reportedly enhance phosphorylation of serine residues of IRS-1 (3, 5, 16). Although S6K1-deficient mice were shown to be resistant to age- and diet-induced obesity and insulin resistance (26), we investigated the acute effect of transient inhibition of Raptor on the impaired insulin signaling and glucose intolerance of K/KAy mice with genetic obesity-associated insulin resistance. In the K/KAy mice, one of the obese rodent models, IRS-1 S307 and IRS-1 S636/S639 phosphorylations are elevated (26).

Raptor contains a highly conserved, amino-terminal domain followed by several HEAT repeats and seven carboxy-terminal WD40 repeats (4), and acts as an adaptor to recruit substrates, p70S6K and 4E-BP1, to mTOR (2, 12, 23). The domains in Raptor and mTOR that interact with each other have been clearly demonstrated, and suggest multiple contact sites between these two proteins (4, 10), in contrast with the selective binding of p70S6K to the N-terminal portion of Raptor (12). We were unable to detect the associations of Raptor and C terminally deleted Raptor (Raptor-ΔCT) with endogenous S6K (data not shown). However, it was demonstrated that Raptor-ΔCT binds to a far smaller amount of mTOR but not to IRS-1, while wild-type Raptor binds to both. Indeed, IRS-1 phosphorylation at Ser 636/639 was markedly decreased by Raptor-ΔCT overexpression. These findings suggest that Raptor-ΔCT functions as a

dominant-negative protein for mTOR/S6K or mTOR/IRS-1 signaling.

Interestingly, we found that 4E-BP1 phosphorylations of both Thr37/46 and Thr70 in the liver were significantly increased by Raptor-ΔCT overexpression. Thus, the inhibitory effect of Raptor-ΔCT is specific for S6 kinase. This result was unexpected but is hoped to provide useful information regarding how the Raptor/mTOR complex recognizes individual downstream molecules. We speculate that S6 kinase, but not 4E-BP1, preferentially associates with Raptor-ΔCT to full-length Raptor. If so, Raptor-ΔCT overexpression would inhibit S6 kinase binding, but not that of 4E-BP1, with the mTOR/Raptor complex. It is also possible that some unidentified molecule is required for this association between S6 kinase and the Raptor/mTOR complex, and that Raptor-ΔCT binds to this as yet unknown molecule. In this case, S6 kinase cannot bind the mTOR complex in the Raptor-ΔCT-overexpressing cells, while 4E-BP1 phosphorylated is unaffected. Further study is necessary to resolve this issue.

In this study, hepatic overexpression of Raptor-ΔCT strongly inhibited insulin induced p70S6K activation and improved glucose intolerance and hyperinsulinemia. Importantly, Akt phosphorylation was markedly enhanced not only under insulin-stimulated but also basal conditions. Decreased IRS-1 Ser307 and Ser636/639

phosphorylations and the resulting increases in tyrosine phosphorylation of IRS-1 and subsequent PI 3-kinase activity can account for the increased Akt phosphorylation under insulin stimulated conditions. However, this may not fully explain the mechanism leading to markedly increased basal Akt phosphorylation, since basal PI 3-kinase activity was not altered by Raptor-ΔCT. Thus, it is possible that other mechanisms, such as increased PDK and/or Rictor activity, or even suppression of Akt dephosphorylation, are involved in the increased basal Akt phosphorylation. Indeed, it has been reported that Raptor/mTOR and Rictor/mTOR complexes regulate Akt phosphorylation in a reverse manner (22). Further study is necessary to clarify whether suppression of the Raptor/mTOR complex via overexpression of Raptor-ΔCT leads to elevated Rictor/mTOR activity or suppressed Akt dephosphorylation.

In summary, we demonstrated that hepatic p70 S6 kinase inhibition in diabetic mice improves glucose tolerance by enhancing both basal and insulin-stimulated Akt phosphorylations. Although further experiments are needed to clarify the molecular mechanisms of increased basal Akt phosphorylation, our results suggest that mTORC1 inhibition is a potential treatment strategy for obesity-related insulin resistance.

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Table 1

	Control(LacZ)	Raptor-ΔCT
Body Weight(day1,g)	36.3±1.04	36.6±1.2
Body Weight(day5,g)	36.9±1.1	37±1.27
Liver/BWx10^2(day5)	4.87±0.44	5.12±0.34
Fat/BWx10^2(day5)	4±0.62	3.9±0.32
Heart/BWx10^2(day5)	0.37±0.25	0.48±0.01
Kidney/BWx10^2(day5)	0.94±0.62	1.28±0.02
FBS(day1, mg/dl)	122±7.48	126±9.03
FBS(day5, mg/dl)	160±6.31	140±8.37
T-cho(day5, mg/dl)	123±10.8	125±7.96
TG(day5, mg/dl)	197±83.2	200±48.2
NEFA(day5, mEq/l)	0.79±0.13	0.88±0.22
Insulin(day5, ng/ml)	2.36±1.78	0.91±0.34

Table and Figure Legends

Table 1. Weights and Metabolic Profiles of control (LacZ) and Raptor-ΔCT overexpressing mice. The body weights, major organ weights, blood glucose levels, and lipid concentrations of control (LacZ) and Raptor-ΔCT mice, before and 4 days after adenovirus injection. FBS: fasting blood sugar, T-cho:total cholesterol, TG: triglyceride, NEFA: non-esterified fatty acid   Control (LacZ): n=8, Raptor-ΔCT: n=8

Fig. 1. The adenovirus of dominant-negative Raptor, C terminally deleted Raptor (Raptor-ΔCT). *A*: The expression levels of endogenous Raptor and overexpressed Raptor-ΔCT in the livers of K/KAy mice and controls. *B*: Immunoblotting of overexpressed Raptor-ΔCT in various tissues with anti-Flag tag antibody. Each tissue (30μg), from Raptor-ΔCT overexpressing mice, was electrophoresed and immunoblotted with anti-flag tag antibody. 1: Brain, 2: Lung, 3: Heart, 4: Spleen, 5: Pancreas, 6: Kidney, 7: Fat, 8: Muscle, 9: Testis, 10: Liver

Fig. 2. C terminus of Raptor is essential for binding with mTOR and IRS-1.

For wild-type Raptor, Raptor-ΔCT and LacZ gene transfer into HepG2 cells, the cells were incubated for 1 hour in DMEM containing recombinant adenovirus. Two days later, the cells were collected and cell lysates were immunoprecipitated with flag-tag antibody. Cell lysates and anti-flag tag immunoprecipitates were immunoblotted with each (IRS-1, mTOR, and flag) antibody as a probe. Representative results are shown in the panel. LacZ:n=3, Raptor:n=3, Raptor-ΔCT:n=3

Fig. 3. Insulin-induced p70S6K activity in hepatic Raptor-ΔCT mice. The effects of Raptor-ΔCT overexpression on p70S6K and 4E-BP1 in the liver were investigated. *A*: Immunoblotting of liver lysates with S6K and phospho-S6K (Thr389) antibodies revealed that insulin-induced activation of p70S6K was significantly depressed in the livers of Raptor-ΔCT mice. Three independent experiments were performed and the panel shown is representative of the results. *B*: S6 kinase assay showed insulin-induced activation of p70S6K to be markedly suppressed in the livers of Raptor-ΔCT overexpressing mice. LacZ:n=8 (insulin+:n=4, insulin-:n=4), ΔCT:n=8 (insulin+:n=4, insulin-:n=4), **:$p<0.01$ *C*:Liver lysates were immunoblotted with phospho-4E-BP1(Thr 37/46 and Thr 70) antibodies in three independent experiments and representative results are shown in the

panel. Both phosphorylations of 4E-BP1 are significantly enhanced by Raptor-ΔCT overexpression.

Fig. 4. Significantly lower glucose levels in Raptor-ΔCT mice after GTT

Mice were fasted for 14h followed by blood sampling and intraperitoneal injection of glucose (2g per kg body weight). *A*: Whole venous blood was obtained from the tail vein at the indicated time points after the glucose load. *B*: AUCs (areas under the curve) for glucose for each group were calculated and compared using the t-test. Intraperitoneal GTT revealed hepatic Raptor-ΔCT overexpression to improve glucose tolerance.

Control(LacZ): n=4, Raptor-ΔCT: n=4, *:$p<0.05$

Fig. 5. Insulin-induced IRS-1 tyrosine residue, Ser307 and Ser636/639 phosphorylations in hepatic Raptor-ΔCT mice. Four days after adenovirus injection, the livers were removed after insulin or saline administration, followed by immunoprecipitation with IRS-1 antibody. SDS-PAGE and immunoblotting were then performed using the appropriate antibody as a probe. *A*: There was no difference between Raptor-ΔCT and control mice, in the expression of IRS-1 protein. *B*: Insulin-induced IRS-1 tyrosine

phosphorylation was significantly increased in Raptor-ΔCT mice. *C,D*: Insulin-induced IRS-1 Ser307 and Ser636/639 phosphorylations were markedly depressed in Raptor-ΔCT mice. LacZ:n=8 (insulin+:n=4, insulin-:n=4), ΔCT:n=8 (insulin+:n=4, insulin-:n=4), *:$p<0.05$, **:$p<0.01$

Fig. 6. Insulin-induced PI3Kinase activity in hepatic Raptor-ΔCT mice. For PI 3-kinase assay, supernatants containing equal amounts of protein were immuno- precipitated for 2 h at 4 degrees C with anti-IRS-1 or 4G10 antibody and protein A- or G-Sepharose. PI 3-kinase activities in the immunoprecipitates were assayed. *A,B*: Insulin induced tyrosine phosphorylation-associated PI3K activity and IRS-1-associated PI3K activity, were both increased to approximately double those of LacZ mice. LacZ: n=8 (insulin+:n=4, insulin-:n=4), ΔCT: n=8 (insulin+:n=4, insulin-:n=4), *:$p<0.05$, **:$p<0.01$

Fig. 7. Insulin induced Akt phosphorylation in hepatic Raptor-ΔCT mice. Liver lysates were immunoblotted with Akt and phospho-Akt Ser473 and Thr308 antibody. *A*: There was no difference between these mice in Akt protein expression levels. *B,C*: Basal Akt Ser473 and Thr308 phosphorylation as well as insulin-induced Akt Ser473 and Thr308

phosphorylation, were also markedly increased in Raptor-ΔCT mice.

LacZ:n=8 (insulin+:n=4, insulin-:n=4), ΔCT:n=8 (insulin+:n=4, insulin-:n=4),

*:$p<0.05$, **:$p<0.01$

Table 1

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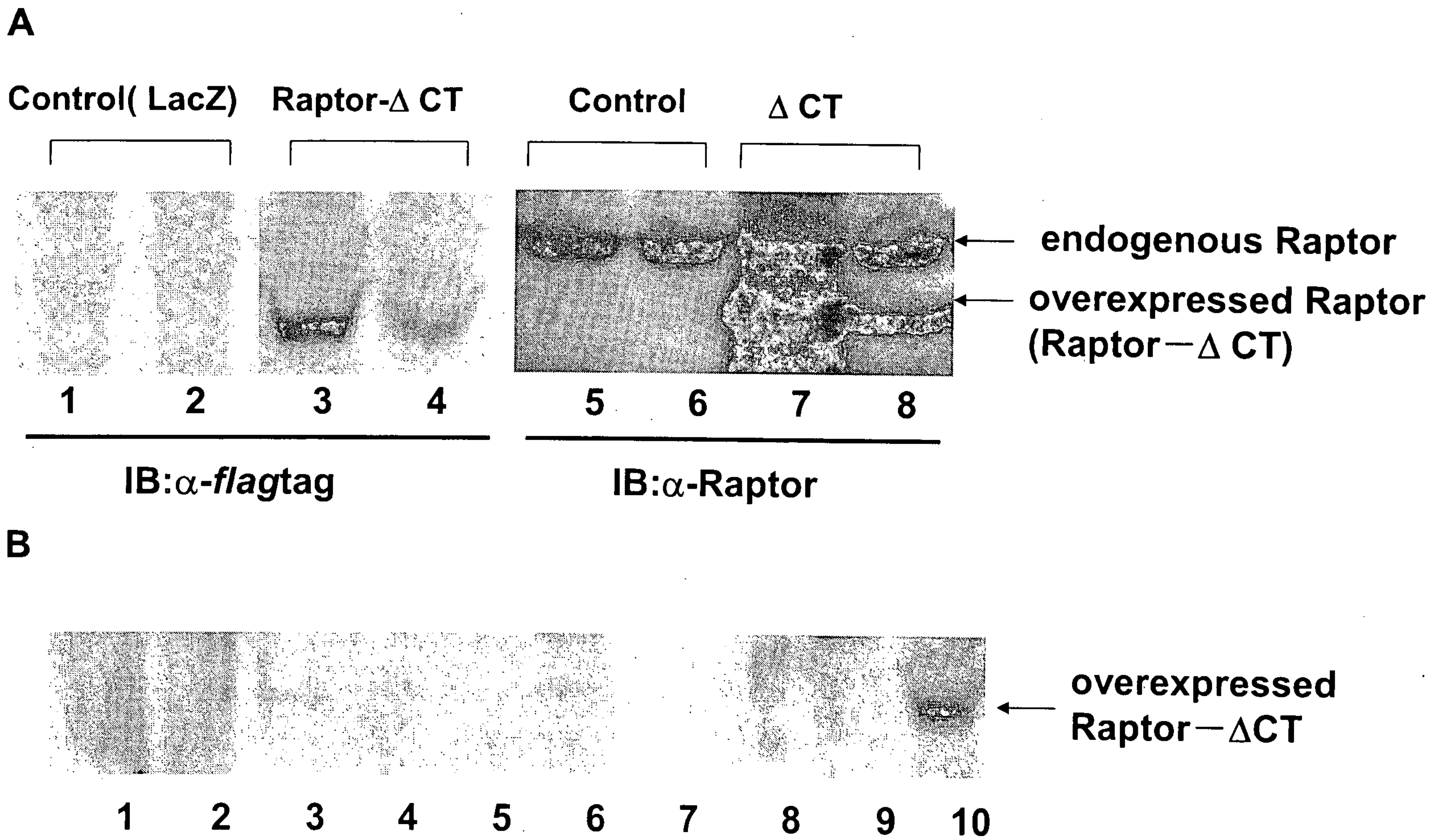
Fig.1.



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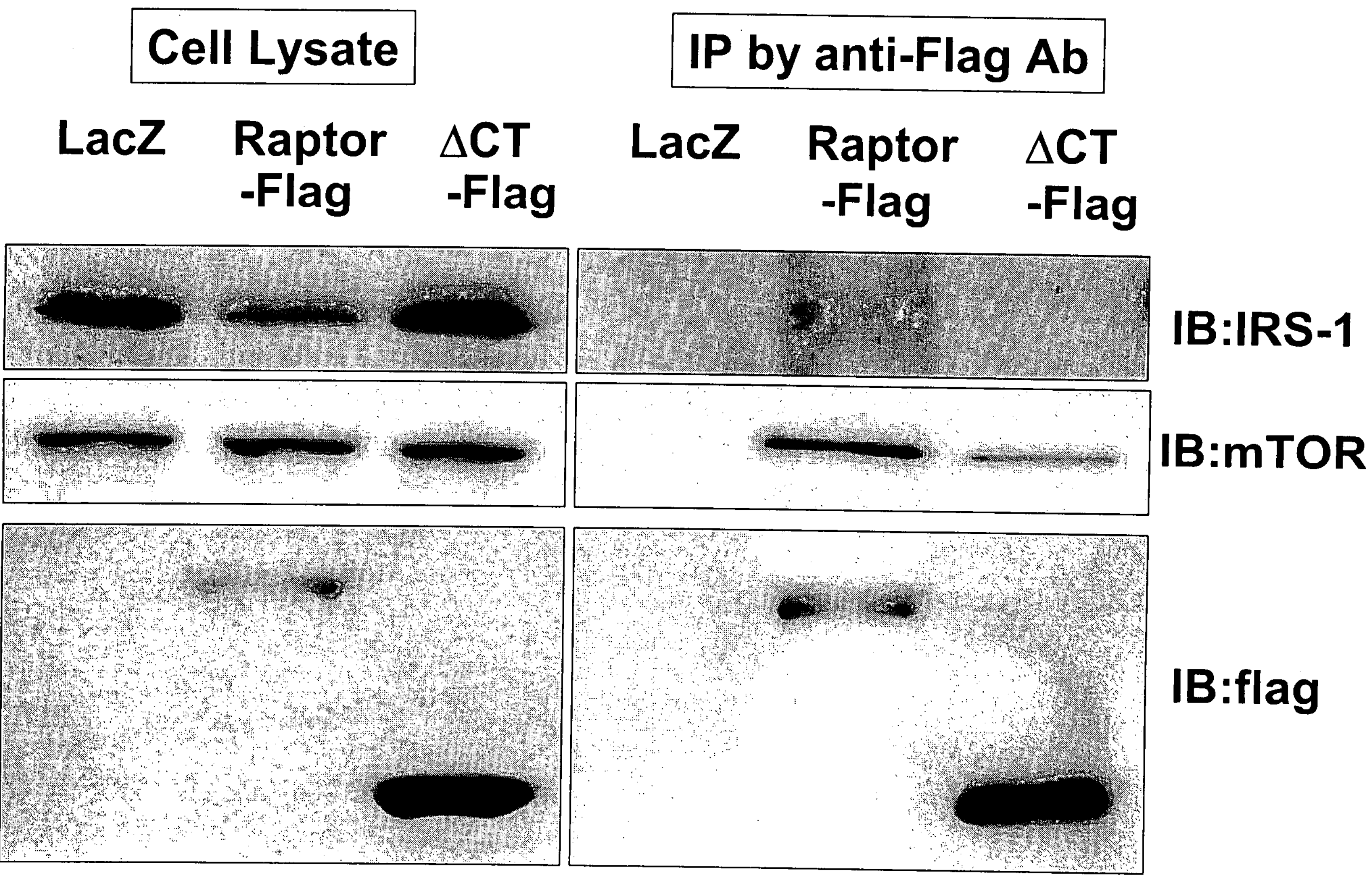

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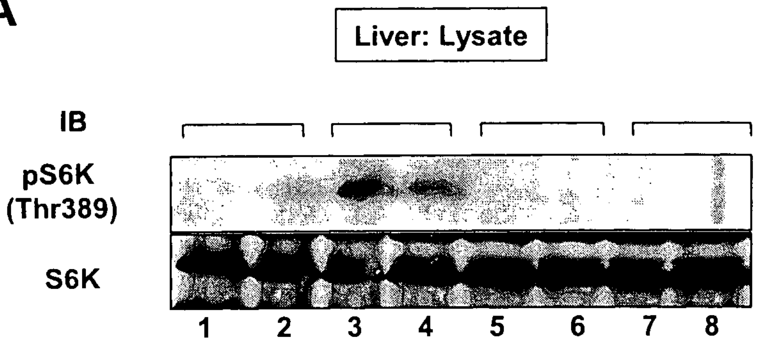

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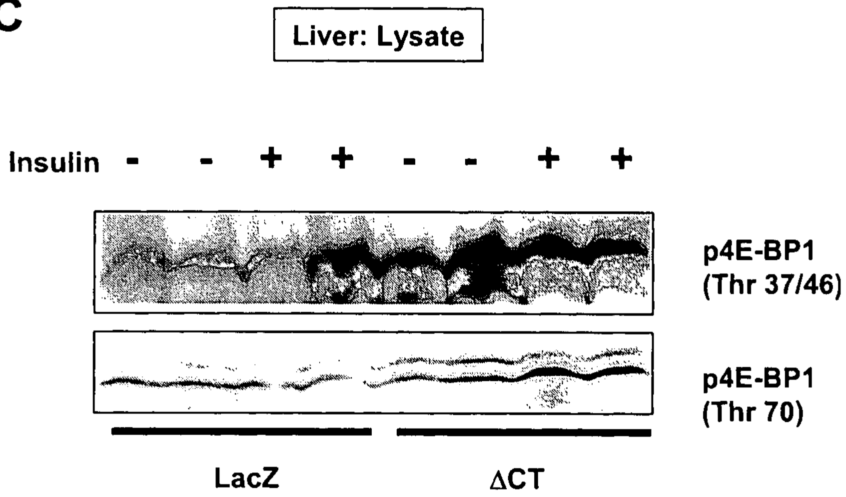

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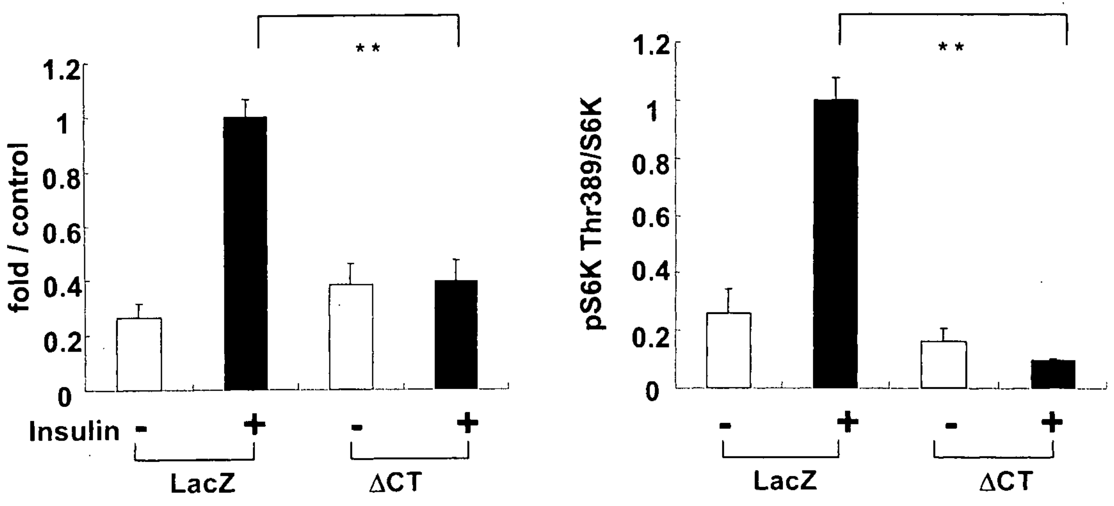

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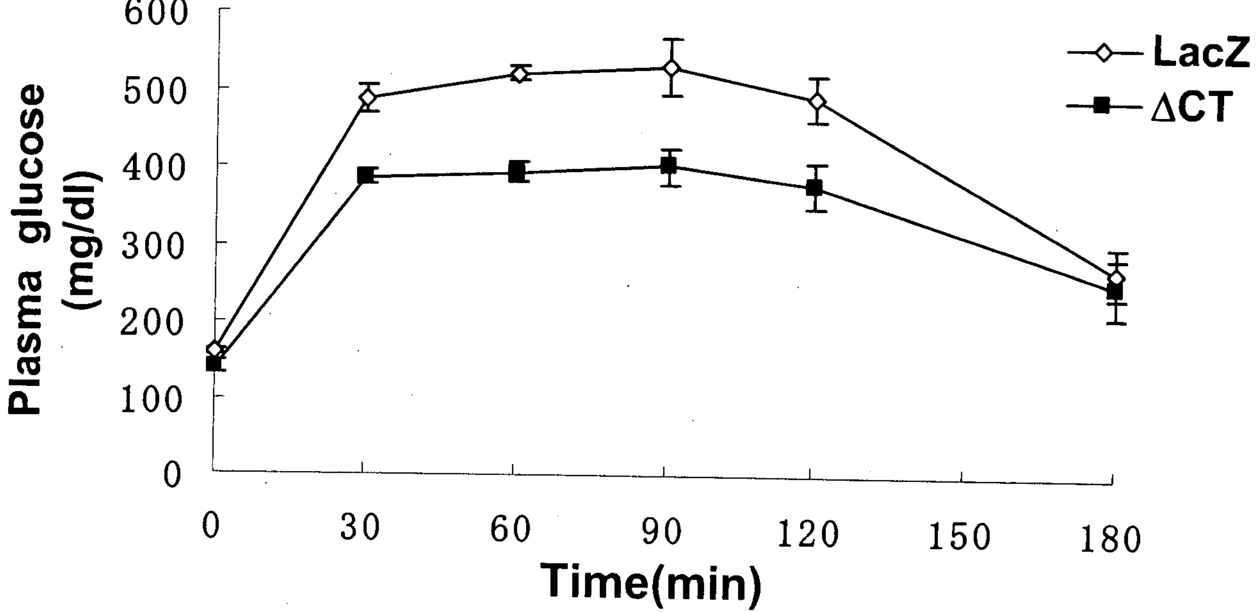
A



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