# Physical Exercise Enhances Protein Kinase C δ Activity and Insulin Receptor Tyrosine Phosphorylation in Diabetes-Prone *Psammomys obesus*

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**We recently reported that physical exercise prevents the progression of type 2 diabetes mellitus in *Psammomys obesus*, an animal model of nutritionally induced type 2 diabetes mellitus. In the present study we characterized the effect of physical exercise on protein kinase C δ (PKCδ) activity, as a mediator of the insulin-signaling cascade in vivo. Three groups of *Psammomys obesus* were exposed to a 4-week protocol: high-energy diet (HE/C), high-energy diet and exercise (HE/EX), or low-energy diet (LE/C). None of the animals in the HE/EX group became diabetic, whereas all the animals in the HE/C group became diabetic. After overnight fast, intraperitoneal (IP) insulin (1U) caused a greater reduction in blood glucose levels in the HE/EX and LE/C groups compared to the HE/C group. Tyrosine phosphorylation of insulin receptor (IR), insulin receptor substrate-1 (IRS-1), and phosphatidylinositol 3 kinase (PI3 kinase) was significantly higher in the HE/EX and LE/C groups compared with the HE/C group. Finally, IR-associated PKCδ was higher in the HE/EX and LE/C groups compared to the HE/C group. Coprecipitation of PKCδ with IR was higher in the HE/EX and LE/C groups compared to the HE/C group. Thus, we suggest that 4 weeks of physical exercise results in improved insulin-signaling response in *Psammomys obesus* accompanied by a direct connection between PKCδ and IR. We conclude that this mechanism may be involved in the preventive effect of exercise on type 2 diabetes mellitus in *Psammomys obesus*.**


TYPE 2 DIABETES MELLITUS is the most common disease in the western world.[1] Improvement in treatment and, in particular, methods of prevention are therefore being sought continuously. In general, physical exercise is known to improve glucose uptake and is recommended for type 2 diabetics or type 2 diabetes-prone patients.[2,3] Some studies have found that skeletal muscle responsiveness to insulin increases following exercise training.[2,4,5] One potential mechanism of this increase in insulin responsiveness could involve the ability of exercise to improve insulin signaling via increased tyrosine phosphorylation of key proteins in the insulin signaling cascade, specifically insulin receptor (IR), insulin receptor substrate 1 (IRS-1), and phosphatidylinositol 3 kinase (PI3 kinase). However, studies in this area have yielded inconsistent findings: some showed no changes and even lower activity in the insulin signaling proteins after exercise,[6] whereas others indicated a significant increase in the response of the proteins after exercise.[7] Moreover, the association between the response of the insulin signaling proteins to the adaptive effects of exercise training in prevention of type 2 diabetes mellitus in a diabetes-prone model has not been extensively studied.

We recently found that exercise training prevents the progression of type 2 diabetes mellitus in *Psammomys obesus* (sand rat).[8] This animal model may be considered a model for nutritionally induced insulin resistance.[9,10] When transferred to a high-energy laboratory diet it develops type 2 diabetes mellitus within several days to 2 weeks.[11] Four generally consecutive stages (A, B, C, D) of progression to diabetes have been described in this model: (A) the original stage—normoglycemia and normoinsulinemia; (B) hyperinsulinemia only, which is sufficient to maintain normoglycemia; (C) hyperinsulinemia and hyperglycemia (blood glucose level $>$ 11.1 mmol/L); (D) hyperglycemia and hypoinsulinemia, due to loss of β-cell insulin secretion capacity. Stage D is irreversible and, unless treated with insulin, the animals eventually die from severe ketoacidosis.[9] We demonstrated for the first time that exercise training prevents the progression of diabetes in most of the exercising animals.[8] Most of the animals in this group remained at the B hyperinsulinemic-normoglycemic stage for at least 4 weeks in spite of the high-energy diet, whereas most of the control nonexercising group became severely diabetic after 1 week. We also showed that protein kinase C δ (PKCδ) activity was higher in the exercising group compared with the diabetic group. Additional studies showed that activated PKCδ mediates insulin-induced glucose uptake in rat skeletal muscle in vitro and that PKCδ plays an important role in early IR signaling.[12] We therefore hypothesized that increased activation of PKCδ may play an important role in the mechanism of prevention of diabetes in prone animals by exercise training via amelioration of the insulin signaling response. The purpose of the present study was, therefore, to elucidate the possible association of PKCδ with the insulin receptor and its major substrates after 4 weeks of exercise training, using in vivo insulin stimulation.

## MATERIALS AND METHODS

### *Animals*

Thirty male *Psammomys obesus* aged 6 weeks (3 weeks after weaning) from the Hebrew University, Hadassah Medical School Animal Farm were used in the present study. The animals were housed in suitable cages (5 animals per cage) in a temperature (22 to 25°C) and light (12:12-hour light-dark cycle) controlled room. The animals were randomly assigned to 3 groups of 10 animals each: HE/EX—exercising animals consuming a high-energy diet; HE/C—control animals con-

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suming the same high-energy diet; and LE/C—control animals consuming a low-energy diet, which does not induce diabetes.[10] The composition of *Psammomys obesus* diets was previously described.[13] The high-energy diet was composed of 23.6% protein, 2.4% fat, 68% carbohydrate, and 6% ash; 12.27 Kj/g (Weizmann Institute, Rehovot, Israel). The low-energy diet was composed of 16.7% protein, 3.1% fat, 70% carbohydrate, and 10.2% ash; 9.97 kJ/g (Weizmann Institute). Food and water were supplied ad libitum. All experimental procedures were authorized by the institutional animal care committee.

### *Materials*

Antiphosphotyrosine was obtained from Upstate Biotechnology, Lake Placid, NY. Anti-IR and anti-PI3 kinase were obtained from Santa Cruz (Santa Cruz, CA). Anti–IRS-1 was obtained from Transduction Laboratories, Lexington, KY. Anti PKCδ was obtained from Promega, Madison, WI.

### *Experimental Protocol*

The exercise training protocol was previously described.[8] Briefly, during the 4-week protocol, the animals ran on a treadmill (Quinton Q55, Seattle WA; 2.25 km/h, 6% slope) 5 days per week, 90 minutes per day (45 minutes in the morning and 45 minutes in the afternoon). The other groups, HE/C and LE/C, were held sedentary, consuming high-energy or low-energy diets, respectively. After 4 weeks the animals went through an overnight fast, and blood glucose levels were measured before and 30 minutes after 1 U of insulin injection IP. The animals were then anaesthetized (sodium pentobarbital 0.03 mg/g IP); the left quadriceps muscle was excised, immediately frozen in liquid nitrogen, and then stored at −70°C. Blood (2 mL) was taken directly from the left ventricle cavity, centrifuged for 15 minutes, and the serum kept at −70°C for later analysis. The animals were then killed by an overdose of sodium pentobarbital.

### *Physiological Measurements*

The animals were weighed at baseline and once a week thereafter for the duration of the study. Blood for glucose determinations was taken at baseline and once a week from the tail vein, using the glucometer Elite (Bayer, Kyoto, Japan) (in the HE/EX group blood was taken 24 hours after the preceding exercise). Serum insulin concentration was measured by radioimmunoassay (RIA) using the standard 18-hour incubation double-antibody assay. Primary (guinea pig) and secondary (goat anti-guinea pig) antisera were from Linco Research (St Charles, MO). Human insulin standard (Novo Nordisk, Bagsvaerd, Denmark) was used for *Psammomys obesus* insulin RIA; cross-reactivity and dilution linearity were previously determined.[9] The minimum detectable concentration was 11 pmol/L; routine intra-assay coefficient of variation (CV) was 4% to 6%, and interassay CV was 6% to 10%.

### *Preparation of Muscle Tissue*

Muscle tissue samples were washed with $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline (PBS) to remove excess blood cells, and then mechanically lysed in RIPA buffer (Tris HCl, pH 7.4, 50 mmol/L, NaCl, 150 mmol/L; EDTA 1 mmol/L; NaF, 10 mmol/L; Triton x100, 1%; sodium dodecyl sulfate [SDS], 0.1%; Na deoxycholate, 1%) containing a cocktail of protease inhibitors (leupeptin, 20 μg/mL; aprotinin, 10 μg/mL; phenylmethylsulfonyl fluoride [PMSF], 0.1 mmol/L; dithiothreitol [DTT], 1 mmol/L) and phosphatase inhibitors (orthovanadate, 200 μmol/L; pepstatin, 2 μg/mL) (Sigma, St Louis MO). After a 30-second homogenization, the preparation was centrifuged at 20,000 × *g* for 20 minutes at 4°C. The supernatant, containing all the tissue's proteins, was then stored at −70°C for later analysis.

### *Biochemical Measurements*

The protein content in each sample was measured using Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad, Richmond, CA).

### *Immunoprecipitation*

Twenty-five microliters of protein A/G Sepharose was added to 0.3 mL of the lysate, and the suspension was rotated continuously for 30 minutes at 4°C. The preparation was then centrifuged at 20,000 × *g* at 4°C for 10 minutes, and 30 μL of A/G Sepharose was added to the supernatant along with specific monoclonal or polyclonal antibodies to various antigens. The suspension was rotated overnight at 4°C and then centrifuged at 20,000 × *g* for 10 minutes at 4°C, and the pellet was washed twice as above with RIPA buffer. The beads were eluted with 25 μL of sample buffer (0.5 mol/L Tris HCl pH 6.8; 10% SDS; 10% glycerol; 4% 2-beta-mercaptoethanol; 0.05% bromophenol blue). The suspension was again centrifuged at 15,000 × *g* (4°C for 10 minutes) and washed 4 times in Tris-buffered saline Tween (TBST). Sample buffer was added and the samples were boiled for 5 minutes and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

### *Western Blotting*

Protein, 20 to 25 μg, was electrophoresed through SDS-polyacrylamide gels (7.5% or 10%) and electrophoretically transferred onto Immobilon-P (Millipore, Bedford, MA) membranes. Following transfer, the membranes were subjected to standard blocking and incubation procedures, and were incubated with specific monoclonal or polyclonal antibodies to the various proteins. The membranes were washed 4 times for 15 minutes in TBST and then further incubated for 20 minutes at room temperature with horseradish peroxidase (HRP)-labeled secondary antibody (goat anti-rabbit or mouse IgG) diluted 1:10,000 in blocking buffer. Following 3 washes (1 × 15 minutes and 2 × 5 minutes) in TBST, the membranes were treated with enhanced chemoluminescence (ECL) reagent for 1 minute, then exposed on x-ray film (Kodak, Rochester, NY) for the required times (5 to 30 seconds), developed, and quantified by densitometry.

### *Activity Assay*

PKCδ activity was measured after immunoprecipitation with anti-PKCδ antibody as described above. The lysates were prepared in RIPA buffer without NaF. Activity was measured with the use of the SignaTECT protein kinase C assay system (Promega). This kit contains all necessary cofactors and utilizes a highly specific biotinylated substrate (Neurogranin).

In preliminary experiments we demonstrated that immunoprecipitation was specific for the PKCδ isoform. In other words, immunoblotting of the immunoprecipitated PKCδ with specific antibodies to other isoforms did not reveal the presence of isoforms other than the one specifically immunoprecipitated.

### *Statistical Analysis*

Results were analyzed using analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. *P* values less than .05 were considered significant. Data are presented as the mean ± SE.

## RESULTS

### *Physiological Profiles of HE/C, HE/EX, and LE/C Groups During the Experiment*

The physiological results after 4 weeks are summarized in Table 1. After 4 weeks none of the animals in the HE/EX groups was diabetic (average blood glucose level, 4.6 ± 0.3

**Table 1. Summary of Physiological Measurements in the Fed State of the HE/C, HE/EX, and LE/C Groups of *Psammonys obesus* After 4 Weeks**

| Measure | HE/C (n = 10) | HE/EX (n = 10) | LE/C (n = 10) |
|---|---|---|---|
| Glucose (mmol/L) | 22 ± 0.5*† | 4.6 ± 0.3 | 3.4 ± 0.4 |
| Insulin (pmol/L) | 4,900 ± 1,200† | 4,600 ± 1,000† | 300 ± 100 |
| Body weight gain (g) | 60 ± 7 | 71 ± 7 | 71 ± 3 |

*HE/EX ($P < .01$).
†LE/C ($P < .01$).

mmol/L), whereas all the animals in the HE/C group became diabetic (average blood glucose level, 21 ± 0.4 mmol/L). As expected, no animal in the LE/C group became diabetic (average blood glucose level, 3.38 ± 0.38 mmol/L). The physiological results as presented in Table 1 clearly point to the fact that the animals in the HE/C group were in the hyperinsulinemic-hyperglycemic stage (C) of the disease, while those in the HE/EX group remained in the hyperinsulinemic-normoglycemic stage (B) of the disease.

*Response to Insulin Injection*

After an overnight fast, blood glucose levels of the various groups were not significantly different (4.6 ± 0.7 mmol/L in the HE/C group, 4.0 ± 0.2 mmol/L in the HE/EX group, and 4.2 ± 0.3 in the LE/C group; $P$ = not significant [NS]) (Fig 1). Insulin injection reduced blood glucose levels in all the groups, but the decrease was significantly greater in the HE/EX and LE/C groups compared to the HE/C group (by 1.5 ± 0.1 mmol/L, by 1.5 ± 0.2 mmol/L, and by 0.6 ± 0.1 mmol/L, respectively) ($P < .02$). These results reflect the higher in vivo response to insulin in the HE/EX and LE/C groups.

*Insulin Signaling Cascade*

In previous studies, we found no differences in the expression and tyrosine phosphorylation of IR, IRS-1, and PI3 kinase in the basal state (data not shown). However, as in the present study, insulin stimulation significantly increased the level of tyrosine phosphorylation of IR, IRS-1, and PI3 kinase in the HE/EX group compared with the 2 other groups. Insulin-induced tyrosine phosphorylation of these proteins was also higher in the LE/C group compared to the HE/C group (Figs 2 through 4). No differences were found in total expression of the phosphorylated proteins (results are presented for the IR only) (Fig 2).

*Effects of Insulin on PKCδ*

Earlier studies have shown that insulin stimulation of skeletal muscle induces an increase in activity of PKCδ.[12] Accordingly, we examined PKCδ activity in muscle removed from the various groups following insulin injection. PKCδ activity 30 minutes after insulin injection was higher in the HE/EX and LE/C groups compared with the HE/C group (708 ± 60 cpm, 643 ± 70 cpm, and 566 ± 70 cpm, respectively) ($P < .05$), with no significant difference between the 2 former groups. Recent studies on primary cultures of rat skeletal muscle have pointed to the involvement of insulin-induced physical association between IR and PKCδ in the increase in glucose transport in response to this hormone.[12,14] We therefore analyzed the amount of PKCδ precipitated with IR in the different groups after insulin administration in vivo. Figure 5 shows that PKCδ

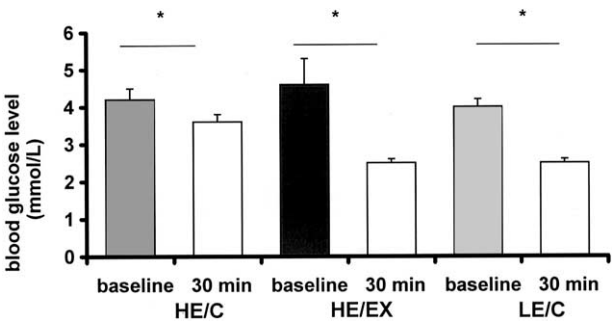
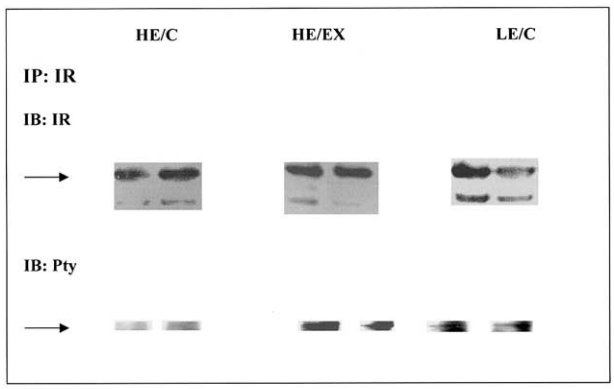



**Fig 1. Blood glucose levels after overnight fast (baseline) and 30 minutes after insulin injection in the HE/C, HE/EX, and LE/C groups of *Psammomys obesus* after 4 weeks. *$P < .01$; **$P < .05$.**



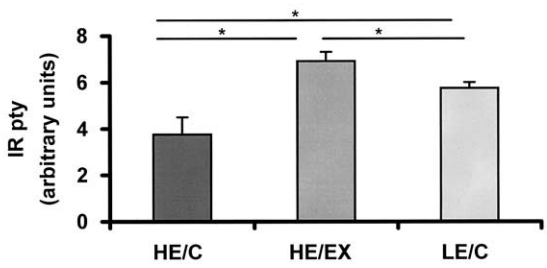




**Fig 2. Muscle IR β-subunit expression (IB) and tyrosine phosphorylation (Pty) 30 minutes after insulin injection in HE/C, HE/EX, and LE/C groups of *Psammomys obesus* after 4 weeks. Each lane represents 1 animal from each group. The quantified data from multiple experiments are given in the graph below. *$P < .05$**

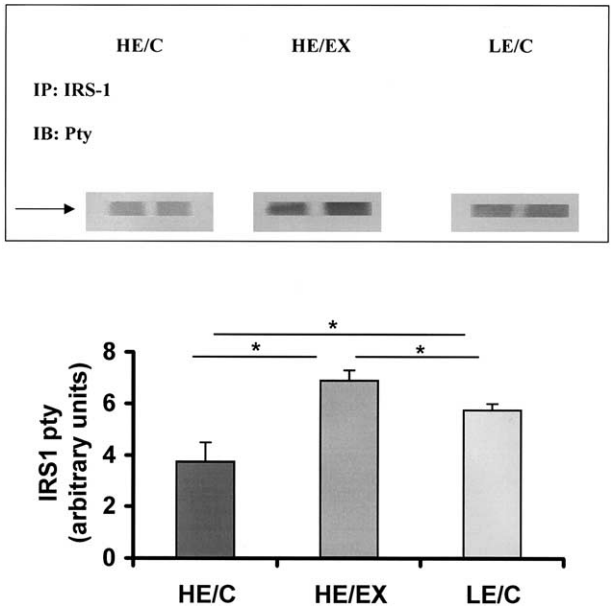



**Fig 3. Muscle IRS-1 tyrosine phosphorylation (Pty) 30 minutes after insulin injection in HE/C, HE/EX, and low-energy LE/C groups of *Psammomys obesus* after 4 weeks. Each lane represents 1 animal from each group. Tyrosine phosphorylation was analyzed on the same amount of IRS-1 protein. The quantified data from multiple experiments are given in the graph below. *$P < .01$.**

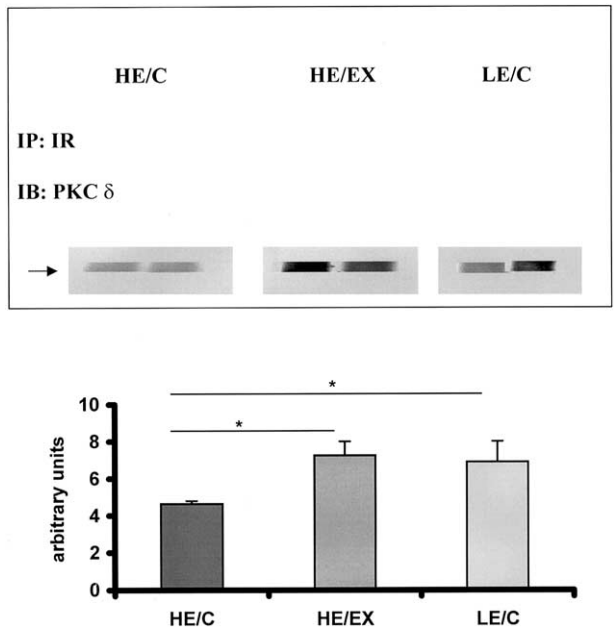



**Fig 5. Muscle PKC$\delta$ immunoprecipitation with IR 30 minutes after insulin injection in HE/C, HE/E, and LE/C groups of *Psammomys obesus* after 4 weeks. Each lane represents 1 animal from each group. No differences were found in the expression of IR. The quantified data from multiple experiments are given in the graph below. *$P < .02$.**

co-immunoprecipitation with IR following insulin stimulation was significantly higher in the HE/EX and LE/C groups compared to the HE/C group. Furthermore, in order to negate the possibility that PKC$\delta$ was activated by the preceding exercise bout, and to isolate the short-term (or acute) effect of exercise, we also analyzed PKC$\delta$ activity after acute exercise in 10 normoglycemic-normoinsulinemic animals. No influence of acute exercise on PKC$\delta$ activity was found (data not shown).

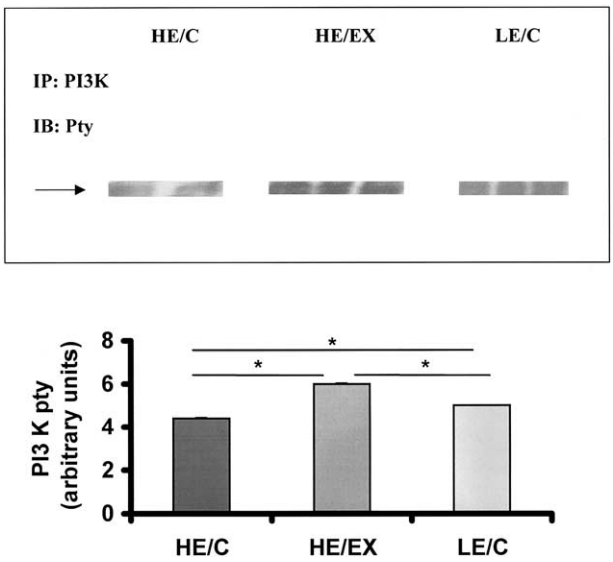



**Fig 4. Muscle PI3 kinase tyrosine phosphorylation (Pty) 30 minutes after insulin injection in HE/C, HE/EX, and LE/C groups of *Psammomys obesus* after 4 weeks. Each lane represents 1 animal from each group. Tyrosine phosphorylation was analyzed on the same amount of PI3 kinase protein. The quantified data from multiple experiments are given in the graph below. *$P < .01$.**

## DISCUSSION

The purpose of the present study was to examine the possibility that certain elements in the insulin-signaling cascade may be involved in the prevention of type 2 diabetes mellitus by long-term exercise training in *Psammomys obesus*. We hypothesized that the improved insulin responsiveness in skeletal muscles as a result of the adaptive effects of exercise would increase the tyrosine phosphorylation of the IR and its downstream signaling key proteins. We further hypothesized, based on previous studies,[8,12,14] that PKC$\delta$ might be involved in this mechanism. Our study demonstrated that exercise training enhanced the hypoglycemic response to insulin administration and increased tyrosine phosphorylation of IR, IRS-1, and PI3 kinase. We also found increased PKC$\delta$ activity and its physical connection with IR in the trained group. The enhanced glycemic response to insulin and the insulin responsiveness in the HE/EX group were observed even though the animals were in stage B of the disease. Weight gain was similar among the different groups, a finding that demonstrates that the effects of exercise were not mediated by weight reduction.[8] There are several possible explanations to these observations. One is that physical exercise might enhance the glucose uptake through mechanisms unrelated to insulin signaling.[15] This physiological

effect of improved glucose uptake would lead to normalized blood glucose levels and prevent various possible effects of the exposure to hyperglycemia on insulin signaling,[16] and possibly on PKCδ. Another possibility is that the IR tyrosine kinase activity might be decreased in the diabetic state by an as yet undetermined mechanism,[17] and that the physical activity might prevent the decreased function of IR. This possibility, however, is unlikely because we did not observe differences in IR tyrosine phosphortylation in the basal state. It should be noted that studies regarding the effect of exercise on insulin signaling appear to be inconsistent. On the one hand it was shown that exercise increased the expression of IRS-1 and the activity of PI3 kinase after insulin injection in human skeletal muscle.[7] On the other hand, it was found that increased tyrosine phosphorylation of IRS-1 and PI3 kinase was correlated with the rate of glucose transport in response to insulin in human skeletal muscle of trained individuals compared with the control group exposed to the same exercise protocol.[18] Other studies in rat skeletal muscle showed that exercise enhanced insulin stimulation of PI3 kinase activity, while no differences were found in IRS-1.[19] Still other studies reported that exercise did not change the skeletal muscle IR tyrosine kinase activity in response to insulin in rats[20] and humans,[21] although the responsiveness to insulin was increased. Another study even pointed to a paradoxical reduction in the tyrosine phosphorylation of IR, IRS-1, and PI3 kinase in rat skeletal muscle in response to insulin after exercise.[22] The findings in our study demonstrate an increased tyrosine phosphorylation of the IR and its major signaling proteins. These results may represent the effect of prolonged (4 weeks) physical training. A third explanation that should be considered is that the increased PKCδ activity and its binding to the IR may increase the IR tyrosine kinase activity and therefore its metabolic effects (Fig 6). Indeed, the higher activation of PKCδ in the HE/EX group and the higher physical association between the PKCδ and the IR, as was demonstrated by their coprecipitation, confirm results from previous studies using primary cultures of rat skeletal muscle.[14] These results showed that insulin stimulation induced a rapid and strong interaction between IR and PKCδ. It was suggested, therefore, that PKCδ is involved in the regulation of IR activity and, in subsequent steps, in the IR signaling cascade. PKC is also known to induce serine phosphorylation of IR, a mechanism that may decrease the receptor tyrosine kinase activity.[23] Nevertheless, serine phosphorylation of IR has also been shown to be an important step in IR routing.[24-26] We therefore assume, according with previous studies, that serine phosphorylation per se does not necessarily prevent tyrosine phosphorylation of IR. Rather, phosphorylation of IR on serine residues may play a role in the internalization of tyrosine phosphorylated IR to the internal membrane.[14] We also showed that acute exercise alone has no significant influence on PKCδ activity and that insulin stimulation is indeed required. Thus, increased PKCδ activity secondary to long term physical training may contribute to the activation of the IR signaling cascade in skeletal muscle.

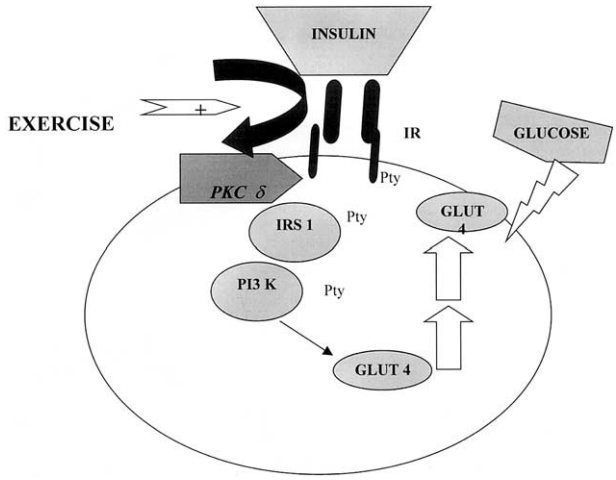



**Fig 6. The suggested mechanism depicting exercise training ameliorate insulin signaling response via activation of PKCδ. According to the results, physical exercise activates PKCδ and insulin signaling response in the presence of insulin in vivo. Activated PKCδ connects to IR and facilitates the signal. The endpoint is augmentation of insulin signaling, GLUT 4 translocation, glucose uptake, and preservation of the normoglycemic state despite the high insulin resistance.**

In summary, our findings demonstrate that long-term exercise training is associated with improved insulin signaling cascade, a mechanism that may be involved in the prevention of type 2 diabetes mellitus in *Psammomys obesus*. We further suggest that activation of PKCδ may be a possible mechanism connected to enhanced upstream insulin signaling response in skeletal muscle after exercise training. Further studies are needed to characterize the mechanism of action of PKCδ in this phenomenon.

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