lavage fluid in experimentally induced pulmonary inflammation, but is most abundant in adipose tissues [13]. RELMβ is specifically expressed in the intestinal tract, especially abundantly in the colon [14] and has been suggested to be related to bacterial colonisation [24]. Injecting RELMβ reportedly induced insulin resistance in rats [16]. RELMγ is expressed in the colon, bone marrow, spleen and lung [17–19] and reportedly increases the proliferation rate of promyelocytic cells and modulates their differentiation [18]. RELMγ is also expressed in rat nasal respiratory epithelium, and is altered by cigarette smoke [17]. Although the physiological roles of these isoforms are still unclear, a series of previous reports seems to suggest their interaction with inflammatory processes and/or insulin resistance.

In this study we showed that RELMγ overexpressed in and secreted by COS7 cells forms a homodimer. Homodimerisation of RELMβ and resistin, but not RELMα, was previously reported and conserved cysteines (Cys26 of resistin and Cys25 of RELMβ) were considered to be required for disulphide bond formation [23, 24]. In RELMγ, however, the corresponding cysteine is not conserved. Therefore, Cys11 or Cys45 in RELMγ may be regarded as critical for dimer formation. Considering that Cys11 is conserved while Cys45 is missing from RELMα, which is not capable of forming a homodimer, Cys45 in RELMγ is very likely to be involved in dimerisation. Interestingly, we also found that RELMβ and RELMγ secreted by COS7 cells and also endogenously expressed in colonic tissues partially heterodimerised (Fig. 4).

RELMγ reportedly binds α-defensin, a cysteine rich 3–4-kDa antimicrobial peptide stored in the cytoplasimic granules of neutrophils, some macrophages and intestinal Paneth cells [19]. Thus, it is possible that some protein(s), like α-defensin, which bind to RELMγ, also bind to RELMβ and may form polymers with disulphide bonds. In this case, this heteromeric formation may modulate the antimicrobial effects of defensin. In addition, homodimerisation and heterodimerisation of RELMγ would affect binding to other molecules such as their receptor(s) and their resultant functions. Thus, whether or not RELMγ heterodimers and homodimers have different roles in insulin resistance and/or inflammatory processes is important. Further analysis, considering the crystal structure data of resistin and RELMβ, is necessary to clarify the mechanism of homo/heterodimer formation of resistin family proteins [20].

This is the first study to investigate the serum concentrations and tissue contents of RELMβ and RELMγ in insulin-resistant animals, while resistin expression has been analysed in several models of obesity and diabetes [8, 11, 25–30]. In high-fat-fed mice and *db/db* mice, serum levels of RELMβ and RELMγ were apparently increased. Taking into consideration that RELMβ causes insulin resistance by impairing hepatic glucose production [16], it is reasonable to consider the increased serum concentrations of RELMβ and RELMγ to be among the molecular mechanisms underlying the insulin resistance in these diabetic mice. Furthermore, we demonstrated that elevated serum concentrations of RELMβ and RELMγ are attributable to increased production in the colon (both RELMβ and RELMγ) and bone marrow (only RELMγ). Since the heterodimer consisting of serum RELMβ and RELMγ was also increased, a considerable portion of the RELMβ and RELMγ in serum appears to be derived from the colon and ileum.

We observed that the PPARγ agonist rosiglitazone had no effect on expression levels (10 mg kg^{-1} day^{-1} for 2 weeks, orally; data not shown). We can suggest two possible mechanisms of RELMβ and RELMγ upregulation in the insulin-resistant diabetic mouse. One involves the expression of these isoforms being regulated by exposure of intestinal and bone marrow cells to nutrients and/or factors such as glucose, lipid, insulin or certain cytokines or hormones. The other involves signals related to inflammation triggering increased RELMβ and RELMγ expression. Since inflammation has mechanistic significance in obesity and insulin resistance [8, 31–34] and some resistin family proteins have been implicated in the inflammatory response [13, 17], increased expression of RELMβ and RELMγ may be involved in the mechanisms connecting inflammation and insulin resistance, which are associated with obesity and/or diabetes. In this regard, a transcriptional factor may be involved in the inflammation-induced increased expression of RELMβ and RELMγ. Indeed, the promoter region of RELMβ contains a binding sequence of nuclear factor κB and signal transducer and activator of transcription 6 [24] and *RELMγ* is reportedly a target gene of CCAAT/enhancer binding protein ε mediating the role of promyelocytic cell development [19]. Our other interesting finding was that the expression of RELMγ was unchanged in the spleen, lung and pancreas in the insulin-resistant as compared with the control mice. A similar observation was that RELMα expression in adipose tissue responds to food deprivation, except in the lung [16]. Thus, it seems that organs specifically responding to nutritional or inflammatory conditions have a system which allows expression of RELMα and RELMγ to be regulated, although the underlying molecular mechanism is unclear.

In summary, adipose tissue and the intestinal tract are major tissues which produce RELM family proteins (resistin and RELMα from adipose tissue, and RELMβ and RELMγ from the intestinal tract). The production of RELMβ and RELMγ in the intestinal tract was clearly demonstrated to be increased in diet-induced and genetically obese mice, with elevated serum concentrations suggesting a hormonal link between the intestinal tract and insulin sensitivity. Furthermore, this link may respond to the total calorie and/or nutrient content of the food ingested. Future studies will examine whether the serum concentrations of RELMβ and RELMγ are also increased in obese or insulin-resistant diabetic human subjects. If so, the measurement of serum RELMβ and RELMγ concentrations may be diagnostically useful. We can also suggest the possibility that RELMβ and RELMγ are potential molecular targets for future antidiabetic drugs, and additional studies are needed to investigate these possibilities.

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Alterations in Mitogen-Activated Protein Kinase Kinase and Extracellular Regulated Kinase Signaling in Theca Cells Contribute to Excessive Androgen Production in Polycystic Ovary Syndrome

Velen L. Nelson-Degrave, Jessica K. Wickenheisser, Karen L. Hendricks, Tomoichiro Asano, Midori Fujishiro, Richard S. Legro, Scot R. Kimball, Jerome F. Strauss, III, and Jan M. McAllister

Departments of Cellular and Molecular Physiology (V.L.N.-D., J.K.W., K.L.H., S.R.K., J.M.M.) and Obstetrics and Gynecology (R.S.L., J.M.M.), Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033; the Department of Diabetes and Metabolism (T.A., M.F.), Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-8655, Japan; and the Center for Research on Reproduction and Women's Health (J.F.S.), University of Pennsylvania, Philadelphia, Pennsylvania 19104

We have investigated the involvement of the MAPK signaling pathway in increased androgen biosynthesis and *CYP17* gene expression in women with polycystic ovary syndrome (PCOS). A comparison of MAPK kinase (MEK1/2) and ERK1/2 phosphorylation in propagated normal and PCOS theca cells, revealed that MEK1/2 phosphorylation was decreased more than 70%, and ERK1/2 phosphorylation was reduced 50% in PCOS cells as compared with normal cells. Infection with dominant-negative MEK1 increased CYP17 mRNA and dehydroepiandrosterone (DHEA) abundance, whereas constitutively active MEK1 reduced DHEA production and CYP17 mRNA abundance. Similarly, the MEK inhibitor, PD98059, increased CYP17 mRNA accumulation and *CYP17* promoter activity to levels observed in PCOS cells. Remarkably, in theca cells maintained in the complete absence of insulin, ERK1/2 phosphorylation was decreased in PCOS theca cells as compared with normal theca cells, and CYP17 mRNA and DHEA synthesis were increased in PCOS theca cells. These studies demonstrate that in PCOS cells reduced levels of activated MEK1/2 and ERK1/2 are correlated with increased androgen production, irrespective of the insulin concentration. These findings implicate alterations in the MAPK pathway in the pathogenesis of excessive ovarian androgen production in PCOS. (*Molecular Endocrinology* 19: 379–390, 2005)

POLYCYSTIC OVARY SYNDROME (PCOS) is a disorder that affects approximately 5–10% of reproductive aged women and is characterized by excess androgen production and infertility (1–3). The presence of an elevated level of circulating free testosterone, primarily from increased production of androgens by the ovaries, is the classical endocrine phenotype of women with PCOS (2). There is general agreement that the ovarian theca cell is the primary source of excess androgen biosynthesis in women with PCOS (4–6). Theca cells express a variety of genes encoding components of the steroidogenic pathway that are involved in androgen biosynthesis, in response to the pituitary gonadotropin, LH (7). Of these, the expression of the cytochrome P450 17α-hydroxylase (*CYP17*) gene, which encodes a single cytochrome P450 (P450c17) with both 17α-hydroxylase and C17, 20 lyase activities, is essential for the production of androgens in theca cells (8).

We have previously reported that basal and forskolin-stimulated androgen production is elevated in theca cells isolated from the ovaries of women with PCOS and propagated for successive population doublings (9, 10). We established that increased CYP17 gene transcription, mRNA accumulation, and associated increases in P450 17α-hydroxylase/C17,20 lyase activity are stable characteristics of PCOS theca cells that persist in long-term culture, and thus do not appear to be a consequence of the hormonal milieu that the cells were exposed to *in vivo* (9–12).

To examine the molecular mechanism(s) involved in increased androgen biosynthesis and *CYP17* gene expression in women with PCOS, we have begun to investigate specific components of the MAPK signaling cascade in ovarian theca cells propagated from normal cycling women and women with PCOS. Although regulation of steroidogenic enzyme expression

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Abbreviations: CA-MEK1, Constitutively active MEK1; *CYP17*, gene encoding cytochrome P450c17; DHEA, dehydroepiandrosterone; DN-MEK1, dominant-negative MEK1 molecule; FBS, fetal bovine serum; β-Gal, β-galactosidase; LUC, luciferase; MEK1/2, MAPK kinase; MKP-1, MAPK phosphatase-1; P450c17, P450 17α-hydroxylase/17,20 lyase; PCOS, polycystic ovary syndrome; PD, PD98059; pfu, plaque forming-units; PKB, protein kinase B; TBP, TATA-box binding protein.

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in theca cells has been largely attributed to LH-dependent increases in adenylate cyclase, more recent studies have provided evidence that alternative signaling pathways, including the MAPK and protein kinase B (AKT) pathways, are associated with LH-induced changes in steroid biosynthesis (13). Similar studies have also demonstrated a role for MAPK signaling in FSH-induced steroid biosynthesis and steroidogenic acute regulatory protein (*STAR*) gene expression in granulosa cells (14–16).

The MAPKs are mediators of signal transduction from the cytosol to the nucleus (17). MAPKs are all proline-directed, serine-threonine kinases that are phosphorylated (*i.e.* activated) on threonine and tyrosine in response to a wide variety of stimuli, including cytokines, growth factors, hormones, cellular stress, and cell adherence (18–20). The signals that link G protein-coupled receptors to the MAPK pathway are complex and may involve the Ras pathway as well as other convergent protein kinase cascades (21). The Ras/MAPK kinase (MEK)/ERK pathway is an important signaling cascade involved in the control of cell proliferation and differentiation. Activation of the ERK1/2 or p42/44 MAPK signaling cascade results from the phosphorylation and activation of MEK1/2 (21). Activation of the ERK pathway stimulates the expression and activity of a number of transcription factors, including members of the Jun, and Fos families, in a cell type-specific manner (17, 20, 22, 23). Pharmacological inhibitors of MEK1 [*i.e.* PD98059 (PD) and U0126], which prevent the activation of ERK1/2 by MEK1/2, have been widely used throughout the literature to examine the role of the ERK1/2 pathway in cellular signaling and differentiation (21, 24, 25).

Although some investigators have suggested that cAMP-stimulated activation of the MEK/ERK signaling cascade augments ovarian steroid biosynthesis (13, 15), others have demonstrated that inhibition of the MEK/ERK signaling cascade is associated with increased steroid biosynthesis (16, 26). In human adrenocortical H295 cells, a reduction in the activation state of the ERK1/2 has also been associated with increased *CYP17* gene expression (27, 28). In the present study, we have begun to evaluate the extent that components of the MEK/ERK signaling cascade may be involved in increased androgen biosynthesis and *CYP17* gene expression in normal and PCOS theca cells. We compared the phosphorylation states of MEK1/2 and ERK1/2 in theca cells propagated from multiple normal and PCOS patients. We used an adenovirus-mediated transfection/infection procedure to over express both the dominant-negative and constitutively active forms of these MEK mutants in theca cells to evaluate the effects of the resultant state of MAPK activation on androgen biosynthesis and CYP17 mRNA abundance. We also examined the effects of the pharmacological MEK1/2 inhibitor, PD, on *CYP17* gene transcription. Because insulin resistance and hyperinsulinemia in PCOS have been hypothesized to contribute to increased androgen production by the ovary, we also examined the extent to which insulin contributes to ERK1/2 phosphorylation in normal and PCOS theca cells.

RESULTS

Phosphorylation of MEK1/2 and ERK1/2 Is Reduced in PCOS Theca Cells

To investigate whether there are differences in MEK/ERK signaling in normal and PCOS theca cells maintained in long-term culture, we compared the phosphorylation states of MEK1/2 and ERK1/2 in fourth-passage theca cells. We examined both the phosphorylated and total forms of MEK1/2 (Fig. 1) and ERK1/2 (Fig. 2) by Western blot analysis in cells that were treated with and without forskolin (20 μM) for 24 h. Our analysis of whole cell lysates harvested from theca cells isolated from five independent normal and PCOS patients, showed that the phosphorylation state of MEK1/ 2 was decreased more than 70% in PCOS theca cells as compared with normal theca cells (Fig. 1). We observed a 50% reduction in the phosphorylation state of ERK1/2 in PCOS cells as compared with normal cells (Fig. 2). ERK1/2 phosphorylation was observed to be decreased in response to forskolin treatment in both normal and PCOS theca cells.

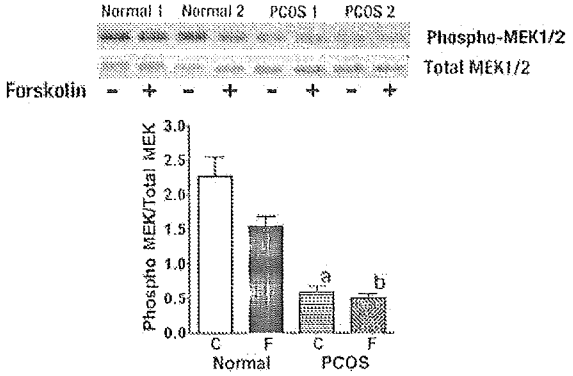

Fig. 1. The Activation State of MEK1/2 Is Decreased in PCOS Theca Cells as Compared with Normal Theca Cells

Fourth-passage theca cells propagated from normal and PCOS patients were grown to confluence and transferred into serum-free medium with vehicle (C) or 20 μM forskolin (F). 24 h thereafter the cells were harvested, and immunnoblot analysis was performed using 35 μg of whole cell extract and antibodies specific for phosphorylated MEK1/2 (Phospho-MEK 1/2) and total MEK. *Upper panel,* Representative immunoblot data of whole cell extracts prepared from theca cells isolated from normal and PCOS patients. *Bottom panel,* Quantitation of the activation state of MEK1/2 in theca cells isolated from five independent normal and PCOS patients is presented. Both basal (a, $P < 0.05$) and forskolin (b, $P < 0.05$)-stimulated MEK1/2 phosphorylation was decreased in PCOS theca cells as compared with normal cells (*, $P < 0.05$).

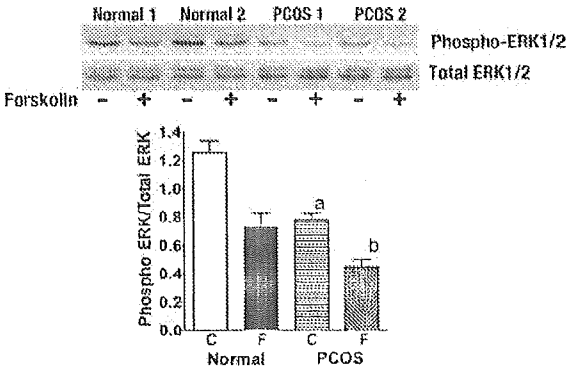

Fig. 2. The Activation State of ERK1/2 Is Decreased in PCOS Theca Cells as Compared with Normal Theca Cells

Fourth-passage theca cells propagated from normal and PCOS patients were grown to confluent and transferred into serum-free medium with vehicle (C) or 20 μM forskolin (F). Twenty-four hours thereafter, the cells were harvested, and immunoblot analysis was performed using 35 μg of whole cell extract and antibodies specific for phosphorylated ERK1/2 (phospho ERK1/2) and total ERK1/2 (ERK1/2). *Upper panel,* Representative immunoblot data of whole cell extracts prepared from theca cells isolated from normal and PCOS patients. *Bottom panel,* Quantitation of the activation state of ERK1/2 in theca cells isolated from five independent normal and PCOS patients is presented. Forskolin treatment resulted in the inhibition of ERK1/2 phosphorylation in both normal and PCOS theca cells. Both basal (a, $P < 0.05$) and forskolin (b, $P < 0.05$)-stimulated ERK1/2 phosphorylation was decreased in PCOS theca cells as compared with normal cells.

MEK and ERK Regulate Thecal Androgen Synthesis

To determine whether the MEK and ERK signaling pathways influence thecal androgen biosynthesis, an adenoviral transfection/infection system was used to examine the effects of a dominant-negative MEK1 molecule (DN-MEK1) (29) on dehydroepiandrosterone (DHEA) production and CYP17 mRNA accumulation in normal theca cells. In these experiments, a half-maximal dose of 7.5 μM forskolin was used to examine possible stimulatory effectors (9). As shown in Fig. 3A, there was a dose-dependent increase in DHEA biosynthesis in response to infection with DN-MEK1 under both basal and forskolin-stimulated conditions, as compared with a control adenovirus expressing β-galactosidase (β-Gal). At a dose of 10–30 plaque forming-units (pfu) of DN-MEK1, which was found to maximally stimulate DHEA biosynthesis 5-fold over forskolin-stimulated conditions, we observed a 50% reduction in ERK1/2 phosphorylation under both basal and forskolin-stimulated conditions (Fig. 3B). We also observed an approximately 2-fold increase in total ERK1/2 after infection with DN-MEK1/2, which effectively reduced the phosphorylation state of ERK1/2. In parallel studies, infection with 10–30 pfu of DN-MEK1 adenovirus significantly increased both basal and forskolin-stimulated CYP17 mRNA abundance (Fig. 3C) and 17α-hydroxylase enzyme activity approximately 2-fold (Fig. 3D).

We used an adenovirus expressing constitutively active MEK1 (CA-MEK1) to examine whether an increase in ERK1/2 activity could reduce DHEA synthesis and CYP17 mRNA accumulation. These studies required using a different adenoviral vector system than that used for DN-MEK1 (Fig. 3) (30) and therefore required using a LacZ control adenovirus. These experiments were performed with a maximal stimulatory dose of 20 μM forskolin so that we could more fully examine the possible inhibitory effects of the MEK1 signaling pathway. As shown in Fig. 4 (*upper panel*), infection of theca cells with CA-MEK1 resulted in an increase in ERK1/2 phosphorylation and a 50% reduction in total ERK1/2. Both basal and forskolin-stimulated DHEA production and CYP17 mRNA abundance were significantly decreased more than 95% and more than 70%, respectively, after infection with CA-MEK1 as compared with control LacZ adenovirus (Fig. 4, *lower* and *middle panels*). These data demonstrate that activation of ERK1/2 directly inhibits thecal CYP17 mRNA accumulation and androgen biosynthesis. Similar results were obtained after infection of PCOS theca cells with CA-MEK1 (data not shown).

To investigate whether inactivation of ERK1/2 signaling directly affects *CYP17* gene expression, we examined the effects of the MEK/ERK pathway-specific pharmacological inhibitor, PD (24) on CYP17 mRNA abundance and promoter function. Evaluation of CYP17 mRNA abundance after PD treatment demonstrated a time-dependent increase in CYP17 mRNA abundance, which was consistent with the results observed after infection with DN-MEK1 (Fig. 3). CYP17 mRNA abundance increased approximately 2-fold in response to 24 h of PD treatment (Fig. 5, *upper panel*). Experiments were performed to assess the effects of PD treatment on DHEA production, however in agreement with the studies of Munir *et al.* (31), we found that PD treatment of theca cells either had no effect or mildly inhibited 17α-hydroxylase enzyme activity as well as DHEA or 17α-hydroxyprogesterone production. From our analysis, it appears that unlike DN-MEK1, PD treatment may nonspecifically inhibit some component of the cytochrome P450 complex.

To examine the effects of PD on the transcriptional regulation of the *CYP17* gene, normal theca cells were transiently transfected with a luciferase (LUC) reporter plasmid containing −750/+44 bp of the 5′ promoter of the human *CYP17* gene. After transfection, the cells were treated with increasing concentrations of PD (0.03–30.0 μM) in the presence and absence of a half-maximal dose of forskolin (7.5 μM). As shown in Fig. 5 (*lower panel*), PD treatment stimulated both basal and forskolin-stimulated *CYP17* promoter activity in a dose-dependent manner with an ED_{50} of approximately 0.5 μM. These experiments were performed in triplicate cultures of

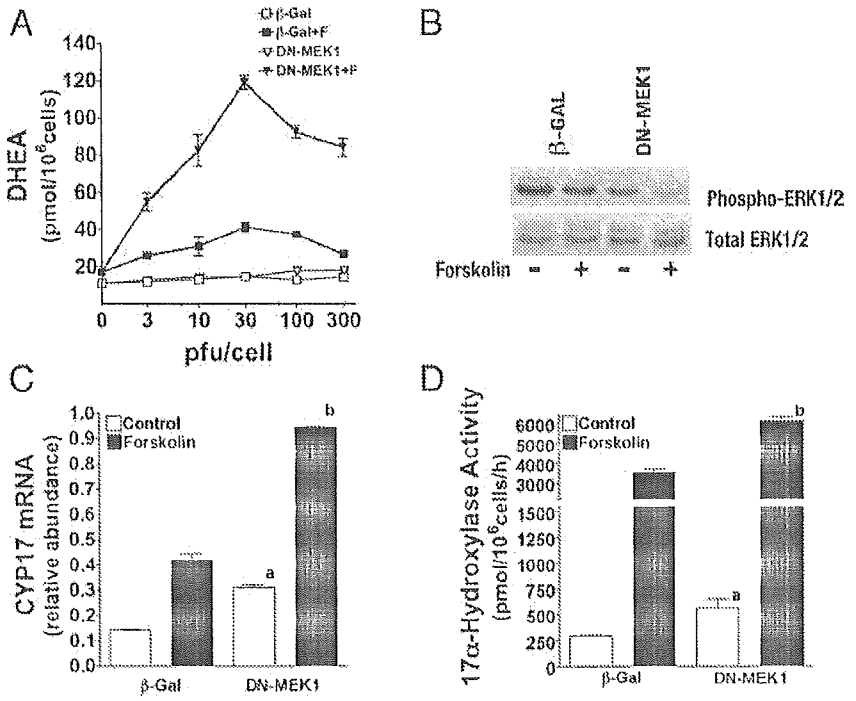

Fig. 3. Inhibition of MEK/ERK Pathway Using a Dominant-Negative MEK1 Adenovirus Augments Thecal Androgen Biosynthesis, 17α-Hydroxylase Enzyme Activity, and CYP17 mRNA Expression

A, To examine the effect of increasing amounts of an adenovirus encoding DN-MEK on thecal androgen biosynthesis, fourth-passage theca cells were grown until subconfluent and were infected with increasing concentrations (3–300 pfu) of either DN-MEK adenovirus or a control β-Gal. Seventy-two hours after treatment with and without forskolin (7.5 μM), the media were collected, and DHEA production was evaluated by RIA. Data were normalized to cell number and results are presented as the mean ± SEM of steroid levels from triplicate theca cell cultures. B, To examine ERK1/2 phosphorylation, fourth-passage theca cells were infected with 10 pfu of either a recombinant adenovirus expressing as a control (β-Gal) or DN-MEK1 and treated with and without 7.5 μM forskolin for 24 h. Thirty-five micrograms of protein isolated from whole cell lysates were subjected to SDS-PAGE and then immunoblotted with antiphospho-ERK (Thr202/Tyr204) antibody. Representative immunoblots from four independent experiments are shown. C, In parallel studies to those performed above, at 24 h CYP17 mRNA abundance was evaluated using quantitative real-time PCR analysis. mRNA accumulation was normalized by TBP mRNA abundance and is depicted graphically as the mean ± SEM. Infection with a DN-MEK1 adenovirus increased both basal (a, $P < 0.05$) and forskolin- (b, $P < 0.05$) stimulated CYP17 mRNA abundance. D, 17α-Hydroxylase enzyme activity was evaluated after infection with DN-MEK or β-Gal adenovirus followed by treatment with and without 7.5 μM forskolin for 72 h, as described in *Materials and Methods*. Infection with a DN-MEK1 adenovirus increased both basal (a, $P < 0.05$) and forskolin- (b, $P < 0.05$) stimulated 17α-hydroxylase enzyme activity.

theca cells isolated from four independent normal patients. Similar results were observed with the MEK1/2 inhibitor U0126 (data not shown) (25).

Inhibition of the ERK1/2 MAPK Pathway with the MEK1 Inhibitor, PD, Differentially Regulates CYP17 Promoter Activity in Normal and PCOS Theca Cells

In view of our data demonstrating that the phosphorylation states of MEK1/2 and ERK1/2 are decreased in PCOS theca cells compared with normal theca cells, we investigated whether pharmacological inhibition of ERK1/2 using PD had similar or differential effects on *CYP17* promoter regulation in normal and PCOS theca cells. In these experiments, normal and PCOS theca cells propagated from five independent normal and PCOS patients were transiently transfected with a pGL3 LUC construct containing −750 to +44 bp of the *CYP17* promoter (−750 CYP17/LUC). After transfection, the cells were treated in the presence or absence of a half-maximal dose of forskolin (7.5 μM), with and without PD (25 μM). In agreement with our preliminary data presented in Fig. 6 (*upper panel*), both basal and forskolin-stimulated CYP17 reporter activity were increased in PCOS theca cells as compared with normal theca cells (11). Treatment of normal cells with PD resulted in an approximately 3-fold increase in basal, and a more than 2-fold increase in forskolin-stimulated reporter activity. In contrast, in PCOS theca cells PD treatment resulted in a less than 1.5-fold increase in both basal and forskolin-stimulated reporter activity. These data demonstrate that *CYP17* gene expression in normal theca cells involves

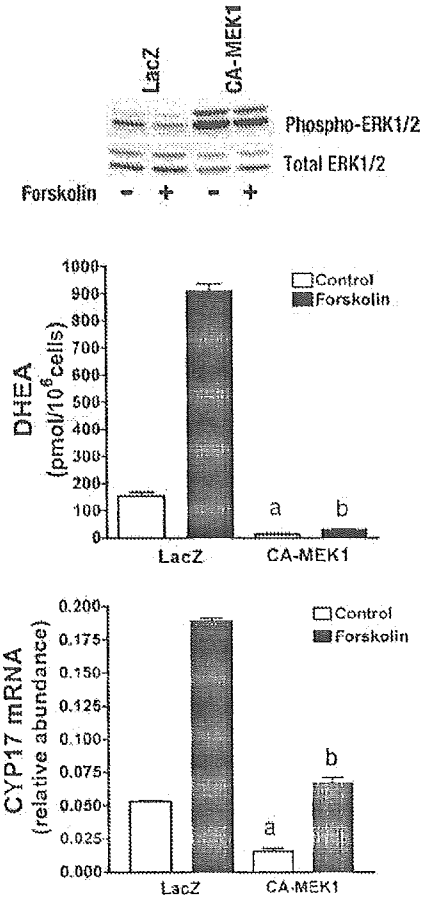

Fig. 4. Constitutively Active MEK Stimulates ERK Phosphorylation and Inhibits DHEA and CYP17 mRNA Abundance

Fourth-passage theca cells were infected with a recombinant adenovirus containing LacZ (control) or CA-MEK1 and treated with and without 20 μM forskolin. *Upper panel*, Twenty-four hours after treatment, the cells were harvested and 35 μg of protein isolated from whole cell lysates were subjected to SDS-PAGE and then immunoblotted with anti-phospho-ERK (Thr202/Tyr204) antibody. Representative immunoblots from four independent experiments are shown. *Middle panel*, Seventy-two hours after treatment, the media were collected and DHEA production was evaluated by RIA. Data were normalized to cell number and are presented as the mean ± SEM from triplicate theca cell cultures that are representative of at least four experiments. Infection with CA-MEK1 inhibited both basal (a, $P < 0.05$) and forskolin-stimulated (b, $P < 0.05$) DHEA production. *Bottom panel*, Fourth-passage theca cells were infected with a recombinant adenovirus containing LacZ or CA-MEK1 and treated with and without 20 μM forskolin. At 24 h, the cells were harvested and CYP17 abundance was evaluated using quantitative real-time PCR analysis. mRNA accumulation was normalized by TBP mRNA abundance and is depicted graphically as the mean ± SEM. Infection with a CA-MEK1 adenovirus inhibited both basal (a, $P < 0.05$) and forskolin (b, $P < 0.05$) stimulated CYP17 abundance.

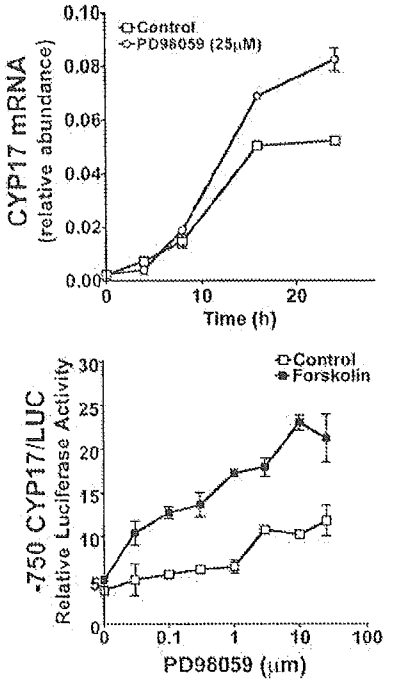

Fig. 5. Inhibition of the ERK1/2 Pathway with the MEK1 Inhibitor PD Augments CYP17 mRNA Accumulation and Promoter Function

Upper panel: Fourth-passage theca cells were treated with and without the MEK1/2 inhibitor PD (25 μM). At 4, 8, 16, 24, and 48 h, the cells were harvested and CYP17 abundance was evaluated using quantitative real-time PCR analysis. mRNA accumulation was normalized by TBP mRNA abundance and is depicted graphically as the mean ± SEM. *Bottom panel:* Fourth-passage theca cells were transiently transfected with a pGL3 LUC construct containing −750 to +44 bp of the *CYP17* promoter (−750 CYP17/LUC). After transfection, the cells were treated with an increasing concentration of PD (0.03–30 μM) with and without a submaximal dose of forskolin (7.5 μM). Seventy-two hours thereafter, the cells were harvested and LUC activity assayed. Data are presented as relative LUC activity that has been corrected for β-Gal activity. Data represent the mean ± SEM of experiments performed with triplicate cultures of theca cells isolated from four normal patients. Inhibition of ERK1/2 signaling using the MEK1 inhibitor PD augmented basal mRNA accumulation and both basal and forskolin-stimulated −750 CYP17/LUC promoter activity in a time and dose-dependent manner ($P < 0.05$).

direct regulation by the ERK pathway. In effect, PD treatment of normal theca cells increased CYP17 promoter activity to the levels observed in PCOS theca cells. In agreement with these findings, in parallel studies PD treatment was observed to inhibit ERK1/2 phosphorylation in normal theca cells to a greater extent than in PCOS theca cells (Fig. 6, *lower panel*).

The Activation State of ERK1/2 Is Decreased, and CYP17 mRNA Abundance and DHEA Biosynthesis Are Increased in PCOS Theca Cells as Compared with Normal Theca Cells Irrespective of the Insulin Concentration

Given the controversial role of insulin in regulating androgen biosynthesis in normal and PCOS theca

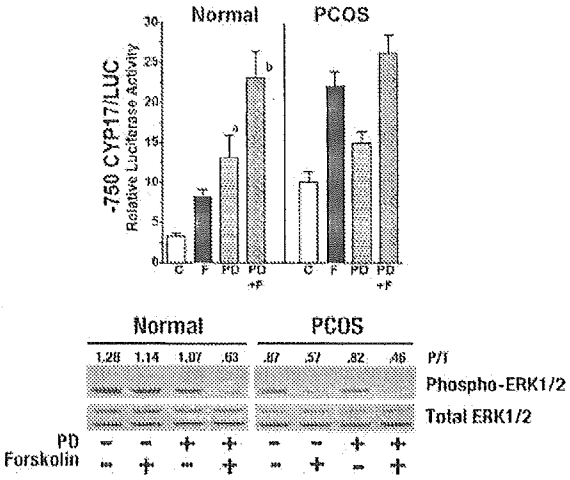

Fig. 6. Inhibition of the ERK1/2 MAPK Pathway with the MEK1 Inhibitor PD Differentially Regulates *CYP17* Promoter Activity in Normal and PCOS Theca Cells

Upper panel: Fourth-passage theca cells isolated from normal or PCOS patients were transiently transfected with a pGL3 LUC construct containing −750 to +44 bp of the *CYP17* promoter. After transfection, the cells were treated with vehicle (C) or 7.5 μM forskolin (F) with and without the 25 μM PD. Seventy-two hours thereafter, the cells were harvested and LUC activity assayed. Data are presented as relative LUC activity that has been corrected for β-Gal activity. Data represent the mean ± SEM of experiments performed with triplicate cultures of theca cells isolated from five independent normal and PCOS patients. In normal theca cells, inhibition of ERK1/2 signaling using PD augmented both basal (a, $P < 0.05$) and forskolin (b, $P < 0.05$) stimulated −750 CYP17/LUC promoter activity. In contrast, −750 CYP17/LUC promoter activity was not significantly increased in response to PD treatment in PCOS theca cells. *Bottom panel:* To compare the effects of PD on ERK1/2 phosphorylation in normal and PCOS theca cells, fourth-passage theca cells propagated from normal and PCOS patients were grown to confluent and transferred into serum-free medium with vehicle (C) or 7.5 μM forskolin (F) in the presence or absence of 25 μM PD. Twenty-four hours thereafter, the cells were harvested, and immunoblot analysis was performed using 35 μg of whole cell extract and antibodies specific for phosphorylated ERK1/2 (Phospho-ERK 1/2) and total ERK1/2.

cells, experiments were performed to assess the effects of insulin on ERK phosphorylation and overall androgen biosynthesis. In these experiments, theca cells propagated from multiple normal and PCOS patients were grown to subconfluent, and transferred into serum-free medium without insulin for 24 h, then treated for an additional 24 h with serum-free medium, with and without 2 or 20 nM insulin, in the presence and absence of 7.5 μM forskolin. An insulin concentration of 2 nM (*i.e.* 10 ng/ml) is equivalent to the normal plasma concentration of insulin. As shown in Fig. 7, insulin treatment did not significantly affect the phosphorylation state of ERK1/2 in normal or PCOS theca cells. ERK1/2 phosphorylation was decreased in PCOS theca cells as compared with normal theca

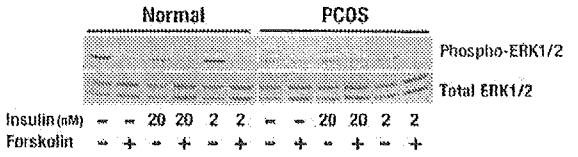

Fig. 7. The Activation State of ERK1/2 Is Decreased in PCOS Theca Cells as Compared with Normal Theca Cells Irrespective of the Insulin Concentration

Fourth-passage theca cells propagated from normal and PCOS patients were grown to confluent, transferred into serum-free medium without insulin for 24 h, and then treated with serum-free medium with vehicle (C) or 7.5 μM forskolin (F), without or with 2 or 20 nM insulin. Twenty-four hours thereafter, the cells were harvested, and immunoblot analysis was performed using 35 μg of whole cell extract and antibodies specific for phosphorylated ERK1/2 (Phospho-ERK1/2) and total ERK1/2. Representative immunoblot data of whole cell extracts prepared from theca cells isolated from normal and PCOS patients. Insulin treatment did not significantly affect the phosphorylation state of ERK1/2 in normal or PCOS theca cells.

cells, at both concentrations of insulin tested, and in the absence of insulin. In agreement with the data presented in Fig. 2, treatment with 7.5 μM forskolin reduced ERK phosphorylation approximately 50% in both normal and PCOS theca cells in the absence and presence of insulin. Of significant interest is the observation that in the absence of insulin treatment, CYP17 mRNA abundance (Fig. 8, *middle panel*), DHEA accumulation (Fig. 8, *lower panel*), and 17α-hydroxylase enzyme activity (data not shown) were increased in PCOS theca cells as compared with normal theca cells. These data suggest that alterations in MEK/ERK signaling, CYP17 mRNA accumulation, and androgen biosynthesis may not be directly associated with insulin action.

DISCUSSION

The study of human theca cells maintained in long-term culture provides an opportunity to compare the steroidogenic abnormalities and their underlying causes in PCOS theca cells (9, 10). In addition to augmented transcription of the *CYP17* gene (11, 12), recent microarray analysis of differential gene expression in normal and PCOS theca cells demonstrated that dysregulation of androgen biosynthesis is associated with selective differences in several gene networks that are involved in steroid hormone biosynthesis as well as insulin and glucose homeostasis (32–34)

In an attempt to examine the molecular mechanisms underlying dysregulated gene expression in the PCOS ovary, we have extended our studies on MAPK signaling in normal and PCOS theca cells. In this report, we present data to support the concept that increased *CYP17* gene expression and overall androgen biosynthesis are associated with diminished MEK1/2 and

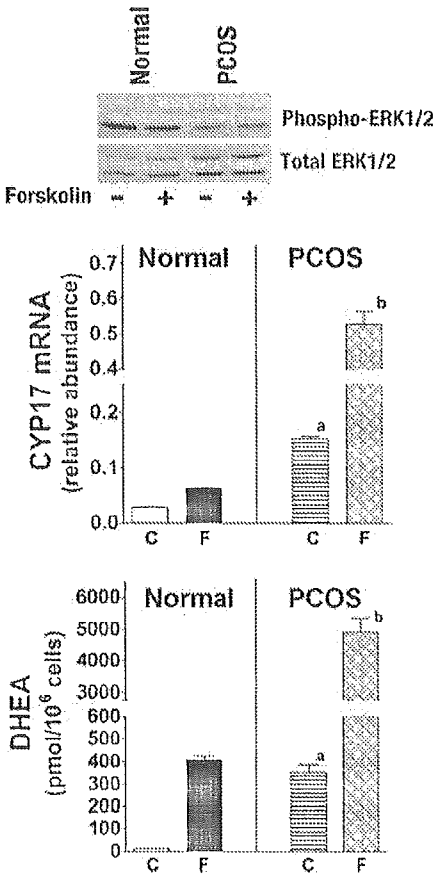

Fig. 8. CYP17 mRNA Abundance and DHEA Biosynthesis Are Increased in PCOS Theca Cells as Compared with Normal Theca Cells in the Absence of Insulin

Fourth-passage theca cells propagated from normal and PCOS patients were grown to subconfluent, transferred into serum-free medium in the absence of insulin for 24 h, and then treated with serum-free medium with vehicle (C) or 20 forskolin (F) in the absence of insulin. *Upper panel*, For analysis of ERK1/2 phosphorylation, 24 h thereafter the cells were harvested, and immunoblot analysis was performed using 35 μg of whole cell extract and antibodies specific for phosphorylated ERK1/2 (Phospho-ERK1/2) and total ERK1/2. Representative immunoblot data of whole cell extracts prepared from theca cells isolated from four independent normal and PCOS patients. *Middle panel*, At 24 h the cells were harvested and CYP17 mRNA abundance was evaluated using quantitative real-time PCR analysis. mRNA accumulation was normalized by TBP mRNA abundance and is depicted graphically as the mean ± SEM. In the absence of insulin treatment, both basal (a, $P < 0.05$) and forskolin (b, $P < 0.05$)-stimulated CYP17 mRNA accumulation were significantly increased in PCOS theca cells as compared with normal theca cells. *Bottom panel*, Seventy-two hours after treatment, the media were collected and DHEA production was evaluated by RIA. Data were normalized to cell number and are presented as the mean ± SEM from triplicate theca cell cultures from four independent normal and PCOS patients. In the absence of insulin treatment, both basal (a, $P < 0.05$) and forskolin (b, $P < 0.05$)-stimulated CYP17 mRNA accumulation were significantly increased in PCOS theca cells as compared with normal theca cells.

ERK1/2 signaling in the PCOS ovary. We compared the phosphorylation states of MEK1/2 and ERK1/2 in the same normal and PCOS theca cell preparations that we previously examined in our *CYP17* transcriptional studies (11, 12) and microarray analysis (32). The comparison of the phosphorylation states of MEK1/2 and ERK1/2 in normal and PCOS theca cells propagated in long-term culture revealed a gross reduction in the tone of MEK/ERK signaling in PCOS cells.

Infection of normal theca cells with a dominant-negative MEK1 adenovirus resulted in increased DHEA production, CYP17 mRNA accumulation, and 17α-hydroxylase enzyme activity, and treatment with the pharmacological MEK1 inhibitor, PD, augmented *CYP17* gene transcription. In contrast, infection with a constitutively active MEK1 inhibited both DHEA synthesis and CYP17 mRNA accumulation. In our comparison of *CYP17* promoter function in normal and PCOS theca cells, the observed lack of a response of PCOS theca cells to PD further supports the idea that the suppression of the MEK/ERK signaling pathway plays a pivotal role in regulating androgen synthesis in the PCOS ovary.

Although several investigators have examined the mechanisms by which insulin and IGF acutely stimulate the ERK1/2 signaling pathway in porcine and human theca cells (31, 35, 36), there is no information available with respect to the intracellular signaling pathways mediating androgen biosynthesis in normal and PCOS theca cells under basal and cAMP-stimulated conditions. In addition, most studies have focused on the acute regulation of MEK or ERK phosphorylation in response to a variety of stimuli, rather than examining MAPK signaling under steady-state conditions in differentiated cells. In contrast, we compared MAPK signaling using conditions in which normal and PCOS theca cells are fully differentiated. Data from array analysis suggests that cross talk between several signaling pathways, including the MAPK pathway, may be dysregulated in the PCOS ovary. For instance tribble 3, which inhibits Akt/protein kinase B (PKB) phosphorylation, exhibits decreased gene expression in PCOS theca cells, and cAMP-GEFII, which augments Akt/PKB phosphorylation, exhibits increased gene expression in PCOS theca cells (32).

PCOS is also associated with insulin resistance, obesity, and type II diabetes (37). Insulin resistance and hyperinsulinemia in PCOS have been hypothesized to contribute to increased androgen production by the ovary. Although several investigators had initially proposed that hyperinsulinemia, resulting from the insulin-resistant state, produces ovarian hyperandrogenism by spillover occupancy and activation of the IGF-I receptors (38), the studies of Nestler *et al.* (6) have conclusively demonstrated that PCOS theca cells are not insulin resistant and are responsive to insulin. However, there are no apparent differences in insulin sensitivity between normal and PCOS theca cells, and insulin appears to act via its own receptor rather than through spillover occupancy of the IGF-I

receptor (6). No differences were found in the ED_{50} of insulin-stimulated steroid biosynthesis in freshly isolated granulosa cells (39, 40) obtained from normal and PCOS ovaries. These data are in agreement with our data demonstrating that insulin treatment does not differentially affect ERK1/2 phosphorylation in normal or PCOS theca cells. Furthermore, in the absence of insulin, CYP17 mRNA abundance and DHEA accumulation was increased in PCOS theca cells as compared with normal theca cells, and the phosphorylation state of ERK1/2 was decreased in PCOS theca cells, as compared with normal theca cells.

In human adrenal cells, Sewer *et al.* (41) has established that there is cross talk between the MAPK and ACTH/cAMP pathways in the regulation of CYP17 mRNA synthesis. These authors demonstrated that a reduction in the activation state of the ERK1/2 is associated with increased *CYP17* gene expression in adrenal cells (41). These authors also showed that protein kinase A phosphorylates and activates a dual-specificity phosphatase, MAPK phosphatase-1 (MKP-1), which mediates *CYP17* gene transcription in response to ACTH/cAMP (28, 41). However, these investigators did not report the role of ERK1/2 or MKP-1 in overall adrenal androgen biosynthesis. Therefore, the possible involvement of MKP-1 in augmented *CYP17* gene expression and increased androgen biosynthesis in normal and PCOS theca cells is unclear and will require future examination.

We are in the process of examining the relationship between the cohort of multiple signaling pathways and transcription factors that confer increased *CYP17* gene expression in PCOS theca cells. The reduction in MEK1/2 and ERK1/2 phosphorylation in PCOS cells is also in agreement with our previously published data demonstrating that a relief of transcriptional repression may play a role in augmented transcriptional regulation of the *CYP17* gene in the PCOS ovary (11). Analysis of the *CYP17* promoter function in normal and PCOS theca cells demonstrated that a 16-bp sequence, spanning −174 to −158 bp of the *CYP17* promoter confers increased expression of the *CYP17* promoter in PCOS theca cells (11). We reported that transcription factor, NF-1 C, bound to this element and was able to repress *CYP17* promoter function (11). Identification of the cohort of transcription factors that regulate this element will further our knowledge of the underlying cause(s) of increased *CYP17* gene expression in PCOS theca cells and the extent to which defects in MAPK signaling and other signaling pathways are involved in the pathogenesis of PCOS.

In patients with insulin resistance and type II diabetes, there are reports of alterations in multiple components of the phosphoinositol 3-kinase/PKB and Ras/MAPK signaling pathways (42–51). Yet, there are very few reports of the specific signaling defects observed in PCOS patients. In patients with PCOS and insulin resistance, there are reports to suggest that D-chiro-inositol signaling is altered (6, 52–55). Dunaif *et al.* and Li *et al.* (56, 57) have reported that insulin receptor autophosphorylation is decreased in fibroblasts in approximately 50% of patients with PCOS. Whereas in PCOS adipocytes, Ciaraldi *et al.* (58, 59) reported that insulin resistance is accompanied by normal function of the insulin receptor, but involves a novel postreceptor defect in the insulin signal transduction between the receptor kinase and glucose transport. With respect to the ovary, although it is well recognized that LH-, FSH-, and insulin receptor sensitivity are increased in ovarian cells isolated from PCOS patients (5, 38–40, 60–62), there are few data that describe defects in cellular signaling in PCOS ovarian cells other than those demonstrating decreased abundance of insulin receptor substrate-2 mRNA and protein in PCOS granulosa and theca cells (63).

The data presented in this report are the first to suggest that MAPK signaling in normal and PCOS cells is distinct. These studies have demonstrated that decreased MEK/ERK phosphorylation are directly associated with increased thecal *CYP17* gene expression and androgen biosynthesis. Moreover, our findings reveal that in the absence of insulin treatment, phosphorylation state of ERK1/2 is decreased, and CYP17 mRNA accumulation and DHEA biosynthesis are increased in PCOS cells as compared with normal cells. We are currently characterizing the extent to which other components of the MAPK signaling pathway regulate increased *CYP17* gene expression and androgen excess in the PCOS ovary. As a consequence of future studies we hope to further our understanding of the underlying cause of increased *CYP17* gene expression and possibly other abnormalities in PCOS, *e.g.* insulin resistance, obesity, arrested follicular development, and endometrial cancer.

MATERIALS AND METHODS

Theca Cell Isolation and Propagation

Human theca interna tissue was obtained from follicles of women undergoing hysterectomy, under a protocol approved by the Institutional Review Board of the Pennsylvania State University College of Medicine. Individual follicles were dissected away from ovarian stroma. The isolated follicles were size selected for diameters ranging from 3–5 mm so that theca cells derived from follicles of similar size from normal and PCOS subjects could be compared. The dissected follicles were placed into serum-containing medium and bisected. Under a dissecting microscope, the theca interna was stripped from the follicle wall, and the granulosa cells were removed with a platinum loop. The cleaned theca shells were dispersed with 0.05% collagenase I, 0.05% collagenase IA, and 0.01% deoxyribonuclease, in medium containing 10% fetal bovine serum (FBS) (64). Dispersed cells were placed in culture dishes that had been precoated with fibronectin by incubation at 37 C with culture medium containing 5 μg/ml human fibronectin. The growth medium used was a 1:1 mixture of DMEM and Hams F-12 medium containing 10% FBS, 10% horse serum, 2% UltroSer G, 20 nM insulin, 20 nM selenium, 1 μM vitamin E, and antibiotics. From each follicle, twelve 35-mm dishes of primary theca interna cells were grown until confluent, removed from the dish with neutral protease (pronase-E; protease type XXIV, Sigma, St.

Louis, MO) in DMEM/F12 (1:1), frozen, and stored in liquid nitrogen (one 35-mm dish per vial) in culture medium that contained 20% FBS and 10% dimethylsulfoxide. In all experiments, cells were thawed and propagated in the growth medium described above. To obtain successive passages of normal and PCOS theca cells, cells were thawed, propagated, and frozen at consecutive passages. The cells were grown in 5% O_2, 90% N_2, and 5% CO_2. Reduced oxygen tension and supplemental antioxidants (vitamin E and selenium) were employed to prevent oxidative damage.

The PCOS and normal ovarian tissue came from age-matched women, 38–40 yr old. The diagnosis of PCOS was made according to established guidelines (2, 65–67), including hyperandrogenemia, oligoovulation, and the exclusion of 21α-hydroxylase deficiency, Cushing's syndrome, and hyperprolactinemia. All of the PCOS theca cell preparations studied came from ovaries of women with fewer than six menses per year and elevated serum total testosterone or bioavailable testosterone levels, as previously described (2, 9). Each of the PCOS ovaries contained multiple subcortical follicles of less than 10 mm in diameter. The control (normal) theca cell preparations came from ovaries of fertile women with normal menstrual histories, menstrual cycles of 21–35 d, and no clinical signs of hyperandrogenism. Neither PCOS nor normal subjects were receiving hormonal medications at the time of surgery. Indications for surgery were dysfunctional uterine bleeding, endometrial cancer, and pelvic pain. The passage conditions and split ratios for all normal and PCOS cells were identical. Experiments comparing PCOS and normal theca were performed using fourth-passage (31–38 population doublings) theca cells isolated from size-matched follicles obtained from age-matched subjects. Sera and growth factors were obtained from the following sources: FBS and DMEM/F12 were obtained from Irvine Scientific (Irvine, CA); horse serum was obtained from Hyclone (Logan, UT); UltroSer G was from Reactifs IBF (Villeneuve-la-Garenne, France). Other compounds were purchased from Sigma.

Western Blot Analysis

Fourth-passage normal and PCOS theca cells were grown until subconfluent and transferred into serum-free medium with and without forskolin for 24 h. After treatment, theca cells were harvested in ice cold modified RIPA buffer (30 mM Tris, 150 mM NaCl, 50 mM Na F, 0.5 mM EDTA, 0.5% deoxycholic acid, 1.0% Nonidet P-40, 0.1% sodium dodecyl sulfate) containing 1 mM sodium orthovanadate, 0.1 mM phenymethylsufonyl fluoride, 1 mM dithiothreitol, 0.2 mM benzamidine, 1 μM microcystin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Whole cell lysates (35 μg/lane) were separated on a 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and Western analysis was performed as previously described (68). Total and phosphospecific MEK1/2 and ERK1/2 used in Western analysis were obtained from Cell Signaling Technology (Beverly, MA).

Replication-Deficient Adenovirus Infections

Adenoviruses expressing constitutively active MEK1 (Ser218 and Ser222 to Glu, CA-MEK1), and/or dominant-negative MEK1 (Ser222 to Ala, DN-MEK1) were used as previously described (29, 30, 69). All recombinant adenoviral vectors were plaque purified, expanded, and titered by detection of formation of visible plaques in a HEK293 monolayer of cells using the agarose gel overlay method. Adenoviral infections involved growing fourth-passage theca cells to 95% confluence, rinsing the cells with PBS, and layering of the adenovirus on the cells in 50% of the normal treatment volume of serum-free medium for 1 h. Subsequently, the cells were cultured in serum-free media with and without treatment as indicated. Using these conditions, >98% of cells showed positive β-Gal staining after infection with the LacZ adenovirus.

Steroid Biosynthesis

For evaluation of steroid production, theca cells were grown until subconfluent and transferred into serum-free medium in the presence or absence of forskolin (20 μM) with and without varying concentrations of adenovirus. At 72 h, the media was collected and RIAs for DHEA were performed without organic solvent extraction using Diagnostic Products (Los Angeles, CA) as previously described (68).

Quantitation of CYP17 mRNA

For quantitative real-time PCR, total mRNA was isolated (9) from fourth-passage theca cells that were grown to subconfluence, transferred into serum-free medium, and treated as indicated. RNA (1 μg) samples were then reverse transcribed using oligo deoxythymidine, and 200 units Stratascript Reverse Transcriptase (Stratagene, Cedar Creek, TX), and CYP17 mRNA abundance was determined by quantitative real-time PCR as previously described (68). The gene-specific two-step PCR was carried out in triplicate for each cDNA sample and for a series of serial dilutions in an Mx4000 Thermocycler (Stratagene, Cedar Creek, TX) using the Mx4000 Multiplex Quantitative PCR system according to manufacturer's instructions. TATA-box binding protein (TBP) mRNA abundance was used for data normalization.

17α-Hydroxylase Enzyme Activity

For evaluation of 17α-hydroxylase enzyme activity, normal theca cells were grown until subconfluent, then infected with adenovirus and transferred to serum-free medium and treated as indicated for 72 h. The cells were then transferred into medium containing a saturating concentration of 1.0 μM [1,2,6,7-^{3}H]progesterone and the rate of conversion to 17α-hydroxyprogesterone and 17α, 20α-dihydroprogesterone was determined as we have previously reported (9, 10). Cell number was estimated using a Coulter counter (Coulter Electronics, Hialeah, FL) after dispersal of cells with trypsin. Steroidogenic enzyme activities are expressed as picomoles per 10^6 cells/hour.

Transient Transfection Assay

Subconfluent cultures of theca cells were transfected with reporter gene constructs as we have previously described (11, 12) using the modified calcium-phosphate method of Graham and Vander Eb (70). One hour before transfection, the cells were transferred into DMEM high-glucose medium containing 20 mM HEPES and 2% heat inactivated calf serum, and moved to a 3% CO_2, 95% ambient air 37 C incubator. DNA/Ca2P04 solution containing 20 μg of reporter plasmid, and 1 μg of pSV-β-Gal/100-mm dish in HEPES phosphate buffer was added to the media. After incubation for 6 h, cells were transferred into 2% calf serum in DMEM containing 20 mM HEPES and treated as described. Seventy-two hours after forskolin treatment, the cells were harvested using trypsin/EDTA, pelleted, and resuspended in reporter lysis buffer for LUC assays. LUC assays were performed using the LUC assay system (Promega, Madison, WI). β-Gal activities were determined by Galacton Light Plus chemiluminescent assay (Tropix, Bedford, MA) and used to normalize LUC activities.

Statistical Analysis

Each experiment was performed using triplicate dishes. After combining the results from individual experiments, nonparametric Mann Whitney tests were performed using PRISM 4.0 from SAS Institute, Inc. (Cary, NC). $P < 0.05$ was considered statistically significant.

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Address all correspondence and requests for reprints to: Jan M. McAllister, Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine 500 University Drive H166, Hershey, Pennsylvania 17033. E-mail: jmcallister@psu.edu.

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ARTICLE

T. Sasaoka · K. Fukui · T. Wada · S. Murakami · J. Kawahara · H. Ishihara · M. Funaki · T. Asano · M. Kobayashi

Inhibition of endogenous SHIP2 ameliorates insulin resistance caused by chronic insulin treatment in 3T3-L1 adipocytes

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Abstract *Aims/hypothesis:* SHIP2 is a physiologically important negative regulator of insulin signalling hydrolysing the PI3-kinase product, PI(3,4,5)P3, which also has an impact on insulin resistance. In the present study, we examined the effect of inhibiting the endogenous SHIP2 function on the insulin resistance caused by chronic insulin treatment. *Methods:* The endogenous function of SHIP2 was inhibited by expressing a catalytically inactive SHIP2 (ΔIP-SHIP), and compared with the effect of treatments designed to restore the levels of IRS-1 in insulin signalling systems of 3T3-L1 adipocytes. *Results:* Chronic insulin treatment induced the large (86%) down-regulation of IRS-1 and the modest (36%) up-regulation of SHIP2. Subsequent stimulation by insulin of Akt phosphorylation, PKCλ activity, and 2-deoxyglucose (2-DOG) uptake was markedly decreased by the chronic insulin treatment. Coincubation with the mTOR inhibitor, rapamycin, effectively inhibited the proteosomal degradation of IRS-1 caused by the chronic insulin treatment. Although the coincubation with rapamycin and advanced overexpression of IRS-1 effectively ameliorated subsequent insulin-induced phosphorylation of Akt, insulin stimulation of PKCλ activity and 2-DOG uptake was partly restored by these treatments. Similarly, expression of ΔIP-SHIP2 effectively ameliorated the insulin-induced phosphorylation of Akt without affecting the amount of IRS-1. Furthermore, the decreased insulin-induced PKCλ activity and 2-DOG uptake following chronic insulin treatment were ameliorated by the expression of ΔIP-SHIP2 more effectively than by the treatment with rapamycin. *Conclusions/interpretation:* Our results indicate that the inhibition of endogenous SHIP2 is effective in improving the state of insulin resistance caused by chronic insulin treatment.

Keywords Akt · Glucose uptake · Insulin · Insulin resistance · PKCλ · SHIP2

Abbreviations 2-DOG: 2-deoxyglucose · Glut4: glucose transporter 4 · IRS-1: insulin receptor substrate-1 · mTOR: mammalian target of rapamycin · PDGF: platelet-derived growth factor · PI3-kinase: phosphatidylinositol 3-kinase · PI(3,4)P2: phosphatidylinositol 3,4-bisphosphate · PI(3,4,5)P3: phosphatidylinositol 3,4,5-triphosphate · PKC: protein kinase C · SHIP2: SH2-containing inositol 5′-phosphatase 2

T. Sasaoka (✉)
Department of Clinical Pharmacology, Toyama Medical and Pharmaceutical University,
2630 Sugitani,
Toyama, 930-0194, Japan
e-mail: tsasaoka-tym@umin.ac.jp
Tel.: +81-76-4347551
Fax: +81-76-4345067

K. Fukui · T. Wada · S. Murakami · J. Kawahara · M. Kobayashi
First Department of Internal Medicine, Toyama Medical and Pharmaceutical University,
2630 Sugitani,
Toyama, 930-0194, Japan

H. Ishihara
Sainou Hospital,
Toyama, 930-0887, Japan

M. Funaki · T. Asano
Department of Internal Medicine, Graduate School of Medicine, University of Tokyo,
Tokyo, 113-8655, Japan

Introduction

The activation of phosphatidylinositol 3-kinase (PI3-kinase) is known to be important to the various metabolic actions of insulin [1–4]. PI(3,4,5)P3 produced by activated PI3-kinase is thought to function as a key lipid second messenger in insulin signalling to further downstream molecules [3–5]. We and others identified SH2-containing inositol 5′-phosphatase 2 (SHIP2) as a lipid phosphatase possessing 5′-phosphatase activity to hydrolyse PI(3,4,5)P3 to PI(3,4)P2 [6, 7]. Previous reports have indicated that overexpression of SHIP2 inhibits insulin-induced glucose uptake and glycogen synthesis via its 5′-phosphatase activity in 3T3-L1 adipocytes and L6 myocytes [8, 9]. Targeted

disruption of the SHIP2 gene in mice increased sensitivity to insulin without affecting other biological systems [10]. These findings indicate that SHIP2 is a physiologically important negative regulator that is relatively specific to insulin signalling. In addition, expression of SHIP2 protein is enhanced in the skeletal muscle and fat tissue of diabetic db/db mice [11]. Treatment with the insulin-senzsitizing thiazolidinedione, rosiglitazone, lowered the elevated levels of SHIP2 in the db/db mice [11]. Furthermore, a deletion in the 3′ untranslated region within the motifs implicated in the control of protein synthesis leading to the possible increase in expression of SHIP2 protein was identified in the UK and Belgian population of individuals with type 2 diabetes [12]. Therefore, SHIP2 is implicated in insulin resistance as a cause of type 2 diabetes in addition to the physiological importance in insulin signalling. Based on these findings, inhibition of endogenous SHIP2 function may be a target for ameliorating insulin signalling in the state of insulin resistance.

Hyperinsulinaemia is a hallmark of insulin resistance [13–15]. Chronic hyperinsulinaemia causes a desensitization to subsequent insulin responses, which appears to be part of the vicious cycle involved in the pathogenesis of type 2 diabetes [16–18]. In this regard, chronic treatment with insulin is known to facilitate the proteosomal degradation of IRS-1 leading to the down-regulation of insulin signalling at IRS-1 in 3T3-L1 adipocytes [17–19]. However, it is unknown whether SHIP2 is also involved in the resistance caused by chronic exposure to insulin. In the present study, the change in SHIP2 expression following chronic insulin treatment was investigated in 3T3-L1 adipocytes. In addition, the effect of inhibition of endogenous SHIP2 function using adenovirus-mediated gene transfer of a dominant-negative SHIP2 (ΔIP-SHIP2) on the possible amelioration of decreased insulin signalling caused by the chronic insulin treatment was investigated. The down-regulation of insulin signalling at the level of IRS-1 caused by the chronic insulin treatment can be ameliorated by pretreatment with rapamycin, which is an inhibitor of mTOR-dependent proteosomal degradation of IRS-1 [20, 21]. Alternatively, the decrease of IRS-1 can be prevented by overexpression of IRS-1 through adenovirus-mediated gene transfer [22]. Finally, the effects of the amelioration at the level of IRS-1 and SHIP2 on the chronic insulin treatment-induced down-regulation of insulin signalling were compared.

Materials and methods

Materials Human crystal insulin was provided by Novo Nordisk Pharmaceutical (Copenhagen, Denmark). [γ-^{32}P] ATP (111 TBq/mmol) and 2-[^{3}H]deoxyglucose (DOG; 3,330 GBq/mmol) were purchased from NEN Life Science Products (Boston, MA, USA). The two polyclonal anti-SHIP2 antibodies were described previously [7]. A polyclonal anti-PKCλ antibody was kindly provided by Dr W. Ogawa (Kobe University, Japan) [22]. A monoclonal anti-phosphotyrosine antibody (PY99) was from Transduction Laboratories (Lexington, KY, USA). A polyclonal anti-Thr^{308} phospho-specific Akt antibody, a polyclonal anti-Ser^{473} phospho-specific Akt antibody, and a monoclonal anti-PKCλ antibody were from Cell Signalling (Beverly, MA, USA). A polyclonal anti-Akt antibody and a polyclonal anti-Glut4 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A polyclonal IRS-1 antibody and a polyclonal anti-PDGF β receptor antibody were from Upstate Biotechnology (Lake Placid, NY, USA). Enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM) vitamin mixtures, and MEM amino acid solutions were from Gibco BRL Japan (Tokyo, Japan). All other reagents were of analytical grade and purchased from Sigma Chemical (St Louis, MO, USA) or Wako Pure Chemical Industries (Osaka, Japan).

Construction of adenoviral vectors A cDNA encoding a phosphatidylinositol 5′-phosphatase-defective mutant of SHIP2 (ΔIP-SHIP2) containing Pro^{687} to Ala, Asp^{691} to Ala, and Arg^{692} to Gly changes was subcloned into the vector pAxCAwt, and transferred to recombinant adenovirus by homologous recombination utilizing an Adenovirus Expression Vector Kit (Takara Biomedicals, Tokyo, Japan) as described previously [8]. The adenoviral vector encoding IRS-1 was also described previously [23].

Cell culture and infections with adenovirus 3T3-L1 fibroblasts were grown and passaged in DMEM supplemented with 10% donor calf serum. Cells at 2–3 days postconfluence were used for differentiation. The differentiation medium contained 10% fetal bovine serum (FBS), 250 nmol/l dexamethasone, 0.5 mmol/l isobutyl methylxanthine, and 500 nmol/l insulin. After 3 days, the differentiation medium was replaced with postdifferentiation medium containing 10% FBS and 500 nmol/l insulin. After 3 more days, the postdifferentiation medium was replaced with DMEM supplemented with 10% FBS. ΔIP-SHIP2 and IRS-1 were transiently expressed in differentiated 3T3-L1 adipocytes by means of adenovirus-mediated gene transfer. A multiplicity of infection (m.o.i.) of 10–40 pfu/cell was used to infect 3T3-L1 adipocytes in DMEM containing 2% FBS, with the virus being left on the cells for 16 h prior to removal. Subsequent experiments were conducted 24–48 h after initial addition of the virus [8]. The efficiency of adenovirus-mediated gene transfer of ΔIP-SHIP2 and IRS-1 was approximately 95%.

Measurements of PI(3,4,5)P3 and PI(3,4)P2 levels in vivo The same numbers of 3T3-L1 adipocytes transfected with LacZ or ΔIP-SHIP2 were starved of phosphate overnight in phosphate-free DMEM (Life Technology), then starved of serum for 3 h. [^{32}P]Orthophosphate (3.7 MBq/ml) was added, and the cells were cultured for an additional 2 h. Following the labelling period, the cells were incubated with or without 1 μmol/l insulin for 15 min. The reaction was terminated by washing once with ice-cold PBS, followed by the addition of methanol and 1 N HCl (1:1). The labelling of the cells with [^{32}P]orthophosphate was con-

ducted at the same time in both sets of transfected cells. Phospholipids were then extracted with chloroform. The extracted lipid was deacylated and subjected to amino-exchange high-performance liquid chromatography (HPLC) using a Partisphere strong anion-exchange column (Whatman) as described previously [8]. The PI(3,4)P2 and PI(3,4,5)P3 levels in the same sample for each line were measured within a single HPLC run. The radioactivity was detected with an online radiochemical detector.

Chronic insulin treatment 3T3-L1 adipocytes grown in 6-well multiplates were incubated with DMEM containing 0.1% FBS with or without 100 nmol/l insulin at 37°C for various periods. For experiments with rapamycin treatment, 20 nmol/l rapamycin was added for 30 min before the addition of insulin. At the end of the chronic treatment with insulin, the cells were washed with PBS, incubated in serum-free DMEM for 30 min, and washed again with PBS. The cells were then treated with or without 17 nmol/l insulin for 5 min.

Plasma membrane fractionation The cells were washed twice with PBS and once with HES buffer (255 mmol/l sucrose, 20 mmol/l HEPES, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulphonyl fluoride [PMSF], 1 mmol/l Na_3VO_4, 2 μg/ml of aprotinin, and 50 ng/ml of okadaic acid, pH 7.4) and immediately homogenized by 20 strokes with a motor-driven homogenizer in HES buffer at 4°C. The homogenates (two 10-cm-diameter dishes per condition) were subjected to subcellular fractionation as described previously to isolate the plasma membrane (PM) [21]. In brief, the homogenates were centrifuged at 19,000 *g* for 20 min. The pellet obtained from the spin was resuspended in HES buffer, layered onto a 1.12 mol/l sucrose cushion, and centrifuged at 100,000 *g* in a swing rotor for 60 min. A white fluffy band at the interface was collected, resuspended in HES buffer, and centrifuged at 40,000 *g* for 20 min, yielding a pellet of PM. All fractions were adjusted to a final protein concentration of 1–3 mg/ml, which was measured by the Bradford method, and stored at −80°C until use.

Immunoprecipitation and western blotting The cells or the plasma membrane preparation were lysed in a buffer containing 20 mmol/l Tris, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 2.5 mmol/l sodium deoxycholate, 1 mmol/l β-glycerophosphate, 1% Triton X-100, 1 mmol/l PMSF, 1 mmol/l Na_3VO_4, 50 mmol/l sodium fluoride, 10 μg/ml of aprotinin, and 10 μmol/l leupeptin, pH 7.4, for 30 min at 4°C. The lysates were centrifuged to remove insoluble materials. The supernatants (100 μg of protein) were immunoprecipitated with antibodies for 2 h at 4°C. The precipitates or the lysates were then separated by 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDM) using a Bio-Rad Transblot apparatus. The membranes were blocked in a buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, 0.1% Tween 20, and 2.5% bovine serum albumin (BSA) or 5% non-fat milk, pH 7.5, for 2 h at 20°C. They were then probed with antibodies for 2 h at 20°C or for 16 h at 4°C. After the membranes were washed in a buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, and 0.1% Tween 20, pH 7.5, blots were incubated with a horseradish peroxidase-linked secondary antibody and subjected to enhanced chemiluminescence detection using ECL reagent according to the manufacturer's instructions (Amersham) [8]. In each experiment, the intensity of the band derived from control cells was assigned a value of 1 arbitrary unit, and the intensity of all treated groups was expressed as a fold value of control.

Measurement of PKCλ activity The cells were washed with ice-cold PBS and lysed with PKCλ buffer containing 50 mmol/l MOPS–HCl, 0.5% Triton X-100, 10% glycerol, 5 mmol/l EDTA, 5 mmol/l EGTA, 20 mmol/l NaF, 50 mmol/l β-glycerophosphate, 2 mmol/l Na_3VO_4, 2 mmol/l DTT, 1 μg/ml of leupeptin, and 2 mmol/l PMSF, pH 7.5. The lysates were centrifuged at 15,000 *g* for 20 min. The protein concentration in the resulting supernatants was determined with the use of bicinchoninic acid protein assay reagent (Pierce), and equal amounts of protein were subjected to immunoprecipitation with anti-PKCλ antibody. The immunoprecipitates were washed twice with PKCλ buffer containing 0.1% BSA, once with PKCλ buffer containing 0.1% BSA and 1 mol/l NaCl, and once with a solution containing 20 mmol/l Tris–HCl, 10% glycerol, 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 20 mmol/l 2-mercaptoethanol, 10 μg/ml of leupeptin, and 2 mmol/l PMSF, pH 7.5. Then, the precipitates were incubated for 14 min at 30°C with 14.8 kBq of [γ-^{32}ATP] in a reaction mixture (25 μl) containing 35 mmol/l Tris, pH 7.5, 10 mmol/l $MgCl_2$, 0.5 mmol/l EGTA, 0.1 mmol/l $CaCl_2$, 40 μmol/l unlabelled ATP, 100 μg/ml of phosphatidylserine, and 30 μmol/l myelin basic protein (MBP) as a substrate. Kinase reactions were terminated by the addition of SDS sample buffer, and the samples were then fractionated by SDS-PAGE [8, 22]. The radioactivity incorporated into substrates was determined with a Fuji BAS 2000 image analyser.

Measurement of 2-DOG uptake 3T3-L1 adipocytes grown in 6-well multiplates were serum-starved for 3 h. The cells were treated with or without rapamycin for 30 min and further incubated with 17 nmol/l insulin for 6 h. The cells were washed once with PBS, three times with Krebs–Ringer phosphate (KRP)–HEPES buffer, 10 nmol/l HEPES, 131.2 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, and 2.5 mmol/l NaH_2PO_4, pH 6.0, and once with KRP–HEPES buffer containing 1% BSA, pH 7.4. The cells were then incubated with the same KRP–HEPES buffer for 1 h at 37°C. The cells were subsequently stimulated with various concentrations of insulin. Following 15 min of insulin treatment, 3.7 kBq of 2-[^{3}H] DOG was added for 4 min. The reaction was stopped by the addition of 10 μmol/l cytochalasin B. The cells were washed three times with PBS and solubilized with 0.2 mmol/l SDS–0.2 N NaOH [8]. The radioactivity incorporated into the cells was measured by liquid scintillation counting.

Statistical analysis The data are represented as means ± SEM. p Values were determined using Student's t test, and $p<0.05$ was considered statistically significant.

Results

Effect of chronic insulin treatment on IRS-1 and SHIP2 Chronic treatment with insulin facilitates the proteosomal degradation of IRS-1 [20, 21, 24, 25]. Treatment with insulin reduced the amount of IRS-1, but not Akt, in a time-dependent manner in 3T3-L1 adipocytes. After 12 h of treatment, the amount of IRS-1 was 13.6±2.6% of the control level (Fig. 1a). In accordance with the reduced amount of IRS-1, the subsequent insulin-stimulated tyrosine phosphorylation of IRS-1 following chronic insulin treatment was markedly decreased to 13.2±1.8% (Fig. 1b). Thus, chronic insulin treatment caused an impairment of insulin signalling, at least, at the step involving IRS-1. Since SHIP2 is an important negative regulator of insulin signalling, alteration of its expression may cause insulin resistance [8–12]. In this regard, the amount of SHIP2 protein was relatively high after chronic treatment with insulin. Following 6 h of insulin treatment, the amount of SHIP2, but not Akt, was increased by 36.4±7.4% (Fig. 1c). Membrane targeting of SHIP2 is known to be important to its function to hydrolyse PI(3,4,5)P3 in insulin signalling [26]. The extent of insulin-induced translocation of SHIP2 to the plasma membrane fraction was increased by 33.4±8.3% compared to that without chronic insulin treatment. To assure equal amounts of protein were loaded among the samples, the PM fraction was immunoblotted with anti-PDGF β receptor antibody (Fig. 1d). These results indicate that chronic insulin treatment elicits insulin resistance, at least in part, at the level of SHIP2 as well as IRS-1.

Inhibition of endogenous SHIP2 function by expression of ΔIP-SHIP2 Because an elevated amount of SHIP2, especially at the plasma membrane, appears to be involved in the insulin resistance caused by the chronic insulin treat-

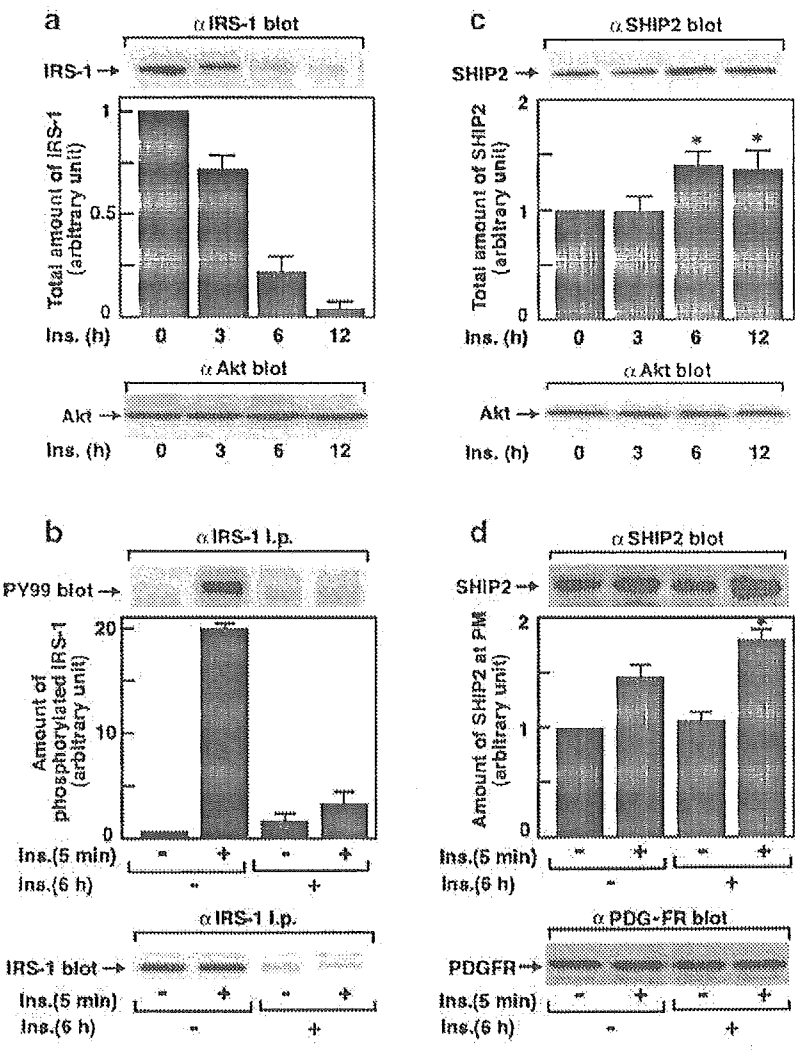


Fig. 1 Effect of chronic insulin treatment on IRS-1 and SHIP2. 3T3-L1 adipocytes were treated with 17 nmol/l insulin for the periods indicated (chronic insulin treatment). **a** The cells were lysed, and the proteins were separated by SDS-PAGE and immunoblotted with anti-IRS-1 antibody or anti-Akt antibody. **b** After chronic insulin treatment, the cells were washed with PBS and incubated in insulin-free medium for 30 min, then stimulated with 17 nmol/l insulin for 5 min (acute insulin treatment). The cell lysates were immunoprecipitated with anti-IRS-1 antibody. The precipitates were subjected to SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody (PY99) or anti-IRS-1 antibody. **c** The cell lysates were separated by SDS-PAGE and immunoblotted with anti-SHIP2 antibody or anti-Akt antibody. *$p<0.05$ versus amounts of SHIP2 without chronic insulin treatment. **d** The cells were homogenized and subjected to subcellular fractionation to yield the plasma membrane (PM) fraction. Samples in the PM fraction were separated by SDS-PAGE and immunoblotted with anti-SHIP2 antibody or anti-PDGF β receptor antibody. *$p<0.05$ versus amounts of SHIP2 at PM following 5 min of insulin stimulation without chronic insulin treatment. Results are means ± SEM of three separate experiments.

ment, expression of ΔIP-SHIP2, which acts in a dominant-negative manner, may ameliorate the impaired insulin signalling. Adenovirus-mediated gene transfer produced an eightfold increase in ΔIP-SHIP2 expression compared to the endogenous level of SHIP2 (Fig. 3b). The expression of ΔIP-SHIP2 increased insulin-induced generation of PI(3,4,5)P3, whereas the amount of PI(3,4)P2 was decreased (Fig. 2a). Thus, the expression of ΔIP-SHIP2 in fact functions to inhibit the endogenous 5′-phosphatase activity of SHIP2 in 3T3-L1 adipocytes. In addition, insulin-induced increase in the levels of PI(3,4,5)P3 was decreased to 25% after chronic insulin exposure. ΔIP-SHIP2 expression ameliorated the reduced levels of PI (3,4,5)P3 to 61% of the control level (data not shown). Thus, expression of ΔIP-SHIP2 appears to effectively ameliorate the decreased PI(3,4,5)P3 levels caused by chronic insulin treatment.

Effect of ΔIP-SHIP2 and IRS-1 expression, and pretreatment with rapamycin on insulin-induced phosphorylation of Akt after chronic insulin treatment Treatment with PI3-kinase inhibitor LY294002 effectively inhibited the chronic insulin treatment-induced degradation of IRS-1. Expression of ΔIP-SHIP2 partly abolished the inhibitory effect of LY294002 on the IRS-1 degradation, because SHIP2 is located downstream of PI3-kinase (data not shown). These results indicate that chronic insulin treatment induces the degradation of IRS-1 via PI3-kinase dependent mechanism. mTOR is a downstream molecule of PI3-kinase, and rapamycin is known to efficiently inhibit mTOR-dependent proteosomal degradation of IRS-1 in 3T3-L1 adipocytes [19–21]. Thus, the decrease in the amount of IRS-1 induced by degradation after chronic insulin treatment was effectively prevented by pretreatment with rapamycin. In contrast, expression of ΔIP-SHIP2 alone did not affect the loss of IRS-1 caused by the chronic insulin treatment. In addition, the preventive effect of rapamycin was not affected by the expression of ΔIP-SHIP2, because SHIP2 is located upstream of mTOR [8]. The decrease in IRS-1 caused by chronic insulin treatment can also be prevented by advanced overexpression of IRS-1. Thus, overexpression of IRS-1 in advance prevented the decrease in IRS-1 caused by chronic insulin treatment in an m.o.i.-dependent manner (data not shown). At an m.o.i. of 10 pfu/cell, the amount of IRS-1 after chronic insulin treatment was similar to that without chronic insulin treatment. The amount of protein loaded among the samples was confirmed to be identical by immunoblotting with anti-Akt antibody (Fig. 3b). As a result, rapamycin treatment and IRS-1 overexpression ameliorated the decreased levels of PI(3,4,5)P3 by chronic insulin treatment to 52% and 58%, respectively, of the control level (data not shown). Akt is an important mediator of the metabolic actions of insulin, and the activation of Akt is induced by the phosphorylation at Thr^{308} and Ser^{473} [8, 26–28]. Chronic insulin treatment decreased the subsequent insulin-stimulated phosphorylation of Akt at Thr^{308} and Ser^{473} to 23.8 ±2.0% and 28.5±2.1%, respectively. The reduction can be caused by the alteration of IRS-1 and/or SHIP2 following chronic insulin treatment. In this regard, pretreatment with rapamycin ameliorated the insulin-induced phosphorylation of Akt at Thr^{308} and Ser^{473}. Similarly, overexpression of ΔIP-SHIP2 effectively ameliorated the phosphorylation of Akt at Thr^{308} and Ser^{473} in an m.o.i.-dependent manner (data not shown), and it was most effectively ameliorated at an m.o.i. of 40 pfu/cell. The amelioration was more apparent and almost fully restored to the control level by both pretreatment with rapamycin and expression of ΔIP-SHIP2. In addition, the effective restoration of insulin-stimulated phosphorylation of Akt following chronic insulin treatment was also seen after advanced overexpression of IRS-1 (Fig. 3a).

Effect of ΔIP-SHIP2 and IRS-1 expression, and pretreatment with rapamycin, on insulin-induced activation of PKCλ after chronic insulin treatment Another important molecule downstream of PI3-kinase for metabolic insulin signalling is atypical PKC in 3T3-L1 adipocytes [22, 29]. In accordance with the results of insulin-induced phosphorylation of Akt, insulin stimulation of PKCλ activity was decreased to 23.8±3.9% of the control level after chronic insulin treatment. Pretreatment with rapamycin

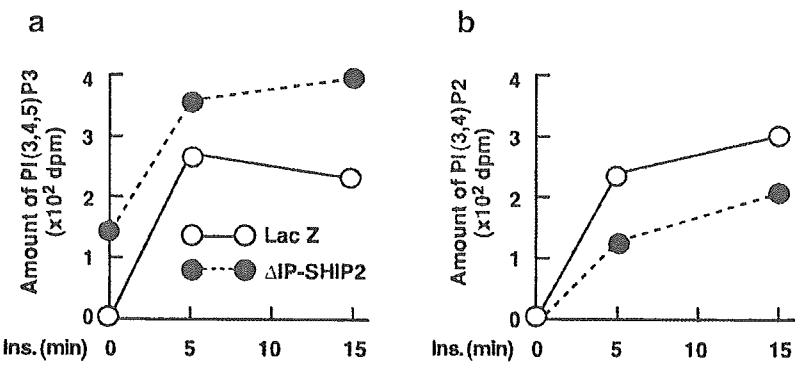
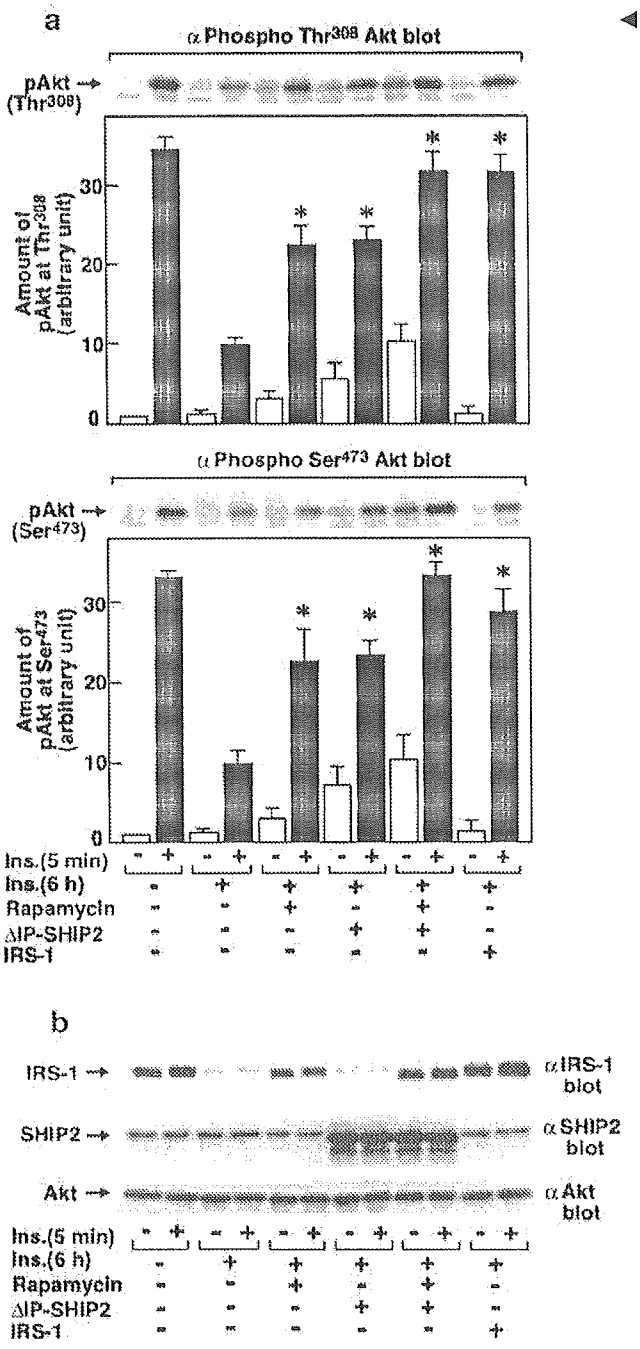


Fig. 2 Inhibition of endogenous SHIP2 function by expression of ΔIP-SHIP2. 3T3-L1 adipocytes were transfected with LacZ or ΔIP-SHIP2 at an m.o.i. of 40 pfu/cell. The cells were labelled with [^{32}P] orthophosphate for 2 h and incubated with or without insulin, and lipids were extracted with chloroform. The extracted lipids were analysed by HPLC after being deacylated. The amounts of ^{32}P-labelled PI(3,4,5)P3 (**a**) and PI(3,4)P2 (**b**) generated were determined with an online radiochemical detector. Results are means of two separate experiments.



◀ **Fig. 3** Effect of ΔIP-SHIP2 and IRS-1 expression, and pretreatment with rapamycin on insulin-induced phosphorylation of Akt after chronic insulin treatment. 3T3-L1 adipocytes were transfected with LacZ and ΔIP-SHIP2 at an m.o.i of 40 pfu/cell, or IRS-1 at an m.o.i. of 10 pfu/cell. Serum-starved transfected cells were incubated with vehicle or 20 nmol/l rapamycin for 30 min, and treated with 17 nmol/l insulin for 6 h. The cells were washed with PBS and incubated in insulin-free medium for 30 min, and the cells were stimulated with 17 nmol/l insulin for 5 min. **a** The cells were lysed and the lysates were separated by SDS-PAGE and immunoblotted with anti-Ser^{473}-phospho-specific or anti-Thr^{308}-phospho-specific Akt antibody. The amount of Akt phosphorylated at Ser^{473} and Thr^{308} was quantitated by densitometry. Results are means ± SEM of four separate experiments. $^*p<0.05$ versus amounts of phosphorylated Akt in LacZ-transfected cells with chronic insulin treatment. **b** The cell lysates were separated by SDS-PAGE, and immunoblotted with anti-IRS-1 antibody, anti-SHIP2 antibody, or anti-Akt antibody.

only partly restored the decreased PKCλ activity to 49.8± 3.8%. Although the effect was still partial, expression of ΔIP-SHIP2 relatively efficiently ameliorated the reduced PKCλ activity caused by the chronic insulin treatment to 65.0±7.5% of the control level. A combination of rapamycin treatment and expression of ΔIP-SHIP2 more effectively restored the insulin-induced activation of PKCλ. In contrast to the results for the phosphorylation of Akt, the restoration of PKCλ activity was still partial, and was 75.2±4.1% of the control level. The partial amelioration of insulin-stimulated PKCλ activity after chronic insulin treatment was also seen with advanced overexpression of IRS-1 (Fig. 4a). The amount of PKCλ protein was not

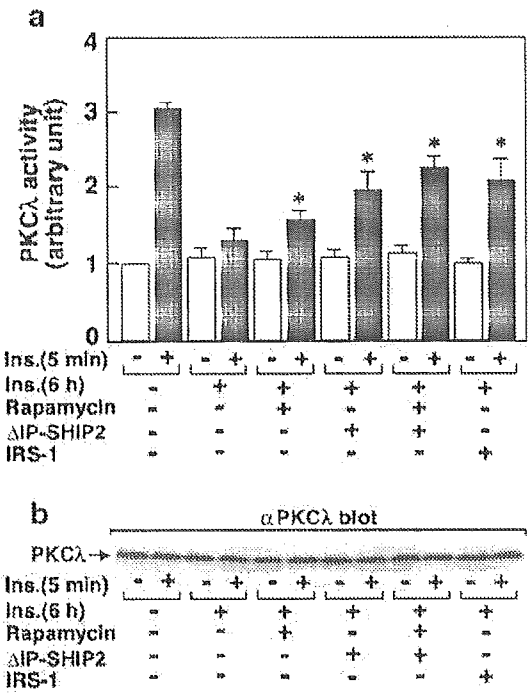


Fig. 4 Effect of ΔIP-SHIP2 and IRS-1 expression, and pretreatment with rapamycin, on insulin-induced activation of PKCλ after chronic insulin treatment. 3T3-L1 adipocytes were transfected with LacZ and ΔIP-SHIP2 at an m.o.i. of 40 pfu/cell, or IRS-1 at an m.o.i. of 10 pfu/cell. Serum-starved transfected cells were incubated with vehicle or 20 nmol/l rapamycin for 30 min, and treated with 17 nmol/l insulin for 6 h. The cells were washed with PBS, incubated in insulin-free medium for 30 min, and stimulated with 100 nmol/l insulin for 5 min. They were then lysed and immunoprecipitated with anti PKCλ antibody. Kinase reactions were conducted, and samples were fractionated by SDS-PAGE. **a** The radioactivity incorporated into substrates was determined with a Fuji BAS 2000 image analyser. Results are means ± SEM of three separate experiments; $^*p<0.05$ versus insulin-stimulated PKCλ activity in LacZ-transfected control cells with chronic insulin treatment. **b** The cell lysates were separated by SDS-PAGE, and immunoblotted with anti-PKCλ antibody.