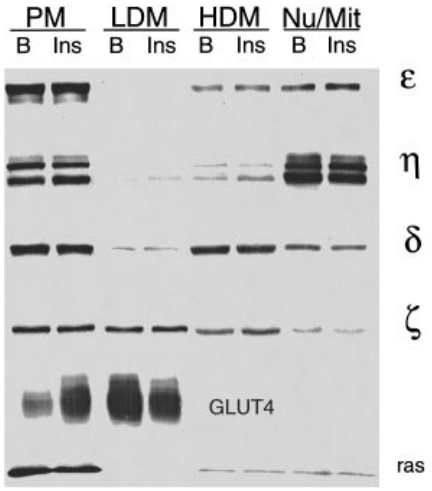

Figure 4 Effect of insulin treatment on the subcellular distribution of PKC isoforms

Western blots of subcellular fractions (PM, LDM, HDM, NU/Mit; 20 μg of protein/lane) from isolated rat adipocytes prepared by differential centrifugation as described in the Materials and methods section. B, basal; Ins, 10 min treatment with 80 nM insulin. The PKC-isoform-specific antiserum used is specified on the right-hand side of each blot. To demonstrate the effects of insulin treatment, PM and LDM fractions were incubated with GLUT4-specific antiserum. To characterize the fractionation procedure, the fractions were incubated with antiserum against the PM marker p21^{ras}. For each of two independent experiments, membranes were prepared from adipocytes pooled from six male rats (176–200 g body weight).

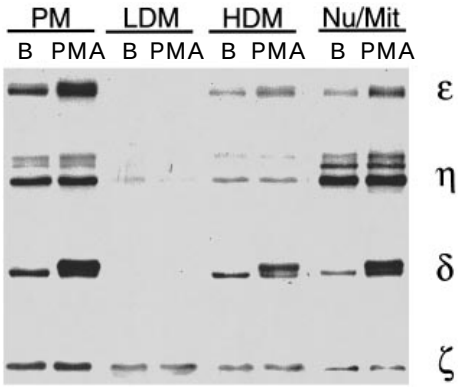

Figure 5 Effects of PMA on the subcellular distribution of PKC isoforms

Western blots of subcellular fractions (PM, LDM, HDM, Nu/Mit; 20 μg of protein/lane) from isolated rat adipocytes (pooled from six male mice, 176–200 g body weight) prepared by differential centrifugation as described in the Materials and methods section. B, Basal; PMA, 10 min of treatment with 1 μM PMA. The PKC-isoform-specific antiserum used is specified on the right-hand side of each blot.

LDM fraction with very low levels in the HDM and Nu/Mit fractions, indicating almost no contamination of internal membranes with PM.

Subcellular fractionation was performed after 10 min stimulation with insulin, and in other experiments cytosol and total membranes from mouse adipocytes were also prepared after 5 and 30 min of insulin (80 nM) treatment (results not shown). There was no translocation of any PKC isoform at any of these time points of insulin stimulation. To be sure that our technique of subcellular fractionation can detect PKC translocation, isol-

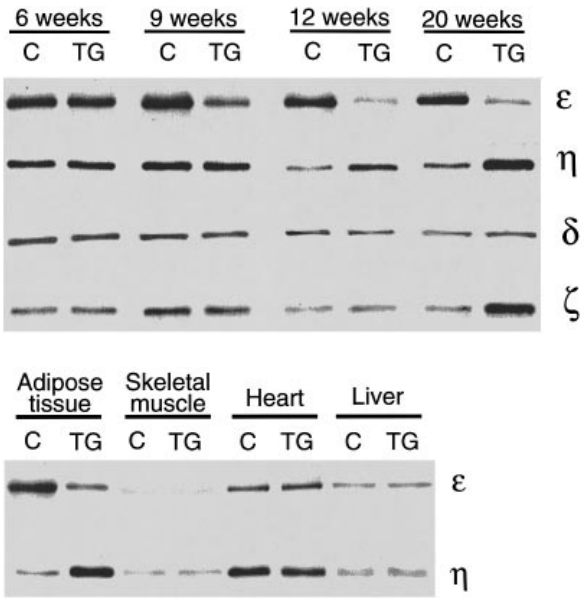

Figure 6 Changes in expression of PKC isoforms with progressive obesity and insulin resistance in tissues from the BAT-deficient mouse model

Top, Western blots of total membranes from isolated mouse adipocytes (TG, transgenic BAT-deficient mice; C, non-transgenic littermate controls) at different ages. The PKC-isoform-specific antiserum used is specified on the right-hand side of each blot. Isolated adipocytes were prepared from several animals of the same age. Each lane represents cells pooled from at least three mice. Western blots are representative of two (6 and 9 weeks of age) or three (12 and 20 weeks of age) independent experiments. Bottom, Western blots of membranes from different tissues of 20-week-old BAT-deficient mice (TG) and control littermates (C). Tissues from two animals were pooled in each of two independent experiments.

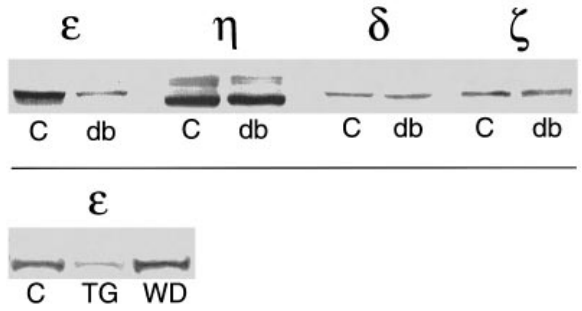

Figure 7 Changes in expression of PKC isoforms in adipocytes from the db/db and Western-diet-fed mouse models

Top, Western blots of total membranes from isolated mouse adipocytes (db, C57BL +db/+db; C, m+/+db controls). The PKC-isoform-specific antiserum used is specified above the bands. Adipocytes from two C57BL +db/+db mice and four m+/+db controls were pooled. Bottom, Western blot of adipocyte total membranes from control FVB mice on a chow diet (C), transgenic BAT-deficient mice on a chow diet (TG) and FVB mice on a Western diet (WD), all at 12 weeks of age. Pooled cells from three mice are represented in each lane. This Western blot is representative of three blots on three independent experiments using a total of nine mice for each condition.

ated rat adipocytes were stimulated with PMA. Figure 5 shows the effect of this treatment on the subcellular distribution of the isoforms. PKC-ϵ shows an increase in immunoreactivity in the PM, HDM and Nu/Mit fractions with PMA treatment. PKC-δ increases and shifts partially to a higher molecular mass in the PM, HDM and Nu/Mit fractions after PMA treatment, whereas PKC-η and -ζ show no change.

Isoform-specific regulation of PKC expression in insulin-resistant states

As there have been reports of changes in PKC isoform expression in insulin-resistant states in liver [16] and skeletal muscle [15], we looked at this expression at different ages in adipocytes of BAT-deficient mice. These mice have been shown to be a model for progressive insulin resistance and diabetes [20]. As shown in Figure 6 (top), expression of PKC-ϵ decreases as early as 9 weeks of age and declines further at 12 and 20 weeks. In contrast with this, expression of PKC-η and -ζ increases in the BAT-deficient mice compared with normal controls starting at 12 and 20 weeks respectively. PKC-δ expression is not altered at any age. In skeletal muscle, heart and liver of 20-week-old transgenic animals no differences in PKC-ϵ and -η expression were observed (Figure 6, bottom). Three different immunoblots of total membranes from adipocytes of 20-week-old BAT-deficient mice and control littermates were quantified. Compared with control values (100%), PKC-ϵ immunoreactivity decreased to 38 ± 7% and PKC-η and -ζ immunoreactivity increased to 209 ± 12% and 188 ± 3% (means $\pm$ S.E.M.) respectively in transgenic animals of this age.

As some of these changes might be unique to the BAT-deficient model, we assessed PKC expression in a different well-established model of obesity and diabetes, the db/db mouse (Figure 7). Immunoreactivity for PKC-ϵ is again substantially reduced in isolated adipocytes of the 16-week-old insulin-resistant diabetic mice, while all other isoforms are expressed at the same level in db/db and control animals.

As in most obese states, both BAT-deficient mice and db/db mice exhibit a marked increase in fat-cell size. To determine whether PKC-ϵ down-regulation is directly related to this increase in cell size, we studied normal mice fed on a Western diet for 9 weeks after weaning [21% (w/w) fat = 40.8% of calories, 49.2% (w/w) carbohydrate = 42.2% of calories and 19.8% (w/w) protein = 17% of calories; compared with chow 6.5% (w/w) fat = 17.3% of calories, 47% (w/w) carbohydrates = 55.1% of calories and 23.5% (w/w) protein = 27.6% of calories]. Cell size in these non-transgenic animals on a Western diet (0.73 ± 0.01 μg/cell) and in age-matched BAT-deficient mice (0.81 ± 0.02 μg/cell) is significantly increased compared with age-matched non-transgenic control animals on a chow diet (0.43 ± 0.02 μg/cell; mean $\pm$ S.E.M.; $n = 3$). Whereas both BAT-deficient mice on a chow diet and non-transgenic mice on a Western diet show increased fat-cell size, only the BAT-deficient mice show *in vivo* and *in vitro* insulin resistance as reflected by a 50-fold increase in fed insulin levels [28] and decrease in insulin-stimulated glucose uptake in isolated adipocytes (results not shown). Mice on a Western diet have normal fed insulin and glucose levels as well as normal basal and insulin-stimulated glucose uptake in adipocytes. As shown in the lower panel of Figure 7, PKC-ϵ expression is not changed in these animals compared with chow-fed littermates. Thus the decrease in PKC-ϵ expression does not appear to be due to increased cell size.

DISCUSSION

The overall goal of our study was to determine whether the Ca^{2+}-independent PKC isoforms could potentially be involved in insulin action or insulin resistance. We began by determining the tissue distribution at the protein level. Although some data for the tissue distribution (not including adipose tissue) of the PKC

isoforms ϵ, η, δ and ζ are available at the RNA level [29–31], data for the protein level are scarce, scattered and often restricted to the membrane or cytosolic fraction of a few tissues or cell types [32–34]. The more extensive tissue distribution and assessment of relative abundance in membranes versus cytosol in this study fills this gap and shows the interesting finding that the ratio of the cytosolic form to the membrane-bound (and therefore probably active) form of each PKC isoform *in vitro* varies in a tissue-specific manner. This tissue specificity might be caused by differences in expression of anchoring proteins and PKC-activating cell surface receptors or accessibility to hormones and growth factors.

The insulin-sensitive tissues express all investigated isoforms, and as shown for the fed state in Figure 2, considerable amounts are associated with the membrane *in vivo*. Even prolonged food deprivation up to 36 h did not change the total amount or the ratio of the membrane-associated to the cytosolic form for each of the isoforms in mouse adipose tissue or skeletal muscle (results not shown). Since insulin levels fall significantly with starvation, this observation provides evidence against profound *in vivo* regulation of the expression or the subcellular localization (presumably reflecting activation) of these isoforms by insulin.

The lack of translocation to any membrane compartment with direct insulin stimulation of adipocytes shown in Figure 4 provides a second line of evidence against stimulation of the investigated PKC isoforms by insulin. These findings are in contrast with another report [18] that showed PKC-ζ translocation on treatment with insulin for 2–20 min. As identical antisera were used in these studies, the different findings might result from differences in adipocyte handling and homogenization. As insulin caused a typical translocation of GLUT4 and PMA caused translocation of PKC-ϵ and -δ from the cytosol to a membrane fraction, we are sure that insulin was present in sufficient amounts and PKC translocation is detectable in the experiments we performed.

PMA-stimulated adipocytes showed translocation of PKC-ϵ and -δ. The lack of response of PKC-ζ to PMA was expected, as this isoform is predicted to be insensitive to PMA because of a modified C1 domain compared with other PKCs. PKC-η was not translocated on PMA stimulation. However, even in the unstimulated adipocyte, we found PKC-η to be located almost exclusively in the membrane fraction. It has to be determined whether this indicates permanent and persistent activation or whether some isoforms can be stimulated without translocation within the cytosol or membrane fraction, as suggested by Heidenreich et al. [35]. If it is possible to activate membrane-associated PKCs, then the exact subcellular localization is of importance. Data on this are few and limited to immunocytochemical studies in cell lines [36–38]. Our data in Figures 4 and 5 show isoform-specific variation of the intracellular localization, again suggesting specific roles for each isoform rather than redundancy. Whereas some studies agree with the subcellular distribution that we see, others do not [36–38]. It has previously been suggested that conflicting data on the localization of specific PKC isoforms could result from cell-type differences or from different cell culture conditions [36]. In addition, overexpression of these proteins above endogenous levels in most other studies by stable or transient transfection might modify the distribution within the cell.

As pointed out in the Introduction, the investigated PKC isoforms are possible direct downstream targets of PI3-K. Whereas platelet-derived-growth-factor-stimulated PI3-K activity is primarily localized in the PM fraction, insulin-stimulated PI3-K activity can also be found in the LDM fraction [39], interestingly the fraction where the insulin-responsive glucose transporter GLUT4 is localized. As platelet-derived growth factor minimally stimulates GLUT4 translocation, the LDM-associated PI3-K activity might play an important role in mediating the effect of insulin on glucose transport. If PKCs are involved in insulin-stimulated glucose transport and are direct downstream targets of PI3-K, they are likely to be localized in the LDM fraction. Our results (Figure 4) show PKC-ζ and only traces of PKC-ϵ, -η and -δ in the LDM fraction. However, activation of the investigated PKCs by phosphatidylinositol phosphates might also take place in the PM fraction, where all isoforms are localized.

The isolated adipocytes expressed all the isoforms investigated, which has been demonstrated previously for PKC-ϵ, -δ, and -ζ [18] but not for PKC-η. 3T3-L1 adipocytes are a useful cell line for the study of insulin effects, as they share many properties with adipocytes [40]. Data on PKC expression in these cells are not available to our knowledge. We show that, whereas the level of expression of PKC-ϵ and -ζ is comparable with that in murine adipocytes, PKC-η and -δ are not expressed in 3T3-L1 adipocytes. This difference between primary rodent adipocytes and the cultured cell model is not necessarily surprising. One major difference is the ability of cultured cells to proliferate and differentiate, and PKCs have been shown to be involved in this regulation in other cell types [41]. Whether the lack of PKC-η and -δ is necessary for or rather a consequence of growth in culture has to be determined. The relative increase in PKC-ϵ during differentiation from fibroblast to adipocyte may suggest a specific role in adipocyte physiology.

Our data do not suggest a direct involvement of the observed PKC isoforms in the acute action of insulin in adipocytes, at least with translocation of immunoreactivity as the indicator of activation. However, our results in BAT-deficient mice demonstrate a tissue-specific divergent regulation of PKC isoform expression in adipocytes in this model of insulin resistance and diabetes. Whereas the decrease in PKC-ϵ expression occurs as early as 9 weeks of age, when the mice are insulin resistant and moderately obese but not hyperglycaemic [28], the increase in PKC-η and -ζ occurs between 12 and 20 weeks of age, when the mice develop severe obesity and hyperglycaemia. Whereas the increase in PKC-η and -ζ expression seems to be restricted to the BAT-deficient model, a substantial decrease in PKC-ϵ expression was found in adipocytes from both the BAT-deficient mice and the db/db mice. However, no change in PKC-ϵ expression was detected after feeding normal mice on a Western diet, indicating that the observed down-regulation in BAT-deficient mice and db/db mice is not merely an effect of increasing cell size. It will be interesting to determine if this decrease in expression of PKC-ϵ can also be found in humans with non-insulin-dependent diabetes mellitus and what consequence this down-regulation of PKC-ϵ has for the metabolic regulation of the adipocyte.

In summary, the PKC isoforms ϵ, η, δ and ζ are expressed in primary mouse adipocytes and show an isoform-specific subcellular distribution *in vivo*. In contrast, 3T3-L1 fibroblasts/adipocytes express only PKC-ϵ and -ζ, and PKC-ϵ expression increases twofold during differentiation. In murine adipocytes, insulin treatment for 5, 10 or 30 min does not lead to a translocation of immunoreactivity to the membrane fraction and thus does not seem to activate any of these isoforms. In contrast, PMA activates PKC-ϵ and -δ. In two rodent models of obesity and non-insulin-dependent diabetes mellitus we find decreased PKC-ϵ expression in adipocytes, and in one model we find up-regulation of PKC-η and -ζ. The tissue-specific nature of this regulation and its onset during progressive development of insulin resistance suggests a role for these changes in PKC

expression in the metabolic alterations in the adipocyte associated with obesity and insulin resistance.

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